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Quantitative estimation of Embelin from *Embelia tsjeriam-Cottam* A.DC. and *in vitro* multiplication for production of quality planting stock

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Abstract *- The genus Embelia tsjeriam-Cottam A.DC. belongs to the family Myrsinaceae. It is a shrub in nature. Globally the species is recorded from India, Myanmar, and Sri Lanka. It is found in India found in Maharashtra, Karnataka, Kerala, Tamil Nadu, Chhattisgarh, and Andhra Pradesh. Embelin, a benzoquinone, and gallic acid 2, polyphenol compounds are reported in species as antioxidant and anticancer properties. Presently the species is under the rare category. Therefore, it is an urgent need to develop an appropriate propagation technology for its conservation and multiplication. This paper highlights the quantitative estimation of bioactive compound Embelin through HPLC for the identification of quality germplasm. The identified quality planting material was subjected to their in vitro multiplication.*

Germplasm of Borivali forest area, Maharashtra showed optimum 4.88% Embelin. Optimum in vitro morphogenetic response in terms of shoot multiplication was achieved when the MS culture medium was supplemented with BAP 3.0mg/lt + IAA 1.0mg/lt. Average 22 green and healthy shoots with 6.16cm length were induced. The in vitro regenerated shoots were shifted forex vitro rooting. IBA 100ppm solution showed an excellent rooting response. After rooting, the plants were acclimatized and hardened in a greenhouse with more than 74% plant recoveries.

Keywords: *MS, bioactive compound, Embelin, in vitro, nodal, explants*.

INTRODUCTION

Embelia tsjeriam-Cottam A.DC. – *Embelia tsjeriam-Cottam* belongs to the family Myrsinaceae. The synonyms of this species are Embelia basaal, Embelia robusta Malabar. It is a shrub in nature. In India, it is distributed in Maharashtra, Karnataka, Kerala, Tamil Nadu, some parts of Chhattisgarh and Andhra Pradesh (http://envis.frlht.org/index.php/bot_ search) (Fig.1).

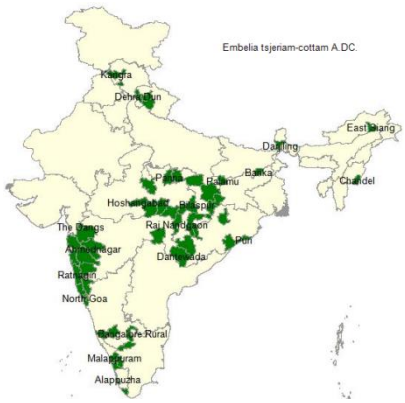

Fig 1 – Distribution of *Embelia tsjeriam-Cottam*

Leaves are generally at the end of branches, ovate, pointed, with entire margins. Flowers are borne in lateral racemes, which are 3 times shorter than the leaves. Flowers are very small, greenish-yellow, with petals expanded and pointed. Berries are round, red when ripe, and sweet. The plant flowers in summer. It is widely used in Ayurveda, Unani and Folk medicine. The plant is highly esteemed in Ayurvedic medicine as a powerful anthelmintic and an important ingredient number of formulations. A decoction is widely used in the treatment of insanity and heart diseases. The fruits are anthelmintic, antispasmodic, and carminative. The finely powdered berries are formulated as an ointment for treating several diseases. The bark of the root is used to treat toothache. In combination with ginger (Zingiber officinale), the leaves are used as a gargle for remedying sore throats [1]. The plant contains Embelin (Fig.2). It is a member of the class of dihydroxy-1,4-benzoquinones that is 2,5-dihydroxy-1,4-benzoquinone, which is substituted by an undecyl group at position 3 and Isolated from *Lysimachia punctata* and *Embelia Ribes*. It exhibits antimicrobial, antineoplastic, and inhibitory activity towards hepatitis C protease. It has a role as a hepatitis C protease inhibitor, an antimicrobial agent, an antineoplastic agent, and a plant metabolite[1].

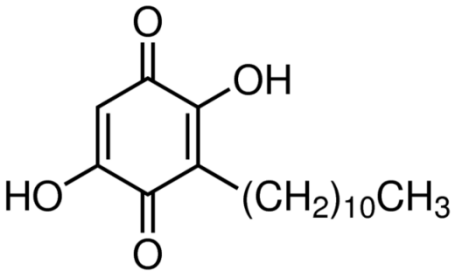

Fig.2 – Embelin

A seed is used as a vermifuge, and decoction of leaves is also used in making a soothing ointment. Due to vast applications in herbal preparation, the natural population is declining day by day, and today the species is reported to be a rare category. Large numbers of medicinal and aromatic plants have been successfully propagated and multiplied through tissue culture techniques [3], [6]. Identifying quality planting material based on morphological traits is very difficult and impractical in medicinal and aromatic plants. Photochemical analysis through chemoprofiling using High-performance liquid chromatography (HPLC) is the best technique for the quantitative determination of bioactive compounds and helps identify the best quality planting stocks [8].

This paper highlights the scientific approach for the quantitative determination of bioactive compound Embelin from *Embelia tsjeriam-Cottam,* a rare medicinal plant. The quality germplasm was screened out based on the ability of % contraction of Embelin. The identified quality planting materials were subjected to their *in vitro* multiplication for the production of their clones.

MATERIALS AND METHODS

I. Collection of wild germplasm

As per the available literature and engine search, the hot spots of the species were identified. The wild germplasm was collected from different parts of India, as mentioned in table 1. The various plant propagules such as leaves, branch cuttings, and whole plant were collected.

TABLE 1

COLLECTION SOURCE OF GERMPLASM OF *EMBELIA TSJERIAM-COTTAM*

S.N.	Source of Germplasm
State- Maharastra	
1.	Borivali forest area
2.	Tekawari, Range- Murbad,
3.	Naneghat, Range- Murbad,
4.	Maljiwara, Thane
5.	Gambhirgarh, Range- Saywan,
6.	Tungreswer, Range- Saywan,
7.	Mahabeleswer, Range- Mahabeleswer
8.	Sudhagarh, Range- Sudhagarh
State - Chhattisgarh	
1.	Dugali, Range- dugli, District- Dhamtari
2.	Jabarra, Range- dugli, District- Dhamtari
3.	Nagari -1,Range- dugli, District- Dhamtari
4.	Nagari -2,Range- dugli,District- Dhamtari
5.	Range- Dantewara, District- Dantewara
State - Karnataka	
1.	Gopalaswami hill
2.	Gundalupeth

II. Maintenance of germplasm:

The collected germplasm was properly maintained in the mist chamber of the institute for further research work.

III. Quantitative determination of Embelin through HPLC for the identification of quality planting stock

The collected wild germplasm was subjected to identifying quality planting stocks from various locations through High-performance liquid chromatography. The seeds of Embelia tsjeriam-Cottam were washed with tap water and dried in the shade for 15-20 days.

The extraction process of *Embelia tsjeriam-Cottam* (seed) –

(i) Drying of collected germplasm – Temperature for drying of collected samples

Different methods of drying were applied to the collected samples after their harvesting. Collected samples were left for drying by spreading on filter paper under sunlight and shade at room temperature to avoid fungal infection. After this, the samples were dried under a hot air oven (Table 2). After drying, the samples were powdered with mortar pestle and filtered with a fine sieve. After this, the samples were ready for the extraction process.

TABLE 2

DRYING TEMPERATURES AND PERIOD OF DRYING *EMBELIA TSJERIAM-COTTAM*

S.N.	Method of drying	Time is taken for drying
1	Spreading material on filter paper and dry at room temperature	20-25 days
2	Drying in the oven at 35^0C	15-20 days
3	Drying in the oven at 45^0C	10-15 days
4	Drying in the oven at 60^0C	3-5 days

a. Chemicals and reagents for HPLC analysis

Solvents used for chromatographic analysis were Acetonitrile and HPLC grade water (E-Merck, India). Hexane, chloroform, 3% HCl solution, 25% ammonia solution, and anhydrous sodium sulfate were used for sample preparation. Samples were filtered millipore filtration unit. Acetonitrile was used as a mobile phase, and ultra sonicator (Flexit, Pune) was used for degassing.

b. Chromatographic Conditions

A Chromatography Instrument Company (CIC, Baroda, India) modular HPLC system was used. CIC software Autochro- WIN ver 2.0 was used to control the unit and analysis of HPLC data. The analysis was performed on a reverse-phase C-18 ODS column. The detector wavelength was 254 nm, and the column temperature was ambient (35^0C).

The extraction process for *Embelia tsjeriam-Cottam*

Soxhlet extraction

2 gm dried samples of the species mentioned above were crushed, and 500ml of various solvent mixtures were taken in the soxhlet apparatus and refluxed for 5-6 hours. Then samples were loaded on Rota-vapour and heated approximately till their boiling point. The remaining concentrated material with some impurities treated with hexane extract was discarded, and the aqueous portion was washed 3-4 times with chloroform through the separatory

funnel. The dark portion was discarded, and the combined aqueous extract was transferred to a conical flask. Anhydrous Sodium Sulphate was added to this extract, then filtered and washed with chloroform. A 20 ml appropriate solvent or solvent mixture was then added and filtered with Millipore for the species. The mobile phase consisting of Acetonitrile: HPLC grade water (10:90) was used [2]. (Table 3).

TABLE 3
LIST OF SOLVENTS AND THEIR MIXTURES TRIED FOR SAMPLE PREPARATION

SN	Solvent and their mixtures
1	Pure Methanol
2	Methanol: Water (80:20)
3	Pure Acetonitrile
4	Acetonitrile: Water (10:90)
5	90% Acetonitrile
6	80% Acetonitrile
7	70% Acetonitrile
8	60% Acetonitrile
9	90% Methanol
10	80% Methanol
11	70% Methanol
12	60% Methanol

c. Sample extraction

The dried plant samples were finely powdered, and 2 gm of dried material with 200 ml of acetonitrile was taken in soxhlet apparatus and were refluxed for 6-7 hours. It was then loaded on rotor-vapor and heated at 80-85^{0}C. The remaining concentrated material was Embelin with some impurities. It was then defatted with hexane 3-4 times to remove fatty acids. The hexane extract was discarded, and the aqueous portion was washed 3-4 times with 3% Hydrochloric acid solution. The solution was filtered, heated in a water bath, and 25% ammonia solution is added. The pH of the solution was adjusted to 7.0. The solution was extracted with chloroform through a separatory funnel 3-4 times. The dark portion was discarded, and the combined aqueous extract was transferred to a conical flask. Anhydrous Sodium sulfate was added to this extract then filtered and washed with chloroform. Dragon Droff's reagent confirmed extracted alkaloids. The sample was ready for HPLC analysis [7].

d. Quantitative determination of Embelin through HPLC

Preparation of standard solution - A standard solution of Embelin (Natural remedy, India) was prepared by dissolving 1 mg in 1 ml of acetonitrile. The 10 µl of Embelin standard solution was injected in10 replicates in HPLC to plot the calibration curve. Similarly, 10 µl of prepared plant samples were also injected in 10 replicates and were calculated to estimate the percent concentration of Embelin with the following formula.

$$\%\text{Concentration} = \frac{\dfrac{\text{Peak area of sample}}{\mu\text{l injection}} \times \text{Wt. of Standard gm/ml}}{\dfrac{\text{Peak area of Stadard}}{\mu\text{l injection}} \times \text{Wt. of sample gm/m}} \times 100$$

IV. Maintenance of identified germplasm under mist conditions

The screened out material through HPLC was properly maintained in the mist chamber to collect young and healthy explants from them.

V. *In vitro* multiplication of *Embelia tsjeriam-Cottam* for the production of quality planting stock

Nodal segments (1.0 to 2.0 cm length) as a source of explants were collected from mist grown plants during morning time in glass bottles under tap water. The collected explants were thoroughly washed with running tap water for 10 minutes to remove the dust particles. The explants were treated with 1% liquid detergent solution Extran (Merck, India) for 2 to 3 minutes. Then the Extran was properly removed from the surface of the explant with the help of double-distilled water. The explants were transferred in a 1% solution of Bavistin (BASF, Mumbai, India), a broad-spectrum fungicide, for 5 to 6 minutes to minimize the explants' microbial load and then washed 3 to 4 time with double distilled water. The explants were transferred in pre-sterilized glass culture bottles under a laminar airflow cabinet and sterilized with surface sterilizing agent Mercuric chloride ($HgCl_2$) (Hi-Media, Mumbai, India) for different time durations. The Mercuric chloride was properly removed from the explant's surface by washing 3 to 4 times with sterilized double-distilled water. The explants' exposed ends were cut with a sterilized scalpel.

Murashige and Skoog [5] culture medium was used for *in vitro* multiplication supplemented with various combinations and plant growth regulators' concentration. The pH of the medium was adjusted to 5.7 and autoclave at 121 ± 2^{0}C for 30 minutes. The prepared explants were inoculated horizontally in a culture test tube (25×150mm) containing culture medium under aseptic conditions in the laminar air flow for fresh cultivation. The cultures were subcultured in culture bottles (500ml) for further multiplication. The cultures were maintained in the culture room at 25±2^0 C, provided cool fluorescent light for 16 hours for photomorphogenesis. Observations were recorded weekly. The *in vitro* regenerated shoots were carefully removed from the bottles, and the extra agar-agar around the shoots was washed properly. The shoots were shifted in poly bags with a mixture of (1:1:1) sand, soil, and FYM and were maintained in a moist chamber for*ex vitro* rooting, acclimatization and hardening. After hardening, the plants were maintained in green net conditions.

RESULTS AND DISCUSSION

A. Quantitative determination of Embelin:

Today chemoprofiling of medicinal plants through HPLC analysis is a widely used technique. High-performance liquid chromatography is the best option for the quantification of secondary metabolites with great precision. It generates peaks for the compound being quantified in correlation to its concentration in each sample, which are finally quantified based on the standard curve generated from known concentrations of the

reference compound. Thus, the germplasm that shows the highest bioactive compound concentration can be referred to as quality planting stock, particularly in medicinally important plant species. Considering these facts, HPLC was performed to quantify Embelin in *Embelia tsjeriam-Cottam* to identify the quality planting stocks.

The compound's identification has been done based on retention time and peak area of standard and samples. The chromatogram of standard Embelin (Fig. 3) and the five samples' chromatogram were generated (Fig. 4, 5, 6, 7, 8).

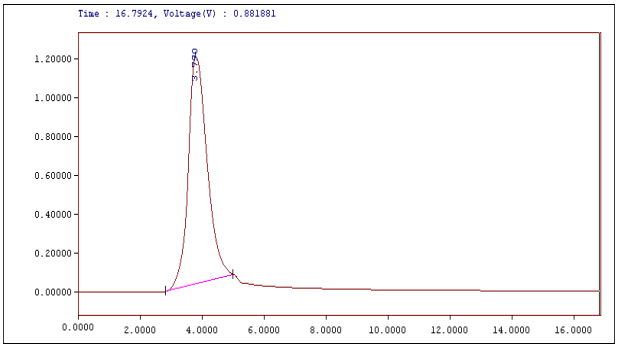

Fig. 3 Estimation of Embelin from *Embelia tsjeriam-cottam*

Standard chromatogram of Embelin

RT(min)	Peak name	Area (Mv*sec)
3.790	standard	49585.680

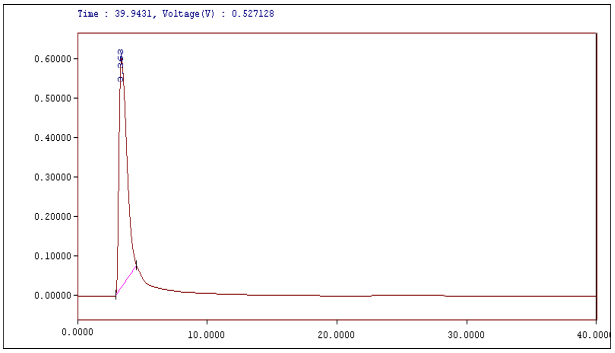

Fig. 4 Chromatogram of sample-1, Borivali forest area, MH

RT(min)	Peak name	Area (Mv*sec)
3.363	sample	24238.844

%concentration = **4.88**

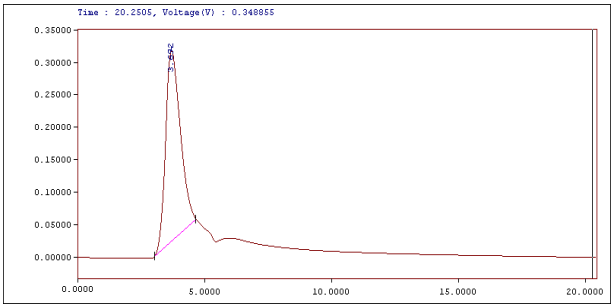

Fig. 5 - Chromatogram of Sample-2, Tekawari range, Murbad (M.H.)

RT(min)	Peak name	Area (Mv*sec)
3.692	sample	11866.343

%concentration = 2.30

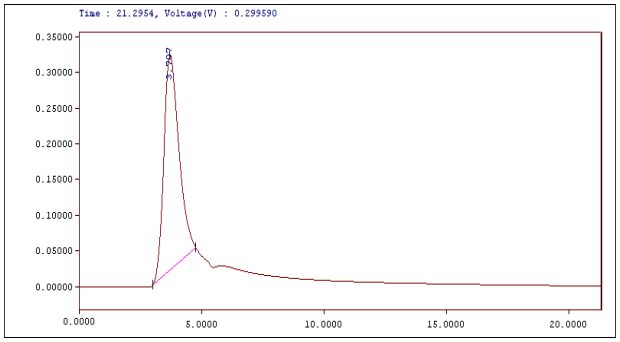

Fig. 6 - Chromatogram of Sample-3, Naneghat range, Murbad (M.H.)

RT(min)	Peak name	Area (Mv*sec)
3.707	sample	12935.913

%concentration = 2.60

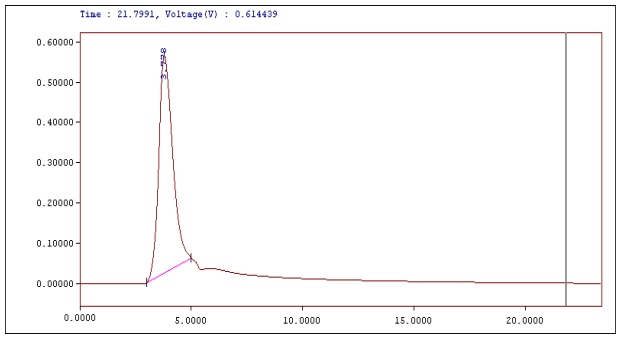

Fig. 7 - Chromatogram of Sample-4, Dugali, Dhamtari (C.G.)

RT(min)	Peak name	Area (Mv*sec)
3.778	sample	23384.992

%concentration = 4.71

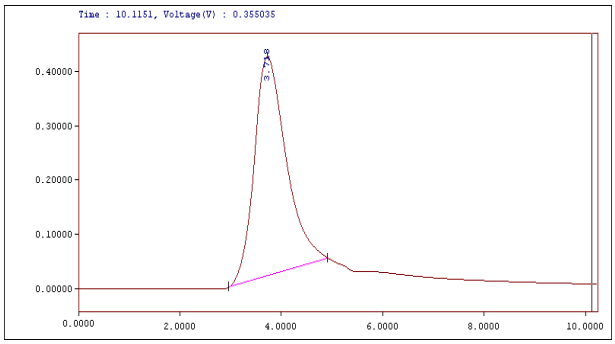

Fig. 8 - Chromatogram of Sample-5, Jabarra range, Dugli, Dhamtari (C.G.)

RT(min)	Peak name	Area (Mv*sec)
3.718	sample	17616.549

%concentration = 3.55

After HPLC analysis, the samples of maximum percent concentration of Embelin was found **in Borivali forest area, Maharashtra (4.88%),** and minimum was

found (2.30%) in Tekarwari, Range- Murbad, Maharashtra, as mentioned in table 4.

TABLE 4
% CONCENTRATION OF EMBELIN FROM DIFFERENT AREAS

S.N	Provenances	% concentration of Embelin
1	Borivali forest area, Maharastra	**4.88**
2	Tekawari, Rang- Murbad, Maharastra	2.30
3	Naneghat, Rang- Murbad, Maharastra	2.60
4	Dugali, Rang- dugli, District- Dhamtari, Chhattisgarh	4.71
5	Jabarra, Rang- dugli, District- Dhamtari, Chhattisgarh	3.55

B. In vitro multiplication through nodal segments of Embelia tsjeriam-Cottam

In vitro mutilation of plant species is one of the best options for large-scale multiplication, especially for those difficult to propagate by conventional propagation methods and recalcitrant or which comes under RET categories. Large numbers of medicinal and aromatic plants have been successfully multiplied by using tissue culture techniques. Keeping the vast potential of tissue culture *in vitro* propagation technique has been standardized for Embelia tsjeriam-cottam through nodal explants from identified sources to produce quality planting stocks.

Several factors were considered in this study: the establishment of aseptic cultures, various combinations and concentrations of plant growth regulators, extensive morphogenetic response in terms of the high rate of multiplication, and establishment of plantlets under *ex vitro* conditions.

It was observed that the treatment time and concentration of $HgCl_2$ plays a vital role in fresh culturing. 100% of contamination-free cultures were established when treated with $HgCl_2$ 0.1% for 2 minutes. The explants were also green and healthy.

Establishment of aseptic cultures:

It is a rare medicinal herbal plant useful in preparing several herbal-based medicines due to its importance and overexploitation, its wild genetic resource depleting day by day. Therefore, it is an urgent need to domesticate and conserve this species by biotechnological interventions. Tissue culture techniques play a vital role in the multiplication and conservation of such rare species. In this project, attempts have been made for the standardization of the micropropagation protocol of this species. The nodal explants from stem branch cuttings were obtained from the mist chamber from March to July. The nodal segments and auxiliary buds were inoculated on MS [4] culture medium with various combinations and concentrations of phytohormones.

In the present study, it was observed that when MS [4] culture media was supplemented alone with BAP or Kn, various concentrations showed no morphogentic response. This may be due to a lack of auxin hormones in culture media.

TABLE 5
EFFECT OF IAA+BAP ON THE MORPHOGENETIC RESPONSE FROM NODAL EXPLANTS OF *EMBELIA TSJERIAM-COTTAM*.

Cytokinin (mg/lit.)	Avg. no. of shoots/ explants	Avg. shoot length (cm)	% Response
Control	Nil	Nil	Nil
IAA+ BAP (0.5+1.0)	Nil	Nil	Nil
IAA+ BAP (0.5+2.0)	Nil	Nil	Nil
IAA+ BAP (1.0+3.0)	**22.02± 0.09**	**6.16± 0.06**	**82**
IAA+ BAP (1.0+4.0)	Nil	Nil	Nil
IAA+ BAP (2.0+5.0)	3.53± 0.10	2.01± 0.09	36

IAA = Indole 3 Acetic acid, BAP = 6-Benzylaminopurine

From the above table, it was observed that when the MS [4] medium was supplemented with a combination of IAA and BAP, the excellent morphogenetic response in terms of shoot induction (figure no. 9 & 10) were recorded after 2 weeks of the culture period. The same medium composition was used for the multiplication of this species. This combination of phytohormones helped to increase the number of shoots as well as their length. On average, 22 green and healthy shoots with 6.16 cm average height were observed after 2-3 weeks of the culture period. (Figure No. 11).

Figure 9 - Fresh culturing of *Embelia tsjeriam-Cottam*

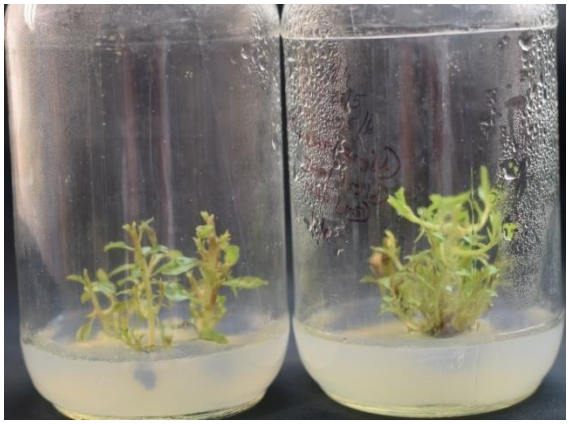

Figure 10 - *Embelia tsjeriam-Cottam* under multiplication stage.

Figure 11 - *Embelia tsjeriam-Cottam* under multiplication stage.

Another combination was also used for the multiplication of *Embelia tsjeriam-Cottam*. It was observed when the MS culture media was supplemented with a combination of IAA and Kn, moderate to poor morphogenetic response in terms of shoots multiplication was recorded as mention in table no. 6.

TABLE 6

EFFECT OF IAA + KN ON THE MORPHOGENETIC RESPONSE FROM NODAL EXPLANTS OF *EMBELIA TSJERIAM-COTTAM* -

Cytokinin (mg/lit.)	Avg. no. of shoots/ explants	Avg. shoot length (cm)	% Response
IAA+ Kn (0.5+1.0)	Nil	Nil	Nil
IAA+ Kn (0.5+2.0)	Nil	Nil	Nil
IAA+ Kn (1.0+3.0)	**3.53± 0.10**	**2.01± 0.09**	**36**
IAA+ Kn (1.0+4.0)	Nil	Nil	Nil
IAA+ Kn (2.0+5.0)	Nil	Nil	Nil

IAA = Indole 3 Acetic acid, Kn = Kinetin

After shoot multiplication, the *in vitro* regenerated shoots were harvested and shifted in a mist chamber under the high humid condition for*ex vitro* rooting. When the in vitro regenerated shoots were treated with IBA 100ppm solution for 10 minutes, it was observed that they showed excellent rooting response within 20 days. After rooting, the plants were shifted to the greenhouse for acclimatization and hardening. More than 74% of plant recoveries were recorded (Fig. 12). Micropropagation studies have been conducted[5] and concluded that an average of 18 shoots was induced from explants.

Figure No. 12 Hardened plants of *Embelia tsjeriam-Cottam*

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REFERENCES

[1] Babu Ganesan, Chemical constituents of Embelia basaal (Roem. & Schult.) DC. Journal of Pharmacy Research 2(10) (2009), 1575-1578.

[2] Basak UC & Mohapatra M, Quantitative reckoning of embelin from fruits of Embelia tsjeriam-Cottam using water bath process as an alternate method of extraction, Indian Journal of Pharmaceutical and Biological Research 3(03) (2015).

[3] Malik Sonia, Sharma Madhu & Ahuja Paramvir Singh, An efficient and economic method for in vitro propagation of Arnebia euchroma using the liquid culture system American Journal of Biotechnology Medical Research DOI: 10.5455/ajbmr., 20160501100040, (2016).

[4] Murashige Toshio & Skoog Folke, A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures, Physiologia Plantarum, 15, (1962).

[5] Rajan Dilna RA, Raghur V, Hrideek TK, & Muralidharan EM, Micropropagation of Embelia tsjeriam-Cottam - an endangered medicinal plant of western ghats, Proceedings of the 8th National Seminar on Medicinal plants. KFRI, Kerala (2013).

[6] Tiwari SK, Krishnamurthy G, Goswami MP, Pandey Amit & Singhal PK, In Vitro Propagation of Litsea glutinosa (Lour) C.B. Robinson An Endangered Medicinal Tree in Madhya Pradesh, India. International Journal of Current Research in Biosciences and Plant Biology, 2(12)(2015), 75-79.

[7] Subramaniam, Sudhakar Raja, Kuzhiumparambil, Unnikrishnan, Ravindran, PN & Balachandran, I. Determination of embelin Embelia Ribes and Embelia tsjeriam-Cottam by HPLC. Indian J. Pharm. Sci,. 67(2005), 734-736.

[8] Tiwari SK, Pandey Amit, Mishra Shweta & Pandey Rashi Quantitative determination of active alkaloid in Withania sominifera through high-performance liquid chromatography the production of quality planting stock. Journal of Tropical Forestry 28(3)(2012), 47-51.