

RESEARCH ARTICLE

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 $8.9 \mu\text{mol}\cdot\text{L}^{-1}$ BA and $9.1 \mu\text{mol}\cdot\text{L}^{-1}$ 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 countries 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^[1–3]. 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^[4–12]. 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^[13,14]. 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^[15] and have a high regeneration rate^[16], making them ideal for genetic engineering of banana^[14,17]. 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.

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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), Gongjiao banana (*Musa acuminata* AA group, cv. Mas), Red banana (*Musa corniculata* AAA group), Rose banana (*M. acuminata* L. AA group) and Xinglongnajibao 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 $8.9 \mu\text{mol}\cdot\text{L}^{-1}$ benzylaminopurine (BA) and one of four plant growth regulators; $9.3 \mu\text{mol}\cdot\text{L}^{-1}$ kinetin, $9.1 \mu\text{mol}\cdot\text{L}^{-1}$ zeatin, $9.1 \mu\text{mol}\cdot\text{L}^{-1}$ thidiazuron (TDZ) or $8.9 \mu\text{mol}\cdot\text{L}^{-1}$ 1-naphthaleneacetic acid (NAA). Every 15 d, explants were transferred to fresh media. After 4 months, the number and fresh weight of regenerated, strong 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 cut and transferred to unamended MS medium for 1 month. Then when plantlets roots grew up to 50–80 mm long they were transplanted into a coconut coir medium. MS basal medium was used for all plant tissue cultures. Solid MS medium was prepared with $7 \text{ g}\cdot\text{L}^{-1}$ of agar (Solarbio, Japan) and $40 \text{ g}\cdot\text{L}^{-1}$ sucrose adjusting the pH to 5.8. After the preparation of media, autoclaving was performed at 120°C for 15 min. All cultures were incubated at $28\pm 1^\circ\text{C}$.

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 ethanol 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 ($56\text{--}57^\circ\text{C}$). Embedded samples were cut into $8 \mu\text{m}$ thin sections on a rotary microtome (Leica SP9000, Germany). Slices were stained with hematoxylin-eosin 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 \pm 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 showed little difference (Table 1). Furthermore, Gongjiao explants regenerated the most shoots on BA and TDZ medium, followed by Red banana, Rose banana, Baxi, and Xinglongnajibao (Table 1; Fig. 1).

3.2 Anatomical observation of shoot regeneration

As shown in Fig. 2a and Fig. 2b, when explants

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

M	Baxi		Gongjiao		Red banana		Rose banana		Xinglongnajibao	
	A	B/g	A	B/g	A	B/g	A	B/g	A	B/g
I	4.00 ± 1.30^c	$0.50\pm 0.03^{a*}$	$16.00\pm 0.80^{c*}$	$0.82\pm 0.03^{a*}$	$12.00\pm 1.20^{c*}$	$0.70\pm 0.03^{ab*}$	$12.00\pm 1.20^{b*}$	0.22 ± 0.03^b	6.00 ± 1.20^b	$0.59\pm 0.05^{bc*}$
II	9.00 ± 0.80^b	$0.53\pm 0.10^{a*}$	$22.00\pm 2.40^{b*}$	$0.79\pm 0.06^{a*}$	$23.00\pm 0.80^{b*}$	$0.70\pm 0.01^{ab*}$	$15.00\pm 2.10^{b*}$	0.27 ± 0.01^a	7.00 ± 0.80^{ab}	$0.67\pm 0.03^{ab*}$
III	13.00 ± 0.80^a	0.43 ± 0.08^a	$38.00\pm 2.10^{a*}$	$0.74\pm 0.07^{ab*}$	$31.00\pm 2.60^{a*}$	$0.73\pm 0.10^{a*}$	$20.00\pm 2.40^{a*}$	0.28 ± 0.01^a	10.00 ± 1.20^a	$0.74\pm 0.09^{a*}$
IV	2.00 ± 0.80^d	$0.35\pm 0.03^{a*}$	4.00 ± 0.80^d	$0.63\pm 0.03^{b*}$	$7.00\pm 1.70^{d*}$	$0.54\pm 0.02^{b*}$	4.00 ± 1.20^c	0.14 ± 0.01^c	2.00 ± 0.80^c	$0.46\pm 0.05^{c*}$

Note: M, Murashige and Skoog media supplemented with $8.9 \mu\text{mol}\cdot\text{L}^{-1}$ benzylaminopurine and $9.3 \mu\text{mol}\cdot\text{L}^{-1}$ kinetin (I), $9.1 \mu\text{mol}\cdot\text{L}^{-1}$ zeatin (II), $9.1 \mu\text{mol}\cdot\text{L}^{-1}$ thidiazuron (III), or $8.9 \mu\text{mol}\cdot\text{L}^{-1}$ 1-naphthaleneacetic acid (IV), respectively; A, number of shoots/explant; B, fresh weight of each shoot. Data are presented as means \pm 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 (*, $P < 0.05$).

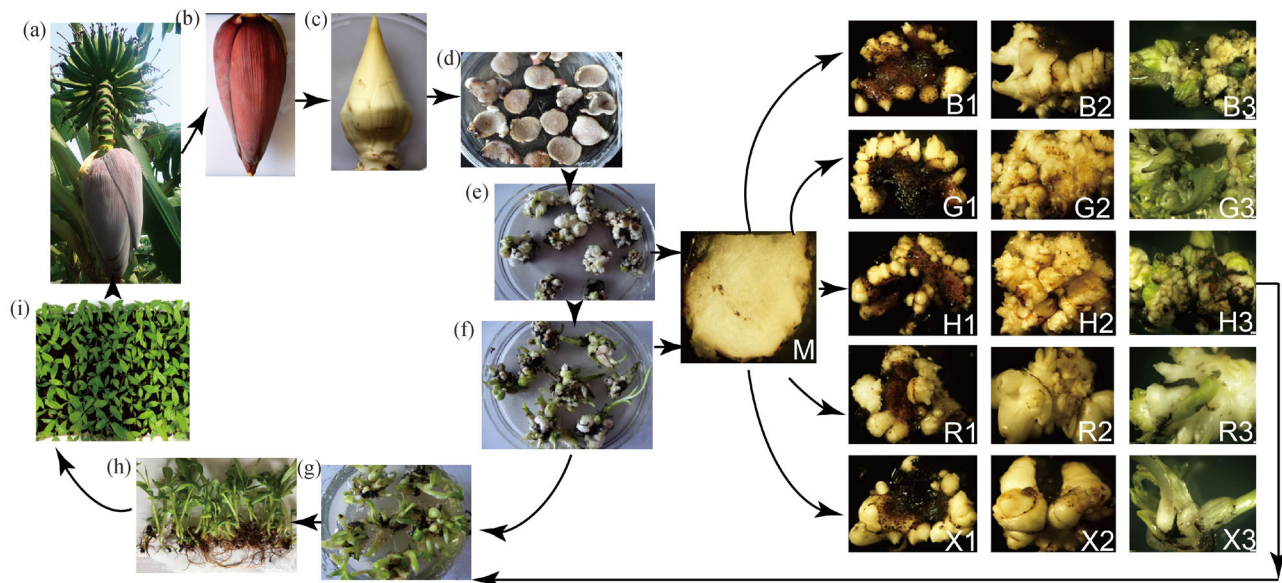


Fig. 1 Regeneration of five *Musa* genotypes. (a–b) Male banana flower; (c) male banana flower (30 mm); (d) tissue slices (1–2 mm); (e–f), shoots regenerated from slices; (g) regenerated banana roots; (h) transplantation; (i) plantlets; M, slices cut from (e–f); B1–B3, different stages of Baxi regeneration; G1–G3, different stages of Gongjiao regeneration; H1–H3, different stages of Red banana regeneration; R1–R3, different stages of Rose banana regeneration; X1–X3, different stages of Xinglongnajibiao regeneration.

(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).

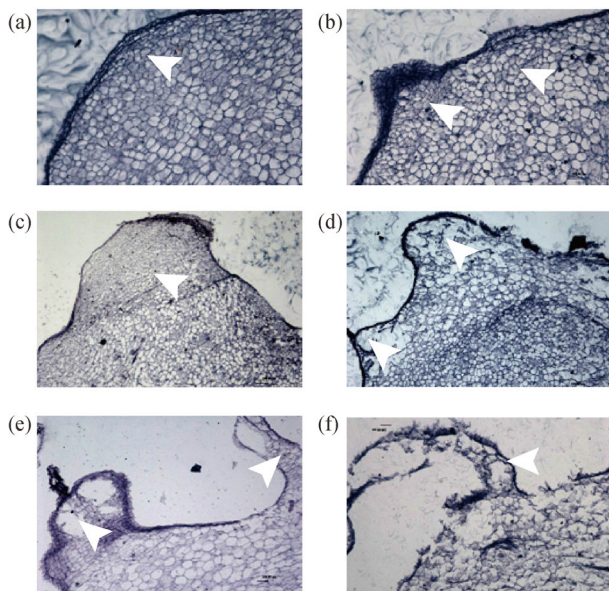


Fig. 2 Cross sections of regenerated banana shoots. (a) Initiation of cell division; (b) vigorous cell division; (c–d) outward formation of bud primordia; (e–f) adventitious shoot regeneration. Arrow, cortical parenchymal cells.

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 $8.9 \mu\text{mol} \cdot \text{L}^{-1}$ BA and $9.1 \mu\text{mol} \cdot \text{L}^{-1}$ 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



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

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. 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 Ndiizi. In contrast, we report a simple method that uses only one kind of growth medium (MS with $8.9 \mu\text{mol} \cdot \text{L}^{-1}$

BA and $9.1 \mu\text{mol} \cdot \text{L}^{-1}$ 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 Xinglongnajibiao, respectively (Table 1; Fig. 3), which amply met the transformation demand.

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.

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References

1. Sági L, Remy S, Pérez Hernández J B, Swennen R. Production of transgenic banana (*Musa species*). In: Khachatourians G G, McHughen A, Scorza R, Nip W K, Hui Y H, eds. *Transgenic Plants and Crops*. New York: Marcel Dekker Inc, 2002, 359–369
2. Pillay M, Tripathi L. Banana breeding. In: M S Kang and P M Priyadarshan, eds. *Breeding major food staples*. New York: Blackwell Science, 2007
3. Santos E, Remy S, Thiry E, Windelinckx S, Swennen R, Sági L. Characterization and isolation of a T-DNA tagged banana promoter active during in vitro culture and low temperature stress. *BMC Plant Biology*, 2009, **9**(1): 77
4. Dhed A D, Dumortier F, Panis B, Vuylsteke D, Langhe E D. Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoe (*Musa* sp. ABB group). *Fruits*, 2001, **46**(2): 125–135
5. Novak F J, Afza R, Van Duren M, Perea-Dallos M, Conger B V, Xiaolang T. Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (AAB) bananas (*Musa* spp.). *Nature Biotechnology*, 1989, **7**(2): 154–159
6. Wei Y R, Huang X L, Li J, Huang X, Li Z, Li X J. Establishment of embryogenic cell suspension culture and plant regeneration of edible banana *Musa acuminata* cv. Mas (AA). *Chinese Journal of Biotechnology*, 2005, **21**(1): 58–65
7. Strosse H, Schoofs H, Panis B, Andre E, Reyniers K, Swennen R. Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa* spp). *Plant Science*, 2006, **170**(1): 104–112
8. Huang X, Huang X L, Xiao W, Zhao J T, Dai X M, Chen Y F, Li X J. Highly efficient *Agrobacterium*-mediated transformation of embryogenic cell suspensions of *Musa acuminata* cv. Mas (AA) via a liquid co-cultivation system. *Plant Cell Reports*, 2007, **26**(10): 1755–1762
9. Ghosh A, Ganapathi T R, Nath P, Bapat V A. Establishment of embryogenic cell suspension cultures and *Agrobacterium*-mediated transformation in an important Cavendish banana cv. Robusta (AAA). *Plant Cell, Tissue and Organ Culture*, 2009, **97**(2): 131–139
10. Khanna H K, Paul J Y, Harding R M, Dickman M B, Dale J L. Inhibition of *Agrobacterium*-induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of Banana. *Molecular Plant-Microbe Interactions*, 2007, **20**(9): 1048–1054
11. Xu C, Takáč T, Burbach C, Menzel D, Šamaj J. Developmental localization and the role of hydroxyproline rich glycoproteins during somatic embryogenesis of banana (*Musa* spp. AAA). *BMC Plant Biology*, 2011, **11**(1): 38
12. Sreedharan S, Shekhawat U K, Ganapathi T R. Transgenic banana plants overexpressing a native plasma membrane aquaporin MusaPIP1; 2 display high tolerance levels to different abiotic stresses. *Plant Biotechnology Journal*, 2013, **11**(8): 942–952
13. Tripathi J N, Muwonge A, Tripathi L. Efficient regeneration and transformation of plantain cv. Gonja manjaya (*Musa* spp. AAB) using embryogenic cell suspensions. *In Vitro Cellular & Developmental Biology. Plant*, 2012, **48**(2): 216–224
14. Hu C, Dou T, Wei Y, Sheng O, Kuang R, Yang Q, Li C, Yi G. Establishment of *Agrobacterium*-mediated transformation of multiple buds slices of Banana. *Molecular Plant Breeding*, 2014, **12**(6): 1195–1200 (in Chinese)
15. Huang X, Huang X L, Wang H H, Li X J. Studies on the plant regeneration from the micro cross sections of banana. *Acta Horticulturae Sinica*, 2001, **28**(1): 19–24 (in Chinese)
16. Okole B N, Schulz F A. Micro-cross sections of banana and plantains (*Musa* spp.): Morphogenesis and regeneration of callus and shoot buds. *Plant Science*, 1996, **116**(2): 185–195
17. Huang Y, Yi G, Zhou B, Zeng J, Wu Y. Research advances about genetic engineering of banana. *Acta Botanica Boreali-Occidentalia Sinica*, 2006, **26**(10): 2179–2185 (in Chinese)
18. Paul J Y, Becker D K, Dickman M B, Harding R M, Khanna H K, Dale J L. Apoptosis-related genes confer resistance to Fusarium wilt in transgenic ‘Lady Finger’ bananas. *Plant Biotechnology Journal*, 2011, **9**(9): 1141–1148
19. Tripathi J N, Oduor R O, Tripathi L. A High-throughput regeneration and transformation platform for production of genetically modified banana. *Frontiers in Plant Science*, 2015, **6**: 1025
20. Hrahsel L, Basu A, Sahoo L, Thangjam R. In vitro propagation and assessment of the genetic fidelity of *Musa acuminata* (AAA) cv. Vaibhalha derived from immature male flowers. *Applied Biochemistry and Biotechnology*, 2014, **172**(3): 1530–1539