Efficient regeneration system applicable to five Musa cultivars

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Abstract  Banana (Musa spp.) is an important staple food, economic crop, and nutritional fruit worldwide. Hybridization is seriously hampered by the long generation time, polyploidy, and sterility of most cultivars. Establishment of an efficient regeneration and transformation system for banana is critical for their genetic improvement. An efficient and reproducible transformation system for banana using direct organogenesis was developed. Media containing benzylaminopurine (BA) combined with one of four other growth regulators was evaluated for the regeneration efficiency of five Musa cultivars and the ability to induce/support development of new banana shoots. The result indicated that the greatest number of shoots per explant for all five Musa cultivars was obtained using MS medium supplemented with 8.9 μmol L⁻¹ BA and 9.1 μmol L⁻¹ thidiazuron (TDZ). In 240–270 d, one immature male flower could regenerate between 380 and 456, 310–372, 200–240, 130–156, and 100–130 well-developed shoots for Gongjiao, Red banana, Rose banana, Baxi, and Xinglongnaijiao, respectively. Such a system will facilitate molecular breeding and functional genomics of banana.

Keywords  banana (Musa spp), system, regeneration

1 Introduction

Bananas (Musa spp.) are one of the most important food and commercial crops in tropical and subtropical developing countries. The environments in these counties although ideal for growing bananas also lead to high rates of plant disease, pest pressure, and abiotic stresses, which all significantly affect the yield and quality of crops. Therefore, in order to overcome the low disease resistance and stress tolerance in banana plants, the importance of developing new banana cultivars is highlighted. Furthermore, sexual hybridization is seriously hampered by the complex and polyploid nature of the Musa genome, as well as the high degree of sterility. Recent developments in transgenics have provided an effective means for banana breeding; however, integration of such tools, including high efficiency of regeneration and genetic transformation, into banana improvement programs is imperative.

Most previous studies on regeneration and transgenics of banana have involved mainly embryogenic cell suspension systems. However, induction and regeneration of embryogenic cells needs more than 12 months, which is time-consuming and has a low frequency of spontaneous mutation and regeneration. Moreover, embryogenic cells are heavily restricted by genotypes. Thin cell layers originating from the shoot tip represent newly developed receptor material for genetic transformation of banana. Compared to embryogenic cell suspension systems, thin cell layer explants from immature male flower produce few chimeras and have a high regeneration rate, making them ideal for genetic engineering of banana. However, thin cell layer regeneration systems require the use of different media at different culture stages which complicates the process.

Here, we report the development of a simple and efficient regeneration system using a single medium, which was demonstrated to be suitable for the five Musa cultivars tested.
2 Materials and methods

2.1 Plant material

Fresh, immature, male banana flowers were obtained from five sources, Baxi banana (Musa AAA group, cv. Brazilian), Gonggiao banana (Musa acuminata AAA group, cv. Mas), Red banana (Musa corniculata AAA group), Rose banana (M. acuminata L. AA group) and Xinglongnaijiao banana (Musa AAB group), from the Institute of Tropical Bioscience and Biotechnology banana plantation (Chengmai County, Hainan Province, China). The flowers were peeled layer by layer until 100 mm long and then transferred to a clean bench to continue peeling until 30 mm long before being cut transversely into slices 1–2 mm thick (one flower was cut into 10–12 slices).

2.2 Regeneration

The banana flower slices were used as explants and cultured on Murashige and Skoog (MS) media in Petri dishes (90 mm diameter) to induce shoot growth. The media was supplemented with 8.9 mmol L\(^{-1}\) benzylaminopurine (BA) and one of four plant growth regulators; 9.3 mmol L\(^{-1}\) kinetin, 9.1 mmol L\(^{-1}\) thidiazuron (TDZ) or 8.9 mmol L\(^{-1}\) 1-naphthaleneacetic acid (NAA). Every 15 d, explants were transferred to fresh media. After 4 months, the number and fresh weight of regenerated shoots from each supplemented media were calculated to determine the best combination of growth regulators. To regenerate roots, when shoot lengths reached 30–50 mm they were transplanted into unamended MS medium for 1 month. Then when plantlets grew up to 50 mm, they were transplanted into a coconut coir medium. MS basal medium was supplemented with 8.9 mmol L\(^{-1}\) thidiazuron (TDZ) or 8.9 mmol L\(^{-1}\) 1-naphthaleneacetic acid (NAA). Every 15 d, explants were transferred to fresh media. After 4 months, the number and fresh weight of each shoot and root were measured. Asterisks indicate significant difference between the five Musa cultivars (*, P < 0.05).

2.3 Anatomy

To observe microscopically the progress of adventitious shoot formation, samples were fixed in FAA solution (90 mL of 70% ethanol, 5 mL acetic acid and 5 mL of 36% formaldehyde), washed repeatedly in 70% ethanol, dehydrated with a series of ethanol dilutions (80%, 90% and 95%), and then dealcoholized with a series of absolute alcohol and xylene mixtures (3:1, 1:1 and 1:3). Samples were infiltrated with a mixture of liquid paraffin and xylene (9:1) for 24 h and then immersed in pure liquid paraffin for 1 h before embedding with pure paraffin (56–57°C). Embedded samples were cut into 8 μm thin sections on a rotary microtome (Leica SP9000, Germany). Slices were stained with hematoxylin-cosin and observed with a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan).

2.4 Statistical analysis

All experiments were repeated three times, and data presented as means ± standard error. One-way analysis of variance was performed and interactions between means were separated by least significant difference at P = 0.05.

3 Results

3.1 Regeneration efficiency of five Musa cultivars

After six months, shoots were induced from the wounded edges of banana flower explants from the five different Musa cultivars, and strong shoots were cut transversely into slices. Three to four months later, shoots were induced again from the wounded edges of slices. The number of shoots in each explant and fresh weight of each shoot are given in Table 1 and images in Fig. 1. The greatest number of shoots per explant for the five cultivars was obtained with BA and TDZ. However, the fresh weight of each shoot showed little difference (Table 1). Furthermore, Gonggiao explants regenerated the most shoots on BA and TDZ medium, followed by Red banana, Rose banana, Baxi, and Xinglongnaijiao (Table 1; Fig. 1).

3.2 Anatomical observation of shoot regeneration

As shown in Fig. 2a and Fig. 2b, when explants were prepared with 7 g L\(^{-1}\) of agar (Solarbio, Japan) and 40 g L\(^{-1}\) sucrose adjusting the pH to 5.8. After the preparation of media, autoclaving was performed at 120°C for 1 h before embedding with pure paraffin (56–57°C). Embedded samples were cut into 8 μm thin sections on a rotary microtome (Leica SP9000, Germany). Slices were stained with hematoxylin-cosin and observed with a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan).

Table 1  Regeneration analysis of five Musa cultivars using four types of media

<table>
<thead>
<tr>
<th>M</th>
<th>Baxi B/g</th>
<th>Gongjiao B/g</th>
<th>Red banana B/g</th>
<th>Rose banana B/g</th>
<th>Xinglongnaijiao B/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.00±1.30(^a) 0.50±0.03(^a)</td>
<td>16.00±0.80(^a) 0.82±0.03(^a)</td>
<td>12.00±1.20(^a) 0.70±0.03(^ab)</td>
<td>12.00±1.20(^a) 0.22±0.03(^b)</td>
<td>6.00±1.20(^a) 0.59±0.05(^bc)</td>
</tr>
<tr>
<td>II</td>
<td>9.00±0.80(^b) 0.53±0.10(^b)</td>
<td>22.00±2.40(^b) 0.79±0.06(^b)</td>
<td>23.00±0.80(^b) 0.70±0.01(^ab)</td>
<td>15.00±2.10(^b) 0.27±0.01(^a)</td>
<td>7.00±0.80(^b) 0.67±0.03(^ab)</td>
</tr>
<tr>
<td>III</td>
<td>13.00±0.80(^c) 0.43±0.08(^c)</td>
<td>38.00±2.10(^c) 0.74±0.07(^abc)</td>
<td>31.00±2.60(^c) 0.73±0.10(^c)</td>
<td>20.00±2.40(^c) 0.28±0.01(^a)</td>
<td>10.00±1.20(^c) 0.74±0.09(^c)</td>
</tr>
<tr>
<td>IV</td>
<td>2.00±0.80(^d) 0.35±0.03(^d)</td>
<td>4.00±0.80(^d) 0.63±0.03(^d)</td>
<td>7.00±1.70(^d) 0.54±0.02(^d)</td>
<td>4.00±1.20(^d) 0.14±0.01(^d)</td>
<td>2.00±0.80(^d) 0.46±0.05(^d)</td>
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</table>

Note: M, Murashige and Skoog media supplemented with 8.9 mmol L\(^{-1}\) benzylaminopurine and 9.3 mmol L\(^{-1}\) kinetin (I), 9.1 mmol L\(^{-1}\) zeatin (II), 9.1 mmol L\(^{-1}\) thidiazuron (III), or 8.9 mmol L\(^{-1}\) 1-naphthaleneacetic acid (IV), respectively; A, number of shoots/explant; B, fresh weight of each shoot. Data are presented as means ± standard error of n = 3 biological replicates. Means denoted by the same letter do not significantly differ at P < 0.05 as determined by least significant difference. Asterisks indicate significant difference between the five Musa cultivars (*, P < 0.05).
(M in Fig. 1) were induced with BA plus TDZ, the cortical parenchymal cells under the epidermis quickly recovered the capacity to divide and rapidly increased in number, resulting in the outward protuberance of bud primordia (Fig. 2c, Fig. 2d). The outward-growing primordia continued to grow, and regenerated adventitious shoots (Fig. 2e, Fig. 2f).

### 3.3 Plants regeneration

After transplanted into a coconut coir medium to grow on for three months, plants of the five cultivars were successfully regenerated. All the plants were growing well and ready for field growth at this stage (Fig. 3).

### 4 Discussion

Genetic transformation of banana has been an important tool for molecular breeding and identification of gene function in recent years\(^{[12,18]}\). However, the dependency of regeneration on banana plant genotype has made it difficult to repeat published results. Here, we report development of an efficient banana regeneration system that has four major advantages over previously reported techniques.

First, our regeneration system is very simple, using only one medium (MS with 8.9 μmol·L\(^{-1}\) BA and 9.1 μmol·L\(^{-1}\) TDZ) for induction and differentiation of adventitious shoots. High-throughput regeneration and transformation using embryogenic cell suspension as reported by Tripathi et al.\(^{[19]}\) for example, required six different media to derive well-developed shoots from immature male flowers. Moreover, Hrahsel et al.\(^{[20]}\) reported in vitro propagation of *M. acuminata* (AAA) cv. Vaibalhla from the immature male flower through direct shoot regeneration that required two different media. To our knowledge, all previously reported banana regeneration and transformation studies have required two or more kinds of media for the different culture stages, making ours the first to successfully use
only one kind of medium.

Second, our regeneration system is reproducible. Most problems with banana regeneration stem from poor repeatability due to variety dependency. Generally speaking, only one method is suitable for use with one variety. For example, Hu et al.\(^{[14]}\) established an efficient propagation protocol for Baxi banana using multiple bud clump explants, while Santos et al.\(^{[3]}\) established a method for a plantain-type banana cv., Three Hand Plenty (AAB genomic group, International Transit Center accession: ITC.0185). Paul et al.\(^{[18]}\) established an effective method for lady finger banana, Sreedharan et al.\(^{[12]}\) for banana cv. Rasthali, and Wei et al.\(^{[6]}\) for the edible banana \(M. \text{acuminata}\) cv. Mas (AA group). The most versatile method we found in the literature was reported by Tripathi et al.\(^{[19]}\) which was suitable for three banana cultivars, including Cavendish Williams, Gros Michel, and Sukali Ndizi. In contrast, we report a simple method that uses only one kind of growth medium (MS with 8.9 \(\mu\)mol·L\(^{-1}\) BA and 9.1 \(\mu\)mol·L\(^{-1}\) TDZ) for regeneration of five kinds of immature male banana flowers that is reproducible and might overcome the problems associated with genotype dependency.

The third major benefit of our method is that it substantially shortens the banana plant growth cycle. Another problem with banana regeneration is that it is time-consuming. Our method was able to produce well-developed plantlets from immature flowers in only 240–270 d; the shortest growth cycle using other methods was 344–478 d\(^{[19]}\). Hence, our method would serve to greatly improve regeneration efficiency.

Lastly, our regeneration system was highly efficient. One immature male banana flower could regenerate 380–456, 310–372, 200–240, 130–156, and 100–130 well-developed shoots and plants for Gongjiao, Red banana, Rose banana, Baxi, and Xinglongnaijiao, respectively (Table 1; Fig. 3), which amply met the transformation demand.

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Fig. 3 Plant regeneration by five cultivars after three months growth in coconut coir medium. (a) Baxi; (b) Gongjiao; (c) Xinglongnaijiao; (d) Red banana; (e) Rose banana.
5 Conclusions

An efficient regeneration system suitable for five Musa cultivars through direct organogenesis using only one medium was established in this study.

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Compliance with ethics guidelines Juhua Liu, Peiguang Sun, Jing Zhang, Jiashui Wang, Jianbin Zhang, Jingyi Wang, Caihong Jia, Pengzhao Gao, Biyu Xu, and Zhiqiang Jin declare they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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