Chapter 32

Thermotherapy, Chemotherapy, and Meristem Culture in Banana

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Abstract

Bananas that provide a staple food to the millions of people are adversely affected by several viruses such as Banana bunchy Top Virus (BBTV), Banana Streak Virus (BSV), and Cucumber Mosaic Virus (CMV). These viruses are known to have a devastating effect on crop production and constraint to the international exchange and conservation of banana germplasm—a cornerstone for breeding new cultivars. The viruses are particularly problematic in vegetative propagated crops, like bananas, because of their transmission in the planting material. Different virus eradication techniques have been developed, such as thermotherapy, chemotherapy, and meristem culture for providing virus-free planting material. Meristem culture proved to be the most effective procedure to eradicate phloem-associated viruses. This method requires isolation of meristematic dome of plant under the aseptic conditions and culture in an appropriate nutrient medium to develop new virus-free plants. Thermotherapy is another widely used virus eradication technique, which is initially carried out on in vivo or *in vitro* plants and eventually combined with meristem culture technique. The plantlets are initially grown at 28°C day temperature and increase it by 2°C per day until reaches 40°C and the night temperature at 28°C; maintain plants at 40°C for 4 weeks; excise meristem and culture onto the regeneration medium. In chemotherapy technique, antiviral chemical compound Virazole® is applied on meristem culture. Combination of these techniques is also applied to improve the eradication rate.

Key words: *Musa*, Virus eradication, Vegetative propagated plant, Tissue culture

1. Introduction

Viruses have most devastating effect on crop production. They are a major problem in vegetative propagated crops, such as bananas. The viruses transmit in the planting material during vegetative propagation using suckers or during *in vitro* multiplication. The availability of virus-free planting material is highly dependent on the availability of efficient virus eradication techniques. Banana is severely affected by several viruses such as Banana Bunchy Top

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Virus (BBTV), Banana Streak Virus (BSV), and Cucumber Mosaic Virus (CMV). Different virus eradication protocols were established, such as thermotherapy, chemotherapy, and meristem culture. The combination of these techniques is also applied to improve the eradication rate.

The meristem culture is based on the observation, frequently made in virus–host combination studies showing that the meristem often remains virus-free. Different researchers have shown that the rate of obtaining a virus-free regenerated plant is inversely related to the size of the isolated meristem (1). However, the capacity of the meristem to regenerate into a full plant is also directly related to the size of the explant. Hence, a balance between successful virus elimination and the probability for plant development is needed. Thermotherapy is another widely used virus eradication technique that can be carried out on in vivo or *in vitro* plants and which eventually could be combined with meristem culture. Relatively little is known about the mode of action of thermotherapy despite its widespread use. This technique is highly effective in virus eradication rates, which depends on the virus type. Moreover, it is time-consuming and *in vitro* plant material shows high mortality rate. The third virus eradication technique is chemotherapy, which uses chemical compounds applied to *in vitro* plant or meristem cultures. A wide range of potentially antiviral molecules has been tested. All of them have shown phytotoxicity and negative effects on meristem growth (2–6). Thus, it is necessary to test varying concentrations and treatment time to establish an optimal balance between the virus elimination rate and the plant survival rate. Among the antiviral substances tested, Virazole® (Ribavirin, 1-B-D-ribofuranosyl-1-2-4-triazole-3 carboxamide), a synthetic analogue of guanosine, is the most frequently used. Virazole acts on virus synthesis rather than through a direct inactivation of the existing virus. It is thus necessary to apply virazole for extended periods of time to eradicate viruses from infected tissues. During the treatment, virazole acts directly or indirectly preventing synthesis of new virus particles while existing virus particles are degraded in the course of their ontogeny (7).

Meristem culture is the most effective procedure for the eradication of phloem-associated viruses. Indeed, virus particles located in the phloem probably cannot invade the meristematic tissues because there is no cell differentiation in this zone. In case of BBTV, known to be phloem-associated (8), meristem culture from *in vitro* banana (*Musa acuminata*, AAA, cv Williams) plants turned out to be very efficient, resulting in 99% disease-free plants. The eradication rate was only 4% when meristems were excised from *in vivo*-grown plants. For BSV, eradication rates after meristem culture reached 76% and 41% by excising meristems from *in vivo*- or *in vitro*-grown banana plants, respectively (*Musa acuminata*, AAA, cv Williams). Thermotherapy combined with meristem

culture slightly increased the number of BSV-free plants up to 79% and 67%, respectively, when meristems were excised from heat-treated in vivo- and *in vitro*-grown plants (Helliot, unpublished results). The successful treatment of BSV-infected plants with three different acyclic nucleoside phosphonate analogues was also reported (6). With reference to CMV eradication, meristem culture alone is not very effective since only 1% and 7% plants were virus-free with the excision of meristems from in vivo- and *in vitro*-grown plants, respectively (9). This low eradication efficiency could be due to the localization of CMV particles within the banana meristematic dome (10). However, the CMV eradication frequency could be enhanced with more severe treatments. CMV eradication was achieved by Gupta (11) in approximately 100% regenerated plants by using meristem culture in combination with a 2-week heat therapy (38–40°C). Helliot et al. (9) obtained an eradication frequency 38% by excising meristems from in vivo-grown plants, and 70% from meristems—sourced from *in vitro*-grown plants, both combined with thermotherapy. Despite a diurnal alternating heat treatment, 60% mortality was still seen among the *in vitro*-grown plants, while in vivo plant material showed less sensitivity (4% mortality). The CMV eradication rate on banana, obtained with virazole chemotherapy, reached 24% (9). Previously, Long and Cassels (12) obtained 100% elimination of CMV on tobacco explant cultures treated with virazole.

All these results demonstrate that the virus eradication technique to be applied depends on (a) the virus characteristics, (b) the type of tissues treated, and (c) the plant species. Furthermore, our results concluded that banana genotype is the fourth major factor influencing the efficiency of a virus eradication technique.

2. Materials

2.1. Instruments and In Vivo Plant Material

1. Sterile distilled water.
2. Ethanol 70% (v/v).
3. Sodium hypochlorite (NaClO, 5%).
4. Dissecting instruments: scalpels with disposable blades and forceps.
5. Sterile plasticware (e.g., Petri dish) or glassware.
6. Laminar air-flow cabinet.
7. Flame to sterilize instruments or glass bead sterilizer.

2.2. Culture Media

1. Media based on the formulation of Murashige and Skoog (MS; (13)) (Duchefa, Haarlem, The Netherlands) (see Note 1).
2. Sucrose as carbon source.

3. Indole-3-acetic acid (IAA) as auxin.
4. 6-Benzylaminopurine (BAP) as cytokinin.
5. Ascorbic acid as antioxidant.
6. Gelrite® or another comparable solidifying agent.
7. Sterile distilled water.
8. Concentrated NaOH (1 M).
9. Concentrated HCl (1 M).
10. pH-meter.
11. Analytical (Ohaus-Adventurer) and precision balance (Sartorius).
12. Hotplate/stirrer.
13. Refrigerator to store prepared media, stock solutions, and some chemicals.
14. Autoclave.
15. Aluminum foil.
16. Screw-capped glass test tubes, 50–100 mL capacity.
17. Tube holders (metallic or plastic racks).
18. For chemotherapy:
    - Virazole® (Ribavirin, 1-B-D-ribofuranosyl-1-2-4-triazole-3 carboxamide; Duchefa, Haarlem, The Netherlands).
    - Syringe with syringe filter unit, pore size 0.2 μm (Acrodisc, VWR, Belgium).

2.3. Meristem Culture, Thermotherapy, and Chemotherapy Combined to Meristem Culture

1. Laminar air-flow cabinet.
2. Dissecting instruments: scalpel with disposable blades (min. two sizes) and forceps (min. two sizes, a longer round tipped and a short fine tipped).
3. Ethanol 70%.
4. Flame to sterilize instruments or glass bead sterilizer.
5. Petri dishes (∅ 90 mm).
6. Parafilm.
7. Binocular microscope with light source.
8. Culture room.
9. Incubator.

2.4. Plant Acclimatization in the Greenhouse

1. Tap water.
2. Greenhouse.
3. Clear plastic for tunnel.
4. Vermiculite.
5. Non-sterilized soil.
6. Plant pots (from ∅ 7 cm).

3. Methods

Among virus eradication protocols (meristem culture, thermotherapy, and chemotherapy), the selection of the most appropriate procedure is made according to the virus type (BBTV, CMV, or BSV). However, eradication efficiency varies depending on the banana genotype and alternative methods may have to be tested when the reference treatment fails. Furthermore, particular attention should be given to BSV. This is due to the fact that the activation of specific integrated virus sequences is associated with some genome of cultivated *Musa* (14). Eradication of viral particles could be counter balanced by the activation of these integrated sequences induced by the stressful eradication treatment and the *in vitro* procedure. A schematic representation of banana virus eradication process is shown in Fig. 1.

3.1. Aseptic Working Conditions

A critical step during the *in vitro* process is the maintenance of aseptic conditions and general precautions, presented hereafter, should be respected (see Note 2).

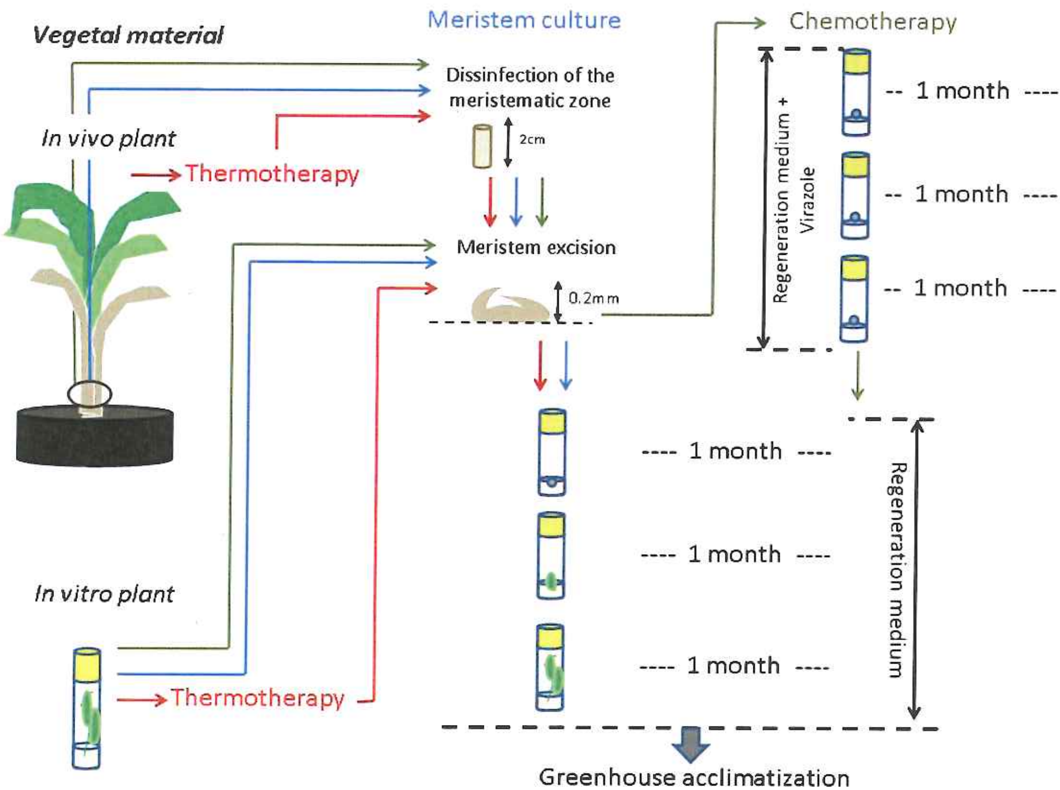

Fig. 1. Schematic representation of virus eradication processes for banana. The *green, blue,* and *red arrows* represent the process of chemotherapy, meristem culture, and thermotherapy, respectively.

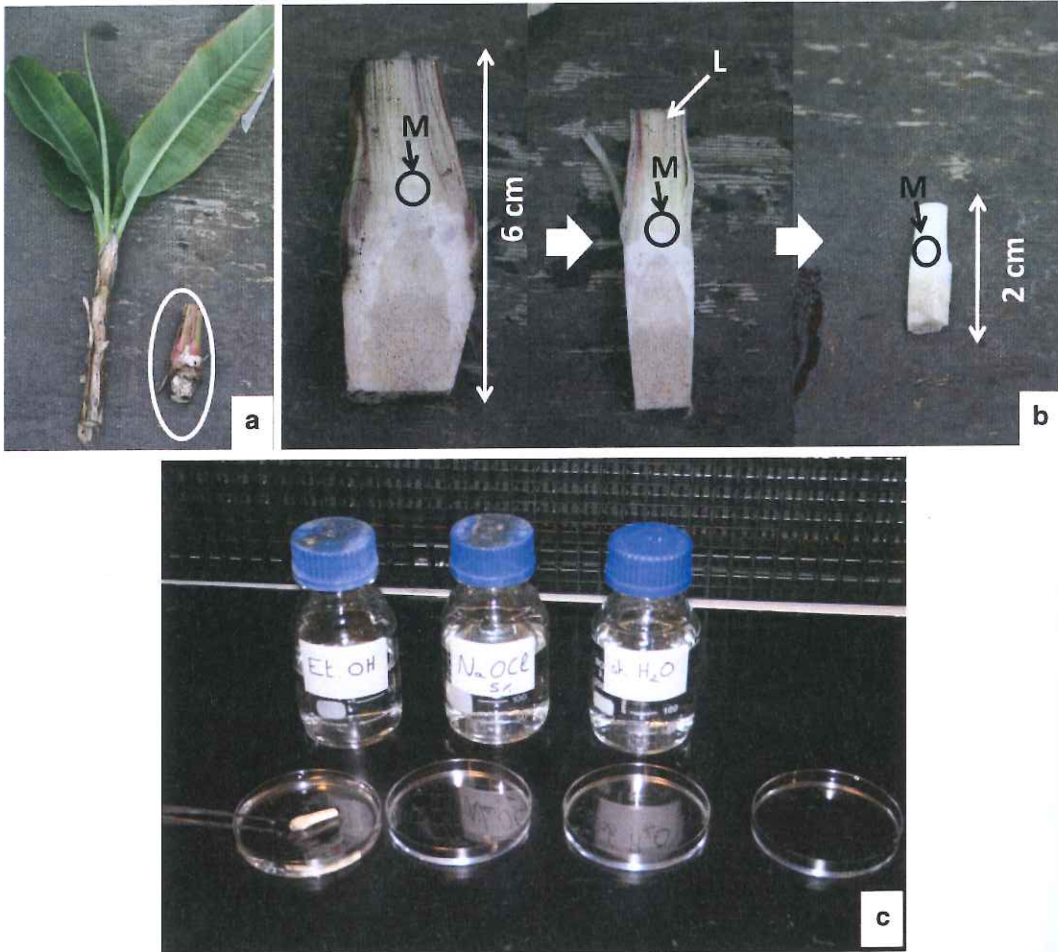

Fig. 2. Explant preparation and disinfection procedure for in vivo banana plants. (**a**) Separation of the meristematic zone from the pseudostem with a non-sterile knife; (**b**) successive trimming tissues to obtain a 1 × 1 × 2 cm cylinder, containing the meristematic zone; (**c**) disinfection of the cylinder (*M* meristematic zone localization; *L* successive leaf sheats).

1. Regularly disinfect thoroughly operator hands with ethanol 70% or disinfecting soap.
2. Clean and disinfect the meristem transfer area and all the equipment needed in the meristem transfer area such as the binocular microscope, light source, etc., with ethanol 70%.
3. Use only sterile plasticware and glassware.
4. Use only sterilized media and products.
5. Autoclave dissecting instruments. They are repeatedly sterilized during manipulation, either by soaking in 70% ethanol followed by flaming or by using glass bead sterilizer if available.
6. Sterilized parts of instruments and plant material should not be touched by hands.

3.2. Culture Media Preparation

1. Fill a beaker with 800 mL distilled water.
2. Start heating and stirring.
3. Add powdered MS (including micro-, macro-elements, and vitamins) sourced from a commercial supplier (e.g., Duchefa, Haarlem, The Netherlands) (see Note 1).
4. Add plant growth regulators: for both regeneration and proliferation media, use 1 mL/L IAA and 1 mL/L BAP from stock solutions of 10^{-3} M, stored at 4°C (growth regulator concentration in the medium: 1 μM).
5. Add 1 mL/L ascorbic acid from a stock solution of 10 mg/mL, stored in darkness at 4°C, and packed in aluminum foil from no more than 1 month.
6. Add 3% (30 g/L) sucrose.
7. Add 3 g/L gelrite.
8. Add distilled water to a volume of 1 L.
9. Adjust pH to 5.8 using concentrated NaOH (1 M) or HCl (1 M).
10. Heat medium until a transparent solution is obtained.
11. Put 10–15 mL medium into each of the glass tubes.
12. Close the tubes with the caps, put them in tube holders (racks).
13. Sterilize by autoclaving at 120°C for 20 min.
    For chemotherapy, modify the previous protocol as follows:
14. Autoclave the bottle containing the medium just after the pH adjustment at 5.8.
15. Freshly prepare an antiviral stock solution by dissolving the antiviral molecule (virazole) in sterile distilled water at a final concentration of 10 mg/mL.
16. Leave the medium to cool to about 50°C and add the antiviral solution via filter sterilization with a syringe filter 0.2 μm, under aseptic conditions, to obtain a final concentration of 50 mg/L.
17. Put 10–15 mL antiviral regeneration media in sterile glass tubes.

3.3. Preparation and Disinfection of In Vivo Material for Meristem Excision

Culturing meristems includes a process of surface sterilization when isolated from in vivo plants. This plant material might be contaminated with microorganisms which could grow very rapidly and kill the plant *in vitro* cultures. When the meristem is isolated from *in vitro*-grown plants, disinfection is not needed and excise meristem immediately under the aseptic conditions.

1. Select *in vivo*-grown plants.
2. Reduce the size of the pseudostem to the meristematic zone and surrounding tissue by cutting the plant with a sharp knife (see Note 3; Fig. 2a).

3. Remove superfluous tissue by successive trimming away the outer leaf sheats, leaf bases, and corm tissue until obtaining a $1 \times 1 \times 2$ cm cylinder, enclosing the meristematic zone (see Note 4; Fig. 2b).
4. Wash the tissue cube under tap water.
   Subsequent steps should be conducted in aseptic conditions:
5. Rinse the tissue cube by immersion (e.g., in a Petri dish) 70% ethanol for 2 min (Fig. 2c).
6. Wash the explants with sterile distilled water and put it in a solution of NaOCl 5% for 20 min.
7. Rinse the explants two times for 5 min and two times for 10 min with sterile distilled water.

   The cubes of tissues are now ready for excision of meristems.

3.4. Culture of Meristem

The meristem is the active growing point of the plant shoot. It is a small zone composed of dense, meristematic cells, which divide very fast. Meristem culture includes a process of tissues surface sterilization, the excision or isolation of the meristem, and its culture in a media under adequate conditions. The dome of the apical bud contains the real meristematic cells and is surrounded by leaf primordia and young leaves (Fig. 3). Isolation of the meristematic dome in aseptic conditions and its culture in an adequate aseptic nutrient medium leads to the development of plantlets. The meristematic cells divide and the differentiation of new tissues continues.

3.4.1. Isolation of Meristems

This step is delicate requiring patience, skill, and a lot of practice (see Note 5). Moreover, it is recognized that every meristem-explanting person develops his proper method of working. The whole processes (Fig. 4) should be performed in aseptic conditions as previously described (Fig. 5a).

1. Sterile instruments (Fig. 5b): minimum two scalpel holders with disposable blades and two forceps, and maintain their sterility.
2. Place the disinfected cylinder of tissue or the *in vitro* plantlets in a sterile Petri dish and hold it with the forceps (Fig. 5c).
3. Carefully remove the successive leaves, overlapping the meristem, by cutting with a scalpel through the circular insertion of each ones (Fig. 4). When the explant becomes too small, use a binocular microscope for a precise excision (see Note 4; Fig. 5c, d).

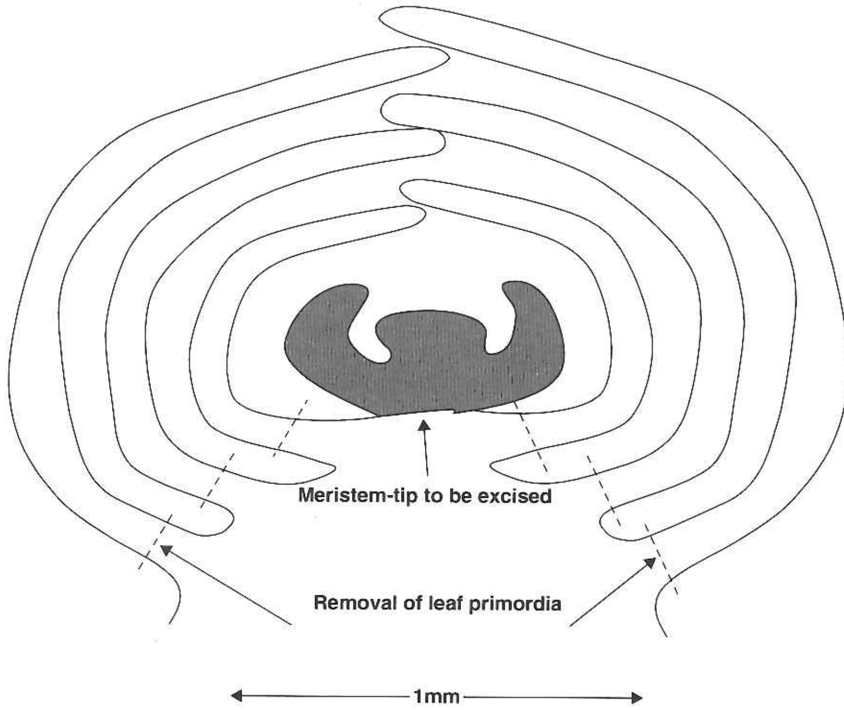

Fig. 3. Meristem-tip region.

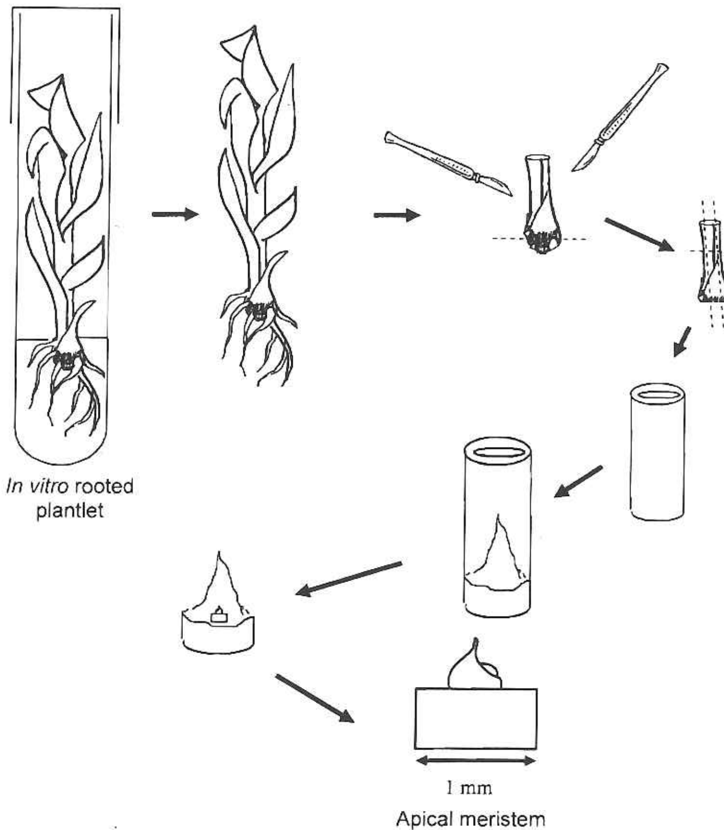

Fig. 4. Schematic representation of meristem excision from in vitro plantlets.

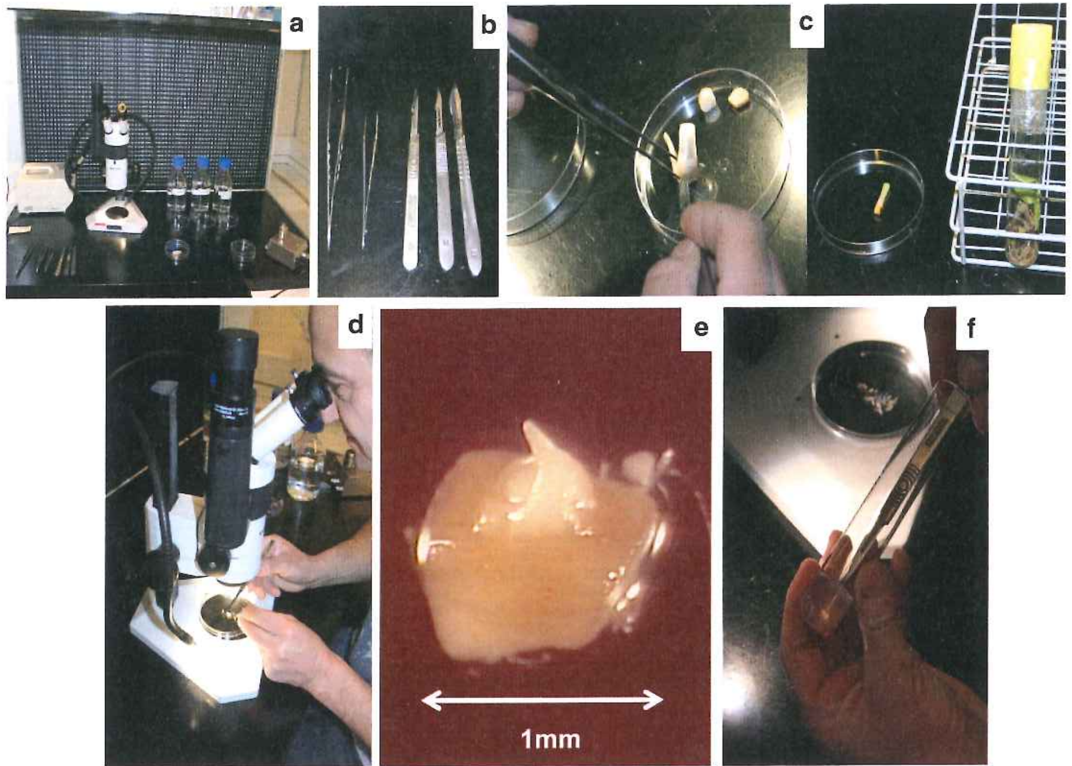

Fig. 5. Meristem culture. (**a**) equipment and supplies needed in the culture preparation area. From left back to right front: lamp, dissecting microscope binocular, ethanol with Petri dish, sodium hypochlorite with Petri dish, sterile water with Petri dish, dissecting tools, Petri dish containing explants from in vivo plant, Bunsen burner; (**b**) dissecting tools (forceps and scalpels of different size); (**c**) meristem excision from in vivo disinfected explant, or from an in vitro plantlet, using sterile forceps and scalpels; (**d**) meristem excision under a binocular microscope; (**e**) excised apical dome; (**f**) meristem tip transfer on sterilized medium.

4. Excise the apical meristem tip (< 1 mm diameter; Fig. 5e) consisting of the meristematic dome with 1–2 leaf primordia (see Note 6) with the second sterile scalpel (see Note 7).

3.4.2. Meristem culture

1. Transfer immediately meristem tips from the scalpel to the glass tubes with screw caps (Fig. 5f); this operation is performed by sticking the scalpel in the sterilized culture media and slowly withdrawing it.
2. Cover the cap with parafilm.

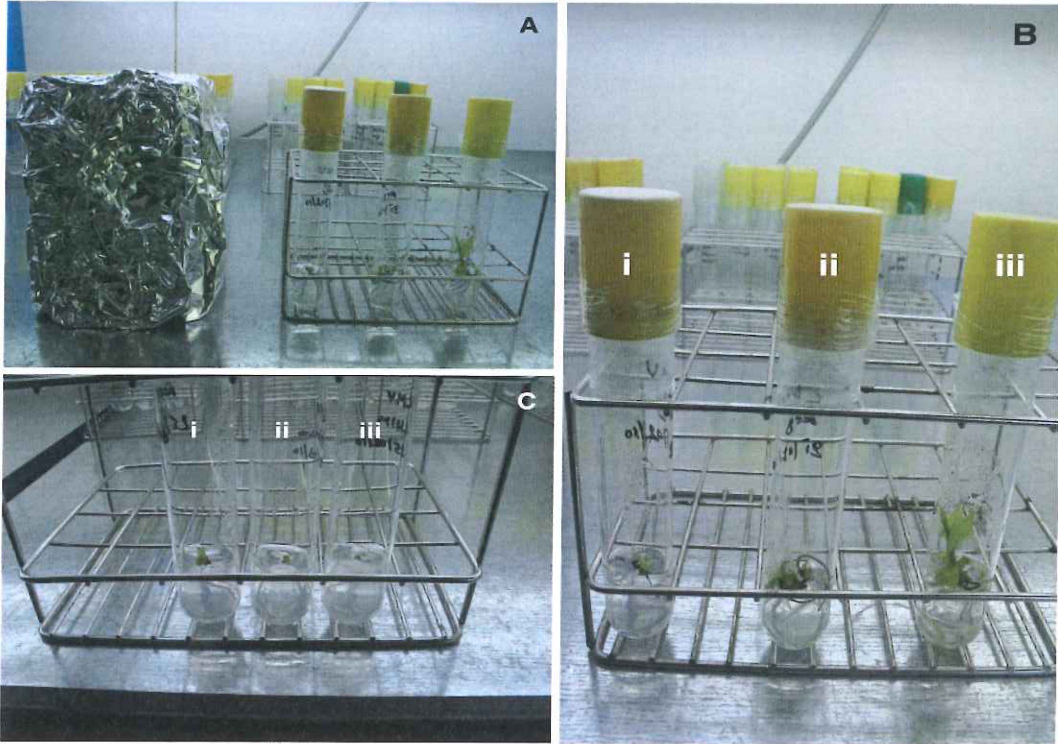

Fig. 6. Meristem cultures in the growth room. (**a**) Test tube rack covered with aluminum foil to obtain dark culture conditions, and without aluminum foil under a 16 h light/8 h dark cycle; (**b**) basket showing three tubes with banana meristem cultures at different stages of growth on the regeneration medium (i, after 1 month; ii, 2 months; iii, 3 months); (**c**) one-month-old cultures of banana meristems, excised from *in vitro* cultures subjected to a therapeutic treatment (i, after meristem culture only; ii, after thermotherapy; iii, after chemotherapy).

3. Place the tubes in a growth culture room at 24 ± 1°C in dark conditions (see Notes 8 and 9) for 7 days, followed by a 16 h light/8 h dark cycle for 24 h (see Note 10; Fig. 6a).
4. During minimum 3 months (see Note 11), transfer monthly the dissected meristem to a new glass tube containing sterilized fresh culture media with sterile forceps (see Note 12; Fig. 6b/i,ii).
5. When plantlets have two or three leaves and some developed roots (Fig. 6b/iii), they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

3.5. Chemotherapy Combined to Meristem Culture

All these steps are performed in aseptic conditions.

1. Excise the meristem and transfer into the screw-capped glass tubes containing the antiviral regeneration medium (Fig. 6c).
2. Cover the cap with parafilm.
3. Place tubes in a growth cabinet (culture room) at 24 ± 1°C in darkness for 7 days, followed by a 16 h light/8 h dark cycle per 24 h.

4. Using sterile forceps, transfer monthly for three times the dissected meristem to new glass tubes, containing sterilized antiviral regeneration media (see Note 13).
5. Transfer plantlets to fresh regeneration media, free of the antiviral compound, to allow their growth.
6. Transfer plantlets monthly to new glass tubes, containing sterile regeneration media.
7. When plantlets have two or three leaves and well-developed roots, they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

3.6. Thermotherapy Combined with Meristem Culture

1. Place *in vivo* or *in vitro* plantlets in a growth cabinet (culture room) under a 16 h light/8 h dark cycle at 28°C.
2. Allow the temperature to increase by 2°C per day from 28 ± 1°C to 40 ± 1°C during the 16 h light period. Maintain always the temperature of the 8 h dark period at 28 ± 1°°C.
3. Once 40 ± 1°C is reached, leave the plant material at this daily temperature (40 ± 1°C during 16 h of light, and 28 ± 1°C during 8 h in the dark) for 4 weeks.
4. Excise the meristem.
5. Transfer the meristem onto regeneration medium (Fig. 6c).
6. When plantlets have two or three leaves and well-developed roots, they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

3.7. Plant Acclimatization

1. Remove plantlets from the glass tubes. Gently wash with tap water the adhering agar from the roots, paying attention to avoid any damage.
2. If more than one plant is growing in the tube, separate them into individual plant with a scalpel or put them together in the same acclimatization pot (Fig. 7a).
3. Carefully transplant the plants in soil pots (see Note 14), inside a greenhouse, without damaging the roots. Water them immediately (Fig. 7b).
4. Cover the box with a clear plastic cover for 30 days to maintain a high air humidity; ensure that the soil always remains moist (Fig. 7c). Potted plants stay over a vermiculite bed (Fig. 7d).
5. Do not expose plants to direct sunlight during the acclimatization step.
6. After 1 month, plants should be grown enough to be transplanted in larger pots and transfer to standard greenhouse conditions (Fig. 7e).

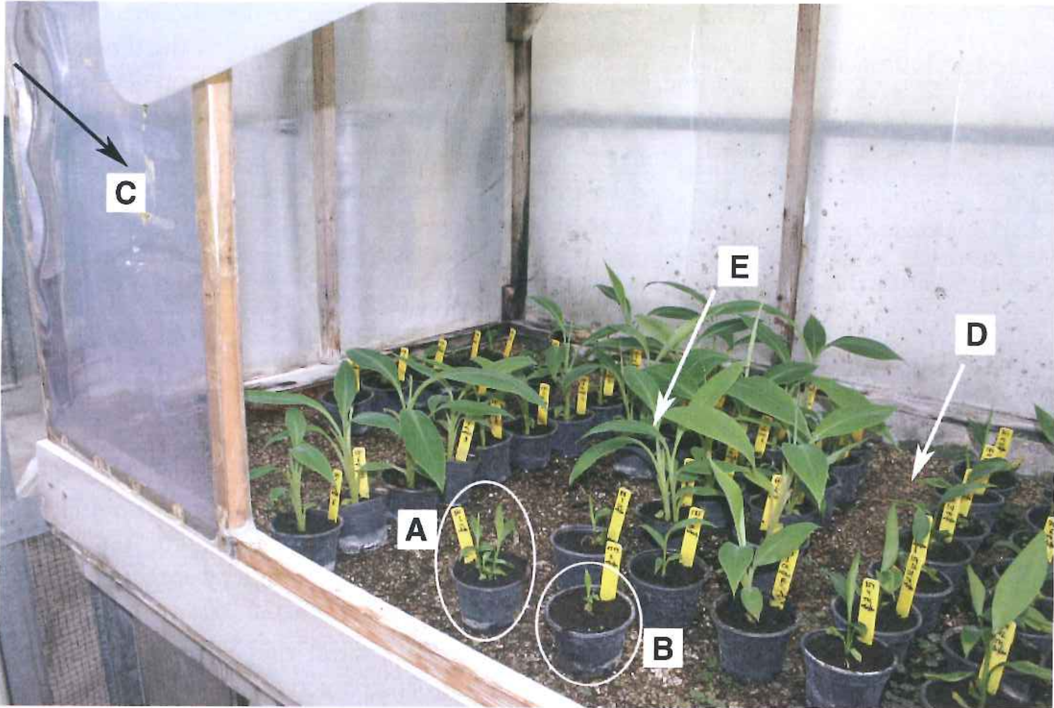

Fig. 7. Greenhouse acclimatization. (**a**) three plantlets acclimatized together; (**b**) plantlet size required for acclimatization; (**c**) plastic box with reclosable plastic door; (**d**) vermiculite; (**e**) plant size required to be transferred in standard greenhouse growing conditions.

4. Notes

1. MS medium could be obtained from suppliers as premixed powdered mixtures. This saves a great deal of time and efforts. However, MS medium could also be prepared by dissolving appropriate quantities of macro-, micro-components, and vitamins in distilled water. This protocol is explained in detail for *Musa* by Vuylsteke (15).
2. To reduce the risk of contamination, it is recommended to excise meristems in a laminar air-flow cabinet.
3. No need to work in aseptic conditions at this stage.
4. Care should be taken to avoid cutting the meristem during this procedure.
5. Some aspects of the excision process are critical; among them the maintenance of sterile conditions, the prevention of virus transmission through the excision tools, and the removal of the apical dome without damaging it, in order to ensure its survival.

6. Size of explants is an important factor for the success of the process. Very small explants consisting of only the meristematic dome increase the production of virus-free plants, but they show high mortality rates and grow very slowly.
7. It should be noted that once the dissection process is finished, the meristem begins to lose water rapidly. Therefore, the final steps in the excision process must be completed rapidly in order to avoid excessive dehydration.
8. Dark conditions are required to avoid excessive blackening of tissues caused by photo-oxidation of phenolic compounds. If dark conditions cannot be applied in the room, cover the glass tubes containing the meristems with an aluminum foil.
9. Environmental control is preferred. However, as bananas and plantains can be cultured under a relatively wide range of environmental conditions, complete control is not absolutely necessary. An air-conditioning unit is suitable for temperature control. It is important that the temperature does not fall below 20°C and does not exceed 35°C (15).
10. One or two weeks after excision, the meristems should be inspected under the binocular microscope. Discard contaminated and dead ones. Dead meristems can be recognized by their black appearance and lack of new growth. If contamination exceeds 5%, the disinfection and excision process should be reviewed. Success in meristem-tip culture depends on a large number of factors and is hard to predict. Normally, 2–5% meristems can die as a result of dissection damage and/or a too small size of explants, depending on the skill of the handling person
11. The regeneration process takes at least 3 months or longer according to the plant genotype, meristem size, and culture conditions.
12. Regular subculturing on fresh medium prevents nutrient deficiency, medium dehydration, pH modifications, and accumulation of secondary metabolites which can slow down the growth of meristems.
13. Meristems do not grow fast when transferred onto chemotherapy media and the meristem size remains stable during the 3-month treatment.
14. A sterile soil is not recommended for successful plant establishment. However, a contaminant-free soil should be used.

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