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Morphological Development and Ultrastructural Changes During Somatic Embryogenesis of Popular Banana Cultivars

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A B S T R A C T

Somatic embryogenesis based plant regeneration is a pre-requisite for genetic transformation of banana. Mass multiplication of banana through somatic embryogenesis is also viewed as an economically viable alternative to commonly used shoot tip based method. Banana somatic embryogenesis response was studied in three popular banana cultivars, Grand Naine, Rasthali and Hill banana 'Virupakshi'. Immature male flower bud explant was used for embryogenic callus induction. Different morphological types of calli were produced during callus induction that includes both embryogenic and non-embryogenic calli. White translucent pro-embryogenic calli were found to be suitable for establishment of embryogenic cell suspension (ECS). Regeneration potential of ECS was assessed and the development of different stages of embryo *viz.*, globular, scutellum, and coleoptillar stages was observed under scanning electron microscope to understand their ultra-structure. The differences in the duration of developmental stages and regeneration potential could be attributed to the genomic differences of cultivars involved.

Introduction

Banana (*Musa spp.*) is an important fruit crop grown in many developing countries. The global banana production was estimated at around 192 million tons in 2017 (FAO, 2019). Conventionally, bananas are propagated by suckers which may carry numerous disease pathogens including fungus, viruses and nematodes. The multiplication rate of suckers is low and takes a long time to produce a large amount of planting materials (Sagi *et al.*, 1998 and Singh *et al.*, 2011). Alternative to conventional means of propagation is micro-propagation through tissue culture which enables production of a large number of disease-free plants (Drew and Smith, 1990; Sheela and Nair, 2001). Somatic embryogenesis is one of the methods of micro-propagation with high potential multiplication index (Hussein *et al.*, 2006 and Wirakarnain *et al.*, 2008). Plant regeneration from immature male flower bud derived embyrogenic cell suspensions has been reported by several earlier workers and this method appears to be a promising approach

for large scale multiplication (Cöte *et al.*, 1996; Ganapathi *et al.*, 1999, Grapin *et al.*, 1998). Somatic embryogenesis is being widely used *in vitro* mass propagation, germplasm conservation, and genetic enhancement of woody plants (Merkle and Dean, 2000; Chiancone and Germanà, 2013; Ozudogru and Lambardi, 2016). Both somatic embryo and zygotic embryos display similar pattern of developmental stages viz., globular, torpedo, and cotyledonary stages in dicots, or globular, scutellar, and coleoptillar stages in monocots (Mordhorst *et al.*, 1997).

Somatic embryo formation from callus is termed indirect somatic emrbyogenesis, which involves a multi-step process starting with a pro-embryogenic mass (PEM), followed by somatic embryo development, maturation, and germination (von Arnold *et al.*, 2002). The present study is an attempt to characterize somatic embryogenesis process in three popular banana cultivars grown in Tamil Nadu *viz*., Grand Naine (Cavendish group, AAA), Rasthali (Silk-sub group, AAB) and Hill banana 'Virupakshi' (Pome group, AAB). While the cv. Grand Naine offers high yield, the cvs Rasthali and Hill banana 'Virupakshi' are popular due to their unique flavor and taste.

Materials and Methods

The immature male inflorescences of banana cultivars 'Grand Naine' and 'Rasthali', were collected from University orchard while the male buds of cv. 'Virupakshi' was collected from Horticultural Research Station, Thadiyankudisai and used as explants. The male inflorescence (10 weeks after of shooting) was cut using sharp knife at 15 cm below the developing fruit hands. From the male inflorescence, several outer layers of bracts were removed and base of the stalk was cut until 15 cm using sterile scalpel. Then explants were disinfected using 70 % ethanol for a minute followed by washing with sterile water thrice under aseptic conditions. The floral primordia were excised by removing inner layer of bract whorl from positioned 16th to 1st bract whorl from terminal position of the inflorescence of immature male flower buds and used for initiating callus cultures. A total of 106, 100 and 102 male flower buds of Rasthali', 'Grand Naine' and 'Hill banana 'Virupakshi'' respectively were inoculated in callus induction medium [MS basal salt (Murashige and Skoog, 1962) containing 2, 4-D 4.0 mg L^{-1}, IAA 1.0 mg L^{-1}, NAA 1.0 mg L^{-1}, Biotin 1 mg L^{-1}, Ascorbic acid 10 mg L^{-1}, Sucrose 30 g L^{-1}, Agarose 7 g L^{-1}]. The cultures were incubated at 26 ± 2 ºC under dark condition. Sub-culturing was done after 3 weeks of inoculation and the cultures were maintained in same media without sub-culturing until embryogenic callus was induced. After 3-6 months of incubation, the cultures were observed for developmental morphology of embryogenic and non-embryogenic calli under stereomicroscope. Observations on influence of different types of calli were carried out to find out suitable callus type for embryogenic callus development. In the present study, observations on days taken for callus induction, days taken for embryogenic callus development, callus induction frequency (CIF), embryogenic callus frequency (ECF), callus mortality (%) were carried out.

The callus induction frequency (CIF) was determined by number of explants produced callus /number of explants inoculated X 100, embryogenic calli frequency (ECF-calculated by number embryogenic calli developed / total numbers of callus produced X 100) were recorded. During the developmental period of callus, different types of calli were morphologically characterized based on colour and texture under a Stereomicroscope (Leica, Switzerland) to identify embryogenic and non-embryogenic calli.

Different stages of somatic embryo development were also observed in Scanning Electron microscope (SEM, Model: Quanta 250, FEI) with operating voltage at 20 kV and various magnifications. The samples were fixed in ½ Karnovsky solution [glutaraldehyde (2.5%) and paraformaldehyde (1.6%) in cacodylate buffer (0.2 M)], pH 7.2 for 24 h at room temperature. The samples were then washed in 0.05 M cacodylate buffer (three times, 10 min interval) and subsequently immersed in 1% osmium tetroxide for 4 h.

The samples were dehydrated by serially washing in different concentrations of acetone (30 %, 50 %, 70 %, 90 % and 100%) and were dried using the critical-point method CPD 030 using liquid CO_2. Prior to imaging, a thin layer of gold was sputtered over the samples to get better resolution by EMITECH sputter coater. Each sample was attached to the SEM stub with the help of double coated conducting carbon tape. Descriptive analysis statistical tool available in MS Excel programme was used to calculate the mean and standard error.

Results and Discussion

Formation of callus began a month after inoculation of immature male floral whorls in callus induction medium (Fig. 1) and subsequently callus started to proliferate actively. After 3 months of culturing, embryogenic calli started to develop and continue to proliferate till formation of pro-embryos. The calli continued to proliferate after 6 to 7 months of culturing and different morphological types of calluses such as yellow nodular, white compact, embryogenic calli, non-embryogenic calli, watery and brown calli were observed in all three cultivars (Fig. 2). Similar observations were also recorded by Ribeiro *et al.,* (2012) and Padua *et al.*,(2015) in banana cv. Prata-Anã; and Youssef *et al.,* (2010) in Cavendish banana cultivars (Grand Naine' and 'Williams).

The white translucent callus containing pro-embryogenic mass on the surface was considered as embryogenic calli and used for establishment of cell suspension cultures as suggested by earlier workers (Ganapathi *et al.,* 1999; Cöte *et al.,* 1996; Khalil *et al.,* 2002; Morais-Lino *et al.,* 2016; Kumaravel *et al.,* 2017; Vargas *et al.,* 2018; Enriquz-Valencia *et al.,* 2018).

Among the three cultivars studied, Grand Naine had taken fewer days (81.28 days) for callus induction followed by Rasthali (85.80 days) and Hill banana 'Virupakshi' (91.76 days) (Table 1). In case of callus induction frequency (CIF %) and embryogenic calli frequency (ECF %), Grand Naine had recorded higher frequency than Rasthali and Hill banana 'Virupakshi'. For embryogenic calli development, Grand Naine took shorter duration compared to Rasthali and Hill Banana. Callus mortality percentage was also found to be low in case of cv. Grand Naine.

Different developmental stages of somatic embryogenesis in three cultivars of banana were observed through scanning electron microscope (SEM) (Fig. 3).

In this present study, white translucent pro-embryogenic mass cells formed globular, scutella and coleoptillar stages of embryos during process of somatic embryogenesis.

After the globular stage development, structure like club-shaped embryo formation was observed during the transition stage of embryo development Enriquz-Valencia *et al.*, (2018) also noticed globular, pear-shaped and early and late coleptillar embryos showing emergence of plumule in banana cv. Manzana under scanning electron microscope.

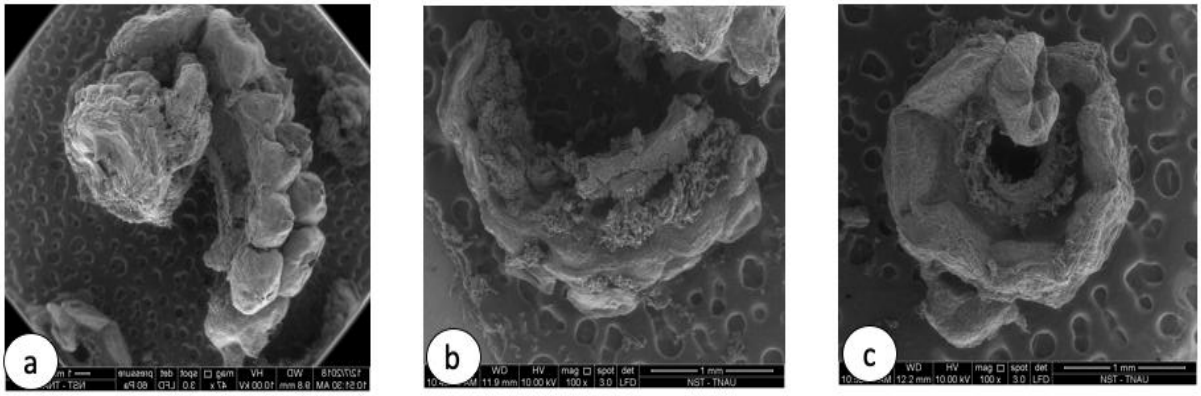


Fig. 1. One-month old scanning electron micrograph of immature male floral whorls [a. Grand Naine, b. Rasthali, and c. Hill banana 'Virupakshi']

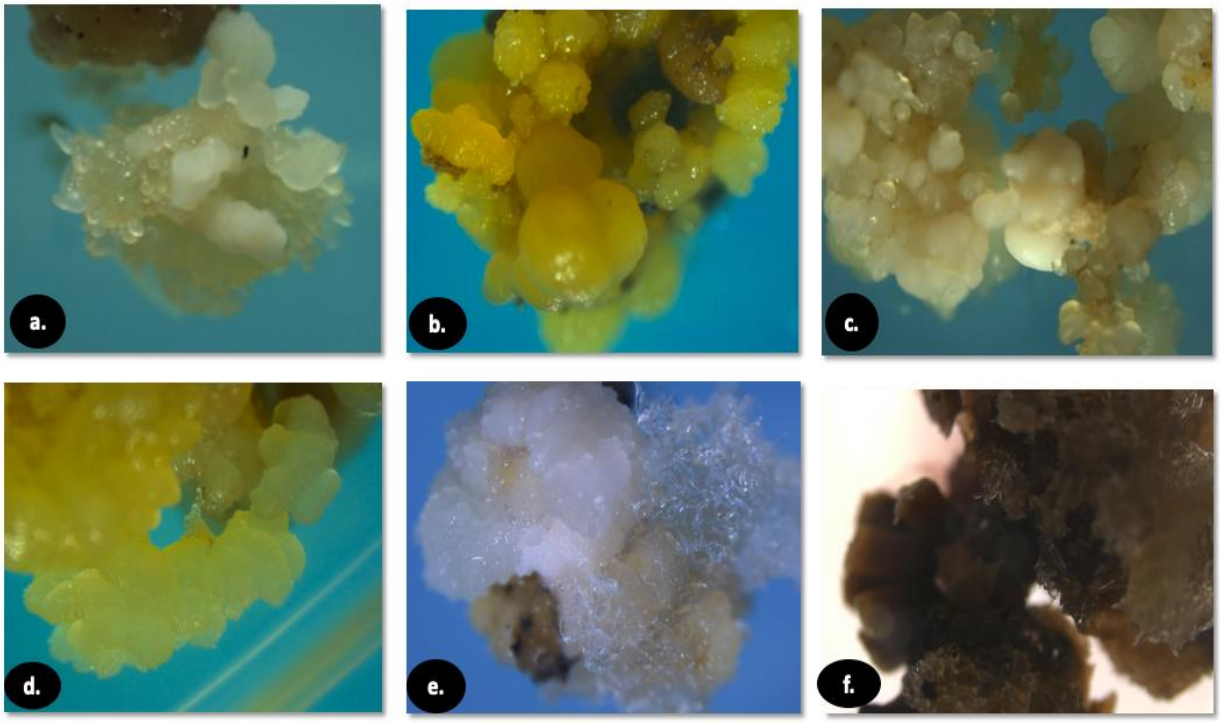
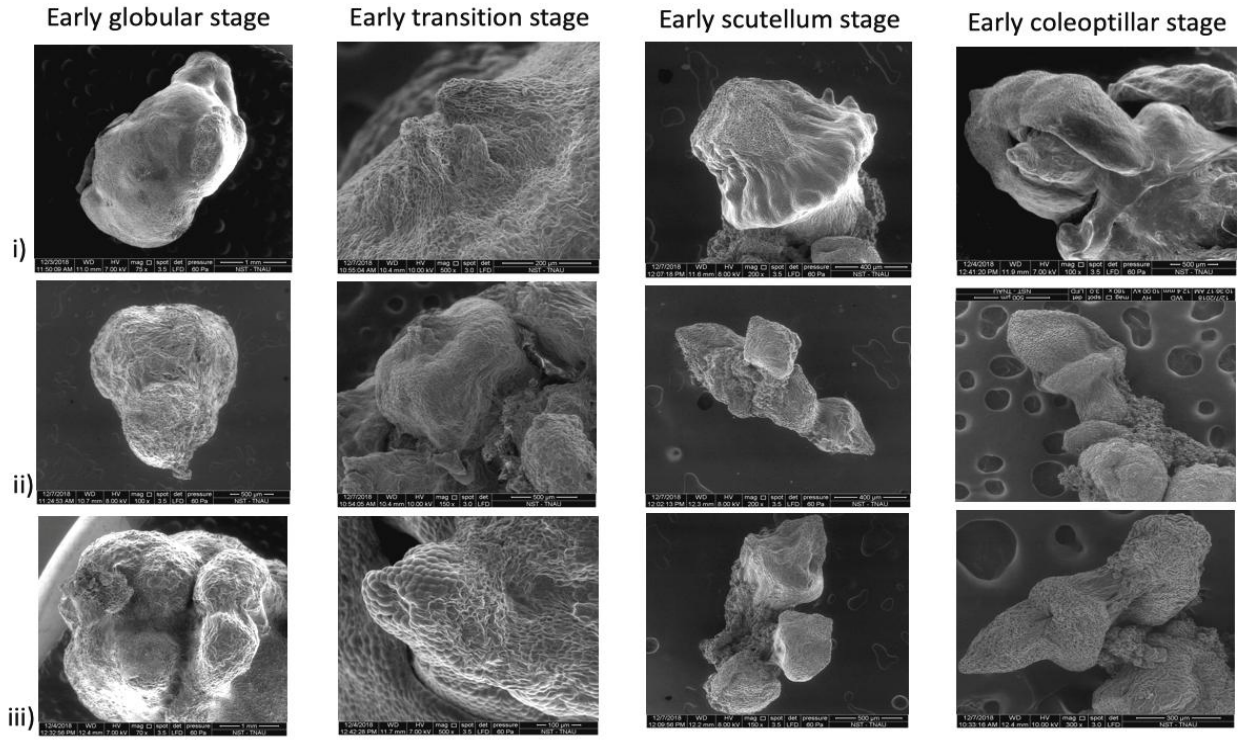


Fig. 2. Types of callus derived from immature male flower buds [a. Embryogenic callus, b. yellow nodular callus, c. white compact callus, d. non-embryogenic callus, e. watery callus and f. brown callus]

Fig.3 Micrograph of different developmental stages of somatic embryogenesis of banana cultivars observed under scanning electron microscope [i) Grand Naine; ii) Rasthali and iii) Hill banana 'Virupakshi']



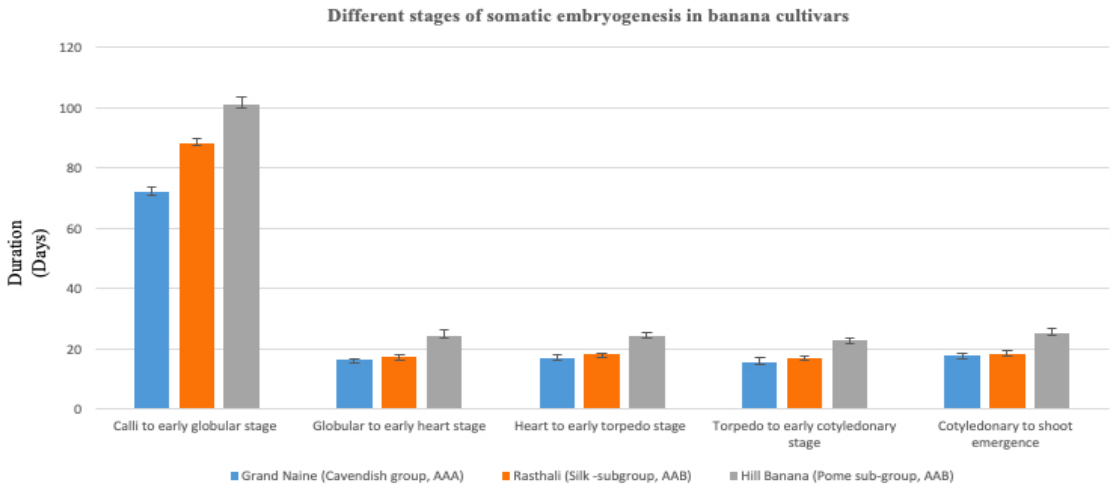


Fig. 4. Different stages of somatic embryogenesis in banana

Table.1 Duration and induction frequency of somatic embryogenesis of banana cultivars

Observations	Grand Naine (Cavendish group, AAA)	Rasthali (Silk - subgroup, AAB)	Hill banana 'Virupakshi' (Pome sub-group, AAB)
	Mean ± SE	Mean ± SE	Mean ± SE
Days taken for embryogenic callus induction	81.28 ± 1.30	85.80 ± 1.48	91.76 ± 3.85
Callus induction frequency (%)	89.84 ± 1.83	87.62 ± 5.02	86.15 ± 5.86
Embryogenic calli frequency (%)	7.99 ± 3.16	7.57 ± 2.48	5.67 ± 2.30
Days taken for EC development	151.05 ± 3.24	177.93 ± 2.20	205.46 ± 2.58
Callus mortality (%)	10.16 ± 1.83	12.38 ± 5.02	13.85 ± 5.86

A high degree of variation of embryo formation was observed in banana cultivars (Mordhorst *et al.,* 1997). Monocot crops produce spherical or club-shaped embryos without any tissue or organ differentiation (Johnson, 1945). Development of special structures such as scutellum, coleoptile and presence of several leaf primordial was also reported in crops like Maize (Van Lammeren, 1986) and barley *(Hordeum vulgare;* Norstog, 1972; Engell, 1989). The duration of different developmental stages of somatic embryogenesis of Grand Naine, Rasthali and Hill banana 'Virupakshi' is presented in table 1; Fig. 4). The duration for development of calli to globular stage, globular to transition stage, early scutellar stage and early coleptillar stage, coleoptillar stage to early shoot emergence was found to be relatively shorter in cv. Grand Naine (having AAA genome) as compared to the cvs Rasthali and Hill banana 'Virupakshi' which have AAB genome in their genetic makeup.

Conclusion

The present study indicates that there are differences in the duration taken for somatic embryogenesis in banana cultivars. It could be observed that presence of 'B' genome (*Musa balbisiana*) delays the process as well as reduces the frequency of embryogenic calli formation in cvs Hill banana 'Virupakshi' (AAB) and Rasthali (AAB). The study confirmed the earlier findings that it is necessary to select suitable calli for enhanced somatic embryogenesis. Further it is necessary to suitably modify the protocols for development of reliable protocols for enhancing somatic embryogenesis and ECS cultures for commercial banana genotypes such as Rasthali and Hill Banana having *Musa balbisiana* geneome. Although the somatic embryogenesis frequency was low in the cv. Hill banana 'Virupakshi', the possibility offers scope for development of BBTV resistant 'Virupakshi's through genetic transformation of somatic embryos.

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