Haploid induction: androgenesis in *Musa balbisiana*

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Abstract — Introduction. This protocol describes the production of haploid and dihaploid plants from *in vitro* anther cultures of *M. balbisiana*. 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, anther isolation and culture. **Results**. White, creamy calli emerge from anthers about (6 to 15) weeks after the beginning of the culture.

France (Guadeloupe) / *Musa balbisiana* / methods / plant biotechnology / haploidy

Induction d'haploïdes : androgenèse chez *Musa balbisiana.*

Résumé — Introduction. Le protocole décrit la production de plantes haploïdes et dihaploïdes par culture *in vitro* d'anthères de *M. balbisiana*. Le principe, les principaux avantages, 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, le prélèvement et la culture des anthères. **Résultats**. Des cals blanc-crémeux se forment sur les anthères environ (6 à 15) semaines après le début de la culture.

France (Guadeloupe) / *Musa balbisiana* / méthode / biotechnologie végétale / haploïdie

1. Introduction

Application

This protocol is recommended to produce haploid and dihaploid plants from *in vitro* anther cultures of *Musa balbisiana*.

Principle

The method is derived from Bakry and Horry [1], Bakry *et al.* [2, 3] and Assani *et al.* [4]. Anthers containing microspores in the uninucleate stage are isolated from immature male flowers and incubated on a modified MS medium [5] in the dark to induce callus formation. Calli are separated from anthers and subcultured to induce embryos, which are regenerated. Plant regeneration occurs in the dark.

Key advantages

The protocol facilitates improvement due to the accelerated release of lines from crosses between *M. balbisiana* clones. It allows one to obtain haploid plant material to conduct genetic studies and somatic hybridization.

Starting material

Male buds (*figure 1*) must be harvested about 1 month after bunch shooting. Male buds contain male flowers at all developmental stages (immature and mature flowers). Reactive anthers for androgenesis must

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be excised from immature male flowers positioned in bracts No. 21 or No. 22. At this stage, which corresponds to about 21 days before anthesis, the immature anthers usually contain highly vacuolated uninucleate microspores.

Note: The following *M. balbisiana* clones have been found to be reactive with the described protocol: Batu, Tani, Pisang Wulung and Pisang Klutuk Wulung.

Time required

One hour is necessary for the male bud harvest and the identification of the right flower stage development in the lab; 2 h, to extract about 300 anthers from male flowers and to inoculate them on culture medium; 2 to 4 months, for callus induction; 30 to 60 days, to complete the plant regeneration from isolated calli.

Expected results

About 3% to 9% of anthers producing at least one callus can be obtained; plant regeneration is expected from 60% of the calli; an average of 30% haploid and 70% spontaneous diploid plants regenerated from calli.

2. Materials and methods

Laboratory materials

The protocol requires:

– sterile 200-mL beaker,

– 95% (v/v) ethanol,

– clean microscope slides and cover glass,

– 1% (w/v) aqueous solution of acetocarmine or 2.8% (w/v) NaH_2PO_4 solution,

– sterile stainless steel forceps and scalpels,

– sterile dish paper,

– standard binocular stereomicroscope (objective magnification ×20),

– sterile 9-cm petri dishes, sterile 2.5 cm × 15 cm cap sealed culture tubes for plantlet rooting,

– plastic film for closing petri dishes and culture tubes,

– incubator or culture room regulated at (27 ± 2) °C for incubation in the dark or in the light.

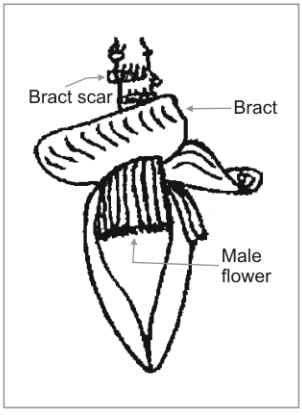

Figure 1.
Schematic presentation of the male flower bud of banana.

Note: for preparation of the acetocarmine solution, boil, for 2 h, 1 g of carmine in 100 mL of 45% acetic acid in a balloon-flask with an ascending cooler, then filter and pour the solution into a dark bottle. The solution can be kept in the darkness at a cold temperature for several months.

Medium preparation

• Step1

Prepare the callus induction medium, and the regeneration and the growth medium (*tables I, II*). Pour 30 mL of callus induction medium into 9-cm petri dishes. Fill tubes with 20 mL of regeneration and growth medium.

Note: media are derived from the basal medium of Murashige and Skoog [3]; the callus induction and the regenerating medium are supplemented with growth regulators. After autoclaving, media can be stored for 3 weeks in the dark.

Anther isolation and culture

Preparation of the male bud

• Step 2

Collect the male bud in the morning and bring the male bud to the lab with no delay after harvest. Mark the open bract as No. 0 (*figure 1*). Open and strip away bracts from No. 1 to No. 10 together with their male flowers from the rachis.

Note: bracts are numbered from the oldest (No. 1) to the youngest (No. 10).

Male bud surface sterilization

• Step 3

In the laminar flow hood, immerse the male bud in 100 mL of 95% ethanol. Sterilize the bud by flaming away the ethanol from the beaker. Repeat sterilization by flaming a second time. Place the bud on sterile paper.

Identification of the microspore developmental stage

• Step 4

Strip away and discard bracts with male flowers from No. 11 to No. 16 with a pair of sterile forceps and scalpel. Strip away bracts with flower clusters at the base from

Table I.
Composition of culture media (callus induction, regeneration and growth) used for the production of haploids in *Musa balbisiana.* 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	25000
pH indicator	Bromocres of purple	5

Note: No casein hydrolyzate is added to the growth medium.

Table II.
Plant growth regulators and gelling agents added to the culture media used for the production of haploids in *Musa balbisiana.*

Type of medium	Plant growth regulators ($mg \cdot L^{-1}$)		Gelling agent ($g \cdot L^{-1}$)	
	Benzylaminopurine (BAP)	Indoleacetic acid (IAA)	Agarose (type II, Sigma)	Gelrite
Callus induction	1.0	0.4	6.0	0
Regeneration	0.5	0.4	7.5	0
Plantlet growth	0	0	0	2

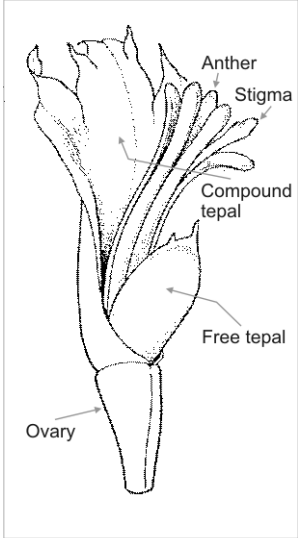

Figure 2.
Schematic presentation of the male flower of banana.

No. 17 to No. 26 and keep them ranked in the laminar flow. Take a median flower with a pair of forceps from each cluster on the bracts and place them on sterile paper. Cut the top of the compound tepal and the junction between the ovary and tepals with a scalpel (*figure 2*). Make a longitudinal incision on the compound tepal and open the flower to remove one anther. Slightly squash the anther in a drop of 1% aceto-carmine solution or 2.8% NaH_2PO_4 solution, between a microscopic slide and a cover glass. Check with the microscope (×200 magnification) the developmental stage of the microspores. Identify the anther containing highly vacuolated microspores with a nucleus in a peripheric position (*figure 3*) and mark the corresponding flower cluster (bract No. *x*).

Note: in *M. balbisiana,* flowers at the uninucleate microspore stage are usually located in bracts with No. 21 or No. 22. Nevertheless, this stage may vary according to the clone and the environmental conditions. Therefore, it is recommended to check clusters of higher and lower ranks to find anthers in the right microspore developmental stage.

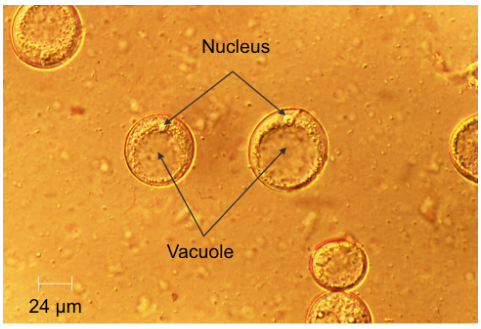

Figure 3.
Highly vacuolated uninucleate microspores of banana with the nucleus in a peripheric position.

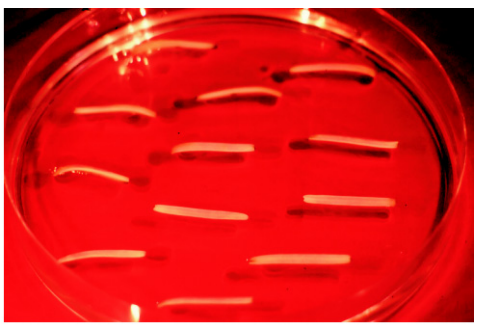

Figure 4.
Anthers of *Musa balbisiana* (clone: Pisang Batu) inoculated on callus induction medium in petri dishes.

Anther inoculation

• Step 5
Collect flowers from bracts No. *x*. Open the flowers and isolate anthers as described above.

Note 1: carefully use the tip of the scalpel blade. Immediately transfer the anthers onto the callus induction medium, the connective filament in contact with the medium (*figure 4*).

Note 2: ten anthers from two flowers can be inoculated in one petri dish. Repeat the same procedure with flowers from bracts No. (*x*–1) and No. (*x*+1). Seal the petri dish with plastic film and incubate at 27 °C in the dark.

Anther culture

• Step 6
Maintain the cultures in the dark until callus induction (about 5–6 weeks).

Note: do not subculture the anthers before the callus becomes visible in order to avoid anther tissue necrosis. Isolate and transfer the induced calli with a size of 2–4 mm (*figure 5*) to the regeneration medium and incubate at 27 °C in the dark until shoot formation (about 2 months).

Plant growth and ploidy evaluation

• Step 7
Isolate plantlets from the callus and transfer to growth medium for (30 to 40) days in an illuminated culture room (16-h photoperiod, provided by "daylight" fluorescent tubes, 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Note: after the growing stage, plantlets can be subcultured *in vitro* or transferred to the nursery for acclimatization. Determine the ploidy level of the regenerated plantlets by flow cytometry or chromosome counts.

Troubleshooting

Two main problems can occur:

(a) Cultured anthers blacken, which can result from:

– The desiccation of the anthers before culture.

Solution: protect the extracted anthers from the direct airflow and speed up the inoculation to the callus induction medium.

– Excess light during culture.
Solution: keep the culture in the dark without subculture and do not move the petri dishes for at least 5 weeks.

– The anther culture is from *M. acuminata* instead of *M. balbisiana*.
Solution: Check the variety used as donor plant material.

(b) No callus is induced. The cause would be that the anthers inoculated are at an inadequate developmental stage.
Solutions: select flowers with anthers with microspores in the highly vacuolated uninucleate stage only. Use only young male buds from 3 weeks to 1 month after bunch shooting. Increase the number of anthers inoculated.

3. Typical results obtained

White, creamy calli emerge from anthers are obtained about (6 to 15) weeks after the beginning of the culture (*figure 5*).

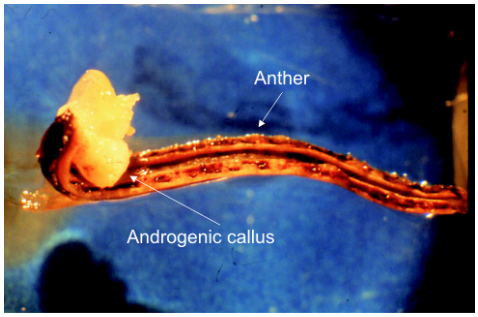

Figure 5.
Callus induction on a cultured anther of *Musa balbisiana* (clone: Pisang Batu).

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