Journal of Genetic Engineering and Biotechnology 16 (2018) 587–598

HOSTED BY

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

Original Article

Plant regeneration, developmental pattern and genetic fidelity of somatic embryogenesis derived *Musa* spp.

Natarajan Nandhakumar [a], Krish Kumar [b], Duraialagaraja Sudhakar [b], K. Soorianathasundaram [a,*]

[a] Department of Fruit Crops, Faculty of Horticulture, Tamil Nadu Agricultural University, Coimbatore, India
[b] Department of Plant Biotechnology, Centre for Plant Biotechnology and Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India

ARTICLE INFO

Article history:
Received 22 January 2018
Received in revised form 29 August 2018
Accepted 1 October 2018
Available online 10 December 2018

Keywords:
Banana
Immature male flower bud
Somatic embryogenesis
Genetic fidelity

ABSTRACT

Multiplication of banana cvs. Grand Naine (*Musa* AAA, Cavendish-sub group) and Rasthali (*Musa* AAB, Silk-sub group) were attempted through somatic embryogenesis. The influence of position of male flower buds, amino acid supplements in the induction of somatic embryogenesis and field performance of embryogenic cell suspension (ECS) derived banana plants were studied. Differentiated immature male flower buds positioned at 6–8 th bract whorl as explants showed better callus induction and somatic embryogenesis. Supplementation with glutamine at 400 mg L^{-1} along with 20:20 g L^{-1}sucrose: maltose in maturation media induced a 10-fold increase in somatic embryo formation compared to control. Cotyledonary stage somatic embryos desiccated for 2 h showed higher germination compared to non-desiccated embryos. The plantlets generated were hardened, and the genetic fidelity of the plantlets was confirmed using ISSR marker. To check the field performance of ECS derived plants, plantlets were hardened and planted in the field along with meristem and sucker. During the field growth, these ECS derived plants were morphologically similar to those of control plants. In this experiment, it was observed that ECS derived banana plants displayed normal phenotype as that of plants grown from meristem and sucker. The protocol developed could be useful highly for large-scale micropropagation or genetic manipulation studies in these commercially important banana cultivars.

1. Introduction

Banana is one of the oldest fruits known to mankind and also an important food for man. The global production of bananas was estimated at nearly 148 million tons in 2016 [1]. Among the dessert bananas grown in the tropical regions of India and in Tamil Nadu, the varieties Grand Naine (*Musa* AAA, Cavendish-sub group) and Rasthali (*Musa* AAB, Silk-sub group) are very popular. Nearly 50 percent of the cultivated area in Tamil Nadu is occupied by Grand Naine and Rasthali. However, both are highly susceptible to the wilt caused by *Fusarium oxysporum* f. sp. *cubense* [2] and Banana Bunchy Top Virus [3]. Propagation through conventional planting materials in banana takes longer time periods due to a low number of suckers which could be also a potential source of dissemination of fungal pathogens, nematodes, weevils, and viruses [4]. Alternatively, rapid production of healthy planting material of desired clones within a short time period can be facilitated by large-scale micropropagation through tissue culture using shoot tips. Even though several such reports in a banana are available, commercial micropropagation of AAB clones *in vitro* is limited due to poor multiplication rates as compared to AAA clones.

Development of *in vitro* plant regeneration protocols is a prerequisite for imparting virus resistance as the manipulation is being carried out under laboratory conditions. Plant propagation by tissue culture using shoot-tip meristems has already been standardized in many cultivars of banana [5–7]. Standardization of somatic embryogenesis protocols is considered much more advantageous for genetic manipulations as the plants derived through somatic embryos are non-chimeric and if the expression of the gene function is elicited in the somatic embryos then they can be rapidly propagated [8]. Different types of explants such as shoot tip, zygotic embryos, proliferating meristems, scalps, female flowers and male flowers have been tried by many workers to develop plants from embryogenic cell suspension (ECS) [9,10,5,11,12]. Of these explants, immature male flowers (IMF) appeared to be the

Peer review under responsibility of National Research Center, Egypt.
* Corresponding author.
E-mail address: sooria@tnau.ac.in (K. Soorianathasundaram).

https://doi.org/10.1016/j.jgeb.2018.10.001
1687-157X/

most responsive starting material for initiating ECS and plant regeneration. In addition, ECS is the most suitable material for genetic manipulation through transformation [13]. However, to our knowledge, so far there are very few studies been taken up on field characteristics of ECS derived plants and their comparison with plants produced from meristem and sucker. The present study was attempted to find out the influence of the position of a male flower bud, amino acid supplements and field performance of embryonic cell suspension derived from IMFs in the economically important banana cvs. Grand Naine and Rasthali, so as to establish an efficient system for virus free plantlet production of banana through somatic embryogenesis.

2. Materials and methods

2.1. Plant material

The banana cultivars 'Grand Naine' and 'Rasthali' were maintained with optimal inputs at the University Orchard for the collection of IMF. The terminal portion of the inflorescence bearing male flowers enclosed in the compact bracts was cut intact along with the portion of peduncle stalk using a sharp knife at 15 cm after the node from which emergence of the last hand was observed 10 weeks after the plants started shooting.

2.2. Initiation of embryogenic callus from IMF

IMF buds were used as initial explants for callus induction. IMF buds of banana cvs. Grand Naine (*Musa* AAA, Cavendish-sub group) and Rasthali (*Musa* AAB, Silk-sub group) excised from the 16th to 1st bract whorl from the terminal position of the inflorescence (bunch) in male phase were used as explants for callus induction. The male flower buds were reduced (bracts were removed one by one) to an optimum level (6–8 cm) and surface-sterilized with 70% ethanol for 2 min followed by three time washed with sterile water under the sterile hood. A total of 100 male flower explants were inoculated in callus induction (M1) medium (MS basal medium [14] with 10: 30 g L^{-1}sucrose: maltose, 1 mg L^{-1} biotin, 1 mg L^{-1} indole-3-acetic acid, 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 1 mg L^{-1} naphthleneacetic acid, 50 mg L^{-1} melatonin, 100 mg L^{-1} glutamine, 4 g L^{-1} agarose (Seakem®)). The cultures were kept in the dark at 26 ± 2 °C without sub culturing until embryogenic callus was induced. Routine checking was carried out for development of friable embryogenic calli. The response of explants at different positions on embryogenic callus induction was studied (Table 1). Callus induction frequency (CIF) was calculated by a number of explants produced embryogenic callus/total number of explants × 100.

2.3. Establishment of embryogenic cell suspension (ECS)

The 4–5 months old embryogenic calli were used for initiation of cell suspension. The embryogenic calli were recognized by the appearance of white translucent pro-embryos on the surface of the calli. Approximately 100 mg of embryogenic calli were transferred to 100 ml conical flasks containing 10 ml of liquid medium (M2). The flasks were incubated on incubator shaker (INNOVA 4330, New Brunswick Scientific, U.S.A) at 80 rpm at 26 °C and the cultures were maintained under dark conditions. The cultures were maintained for 2–3 months with periodical replacement (at 15 d interval) with fresh medium. At the time of every subculture, the cell suspension was observed under a light microscope to identify ECS. The cells of the ECS are spherical with dense starch granules whereas the non-embryogenic cells are irregular and elongated in size with no granule deposition. Different media composition was tested to establish ECS (Table 2). The viability of cell suspension cultures was examined to trace the ontogeny of pro-embryogenic mass by fluoresein diacetate (FDA) stain and viewed under a fluorescent microscope as described by Nandhakumar et al. [15].

2.4. Somatic embryo growth and development

In this study, the influence of different concentrations of L-glutamine, L-proline, and L- asparagine on induction of somatic embryogenesis was studied by involving the proliferating cultures placed in maturation (M3) medium. M3 medium consisted of SH basal medium [16] supplemented with 20:20 g L^{-1} sucrose: maltose, 0.2 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, 0.2 mg L^{-1} zeatin and 0.14 mg L^{-1} 2-isopentyladenine (2ip) and solidified in 3 g L^{-1} phytagel. Different concentrations of glutamine or proline at 100, 200,

Table 1
Influence of position of immature floral hands in callus induction frequency in banana.

Sl. no.	Position of floral hands	Grand Naine Embryogenic calli (%)	Rasthali Embryogenic calli (%)	Nature of callus
1	0–2	0^{d}	0^{d}	–
2	3–5	9.0 ± 0.65^{c}	6.0 ± 0.00^{c}	Milky white viscous and compact calli
3	6–8	50.0 ± 0.54^{a}	48.0 ± 1.67^{a}	Yellow globular friable calli
4	9–11	16.0 ± 0.67^{b}	17.0 ± 1.33^{b}	yellow friable and mucilaginous
5	12–15	5.9 ± 0.75^{c}	6.0 ± 0.00^{c}	Whitish friable and mucilaginous
6	Above 16	0^{d}	0^{d}	–

*Each value represents the treatment means of three independent replicates. Values within a column, means followed by a common letter are not significantly different ($P = 0.05$) by DMRT.

Table 2
Different media used to initiate banana embryogenic cell suspension.

Media	MS salts	2,4-D (mg/l)	BAP (mg/l)	Ascorbate (mg/l)	Zeatin (mg/l)	Sucrose: Maltose (g/l)
ESIM1	+	0	0	0	0	20:20
ESIM2	+	2	0	10	0.5	20:20
ESIM3	+	4	0	10	0.5	20:20
ESIM4	+	6	0	10	0.5	20:20
ESIM5	+	10	0	10	0.5	20:20
ESIM6	+	0	0.5	10	0.5	20:20
ESIM7	+	0	1.0	10	0.5	20:20
ESIM8	+	0	1.5	10	0.5	20:20

300 and 400 mg L^{-1} and L-asparagine at 50, 100, 150, 200 mg L^{-1} added to the medium were tested. The cultures were kept in the dark at 26 ± 2 °C with regular subculturing, once in a month. The experiments were carried out two times with three replications for each treatment.

2.5. Desiccation of embryos and conversion of plantlets

To study the effect of duration of desiccation (0, 0.5, 1, 2, 3, 4 and 5 h) on germination response, 50 numbers of cotyledonary-stage somatic embryos were transferred to each empty sterile Petri plates (90 × 15 mm) and placed in dark at 26 ± 2 °C. Desiccated somatic embryos were later transferred to germination (M4) medium (MS basal medium with Morel vitamins [17], 1 mg L^{-1} 6-benzylaminopurine (6-BA), 1 mg L^{-1} IAA, 30 g L^{-1} sucrose, 2 g L^{-1}gelrite). The cultures were maintained in the germination medium for further two months with regular subculturing, once in a month. The light was provided by fluorescent lamps (16 h light/8 h dark) at a photosynthetic photon flux of 50 μ mol m^{-2} s^{-1} to induce conversion of somatic embryos to plantlets.

2.6. Rooting of plantlets in vitro

Proliferated and non-hyperhydrified shoots were dissected out carefully from the base of multiple shoot stock and transferred to rooting in both semi-solid and liquid media. Healthy shoots longer than 5 cm were found ideal for the induction of adventitious roots. Rootable shoots were cultured in half-strength MS medium with IBA (1, 2, 2.5 and 5.0 mg L^{-1}) and NAA (0.5 and 1.0 mg L^{-1}). The effect of half-strength MS medium devoid of any growth regulator served as control. Rooting was also attempted in hydroponics system containing liquid half-strength MS medium supplemented with IBA (5 mg L^{-1}) and NAA (0.5 mg L^{-1}). Rooting intensity, percentage and root length (cm) were recorded after two weeks of culture in the same medium.

2.7. Primary and secondary hardening

Rooted plantlets with varying shoot lengths were carefully taken out of the jam bottle, and the roots were washed thoroughly in water and transferred to small cups (15 days) followed by pots of 8 cm diameter containing the potting mixture. It contained smooth and coarse sand mixed with autoclaved garden soil: 10% organic matter (3:2). To study the effect of potting mixtures such as an addition of pre-sterilized soilrite mix and Monoammonium phosphate (MAP) (5 and 10%) in the potting medium was carried out. The plantlets maintained under same conditions were transferred to mist chamber for secondary hardening. This step minimizes transplanting shock. As a control, some plants were directly transferred to mist chamber and then to the greenhouse.

2.8. Field performance of somatic embryogenesis derived plants

ECS derived banana plants were planted in the field along with those plants produced by meristem and sucker as a control. After acclimatized in the greenhouse, the plants were 45–50 cm tall, were transferred to the main field and an average of 300 plants was used. Observations and data were recorded for pseudostem height, pseudostem girth, number of leaves, leaf area, days to harvest, bunch weight, number of hands, finger length and finger weight were measured as described by Kumar et al. [18].

2.9. ISSR analysis

Plant genomic DNA was isolated using modified CTAB method [19] from young leaves (100 mg) of 24 randomly selected banana (vegetative and reproductive stage) plantlets of cvs. Grand Naine and Rasthali, along with mother plant for assessment of the genetic fidelity using ISSR marker [20]. ISSR-PCR amplifications were carried out with 10 ISSR (NCS-TCP, Department of Biotechnology, Government of India, New Delhi) primers performed in a Thermal Cycler (Bio-Rad, C 1000™) with the following conditions: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 37 °C, 1.5 min at 72 °C and a final extension step 10 min at 72 °C. PCR reactions were carried out in a 20 μl reaction volume containing 50 ng of template DNA, 0.5 μl of 10 mMdNTP mix, 0.6 μM of random primers, 1X *Taq* DNA polymerase buffer containing 15 mM $MgCl_2$ and 1.0 U of *Taq*polymerase (GeNei). The PCR amplified products were resolved by electrophoresis on a 1.7% (w/v) agarose gel ran in 1X TAE buffer. The gel was photographed using a gel documentation system (Syngene) and the resulting fragments were scored. Only the distinct, reproducible and well-resolved fragments were scored as present (1) and it's absent as (0). Less resolving bands were excluded from the scoring process.

3. Histological analysis

For histological examination, samples were dehydrated, embedded and sectioned using a rotary microtome as described by Johansen [21], the details of which are given below.

3.1. Fixative materials

Tissues for sectioning includes pieces of primary IMF explants (Rasthali), 4-months-old embryogenic calli from M1 medium, somatic embryos at different developmental stages of ECS raised in M2, M3 and M4, respectively.

The aim of fixation is to kill and preserve plant cells and their contents so that they are not distorted in any way. In fact, it helps to conserve the morphology of different structures, stabilize the chemical constituents by preventing the alterations and degradation, kill the cells in order to arrest the process of autolysis i.e. alteration of the lysozomes and liberation of hydrolases, favor easy penetration during embedding and for proper staining and softening of the materials. It is very important that the fixative should penetrate the plant cells quickly, as sooner the cells are killed and fixed, the more natural the appearance. This was made possible by degassing the material in the fixatives. For the present study, Formalin-Aceto-Alcohol (FAA) fixative was used. It is composed of 5 parts of 35% formalin, 5 parts of glacial acetic acid and 90 parts of 70% EtOH. Materials left in this mixture indefinitely have many advantages: Acetic acid fixes the chromosomes well and formalin acts upon the cytoplasmic proteins while EtOH enhances easy penetration. The sampled materials were immersed in FAA for 24 h. Then the leaf bits were stored in 70% EtOH.

3.2. Staining

Slides were washed in running tap water to remove traces of EtOH and then immersed in 4% mordent for 5 min. Slides were again thoroughly washed then dipped in haematoxylin stain for 2 min, washed in water and then passed onto picric acid for 2 min. Traces of picric acid were removed after washing through a series of EtOH-xylol mixture: distilled water for 2 min; EtOH 70% for 2 min; EtOH 90% for 2 min, EtOH 100% for 2 min and EtOH: xylol (1:1) for 2 min and xylol 10 min. Slides were taken out and made permanent by mounting with DPX mountant, and kept for drying and observed in a microscope (ZEISS, Germany or Leica, Switzerland) after 1 or 2 days.

3.3. Statistical analysis

Data collected were subjected using SPSS version 20 software (SPSS Inc). The means were compared with Duncan's Multiple Comparison Test (DMCT) at 0.05% possibility for statistical significance.

4. Results and discussion

4.1. Effect of position of floral hands on callus induction

Establishment of high-frequency regeneration protocol in the two selected varieties of banana is a pre-requisite for developing an efficient method for production of virus-free and mass multiplication of banana. It is well known that banana is a recalcitrant crop to regeneration. In the present study was attempted to find out the influence of position of male flower bud for callus induction, the establishment of cell suspension culture and regeneration through somatic embryogenesis. IMF bud was found to be suitable explants for callus induction in both cultivars tested. Male inflorescences collected from the mother plant at the right stage (10 weeks after the plants start shooting) were used in the present study for isolation of IMFs for callus induction. In this study, 100 male flower buds (16th to 1st bract whorl position from inflorescence terminal) of banana cvs. Grand Naine (*Musa* AAA, Cavendish-sub group) and Rasthali (*Musa* AAB, Silk-sub group) were used to initiate embryogenic calli. The explants became brown at the base within a week of culture initiation and began to swell and increase in size after a month. The brown colour that frequently develops in callus cultures of banana is due to formation of phenols which are well known to be inhibitory to plant cellular growth. When culturing these species of plants, special precautions are necessary for preventing the accumulation of toxic products resulting from phenolic oxidation. The main problem in callus induction was the exudation of phenolics into the medium. This leads to rapid browning, a decay of the explants and calli. During decay, the calli were watery and hyperhydricity was clearly visible. Phenolic accumulation in the medium affecting callus induction has been reported by Kosky et al. [22]. The higher amount of phenolic accumulation in banana may be due to its perennial nature of cultivation. In order to overcome this problem, anti-oxidant like melatonin (50 mg L^{-1}) and L-Glutamine (100 mg L^{-1}) added to the callus induction medium containing 4 g/l Seakem®agarose resulted in significant improvement in callus induction frequency. Gelling agent and consistency of the gel are considered to be important for the initial establishment of cultures. In contrast to the lower amount of gelling agent used in the present study, earlier workers have reported the use of agar, agarose or gelrite as gelling agents at higher concentration in the callus induction medium [23]. It was observed that use of lower percent of gelling agent aided the diffusion of phenolic compounds away from the explants, thereby reducing the ill effect of phenolics on callus induction. Maintenance of cultures in the dark is also helpful because illumination is stimulatory to the production of phenolics. None of the earlier reports have indicated the use of melatonin (50 mg L^{-1}) even though it is well known for preventing phenolic accumulation in other crops. An interesting observation was made that the frequency of callus formation varied significantly with bract position of floral explants. While embryogenic callus formation was observed from flower bud explants excised from 1st to 16th bract whorl positions, only those from 6 to 8th bract position were found to respond highest with the production of yellow globular friable callus (50.0 ± 0.54 and 48.0 ± 1.67%) after five months of culture initiation. Floral buds from position 3–5 (9.0 ± 0.65 and 6.0%) produced milky white viscous and compact calli, whereas, those explants from position 9–11 (16.0 ± 0.67 and 17.0 ± 1.33%) produced yellow friable calli with mucilage and explants at 12–15 (5.9 ± 0.75 and 6.0%) produced whitish friable with mucilage callus. The primordial floral explants from the position below 3 and above 15 failed to induce callus (Table 1).

The higher rate of embryogenic calli was obtained in explants collected from 6th to 8th position, followed by 9th to 11th position. Therefore, the result indicates that the right stage of physiological maturity is crucial for the response. Subsequent experiments were carried out using IMFs isolated from 6th to 11th position. In the present investigation, a different morphological appearance of callus such as brown compact callus, milky white viscous and compact callus, yellow friable callus, pale yellow friable, mucilaginous and white yellow mucilaginous callus was observed (Fig. 1). It was also

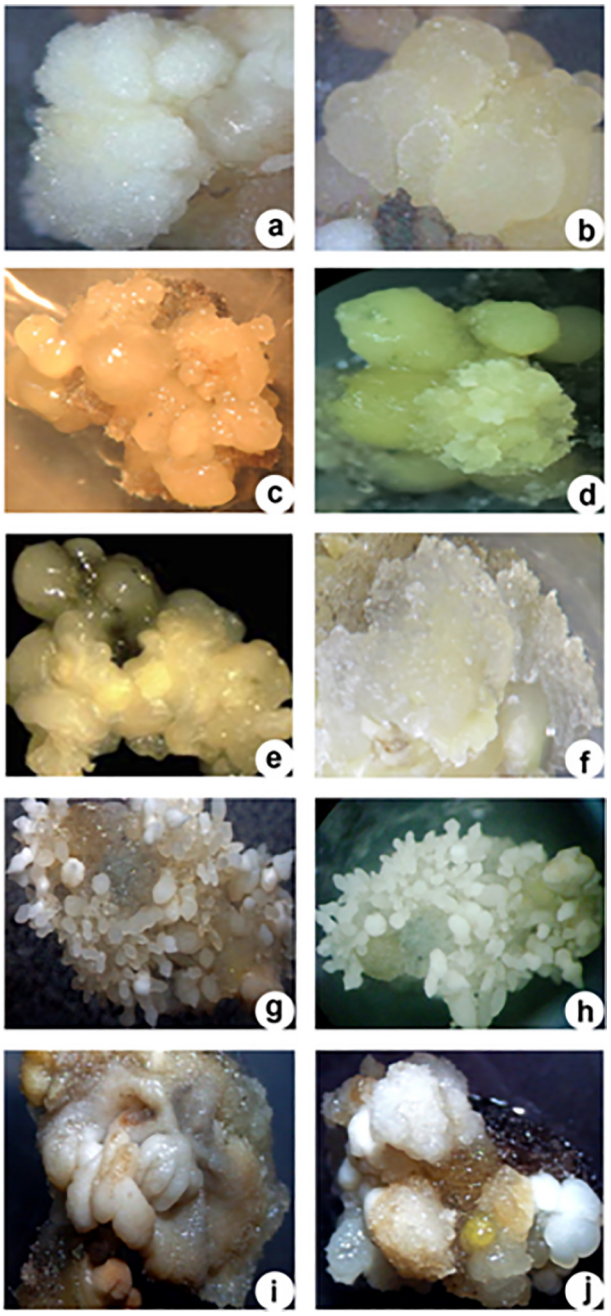

Fig. 1. Morphology of IMF bud derived calli in banana. a–b: Milky white viscous and compact calli; c–d: Pale yellow mucilaginous calli; e: Brown compact calli; f: Watery translucent calli; g–h: Friable embryogenic calli; i–j: Abnormal calli.

Table 2a
Effect of media compositions on initiation of embryogenic cell suspension in banana.

Media	Embryo suspension induction medium (ESIM)					
	Globular-stage (%)		Heart-stage (%)		Torpedo-stage (%)	
	Grand Naine	Rasthali	Grand Naine	Rasthali	Grand Naine	Rasthali
ESIM1	0^{h}	0^{g}	0^{f}	0^{f}	0^{e}	0^{f}
ESIM2	9.70 ± 1.07^{f}	5.90 ± 0.92^{f}	0^{f}	0^{f}	0^{e}	0^{f}
ESIM3	13.5 ± 0.73^{e}	12.2 ± 0.43^{d}	6.80 ± 1.12^{d}	4.60 ± 0.56^{e}	9.10 ± 0.95^{c}	5.80 ± 0.83^{d}
ESIM4	20.3 ± 0.23^{c}	19.2 ± 0.92^{c}	15.4 ± 0.54^{b}	11.9 ± 0.71^{b}	13.8 ± 1.08^{b}	12.7 ± 1.04^{b}
ESIM5	37.7 ± 0.68^{a}	36.1 ± 0.59^{a}	29.4 ± 0.63^{a}	26.9 ± 0.46^{a}	25.4 ± 0.63^{a}	22.3 ± 0.74^{a}
ESIM6	25.3 ± 0.67^{b}	23.1 ± 0.18^{b}	16.4 ± 0.81^{b}	12.0 ± 0.61^{b}	10.4 ± 0.56^{c}	9.70 ± 1.23^{c}
ESIM7	18.6 ± 1.15^{d}	12.3 ± 0.67^{d}	7.8 ± 0.73^{c}	6.4 ± 0.43^{c}	3.70 ± 0.43^{d}	2.20 ± 0.67^{e}
ESIM8	7.9 ± 1.02^{g}	6.70 ± 0.91^{e}	3.60 ± 0.82^{e}	3.40 ± 0.61^{d}	0^{e}	0^{f}

*Each value represents the treatment means of three independent replicates. Values within a column, means followed by a common letter are not significantly different ($P = 0.05$) by DMRT.

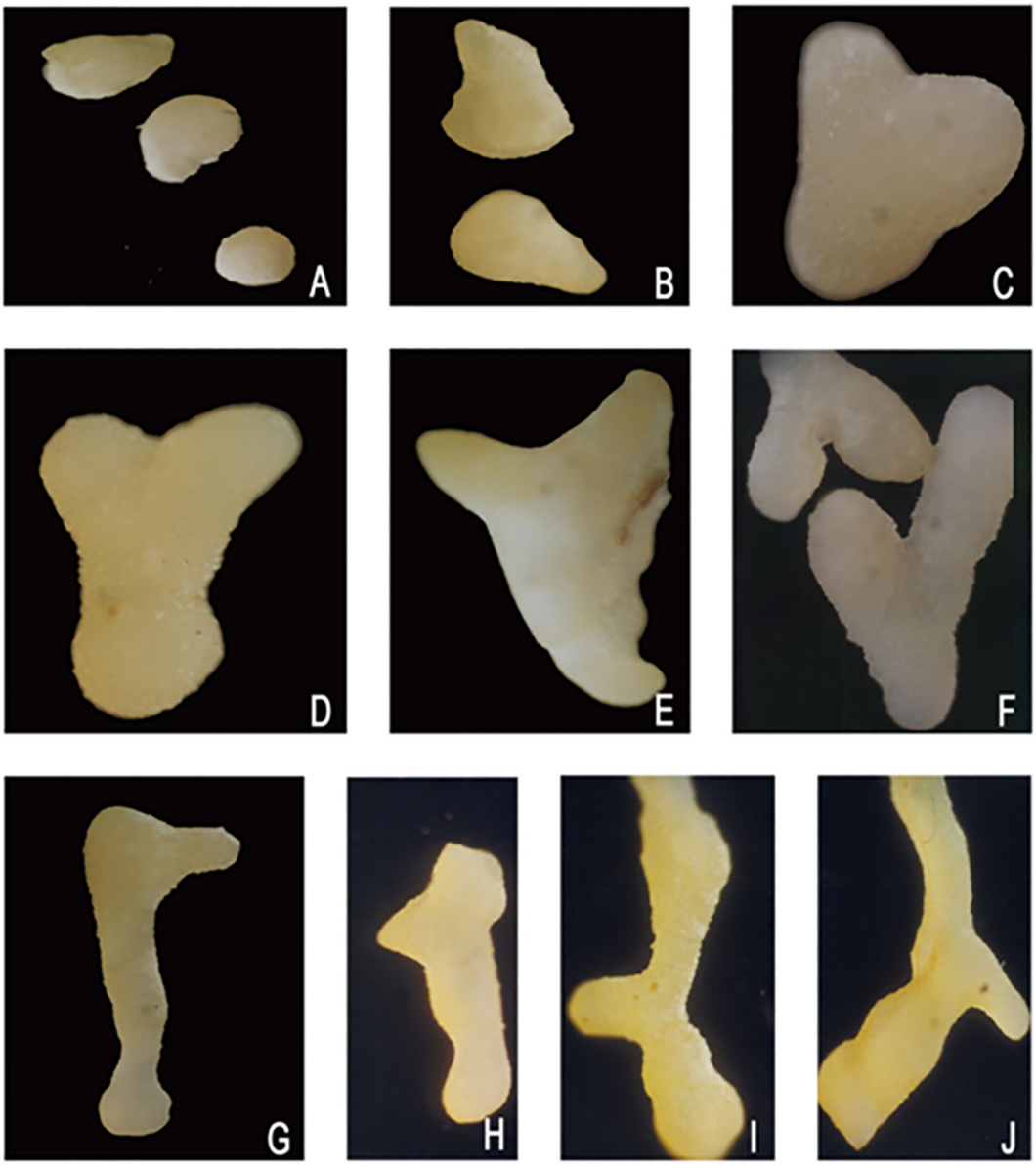

Fig. 2. Somatic embryos recovered from ECS of banana. A: Globular-stage; B: Oblong-stage; C: Heart stage; D: Torpedo-stage; E: Matured torpedo-stage; F: Coteledonary-stage; G: Mature coteledonary-stage; H: Secondary somatic embryos developed from coteledonary-stage.

Table 3
Effect of different amino acids on somatic embryogenesis in banana.

Concentration (mg/l)	Number of somatic embryos per cultures			
	Grand Naine		Rasthali	
	Primary embryos	Secondary embryos	Primary embryos	Secondary embryos
Control	100 ± 0.33[l]	222 ± 0.26[i]	120 ± 0.01[l]	356 ± 0.20[i]
L-Proline				
100	140 ± 0.12[k]	220 ± 0.00[j]	160 ± 0.07[k]	230 ± 0.14[j]
200	360 ± 0.08[e]	630 ± 0.15[f]	380 ± 0.03[e]	680 ± 0.26[f]
300	600 ± 0.15[c]	890 ± 0.03[d]	680 ± 0.16[c]	982 ± 0.14[d]
400	220 ± 0.08[i]	500 ± 0.18[h]	200 ± 0.10[i]	480 ± 0.18[h]
L-Glutamine				
100	178 ± 0.09[j]	630 ± 0.26[f]	186 ± 0.01[j]	654 ± 0.31[f]
200	535 ± 0.08[d]	1297 ± 0.36[c]	539 ± 0.12[d]	1352 ± 0.37[c]
300	1015 ± 0.24[b]	2950 ± 0.68[b]	1040 ± 0.13[b]	2597 ± 0.45[b]
400	1680 ± 0.45[a]	3597 ± 0.65[a]	1850 ± 0.12[a]	3270 ± 0.65[a]
L-Asparagine				
50	110 ± 0.00[l]	353 ± 0.21[i]	120 ± 0.13[l]	366 ± 0.18[i]
100	300 ± 0.15[f]	843 ± 0.24[e]	332 ± 0.12[f]	853 ± 0.08[e]
150	240 ± 0.00[g]	222 ± 0.17[f]	240 ± 0.16[g]	643 ± 0.26[f]
200	200 ± 0.06[h]	220 ± 0.26[g]	210 ± 0.05[h]	532 ± 0.13[g]

*Each value represents the treatment means of three independent replicates. Values within a column, means followed by a common letter are not significantly different ($P = 0.05$) by DMRT.

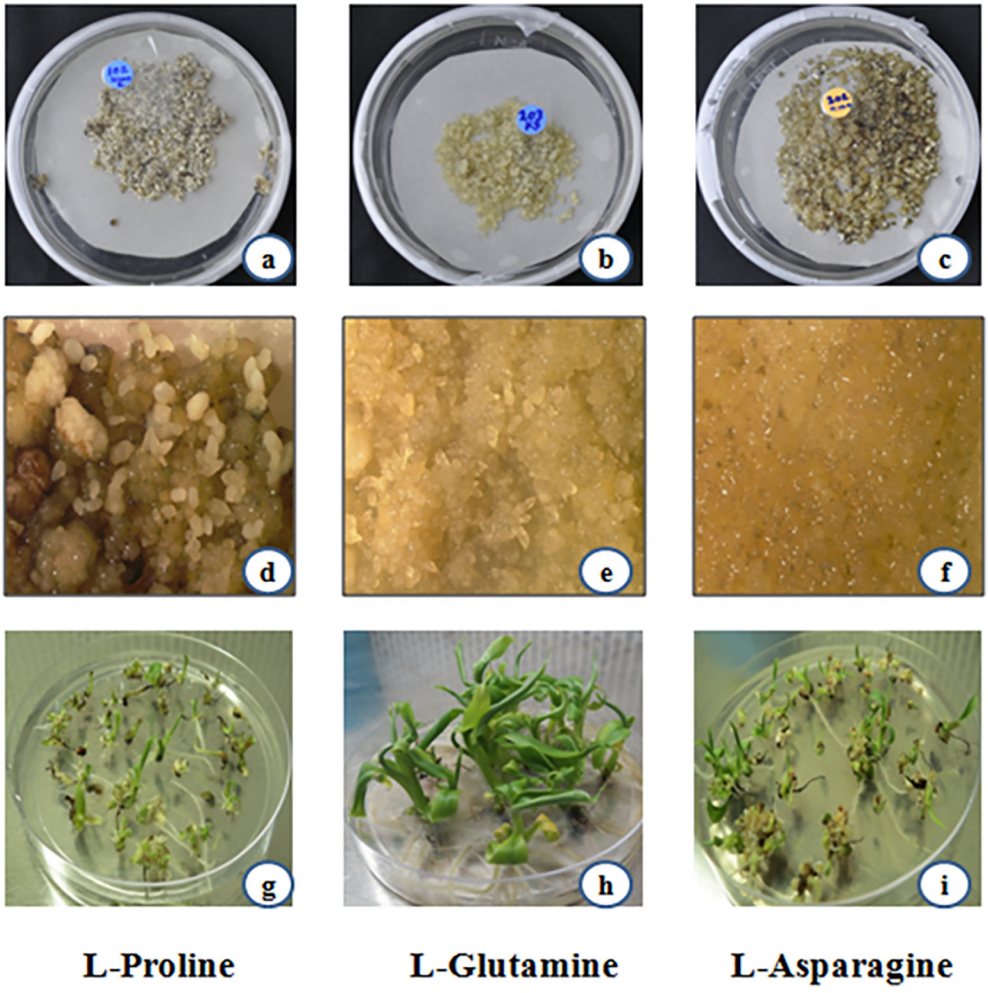

Fig. 3. Effect of different amino acids on maturation of somatic embryos. a–c: Homogenous cell suspension placed on M3 media; d–f: Matured somatic embryos from ECS; g–i: Germination of somatic embryos.

observed that embryogenic calli grew slowly compared to non-embryogenic calli [23]. Hence, present study showed that embryogenic calli formation direct proportion to the position of the male flowers (16th to 1st) and media composition.

4.2. Establishment and maintenance of cell suspension

The banana cell suspension is generally used for the generation of a large number of somatic embryo for novel breeding approaches such as transgenic and CRISPR/Cas being tried for crop improvement. Most of the reports on banana transformation have utilized the embryogenic cell suspension for either *Agrobacterium*-mediated transformation or particle gun-mediated method of transformation [13]. Reports on the banana higher rate of regeneration frequency were dependent on the quality of ECS [13,15]. Hence, the present study discovered that quality of the suspension cultures and a higher rate of regeneration were developed. It is critical to select embryogenic calli with pro-embryos for the establishment of quality ECS culture. The suspension media composition commonly used for suspension culture is MS basal medium containing 1 mg L^{-1}2, 4-D [8,10,23]. On the contrary, our results have shows that suspension medium (ESIM5) containing 10 mg L^{-1} 2, 4-D resulted in better formation of the somatic embryo in the cell suspension culture itself thus making it more suitable for further maintenance and multiplication of cell suspension. The MS based ECS medium containing sucrose (20 g L^{-1}), maltose (20 g L^{-1}) and 2,4-D concentration (10 mg L^{-1}) resulted in the rapid multiplication of embryogenic cells. Earlier reports used only sucrose in the cell suspension medium [8,23,15], however, it was observed that addition of maltose was highly beneficial for the formation of ECS in both cultivars. Most of the suspension turned highly embryogenic at the end of the third month [23]. In the present study, different stages (globular, heart and torpedo) of somatic embryos were recorded (Table 2a; Fig. 2). Mostly ECS developed and multiplied in ESIM 5 compared to other media. The embryogenic cells were identified by the presence of dense cytoplasmic starch granule when viewed under a light microscope (Supplementary Fig. 2a).

4.3. Viability of cell suspension by fluorescein diacetate (FDA) staining

The viability of cells in suspensions was examined by FDA staining. ECS were examined to trace the ontogeny of pro-embryogenic mass by FDA stain and viewed under a fluorescent microscope. ECS showed two morphologically distinct cells, namely, spherical and elongated cells, both with visible cytoplasm (Supplementary Fig. 2a). The spherical embryogenic cells underwent a transverse division, resulting in two-cell and four-cell structures. Later, these cells divide further and result in the formation of a clump of cells that are considered proembryo-like-structure, which further divided into multiple planes resulting in the formation of globular embryos within a week.

4.4. Effect of different amino acids on somatic embryo production

Somatic embryogenesis is a complex process where the survival and growth of regenerated plants depend on the genotype and conditions of cultivation [24]. Amino acid supplementation has been known to enhance somatic embryogenesis in several species. Amino acids *viz.*, L - Proline, L- glutamine and L- asparagine were tested to find their ability to induce somatic embryos on maturation (M3) medium. Among the amino acids tested, L- glutamine (400 mg L^{-1}) significantly enhanced both primary and secondary somatic embryos in M3 medium fortified with 0.2 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, 0.2 mg L^{-1} zeatin and 0.14 mg L^{-1} 2- isopentyladenine (2ip), 20 : 20 g L^{-1} sucrose : maltose. This composition registered a greater response of primary embryos (1680 ± 0.45 and 1850 ± 0.12) and secondary embryos (3597 ± 0.65 and 3270 ± 0.65) per 0.5 ml of ECS as compared to supplementation with L-proline and L-asparagine (Table 3, Fig. 3). It was observed that the abnormalities such as an extensive formation of brown cells and retardation of embryogenesis increased with increasing proline and asparagine concentrations. All abnormalities appeared after 1–2 months of culture in M3 media. Abnormal embryos structures became black and later turned necrotic.

The glutamine (400 mg L^{-1}) and carbon source (20 g L^{-1}sucrose and 20 g L^{-1}maltose) were found to be critical for efficient embryo maturation [24]. The addition of glutamine to the M3 media may contribute to some amount of nitrogen source in the medium, which may have helped in improving the rate of somatic embryo maturation. The presence of glutamine facilitated completion of maturation of somatic embryos as reported by Morais-Lino et al. [25]. The results indicated that exogenous supplementation of amino acid can serve as a nitrogen source for the synthesis of the protein which is important for embryo differentiation and maturation. An earlier study done in crop plants such as chestnut, eastern

Table 4
Effect of duration of desiccation on germination of somatic embryos of banana.

Germination response (%)			
Treatment No	Desiccation period (h)	Grand Naine (AAA)	Rasthali (AAB)
1	0	10^{f}	11^{f}
2	0.5	15^{e}	17^{e}
3	1	44^{b}	42^{b}
4	2	66^{a}	65^{a}
5	3	36^{c}	37^{c}
6	4	24^{d}	25^{d}
7	5	13^{e}	15^{e}

*Each experiment was repeated with 50 matured cotyledonary embryos. Values within a column, means followed by a common letter are not significantly different ($P = 0.05$) by DMRT.

Table 5
Influence of auxins on *in vitro* rooting of regenerated plantlets.

Auxin concentration (mg/l)	Rasthali (AAB)			Grand Naine (AAA)		
	Rooting (%)	Number of roots per shoot	Root length (cm)	Rooting (%)	Number of roots per shoot	Root length (cm)
Half MS basal	52.38^{h}	4.19^{h}	3.08^{gh}	50.00^{h}	4.37^{h}	3.08^{h}
Half MS + IBA 1.0 + NAA 0.5	63.63^{ef}	4.37^{g}	3.45^{f}	63.03^{fg}	4.61^{fg}	3.15^{g}
Half MS + IBA 1.0 + NAA 1.0	64.81^{e}	4.78^{e}	3.13^{g}	64.88^{f}	4.78^{f}	3.46^{f}
Half MS + IBA 2.0 + NAA 0.5	60.12^{g}	4.61^{ef}	4.43^{d}	76.92^{e}	5.19^{e}	4.67^{cd}
Half MS + IBA 2.0 + NAA 1.0	86.24^{d}	5.84^{d}	4.82^{c}	78.57^{d}	5.84^{d}	4.70^{c}
Half MS + IBA 2.5 + NAA 0.5	92.15^{a}	7.67^{b}	5.34^{a}	92.50^{a}	8.67^{b}	6.14^{a}
Half MS + IBA 2.5 + NAA 1.0	89.68^{bc}	6.34^{c}	5.14^{b}	86.47^{c}	8.34^{c}	5.34^{b}
Liquid Half MS + IBA 5.0 + NAA 0.5	90.47^{b}	10.24^{a}	4.18^{de}	89.68^{b}	10.24^{a}	4.18^{e}

*Each value represents the treatment means of five independent replicates. Values within a column, means followed by a common letter are not significantly different ($P = 0.05$) by DMRT.

white pine, white spruce and hybrid tea rose also proved that L-glutamine is the best amino acid supplement for somatic embryogenesis [26–29]. The present study demonstrates that banana somatic embryos increased with the incorporation of glutamine and carbon source in the media.

4.5. Desiccation and germination

The rate and degree of desiccation of mature embryos stand crucial for their ability to germinate [30]. To overcome difficulties in the germination, desiccation of somatic embryos was attempted by transferring fifty cream colored well-developed embryos to each empty Petri plates in three sets in the dark for at 26 ± 2 °C at varying duration of 30 min to 5 h. During desiccation, the embryos were observed to lose their water content; shrink in size and such embryos reached physiological maturity early. Furthermore, synthesis and accumulation of storage compounds, especially proteins, and the acquisition of desiccation tolerance [30] are important factors that determine the ability of embryos to grow into plants. Over-desiccated embryos turned brown and never germinated; hence, they were excluded from the evaluation of germination and conversion frequencies. Partial desiccation of mature banana somatic embryos improved the conversion frequencies [30]. In the present study, somatic embryos desiccated for 2 h showed 65% germination response which was significantly higher than other treatments (Table 4). According to Srinivas et al. [30], the germination of banana somatic embryos and their conversion into vigorous plantlets depends on the type and concentration of growth regulators, amino acids, desiccation, the type of cell aggregates in the suspension and the stage of the embryos when they were transferred to the germination medium. The present study reveals that desiccation duration of 2 h could resulted in higher frequency of plant recovery as compared to longer or shorter duration of desiccation.

4.6. Rooting of plantlets in vitro

Spontaneous rooting was noticed in germinated somatic embryos in the M3 medium after 90 d of transfer. However, to increase production of lateral roots from primary roots, germinated somatic embryos were transferred after two weeks to half-strength MS rooting media with different levels of IBA and NAA in combination (Table 5). A complete plantlet of over 5–6 cm height with first two leaves was established and rooted profusely with high-density lateral roots in half-strength MS, 2.5 mg L^{-1} IBA, and 0.5 mg L^{-1} NAA after 10 d of culture compared to basal medium. Rooting efficiency was greater in basal than liquid medium with an increased level of IBA (Table 5). Rooting in liquid medium induced a clump of roots. But longer roots with more adventitious roots were observed in the basal medium. Rooted plantlets were initially acclimatized in mist chamber with successful initiation of fresh leaves after two weeks and then transferred to field for an establishment.

4.7. Primary and secondary hardening for plant establishment

Plantlets subjected to primary hardening and established were transferred to mist chamber. Chlorosis and premature senescence of leaves were observed within 3–5 days of transfer to secondary

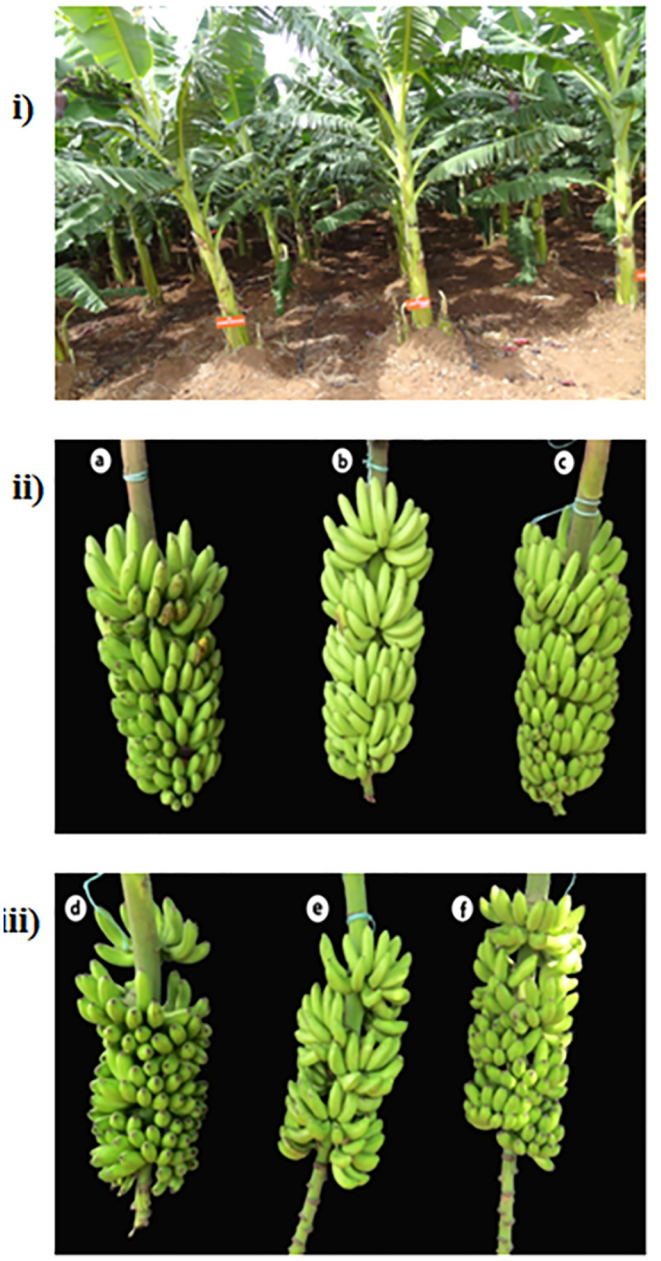

Fig. 4. Bunch traits in ECS derived banana cv. Grand Naine and Rasthali. i: Field view of experimental plots; ii: Banana cv. Grand Naine (a) sucker, (b) ECS, (c) shoot tip; iii: Banana cv. Rasthali (d) sucker, (e) ECS, (f) shoot tip.

Table 6
Comparison of Bunch traits of ECS, TC and conventional sucker derived plants of banana.

Bunch traits	Grand Naine			Rasthali		
	ECS derived plants	TC derived plants	Sucker propagated plants	ECS derived plants	TC derived plants	Sucker propagated plants
Bunch weight (kg)	26.10 ± 0.34	25.88 ± 0.83	25.03 ± 0.48	15.40 ± 0.43	15.13 ± 0.55	14.93 ± 0.39
No of hands per bunch	10.63 ± 0.29	10.13 ± 0.29	9.70 ± 0.28	7.70 ± 0.55	7.40 ± 0.54	7.30 ± 0.61
Average finger weight (g)	173.36 ± 2.06	170.55 ± 2.46	168.96 ± 2.06	117.75 ± 1.11	114.70 ± 1.55	110.03 ± 3.45
Average finger length (cm)	22.05 ± 0.51	21.00 ± 0.46	20.08 ± 0.59	15.88 ± 0.19	15.20 ± 0.21	15.13 ± 0.40

values indicate mean ± standard error.

Table 6a
ISSR markers used in the genetic fidelity testing of somatic embryogenesis derived *Musa* spp.

S. No	Primer Number	Primer sequences
1	USB 807	AGAGAGAGAGAGAGAGT
2	USB 808	AGAGAGAGAGAGAGAGC
3	USB 811	GAGAGAGAGAGAGAGAC
4	USB 818	CACACACACACACACG
5	USB 834	AGAGAGAGAGAGAGAGYT
6	USB 836	AGAGAGAGAGAGAGAGYA
7	USB 840	GAGAGAGAGAGAGAGAYT
8	USB 841	GAGAGAGAGAGAGAGAYC
9	USB 842	GAGAGAGAGAGAGAGAYG
10	USB 868	GAAGAAGAAGAAGAAGAA

hardening in all the plantlets, irrespective of the potting mix. Further growth of plantlets was achieved only when sprayed with 5% of Monoammonium phosphate (MAP). While in rest of the treatments, leaf drying followed by senescence was observed after a week of transfer. By day 15, induction of fresh shoot sprouts was observed in MAP spray at 5% level. The survival percentage and the shoot length were highest when sprayed with 5% MAP. This was followed by elongation and induction of fresh leaves after a week. The entire acclimatization process took six weeks. This may be due to the stress-like phenomena exhibited *in vitro* and in plantlets during the initial phase of acclimatization. Though this phenomenon has been extensively described, less is known about the underlying causal mechanisms [5]. However, induction of fresh leaves was observed after 2 weeks.

4.8. Field evaluation of regenerated plants

ECS derived banana plants were hardened and observed along with plants produced by tissue culture of meristem and conventional suckers. The ECS derived banana plants displayed normal phenotype as that of plants derived from tissue culture and conventionally propagated plants. The bunch appearance and the yield was normal (Table 6, Supplementary Fig. 3a). During the field observation, plants regenerated through ECS in the present study were morphologically normal, suggesting absence of somaclonal variation in morphometric traits. Somaclonal variation can occur due to long duration exposure in culture environment or recurrent subculturing [19] but in the present study there were no such negative influence. Earlier studies carried out in crops such as barley, fescue, and rubber also proved that there was no significant difference between the vegetative and yield characteristics of plants [31–33] Fig. 4.

Production of plantlets from somatic embryos offer scope for successful genetic transformation for imparting desirable attributes such as BBTV, wilt resistance and enhanced shelf life attributes which are at present lacking in commercially important

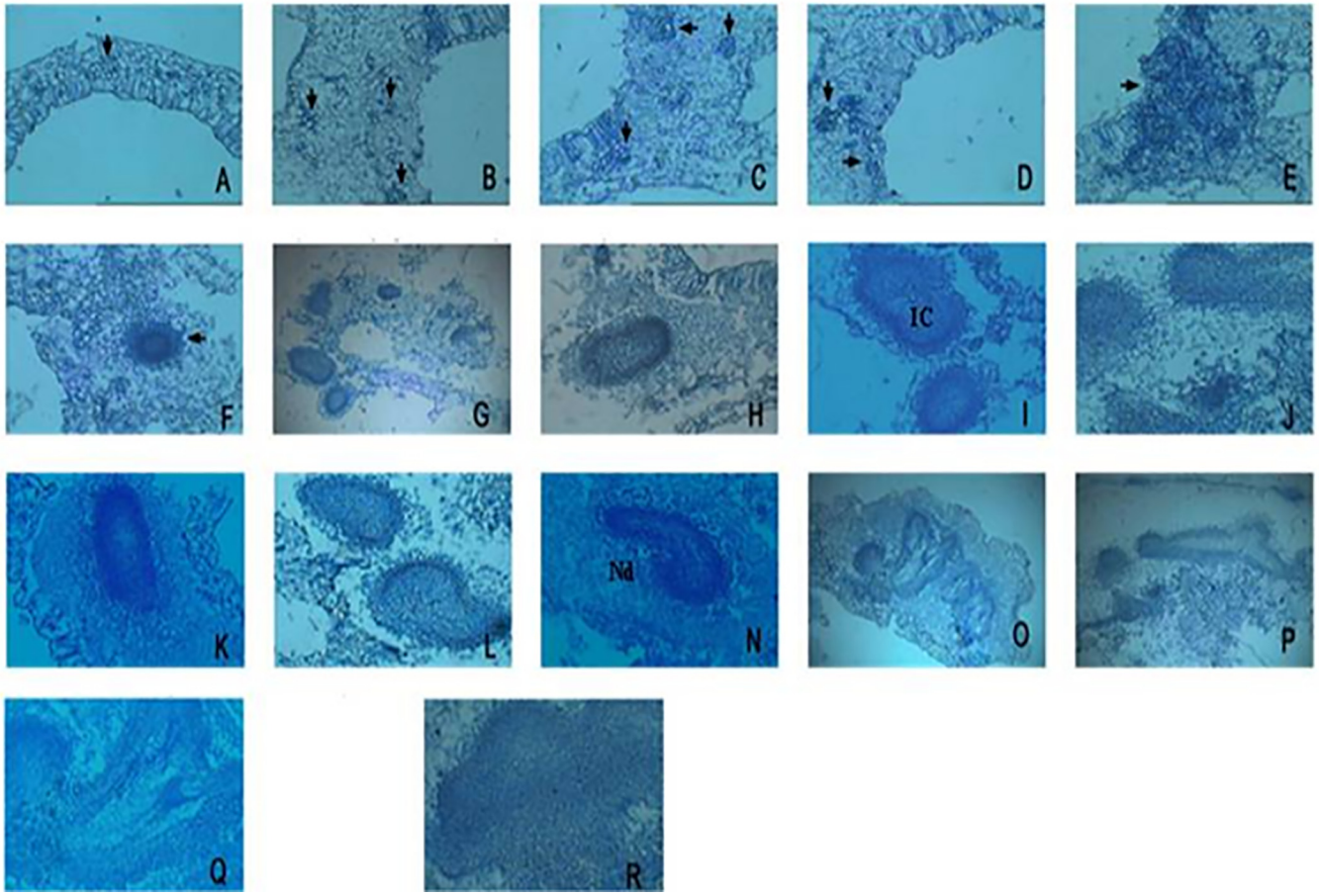

Fig. 5. Developmental pattern of somatic embryos derived from IMF of banana. A–E: Embryogenic calli at 2 months of cultivation on M1 medium; F–J: Isodiametric cells were started at 3 months of cultivation on M1 medium; K–P: Dense pro-embryogenic mass cells were observed at 4–5 months of cultivation on M1 medium; Q–R: Closer view of PEMs. IC- Isodiametric Cells; Nd-Nodules.

banana varieties. These results suggest that the establishment of ECS of banana can be an effective method for production of genetically stable banana plants from a single elite mother plant.

4.9. Genetic fidelity testing using ISSR marker

This is the first in-depth report of genetic fidelity in Rasthali and Grand Naine using ISSR markers. The ISSR marker system has been adapted for a variety of purpose in different crops, including fingerprinting and genetic fidelity studies [20]. As bananas are preferred for their require attributes such as flavor, texture, keeping quality etc. average changes in these features will defeat the objectives of genetic improvement attempts. To verify whether the somatic embryos derived from ECS the banana plants regenerated were subjected to genetic fidelity testing using ISSR markers. Ten ISSR markers (Table 6a) prescribed by the National Certification System for Tissue Cultured Raised Plants (NCS-TCP), Department of Biotechnology, Government of India were used in the study. All ten ISSR primers generated well resolved reproducible banding profiles. These primers produced 62 and 64 scorable band classes, ranging from 200 bp to 2500 bp in size in cv. 'Rasthali' and 'Grand Naine', respectively. A total of 1488 and 1534 bands were generated, giving rise to only monomorphic (USB 807, 808, 811, 818, 834, 836, 840, 841, 842 and 868) pattern across all the plantlets analysed in both cultivars during vegetative and reproductive stage (Supplementary Fig. 3a). Presence of no somaclonal variation in the banding pattern of ECS derived plants in both test cultivars clearly indicates the suitability of this method for quality planting material in banana.

5. Histological studies on the formation of somatic embryos

Histological analysis was carried out at different developmental stages starting from IMF bud to embryogenic calli formation. For histological study, different growth stages of the embryogenic calli (2, 3, 4 and 5 months old) were used. Analysis of two-month-old calli revealed that the cell division on the surface layer of IMF (6–11 position) led to the formation of small proembryogenic cell clumps, which gradually protrude from the IMF as a mass of embryogenic callus (Fig. 5A–E). Three-month-old calli revealed the development of the number of proemrbyogenic mass (PEMs), characterized by small, dense cytoplasmic cells (Fig. 5F–P). After 5 months, these clumps further underwent cycles of growth and fragmentation in the same medium to form large number of a dense mass of embryogenic calli termed as nodules (Fig. 5Q-R), which is composed of spherical (isodiametric) small cells containing dense cytoplasm. Callus induction frequency is depended more on the developmental stage (position of flower hands) of the male bud from which the explants were excised than on the cultivar [34,35].

Four to five-month-old embryogenic calli were used to initiate banana cell suspension culture. From this cell suspension, a further histological study was continued to trace the develop-

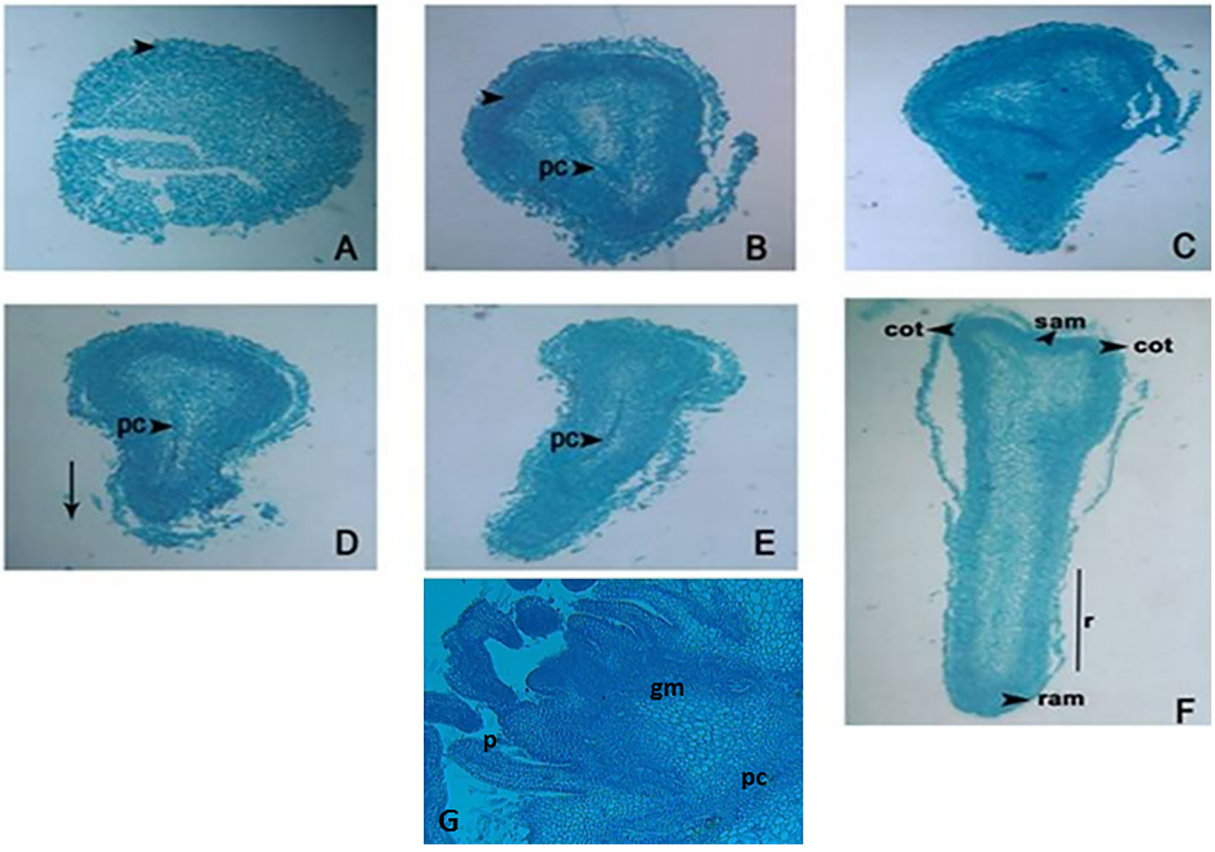

Fig. 6. Apical and basal differentiation patterns in somatic embryo development. A: Globular stage somatic embryos; B–C: Gradual establishment of polarity associated with differentiation of procambial cell layer; D: Oblong stage somatic embryo with procambial cell layer; E: Advanced globular embryo with established polarity. The two meristems are separated by a linear array of juvenile cells; F: Torpedo stage somatic embryos with visible three embryogenic cell domains (arrow heads). The apical domain, derived from the upper tier, has been partitioned into cotyledon (cot), primordial and shoots apical meristem (sam). The basal domain derived from the tier, formed the root (r).

ment of somatic embryogenesis. The study showed that most of the suspensions were single cells with some multi-celled embryogenic clump indicating asynchronous induction at different stages of somatic embryos. Non-embryogenic cells are characterized by large vacuole in the callus cells indicating cell degradation, as vacuole plays a critical role in programmed cell death [36]. The acquisition of embryogenic competence has been attributed to the cells that show meristematic traits during the induction phase [37].The present histological study demonstrates the presence of all important stages during somatic embryogenesis in banana and importance of utilizing the optimal stage for promoting embryogenic competence. ECS containing embryogenic cells were transferred to embryo maturation medium (M3) for further development of somatic embryos. Histological examination with longitudinal sections of the different stages of somatic embryos formed in M3 medium revealed the typical structure of somatic embryos (Fig. 6). The induction of globular stage of somatic embryos marked the beginning of structural differentiation (Fig. 6A). The outer cell layer divided more rapidly in an anticlinal direction, while the inner layers preferentially divided slowly in a periclinal direction to yield ground and vascular tissue (Fig. 6B). Axial elongation of inner isodiametric cells of the globular embryo led to the formation of longitudinal extensions near the basal end of the embryo (Fig. 6B–C), leading to the formation of the oblong-shaped embryo (Fig. 6D). Procambial cell differentiation and polarity establishment (Fig. 6E) were observed at the oblong stage of embryo development. Later the ground meristems of the oblong shape embryo have become vacuolated leading to the formation of a notch region in the protoderm cell layer. This induced the formation of torpedo shape somatic embryos (Fig. 6F). In this transition-stage, three major embryonic tissue systems were visible (Fig. 6G): protoderm (p), ground meristem (gm) and procambium (pc).

The next important morphogenetic event in embryogenesis was the formation of the shoot apical meristem. Longitudinal sections of the mature monocotyledonary somatic embryos revealed an organized development leading to the formation of shoot primordia. These embryos contain a well-defined meristematic dome. The cells within the meristematic dome are tightly-packed with dense cytoplasm and small vacuoles at the apical notch of the meristem. The cells begin to divide mainly in the periclinal direction. The mature embryos are transferred to germination medium (M4) for further growth. After one month of somatic embryo germination, shoot and leaf primordia begin to initiate from the protoderm cell layer in the development of a complete plantlet. The present histological study demonstrates the presence of all important stages during somatic embryogenesis in banana and importance of utilizing the optimal stage for promoting embryogenic competence.

6. Conclusions

As conventional breeding programs in bananas and plantains are generally hampered due to problems of sterility and polyploidy, alternate and novel breeding approaches such as transgenics and CRISPR/Cas being tried for crop improvement. ECS are a useful tool for regeneration and for such breeding approaches. Understanding the factors that contribute to the higher rate of somatic embryogenesis can help in rapid multiplication and in breeding programs using novel approaches. In this study, somatic embryos were successfully induced from the IMF of banana cvs. Grand Naine (*Musa* AAA, Cavendish-sub group) and Rasthali (*Musa* AAB, Silk-sub group). Clonally identical plantlets were regenerated from ECS obtained from the male buds. This study indicated that use of IMF from 6 to 8th bract whorl position from male flower terminal, employing L-glutamine 400 mg L^{-1} and subjecting the embryogenic culture to 2 h of desiccation period under 26 ± 2 °C conditions can enhance the rate of embryogenesis in banana cultivars Grand Naine and Rasthali. Somatic embryogenesis derived plants with agronomic behavior similar to those plants produced by meristem and sucker. This method can be useful generating plantlets in large numbers from a single elite mother plant. This regeneration system can also facilitate future improvement efforts employing genetic transformation. The protocols developed in the study may be useful for other cultivars of banana which have not been experimented for their somatic embryogenic ability with certain modifications.

Acknowledgement

The support of Biotechnology Industry Research Assistance Council (BIRAC), Department of Biotechnology (DBT), New Delhi, India in facilitating this work is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2018.10.001.

References

[1] FAO Stat. FAO Stat accessed vide http://www.fao.org/faostat/en/#data/QC on 12/8/2018.
[2] Mostert D, Molina AB, Daniells J, Fourie G, Hermanto C, Chao CP, et al. PLoS ONE 12(7):e0181630.
[3] Sharman M, Thomas J, Skabo S, Holton TA. Arch Virol 2008. 153-135.
[4] Sagi L, Remy S, Swennen R. PhytoProtection 1998;79:117–20.
[5] Cronauer MSS, Krikorian AP. Plant Cell Rep 1983;2:289–91.
[6] Al-Amin MD, Karim MR, Amin MR, Rahman S, Mamun ANM, Bangladesh J. Agric Res 2009;34:645–59.
[7] Karule P, Dalvi V, Kadu A, Chaudhari R, Subramanium VR, Patil AB. Afr J Biotechnol 2016;15:401–7.
[8] Ganapathi TR, Suprasanna P, Bapat VA, Kulkarni VM, Rao PS. CurrSci 1999;76:1228–31.
[9] Kulkarni VM, Suprasanna P, Bapat VA. CurrSci 2006;90:842–6.
[10] Navarro C, Escobedo RM, Mayo A. Plant Cell Tiss Org 1997;51:17–25.
[11] Grapin A, Schwendiman J, Teisson C. In vitro Cell Dev Biol 1996;32:66–71.
[12] Resmi L, Nair AS. Plant Cell Tiss Org 2007;88:333–8.
[13] Tripathi JN, Richard O, Leena T. Plant Sci 2015;6:1025.
[14] Murashige T, Skoog FA. Plant Physiol 1962;15:473–97.
[15] Nandhakumar N, Kumar KK, Sudhakar D, Soorianathasundaram K. Indian Res J Genet Biotechnol 2017;9(1):10–22.
[16] Schenk RU, Hildebrandt AC. Can J Bot 1972;50:199–204.
[17] Morel G, Wetmore RM. Am J Bot 1951;38:141–3.
[18] Kumar N, Krishnamurthy V, Nalina L, Soorianathasundaram K. Info Musa 2002;11(2):42–3.
[19] Nandhakumar N, Soorianathasundaram K, Sudhakar D, Kumar KK. Flora Fauna 2017;23:25–36.
[20] Nandhakumar N, Soorianathasundaram K, Sudhakar D, Kumar KK. Int J Curr Microbiol App Sci 2017;6:1759–69.
[21] . First ed. New York: McGraw; 1940.
[22] Kosky RG, Silva MF, Perez LP, Gilliard T, Martínez FB, Vega MR, Milian MC, Mendonza EQ. Plant Cell Tiss Org 2002;68:21–6.
[23] Cote FX, Domergue R, Monmarson S, Schwendiman J, Teisson C, Escalant JV. Physiol Plant 1996;97:285–90.
[24] Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L. Plant Cell Tiss Org 2002;69:233–49.
[25] Morais-Lino LS, Santos-Serejo JA, Silva SO, Santana JRF, Kobayashi AK. Pesq Agropec Bras 2008;43:1325–30.
[26] Robichaud RL, Lessard VC, Merkle SA. J Plant Physiol 2004;161:957–69.
[27] Garin E, Bernier-Cardou M, Isabel N, Klimaszewska K, Plourde A. Plant Cell Tiss Org 2000;62:27–37.
[28] Ashihara H, Stasolla C, Loukanina N, Thorpe TA. Plant Sci 2001;160:647–57.
[29] Marchant R, Davey MR, Lucas JA. J.B. Plant Sci 1996;120:95–105.
[30] Srinivas L, Ganapathy TR, Suprasanna P, Bapat VA. Indian J Biotech 2006;5:521–6.
[31] Baillie AMR, Rossnagel BGKK. Can J Plant Sci 1992;72:725–33.
[32] Roylance JT, Hill NS, Parrot WA. Crop Sci 1994;34:1369–72.
[33] Carron MP, Dea BG, Tison J, Leconte A, Keli J. Plantation Res Dev 1997;4:271–3.

[34] Youssef M, James A, Mayo-Mosqueds A, Ku-Cauich J, Grijalva-Arrango R, Escobedo-Gm RM. Afr J Biotechnol 2010;9(15):2216–23.
[35] Padua MS, Lima CD, Paiva LV, Barduche D, Santos BR, Stein VC. Rev Cienc Agrar 2015;58:168–75.
[36] Solís-Ramos LY, Nahuath-Dzib S, Andrade-Torres A, Barredo-Pool F, González-Estrada T, Serna ECL. Biologia 2010;65(3):504–11.
[37] Lam E, Frkuda H, Greenberg J. Plant Mol Biol 2000;44:245–53.