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Regeneration of Plants Through Somatic Embryogenesis in *Emilia zeylanica* C. B. Clarke a Potential Medicinal Herb

[1]*Jayachandran Philip Robinson,* [2] *S. John Britto and* [1] *V. Balakrishnan*

[1]Department of Biotechnology,
K.S. Rangasamy College of Technology, Tiruchengode 637 215, Tamil Nadu, India
[2] The Rapinat Herbarium and Centre fir Molecular Systematics, St. Joseph's College (Autonomous),
Tiruchirappalli - 620002, Tamilnadu, India

Abstract: Tissue culture techniques are useful for *ex situ* conservation of rare, endemic or threatened plant species. This report describes a protocol for somatic embryogenesis of *Emilia zeylanica* (Asteraceae) a rare medicinal plant species, using stem explants. Highest frequency of embryogenic callus formation obtained from stem explants on MS media supplemented with KIN (0.50 mg/l) and 2, 4- D (0.10 mg/l). Fast growing greenish yellow, nodular callus lines containing somatic embryos were established on initiation medium containing KIN (0.50mg/l) and 2,4- D(0.50mg/l). The well developed embryos germinated into complete plantlets on MS medium containing BAP (0.05 mg/l) and ABA (0.10 mg/l). Regenerated plantlets having secondary and tertiary roots were considered to be fully regenerated and were transferred to plastic pots for hardening in a mixture containing Red soil, sand and vermiculate(1:1:1) ratio.

Key words: Callus · Explant · Hardening · Medicinal Plant · Regeneration · Somatic Embryogenesis · Shoot Proliferation · Vermiculate

INTRODUCTION

Genetic diversity is widely recognized as the key component for long term survival of the species. It is the foundation of sustainability because it provides raw material for adoption, evolution and survival of species and individuals, especially under changed environmental and disease conditions [1]. Increasing conversion of natural forests for agriculture and urban development has resulted in the forest fragmentation and declining forest habitat. For forest fragmentation and increased rates of population extinction results in smaller population sizes and increased isolation of populations, particularly among the less common or rare species [2].

There is an increasing concern throughout the world about the uncontrolled exploitation and depletion of the earth's natural resources, especially of those affecting plant biodiversity of tropical forests. The extinction of a species is related to a degree of threat by biotic and a biotic factors. Therefore the need for conservation is exceptionally high and of paramount importance to preserve heritage for posterity of a plant [3]. Current progress in plant genetics and biotechnology is highly adopted upon the use of *in vitro* cultures, hence the establishment of effective *in vitro* plant regeneration systems enabling a rapid production of fertile, genetically 'solid' plants, is of great interest to plant biotechnologists. Among the various *in vitro* systems applied, somatic embryogenesis (SE) is of special value[4]. *In vitro* propagation has the potential for fast multiplication of superior genotypes, allowing the exploitation of maximum genetic gain achieved in the breeding program. When compared to other *in vitro* propagation methods, somatic embryogenesis offers several advantages in improving forest plants including the fact that embryogenic tissue is readily cryopreserved.

Emilia zeylanica (Asteraceae) is a tropical annual herb. *Emilia* a small genus of annual or perennial herb. Seven species occur in India. The genus yields a phyrrolizidine alkaloids an Hepatoprotuctive drug. The plant is used in sudorific, antipyretic, ophthalmic, antiasmatic and it is useful in vitiated conditions of vata, infantile tympanitis, gastropathy, diarrhoea, otalgia and intermittent fevers. The plants were commonly used by

Corresponding Author: Jayachandran Philip Robinson, Department of Biotechnology, K. S. Rangasamy College of Technology, Tiruchengode 637 215, Tamil Nadu, India

the indigenous community of Western Ghats. Owning biotic factors, destruction of habitat, forest fragmentation and prolonged drought, there is fast depletion of its natural population. Regeneration and reestablishment of plants through *in vitro* culture is one of the most effective biological techniques to conserve its diversity. Different species require protocols that have combinations and concentrations of plant growth regulators customized to improve the final yield of normally germinating and functioning embryos.

In, recent years, there has been an increased interest in *in vitro* culture technique which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants [5-7] there has been few reports to date on somatic embryogenesis in the genus using various explants [8-10]. In the present report, a protocol for successful plant regeneration through callus mediated somatic embryogenesis were presented for *Emila zylenica* a rare medicinal herb.

MATERIALS AND METHODS

Plant Material and Disinfection: Stem explants of *Emilia zeylanica* were collected from the shoal forests of Palni hills of Tamil Nadu, India. The excised stem explants were washed with tap water for 30 min. followed by treatment with solutions of 2%(v/v) Teepol (Reckitt Benckinser – India) and 70% (v/v) ethanol for 15s and thereafter washed three to five times with sterilized distilled water. The explants were then surface – disinfected with 0.1 %(w/v) aqueous mercuric chloride solution for 5-6 min. and finally rinsed with sterile water (three to five changes). The stem segments were then trimmed at both ends prior to inoculation on culture media.

Culture Conditions: Single disinfected stem segments were cultured on MS [11] basal medium supplemented with3% (w/v) sucrose (Himedia, Mumbai, India) for culture initiation and served as explant source for subsequent experiments, the p^H of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1N NaoH or 1N Hcl before gelling with 0.8% (w/v) agar (Himedia). In all the experiments the chemicals used were of analytical grade (Himedia, Qualigens, Merkard and Sigma). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa and 121°C for 15 min. the surface disinfected explants were implanted horizontally on the culture medium test tubes (150_X25) containing 50 ml medium and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25+ 2°C under 16 h photoperiod of 45- 50 mol m^{-2} s^{-2} irradiance provided by the cool white fluorescent tubes (Philips, India) and with 55- 60 % relative humidity. All subcultures were done at 3 week intervals.

Initiation, Proliferation and Selection of Embryogenic Callus: Stem segments were placed horizontally on medium comprising MS salts, 100 mg/l myo-inositol, 0.8 g/l agar and 30g/l sucrose and supplemented with 0.05-4.0mg/l BAP, 0.05- 0.2 IAA, 0.05-0.2 IBA, 0.05-0.2 KN and 0.05- 0.2NAA alone and in combination. After 3 weeks of culture, pro- embryonic callus was isolated and sub cultured at weekly intervals on the same medium for another 2 weeks for the induction of embryogenic calli. The embryogenic callus was identified by means of anatomical studies on the basis of its creamy white colour and presence of small less vacuolated and densely filled cytoplasmic cells.

Embryoid Induction, Maturation and Plantlet Regeneration from Somatic Embryos: The embryogenic callus obtained was subcultured on medium containing MS salts, 150mg/l thiamine ABA 0.05-0.2mg/l for accelerating the induction of embryoids, weekly subcultures of embryogenic callus and the removal of dead, dark-brown cells from the callus was carried out. After 4 weeks of subculture, greenish- yellow, globular pro- embryoids were observed and these were selected and subcultured on the same medium for maturation. Matured somatic embryos (heart- and torpedo shaped) were transferred to somatic embryo regeneration medium containing MS salts; 100mg/l myo- inositol, 0.02-0.2mg/l thiamine, 30g/l glucose together with 0.02-0.2mg/l Ga_3 and 0.05-2.0 mg/l BAP were supplemented with alone and in combination. After the complete regeneration of embryoids to plant let(40-60 days), the latter were transferred to plastic pots containing FYM, Red soil and sand in 1:1:2: ratio, for hardening. To maintain the humidity, hardened plants were completely covered with plastic bags, which were progressively removed to aid adoption to normal environmental conditions. The selected plants, then adapted to normal environmental conditions were transferred to earthen pots for further growth and development.

Only in selected regions could proembryogenic calli be identified based on the presence of dense cytoplasm and small cells [12]. The hypocotyl segments responded better than cotyledon explants with respect to

proembryogenic callus induction. While the calli that developed showed variations in colour and texture- including yellowish –green, watery yellow and brownish friable- only the creamy – white calli were pro-embryogenic in nature. The selected pro- embryogenic calli were subcultured on the same medium for a second 2 week period to induce the formation of embryogenic calli and these calli were morphologically similar to those developed [13].

Statistical Analysis: Experiments were set up in a randomized block design and each experiment usually 10 replications and was repeated at least three times. Observations were recorded on the frequency of embryos from the explant and the number of germinating embryos. Means and standard errors were used through out the study and the values were compared using Duncan's multiple range tests according to Gomez and Gomez [14].

RESULTS AND DISCUSSIONS

Induction of Embryogenic Callus: Among the different combinations of growth regulators used only stem explants allowed the proliferation of embryogenic calli. Stem explants developed calli three weeks after inoculation. A highest frequency (35.4%) of embryogenic calli induction was observed on MS medium augumented with 0.50 mg/l KIN in combination with 0.10 mg/l 2,4-D (Tabel-1). The initial primary embryogenic callus was yellowish, nodular and less friable. Individual and concentrations of BAP, KIN and BAP+ 2,4-D induced nodular callus that did not become embryogenic with 4 weeks of culture. Jadhar and Hegale [15] used tender shoots for development of somatic embryos on MS medium supplemented with 4.0 mg/l 2,4-D and 5.0 mg/l KIN.

Development of Somatic Embryos: Embryogenic calli which were separated from the primary culture and subcultured on fresh medium containing MS basal medium supplemented with combinations of BAP(1.50), KIN (0.50) and (0.02-1.00) 2, 4- D. It was reported that in other studies that 2,4-D has been the most commonly used auxin for induction of somatic embryogenesis[16]. The percentage response for embryogenic proliferation increased to 75% in the medium supplemented with KIN(0.50) and 2,4-D(0.10) on medium with 0.50 mg/l KIN and 0.10 mg/l 2,4- D numerous globular and bipolar somatic embryos developed on the surface

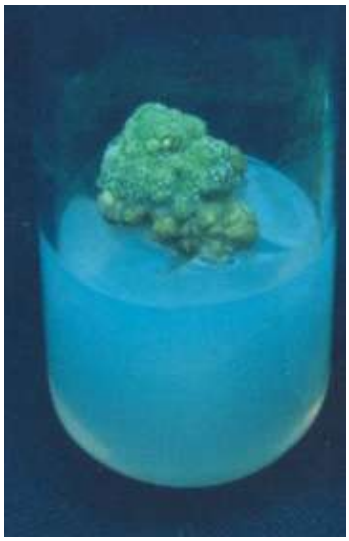

Fig. A: Nodular Callus of *Emilia zylenica* from stem derived callus on MS medium supplemented with KIN 90.50) and 2, 4-D (0.10 Mg/l)

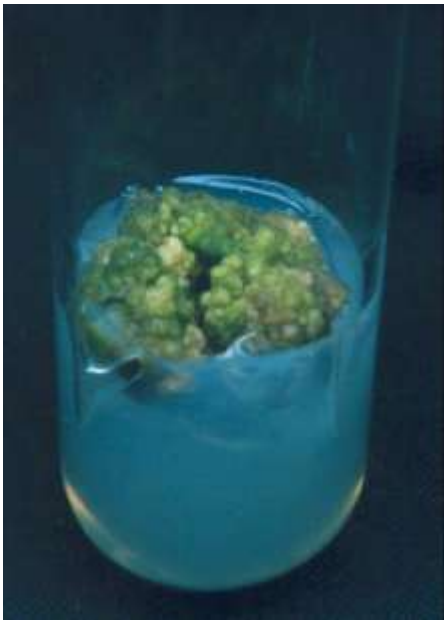

Fig. B: Initiation of somatic embryos from the surface of the callus

of the embryogeneic calli with in 2 weeks of culture repeated secondly. Somatic embryogenesis was achieved by regular subculture. In normal somatic embryogenesis, only part of the callus embryogenic and the non-embryogenic was progressively removed, otherwise if converted the embryogenic calli into non-embryogenic calli.

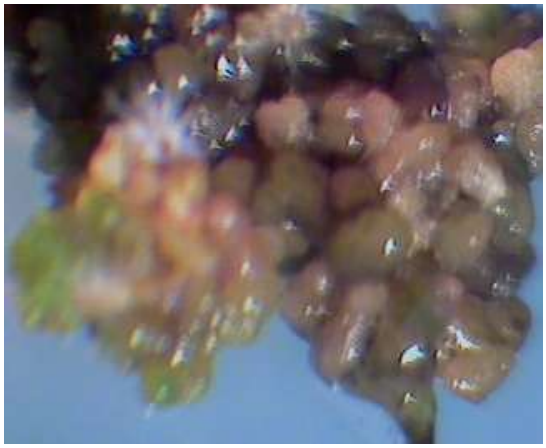

Fig. C: Different stages of somatic embryos on MS medium supplemented with BAP(0.05mg/l) and ABA (0.10mg/l)

Fig. D: Germination of single embryo on liquid medium.

Fig. E: Regenerated plantlet acclimatized in degradable cup

Table 1: Effect of BAP, KIN, 2,4- D and NAA on embryogenic callus production in the stem explants of *Emilia zeylanica* after 3 weeks of culture

Hormone		Total no of explants	Total No. of Explants responded	No. of Callus / explants	Percentage of callusing
BAP					
0.05		50	10e	1.7±1.26	20
0.10		50	16cd	2.7±1.76	32
0.50		50	22a	4.8±2.27	44
1.00		50	18b	2.5±1.28	36
1.50		50	17bc	1.5±1.02	34
KIN					
0.05		50	8	1.6±1.2	16
0.10		50	12	3.3±2.53	24
0.50		50	19	4.5±2.29	38
1.00		50	13	3.8±2.27	26
1.50		50	10	3.2±2.13	20
2,4-D					
0.02		50	20	4.0±1.72	40
0.05		50	29	5.3±2.29	58
0.10		50	21	10.2±4.97	42
0.50		50	15	5.6±4.02	30
1.00		50	10	2.9±2.84	20
BAP	2,4-D				
0.50	0.02	50	13	3.4±1.74	26
0.50	0.05	50	21	10.9±4.00	42
0.50	0.10	50	30	7.6±3.44	60
0.50	0.50	50	19	5.4±3.23	38
0.50	1.00	50	7	3.8±3.21	14
KIN	2,4-D				
0.50	0.02	50	20	18.3±6.18	40
0.50	0.05	50	25	25.1±6.20	50
0.50	0.10	50	42	35.4±4.86	84
0.50	0.50	50	30	19.2±8.07	60
0.50	1.00	50	18	11.4±5.00	36

•Values are mean±SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test (P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the $p<0.05$ level according to Duncan's multiple range test.

The induced embryoids were subcultured for another 3 weeks for complete maturation and the formation of globular heart and torpedo shaped embryos demonstrated the embryo maturation process. The mature somatic embryos were transferred to somatic embryo regeneration medium containing MS salts, B5 Vitamins, 0.2mg/l GA_3 and 1.0 mg/l BA and observed 53% plant

Table 2: Effect of BAP, KIN and 2, 4-D on embryogenic callus production from the stem callus explant

Growth Regulators (mg/l)	Total No. of Explants	No. of explants responded	Mean No. of Embryos per explant	Percentage of Responding cultures
2,4-D				
0.05	250	61 d	4.0±1.72 cd	24.4 d
0.10	250	96 b	5.3±2.79 bc	38.4 b
0.50	250	113 a	10.2±4.97 a	45.2 a
1.00	250	81 bc	5.6±4.02 b	32.4 bc
1.50	250	46 de	2.9±2.84 de	18.4 de
2.00	250	30 ef	0.8±0.97 f	12.0 ef
BAP+2,4-D				
0.50+0.02	250	30 cd	3.4±1.74 de	24 b
0.50+0.05	250	80 a	10.9±4.00 a	32 a
0.50+0.10	250	51 b	7.6±3.44 b	20.4 bc
0.50+0.50	250	40 bc	5.4±3.23 c	16 cd
0.50+1.00	250	19 de	3.8±3.21 cd	7.6 e
0.50+1.50	250	14 ef	1.8±2.35 ef	5.6 ef
KIN+2,4-D				
0.50+0.02	250	97 d	18.3±6.18 cd	38.8 cd
0.50+0.05	250	120 c	25.1 ±6.20 b	48 c
0.50+0.10	250	185 a	35.4±4.86 a	74 a
0.50+0.50	250	146b	19.2±8.07 c	58.4 b
0.50+1.00	250	72 e	11.4±5.00 e	28.8 c
0.50+1.50	250	63 ef	7.8±4.23 ef	25.2 ef

•Values are mean±SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test (P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the p<0.05 level according to Duncan's multiple range test.

Table 3: *Emilia zeylanica*: Effect of growth regulators on germination of directly derived somatic embryos

Growth Regulators (mg/l)	Total no. of Explants	No. of explants responded	Mean No. of Embryos per explants	Percentage of Responding cultures
½ MS	250	6 b	0.9±0.83 b	2.4 b
MS Basal	250	10 a	6.3±2.83 a	184 a
BAP				
0.02	250	18 b	3.3±1.73 b	7 b
0.05	250	80 a	5.4±1.42 a	32 a
0.10	250	7 c	2.7±1.79 bc	2.8 bc
0.50	250	3.5 d	1.1±0.83 d	1.4 cd
1.00	250	2 de	0.6±1.01 de	0.8de
BAP+GA_3				
0.05+0.02	250	18 c	5.2±1.46 bc	7.2 c
0.05+0.05	250	52 b	7.1±2.16 b	20.8 b
0.05+0.10	250	88 a	10.3±4.1 a	35.2 a
0.05+0.50	250	5 cd	2.0±2.23 d	2 cd
0.05+1.00	250	3 de	0.8±1.97 de	1.2 de
BAP+ABA				
0.05+0.02	250	21 de	3.2±1.83 cd	8.4 cd
0.05+0.05	250	104 b	10.2±3.09 b	41.6 b
0.05+0.10	250	167 a	18.0±2.36 a	66.8 a
0.05+0.50	250	58 c	6.5±2.80 bc	23.2 c
0.05+1.00	250	5 f	1.4±1.35 de	2 cd

•Values are mean±SD(n=10) of two independent experiments. Means followed by the same letter in a column are not significantly different as indicated by Duncan's multiple range test (P= 0.05) Mean values within a column having the same alphabet are not statically significant. Means sharing at least one letter are no significantly different at the p<0.05 level according to Duncan's multiple range test.

regeneration from mature somatic embryos after 8 weeks of culture with subculture at 8 day intervals. During somatic embryo germination, some abnormal growth occurred in which there was only shoot or root formation the occurrence of abnormal somatic embryo genesis during somatic embryogenesis reduces the percentage of plant recovered. The present results indicated that ABA promotes maturation and germination of somatic embryos of *Emilia zeylanica* Similar observations have been made in other plant by various authors [17-19].

Regenerated plantlets having secondary and tertiary roots were considered to be fully regenerated and were transferred to plastic pots for hardening in a mixture containing Red soil sand and vermiculate(1:1:1) ratio. The plastic pots were covered with plastic bags that were progressively removed to reach normal atmospheric conditions. The regenerated plants were phenotypically similar, although smaller to the parental lines and in *in vitro* seed derived plantlets. The *in vitro* regenerated plants showed a 100% similar rate after hardening and after 3 weeks, were transferred to earthen pots for the further growth. An increase in the number of somatic embryos and the regenenaration potential of embryoids was also observed.

We also conclude that the protocol for *Emilia zeylanica* somatic embryogenesis described here in reduces the difficulties involved in plant regeneration and will be use ful for medicinal plant germplasm improvement. We suggest the further research can be undertaken to study the effect of this protocol for Agrobacterium mediated transformation studies.

In conclusion, further investigations are necessary enhance the germination of somatic embryos ad subsequent establishment in the green house. This protocol will help in rapid propagation of *Emilia* and other aster members.

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