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High frequency somatic embryogenesis from leaf tissue of *Emblica officinalis* Gaertn. - A high valued tree for non-timber forest products

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Abstract

A simple and one step reproducible protocol was developed for induction of high frequency somatic embryogenesis from *in vitro* derived juvenile leaf tissues of *Emblica officinalis.* Murashige and Skoog (MS) medium supplemented with twelve combinations of auxins and cytokinins involving 2, 4 – dichlorophenoxy acetic acid (2, 4 - D), indole 3- acetic acid (IAA), 6 – benzylaminopurine (BAP) and kinetin (KIN) were tested at various concentrations. Highest percentage of callus (67.5%) was obtained on media containing 0.45 µM 2, 4- D in combination with 22 µM BAP. Three distinct callus types were induced in various combinations of media within 60 days of culture. Maintenance of proliferating callus on their respective media led to high frequency somatic embryogenesis. Of the three distinct types of callus, type III and type II callus recorded 33.8% and 8.3% of somatic embryogenesis respectively. Somatic embryos were matured within two weeks when treated with 3.78 µM ABA. Matured embryos were transferred to MS medium fortified with 0.46 µM KIN for germinating into healthy plantlets. About 90% plantlets transferred to *ex vitro* condition were acclimatized for field establishment.

Keywords: *Emblica officinalis*; somatic embryogenesis; plant regeneration; genetic improvement.
Abbreviations: NTFP_non-timber forest products; MS_Murashige and Skoog; 2, 4-D_2, 4-dichlorophenoxyacetic acid; IAA_indole-3-acetic acid; BAP_6-benzylaminopurine; KIN_kinetin; ABA_abscisic acid.

Introduction

Emblica officinalis Gaertn is a moderate sized deciduous tree, belonging to *Phyllanthaceae*. Distribution of *E. officinalis* covers India, Pakistan, Bangladesh, Sri Lanka, Malaya, China and Mascarene Island (Pathak et al., 1989). In Indian system of herbal medicines, fruit of *E. officinalis* is one of the most important ingredients (Baliga and Dsouza, 2011) for its disease preventive, curative and health restorative properties (Jose and Kuttan, 2000; Ojha et al., 1975). It was reported to have anti-inflammatory (Asmawi et al., 1993), antiviral (El-Mekkawy et al., 1995), pancreas protective (Thorat et al., 1995) and hepatoprotective properties (Bhattacharya et al., 2000), Hence, cultivation of *E. officinalis* is promoted in both traditional and non-traditional areas in Indian Sub-continents to meet the ever increasing demand for the fruit in preparation of a variety of herbal medicines (Pathak, 2003). In India, cultivation of *E. officinalis* is adversely affected by major diseases such as leaf rust (*Ravenelia emblicae* Styd), wilt (*Fusarium* sp.), anthracnose (*Glomerella cingulata* Stoenm), soft rot (*Phomopsis phyllanthi* Punith.) and pest (*Betousa stylophora* Swinhoe), resulting in significant level of crop loss. Unfortunately, conventional breeding is not recommended for genetic improvement of *E. officinalis* due to various factors such as very tiny flower size, variations in chromosome number and complex taxonomy in addition to perennial nature (Pathak, 2003). Thus, genetic improvement of Indian Goosberry is confined only to selection of promising genotypes from seedling progenies. Introduction of novel agronomic traits into *E. officinalis* through genetic transformation is possible only if reliable protocol for regeneration is developed. Though efforts on micro-propagation of this species through adventitious shoot multiplication and somatic embryogenesis were reported (Tyagi and Govil, 1999; Verma and Kant, 1999; Sebastian et al., 2005; Menka and Usha, 2006; Nayak et al., 2010; Madhuri et al., 2011), no viable protocol is available for the mass propagation of *E. officinalis* (Al-Sabah et al., 2012). More recently, various tissues of *E. officinalis* were tested for somatic embryogenesis and leaf tissues were reported to be not suitable for somatic embryogenesis (Al – Sabah et al., 2012). In India, Narendra Aonla (NA – 7) is a popular variety of *E. officinalis*, characterized with tall, semi-drooping branches with dense foliage. It yields medium size fruits with the highest vitamin content of 500 mg/100g (Pathak, 2003). This variety is relatively tolerant to fruit necrosis, suitable for preservation and processing of various food products. This variety is highly promising for cultivation under drought and alkaline soil throughout India. Thus, development of viable protocol for mass propagation through somatic embryogenesis from leaf tissue of this variety becomes an important objective of this study. The utility of the present

Table 1. Effect of auxins and cytokinins on induction of different types of callus of *Emblica officinalis*.

Media (µM)				Type of callus	Callusing (%)	Callus (g)		Embryogenesis (%)	Frequency of embryos per callus
2, 4-D	IAA	BAP	KIN			FW	DW		
--	--	--	--	--	00.00	--	--	NR	NR
0.45	--	0.44		II	30.54^{h}	1.20^{e}	0.019^{cd}	NR	NR
0.45	--	4.40		II	35.10^{f}	1.50^{d}	0.025^{bcd}	8.30	LF
0.45	--	11.0		III	46.72^{d}	2.12^{c}	0.035^{abc}	33.80	HF
0.45	--	22.0		III	67.50^{a}	2.50^{a}	0.050^{a}	NR	NR
--	0.57	0.44		I	21.62^{i}	0.88^{g}	0.010^{d}	NR	NR
--	0.57	4.40		I	18.45^{j}	0.90^{g}	0.020^{cd}	NR	NR
--	0.57	11.0		II	11.87^{k}	0.90^{g}	0.017^{cd}	NR	NR
--	0.57	22.0		III	53.75^{c}	2.35^{b}	0.051^{a}	NR	NR
0.45	--	--	0.46	I	32.12^{g}	0.83^{g}	0.010^{d}	NR	NR
0.45	--	--	4.60	II	30.50^{h}	1.10^{f}	0.017^{cd}	NR	NR
0.45	--	--	11.5	II	41.87^{e}	1.20^{e}	0.030^{bc}	NR	NR
0.45	--	--	23.0	III	57.45^{b}	2.42^{b}	0.040^{bc}	24.30	LF

Mean values within each column followed by same letter in superscript are not significantly different (P< 0.05, by one way ANOVA and Duncan's new multiple range test). Data collected after 8 weeks of culture. LF- Low Frequency, HF- High Frequency, FW- Fresh Weight, DW- Dry Weight, NR – No Response.

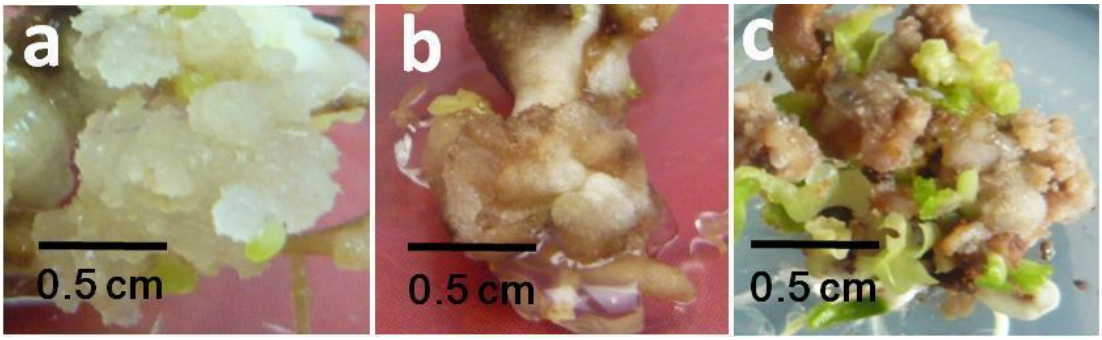

Fig 1. Characteristic features of three different types of callus induced from leaf tissue of *Emblica officinalis*: (a) Whitish, watery non-embryogenic calli. (b) Brownish, compact callus with very slow proliferation, having potency for low frequency embryogenesis. (c) Light brown, high proliferative, friable callus having potency for high frequency somatic embryogenesis.

findings and its possible application in large scale propagation of this tree for conservation and genetic improvement is discussed.

Results

Callus induction

Response of leaf explants on callus induction was varied in various compositions of media (Table 1). Of the twelve different compositions of media, 0.45 µM 2, 4 - D in combination with 22 µM BAP was superior over remaining compositions as it induced the highest percentage of callus (67.5%) followed by 0.45 µM 2, 4 - D and 23 µM KIN with 57.5%. Low concentration of 0.57 µM IAA in combination with 22 µM BAP induced 54.5% callus. The remaining combinations induced callus in varying percentages ranging from 12 to 47%. Combination of 0.45 µM 2, 4 D and 22 µM BAP which induced the highest percentage of callus produced significant quantity of callus (2.5 g), whereas in other combinations, quantity of callus was significantly reduced. Leaf tissues cultured on various compositions of media produced three distinct types of callus based on their colour and rate of proliferation. Type I callus was friable, watery, colourless, highly proliferating and often produced lesser quantity of callus due to slow proliferation (Fig. 1a). Type II callus was distinguishable with brownish and harder callus (Fig. 1b). Type III callus was friable, highly proliferating and found to possess high morphogenic potency for embryogenesis than other two types of callus (Fig. 1c). Of the two auxins tested, 2, 4-D was more effective than IAA for efficient callus induction. Among three types of callus, type II and type III callus were observed in many combinations of media while type I callus was noticed only in three of the combinations. Low concentrations of 0.45 µM 2,4-D either in combination with BAP (0.44 - 4.4 µM) or with KIN (4.6 - 11.5 µM) induced type II callus, while similar concentration of 2,4-D with higher levels of BAP (11 - 22 µM) and 23 µM KIN induced type III callus.

Somatic embryogenesis

Among the twelve combinations, media supplemented with 0.45 µM 2,4 - D and 11 µM BAP was found optimum for inducing the highest frequency embryogenesis (33.80%). Type I callus which produced callus at slow rate did not respond for somatic embryogenesis and remained as non-embryogenic callus after 160 days of culture. However, type II and type III callus responded for low and high frequency somatic embryogenesis respectively (Fig. 2). A large number of somatic embryos were simultaneously emerged from the embryogenic callus of type III (Fig. 3a-d). Cross section of embryogenic callus along with somatic embryos of various developmental stages revealed that most of the embryos were regenerated as cup- like somatic embryos from peripheral region of the callus (Fig. 3e). Somatic embryos were often detached from the callus upon prolonged culture. Type II embryogenic callus was easily distinguishable based on the

Table 2. Characterization of Somatic embryos obtained through Low and High frequency somatic embryogenesis of *Emblica officinalis*

Characteristics	Type I	Type II	Type III
Embryogenic potency	Non-embryogenic	Embryogenic with low frequency	Embryogenic with high frequency
Time required for embryogenesis	--	6-7 weeks	9-10 weeks
Percentage of cultures turned embryogenesis	--	8.30%	33.80%
No. of somatic embryos/ culture	--	Low frequency 15 ± 2.32	High requency 120.28 ± 15.15
Size of somatic embryos (SEs)	--	Smaller (1-2 mm)	Larger (2-4 mm)
Length of SE's (mm)	--	2.12 ± 0.22	4.2 ± 0.24
Rate of germination	--	Faster	Slower
Leaf length	--	Larger 8.82 ± 0.42	Smaller and tiny 3.4 ± 0.32
Leaf width		2.82 ± 0.12	1.92 ± 0.08
Root length	--	Roots are thicker with lateral roots 30.18 ± 2.24 mm	Roots are tiny, highly tapering with many lateral roots 14.32 ± 2.34 mm
No. of lateral roots	--	10 ± 1.32	08 ± 1.22
Time taken for acclimatization	--	55 – 60 days	60 – 80 days

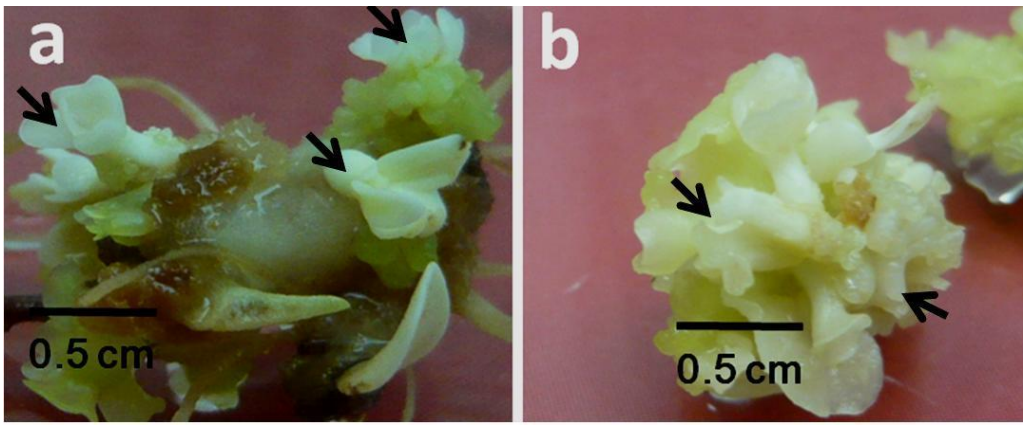

Fig 2. Development of low and high frequency somatic embryogenesis from type II and type III callus respectively from leaf derived callus of *Emblica officinalis.* Arrows indicate emerging of somatic embryos.

time taken for embryogenesis. For instance, type II callus responded for low frequency somatic embryogenesis within 6-7 weeks of culture while type III callus took longer time (9-10 weeks) to respond. However, high frequency somatic embryogenesis was obtained in most of the type III cultures. Type II callus recorded low percentage of embryogenesis (8.3%) while type III callus recorded the highest percentage of embryogenesis (33.8%). Somatic embryos regenerated from type II callus were larger whereas type III callus produced smaller somatic embryos. Variation was observed on germination of somatic embryos derived from type I and II callus. Low frequency somatic embryogenesis obtained from type II callus germinated faster. In contrast, slow germination was noticed among embryos regenerated from type III callus. In general, somatic embryos obtained from type II callus produced larger leaves as compared to leaves produced by the somatic embryos of type III callus. Somatic embryos of type II callus had produced thicker roots with many lateral roots whereas somatic embryos of type III callus produced tiny tapering roots with many lateral roots (Table 2).

Maturation and germination of embryos

Of the various concentrations of ABA, somatic embryos cultured on medium containing 3.78 µM ABA induced minimal germination (5%). At this concentration, enlargement of somatic embryos was observed. In this experiment, 3.78 µM ABA was found to be the optimum concentration for maturation of somatic embryos (Fig. 3f). Lower concentrations of ABA (0.94 - 1.89 µM) were not suitable for inducing uniform maturation as somatic embryos germinated were at different developmental stages (Fig. 4). Somatic embryos cultured at higher concentrations of ABA (9.45 and 18.9 µM) often turned brown. Culture of somatic embryos on MS medium supplemented with 3.78 µM ABA for 45 days was found to be appropriate for inducing

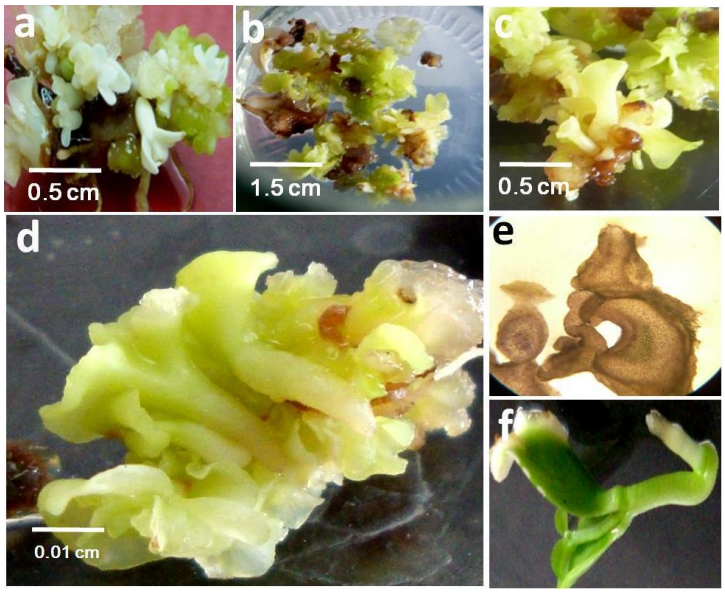

Fig 3. Developmental stages of high frequency somatic embryogenesis from leaf tissues of *Emblica officinalis*: (a) Type III callus showing high frequency somatic embryogenesis. (b) Multiplication of somatic embryos of different developmental stages. (c-d) Mature green somatic embryos detaching from the original callus. (e) Cross section of somatic embryos showing cup-like appearance from the superficial part of callus. (f) Somatic embryos showing slow growth on MS medium supplemented with 3.78 µM ABA.

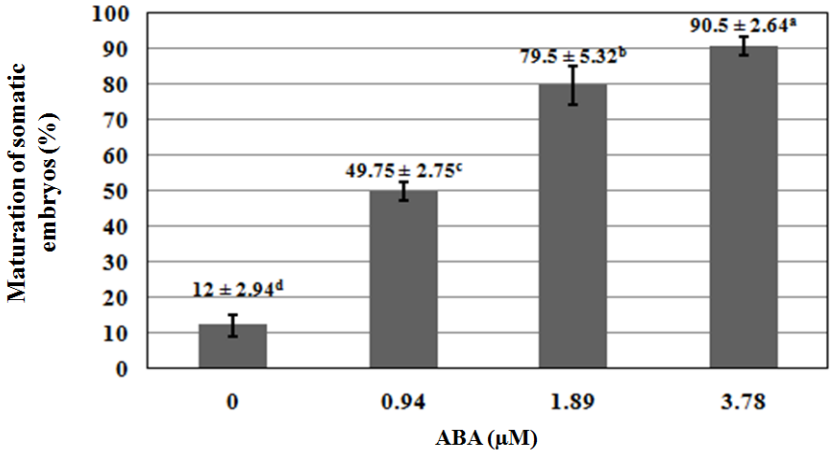

Fig 4. Effect of different concentrations of ABA on germination of somatic embryos: Increase in concentrations of ABA from 0.94 - 18.9 µM sharply decrease the germination by inducing dormancy and highest concentration of ABA at 9.45 and 18.9 µM cause browning of embryos. Duncan's new multiple range test (DMRT) was performed to separate the mean values for determining the significance among the treatments at $p \leq 0.05$.

maturation of somatic embryos (Fig. 5). Higher percentage of germination with longer shoot and root was obtained when mature somatic embryos were germinated on medium containing 0.46 µM KIN (Fig. 6a, b). Somatic embryos developed from type II callus produced thicker shoots and roots. But, high frequency somatic embryos from type III callus produced linear shoots till they attain complete plantlets (Fig. 7a - d). Though type II callus responded for low frequency somatic embryogenesis, conversion of somatic embryos into plantlet was higher with 80% whereas only 33% of high frequency somatic embryos obtained from type III callus could be converted into complete plantlets. About 78% of hardened plantlets were survived (Fig. 7e).

Discussion

Leaf tissues of selected cultivar of *E. officinalis* were responded well with high frequency somatic embryogenesis. A few number of protocols for *in vitro* regeneration of *E. officinalis* from various explants such as endosperm (Sehgal and Khurana, 1985), nodal explants (Goyal and Bhadauria, 2008; Patidar et al., 2010), epicotyls (Nayak et al., 2010) and hypocotyls (Hai-Tao et al., 2006) were already reported. Previously, low frequency embryogenesis was reported in this species (Sebastian et al., 2005; Menka and Usha, 2006) but reproducibility of such protocols was not mentioned. Low frequency embryoid like structure was developed from endosperm tissues of *E. officinalis* in about 16 weeks by employing various combinations of IAA, NAA, KIN, BAP (Sehgal and Khurana, 1985). But, the present protocol required only six weeks for embryogenesis. In our study, a single combination of media containing 0.45 µM 2, 4- D and 11 µM BAP could generate complete plantlets as somatic embryos were germinated without involving rooting hormones unlike the previous work (Sehgal and Khurana, 1985). Critical analysis of embryogenesis revealed that

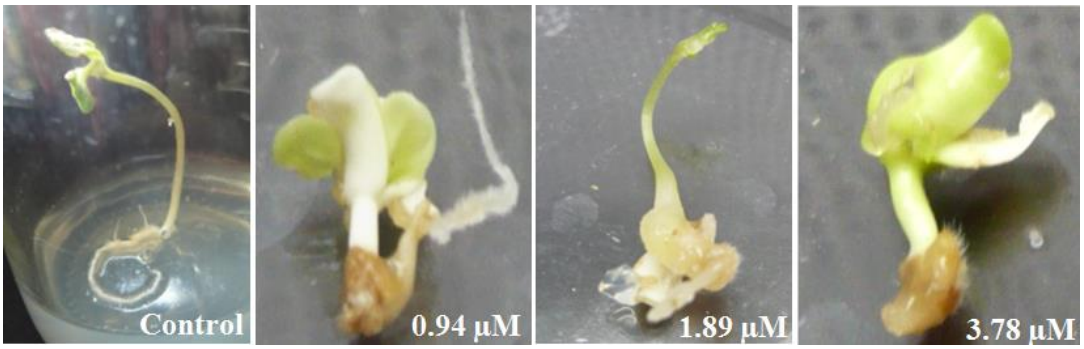

Fig 5. Behaviour of somatic embryos at various concentrations of ABA (0 - 3.78 µM). Increase in concentration of ABA from 0 - 1.89 µM reduce the germination by induction of dormancy/maturation. At 3.78 µM ABA, complete dormancy/maturation was induced with minimal growth as evidenced by enlargement of hypocotyl with cotyledonary leaves.

presence of 2, 4 - D and BAP limits the development of type I callus which is non-embryogenic. Thus, presence of 2,4-D and BAP play a crucial role in embryogenesis than the other combinations. The role of 2,4-D in inducing somatic embryogenesis was emphasized by many workers (Feher et al., 2003; Jimenez, 2005; Moon et al., 2005). In the present study, 3.78 µM ABA induced maturation of somatic embryos but with less germination as ABA inhibits early embryo germination and prevents embryogenesis (Hackman and von Arnold, 1988). Though type III callus shown maximum embryogenic potency (33.8%) with highest frequency of somatic embryos/callus (120.28), germination of somatic embryos were slower than the embryos regenerated from type II callus. However, induction of type III is more appropriate for large scale propagation if the germination of somatic embryos could be improved. Time taken for acclimatization plantlets regenerated from type III callus was 60 – 80 days. In several plant species, explants from field grown plants were used for cloning true-to-type. However, high rate of microbial contamination coupled with lack of morphogenic potency often hinders the embryogenesis. Contrarily, juvenile explants from seedlings are amenable for regeneration due to their high morphogenetic potential. Therefore, a large number of works on genetic transformation was reported utilizing the explants from juvenile plants as reviewed by Cesar and Lorenzo, (2005). Non - availability of efficient regeneration protocol hinders the application of transgenic technology for genetic improvement (Chandra and Pental, 2003). Since embryogenic system is prerequisite for developing transgenic plants (Christou, 1997), the present protocol can be effectively used for genetic improvement of *E. officinalis* through transgenic approaches. Cultivation of Indian Goosberry in India is seriously affected by high incidence of leaf rust disease (*Ravenelia emblicae* Styd.) and shoot gall maker (*Betousa stylophora* Swinhoe) with crop loss of 40% (Lal et al., 1996; Haseeb et al., 1990, 2000). Unfortunately, tolerant cultivars are not available from natural selections. The present protocol (Fig. 8) is expected to be useful for development of tolerant transgenic plants of Indian Gooseberries against major diseases and pests.

Materials and methods

Plant material

Seeds of high yielding variety, popularly known as Narendra Aonla 7 (NA-7) were obtained from Tamil Nadu State Horticulture Department, Government of Tamil Nadu, India for the isolation of zygotic embryos.

Media and culture condition

The basic culture medium used for germination of zygotic embryos, callus induction and somatic embryogenesis includes MS (Murashige and Skoog, 1962) medium supplemented with sucrose (3%). Various plant growth regulators such as 2,4-D, IAA, BAP and KIN (Hi-Media, Mumbai, India) were used in various concentrations either alone or in combination with each other depending upon the experimental design. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-Media, Mumbai India). Molten medium was dispersed into petri dishes, culture tubes or Erlenmeyer flasks (Borosil, Chennai, India) based on experiment. The culture flasks/tubes were capped with either aluminium foil or cotton plugs prior to sterilization at 121^{o}C for 20 min. All the cultures were maintained at 25 ± 1^{o}C under 16 h photo period provided by cool white fluorescent tubes (Phillips, Mumbai, India) with a light intensity of 40 µmol m^{-2} s^{-1}. The number of explants in each treatment was 60 and the duration of experiment ranged from 30-90 days depending upon the experiment.

Development of plantlets from zygotic embryos

Mature seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) for 15 min followed by rinsing with sterile distilled water. Seeds were blotted on sterile filter paper (Hi-Media, Mumbai, India) and zygotic embryos were dissected carefully. MS basal medium supplemented with 3.78 µM ABA was used for the maturation of zygotic embryos for 30 days based on a previously reported protocol (Mercy et al., 2010). Subsequently, matured zygotic embryos were germinated on MS medium supplemented with 0.46 µM KIN for 60 days. Fully expanded leaves from aseptic seedlings were used for induction of callus and embryogenesis.

Callus induction and embryogenesis

Leaf tissues of aseptically grown seedlings were split vertically into three fourth of length. These tissues were inoculated by placing the adaxial surface on media. MS media with 12 combinations of auxins and cytokinins involving 2, 4 - D, IAA, BAP and KIN (Table 1) were used for the present study. The culture was maintained for 90 days with the sub culture after 60 days of primary culture. Data on fresh and dry weight of callus, type of callus, somatic embryogenesis and characteristics of somatic embryos were recorded. Callus along with somatic embryos were sectioned longitudinally and stained with acetocarmine (Qualikems,

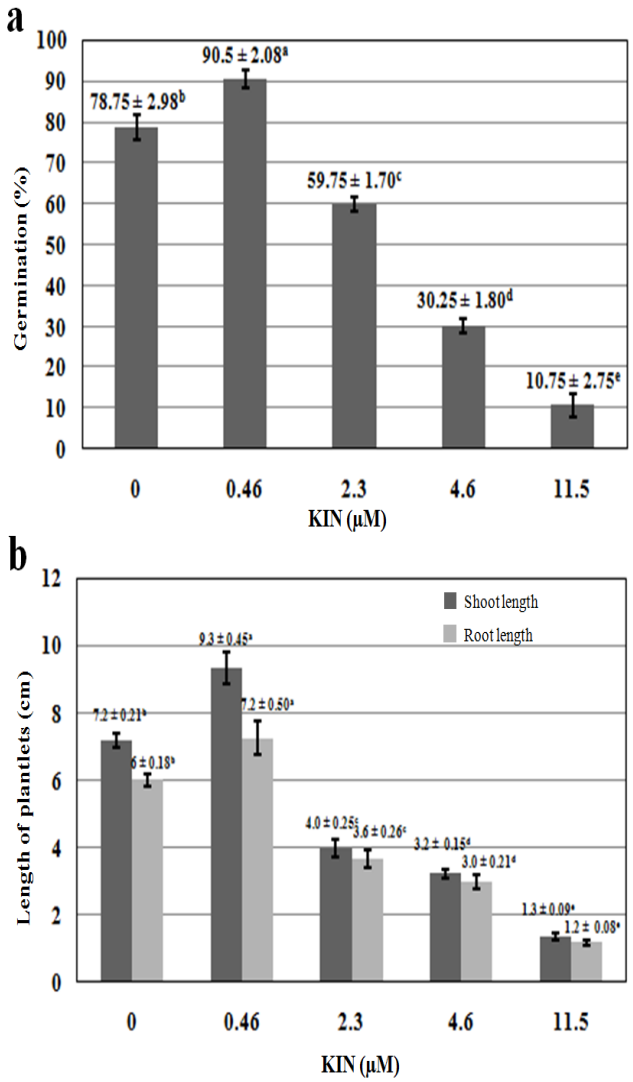

Fig 6. Effect of various concentrations of KIN on germination of somatic embryos. **(**a) Highest percentage of germination as recorded at 0.46 µM KIN and further increase in level of KIN reduce the percentage of germination. (b) Effect of various concentrations of KIN on shoot and root development of somatic embryos. Highest shoot and root length was recorded at 0.46 µM KIN. Increase in concentration of KIN from 2.3 – 11.5 µM significantly reduce the shoot length and root. Duncan's new multiple range test (DMRT) was performed to separate the mean values for determining the significance among the treatments at $p \leq 0.05$.

New Delhi, India) to observe the origin of regeneration of somatic embryos. Sections observed under microscope (Carl Zeiss, Bangalore, India) was documented using digital camera (Sony, Kyoto, Japan) attached with the microscope.

Conversion of somatic embryos into plantlets

Immature somatic embryos were treated with various concentrations of ABA ranging from 0.94 - 18.9 µM to achieve maturation. Somatic embryos cultured on these media were maintained for 30 days. Optimum level of ABA for induction of dormancy was determined on the basis of minimal germination with enlargement of somatic embryos. In another experiment, various concentrations of KIN ranging from 0.46 – 11.5 µM were tested for determining the effective concentration of KIN for germination of somatic embryos. Various parameters such as percentage of germination shoot and root length of plantlets were recorded. Plantlets with 3-4 pairs of leaves were transferred to substrate containing soil, sand and farm yard manure (6:2:1) for acclimatization under polyhouse until they attain sufficient growth for field establishment.

Statistical analysis

For callus induction and somatic embryogenesis, each treatment was consisted of 60 explants with 3 replicates. Data on callus induction was analyzed using one way Analysis of variance (ANOVA). Duncan's new multiple range test (DMRT) was performed to separate the mean values for determining the significance among the treatments at $p \leq 0.05$. All statistical analysis was performed by using SPSS V.16 (Statistical Package for Social Sciences).

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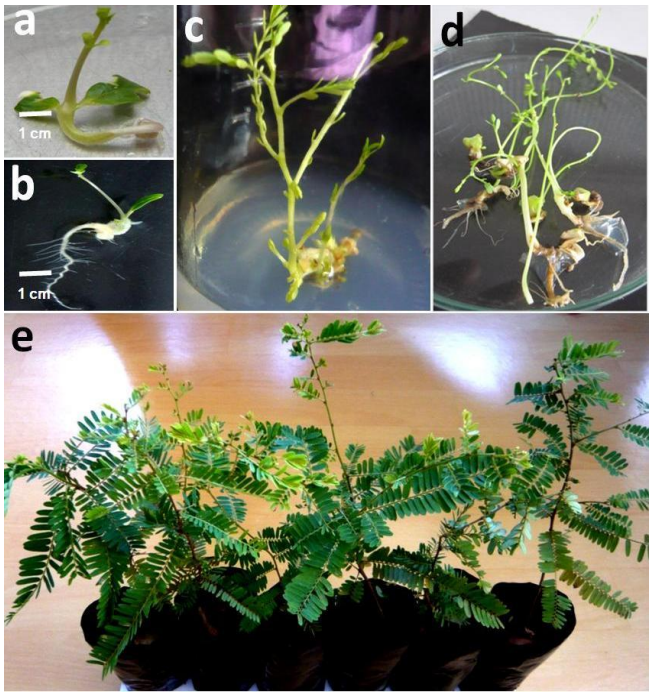

Fig 7. Germination of somatic embryos and development of plantlets: (a - b) Germination of low and high frequency somatic embryo respectively on MS medium supplemented with 0.46 µM KIN. (c) Development of healthy plantlet from low frequency somatic embryos in germination medium containing 0.46 µM KIN. (d**)** Lean and lanky weaker plantlet obtained from high frequency somatic embryos. (e) *Ex vitro* acclimatized plantlets suitable for field planting.

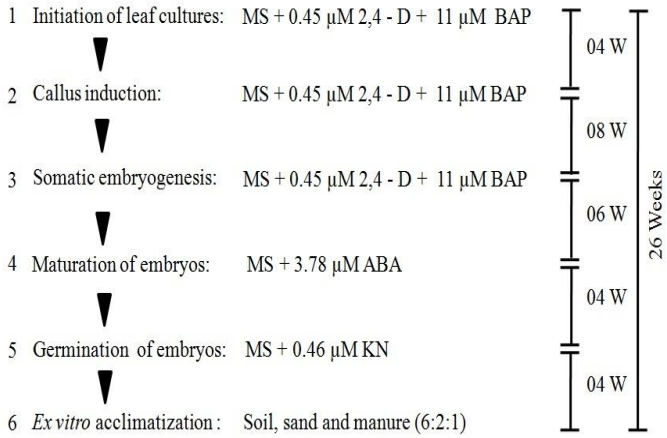

Fig 8. Schematic figure showing sequential steps with corresponding culture conditions and duration of culture for development of complete plantlets from leaf tissues on *Emblica officinalis* through somatic embryogenesis.

author for her Ph. D Degree under the project 'Collection, conservation and molecular characterization of wild and hybrid derivatives of amla (*Phyllanthus emblica*) germplasm in Tirunelveli District, Tamilnadu (No. 33-244/2007 (SR) dated 24 December 2007), sponsored by University Grants Commission, Govt of India, New Delhi.

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