Multiple shoot induction in zygotic embryos: a strategy for acceleration of banana breeding

Suthanthiram Backiyarani1 · Subbaraya Uma1 · Swaminathan Saranya1 · Palani Durai1 · Selvaraj Eugin Perianayagaraj1 · Vadivel Selvaraj1 · Marimuthu Somasundaram Saraswathi1 · Raju Karthic1 · Sathiamoorthy Kalpana1

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Abstract
The presence of residual female fertility in most of the parthenocarpic banana accessions encourages the banana breeder to develop new hybrids through conventional breeding. Desirable trait can be fixed in the first generation of hybrid progenies, but the evaluation of these hybrids in field is the time-consuming process owing to non-availability of uniform suckers/planting material. This can be overcome by developing multiple shoots from single embryo in a short period of time through embryo culture. A protocol for in vitro multiplication of plantlets from zygotic embryos was standardized in seeded accessions. Multiple shoots from zygotic embryos were achieved up to 55.2% and 64.1% in seeded accessions of Musa acuminata and Musa velutina respectively in medium supplemented with 17.76 µM of BAP. The Single shoot derived (only germination) from zygotic embryos was decapitated and the apical meristem were disturbed for further multiple shoot formation in media supplemented with 17.76 µM of BAP. Present studies revealed that in total 75% and 91% of the Musa acuminata and Musa velutina embryos were able to produce multiple shoot from single embryo by manipulating the media composition and decortications technique. The above protocol was applied for zygotic embryos obtained from controlled pollination (18 cross combinations) and open pollination (nine accessions) of various genomic groups (ABB, AAB, AA). The multiple shoots derived from zygotic embryos and plantlet germinated from zygotic embryos was examined for genetic fidelity analysis by SSR markers.

Key message
The protocol for multiple shoot formation from single zygotic embryo under in vitro culture developed in this study will accelerate the banana breeding program.

Keywords Banana breeding · Embryo culture · Regeneration efficiency · Multiple shoots · Decortications

Introduction
Polyploidy and sterility are the major constraints in the improvement of banana and plantain through conventional breeding approach (Laliberté 2016). Although, cultivated banana is a vegetatively propagated crop, the seeded nature of its ancestors and frequent occurrence of mutations in the natural habitats led to the occurrence of broad banana diversity which is being exploited by hybridization program. Some of the commercial cultivars exhibit low residual fertility to some extent possibly due to seeded nature of the predecessors and transfer of this trait through few gametes having complete set of ancestral genome through balanced chromosomal segregation (Heslop-Harrison and Trude Schwarzacher 2007). Developing parthenocarpic varieties with desirable traits has been possible in banana due to occurrence of 2n gametes followed by hybridization (Ortiz 1997). Thus low rate of residual fertility and the occurrence of unreduced gametes offer the scope for the banana breeder to improve the commercial varieties (Ortiz...
This has resulted in releasing many banana hybrids by FHIA, Honduras (Rowe and Rosales 1993) International Institute of Tropical Agriculture, Nigeria and Kenya (Crouch et al. 1999); EMBRAPA, Brazil (Ferreira et al. 2004) and ICAR-NRCB, Tamil Nadu Agricultural University, and KAU-Banana Research Station, India (Uma et al. 2015).

Although residual female fertility is a desirable trait, the poor rate of seed set (Shepherd et al. 1987) and germination (Andrus et al. 1971) limit the chance of developing large number of commercial hybrids. The complicated genetic system of banana with heterozygocity and polyploidy, causes the concurrence of abnormal meiotic recombination or coming togetherness of different sets of chromosomes which resulted in poor seed set and poor germination. It was also reported that an abnormal embryo-endosperm relationship and impermeability of seed coat, along with chemical barriers like growth inhibitor-induced dormancy etc. cause poor germination percentage of hybrid banana seeds (Simmonds 1962; Uma et al. 2011). The problem of seed set can be overcome by choosing the compatible male and female parents, optimal stage and time for pollination and by spraying of antiauxins on the flower bud (Backiyarani et al. 2016). The problem of seed germination can be overcome through embryo culture and embryo rescue (Vuylescke et al. 1990), but the embryo maturity, culture medium (Johri and Rao 1984), positioning of embryo in the culture medium, allelic combination of hybrid embryos are significantly affect the germination rate (Asif et al. 2001). It has been reported that seed germination can be enhanced up to 10% and 30% by supplementing the media with plant growth regulators (Diro and Van Staden 2003; Uma et al. 2011) and combination of seed treatment with GA3 and supplementing with growth regulators (Arun et al. 2013) respectively, but it should be further enhanced for attaining better success in banana breeding (Batte et al. 2019). Apart from the seed germination, hardening of these embryo derived plantlets is another big task due to poor acclimatization, the open pollinated seeds of two seeded accessions of Musa species namely M. acuminata and M. velutina were taken. The best media thus identified for multiple shoot development was tried for seeds derived through cross/ open pollinated commercial cultivars. Hybridization was taken up in the four accessions of ABB genome (Chinia, Karpuravalli, Kothia and Saba) and two accessions of AAB genome (Nendran and Poovan), one Nendran × Pisang Lilin (NPL 33) progeny, one Nendran open pollinated progeny (NOP46) and two accessions of AA genome (Matti, Cultivar Rose) using four male diploid accessions of A genome. Out of four male accessions two were parthenocarpic type (Pisang Lilin, Matti) and two were seeded type (Pisang Jajee, Calcutta 4) (Electronic Supplementary Information: Supplementary Table S1). The open pollinated bunches of triploid accession of ABB genome (Benkela, Boothibale, Chinia, Cuba, EnnaBenian, Saba, Vennuthumannan) and wild diploid accessions of AA genome (Calcutta 4 and Microcarpa) were also taken.

Hybridization among different genomic group

The whole inflorescence of the female parents was bagged before the opening of the first hand of female phase. Simultaneously the male phase of each male parent was bagged immediately after the completion of neutral phase. Each day the pollen grains were collected from the male parents during anthesis stage at 7.00 am. And the collected pollens were dusted over the female flowers which are about to open on that day (i.e., having receptive stigma) of the female parents during 7–10.00 am. Immediately after dusting, the female flower hands were carefully closed using its own bract and then the whole inflorescence was bagged. The next hand of the female flower of the same inflorescence was used for dusting on the following day. Hybridization was carried out in 18 different combinations and each pollinated inflorescence was labeled with tags containing relevant crossing information.
Seed extraction

The artificially and open pollinated bunches were harvested at full matured stage. The days taken for full matured stage (from flowering to harvest) of each female parent is given in supplementary table S2. The bunches were kept in the ripening chamber. The seeds were extracted from the ripened bunches and washed in running tap water. The extracted seeds were soaked in water to separate sunken and floating seeds. Since floating seeds are normally devoid of either endosperm or embryo, sunken seeds alone were subjected to surface sterilization by treating them with 4% sodium hypochlorite for 15 min, followed by dipping in 0.1% mercuric chloride for 15 min. Seeds were rinsed with sterile distilled water upon each treatment for about 2–3 min. In order to break the dormancy, the sterilized seeds were soaked overnight in water supplemented with 10 ppm GA₃ as described by Arun et al (2013). Subsequently debris was removed from the seeds by following the same protocol that was utilized for sterilization. Then seeds were transferred to sterile plate and subjected to embryo dissection.

Embryo initiation and multi shoot formation

To minimize the experimental error, a proper sample size of 100 seeds were taken from the cross combinations that recorded profuse seed setting while among the crosses where seed set was low, all the seeds were utilized for studies. The nature of seeds and embryos of controlled and open pollinated bunches of different accessions are given in the (Electronic Supplementary Information: Supplementary Table S2). A longitudinal fissure was made in GA₃ soaked sunken seeds and the whitish, mushroom- shaped embryo, which consists of a haustorium and a meristematic stalk (Fig. 1b) was excised. The haustorium region of the embryo was placed on the surface of medium. The excised embryos of seeded accessions M. acuminata and M. velutina were initiated in Murashige and Skoog (1962) medium supplemented with various concentration of BAP (4.44 µM, 8.88 µM, 13.32 µM 17.76 µM and 22.2 µM BAP). Initiated embryos were kept in dark condition approximately for a period of 7–10 days until germination took place. The tubes having germinating embryos were shifted to light and dark (16/8-h) condition for regeneration. The regenerated embryos were further subjected for sub culturing in the same medium supplemented with the same BAP concentration. The excised embryos of the seeds collected from artificially and open pollinated bunches of various accessions were placed in the best media composition (medium supplemented with the 17.76 µM BAP concentration) and cultured as per the protocol mentioned above.

Decortications of embryo derived single plantlets

Embryo culture derived single plants were further subjected to decortications to obtain the optimum width of basal shoots. To determine the optimum shoot size, three different sizes of the single plants were selected and the excised shoot tips with the size of 1, 2 and 3 cm³ were decapitated and the apical meristem were disturbed and kept in the same media composition. After three subcultures the proliferated shoots were observed for multiple shoot formation.
Hardening of embryo derived plants

The shoots obtained through direct regeneration from single embryo were sub-cultured in rooting media containing auxin i.e. IBA-4.90 µM and NAA-10.74 µM. After the root formation, the single plantlets were primary hardened in the protray containing the mixture of coir pith with vermicompost. After 45 days, well grown plants were transferred for secondary hardening in a polybag containing a mixture of red soil, vermicompost and coir pith in equal proportion.

Statistical analysis

Experiment on standardization of media for multiple shoot formation was carried out in completely randomized design with three replications per treatment. The germination percentage data was transformed using arc sine transformation and the transformed data were analyzed using SPSS v. 11.09 (IBM Corporation, Armonk, NY, USA). The mean of three replicates of each parameter was expressed as mean ± SD.

Genetic fidelity

Isolation of genomic DNA

Two gram of leaf tissues were collected from cigar leaf (recently emerged leaf which are tightly coiled, whitish, and particularly fragile and still rolled as a cylinder) from the secondary hardened plants (minimum of five plants) of each hybrid and for parents cigar leaf tissues were collected from field grown plants. The leaf tissues were freeze dried using liquid nitrogen and genomic DNA was isolated by cetyl-trimethyl ammonium bromide (CTAB) method (Gawel and Jarret, 1991) with a minor modifications. The isolated DNA was treated with 7 µl RNase (100 mg/ml), and quantified by spectrophotometer at 260 nm and 280 nm and the quality of DNA was checked with the help of agarose gel electrophoresis (0.8%).

PCR amplification

The genetic fidelity of multiple shoots obtained from single embryo was tested through PCR using thirty five in silico polymorphic SSR primers (Electronic Supplementary Information: Supplementary Table S3) retrieved from the Musa transSSRDB- Musa transcriptome derived SSRs database (http://bioinfnrcb.byethost7.com/nrcbbio/) (Backiyarani et al. 2019). Polymerase chain reaction was carried out for a total volume of 10 µl containing 10X PCR buffer, 200 pmol of each forward and reverse primer, 2.5 mM of dNTPs, 0.5 unit of Taq polymerase, 100 ng of DNA plus nuclease free water to make up to the final volume. Amplification was performed on a programmable thermocycler.
Biosystem) with a condition of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at the corresponding annealing temperature for the respective primers employed for 30 s and extension at 72 °C for 1 min, with a final step of final extension at 72 °C for 10 min. PCR products were loaded on 3% agarose gel and the gel was documented using gel doc system (Gelstan, medicare).

Results
To standardize the multiple shoot production in embryo culture, MS media with different concentration of BAP were tried in two seeded accessions of Musa species, *M. acuminata* and *M. velutina*. Irrespective of the species, maximum germination and regeneration percentage were observed in media supplemented with 17.76 µM BAP concentration and the minimum response was observed in 4.44 µM BAP (Table 1). The embryo germination was observed within 3–10 days after initiation irrespective of the BAP concentrations in both *M. acuminata* and *M. velutina*. Early proliferation of shoots was observed in the media composition of MS with 17.76 µM BAP (15–20 days) compared to other concentrations. No multiple shoot formation was obtained in the BAP concentration of 4.44 µM and 8.88 µM in *M. acuminata*, whereas in case of *M. velutina* it was noticed at 8.88 µM. Minimum of five multiple shoots in both the species and the maximum of nine and seven multiple shoots were obtained in *M. acuminata* and *M. velutina* respectively and the highest percentage of multiple shoot formation was observed at 17.76 µM BAP concentration in both the species (55.2% and 64.1%). Manipulation of media composition could not achieve multiple shoot development in all the embryos cultured and it was found that 44.8% and 35.9% of the embryos produced single plantlets in *M. acuminata* and *M. velutina* respectively.

To produce multiple shoots from the single plant developed from embryo, three different sizes (‘A’ type: 3 cm³ girth; ‘B’ type: 2 cm³ and ‘C’ type: 1 cm³) of embryo cultured single plants were used as explants and subjected to initiation of shoot tip culture. Proliferation has been noticed within 30–35 days of sub culturing. The proliferated explants were again sub cultured and an average of seven plantlets were obtained (maximum of ten) among type A followed by ‘B’ type with an average of 5. Continuous sub culture resulted in enhancing the shoot formation and in the third sub culture, highest multiple shoots were obtained in ‘A’ type (10) followed by ‘B’ type (7) but no proliferation was obtained among ‘C’ type explants which only resulted in single plant development. This study revealed that in vitro plantlets with the stem girth of 3 cm³ and 2 cm³ are suitable explants sizes for the production of multiple shoots. It was observed that multiple shoots were induced in 52% and 75% of embryo derived single plantlets of *M. acuminata* and *M. velutina* from the decortication of ‘A’ type explants.

To understand the response to multiple shoot formation in the breeding material, sunken seeds obtained from 18 different cross combinations of different ploidy and genomic group such as AA × AA (2), AAB × AA (9) and ABB × AA (7) and open pollinated seeds of nine different genotypes such as ABB (7) and AA (2) were subjected to embryo culture in the media composition of MS with 17.76 µM BAP.

Minimum and maximum embryo germination percentage was recorded in Matti × Cultivar Rose (23.08%) and Cultivar Rose × Calcutta 4 (84.62%) respectively under controlled pollination whereas in open pollination it ranged from 16.7% (M.ac. ssp. microcarpa) to 80% (Calcutta 4 and Chinia). The germination percentage in the cross combinations 3x × 2x of various genomic groups ranged from 33.3% (Nendran × Pisang Jajee) to 80% (Chinia × Pisang Jajee) (Table 2).

Interestingly 100% regeneration was observed from germinated embryos of most of the cross combinations except in Karpuravalli × Cultivar Rose (50.0%), Chinia × Pisang Jajee (55%), Nendran × Cultivar Rose (66.7%), Cultivar Rose × Calcutta 4 (72.73%) and Kothia × Calcutta 4 (76.92%). Similarly 100% regeneration was observed in all the open pollinated germinated embryos except Enna Bennian (60%) and Veenut Mannan (50%). One month after embryo initiation, the regenerated plants were sub-cultured in the same media composition.

The multiple shoot formation from regenerated embryos varied from 36.36 (Chinia × Pisang Jajee) to 100% (Nendran × Pisang Jajee, Nendran × Pisang Lilin and NPL33 × Pisang Lilin). Among seeds obtained under natural pollination, it ranged from 50 (Saba and Venntutmannan) to 100% (Bankela, *M.ac. ssp. microcarpa*, Cuba) (Table 3). To generate multiple shoots from the remnant single plant derived from single embryo, 3-4 cm girth shoot of these in vitro plantlets were used as explants for decortications which resulted in multiple shoot induction with the range of 50–100% in controlled pollination and 66.67–100% in the open pollination.

Genetic fidelity test of the multiple shoots derived from single embryo of each cross was carried out using in silico polymorphic primers. The parental polymorphic primers were detected by testing against the parents of the each cross combination using 35 primers. Of which 20 showed polymorphism among the parents used for controlled pollination for the respective cross combinations. All the primers showed monomorphic bands for the multiple shoots derived from each embryo irrespective of origin, whether control or open pollinated seeds.
Discussion

The success of any conventional breeding program depends on the production of vast amount of recombinant events among the compatible parents through sexual hybridization. Banana is a recalcitrant crop for sexual hybridization owing to its polyploidy, female and/or male sterility. In spite of these hurdles, owing to its residual female fertility natural seed set has been observed in some of the parthenocarpic genotypes paving way to improve the bananas through hybridization techniques (Uma et al. 2011; Ortiz et al. 1995). Still, the success rate of banana breeding is slow owing to low germination percentage of hybrid seeds (Batte et al. 2019).

Table 2 Efficiency of control pollination with respect to embryo formation, germination, regeneration and multiple shoot formation

| Cross combinations of control pollination | Percentages of Sunken seeds having embryos | Embryo germination | Plant regeneration from germinated embryos | Plant regeneration from sunken seeds | Direct multiple shoot formation | Multiple shoot formation through decortications | Total percentage of multiple shoots |
|------------------------------------------|------------------------------------------|-------------------|--------------------------------------------|----------------------------------|-------------------------------|---------------------------------------------|----------------------------------|
| Chinia × Pisang Jajee                   | 65.79                                    | 80.00             | 55.00                                      | 44.00                            | 36.36                         | 71.43                                       | 81.82                            |
| Karpuravalli × Calcutta 4               | 66.67                                    | 58.33             | 100.00                                     | 58.33                            | 71.43                         | 100.00                                     | 100.00                           |
| Karpuravalli × Cultivar Rose            | 60.71                                    | 70.59             | 50.00                                      | 35.29                            | 50.00                         | 66.67                                       | 83.33                            |
| Karpuravalli × Pisang Jajee             | 34.15                                    | 57.14             | 100.00                                     | 57.14                            | 37.50                         | 60.00                                       | 75.00                            |
| Kothia × Calcutta 4                     | 60.00                                    | 54.17             | 76.92                                      | 41.67                            | 40.00                         | 50.00                                       | 70.00                            |
| Saba × Calcutta 4                       | 52.27                                    | 34.78             | 100.00                                     | 34.78                            | 37.50                         | 60.00                                       | 75.00                            |
| Saba × Pisang Lilin                     | 42.37                                    | 40.00             | 100.00                                     | 40.00                            | 60.00                         | 50.00                                       | 80.00                            |
| Nendran × Pisang Jajee                  | 52.94                                    | 33.33             | 100.00                                     | 33.33                            | 100.00                        | -                                           | 100.00                           |
| Nendran × Calcutta 4                    | 66.67                                    | 50.00             | 100.00                                     | 50.00                            | 66.67                         | 100.00                                      | 100.00                           |
| Nendran × Cultivar Rose                 | 62.50                                    | 60.00             | 66.67                                      | 40.00                            | 50.00                         | 100.00                                      | 100.00                           |
| Nendran × Pisang Lilin                  | 47.06                                    | 50.00             | 100.00                                     | 50.00                            | 100.00                        | -                                           | 100.00                           |
| NOP 46 × Calcutta 4                     | 60.00                                    | 44.44             | 100.00                                     | 44.44                            | 75.00                         | -                                           | 75.00                            |
| NPL33 × Calcutta 4                      | 50.00                                    | 40.00             | 100.00                                     | 40.00                            | 50.00                         | 100.00                                      | 100.00                           |
| NPL33 × Pisang Lilin                    | 44.44                                    | 50.00             | 100.00                                     | 50.00                            | 100.00                        | -                                           | 100.00                           |
| Poovan × Calcutta 4                     | 50.00                                    | 60.00             | 100.00                                     | 60.00                            | 66.67                         | -                                           | 66.67                            |
| Poovan × Pisang Lilin                   | 19.15                                    | 66.67             | 100.00                                     | 66.67                            | 50.00                         | 100.00                                      | 100.00                           |
| Cultivar Rose × Calcutta 4              | 59.09                                    | 84.62             | 72.73                                      | 61.54                            | 62.50                         | 66.67                                       | 87.50                            |
| Matti × Cultivar Rose                   | 61.90                                    | 23.08             | 100.00                                     | 23.08                            | 66.67                         | 100.00                                      | 100.00                           |
| Mean                                     | 53.10                                    | 53.18             | 90.07                                      | 46.13                            | 62.24                         | 60.28                                       | 88.57                            |
| Standard deviation                      | 12.47                                    | 16.13             | 17.39                                      | 11.50                            | 21.11                         | 39.07                                       | 12.60                            |

“-” indicates samples were not subjected to decortications as all the embryos produced multiple shoots
Direct sowing of hybrid seeds in potting mixture resulted in poor germination (1–1.4%) (Swennen et al. 1992; Talengera et al. 1996) with germination extending over a period of 12 months (Uma 2015). The slower and low germination percentage is due to water repellent substances present in the cell walls of the mesotesta (Guan 1991) and the presence of polyphenolic substances which hinder the diffusion of oxygen through the seed coat (Burgos-Hernández et al. 2014). The germination percentage can be improved either by extracting the seeds at the right maturity (Uma et al. 2011) and/or by using stratification and scarification methods (Wattanachaiyingcharoen 1990). But through these approaches, germination percentage could be enhanced only up to 4–16% in wild species (Burgos-Hernández et al. 2014). The germination percentage can be improved either by extracting the seeds at the right maturity (Uma et al. 2011) and/or by using stratification and scarification methods (Wattanachaiyingcharoen 1990). But through these approaches, germination percentage could be enhanced only up to 4–16% in wild species (Burgos-Hernández et al. 2014). While Ahmed et al. (2006) and Dayarani et al. (2014) reported that it can be enhanced up to 40–60% through embryo culture of wild seeds under in vitro conditions. Asif and Othman (2001) reported that apart from enhancing the seed germination, the germination period could also be reduced from 50 days to one week through in vitro embryo culture over direct seed germination.

Survival of plantlets derived from hybrid embryo is also an important factor for the success of banana breeding program. The risk of mortality of the embryo derived plants can be reduced by developing multiple plantlets from a single embryo. This indirectly helps to maintain all the regenerated hybrid events and facilitate to evaluate large number of heterogeneous population in one go which is essential for the selection of best recombinant variants. Uma et al. (2012) developed multiple shoot formation protocol from immature embryos of banana through embryo rescue via callus induction. Similarly, many researchers evidenced that plantlets could be produced through indirect regeneration from the mature zygotic embryos by media augmentation with high 2, 4, D and IAA in various Musa species such as M. ornata (Dayarani et al. 2014) and M. acuminata (Uma et al. 2011) respectively. Sivanesan (2007) and Marco A. Ramírez and Lourdes (2015) reported that plantlets regenerated through indirect organogenesis, showed high genetic and epigenetic variations (Grafi and Barak 2014). Varshney et al. (2001) also reported that the indirect regeneration involving a callus phase is more vulnerable to DNA damage during micro-propagation which led to higher frequency of somaclonal variation (Peschke and Phillips 1992). The occurrence of somaclonal variation could be reduced by avoiding the long-term culture and using axillary shoot induction systems. Zapata et al. (1999) evidenced that direct regeneration is a faster and a time saving approach for obtaining whole plants without the callus interphase. Among the purine type of cytokinin, BAP is preferred to for its stable nature (Klem et al. 2004) and effect in stimulation of multiple shoots (Kadota and Niimi 2003).

**Multiple shoot formation from single embryo**

Variation was observed among the two seeded accessions for days taken for germination. M. acuminata showed early response (3–7 days) than compared with that of M. velutina (3–10 days). Though no specific trend was observed for the days taken for germination with different concentrations of BAP, enhanced germination was noticed with the increased BAP concentrations, in both the species. Similar result was

| Open pollinated accessions | Percentages of | Sunken seeds having embryos | Embryo germination | Plant regeneration from germinated embryos | Plant regeneration from sunken seeds | Direct multiple shoot formation | Multiple shoot formation through decortication | Total percentage of multiple shoots |
|---------------------------|----------------|-----------------------------|-------------------|---------------------------------|---------------------------------|--------------------------------|------------------------------------------|----------------------------------|
| Bankela                   | 60.00          | 33.33                       | 100.00            | 33.33                           | 100.00                          | -                             | -                                          | 100.00                           |
| Boothibale                | 19.05          | 62.50                       | 100.00            | 62.50                           | 60.00                           | 100.00                        | 100.00                                    | 100.00                           |
| Chinia 0087               | 23.26          | 80.00                       | 100.00            | 80.00                           | 62.5                            | 66.67                         | 87.5                                       |                                  |
| Saba                      | 84.38          | 44.44                       | 100.00            | 44.44                           | 50.00                           | 66.67                         | 83.33                                      |                                  |
| Calcutta 4                | 66.67          | 80.00                       | 100.00            | 80.00                           | 62.5                            | 66.67                         | 87.5                                       |                                  |
| Microcarpa                | 60.00          | 16.67                       | 100.00            | 16.67                           | 100.00                          | -                             | 100.00                                    |                                  |
| Cuba                      | 25.00          | 20.00                       | 100.00            | 20.00                           | 100.00                          | -                             | 100.00                                    |                                  |
| VeenutMannan              | 50.00          | 50.00                       | 50.00             | 25.00                           | 50.00                           | 100.00                        | 100.00                                    |                                  |
| Ennanberman               | 32.14          | 55.56                       | 60.00             | 33.33                           | 66.67                           | 100.00                        | 100.00                                    |                                  |
| Mean                      | 46.72          | 49.17                       | 90.00             | 43.92                           | 72.41                           | 55.57                         | 95.37                                     |                                  |
| Standard deviation        | 22.87          | 23.19                       | 20.00             | 24.61                           | 21.42                           | 44.10                         | 7.05                                      |                                  |

“-“ indicates samples were not subjected to decortications as all the embryos produced multiple shoots
also reported by in *Aconitum hererophyllum* (Pandey et al. 2000) and *Amaranthus* sp. (Tiryaki et al. 2009). The supplementation of BAP in the media composition enhances the germination percentage as it is involved metabolically in the stimulation of germination (Dissanayaka et al. 2015) and reduces the chances of mortality as it provides a higher competitive ability (Zhang & Maun 1990).

Franklin et al. (2000) reported that, incubation of embryo in dark followed by light conditions is a prerequisite for the induction of morphogenic potential of cells and plantlets formation (regeneration) respectively. Thus, the germinated culture was shifted to light and dark (16/8-h) condition and shoot elongation was noticed from third day on wards. Proliferation of shoots was observed earlier in the media composition of MS with BAP 17.76 µM (15–20 days) compared to other concentrations. In the present investigation, the normal shoot elongation was observed in all the BAP concentrations except in 22.2 µM where the elongation rate was reduced. Chang et al. (1992) also reported that higher BAP concentration stimulated higher shoot formation but inhibiting its further development.

Interestingly number of multiple shoot increased with increased concentration of BAP up to MS + 17.76 µM and beyond which it is reduced. In this, multiple shoots emerged directly from embryo without any intermediate callus tissue. Similar trend of multiple shoot formation as in the present study were also observed with embryos of barley (Ganesan et al. 2003), rice (Zhang et al. 1996, 1998) groundnut (Palanivelet al. 2002) and papaya (Bhattacharya et al. 2003). Many reports reported that meristematic cells of germinated embryos have the ability to produce multiple shoots under the higher concentration of cytokinins (Sobhakumari and Lalithakumari 2003; Rao et al. 2006), but rosette of distorted leaves with up-normal growth of shoots was observed in higher concentration of BAP (22.2 µM BAP) in *M. acuminate* and *M. velutina*. Maximum of nine shoots with an average of seven shoots from single embryo was obtained in *M. acuminate* and seven shoots with an average of six shoot were obtained in *M. velutina* in the media supplemented with 17.76 µM BAP.

Two types of multiple shoot formation were observed in this study. In the first type, primary apical meristem shoot was developed from the haustorium region of embryo and from these started the initiation of the first leaf. Then 3–4 secondary shoots arose simultaneously around the primary meristem from the same haustorium region (Fig. 1e). In the second type, embryos produce shoots simultaneously and produce very short shoots with big leaves which resulted in a rosette appearance. And normal shoot production was observed by continuous sub culturing in the same media composition. This variation in pattern of multiple shoot formation might be due to the genotype effect i.e. variation in the level of endogenous hormone (Venkatachalam et al. 1999).

Interestingly, except in MS media supplemented with 22.2 µM BAP, root initiation was also observed in all other concentrations. The elongated shoots were excised and subcultured onto MS medium supplemented with the auxin IBA-4.90 µM and NAA-10.74 µM. Overall the present experiment suggested that 17.76 µM BAP is the optimum concentration not only for enhancing the germination and regeneration efficiency but for multiple shoot production too in the short span of time. This phenomenon has been applied for the production of multiple shoots/embryo in NRCB breeding program.
Induction of multiple shoots from embryo derived single plantlets

It was observed that through media manipulation technique, 44.8% and 35.9% of the embryos produced single plantlets in *M. acuminata* and *M. velutina* respectively. Thus, to develop multiple shoots from a single shoot, bud manipulation technique, decapitation of the apical meristem, was performed to remove apical dominance. Suman (2017) reported that the success of shoot tip culture mainly depends on the final size of the explants. But the size of the single plantlets which is ready for primary hardening after removing the sheath is very small (1 cm³) which is not suitable for decortications. Hence to increase the size of the explants, embryo derived single plantlets were subjected to three sub cultures in the same media composition of MS + 17.76 µM BAP. Three different sizes of explants were subjected to decortications followed by sub cultures for the production of shoots and the results revealed that stem girth of diameter 3 cm³ and 2 cm³ width in vitro derived plantlets are suitable size explants for the production of multiple shoots. It was observed that multiple shoots were induced in 52% and 75% of embryo derived single plantlets of *M. acuminata* and *M. velutina* respectively from decortications of A type explants (Data not shown). Both these studies revealed that in total 75% and 91% of the *M. acuminata* and *M. velutina* embryos were able to produce multiple shoot from single embryo by manipulating the media composition and decortications technique (Fig. 2).

Normally for evaluation of hybrid progenies for a specific trait, it needs a minimum of five uniform suckers which could be obtained only after 18–24 months of field planting. Uniform size plantlets of hybrid progenies will be available, if the multiple shoots are regenerated through direct regeneration from embryo culture. Within 6–7 months the hybrid progenies will be ready for field evaluation under replication trial whereas it will take 10–11 months if decortications also involved. These results imply that this technique will reduce the duration of breeding programme by 9–12 months.

Understanding the response of various genomic groups for seed germination and multiple shoot formation

The embryos of open and controlled pollinated seeds were initiated and bulging was observed in 56% of embryos, which confirmed their viable status while 44% failed to show any growth induction in terms of size, colour (green) and callus development and turn into black color. Similar result has also been recorded in cucumber (Ali et al. 1991). The non-germination of embryos i.e. lethality of hybrid seed has been attributed due to interploidy crosses (Scott et al. 1998; Köhler et al. 2003). The low germination percentage in the hybrids seeds might be due to different type of genetic imbalance owing to chromosome mismatch of male and female gametes (Simmonds 1962). This may be occurred due to variation in the chromosome structural heterozygosity, ploidy among the intra specific and inter specific genomic groups (González de León and Fauré 1993). In general, irrespective of the genomic group, higher embryo germination and regeneration percentage was observed in the control pollinated seeds than the open pollinated ones. This could mean that the chances of union of compatible gametes are more in case of controlled pollination as the male parents are highly polleniferous and diploid in nature. The occurrence of variation in the regeneration ability of embryos derived from the same and various genomic groups used for crossing with diploids revealed that reproductive and regeneration potential are not only the genome specific but also genotypic/cultivar dependent. This could be due to specific requirement of plant growth regulators which varies from genotype to genotype (Mamidala and Nanna 2011) and regeneration efficiency could be enhanced by manipulating media using growth regulators (Mensuali-Sodi et al. 1995).

In general, the present investigation revealed that the regeneration ability of germinated embryos having cultivar rose as the male parent was comparatively less with interspecific triploid female parent than the intraspecific AA diploid one. In spite of low rate of embryo germination, 100% regeneration was observed in most of the embryos obtained under controlled pollination and all the open pollinated accessions except Ennabernian. The reason for having low regeneration percentage in some of the cross combinations might be that these embryos could have developed by the union of non-functioning of gametes with more unbalanced chromosome numbers which could have occurred as the result of chromosome assortment encountered in the meiotic process of the triploid (Punyasingham 1947).

In general, there is no specific trend noticed in the multiple shoot formation and genomic composition. Irrespective of the male parents used, all the Nendran cross combinations responded for multiple shoot formation whereas no trend was observed for all other cross combinations. In general, this study revealed that multiple shoot induction through both the techniques, influence of female parent is higher than the male parent in all the cross combinations except in Karpuravalli cross combinations. The multiple shoot formation from all the regenerated embryos of the Nendran based cross combinations revealed that they are highly amenable for multiple shoot induction through media manipulation and shoot tip culture.

Genetic fidelity testing

The SSR marker study was carried out to determine the hybridity of the progenies and genetic integrity of the
multiple shoots derived from single embryo. In case of controlled pollinated progenies visualization of both the parental bands in the respective hybrid confirmed their hybrid nature and monomorphic banding pattern among the multiple shoot derived plants of each embryo confirmed their genetic fidelity (Fig. 3). Whereas in open pollinated derived plantlets nearly 80% of the primers showed monomorphism with that of female parent. But multiple shoots developed from each embryo did not show any polymorphic bands which proved that multiple shoot formation through direct organogenesis/decortications from embryo is considered to be more effective, easy and time saving approach compared to indirect organogenesis and/or normal conventional germination of hybrid seeds in the banana genetic improvement. This results is in concordance with the findings of Venkataramana et al. (2015), Rotchanapreeda et al. (2014); RaviShankar et al. (2013) and Manchanda Pooja and Gosal (2011) who used the SSR markers to confirm the genetic fidelity of tissue culture derived plants of various Musa species and cultivars having various genomic composition developed through direct regeneration protocols.

**Conclusion**

This study proved that media augmented with 17.76 µM BAP concentration enhanced the regeneration efficiency of the pollinated embryos and produced multiple shoots from the regenerated embryos with a range of 36.36–100%. Decortications of in vitro derived plantlets produced multiple shoots with a range of 50–100% in the remaining embryo derived single shoots. Combination of these two techniques could lead to multiple shoot induction up to 66.7–100% in various genomic accessions of both controlled and open pollinated seeds. Production of multiple shoots from single embryo will reduce the risk of mortality of each hybrid event and reduces the duration of the breeding program by 9–12 months. This multiple shoot formation from single embryo technique can be extended in banana improvement program either through chromosome and/or gene manipulation techniques by doubling the chromosome and developing parthenocarpic trait in seeded accessions through antimitotic agents and genetic transformation respectively.

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Declarations

Conflict of interest  The author’s declare that they have no competing interests.

References

Ahmed KZ, Remy S, Sagi L, Swennen R (2006) Germination of Musa balbisiana seeds and embryos. XVII International ACROBAT Meeting, Joinville, Santa Catarina State, Brasil, pp 510–512

Ali N, Skirvin R, Splittoesser WE, George WL (1991) Germination and regeneration of plants from old cucumber seed. HortScience 26(7):917–918

Allam EK, Othman BA, Sawy EL, Thabet SD (2000) Establishment of an aseptic culture of banana micropropagation in vitro. Ann Agric Sci 38:1121–1136

Andras L, Ding Z, Jiang F, Jin B, Ding X, Sun J, Guiyuan L (1971) Induction and identification of hexadecaploid of Pinellia ternate. J Euphytica 186(2):479–488

Arun K, Uma S, Sarawathi MS, Backiyarani S, Durai P (2013) Effects of whole seed priming in the in vitro germination of hybrid banana embryos (Musa spp.). Seed Sci Technol 41:439–451

Asif MJ, Mak C, Othman RY (2001) In vitro zygotic embryo culture of wild Musa acuminate spp. Malaccensis and factors affecting germination and seedling growth. Plant Cell Tiss Org Cult 67:267–270

Backiyarani S, Uma S, Maria Doss A, Sarawathi MS, Selvaraj V, Arun K, Durai P (2016) Enhancing the seed set in banana using antitoxins in National Conference on Fruit Breeding in Tropics and subtropics-An Indian Perspective. ICAR-Indian Institute of horticultural Research, Bengaluru, p 116

Backiyarani S, Chandrasekar A, Uma S, Sarawathi MS (2019) MusatransSSRDB (a transcriptome derived SSR database)—an advanced tool for banana improvement. J Bioso 43:110–116

Batte M, Swennen R, Uwimana B, Akech V, Brown A, Tumuhimbise R, Hovmalm HP, Geleta M, Ortiz R (2019) Crossbreeding East African highland bananas: lessons learnt relevant to the botany of the crop after 21 years of genetic enhancement. Front Plant Sci 10:81

Bhattacharya J, Renukdas NN, Khuspe SS, Rawal SK (2003) Multiple shoot regeneration from immature embryo explants of papaya. Biol Plant 47(3):327–331

Burgos-Hernández M, Castillo-Campos G, Mata-Rosas M, González D, Vovides AP, Murugía-González J (2014) Seed germination of wild banana Musa ornata (Musaceae). Seed Sci Technol 42:16–27

Chang FC, Yang CM (1992) Allolepaphic potential of purple nusetse (Cyperus rotundus L.) and barnyardgrass (Echinochloa crus-galli (L.) Beauv.) on corn (Zea mays L.). weed residues effect on corn emergence and seedling growth. J Agric Res China 41:9–23

Crouch JH, Crouch HK, Tenkuoano A, Ortiz R (1999) VNTR diversity analysis of 2x and 4x full-sib Musa hybrids. Electron J Biotechnol 2:130–139

Dayaran M, Dhanaranjan MS, Arun K, Uma S, Narayani Padma (2014) Embryo culture and embryo rescue studies in wild Musa spp. (Musa ornata). J Appl Hort 16(2):126–130

Diro M, Van Staden J (2003) In vitro regeneration of Ensete ventricosum from zygotic embryos of stored seeds. S Afr J Bot 69:364–369

Dissanayaka NP, Kodikara KAS, Vithanage DS, Krishnarajah SA, Rubasinghe MK, Dayananda TG (2015) Effects of 6-benzylaminopurine (BAP) treatment on seed germination and seedling vigour of endemic herb exacumturinervum L. in Sri Lanka: conservation strategy. J Univ Ruhuna 1:14–20

Ferreira CF, Silva SO, Damasceno Sobrinho LP, Damascena SCS, Alves FSA, Paz OP (2004) Molecular characterization of banana (AA) diploid with contrasting levels of black and yellow sigatoka resistance. Am J Appl Sci 7:273–278

Franklin G, Jeyachandran R, Ignicimuthu S (2000) Factors affecting regeneration of pigeon pea (Cajanus cajan L. Millsp) from mature embryonal axes. Plant Growth Regul 30:31–36

Ganeshan S, Baga M, Harvey BL, Rossnagel BG, Chibbar RN (2003) Production of multiple shoots from thidiazuron-treated mature embryos and leaf-base/apical meristems of barley (Hordeum vulgare). Plant Cell Tiss Org Cult 73:57–64

Gawel NL, Jarret RL (1991) A modified CTAB DNA extraction procedure of Musa and Ipomoea. Plant Mol Biol Report 9:262–266

González de León D, Fauré S (1993) Genetic mapping of the banana diploid genome: toward an integrated approach to the study of the Musa genome and the use of molecular marker technologies in Musa breeding. In: Proceedings of the workshop on “Biotechnology applications for banana and plantain improvement”, San Jose, Costa Rica (edited by INIBAP, Montpellier, France) pp 29

Grafi G, Barak S (2014) Stress induces cell dedifferentiation in plants. BBA-Gene Regul Mech 1849(4):378–384

Guan KL (1991) Studies on complex dormancy of Cornus officinalis seed. Chin J of Bot 3:145–150

Heslop-Harrison TS (2007) Domestication, genomics and the future for banana. Ann Bot 100:1073–1084

Johri BM, Rao PS (1984) Experimental embryology. In: Johri BM (ed) Embryology of angiosperms. Springer-Verlag, Berlin, pp 744–802

Kadota M, Niimi Y (2003) Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in in vitro pear cultivar shoots. Plant Cell Tiss Org Cult 72:261–265

Klem M, Balla J, Machackova I, Eder J, Prochazka S (2004) The uptake and metabolism of benzyl amino purine in tobacco (Nicotiana tabacum L.) and cucumber (Cucumis sativus L.) explants. Plant Growth Regul 31:135–142

Köhler C, Hennig L, Spillane C, Stephane P, Gruissem W, Grossniklaus U (2003) The polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES. Genes Dev 17:1540–1553

Laliberté B (2016) Global strategy for the conservation and use of Musa (banana) genetic resources: a consultative document prepared by the Global Musa Genetic Resources Network (Musa Net). Bioversity International, Rome

Mamidala P, Nanna KS (2011) Effect of genotype, explants source and medium on in vitro regeneration of tomato. Int J Genet Mol Biol 3:45–50

Manchanda P, Gosal SS (2011) Molecular assessment of genetic fidelity of micropropagated banana (Musa acuminate L.) using SSR markers. Int J Plant Res 24(2):91–101

Mensuali-Sodi A, Panizza M, Tognoni F (1995) Endogenous ethylene requirement for adventitious root induction and growth in tomato cotyledons and lavandin microcuttings in vitro. Plant Growth Regul 15:473–497

Ortiz R (1997) Occurrence and inheritance of 2n pollen in Musa. Ann Bot 79:449–453
Pandey H, Nandi SK, Nadeem M, Palni LM (2000) Chemical stimulation
Ortiz R, Vuylsteke D (1995) Factors influencing seed set in triplo-
yielding seed in Aconitum heterophyllum Wall, and A. balfourii Stapf.: important Himalayan species of medicinal value. International Seed Testing Association. Seed Sci Technol 28:39–48
Peschke VM, Phillips RL (1992) Genetic implications of somaclonal
variation in plants. Adv Genet 30:41–75
Punyasongk K (1947) Chromosome numbers in crosses of diploid, triplo-
yielding and tetraploid maize. Genetics 32:541–554
Ramírez-Mosqueda MA, Iglesias-Andreu LG (2015) Indirect organo-
genesis and assessment of somaclonal variation in plantlets of
Vanilla planifolia Jacks. Plant Cell Tiss Org Cult 123(3):657–664
Rao MS, Purohit SD (2006) In vitro shoot bud differentiation and plantlet regeneration in Celastrus paniculatus Wild. Biol Plant 50:501–506
Ravishankar KV, Raghavendra KP, Athani V, Rekha A, Sudeepa K, Bhavya D, Srinivar V, Anand L (2013) Development and characterisation of microsatellite markers for wild banana (Musa balbisiana). J Hort Sci Biotechnol 88:605–609
Rotchanapreeda T, Wongniam S, Swangpol SC, Chareonsap PP, Suk-
kaewmanee N (2019) A rapid protocol for somatic embryogenesis from immature leaflets of groundnut (Arachis hypogaea L.). J Plant Physiol 148:667–671
Rowe PR, Rosales F (1993) Diploid breeding at FHIA and the develop-
ment of Goldfinger (FHIA-01). InfoMusa 2:9–11
Scott RJ, Spielman M, Bailey J, Dickinson HG (1998) Parent-of-origin
effects on seed development in Arabidopsis thaliana. Development 125:3329–3341
Shepherd K (1987) Banana breeding—past and present. Acta Hortic 196:37–43
Simmonds NW (1962) The evolution of the bananas. Tropical science series. Longmans, London, p 170
Sivasesan I (2007) Shoot regeneration and somaclonal variation from leaf callus cultures of Plumbago zeylanica. Asian J Plant Sci 6:83–86
Sobhakumari VPP, Lalithakumari D (2003) Direct plant regeneration
from shoot tip cultures of Capsicum annuum L.cv.PL-1. Phyto-
morphology 53:235–242
Suman S (2017) Plant tissue culture: a promising tool of quality mate-
rial production with special reference to micropropagation of banana. Biochem Cell Arch 17:1–26
Swennen R, Vuylsteke D, Hahn SK (1992) The use of simple biote-
technological tools to facilitate plantain breeding. In: Thottappilly G, Monti LM, Mohan Raj DR, Moore AW (eds) Biotechnology: enhancing research on tropical crops in Africa. IITA, Nigeria, pp 69–74
Talengera D, Vuylsteke D, Karamura E (1996) In vitro germination of Ugandan banana hybrids. Musa Africa 10:14
Tiryaki I, Korkmaz A, Nas MN, Ozbay N (2009) Priming combined
with plant growth regulators promotes germination and emergence of dormant Amaranthus cruentus L. seeds. International Seed Test-
ning Association. Seed Sci Technol 33(3):571–579
Uma S, Mustaffa MM, Saraswathi MS, Durai P (2011) Exploitation
of diploids in Indian breeding programmes. Acta Hortic 897:215–223
Uma S, Lakshmi S, Saraswathi MS, Akbar A, Mustaffa MM (2012)
Embryo rescue and plant regeneration in banana (Musa spp.). Plant Cell Tiss Org Cult 105:105–111
Uma S, Saraswathi MS, Backiyurani S, Durai P (2015) Banana breeding—a brief review. Int J Innov Hortic 4(1):11–19
Varshney A, Lakshmikumaran M, Srivastava PS, Dhawan V (2001)
Establishment of genetic fidelity of in vitro-raised lilium bulblets
through rapid markers. Vitro Cell Dev Biol Plant 37:227–231
Vasane SR, Kothari RM (2006) Optimization of secondary harden-
ning process of banana plantlets (Musa paradisiaca L. var. Grand Naine). Indian J Biotechnol 5:394–399
Venkatachalap P, Kavikishor PB, Geetha N, Thangavelu M, Jayaba-
lan N (1999) A rapid protocol for somatic embryogenesis from immature leaflets of groundnut (Arachis hypogaea L.). Vitro Cell Dev Biol Plant 35:409–412
Venkataramana RK, Sampangi-Ramaiah MH, Ajitha R, Khadke GN,
Chellam V (2015) Insights into Musa balbisiana and Musa acumu-
nate species divergence and development of genomic microsatellites by transcriptomics approach. Plant Gene 4:78–82
Wattanachaiyingcharoen D (1990) Viability, germination and dor-
mancy of banana seed (Musa acuminate subsp.). Unpublished Thesis. The Faculty of Natural and Agriculture Science, The University of Western Australia
Zapata C, Srivatanakul M, Park SH, Lee BM, Salas MG, Smith RH (1999) Improvements in shoot apex regeneration of two fiber crops: cotton and kenaf. Plant Cell Tiss Org Cult 56:185–191
Zhang J, Maun MA (1990) Seed size variation and its effects on seedling growth in Agropyron psammophilum. Bot Gaz 151:106–113
Zhang S, Zhang H, Zhang MB (1996) Production of multiple shoots
from shoot apical meristems of oat (Avena sativa L.). J Plant Physiol 148:667–671
Zhang S, Williams Carrier R, Jackson D, Lemaux PG (1998) Expres-
sion of CDC2Zm and KNOTTED1 during in vitro axillary shoot meristem proliferation and adventitious shoot meristem formation in maize (Zea mays L.) and barley (Hordeum vulgare L.). Planta 204:542–549

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