Zygotic embryo rescue in bananas

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Abstract — Introduction. This protocol describes a method for obtaining *in vitro* germination of zygotic embryos from open-pollinated wild seedy bananas and controlled hybridizations. The principle, key advantages, starting plant material, time required and expected results are presented. **Materials and methods**. This part describes the required laboratory materials, medium preparation, embryo extraction and culture. **Results**. Embryos typically begin germination in the dark about 15 days after inoculation on germinating medium. After 30—40 days of culture, plantlets are subcultured on a plant growth medium and placed in the light to achieve plant growth and rooting. The ratios of zygotic embryos obtained vary with the types of crosses achieved between *Musa* sp. genotypes.

France (Guadeloupe) / *Musa* sp. / methods / in vitro culture / seeds / embryo culture / vitroplants

Sauvetage d'embryons zygotiques chez le bananier.

Résumé — Introduction. Le protocole décrit une méthode qui permet d'obtenir la germination *in vitro* d'embryons zygotiques à partir de graines de bananiers sauvages en pollinisation libre et d'hybridations contrôlées. Le principe, les principaux avantages de la méthode, le matériel végétal de départ, le temps nécessaire et les résultats attendus sont présentés. **Matériel et méthodes**. Cette partie décrit le matériel de laboratoire nécessaire, la préparation des milieux et l'extraction des embryons ainsi que leur culture. **Résultats**. Typiquement, les embryons amorcent leur germination à l'obscurité, 15 jours après leur mise en culture sur le milieu de germination. Après (30 à 40) jours de culture, les plantules sont transférées à la lumière et placées sur un milieu de croissance pour obtenir le développement de plantes entières enracinées. Les taux de germination *in vitro* des embryons zygotiques obtenus varient selon les types de croisements réalisés entre les génotypes de *Musa* sp.

France (Guadeloupe) / *Musa* sp. / méthode / culture in vitro / graine / culture d'embryon / vitroplant

1. Introduction

Application

This protocol makes it possible to obtain *in vitro* germination of zygotic embryos from open-pollinated wild seedy bananas and controlled hybridizations.

Principle

The method is derived from Bakry and Horry [1] and Bakry *et al.* [2]. Seeds from mature fruits, as described by Darjo and Bakry [3], are surface-sterilized in aqueous silver nitrate solution, transferred into a sterile sodium chloride solution and then rinsed in sterile distilled water. The seeds are opened in aseptic conditions and embryos extracted under a binocular microscope. Embryos are placed on a derived Murashige and Skoog [4] semi-solid medium supplemented with BA and IAA. Cultures are incubated at 27 °C in the dark until embryo germination. The seedlings are subcultured individually in tubes on a growth medium for 2 months. The rooted plantlets are then transferred to the nursery for acclimatization before field transfer.

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Key advantages

This protocol allows one to:

– save plant material because the germinating ratio is higher with *in vitro* embryo rescue than by seed sowing,

– save time because *in vitro* embryo germination is faster and more reliable than natural seed germination,

– recover healthy plant material because the *in vitro* germinated embryos are free of pest or disease before weaning,

– facilitate hybrid and field management because germinated plants can be stored *in vitro* before further utilization (allowing, for example, progeny groupings to conduct field trials).

Starting material

Seeds from hand-pollinated or open-pollinated bunches are harvested 65–70 days to 120–135 days after flowering.

Note: seeds should be extracted from ripe yellow fruits, washed with tap water and rapidly transferred to the laboratory to avoid seed embryo desiccation.

Time required

Twenty min are necessary for external seed sterilization; 1 h, for extraction of (20 to 30) embryos and embryo inoculation on germinating medium; (30 to 60) days, to complete *in vitro* embryo germination.

Expected results

Healthy *in vitro* germinated embryos ready to develop into banana-rooted vitroplants after subculture on plant growth medium are obtained with a germination rate of from 10% to 99 % according to the maternal donor plant and crosses.

2. Materials and methods

Laboratory materials

This protocol requires:

– 20 to 30 freshly extracted and tap-water washed seeds,

– a sterilized 50-mL beaker,

– a stainless steel forceps,

– a scalpel,

– 20 mL aqueous silver nitrate solution (1% w/v),

– 20 mL aqueous solution of sodium chloride (0.5% w/v) sterilized by autoclaving,

– 100 mL sterilized distilled water,

– sterilized dish paper,

– a binocular microscope with 7 × to 40 × magnification,

– 9-cm petri dishes,

– 2.5-cm / 15-cm cap-sealed culture tubes for vitroplantlet rooting,

– plastic film,

– a laminar flow hood,

– an incubator regulated at (27 ± 2)°C for embryo germination in the dark and a growth culture room at the same temperature under a 16-h (day) photoperiod (light provided by "daylight" fluorescent tubes of 100 µmol·m^{-2}·s^{-1}).

Medium preparation

• Step 1

Prepare the embryo germinating and growth media (*table I, II*) according to classical *in vitro* culture rules; fill up 9-cm petri dishes with 30 mL of germinating medium; fill up tubes with 20 mL of growth medium.

Note: media are derived from Murashige and Skoog [4]; germination medium is supplemented with growth regulator. After autoclaving, media can be kept for 3 weeks in the dark.

Embryo extraction and culture

• Step 2

To achieve the seed sterilization under the laminar flow hood:

– surface-sterilize seeds by stirring them for 10 min in 15 mL of the silver nitrate solution,

– pour out the silver nitrate solution,

– add 20 mL of the sterile sodium chloride solution for 30 sec,

– pour out the solution and the resulting AgCl precipitate,

– wash seeds three times with sterile distilled water.

Table I.
Composition of the basal culture media (germinating medium and plant growth medium) used for the *in vitro* rescue of zygotic embryos in *Musa* sp. For each medium, pH is adjusted to 5.7–5.8 with 0.1 N KOH prior to autoclaving for 20 min at 118 °C.

Components	Elements	Concentration of components ($mg \cdot L^{-1}$)
Murashige and Skoog macroelements	Ammonium nitrate (NH_4NO_3)	1 650
	Potassium nitrate (KNO_3)	1 900
	Calcium chloride ($CaCl_2$, $2H_2O$)	440
	Magnesium sulfate ($MgSO_4$, $7H_2O$)	370
	Potassium phosphate, monobasic (KH_2PO_4)	170
Murashige and Skoog microelements	Manganese sulfate ($MnSO_4$, H_2O)	22.3
	Zinc sulfate ($ZnSO_4$, $7H_2O$)	8.6
	Boric acid (H_3BO_3)	6.2
	Potassium iodide (KI)	0.83
	Molybdic acid (sodium salt) (Na_2MoO_4, $2H_2O$)	0.25
	Cobalt chloride ($CoCl_2$, $6H_2O$)	0.025
	Cupric sulfate ($CuSO_4$, $5H_2O$)	0.025
Morel and Wetmore vitamins	Myo-inositol	100
	Thiamine.HCl	1
	Pyridoxine.HCl	1
	Nicotinic acid	1
	D-Calcium panthothenate	1
	Biotin	0.01
Fe-EDTA	Na_2-EDTA	37.3
	Ferrous sulfate ($FeSO_4$, $7H_2O$)	27.8
Amino acids	Casein hydrolyzate	500
Carbohydrates	sucrose	25 000
pH indicator	Bromocresol purple	8

Table II. Plant growth regulators and gelling agents added to the basal culture media used for the *in vitro* rescue of zygotic embryos in *Musa* sp.

Type of medium	Plant growth regulators ($mg \cdot L^{-1}$)		Gelling agent ($g \cdot L^{-1}$)
	Benzylaminopurine (BAP)	Indole acetic acid (IAA)	Gelrite
Germinating medium	1.0	0.4	1.5
Plant growth medium	0	0	2.0

• Step 3
To achieve the embryo extraction from seeds under the laminar flow hood:
– under a binocular, using forceps, keep the sterilized seeds with the micropyle facing up (*figure 1*). *Note:* maintain seeds on a sterile paper disc during step 3,

– make a first incision on the external side of the seed without damaging the embryo,

– make a second incision on the opposite side of the seed in order to open it,

– take out the embryo carefully with the tip of the blade (*figure 2*),

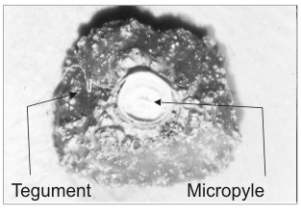

Figure 1.
Banana seed, mycropyle side facing up.

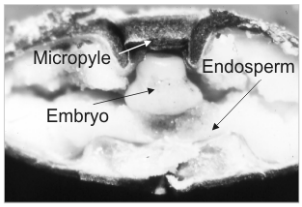

Figure 2.
Open banana seed.

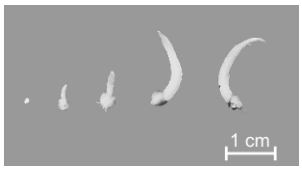

Figure 4.
In vitro embryo evolution in the germinating medium (*Musa acuminata*): on the left, an embryo at inoculation; on the right, two embryos after 30-40 days of culture ready for transfer onto the growth medium.

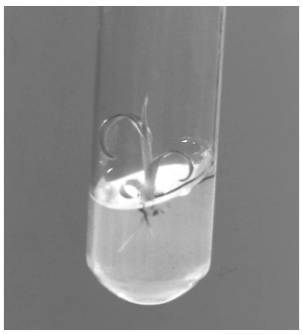

Figure 5.
Typical germinated embryo about 15 days after transfer onto the plant growth medium. Emerged leaves are green and roots elongated.

– immediately transfer the embryo onto the germinating medium, put the cotyledon down in contact with the medium (*figure 3*). *Note:* ten embryos can be inoculated on one petri dish;
– seal the petri dish with a plastic film and place the cultures in the incubator in the dark at 27 °C.

• Step 4
For embryo culture:
– maintain the cultures in the dark until embryos germinate,
– when the germinated embryos reach a size of 1.5–2 cm (*figure 4*), transfer these plantlets onto growth medium for (30 to 40) days in the illuminated growth culture chamber (photoperiod of light 16 h / dark 8 h, artificial light intensity provided by "daylight" fluorescent tubes of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Note: after the growing stage, the plantlets obtained can be subcultured *in vitro* or transferred to the nursery for acclimatization.

Troubleshooting

Three main problems can occur:

(a) Embryos are infected *in vitro* by fungus or bacteria, which can result from an inaccurate sterilization of seeds.

Solution: to improve the seed sterilization procedure, verify the sterility of the sodium chloride solution and of the water used to rinse the seeds.

(b) There is no germination of the embryos due to old seeds with desiccated embryos.

Solution: soak the seeds for 48 h by immersion in sterile water after a first surface sterilization, then proceed to a second surface sterilization before embryo extraction.

(c) Embryos of interspecific *M. acuminata* / *M. balbisiana* genomic constitution are blackening: in that case, the usual procedure could be inadequate.

Solutions:
– do not wait for full bunch maturation, but harvest the pollinated bunches 85–90 days after the beginning of flowering,
– dilute by half the Murashige and Skoog macroelements in the germinating medium and add 120 mg $KH_2PO_4 \cdot L^{-1}$,
– suppress casein hydrolyzate from the germinating medium and add 300 mg glutamine$\cdot L^{-1}$.

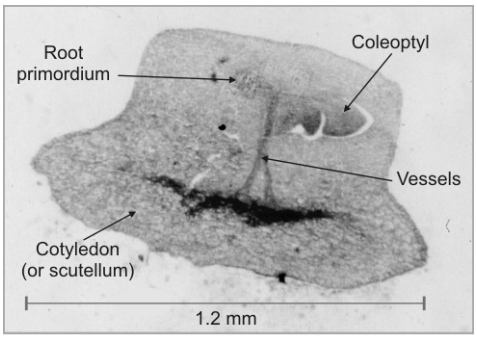

Figure 3.
Anatomy of a *Musa balbisiana* zygotic embryo.

3. Typical results obtained

Embryos typically begin germination in the dark about 15 days after inoculation on germinating medium. After 30-40 days of culture, plantlets are subcultured on a plant growth medium and placed in the light to achieve plant growth and rooting. The ratios of zygotic embryos obtained vary with the types of crosses achieved between *Musa* sp. genotypes (*figures 1* to *5, table III).*

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Table III.
Examples of *in vitro* germination ratios of zygotic embryos obtained with different types of crosses (*Musa* sp.); balb: balbisiana; acum: acuminata.

Female parent	Male parent	Putative genotype of hybrids	Number of inoculated embryos	Number of germinated embryos	% of germination
balb / balb (wild clone: Lal Velchi)	balb / balb (wild clone: Lal Velchi)	balb / balb (inbred)	95	94	99.0
acum / acum (Tha 052 cultivar)	acum / acum (wild clone: Long Tavoy)	acum / acum	86	58	67.5
acum / acum (Pisang Madu cultivar)	acum / acum (Pisang Madu cultivar)	acum / acum (inbred)	120	90	75.0
acum / acum (wild clone: Calcutta 4)	acum / acum / acum / acum (Pisang Lilin tétraploid cultivar)	acum / acum / acum	74	61	82.0
balb / balb (wild clone: Pisang Klutuk Wulung)	acum / acum (SF265 cultivar)	balb / acum	78	36	46.0
acum / acum / balb (plantain cultivar)	acum / acum (wild clone: Calcutta 4)	acum / acum acum / acum / balb acum / acum / acum acum / acum / acum / balb	173	47	27.0
acum / acum / balb (Popoulou cultivar)	acum / acum (wild clone: Calcutta 4)	acum / acum acum / acum / balb acum / acum / acum acum / acum / acum / balb	599	195	32.5