

## Micropropagation of *Embelia ribes* Burm.f. using inflorescence segments

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An efficient micropropagation protocol was developed for a threatened medicinal plant, *Embelia ribes* using inflorescence explants. The immature ovaries of the inflorescence segments proliferated into luxuriant mass of callus on Murashige and Skoog's (MS) medium supplemented with IBA (3.5 mg/L) and Kn (0.5 mg/L). The combination of Kn and NAA in the range of 2.0 to 4.0 mg/L and 0.2 to 0.6 mg/L, respectively provoked the calli to differentiate into both shoot and root initials from the ovary callus. Highest number of regenerants ( $25 \pm 0.81$  per callus) were obtained at the concentration of 3.0 mg/L Kn and 0.4 mg/L NAA. The regenerants were transferred to the pots containing sterilized soil and hardened for a week. A mean of  $21.82 \pm 1.02$  regenerants per harvest were well acclimatized to the natural conditions exhibiting a normal development.

**Keywords:** *Embelia ribes*, inflorescence culture, micropropagation  
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### Introduction

*Embelia ribes* Burm. f. (Myrsinaceae) is a threatened woody shrub sparsely distributed in the moist deciduous forests of the Western Ghats of India, South Asia and Malaysia<sup>1</sup>. The plant is popularly known as 'Vidanga', Bashmak or Krimigna (Sanskrit) and Baberung or Wawrung (Hindi), which is one of the adjuvants in most of the drug preparations. The whole plant is used as an anti-inflammatory agent<sup>2</sup> and anthelmintic<sup>3</sup>.

The fruits contain a quinone derivative embelin (3-undecyl 2, 5-dihydroxy, 1, 4-benzoquinone), and an alkaloid, christembin<sup>4</sup>. The biological activities of this species have been evaluated for antifertility<sup>5</sup> and antihelminthic<sup>6</sup>. The traditional medical practitioners residing in the vicinity of the Lakkinakoppa forest range of Bhadra Wildlife Sanctuary are using the tender leaves to cure critical jaundiced condition. The ethnobotanical literature also mentioned the hepatoprotective properties of the leaves of *E. ribes*<sup>7</sup>.

Rapid progress has been observed in deriving protocols for the micropropagation of medicinal plants using vegetative explants and enhanced production of secondary metabolites<sup>8,9</sup>. Although culture of reproductive organs of angiosperms was initiated 60 years ago<sup>10</sup>, so far there are only limited

reports on the feasibility of subjecting inflorescence explants for *in vitro* research<sup>11-14</sup>. This paper reports rapid regeneration of plantlets from the immature ovary calli attached to inflorescence segments of *E. ribes*.

### Materials and Methods

#### Plant Material

Immature inflorescence segments of *Embelia ribes* were collected from a healthy plant growing in the medicinal plant garden, Kuvempu University, Karnataka, India. The materials were thoroughly washed in running tap water, rinsed in 1% Labolene, a neutral detergent (Qualigens, India) and then rinsed in distilled water 3 to 4 times to remove the surface microflora. The explants were surface sterilized with 0.1% (w/v) aqueous mercuric chloride (HgCl<sub>2</sub>) for 4-5 min. The disinfectant was removed by rinsing the materials with sterilized and cooled distilled water 4-5 times under aseptic conditions and the explants were inoculated onto the culture media.

#### Culture Media

The media consisted of MS basal constituents<sup>15</sup> supplemented with growth regulators IBA, NAA and Kn, tested either alone or in combinations at the range of 1 to 10 mg/L. The combinations of Kn and NAA were also tested between the range of 2.5 and 4.5 mg/L and 0.2 and 0.6 mg/L, respectively. All the media had 3% sucrose and gelled with 0.8% w/v

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agar-agar (Hi media, India). The pH of the media were adjusted between 5.6 and 5.8 prior to autoclaving. About 50 mL of molten medium was dispensed into sterilized culture bottles and autoclaved at 121°C at 15 PSI (1.06 Kg/cm<sup>2</sup>) pressure for 15 to 40 min. Each culture bottle was inoculated with five explants and incubated at 25±2° C, 12 h photoperiod, provided by cool white fluorescent tubes (Philips, India; 1000 Lux) and 65 to 70% relative humidity. The plantlets with fully expanded leaves and well developed roots were first transferred to the plastic cups containing sterilized soil. The regenerated plantlets were hardened by covering them with a thin perforated transparent polythene bag to maintain humidity. Plantlets were watered with 1/10<sup>th</sup> strength MS salts solution and maintained in the culture condition. After two weeks they were transferred to the medicinal plant garden. The number of root intact plantlets recovered per explant were recorded after 8 weeks of culture and the data were analysed statistically by using "Sigmastat" One Way ANOVA using Scientific Statistical Software<sup>16</sup>. The value P<0.005 is considered as significantly different.

## Results and Discussion

The immature inflorescence segments when inoculated onto MS basal nutrient media, blossoming of flower buds was noticed within 5 to 6 d of culture. (Fig. 1a). But they failed to transform into callus or any morphologically different organized structure. On the contrary, the MS medium augmented with the growth regulators IBA and Kn in a range of 2.5 to 4.5 mg/L and 0.1 mg/L to 0.7 mg/L, respectively induced callus formation from immature ovary of the inflorescence explant and it was optimized at the concentration of 3.5 mg/L IBA and 0.5 mg/L Kn. Within a week of incubation defoliation of sepals, petals and anthers occurred in the basal flower buds of the inflorescence. After two weeks of culture the immature ovaries became bulged into ten folds of their normal size, gradually transformed into fleshy green yellowish callus mass (Fig. 1b). On further incubation in the same medium, differentiation of few slender shoot buds was also observed from the ovary callus but these shoot buds were fleshy and dedifferentiated into callus. At this stage the floral buds present at the apex of the recemose axis were blossomed (Fig.1c). Later, these were also transformed into callus mass. After 20 d of culture, the base of axis consisted of callus initiated from the

bulged ovaries. They were excised from the axis and subcultured onto the same medium containing a range of higher concentration of Kn and lower concentration of auxins (2.0 to 4.0 mg/L Kn and 0.2 to 0.6 mg/L NAA, respectively). The interaction of Kn and NAA promoted differentiation of both shoot and root initials from the callus (Fig.1d). After 30 d of incubation proliferation of callus and differentiation of shoot and root initials were profusely increased simultaneously. In a 45-day-old culture a mean of

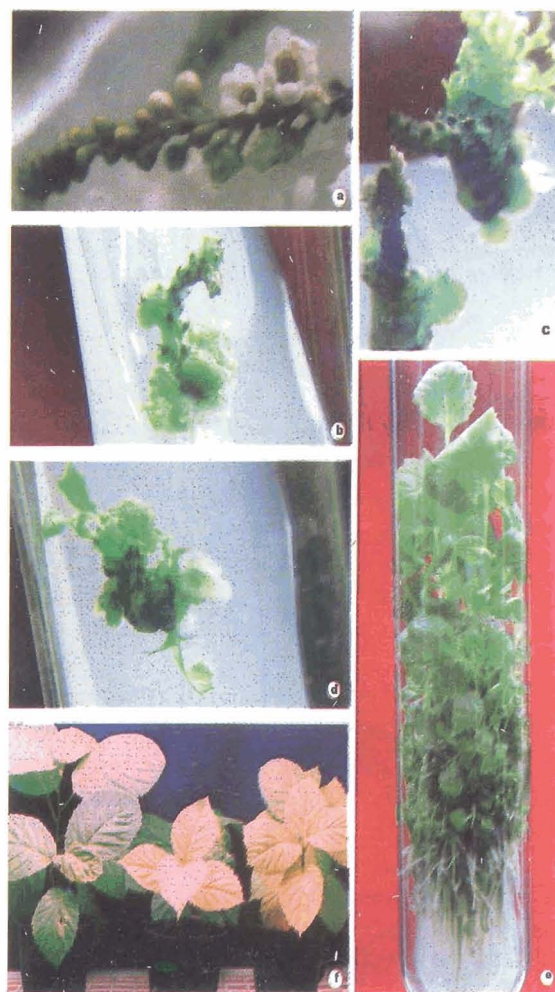


Fig 1—Plant regeneration through inflorescence culture of *E. ribes*: a. *In vitro* blossoming of flower buds on MS basal medium; b. Callus initiation from the bulged ovary of the inflorescence segment on MS medium supplemented with + 3.5 mg/L IBA and 0.5 mg/L Kn; c. Inflorescence segment showing differentiation of shoot buds from the ovary callus and blossoming of flower buds at the apex on MS basal nutrient medium; d. Excised ovary callus showing differentiation of both shoot and root initials on MS medium supplemented with +3.0 mg/L Kn and 0.4 mg/L NAA; e. 60-day-old culture of ovary callus showing differentiation of root intact plantlets on MS medium supplemented with + 3.0 mg/L Kn and 0.4 mg/L NAA; & f. 45-day-old hardened regenerants.

28±0.81 shoot buds noticed per callus and from 60-day-old culture a mean of 25±0.81 root intact plantlets were harvested (Fig. 1e). The frequency of regeneration of shoot and root from calli decreased beyond and above the optimal concentration as shown in Table 1. The root intact plantlets were taken out of the culture vessel, transferred to the soil and subjected to hardening process at culture condition. After two weeks of hardening the plantlets derived from the immature ovary accomplished well to the natural condition and their survival rate was evaluated statistically. A mean of 21.82±1.02 plantlets survived to the natural environment (Fig. 1f). Out of a mean of 25±0.81 plantlets harvested from the optimal concentration of Kn and NAA. Similar report of plant regeneration through inflorescence calli was observed in *Aerva tomentosa*<sup>17</sup>. Though the plantlets were regenerated from the immature ovary calli of the inflorescence segments, it is very difficult to assess whether the regenerants were originated from the megaspore of the ovule or from the diploid tissue of the ovary. Therefore, further studies are required to evaluate the ploidy and genetic variabilities among the regenerants.

Presently, urbanization and shrinking forest base have resulted in significant decline in the number of medicinal species. The real threat is the irreversible

loss of populations of medicinal plants whose size will be too diminished to survive through natural means of sexual reproduction. The *in vitro* technique is a promising area of research in the conservation of threatened plants as it assists in sustainable maintenance of the present day rapidly dwindling germplasm on long-term basis, especially for the medicinal plants viz., *Decalepis hamiltoni*<sup>18</sup>, *Withania somnifera*<sup>19</sup> and *Andrographis alata*<sup>20</sup>. The above protocol is applicable for the *ex situ* conservation of other threatened medicinally important species also.

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### References

- 1 Saldanha C J & Nicolson D H, *Flora of Hassan District, Karnataka, India* (Amerind, New Delhi) 1976, 200-203.
- 2 Kapoor V K, Chawla A S, Kumar M & Kumar P, Search for anti-inflammatory agent in Indian laboratories, *Indian Drugs*, 30 (1983) 481-488.
- 3 Kirtikar K R & Basu B D, *Indian medicinal plants*, vol II (Lalit Mohan Basu, Allahabad) 1987, 1479.
- 4 Tyagi R D, Tyagi M K, Goyal H R & Sharma K, A chemical study on krimiroga, *J Res Indian Med*, 3 (1978) 130-132.
- 5 Arora R B, Ghatak N & Gupta S P, Antifertility activity of *Embelia ribes*, *J Res Indian Med*, 6 (1971) 107-111.
- 6 Wanjari Y S, Jangde C R, Srikhande G B & Vora S C, Efficacy of aqueous extract of *Embelia ribes* seeds against *Ascaridia galli* in white leg horn chicks, *Indian Vet J*, 77 (2000) 73-75.
- 7 Manjunath B K, Krishna V & Pullaiah T, *Floristic composition of Davanagere District, Karnataka*, (Regency Publishers, New Delhi) 2004, 234.
- 8 Neumann K H, Barg W & Reinhard E (eds), *Primary and secondary metabolism in plant cell culture* (Springer-Verlag, Berlin) 1985, 1-377.
- 9 Misawa M, *Plant tissue culture: An alternative for production of useful metabolites* (FAO Agriculture Services Bull., Rome) 1994, 87.
- 10 La Rue C D, Rooting of flowers in sterile culture, *Bull Torrey Bot Club*, 69 (1942) 332-341.
- 11 Bonga J M, Vegetative propagation by tissue and organ culture: Vegetative propagation-Tissue and organ culture as an alternative to rooting cuttings, *J For Sci*, 4 (1973) 253-260.
- 12 Sudarshana M S & Shanthamma C, Plant regeneration from inflorescence culture of *Boerhaavia diffusa* L., *Curr Sci*, 57 (1998) 268-270.
- 13 Ornstrup H, Molgaard J P & Farestveit B, Somatic embryogenesis and plant regeneration from cell suspensions of *Exacum affine*, *Plant Cell Tissue Organ Cult*, 35 (1993) 37-41.
- 14 Kumar S, Agarwal K & Kothari S L, *In vitro* induction and enlargement of apical domes and formation of multiple

Table 1—Effect of Kn and NAA on shoot bud induction and regeneration of plantlets through inflorescence culture of *E. ribes*

Plant growth regulators mg/L		Number of shoot buds/explant	Number of rooted plantlets recovered/explant
Kn	NAA	Mean ± SD	Mean ± SD
2.0	0.2	02.6 ± 4.03	02.1 ± 4.25
2.0	0.4	06.1 ± 0.87	03.1 ± 0.73
2.0	0.6	09.4 ± 1.07	05.0 ± 0.94
2.5	0.2	15.1 ± 0.87	11.7 ± 1.42
2.5	0.4	20.8 ± 0.78	17.0 ± 1.05
2.5	0.6	18.0 ± 1.05	14.2 ± 0.78
3.0	0.2	24.0 ± 0.81	21.7 ± 0.94
3.0	0.4	28.0 ± 0.81	25.0 ± 0.81
3.0	0.6	25.1 ± 0.87	22.0 ± 1.05
3.5	0.2	17.2 ± 1.23	15.1 ± 0.73
3.5	0.4	19.2 ± 0.78	16.3 ± 0.82
3.5	0.6	12.1 ± 0.87	10.2 ± 0.78
4.0	0.2	08.0 ± 0.81	04.3 ± 0.82
4.0	0.4	05.1 ± 0.73	03.0 ± 0.81
4.0	0.6	03.2 ± 0.63	02.2 ± 0.78
F value	379.9		318.0

The value of each concentration consisted of ± S.D. of 10 replicates.

The F value is significantly different at 0.05%.

- shoots in finger millet, *Eleusine coracana* (L.) Gaertn. and crowfoot grass, *Eleusine indica* (L.) Gaertn, *Curr Sci*, 81 (2001) 1482-1485.
- 15 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473-497.
  - 16 Sigmastat, *One Way Analysis of variance for significant F-value confirmation* (Jandel Corporation) 2005.
  - 17 Murgai P, *In vitro* culture of the inflorescence, flowers and ovaries of an apomitic *Aerva tomentosa* Forest, *Nature (Lond)*, 184 (1959) 72-73.
  - 18 Anitha S & Pullaiah T, *In vitro* propagation of *Decalepis hamiltoni*, *J Trop Med Plants*, 3 (2002) 227-232.
  - 19 Govindaraju B, Rao S R, Venugopal R B, Kiran S G, Kaviraj C P & Rao S, High frequency plant regeneration in Ashwagandha (*Withania somnifera* Dunal.): An important medicinal plant, *Plant Cell Biotechnol Mol Biol*, 4 (2003) 49-46.
  - 20 Nagaraja Y P, Krishna V & Maruthi K R, Rapid micropropagation of *Andrographis alata* through leaf culture, *Plant Cell Biotechnol Mol Biol*, 4 (2003) 117-124.