Research Report No. RR - 538

# Propagation and Planting of *Embelia ribes* and *Embelia tsjeriam-cottam* : Two Threatened Medicinal Plants

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# Propagation and plantation of *Embelia ribes* and *Embelia tsjeriam-cottam* – two threatened medicinal plants

(Final Report of Project No. RP 615/2011)

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August 2017

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# Project Proposal

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| 1. | Title Of The Project | : | Propagation and planting of *Embelia ribes* and *Embelia tsjeriam-cottam* - two threatened medicinal plants |
| 2. | Principal Investigator | : | A.V. Raghu |
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| 4. | Date Of Commencement | : | July 2011 |
| 5. | Date Of Completion | : | June 2014 |
| 6. | Funding Agency | : | KFRI Plan Grants |
| 7. | Objective | : | Large- scale multiplication of these two species through *in vitro/ in vivo*methods for planting materialproduction. |

## Abstract

Efficient micropropagation methods for *Embeliaribes* and *E. tsjeriam-cottam*, two medicinally important species from family Myrcinaceae, by axillary bud proliferation were standardized. Effect of plant growth regulators like BAP, Kinetin, IAA and IBA were studied for shoot multiplication and root development. *Ex vitro* rooting was also attempted in *E.tsjeriam-cottam* According to the study, promising results in shoot multiplication were found in the both species by using combinations of Cytokinins and auxins. In*E.ribes*good results was obtained by using Kinetin (1mg/l)with IAA (0.1mg/l) and in *E.tsjeriam-cottam*,it was BAP (1mg/l) with IAA (0.1mg/l).Best root induction was obtained in half strength MS basal media with IBA(1 mg/l) in both the species. The survival rate was low in *E.ribes* due to shoot decayingbut in*E.tsjeriam-cottam* 70% survival was observed in both *ex vitro* and *in vitro* rooted plants.



**Key words**: *E.ribes*, *E.tsjeriam-cottam*, axillary bud proliferation, acclimatization, *ex vitro* rooting.

## 1a. Introduction

The growing interest in therapeutic use of plant derived natural products all over the world leads to a fast growing market for plant based drugs and pharmaceuticals. The realization of health hazards associated with the harmful side effects of synthetic medicines and their indiscriminate use have increased the popularity and acceptance of natural drugs among the people. Use of plants as source of medicines is an ancient practice.It is estimated that more than two thirds of current drugs are derived from plant sources (Coe and Anderson, 1996).

Medicinal plants constitute a very important natural resource of India because she has one of the richest plant based ethno medicinaltraditions in the world going back to 3000 years old medicinal heritage (Rajasekhara and Ganesan, 2002). Even though India is blessed with a full- fledged plant resource, a high level of exploitation and habitat loss with various biotic pressures have led to a considerable decrease in the population level of many of the valuable medicinal plant species.

*Embelia* is one of the most important genera once placed in the family Myrsinaceae, which is now included in Primulaceae. *Embelia* has been investigated for a wide range of applications in Ayurveda, Unani, Siddha, Homeopathy and in several folk medicines. In Kerala, the genus is represented by five species, *Embelia ribes*, *Embelia tsjeriam-cottam*, *Embelia adnata*, *Embelia gardneriana* and *Embelia basaal.* Among the five species *Embelia ribes* is well known for its medicinal value and extensive studies have been carried out for revealing the rich medicinal properties. Studiesare now restricted to the common species *Embelia ribes* and *Embelia tseriam-cottam*. *Embelia ribes* is also one of the 32 important medicinal plants listed by

the Medicinal Plant Board, Govt. of India, New Delhi for large scale cultivation for commercial use.

Medicinal properties of the genus is attributed to the presence of the active phytochemical compound embelin. Genus *Embelia* is the only known natural source of this quinonic compound. In nature this active compound is found in leaves and fruits of the plants belonging to this genus. Embelin was identified as 2-5 dihydroxy –3 undicyl-1 4-benzoquinone. This was first isolated by Warden, from the fruits and studied its chemical and physical properties (Dymock *et al*., 1891). Later several workers have reported the constitution and synthesis of the active principle of *E. ribes* (Fieser and Chemberlin 1948; Murty *et al*., 1976). The pharmacological and clinical investigations by various workers gave promising results about its anti-fertility activity without any side effects (Mitra, 1995; Anonymous, 2002). Embelin has anti-inflammatory and anticancer principle (Chitra*et al*., 1994a; Chitra*et al*., 1994b). Chemical structure of embelin has good resemblance with natural coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanism (Kobaisy *et al*., 2008). Phytochemical analysis of various extracts of fruits of *Embelia ribes*revealed the presence of phytoconstituents other than embelin such as embolic acid, rapanone, vilangin, etc.

*Embelia ribes*grows in semi - evergreen, evergreen, deciduous and shola forests. Natural regeneration of this plant is poor due to over harvesting and exploitation which leads to fragmented population and ultimately results in inbreeding, development of abortive embryo and the slow germination of fertile small seeds. Apart from this poor seed viability, low rate of germination and poor rooting from stem cuttings made conventional propagation methods difficult. Lack of knowledge about its distribution and unknown propagation techniques have resulted in the lack of availability of quality planting materials(QPM) for promoting cultivation

(Mhaskar *et al*., 2011). Misidentification of this species coupled with the use of adulterants and substitutes have further aggravated the problem (Mhaskar *et al*., 2011).

*Embelia tsjeriam-cottam* is distributed throughout India in moist deciduous forests and plains. Its fruit is used as a major adulterant or substitute for *Embelia ribes* fruits in local markets. But *Embelia tsjeriam-cottam* is also significant for its embelin content and it is less threatened as compared to *Embelia ribes*.

Plant tissue culture – a key tool in plant biotechnology can be helpful for the successful propagation and conservation of these important medicinal plants. Especially when natural and conventional regeneration methods are difficult, *in vitro* methods can be helpful. Such important plants can be multiplied by axillary shoot induction or by somatic embryogenesis. Plant tissue culture also offers *in vitro* manipulation of cultures for the production of biologically active compounds in a continuous manner and also to enhance the production of these compounds*in vitro.*

**1b. Objective of the study**

Large scale multiplication of *E. ribes* and *E. tsjeriam-cottam* through *in vitro / in vivo* methods for planting material production.

## 2. Materials and Methods

### 2a. Plant material

Plant material for culture was collected from different regions of Kerala. There were six and sevendifferent accessions of *E. tsjeriam-cottam*(Etc) and *E. ribes*(Er) respectively. Accessions Etc-1, Etc-2, Etc-3, Etc-4, Etc-5 and Etc-6 were collected respectively from Kottakkal, Vellanipacha, Peechi, Pookodu forest area, Kottapara and forest nursery KFRI, Peechi. Different accessions of *E. ribes*, Er-1, Er-2, Er-3, Er-4, Er-6, Er-7 and Er-8 were collected from Kottakkal, Kakkayam, Wayanad and Ponmudi regions.

### 2b. Chemicals

All the chemicals used in this study were of AR grade and double distilled water was used for preparation of media and stock solutions of the mineral salts. Vitamin and hormone stocks were also prepared in double distilled water and stored in refrigerator.

### 2c. Media

The basal media used in all the stage of cultures was Murashige and Skoog media. Carbohydrate source was sucrose and agar (PTC grade, Meron) was used as gelling agent. Required quantities of growth regulators and vitamins were added from the stock solution. After adding sucrose, pH of the media was regulated up to 5.7 using 1N NaOH and 1N HCl solution. For solid media, agar (0.7-0.8%) was added and melted in a microwave oven. 15ml-50ml media was dispensed into test tubes and bottles and sterilized in autoclave.

### 2d. Culture condition

After inoculation cultures were transferred to the growth room where a temperature of 25°C$\pm$2 °C was maintained through air conditioning. The cultures were illuminated with cool day

light fluorescent tubes. A photoperiod of 8 hour light and 16 hour darkness was provided to the cultures. Cultures were subcultured in to a fresh media every 4 weeks.

**2e.Stages in cultures**

***Embelia ribes***

For *in vitro* clonal propagation of *Embelia ribes*,explants were collected from wild population. Nodal segments and shoot tips were subjected to detergent wash followed by treatment in a mixture of 1.5% fungicide and 250ppm antibiotics for 1hour. After treatment, explants were surface sterilized using 0.1% (W/V) $HgCl_2$ for 10minutes followed by washing with sterile distilled water. The explants were then inoculated in test tubes containing solid initiation media consisting of MS basal media supplemented with BAP (1.0 mg/l) with sucrose 3%.

- Standardization of multiplication media

Sprouted shoots were sub cultured on to a fresh media for multiple shoot formation and further multiplication. Solid MS basal media supplemented with BAP, IAA and Kinetin in single and in combination with concentration ranging from 0.5 mg/l to 1.5 mg/l were tried to find the best media for shoot multiplication. Observed the cultures in 10 days interval and transferred to fresh media every 2 weeks.

- Shoot elongation and rooting

Since the length of the shoots obtained in multiplication media was not suitable for persistent root formation, the clumps of the shoot initials were excised from the parent culture and transferred to MS basal medium containing different concentration ranging from 0.5 mg/l to 2 mg /l of BAP and Kinetin in single and in combination for elongation.Shoot elongation was also tested in basal MS media without any growth regulators. After getting sufficient elongation

within 20 days shoots were transferred to induce roots in liquid half strength MS basal media supplemented with IBA (1 mg/l) and sucrose 3%.

- Acclimatization

Rooted plantlets were transferred to cups filled with soil and sand (1:1 v/v mixture) and transferred to mist chamber and monitored daily.

***Embelia tsjeriam-cottam***

For initiating nodal cultures of *Embelia tsjeriam-cottam* explants were taken from 2 – 3 year old plants. Nodal segments and shoot tips were subjected to detergent wash followed by treatment in mixture of 1.5% fungicide and 250ppm antibiotics for 1hour. After treatment explants were surface sterilized using 0.1 % (W/V) $HgCl_2$ for 10minutes followed by washing with sterile distilled water. The explants were then inoculated in test tubes containing solid initiation media consisting of MS basal media supplemented with BAP (1.0 mg/l) and IAA (0.1mg/l) with sucrose 3%.

- Standardization of multiplication media

Sprouted shoots were sub cultured on to a fresh media for multiple shoot formation and further multiplication. Solid MS basal media supplemented with BAP, IAA and Kinetin in single and in combination with concentration ranging from 0.5 mg/l to 1.5 mg/l were used to find the best media for shoot multiplication. Observed the cultures at 10 days interval and transferred to fresh media every 2 weeks.

- Shoot elongation androoting

Since the length of the shoots obtained in multiplication media was not suitable for persistent root formation the clumps of the shoot initials were excised from the parent culture and transferred to growth regulator free MS basal medium for elongation. After getting sufficient

elongation, shoots were transferred to induce roots in liquid half strength MS basal media supplemented with IBA (1 mg/l) and sucrose 3%.

- Acclimatization

Rooted plantlets were transferred to cups consisting of soil and sand (1:1 v/v mixture) and transferred to mist chamber and monitored regularly.

- *Ex vitro* rooting

In order to check the efficiency of tissue cultured shoots to produce the roots in *ex vitro* condition in *Embelia tsjeriam-cottam*, each single elongated shoots from the culture maintained in multiplication media was separated and given a treatment in different concentration IBA solution and transferred into cups containing sterilized vermiculate. The cups were covered and transferred to mist chamber and monitored regularly.

## 3. Results and Discussion

In medicinal plants different tissue culture methods are practiced in order to get optimum multiplication rate, which includes axillary bud proliferation, direct and indirect organogenesis, somatic embryogenesis etc.

### 3a. Explant selection and initiation of culture

Being an important medicinal plant which is difficult to propagate by conventional methods,*invitro* micropropagation studies were attempted by several workers for *E. ribes*. But *in vitro* studies in*E.tsjeriam-cottam* is limited. Study reports for direct and indirect shoot induction from different types of explants are already available for *E. ribes*. Raghu *et al.*(2006) reported an effective system for direct plant regeneration from *in vitro* derived leaf explants of *E. ribes* on basal MS media supplemented with different concentration of TDZ

In 2010, Annapurna and Rathorereported micropropagation system through direct shoot induction from hypocotyl segments of *E. ribes* in MS basal media supplemented with different concentration of TDZ. Using immature ovaries of *E.ribes*, Shankarmurthy and Krishna(2006) reported another method for indirect shoot induction. Plant regeneration from embryogenic and organogenic callus from leaf explants was reported by Raghu *et al.*(2011).In the present study nodal portions of *E.ribes* and *E. tsjeriam-cottam* were used as explants for axillary bud proliferation.In *Embelia ribes*, the explants responded with high frequency of bud break within two weeks on MS basal medium containing BAP 1mg/lt. (fig.1.A)

Where nodal explants of *Embelia tsjeriam-cottam* produced high frequency of sprouting within two weeks on the MS basal medium supplemented with BAP(1.0 mg/l) and IAA (0.1mg/lt), (Fig.2.A), bulging of explant was noted which resulted in a subsequent development of a mass of callus at the base of the shoot cluster.

Preetha *et al.*,(2012) reported that 0.5mg/lt BA was most effective for shoot bud induction from nodal explants of *E.ribes*. In another study carried out by Dhavala *et al.,* (2010) nodal shoot segments exhibited high frequency shoot initiation when cultured on MS basal media supplemented with TDZ at 1.13µM and IBA at 0.49µM.

**3b. Standardization of multiplication media**

Sprouted shoots of *E. ribes* and *E.tsjeriam-cottam* from initiation media were sub cultured onto solid MS basal media supplemented with different combination of BA, IAA and Kinetin. For *Embelia ribes*, considerable number of multiple shoots were observed in MS basal media containing Kinetin 1.0mg/l with IAA (0.1 mg/l) (Fig.1.B).After 3- 4 sub cultures the shoots were split into clusters containing 4-5 shoots each and transferred to fresh media.

In *Embelia tsjeriam-cottam* cultures, considerable number of multiple shoots were noticed in media supplemented with combination of BA and IAA with concentration of 1.0 mg/l and 0.1mg/l respectively, the same media used for initiation of cultures (Fig.2B). After 3-4 sub culture these shoots were split into clusters consisting of 5-6 shoots each and transferred to fresh media.

In their study Preetha *et al.* (2012) achieved multiple shoot induction in MS basal media supplemented with 0.5mg/l BA and 0.1mg/l IAA. Dhavala *et al.*(2010) achieved multiple shoots when transferred to media supplemented with TDZ at 1.13µM concentration and in subsequent sub culturing procedures the inhibition by TDZ for shoot elongation was overcome by transferring onto MS media containing BAP at 11.10 µM concentration.

**3c. Shoot elongation and rooting**

The single shoots of *Embelia tsjeriam-cottam*in MS basal media without any growth regulators attained considerable length within 20 days and this elongated shoots were transferred to half strength MS basal media supplemented with IBA (1mg/l) and 3% sucrose for rooting.

But in the case of *Embelia ribes*, shoot elongation was obtained in MS basal media supplemented with BAP 1mg/l and kinetin 1mg/l (Fig.1.C). The elongated shoots were transferred to half strength MS basal media with IBA (1mg/l) for rooting. The transferred shoots produced roots within one month of incubation for both species. This finding was very much similar to that of Raghu *et al.* (2011). According to him half strength MS basal media with 1mg/l IBA was the best media for root induction of *E.ribes.*

**3d. Acclimatization**

Rooted plantlets were transferred to cups consisting of sand and soil (1: 1 v/v mixture). In the case of *E.ribes* survival rate of rooted plantlets were very low due to the decaying of shoots. But 70% of plants survived in *E. tsjeriam-cottam*. Reasons for decaying is unknown and requires further studies. In*E. tsjeriam-cottam,ex vitro* rooting was also practiced by transferring plants from multiplication stage to rooting in *ex vitro* condition. In *ex vitro* condition the survival rate was about 70% (Fig.2D). It was found that *ex vitro* rooting was very much effective for *E.tsjeriam-cottam.*

In conclusion, a total number of 300 *E.tsjeriam-cottam* plants were produced. 50 plants were planted in arboretum. 147 plantlets are under multiplication state. In the case of *E. ribes* 82 plantlets of *Er*-I are under elongation state and 17 plantlets were hardened and planted. New accession *Er*-8 was initiated. The difficulties faced in rooting state and hardening state of *E.ribes*are bottle neck for large scale production of this species. Future research should concentrate on the two above problems for successful propagation of this important medicinal species.


**Fig. 1. A-F.** *In Vitro* Clonal Propagation of *Embelia ribes*. **A,** Culture initiation on MS with 1.0 mg/ l BAP. **B,** Multiple shoot formation on MS with kinetin (1.0 mg/ l) and IAA (0.1 mg/ l). **C,** Elongation of multiple shoots on BA (1.0 mg/ l) and Kinetin (1.0 mg/ l) containing medium. **D,** Elongation of multiple shoots on BA (2.0 mg/ l) and Kinetin (2.0 mg/ l) containing medium. **E,** Rooted plantlets. **F,** Acclimatized plantlets on poly bag.

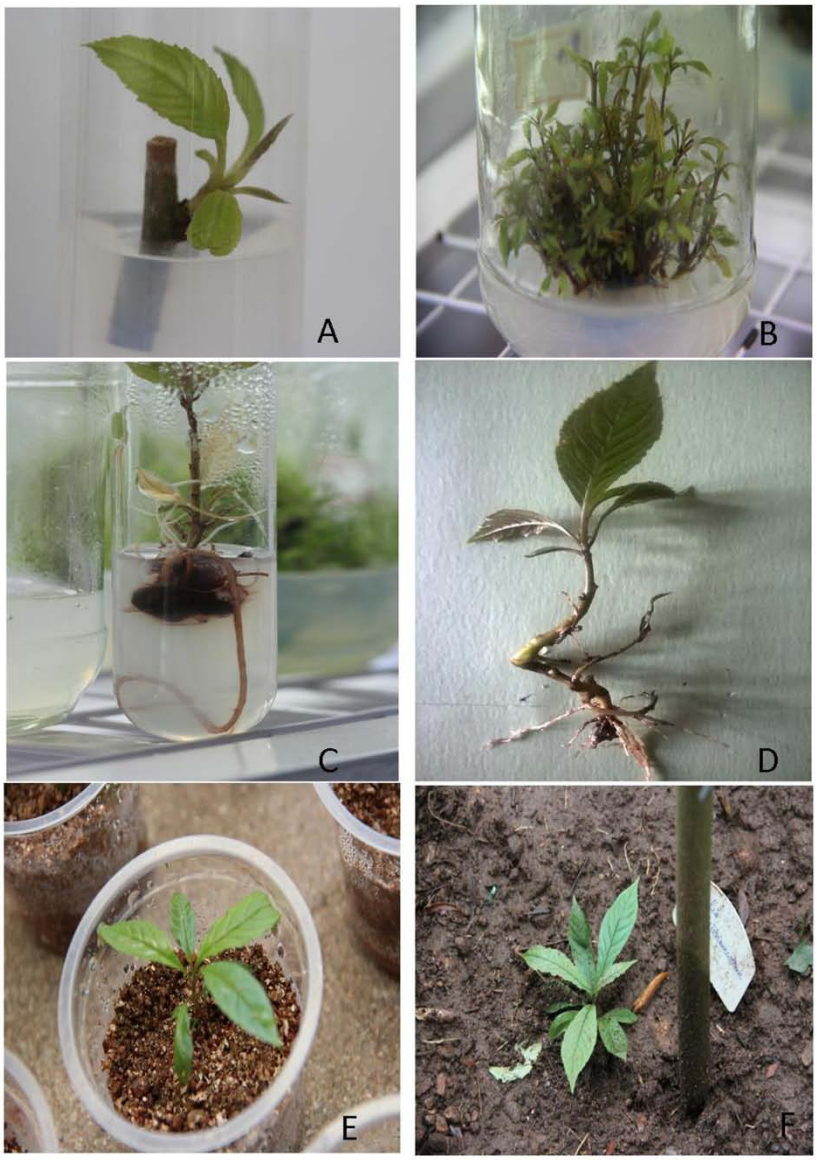


**Fig.2.** *In vitro* clonal propagation of *E.tsjeriam– cottam* **A)** Culture Initiation on MS basal media with 1.0 mg/lt BAP and 0.1mg/lt IAA **B)** Multiple shoot formation in MS basal media with Bap 1mg/lt and IAA 0.1mg/lt, **C)** *In vitro* rooting of elongated shoots in half strength MS basal media with IBA 1mg/lt, **D)** *Ex vitro* Rooted shoots, E) Rooted plants in poly pots for hardening **F)** Hardened plant established in the field.

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