Chapter 2
Gamma Irradiation of Embryogenic Cell Suspension Cultures from Cavendish Banana (*Musa* spp. AAA Group) and *In Vitro* Selection for Resistance to Fusarium Wilt

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Abstract In this chapter, the establishment of embryogenic cell suspension (ECS) cultures using immature male flowers of triploid banana (*Musa* AAA Cavendish subgroup cv. 'Brazil'), followed by somatic embryogenesis and plantlet regeneration is described. Mutation induction is achieved by exposing the ECS to gamma irradiation with the dose of 80 Gy. The mutagenized cell population is transferred to solid long-term suspension culture medium for 96 h to recover from mutagen treatment shock, followed by somatic embryo induction and development medium containing 20% crude culture filtrates from *Fusarium oxysporum* f. sp. *cubense* (Foc). After 90 days, the somatic embryos that survive are transferred to the germination medium containing 25% crude culture filtrates. The surviving mature somatic embryos are transferred to rooting medium after the fourth subculture on the germination medium containing 50% crude culture filtrates. Before transplanting in a Foc infected field, the *in vitro* plantlets are acclimatized and screened for resistance to Foc using a pot-based greenhouse bioassay.

Keywords Cavendish banana · Embryogenic callus · Gamma irradiation · Germplasm resistant to Fusarium wilt

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1 Introduction

Bananas and plantains (*Musa* spp.) are not only the most widely consumed fruits in the world, but also a staple food for over 500 million people. In recent years, the world faced a sharp decline in banana production, due to extreme weather patterns and the outbreak of pests and diseases, especially Fusarium wilt. Today's global banana production is seriously threatened by a new strain of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). There is an urgent need to introduce resistance against Foc TR4 into commercial banana cultivars. The most common cultivars for commercial production belong to *Musa* AAA Cavendish subgroup, which are sterile and seedless. Attempts to develop new banana genotypes resistant to Fusarium wilt using traditional cross breeding techniques face significant hurdles. Thus, induced mutagenesis or genetic engineering offers practical alternatives to create new varieties or novel germplasm and has become a dominant approach for breeding disease-resistant banana (Dita et al. 2018).

Somatic embryos originate from single cells, and the chimera frequency is very low. To accelerate genetic improvement of banana, it is important to establish embryogenic cell suspension (ECS) cultures, followed by plantlet regeneration through somatic embryogenesis. So far, different source explants have been used to establish ECS in banana, including basal leaf sheaths and corm sections (Novák et al. 1989), highly proliferating meristems (Dhed'a et al. 1991; Strosse et al. 2006), zygotic embryos (Marroquin et al. 1993), immature male (Escalant et al. 1994; Côte et al. 1996; Navarro et al. 1997; Becker et al. 2000; Pérez-Hernández and Rosell-García 2008; Kulkarni and Bapat 2012; Elayabalan et al. 2013; Namanya et al. 2014; Morais-Lino et al. 2016) and female flowers (Grapin et al. 2000). Among the various explants used, immature male flowers appear to be the most widely applicable starting material for the establishment of regenerable ECS.

Plant mutation breeding is an effective method for creating novel germplasm. Briefly, seeds, pollen, the whole plant, vegetative organs, or callus are subjected to irradiation, followed by the process of selection and identification of a new variety (Spencer-Lopes et al. 2018). Physical mutagenesis is also applied to improve horticultural crops, it is reported that novel germplasm with agriculturally valuable traits has been developed in apple, peach, pear, and citrus (Source: FAO/IAEA Mutant Varieties Database). Gamma irradiation has been widely used as a physical mutagen for breeding of many crops including banana (Novák et al. 1990; Mak et al. 1996; Guo et al. 2003).

In this chapter, we present a detailed protocol for the establishment of a cell suspension culture and plantlet regeneration *via* somatic embryogenesis of Cavendish banana (*Musa* spp. AAA group). The ECS cultures are then subjected to 80 Gy gamma irradiation, followed by *in vitro* selection for resistance to Fusarium wilt.

2 Materials

2.1 In Vitro *Media for Induction of Embryogenic Callus (MI), Long Term Suspension Culture (ML), Somatic Embryo Induction and Development (MSD), Somatic Embryo Germination (MG), and Rooting of Somatic Embryos (MR)*

1. MS (Murashige and Skoog 1962) basal medium.
2. MS basal medium without vitamins.
3. MS vitamins.
4. SH (Schenk and Hildebrandt 1972) basal medium without vitamins.
5. Morel and Wetmore (1951) vitamins.
6. Biotin.
7. Indole-3-acetic acid (IAA).
8. Naphthalene acetic acid (NAA).
9. 2, 4-Dichlorophenoxyacetic acid (2, 4-D).
10. 6-Benzyl aminopurine (6-BA).
11. Kinetin (KT).
12. Malt extract.
13. Glutamine.
14. Proline.
15. Sucrose.
16. Gelrite.
17. Agar.
18. Distilled water.
19. Analytical balance.
20. pH meter.
21. Autoclave.

2.2 *Materials for the Induction of Embryogenic Callus, Establishment of ECS Cultures, Induction and Maturation of Somatic Embryos*

1. Sterile culture media (MI, ML, MSD, MG, MR).
2. Male inflorescence of Cavendish banana (*Musa* spp. AAA group) (*see* Note 1).
3. 75% ethanol.
4. Sterile distilled water.
5. Stereo microscope.
6. Erlenmeyer flask (100 ml).
7. Petri dish (9.0 cm).
8. Sieve (154 μm and 900 μm).

9. Dissecting instruments (scalpels handle and blades, forceps).
10. Laminar airflow cabinet.
11. Rotary shaker.
12. Growth chamber with environmental control.

2.3 *Mutation Induction of ECS via Gamma-Irradiation*

1. Cobalt-60 source (0-100 Gy) (*see* Note 2).
2. ECS cultures.
3. Sterile ML agar medium.
4. Sterile filter paper.
5. Sterile petri dishes (9.0 cm).

2.4 In Vitro *Selection for Resistance to Fusarium Wilt*

1. Seven-days-old PDA (potato dextrose agar) plate culture of Foc TR4 strain II5 (NRRL 54006, VCG 01213).
2. Haemocytometer.
3. Czapek's Broth (ready to use).
4. Erlenmeyer flasks (250 ml).
5. Rotary shaker.
6. Cheesecloth.
7. Centrifuge.
8. Autoclave.
9. Microporous filters (0.25 μm).
10. Sterile MSD, MG and MR medium.
11. Substrates for *ex vitro* acclimatization of banana plantlets.
12. Greenhouse with environmental control.

3 Methods

3.1 *Preparation of MI, ML, MSD, MG, and MR Medium*

1. MI medium consists of MS basal medium, 1 mg/l biotin, 1 mg/l IAA, 1 mg/l NAA, 1 mg/l 2, 4-D, 100 mg/l glutamine, 100 mg/l malt extract, 30 g/l sucrose, and 7 g/l agar or 2 g/l gelrite.
2. ML medium consists of MS basal medium, 1 mg/l biotin, 1 mg/l 2,4-D, 100 mg/l glutamine, 100 mg/l malt extract, and 45 g/l sucrose.

3. MSD medium consists of SH basal medium without vitamins, MS vitamin, 1 mg/l biotin, 100 mg/l glutamine, 230 mg/l proline, 100 mg/l malt extract, 0.2 mg/l NAA, 0.1 mg/l KT, 45 g/l sucrose, 2 g/l gelrite.
4. MG medium consists of MS basal medium without vitamins, Morel and Wetmore vitamins, 1 mg/l 6-BA, 0.2 mg/l IAA, 30 g/l sucrose, 2 g/l gelrite.
5. MR medium is MG medium without any plant growth regulators.
6. Adjust pH value of MI and ML medium to 5.3, adjust pH value of other media to 5.8.
7. Sterilize all media for 15 min at 120 °C.
8. Allow media to cool prior to use.
9. Store for up to a week in a cold room.

3.2 Isolation of Immature Male Flowers

1. Take a female flower bud that has just completed the fruit set process and use the 10-12 cm long portion at the top end of the bud, that is, the male inflorescence.
2. Keep on removing the outer bract and the male flower under it until the top 1.5 cm long portion of the inflorescence remains.
3. Surface sterilize the inflorescence by immersing in 75% ethanol for 1-2 min, followed by a rinse with sterile distilled water.
4. Under aseptic conditions, remove the inner bract and isolate the immature male flower under it with the aid of a stereo microscope. The one adjacent to the floral apex is rank 1 flower.
5. Rank 1 to rank 15 flowers are used as explant and are placed on MI medium.
6. The cultures are maintained at 28 ± 1 °C in darkness for nearly 5 months.

3.3 Induction of Embryogenic Callus and Development of ECS

1. For initiation of the suspension culture, select loose and fragile light-yellow callus induced on MI medium (*see* Fig. 2.1a).
2. Weigh ≈ 2 g of embryogenic callus and add to a 100 ml Erlenmeyer flask containing 30 ml ML medium. The cultures are incubated on a rotary shaker (110 rpm/min) at 28 ± 1 °C in darkness.
3. In the first month of suspension culture, replace ML medium once a week, and sieve cultures through a 900 μm mesh to remove the non-dispersible large culture particles, including callus clusters, dead tissues, and sometimes pre-embryos.
4. After one month, replace ML medium every 2 weeks. When cultures become dispersed, use a sieve of 154 μm aperture to remove the larger cell clusters.
5. Generally, it takes 3 months to obtain ECS cultures in Cavendish banana (*Musa* spp. AAA group), which are relatively more dispersed and homogeneous (*see* Fig. 2.1b).

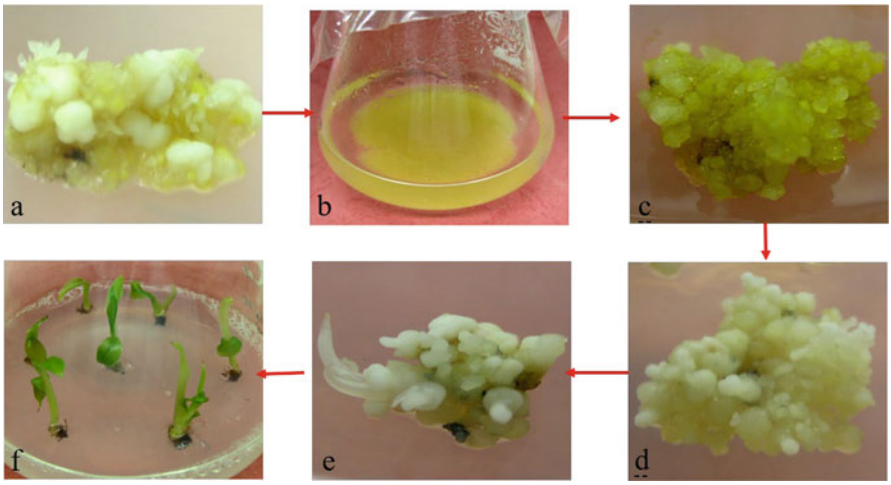

Fig. 2.1 Steps for cell suspension culture and plantlet regeneration *via* somatic embryogenesis of Cavendish banana (*Musa* spp. AAA group cv. 'Brazil'). (**a**) Embryogenic callus induced on MI medium; (**b**) Well established embryogenic cell suspension; (**c**) Induction of somatic embryo on MSD medium; (**d**) Maturation of somatic embryo; (**e**) Germination of somatic embryo; (**f**) Regenerated plantlet

3.4 Induction and Maturation of Somatic Embryos

1. Ten days after subculture, cell clumps are taken from ECS cultures and sieved through a 154 μm mesh.
2. Transfer the cell population onto MSD medium (*see* Fig. 2.1c).
3. The cultures are maintained at 28 ± 1 °C in darkness.
4. Generally, it takes 90 days to promote maturation of somatic embryos (*see* Fig. 2.1d).

3.5 Plantlet Regeneration

1. Mature somatic embryos which have been cultured on MSD medium for 90 days are transferred to MG medium in Petri dishes.
2. The cultures are maintained at 28 ± 1 °C in darkness for 30 days.
3. After germination (*see* Fig. 2.1e), the somatic embryos with light green leaf sheaths are then transferred to MR medium for the development of complete plantlets (*see* Fig. 2.1f).
4. Cultures are maintained at 28 ± 1 °C under a 16 h/8 h photoperiod with 30 μmol m^{-2} s^{-1} from cool white fluorescent lamps.

3.6 *Gamma Irradiation of ECS and* In Vitro *Selection for Resistance to Fusarium Wilt*

3.6.1 Determination of Irradiation Dose

1. Prior to irradiation, the initial density of ECS is adjusted to 1.5% of the packed cell volume (PCV) in 30 ml of medium.
2. One week after subculture, ECS cultures are subjected to γ-ray irradiation treatment. Erlenmeyer flasks containing ECS cultures receive 40, 60, 80, and 100 Gy of irradiation, respectively, at a dose rate of 2 Gy/min. Control ECS cultures are prepared in the same manner but don't receive irradiation.
3. Weigh 0.1 g cell clumps and transfer to Erlenmeyer flask containing 40 ml MSD medium. Each treatment includes 4 replicates. The cultures are incubated in darkness for 90 days.
4. Weigh somatic embryo obtained, then transfer to MG medium. The cultures are incubated in darkness for 30 days.
5. Transfer the germinated somatic embryo to MR medium and calculate the number of regenerated plantlets.
6. Calculate LD_{50} according to the weight of somatic embryo and the number of regenerated plantlets (*see* Note 3).

3.6.2 Preparation of Crude Culture Filtrates from Foc TR4

1. Collect conidia from 7-days-old PDA plate culture of Foc TR4 strain by rinsing with sterile distilled water. Conidia concentration was determined with a haemocytometer and adjusted with sterile distilled water to 3×10^5—4×10^5 conidia /ml.
2. Take 1 ml of the conidia suspension and transfer to a 250 ml Erlenmeyer flask containing 100 ml Czapek's liquid medium. The culture is incubated on a rotary shaker (120 rpm/min) at 25 ± 2 °C for 12 days.
3. Then the culture is filtered through four layers of cheesecloth to remove mycelium, followed by filtrate being centrifuged at 5000 rpm/min for 15 min.
4. The supernatant broth is autoclaved at 121 °C for 20 min to eliminate the effect of enzymes, and then filtered under pressure with a bacteria filter with a 0.25 μm microfiltration membrane. The filtrate is the sterile crude culture filtrates from Foc.

3.6.3 *In Vitro* Selection for Resistance to Fusarium Wilt

1. Put a sterile filter paper on the surface of the ML agar medium, then transfer mutagen treated cells to the medium. The cultures are maintained for 96 h, which allows the cells to recover from mutagen treatment shock.

2. The mutagenized embryogenic cell population is transferred to MSD medium containing 20% crude culture filtrates from Foc.
3. After 90 days, the somatic embryos that survive are transferred to MG medium containing 25% crude culture filtrates.
4. After nearly 30 days, the surviving mature somatic embryos are then sub-cultured at least four times on the same medium containing 50% crude culture filtrates.
5. The regenerated shoots obtained are transferred to MR medium.
6. Before planting in a Foc infected field, the plantlets are acclimatized and screened for resistance to Foc using a pot system in greenhouse (*see* Note 4).

4 Notes

1. Although somatic embryogenesis in banana has been studied for more than 30 years (Cronauer-Mitra and Krikorian 1988), it is still far from being considered a routine technology. In other words, it is difficult to develop an efficient and repeatable protocol for all *Musa* genotypes. Given the fact that cultivars in Cavendish subgroup are the most common cultivars for commercial production around the world, this protocol describes culture establishment for a commercial cultivar 'Brazil' (*Musa* AAA Cavendish subgroup), which can also be applied to other Cavendish cultivars (Xu et al. 2003).
2. The mutagenesis was conducted in Guangdong Irradiation Center, which was only about an hour's ride from the institute. If the irradiation source is far away from the tissue culture lab, the risk of contamination during long-distance transportation require additional precautions, such as the use of dedicated and sterilized container for Erlenmeyer flasks and a temperature-controlled delivery vehicle.
3. It is observed that the growth and regeneration ability of ECS decreases gradually with the increase of irradiation dosage. After ECS cultures receive 80 Gy of irradiation, the weight of somatic embryo and the number of regenerated plantlets is 48.76% and 46.12% of the control, respectively. It is strongly recommended to determine the LD_{50} of the ECS cultures before ECS bulk irradiation.
4. The pot-based greenhouse screening protocol for *Musa* genotypes against Foc was performed as described by Zuo et al. (2018).

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