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In Vitro Production of Embelin from Callus Cultures of *Embelia ribes*

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Abstract:

E. ribes is an extremely valuable medicinal plant, containing Embelin as its main active component. Due to poor natural regeneration and over exploitation this species is red-listed. Traditional methods of propagation of *E. ribes* are not enough to meet the demands of the drug. However, *in vitro* production of Embelin from cell cultures of *E. ribes* is an alternative for obtaining this compound in large amounts, particularly in shorter duration. In order to achieve this, callus initiation from leaf explants of *E. ribes* and its multiplication were studied using varied concentrations and combinations of plant growth regulators in different genotypes. MS medium containing 2,4-D and BAP yielded maximum callusing and friable callus with high proliferation rate. Embelin was extracted from the callus in chloroform, which was then identified by chemical analysis, and quantified spectrophotometrically. The maximum quantity of Embelin produced in leaf induced callus was 0.632% (w/w), which was more than 3.5 times the amount extracted from *in vitro* grown leaves. Among the four genotypes screened, callus from genotype S1 accumulated maximum amount of Embelin on dry weight basis, showing that the production of Embelin is genotype specific. Our study also shows that increased Embelin can be produced by leaf induced callus cultures of *E. ribes* as compared to *ex vitro* and *in vitro* grown leaves. The outcome of this study can be potentially utilised for large scale production of Embelin through cell culture techniques, without depending on extraction from natural populations, thereby aiding in their conservation as well.

Keywords: *Embelia ribes*, Embelin, secondary metabolite, callus culture.

Introduction:

Embelia ribes Burm. f., from the *Myrsinaceae* family, is a highly valuable medicinal plant. It is a woody climbing shrub that is generally called as false black pepper, or “Vidanga” in Sanskrit which is its trade name. It grows at an altitude of 1,500m in the semi-evergreen and deciduous forests of India (1). It is also found in other Asian countries such as China, Srilanka, Malaya and Singapore (2). It is used in about 75 Ayurvedic preparations in numerous forms like asava, churna, aristha, lauha and taila (4, 12). The major bioactive constituent of *E. ribes* is Embelin (6). Other constituents found in *E. ribes* are christembine, quercitol, vilangin and resinoid (4). Since Embelin is non-steroidal and non-hormonal, it does not cause unwanted physiological effects (1). It has shown to possess antioxidant, hypoglycemic, antimicrobial, anthelmintic, anti-inflammatory, antitumour/anticancer, antispermatazoal, antiandrogenic, enzyme

inhibitory, antihyperlipidemic, anticonvulsant, chemopreventive, antiulcer, anti-angiogenesis, carminative and wound healing properties, and therefore *E. ribes* is used as a drug in Ayurveda, Siddha and Unani (3, 5).

Natural propagation of *E. ribes* is low because of uneven distribution of this species, resulting in inbreeding and formation of abortive embryos. Propagation of this species by artificial means is also difficult because the seeds do not germinate easily and cuttings of the stems do not undergo rooting. *E. ribes* is habitat specialist and does not easily get acclimatised to other climatic conditions. (1, 8, 9). The natural populations of *E. ribes* have been overexploited for its medicinal value, leading to its red-listed status as vulnerable in red list data book (7). Because of its commercial value, National Medicinal Plants Board has recognised this species to be very important for large scale cultivation (14). Since natural propagation of *E. ribes* and conventional means of propagation are insufficient to meet its commercial demands, plant tissue culture is necessary for its conservation and propagation (10). There are many reports on vegetative propagation of this species through cuttings, propagation through seeds and through micropropagation. This study has explored the possibility of *in vitro* production of the active component Embelin through callus cultures, which will allow large scale production of the same without harvesting the plant for extraction.

Materials and Methods:

In vitro shoot multiplication and callus induction

The nodal explants of *E. ribes*, from pre-maintained *in vitro* shoots, were aseptically inoculated in MS medium supplemented with additives and growth hormones, and incubated at 25°C for a period of 30 days for shoot multiplication (8). Leaves from *in vitro* multiplied shoots, were cut into segments of 1cm × 0.5cm and placed on the medium with abaxial side facing down, and incubated in dark at 25°C. MS media supplemented with additives (8) was used to study the effect of various concentrations and combinations of PGRs on callus induction from leaf, in 15 treatments (Table 1) with 4 replicates per treatment.

To evaluate the response of genotype on callus induction and Embelin production, leaf segments from 4 different genotypes i.e., 3 samples from Shringeri (S1, S2 and S3) and 1 from Agumbe (Ag) were used. The leaf explants were inoculated in MS media with additives, and 3 different hormone variations (which produced friable callus) as in Table 2, with 4 replicates per treatment.

Extraction of embelin

Embelin was extracted from *in vitro* leaves, *ex vitro* leaves, seeds and leaf induced callus of *E. ribes* with chloroform (13). 2g of shade dried leaves and seeds of *E. ribes* were powdered mechanically with mortar and pestle before extraction; 2g of fresh callus was used for Embelin extraction. The samples were homogenised in chloroform for 10 minutes. The content was then transferred to a stoppered conical flask and extracted in 15ml chloroform by placing the flask in boiling hot water bath for 20 minutes, and the extract was filtered. Extraction was repeated thrice using 15ml chloroform and filtered each time. The clear filtrate was concentrated to dryness in rotary evaporator. The extracts from leaves were dissolved in petroleum ether, and those from seeds and callus were dissolved in hexane, and filtered again. The residue was washed with petroleum ether to remove the chlorophyll content and redissolved in chloroform. The solvent was then evaporated to obtain the dried extract containing golden yellow crystals of Embelin.

Identification of embelin by Chemical Test

The crude extract was dissolved in petroleum ether and then diluted ammonia solution was added to it. Formation of violet precipitate indicated the presence of Embelin (11).

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Quantification of embelin by UV spectrophotometry

Embelin concentration in the extracts was estimated using UV spectrophotometer against chloroform as blank (13). Standard Embelin (Sigma Aldrich) dissolved in chloroform showed maximum absorbance at 289nm. Hence, absorbance of test samples was also measured at 289nm against chloroform blank. The amount of Embelin in test sample was determined using the linear regression equation obtained for Embelin standard. Three replicates per sample were quantified and the mean of the concentrations was calculated.

Culture conditions and statistical analysis

All *in vitro* studies were conducted in well-defined aseptic conditions. The culture room was maintained at 25 ± 2°C temperature at completely dark conditions for callus initiation and multiplication. Completely Randomised Design was employed for each experiment, and data recorded were statistically analysed.

Results and Discussion:

Effect of PGRs on callus induction

Leaf segments of *E. ribes* obtained from the *in vitro* multiplied shoots (Figure 1) were inoculated in MS media with different concentrations and combinations of PGRs, to study their effect on callus induction, and the results obtained after three weeks are tabulated (Tables 3, 4 & 5). Leaf explants started bulging after one week of inoculation. Callus initiation was observed after two weeks in all the treatments, except in the medium containing both 2,4-D and TDZ, in which callus initiated in the third week (Figure 2). It was found that formation of callus started from the mid-rib and at the cut-ends of the leaf explants. As the callus initiated, the abaxial surface of the leaf explants started to turn black. Callus initiation was observed in all the combinations and concentrations of growth regulators, except in hormone free (HF) medium. However, frequency of callus induction, colour and texture of callus formation varied among the different treatments, and there was significant difference

Fig. 1: *In vitro* multiplied shoots of *E. ribes* after 4 weeks of culture in MS medium containing 0.5mg/l BAP + 0.1mg/l IBA

Table 1: Concentration of PGRs used for callus induction

PGR		HF	0.5 mg/l BAP 0.5	Picloram (mg/l)				TDZ (mg/l)	
				1.0	1.5	2.0	0.5	1.0	
2,4-D (mg/l)	0	T1	-	T8	T9	T10	T11	-	-
	0.5	T2	-	-	-	-	-	T12	T13
	1.0	T3	T6	-	-	-	-	T14	T15
	1.5	T4	-	-	-	-	-	-	-
	2.0	T5	T7	-	-	-	-	-	-

Table 2: Treatments for callus induction in different genotypes at various concentrations of PGRs

T. No.	Composition of media	Genotype
G1	1.0 mg/l 2,4-D	S1
G2	1.0 mg/l 2,4-D	S2
G3	1.0 mg/l 2,4-D	S3
G4	1.0 mg/l 2,4-D	Ag
G5	1.0 mg/l 2,4-D + 0.5 mg/l BAP	S1
G6	1.0 mg/l 2,4-D + 0.5 mg/l BAP	S2
G7	1.0 mg/l 2,4-D + 0.5 mg/l BAP	S3
G8	1.0 mg/l 2,4-D + 0.5 mg/l BAP	Ag
G9	2.0 mg/l 2,4-D + 0.5 mg/l BAP	S1
G10	2.0 mg/l 2,4-D + 0.5 mg/l BAP	S2
G11	2.0 mg/l 2,4-D + 0.5 mg/l BAP	S3
G12	2.0 mg/l 2,4-D + 0.5 mg/l BAP	Ag

Table 3: Callus induction percentage (± SE) in media containing 2,4-D with BAP

PGR		BAP (mg/l)	
0		0.5	
2,4-D (mg/l)	0	0	-
	0.5	25±5.00	-
	1.0	40±18.26	25±9.57
	1.5	20±8.16	-
	2.0	20±10.00	35±12.58
Mean		21.00	30.00

Table 4: Callus induction percentage (±SE) in media containing 2,4-D with TDZ

PGR		TDZ (mg/l)		Mean
0.5		1.0		
2,4-D (mg/l)	0.5	70±12.91	80±20.00	75.00
	1.0	85±9.57	95±5.00	90.00
Mean		77.50	87.50	82.50

Table 5: Callus induction percentage in different concentrations of Picloram

Concentration of Picloram (mg/l)	Callus induction (% ± SE)
0.5	40±18.26
1.0	15±5.00
1.5	30±12.91
2.0	55±9.57
Mean	35.00

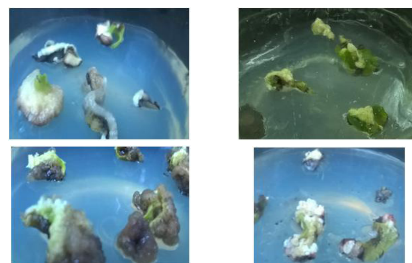

Fig. 2: Callus induction in media containing (i) 1mg/l 2,4-D + 1mg/l TDZ (ii) 2mg/l Picloram (iii) 2mg/l 2,4-D + 0.5mg/l BAP (iv) 1mg/l 2,4-D

Table 6: Variation in callus proliferation and morphology in different treatments

T. No.	Concentration of PGR	Callus proliferation			Colour of callus	Morphology of callus
		After 1st subculture	After 2nd subculture	After 3rd subculture		
T1	HF	-	-	-	-	No callus
T2	0.5 mg/l 2,4-D	+	+	+	White	Compact
T3	1.0 mg/l 2,4-D	+	++	+	White	Friable
T4	1.5 mg/l 2,4-D	+	+	+	White	Compact
T5	2.0 mg/l 2,4-D	+	+	+	White	Compact
T6	1.0 mg/l 2,4-D + 0.5 mg/l BAP	++	++	++	Slightly brownish	Friable
T7	2.0 mg/l 2,4-D + 0.5 mg/l BAP	++	+++	+++	Slightly brownish	Friable
T8	0.5 mg/l Picloram	++	+	+	White	Compact
T9	1.0 mg/l Picloram	+	+	+	White	Compact
T10	1.5 mg/l Picloram	++	+	+	White	Compact
T11	2.0 mg/l Picloram	++	+	+	White	Compact
T12	0.5 mg/l 2,4-D + 0.5 mg/l TDZ	+	+	++	White	Compact
T13	0.5 mg/l 2,4-D + 1.0 mg/l TDZ	+	++	++	White	Compact
T14	1.0 mg/l 2,4-D + 0.5 mg/l TDZ	+	++	++	White	Compact
T15	1.0 mg/l 2,4-D + 1.0 mg/l TDZ	+	++	+	White	Compact

Intense +++; Moderate ++; Meagre +

among different growth regulator treatments. Among the different growth regulators tested, 1mg/l 2,4-D with 1mg/l TDZ showed maximum frequency (95%) of callus induction. Among the various treatments tested, when the hormones were used singly, 2mg/l Picloram and 1mg/l 2,4-D showed maximum callus initiation frequency of 55% and 40% respectively.

Effect of PGRs on callus multiplication and morphology

To study the effect of PGRs on callus multiplication and morphology, the initiated callus was subcultured in fresh media, and callus proliferation was scored three weeks after subculturing (Table 6; Figure 3). Since callus started to brown after three weeks of callus initiation, subculturing in fresh medium was done after 21 days. The medium was supplemented with 25mg/l PVP during the subsequent subcultures to reduce browning. It was observed that during subculturing, the callus did not survive when it was broken. Therefore, whole intact callus formed was transferred as such into fresh media each time.

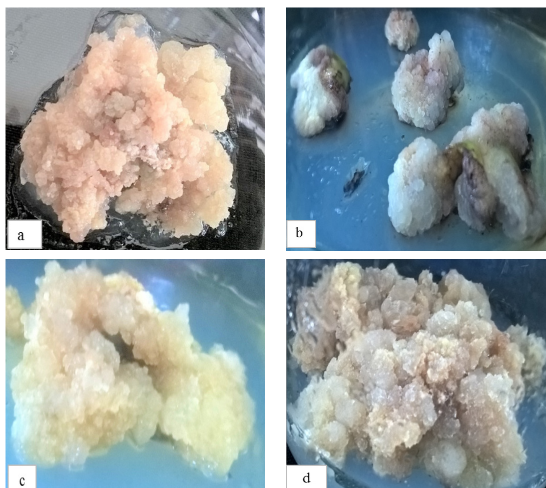

Fig. 3: Callus proliferation and morphology in media containing (a) 2,4-D 1.0 mg/l + BAP 0.5 mg/l (b) 2,4-D 1.0 mg/l + TDZ 1.0 mg/l (c) 2,4-D 1.0 mg/l (d) 2,4-D 2.0 mg/l+ BAP 0.5 mg/l

It was found that the rate of proliferation was the highest in treatment containing combination of 2mg/l 2,4-D + 0.5mg/l BAP. However, Raghu *et al*. (2011) reported optimal callusing response in *E. ribes* on a medium containing 1mg/l 2,4-D + 0.5mg/1BAP (15). MS media containing combination of 2,4-D and BAP produced brownish-white friable callus, whereas, other treatments resulted in whitish compact callus. Among the various concentrations of 2,4-D used, 1mg/l 2,4-D produced white friable callus; however, some explants showed adventitious root development after first subculture. In case of Picloram, one out of five explants showed adventitious root development from the callus induced in media containing 2mg/l Picloram.

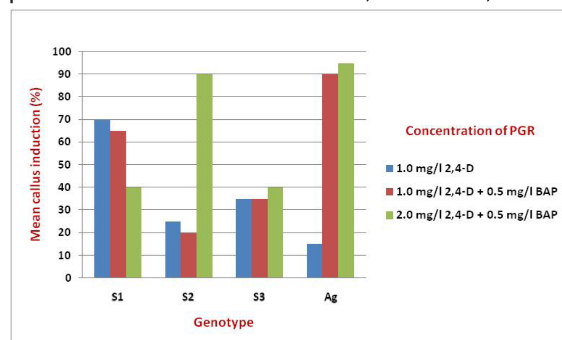

Fig. 4: Callus induction in different genotypes of *E. ribes*

Effect of genotype on callus induction

Since friable callus is better for initiating cell cultures, media containing growth regulators that gave morphologically acceptable callus, as in Table 6, were used to study the effect of genotype on callus induction. Leaf segments of four genotypes (S1, S2, S3 and Ag) were inoculated into the 3 different media, and it was observed that each genotype's response was different with respect to different combinations of plant growth regulators (Figure 4). There was significant difference among the genotypes for a given combination. Genotype Ag gave best callusing response of 95% and 90% in media containing 1mg/l 2,4-D + 0.5mg/l BAP and 2mg/l 2,4-D + 0.5mg/l BAP respectively. Genotype S1 gave best callusing response of 70% in 1mg/l

2,4-D. From the present study, it is concluded that genotype has an impact on callus initiation frequency in *E. ribes*. Similar observations on the influence of genotype on callus initiation have been reported in cotton and *Vaccinium* spp. also (16, 17).

Chemical test for identification of embelin

The presence of Embelin in dried *E. ribes* extracts was ascertained by dissolving it in petroleum ether and adding diluted ammonia solution. Violet coloured precipitate formed in seed, *ex vitro* leaf and callus extracts of *E. ribes*, indicating the presence of benzoquinone. Whereas, *in vitro* leaf sample did not show clear precipitate formation (Figure 5).

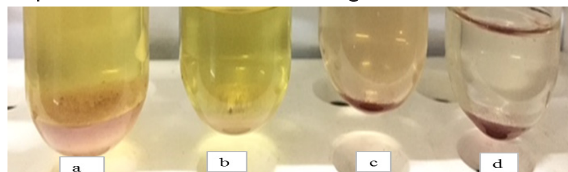

Fig. 5: Embelin identification by chemical test in (a) *Ex vitro* leaf extract (b) *In vitro* leaf extract (c) Callus extract (d) Seed extract

UV spectrophotometric quantification of embelin

UV spectrophotometric scanning of embelin standard dissolved in chloroform showed absorption maxima at 289nm. Hence, spectrophotometric quantification of Embelin in chloroform extracts of *E. ribes* was done at 289nm (Figure 6). Seeds of *E. ribes* showed highest Embelin content of 3.72% (w/w), followed by *ex vitro* leaves with 1.185% (w/w) Embelin. Leaf induced callus produced Embelin in the range of 0.4 to 0.6% (w/w), with maximum Embelin in callus derived from genotype S1, whereas, the *in vitro* leaves produced least Embelin content. The present study indicates that Embelin content was more in seeds and *ex vitro* leaves. This is because field grown plants are exposed to different biotic and abiotic stress conditions, and secondary metabolites are produced in larger amounts, compared to the *in vitro* leaves and callus which are grown under controlled conditions. However, Raghu *et al*. (2011) reported maximum Embelin content in embryogenic callus of *E. ribes* as compared to the berries (15). Also, in this study, fresh callus was used for Embelin extraction compared to the other parts which were weighed after drying. Therefore, extraction from the same weight of dried callus is expected to result in increased Embelin content.

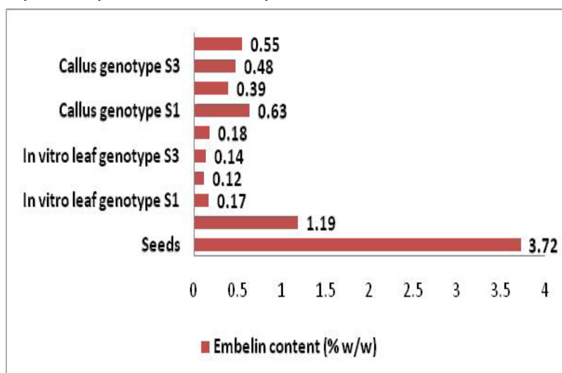

Fig. 6: Concentration of Embelin in different parts of *E. ribes*

Conclusion:

The enhanced understanding about metabolic pathways and advancements in metabolic engineering have led to the increased use of plant tissue culture technology for production of secondary metabolites including medicinally important compounds such as Taxol, Diosgenin, Capsaicin, Camptothecin, Vinblastine, Vincristine etc. (18). Similarly, callus culture also serves as an alternative for producing Embelin in shorter duration of time. This study reports the protocol for initiation and multiplication of callus from *in vitro* leaf explants of *E. ribes*. The presence of Embelin in callus extract, seed extract and *ex vitro* leaf extract was confirmed by phytochemical analysis, and was also quantified spectrophotometrically. Optimum callusing and morphologically acceptable callus was obtained in treatment containing combination of 2mg/l 2,4-D with 0.5mg/l BAP. However, the quantity is less than the amount of Embelin extracted from seeds of *E. ribes*. Hence, further optimisation is

required to enhance the yield of Embelin from callus cultures, after which large-scale Embelin production through cell suspension culture in bioreactors can be achieved. Our study also indicates that a single medium combination standardised for one genotype may not show the same response for other genotypes, in case of *E. ribes*.

Plants from different locations produce different amounts of secondary metabolites. Screening of suitable genotype of *E. ribes* for maximum Embelin production has not been reported so far. The effect of genotype on Embelin production through callus was also studied. Among the genotypes used for callus induction, genotype S1 produced maximum Embelin (0.632% w/w). Considering the many medicinal properties of *E. ribes*, production of Embelin in large quantities is necessary to meet the demands as well as to preserve this species.

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