

HANDBOOK OF PROCEDURES FOR *IN VITRO* GERMPLASM CONSERVATION OF THE GENUS *Manihot*



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HANDBOOK OF PROCEDURES FOR *IN VITRO* GERMPLASM CONSERVATION OF THE GENUS *Manihot*

1. INTRODUCTION

The Program of Genetic Resources (PGR) of the Centro Internacional de Agricultura Tropical (CIAT) maintains and distributes *in vitro* germplasm of the genus *Manihot*. Quarantine regulations for *Manihot* have been implemented since 1980 when it was stated that only *in vitro* plants could be used for exchange throughout the world, and technical guidelines for the safe movement of germplasm were developed (Frison & Feliu, 1991). *In vitro* techniques have been used for a dual purpose: 1) to introduce to CIAT a large number of materials collected in the main centers of variability and kept *in vitro*, and 2) to distribute germplasm selected from CIAT to the national programs and other users. This collection is currently represented by 6,467 materials and is made of three kinds of germplasm: 5,184 materials of cultivated cassava (*Manihot esculenta*) from 28 countries, 883 materials of the *Manihot* wild species (33 species) and 400 CIAT hybrid materials. Since 1994, these materials have been designated to FAO (Food and Agriculture Organization of the United Nations) under the FAO-CGIAR agreement. Since October 2006, they have been recorded in the Multilateral System of Access and Benefit Sharing of the International Treaty on Plant Genetic Resources for Food and Agriculture, within the framework of the agreement between the Governing Body of the Treaty and CIAT, and meet the standards for germplasm banks. Since 1979 when CIAT accepted the global mandate for the crop, 32,195 samples have been distributed through the years up to 2008 (6,106 materials) to 67 countries using *in vitro* techniques.

The different steps identified in the Flowchart (Figure 1) for the Management of *Manihot* germplasm are: a) Introduction b) Quarantine c) Elimination of pests, d) Indexing, e) Micropropagation, f) Conservation, g) Characterization, h) Distribution, and i) Safety duplication. These procedures have been developed for the better management of *in vitro* collection bearing in mind that the use of this germplasm by external recipients is the main objective.

A total of five culture media have been developed for different purposes involved in the management of the collection: 1) culture media for meristem development and nodes (4E) (Roca et al. 1991), 2) culture media for root development and better development of plants that will be brought to greenhouse conditions (17N) (CIAT 1982), 3) culture media for conservation (8S) (CIAT 1984), 4) culture media for minimum growth (SN) (Mafla et al. 2000), and 5) culture media for wild species (12A₃) (Velásquez & Mafla 1999) (see Annexes).

The *in vitro* collection is managed by a working group of two professionals and five technicians who are responsible for all the tasks explained below in this handbook.

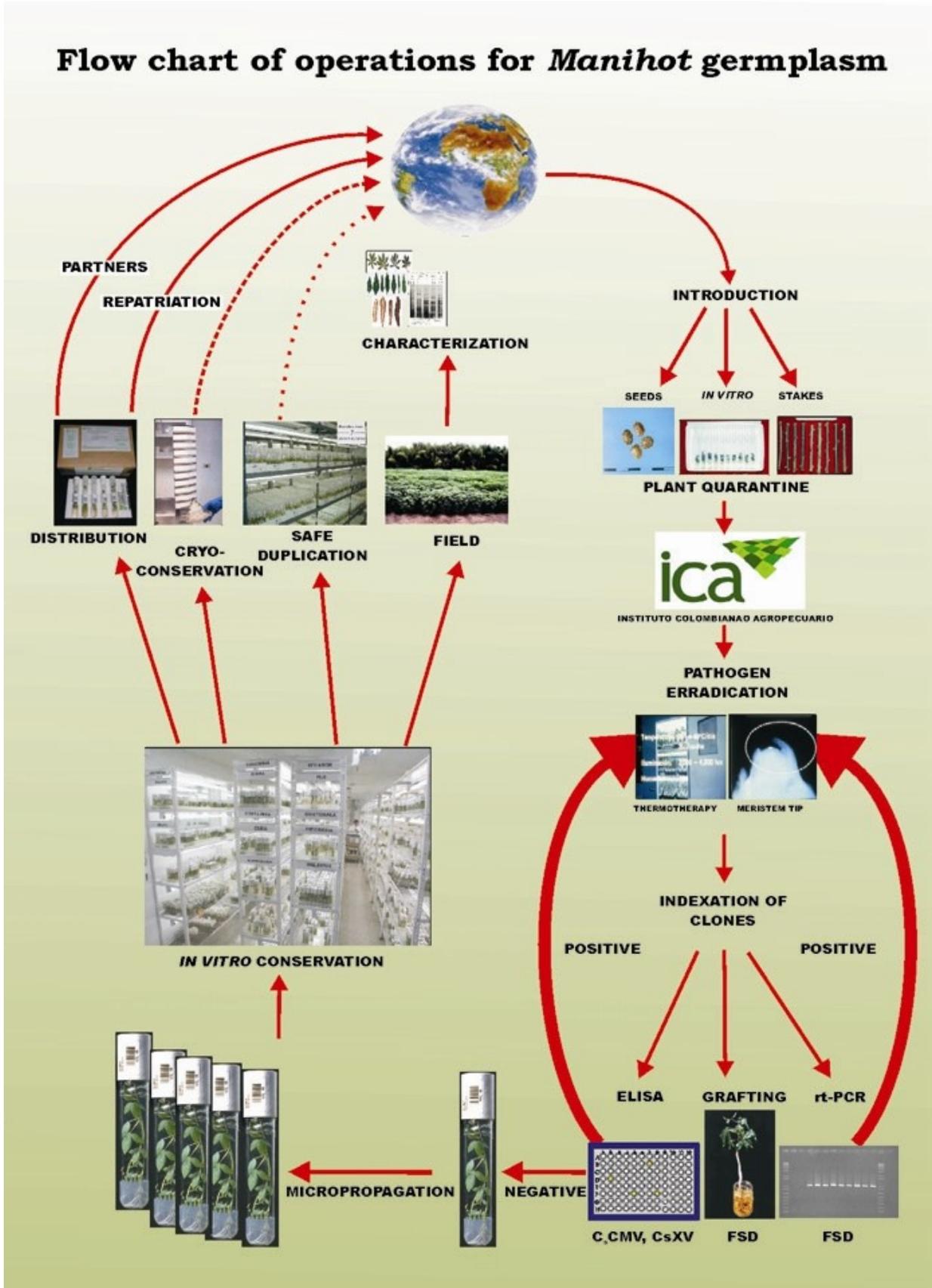


Figure 1. Flowchart of activities for the management of *Manihot* germplasm.

2. FACILITIES

Tissue culture technique is essentially isolating a portion of the plant (explant), and artificially providing the proper physical and chemical conditions for the cells to express their intrinsic or induced potential. It is also necessary to adopt aseptic procedures for maintaining the cultures free of contamination.

Based on this concept, the design of a tissue culture laboratory can vary in size and complexity, depending on the objectives of the laboratory. Bearing this in mind, the following is a description of the tissue culture laboratory of PGR and the areas, equipment and other supplies required for the proper development of the activities assigned to the maintenance of the *in vitro* collection of *Manihot*.

In the PGR, we have established different areas for different activities of the *in vitro* conservation laboratory. Below are the different areas (Figure 2) with their respective purposes and equipment (Table 1).

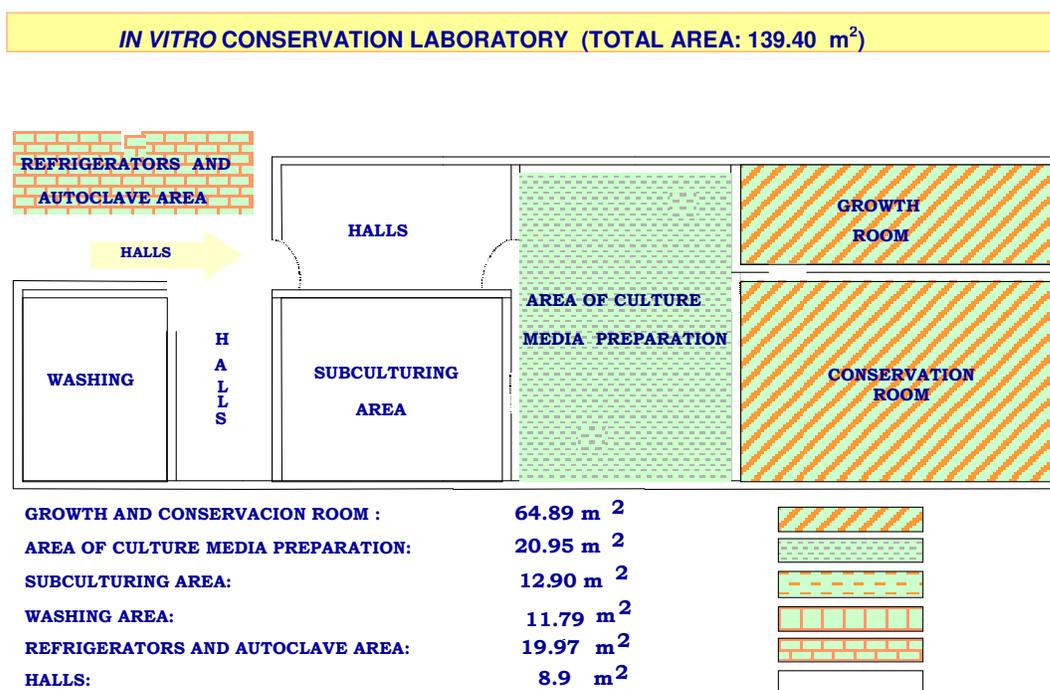


Figure 2. Different areas of the tissue culture laboratory of the Program of Genetic Resources at CIAT.

Table 1. Equipment required in each area of the laboratory.

Area	Equipment	Purpose
Washing	Small autoclave (1) Drying Oven (1) Cabinets (2)	Sterilize tools Dry glasses Store glassware
Refrigerators and autoclave	Refrigerators (5) Autoclave (1) Distiller (1) Incubator (1)	Store culture media Sterilize culture media Provide double-distilled water Research
Preparation of culture media	Dispenser (1) Heating plates with magnetic stirrers (2) pH meter (1) Balance (1) Computer	Filling the tubes and shake media Warm media (agar) Adjust pH of the culture media Weight of different components of media Database-management of collection
Corridor	Extinguisher (1) Air curtain	Security element Asepsis element
Subculturing Room	Laminar flow chambers (6) Dehumidifiers (1) Air conditioning unit Air purification system Fire extinguisher ¹	Subculture process Control relative humidity Cooling Improve air quality Security element
Growth Room	Shelves (16) Hygrothermographer (1)	Locate plant material Register temperature and moisture
Conservation Room	Shelves (41) Dehumidifiers (1) Hygrothermographer (1) Fire extinguishers (1) Smoke and temperature sensors (1) Photoperiod Controller (1) Air conditioning unit (1)	Locate plant material Control relative humidity Register temperature and humidity Security element Security elements Regulate lights on and off Cooling
Room for safety duplicate	Shelf (10) Dehumidifiers (1) Hygrothermographer (1) Fire extinguishers, smoke and temperature sensors (1) Air conditioning unit (1)	Locate the safety duplicate Control relative humidity Register temperature and humidity Security elements Cooling



Health hazard : Care is taken that the extinguisher is light enough to be easily handled by any staff.



Health hazard : Care is taken that the autoclave's pressure is at zero, and the temperature is below boiling point before opening the autoclave.

2.1 Washing area

The whole washing and storage process of glass material, sterile equipment and preparation of different types of waters (ionized, distilled, double-distilled, and double-distilled sterile) needed in the daily activities of the laboratory are done in this area.

This area is provided with a large washer sink with three types of water: cold, hot and double-distilled water used for the final rinse in the washing of glass material (Figure 3).

Also, in this area there are shelves available for storing glass material and double-distilled sterile water to be used in the disinfection process.



Figure 3. Activities in the washing area.

2.2. Refrigerators and autoclave area

Refrigerators to store reagents, stock solutions and the different culture media used in the laboratory are located in this area. The autoclave used for the sterilization of culture media, the water distiller and an incubation chamber used for research purposes are also located in this area (Figure 4).



Figure 4. Refrigerators and autoclave area. The refrigerators are used for storage of culture media.

2.3 Area of culture media preparation

As its name suggests, this is the area for the preparation of culture media, but it also provides a space for storing glassware and plastic material, chemical reagents and double-distilled water. This area has benches for the preparation of media, an analytical balance, pH meter, magnetic stirrers, and media dispensers among others (Figure 5). A computer for the management of the *in vitro* collection is also located in this area.



Figure 5. Area of culture media preparation. You can see the different solutions and equipment used for this purpose.

2.4 Subculturing area

The excision, planting and transferring of explants to the culture media are performed in this area. A high level of asepsis should be maintained in this area; to that end, there is an air purification system (Figure 6).

The air purification system provides reduction of bacteria, mold, odor and VOCs (chemical odors), and uses photohydroionization technology, which utilizes ozonide ions, super oxide ions, and UV light targeted on a hydrated quad-metallic target to develop an advanced oxidation.

The subculturing practices are performed in a laminar flow chamber with the presence of an industrial alcohol (methyl alcohol) burner and sterile tools necessary for this activity. A total of six laminar flow chambers are located in this area (Figure 7).

There are also laboratory trolleys for the location of the culture media, planting material, 70% alcohol dispensers and other required implements for subculturing activity. Plastic recipients for the planting material, inorganic material and glass that are discarded are also available.



Figure 6. Air purification system located in the subculturing area.



Figure 7. Subculturing area where the laminar flow chambers are located

2.5 Growth room

The incubation conditions for growing cassava *in vitro* material are (Roca et al. 1991):

Temperature:	27-28°C
Lighting:	18.5 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$
Photoperiod:	12 hours
Light Quality:	fluorescent lamps, light day type
Relative humidity:	50-70%

The room has a total of 16 white metal shelves, each shelf has five levels and each level allows the location of eight racks, each one is occupied by six materials (5 tubes/material) giving a storage capacity of 3,840 materials.

Each level of the shelf has a platform with holes for a better aeration and of size of 124 x 39cm. The height between each level is 39cm allowing a good light. Each level is provided below with a light day fluorescent lamp (ballasts are installed outside the lab to avoid any increase of temperature in the room). The space between the ground and first level should be between 10 and 20cm to facilitate cleaning work (Figure 8). The temperature control of the air is done through a cooling system, which is connected to temperature sensors that detect its changes. Smoke sensors are also located in this area (Figure 9). A dehumidifier is used to control air relative humidity (Figure 10).



Figure 8. Shelves used in the growth room.



Figure 9. Safety systems to monitor any smoke or rise in temperature in the growth and conservation rooms.



Figure 10. Dehumidifiers used to control relative humidity of the air in the growth and conservation rooms.

2.6 Conservation room

The physical conditions for the *in vitro* cassava germplasm are the following (Roca et al. 1989):

Temperature: 23-24°C
 Lighting: 18.5 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$
 Photoperiod: 12 hours
 Light Quality: fluorescent lamps, light day type
 Relative Humidity: 50-70%

This room has a total of 41 metal shelves, each shelf has five levels and each level allows the location of eight racks, each rack is occupied by six materials (5 tubes/material) giving a conservation potential of 9,840 materials (Figure 11). Security systems similar to the ones used in the Growth Room are used in the Conservation Room (temperature and smoke sensors). In this same area, there is a space for evaluation and the periodic monitoring of crops, using lamps, magnifiers and stereomicroscope among others.

The evaluation is done "in situ" to limit the movement of materials kept in the bank and reduce the different risks that may affect them.

Refrigeration units used to regulate the temperature conditions required in the Growth and Conservation Rooms are operated by an internal and independent system to minimize contamination risks.



Figure 11. Conservation room and racks containing cassava *in vitro* materials.

3. DESCRIPTION OF ACTIVITIES

3.1 Introduction of germplasm

The introduction of materials for the beginning of the conservation process is usually done in three ways:

1. From planting material (cuttings): from experimental centers or collections done in the host country.
2. By *in vitro*: from donor countries.
3. In form of botanic seed and subsequent rescue of embryos, in the case of wild species.

Whatever form of introduction, a unique number is assigned to the material, maintaining always the original data of the materials, which are entered in the database as synonyms. *Manihot esculenta* is identified with the first three letters of the country of origin and then a consecutive number according to the order of entry (for example: ARG 2 is material from Argentina). Materials of wild species are identified with the initials of each species followed by the number of population, and subsequently the genotype number (for example: PER 411- 1, belongs to the species *Manihot peruviana*, population 411 and genotype number 1). Materials from the breeding program are identified with two letters indicating the type of cross (CG, CM, SG, SM), then the number of crossing and then the selected genotype (for example: CG 1141- 1). A basic information recording the passport data is done to allow an efficient documentation. This information is saved in the database of the Program of Genetic Resources (ORACLE V.7.0). The data describe origin, geographical characteristics of the varieties according to the descriptors developed for a better management of information (Gulick et al. 1983).

The basic data provide general information about latitude, longitude and altitude allowing us to know the site where the sample was collected, the name of the germplasm collector and information about the institution making the donation (Figure 12). Passport data are available on line (www.ciat.cgiar.org/urg, www.singer.cgiar.org).

Developer/2000 Forms Runtime for Windows 95 / NT - [INTRODUCCION YUCA]

Acción Edición Consulta Bloque Registro Campo Ayuda

Accesión Género Especie

Características Geográficas Pre Registro Pasaporte Básico Colectores Misiones

SOURCE OF COLLECTION:
 Wildlife habitat Rural market
 Agricultural field Trade market
 Store farmer Institutes
 Horticultural Garden Planting
 Field edges Not applicable
 Not known Other (specify)

TYPE OF SITE
 Plane Slope
 Peak Depression
 Moderate slope
 Steep slope
 Other

HABITAT
 Rainforest
 Rainforest
 Semi-humid forest
 Dry forest
 Very dry forest
 Spiny forest
 Desert scrub
 Desert
 Other

HABIT
 Tree
 Bush
 Creeper
 Erect
 Stoloniferous prostrate
 Stoloniferous creeper
 Other

SOIL TEXTURE
 Sandy
 Intermediate
 Clayey
 Stony
 Organic
 Sandy silty
 Clay silty
 Silt
 Other

SOIL DRAIN
 Excessive
 Good
 Moderate
 Poor
 Swampy
 Other

TOPOGRAPHY
 Sierra
 Flat
 Wavy
 Foothills
 Mountain
 Hills
 Marsh
 Other

URG Banco
 Elites
 Herbario
 Siguiente
 Anterior
 Terminar

Figure 12. Passport information registered in the database.

The introduction of materials from 1) vegetative (cuttings), and 2) from *in vitro* material is explained in section 3.3 of this handbook. Here follows the explanation of procedure when the introduction of material is through botanic seeds.

Protocol for the rescue of embryos of wild *Manihot* species

In vitro culture of zygotic embryos has been used for the introduction of wild species of the genus *Manihot*. Culturing embryos provides a simple technique for breaking dormancy of the seed by ensuring a fairly uniform rate of germination (Biggs et al. 1986).

This technique involves aseptic excision of the embryo of the seed and sowing in sterile culture media (Hoded 1977), providing potential alternatives for research in plants. It is frequently used to save embryos that fail to develop naturally in seeds and fruits, or to save seed embryos from interspecific crosses where defective endosperms are common, or to reduce the long periods of dormancy and to eliminate the physical and chemical barriers present in fruits and seeds.

Disinfection of seeds:

Seeds are superficially disinfected by following the protocol described below:

1. Wash with alcohol 70% for 1 minute, followed by
2. Benlate[®] 0.5 g/l for 15 minutes,
3. Sodium hypochlorite at 2.5% for 15 minutes, finally three rinses with sterile distilled water in laminar flow chamber.

Once we have carried out the protocol for disinfection, the seeds are placed in a sterile solution of 200 ppm of gibberellic acid (GA_3) for 12 hours at room temperature.

Subsequently the solution is withdrawn, and we proceed with the rescue of embryos, for which the seeds are broken transversely using forceps. Once the embryo is identified, it is removed using a blade and planted in the culture medium (17N) (Figure 13). The tubes are labeled, sealed and incubated at 27°C in darkness for 7 days, then transferred to normal growing conditions (temperature 26-28°C, lighting 18.5 $\mu\text{mol.m}^{-2}\text{s}^{-1}$, and photoperiod of 12 hours).

After two weeks one wait for the morphological differentiation of the embryo into a normal plant. Micropropagation follows next, as well as the conservation of the materials using the culture media to that purpose.

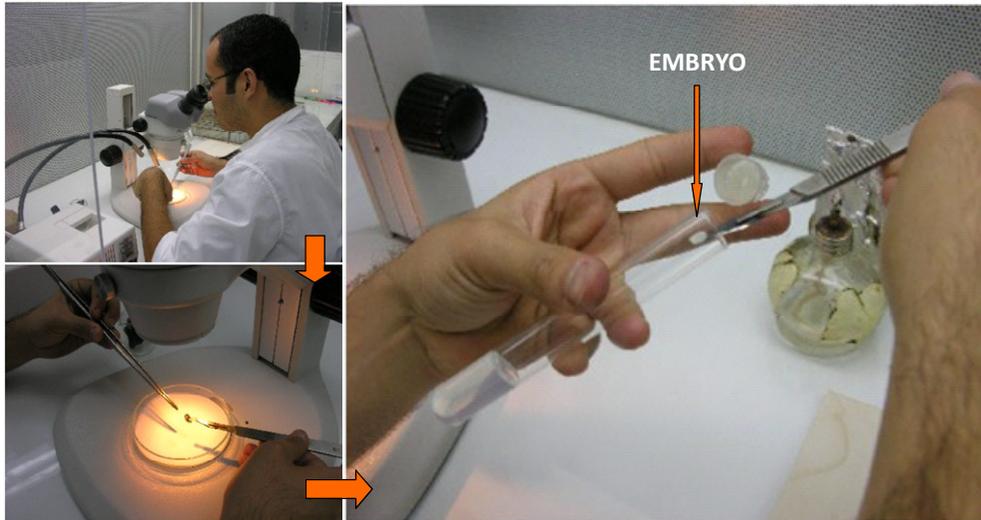


Figure 13. Process of excision and planting in the culture medium developed for zygotic embryos.

3.2 Quarantine

In the case of cassava, the allogamous reproduction and the highly heterozygous genetic constitution are the main reasons to propagate it by cuttings, and not by sexual seed (Ceballos & De la Cruz 2002). However, the distribution of materials through stakes can lead to the distribution of pests, bacterial diseases, and especially viral diseases (Table 2).

The exchange of material can lead to serious problems for the agriculture in a country, since many of the pathogens affecting crops spread through organs such as seeds and tubers among others, and these are the means of introduction of different races of pathogens into production areas (Huertas 1992).

Table 2. List of different cassava pests and diseases (Frison & Feliu 1991).

	CAUSAL AGENT
ARTHROPOD PESTS	<p>Hornworm (<i>Erynnis ello</i>) Green spotted mite (<i>Tetranychus urticae</i>) Green mite (<i>Mononychellus tanajoa</i>) Red spider mite (<i>Tetranychus cinnabarinus</i>) Flat mite (<i>Olygonichus peruvianus</i>) Whitefly (<i>Aleurotrachelus socialis</i>) Mealybugs (<i>Phenacoccus herreni</i>, <i>P. granadensis</i> and <i>P. manihoti</i>) Thrips (<i>Frankliniella williams</i> and <i>Scirtothrips manihoti</i>) Burrower bug (<i>Cyrtomensus bergi</i>) Lacebug (<i>Vatiga manihotae</i> and <i>V. illudens</i>) Stemborer grubs (<i>Chilomina clarkei</i>, <i>Lagochirus araneiformis</i> and <i>Coelosternus</i> spp.) White grubs (<i>Phyllophaga</i> spp. and <i>Leucopholis rorida</i>)</p>
DISEASES	<p>Superelongation (<i>Sphaceloma manihoticola</i>) Brown leafspot (<i>Cercosporidium henningsii</i>) Phonopsis blight (<i>Phoma</i> spp.) Angular leafspot (<i>Xanthomonas campestre</i> pv. <i>cassavae</i>) Cassava anthracnose (<i>Glomerella manihotis</i>) Cassava ash disease (<i>Oidium manihotis</i>) Cassava rust (<i>Uromyces</i> spp.) Diffuse leafspot (<i>Cercospora vicosae</i>) Cassava bacterial blight (<i>Xanthomonas axonopodis</i> pv. <i>manihotis</i>) Glomerella stem rot (<i>Glomerella cingulata</i>) Dry root and stem rot (<i>Diplodia manihotis</i>) Cassava sof rot (<i>Erwinia carotovora</i> p.v. <i>carotovora</i>) Root rot (<i>Phytophthora</i> sp., <i>Rosellinia</i> spp. and <i>Pythium</i> spp.)</p>
VIRUS DISEASES	<p>Cassava Common Mosaic Virus, CCMV (Potexvirus) Frogskin disease, FSD (virus) (Calvert et al. 2008) Cassava X virus (CsXV) African Cassava Mosaic Virus (ACMV) Cassava Vein Mosaic Virus (CVMV) Indian Cassava Mosaic Virus (ICMV) Cassava brown Streak Virus (CBSV)</p>

Most countries have established quarantine regulations and special measures are taken to avoid the entrance of pests and diseases into their territory. In Colombia, there are government regulations for the import and export of plant material implemented through the Colombian Agricultural Institute (ICA).

There is a procedure designed in common through an agreement between CIAT and ICA to manage the germplasm that is introduced in the *in vitro Manihot* collection:

- (i) Review of documentation required for the germplasm that has just arrived:
 1. Import permit issued by the Colombian Agricultural Institute (ICA).
 2. Phytosanitary Certificate issued by the official phytosanitary plant authority of the donor country.
 3. List of shipped varieties.

- (ii) Inspection of plants to determine if there is any plant health problem (fungi and bacteria). If there is presence of these pathogens, the material should be destroyed. No material may be distributed until the whole process of eliminating pathogens and indexing is completed.

The quarantine activity also counts on a documentation system that allows us to better manage the information from ICA-CIAT (Figure 14). The system records all information related to the introduction of materials.

Figure 14. Quarantine documentation system in the PGR database.

3.3. Pathogen eradication

Diseases caused by viruses significantly affect the yield and quality of cassava in the major growing regions of this root crop in the world. In Africa, the most important virus is the one that causes the disease African cassava mosaic virus (ACMV), responsible for yield losses of up to 90% (Bock, 1976). In Latin America there are several viruses and organisms like viruses. Frogskin disease (FSD) reduces the yield by 80% to 100% (Lozano et al. 1983a); the cassava common mosaic virus (CCMV), widespread in Latin America, reduces yield by up to 60% (Costa 1972). Other viruses have been discovered such as cassava X virus (CsXV) and other that causes the disease latent virus, both of considerable importance (CIAT 1986).

The use of meristem culture technique offers the opportunity to eliminate the existing virus in a culture (Roca et al. 1991). The meristem is a small tissue (0.2-0.3 mm) located at the tip of the bud. The apical meristem is formed during embryonic development, and except for dormancy periods, remains active through the life of the plant. Reports show how the meristem culture technique has successfully eliminated in other species such as the potato S virus (Brown et al. 1988) and the yellowing virus in sweet potato (Green & Lo 1989), among others. The technique consists of aseptically isolating the meristematic region of the vegetative apical or axillary bud, together with 1-2 of the younger leaf primordia, and to introduce it in a sterile growing medium.

To explain the health or cleanliness of the meristem several assumptions have been formulated. One of them suggests that due to the absence of vascular tissue in the proximity of the apical meristem; since plasmodesmata linked in the cells of this tissue are very small, the virus moves very slowly toward the meristem. This morphological characteristic tied with the active cell multiplication that occurred there, may explain the low concentration or absence of virus in this tissue (Wu et al. 1960).

The meristem culture techniques and thermotherapy were developed at CIAT with the purpose of eliminating the virus from infected cassava varieties. The development of this technique has greatly contributed to the production of cassava clones completely free of pathogenic organisms (Roca et al. 1991).

The basic principle of thermotherapy is that it can alter the viral synthesis in infected tissue and retard the translocation of virus into the plant, so that the region of apical meristem free of viruses increases in size and, therefore, makes the cleaning more feasible (Roca et al. 1991).

Thermotherapy, by affecting cellular metabolism, appears to alter the synthesis of the virus; therefore, thermotherapy success depends on the ability of the tissue to withstand long periods of high temperatures that inactivate the virus without significantly affecting the growth of plant tissues (CIAT 1982; Roca et al. 1991).

The methods for the cleaning of clones infected by viruses can be applied:

1. to the stem cuttings (in vivo) or,
2. to shoot tips of the *in vitro* materials

3.3.1 Steps from cuttings

Source material:

- Take stem cuttings of 15-20 cm containing vigorous buds.
- Disinfect stem cuttings superficially by submerging them for five minutes in a solution of Dimethoate at 0.3%, then allow them to dry for 1-2 hours in the shade.
- Plant treated stem cuttings in pots containing sterilized substrate (soil: sand 1:2), irrigate with water (Figure 15).

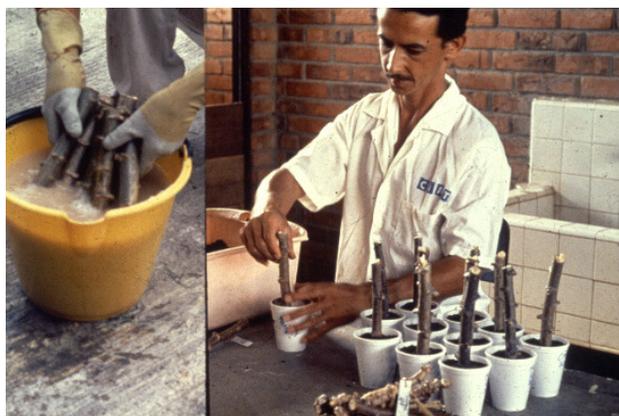


Figure 15. Disinfection process and planting stem cuttings for the initiation of thermotherapy treatment.

Thermotherapy treatment

- Place the pots with cuttings to grow in the thermotherapy chamber at a temperature of 40°C day/35°C night, 3000-4000 lux, high relative humidity (Figure 16).
- Keep the pots with cuttings in this environment for 3 weeks, after this time the buds have sprouted and the meristem growing can start, 2-3 buds can be cut for each stem cutting.
- The buds to be cut have to be the strongest and with the highest elongation. It is important to disinfect the blade with detergent every time buds are cut from one plant to another.



Figure 16. Chamber and required conditions for the thermotherapy process of the stakes.

Preparation of sterile tissue

- Remove the terminal buds of the cuttings and place them in a glass jar covered with a mesh that facilitates the management of these buds in the laboratory.
- In the laboratory under a laminar flow chamber the collected buds are disinfected by the following protocol:
 - a) Dip the buds quickly in 70% alcohol and rinse with sterile water,
 - b) Put the buds in sodium hypochlorite 0.5% for 5 minutes (commercial bleach with 5.25% active ingredient of sodium hypochlorite),
 - c) Make three rinses with double-distilled sterile water (Figure 17).



Figure 17. Materials required for the disinfection process of the buds.

Isolation and meristem culture

This stage of the process consists of the aseptic isolation of meristem (0.3-0.5 mm structure that includes the meristematic region of the vegetative bud, with 1 or 2 of their youngest primordial), and its placing on a nutritional and sterile culture media (4E) (Figure 18).

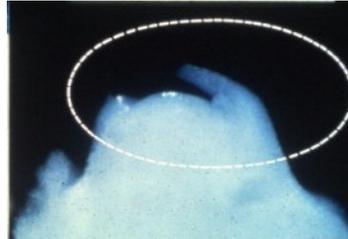


Figure 18. Cassava meristem used for the removal process of virus.

a) Tools for isolation (Figure 19)

- A stereomicroscope (10X) with cool light lamp (optic fiber).
- Two scalpels (blade No. 11).
- Two small tweezers.



Figure 19. Stereomicroscope and tools required for the isolation of the meristem.

b) Sterilization of tools

- The tools were soaked in alcohol 96%, flamed and shaken several times or sterilized in autoclave for 15 minutes.
- Allow the tools to cool down and introduce them in a sterile cardboard.

The plant material can deteriorate and turn dry when tools are still moistened with alcohol or when they are too hot.

Isolation

- Under the visual field of the stereomicroscope (10-40X) the apical bud is taken with the tweezers and one starts removing with the blade of scalpel the appendages (leaves and stipules) that cover the shoot tip until observing a brilliant structure of 0.3-0.5 mm with 1 or 2 leaf primordia. A fine cut is done. This operation should be done in a very fast and careful way to avoid excessive dehydration of the meristem that can cause its death (Figure 20).



Figure 20. Dissecting process of meristems using stereomicroscope.

- Place the explant in the culture media for its growth and development (Media 4E, see Appendix).
- The location of the explant in the culture media is very important. The baseline should be on the surface of the media; if it is on a side over one of the primordia a uniform development of the meristem will not be obtained and is going to benefit the development of leaf primordia.

Incubation of the culture

The tubes are properly sealed and labeled indicating the name of the accession and sowing date, then they are placed in racks and taken to the growth room which is set for the following conditions:

Temperature:	26-28°C
Lighting:	18.5 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$
Photoperiod:	12 hours

Quality of light: Fluorescent lamps, day light type

Relative Humidity: 50-70%

Culture Development

- During the first week of cultivation, little morphological differentiation can be observed. A slight increase in the volume of tissue may happen. The development of a light green pigment may be evident, this being the first indication of survival of the tissue. If, however, the explant has a whitish look, it is an indication that an injury has occurred at the time of excision.
- Over the next 3-4 weeks, there is an increase in growth but it is necessary to pass again the tissue to the media 4E, with a previous cut in its base to remove any callus that is formed.
- After three weeks of being transferred back to the culture media a growth of explants is starting, there is an emission of plant roots and a fully developed plant is formed (Figure 21). With the development of this plant, one can continue the indexing tests (see protocols of Germplasm Health Lab).



Figure 21. *In vitro* cassava plants at the beginning of the indexing process.

3.3.2 Steps from *in vitro* material

Source material

Materials that have been introduced *in vitro* (from countries other than Colombia) must first be registered with the following data: place of origin, date of introduction, number of tubes, identification of varieties, physiological condition (chlorosis, death and other symptoms), the crop phytosanitary condition (fungi and bacteria) and environmental conditions (presence of phenolization, solidification). These data provide information for the evaluation and the arrival status report of the materials.

Once these materials are inventoried, they must undergo a phase of recovery and setting, which takes place in the growth room for two weeks. After this time, one expects a greening of the seedlings and proceeds to make a new assessment of the physiological and phytosanitary status of the plant culture. This information is reported to the Colombian Institute of Agriculture official who is responsible for carrying out the nationalization of the material and therefore gives the approval to proceed with micropropagation.

Once the establishment and recovery of materials have been obtained, then comes the stage of micropropagation, which is performed taking as explants the apical buds of 0.5 cm (which are used for the treatment of thermotherapy explained below), and 0.5 cm nodes (which will serve as a backup until the thermotherapy and indexing process is done). The apical buds are planted in the culture media 17N and nodes are planted in the culture media 4E.

Treatment of *in vitro* thermotherapy

- Test tubes containing the apical buds are transferred to the thermotherapy chamber, which is at a temperature of 37°C day/35°C night, illumination of 18.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and photoperiod of 12 h (Figure 22)
- Under such conditions the apical buds are maintained for a period of 12 days, after this period the apical buds are extracted again and planted in the media 17N. This process is performed 3 times with duration of 12 days each.
- At the end of the third cycle the material is placed in the growth room, it is micropropagated and one or two plants are delivered to germplasm health laboratory to perform the tests for indexing.

With the development of these activities we have achieved the sanitation of clones for diseases with quarantine restrictions (CCMV, CsXV and FSD), showing that the efficiency of control depends not only on the pre-treatment with heat (intensity and duration) of the infected cuttings but also, and significantly, on the size of the meristem which is grown subsequently (Roca et al. 1991).



Figure 22. Thermotherapy chamber for *in vitro* virus cleaning treatment.

3.4 Micropropagation

Micropropagation is the massive multiplication of a species from tissues or organs under *in vitro* conditions to obtain, maintain and multiply genetic materials. In each case the formation of axillary branches that can be separated, and rooting are expected. Theoretically, axillary or lateral shoots can then produce additional axillary branches in perpetuity, as each newly formed branch or each explant of node is subcultured (Krikorian 1991).

The technique of micropropagation is important where few materials are available, where clonal material is required, where large quantities of planting material is necessary. Materials that have proved negative for the different indexation tests need to be micropropagated in order to increase the number of explants for each clone. One can then obtain the 5 tubes per clone that are required to enter into the *in vitro* Germplasm Bank.

The procedure is as follows:

- The negative *in vitro* plant is carried to the laminar flow chamber, where it is extracted from the test tube and then is multiplied by cutting the apical buds and nodes with its respective axillary buds, which are planted later in the media 4E (Figure 23).
- The tubes are sealed and taken to the conditions previously described of the Growth Room.
- After 4-5 weeks, plants have developed as to be sown in the conservation media (8S) (see Annex).

When the materials have been proven positive for any of the viruses, the procedure of elimination of pathogens is restarted and then indexation testing is conducted again.



Figure 23. Micropropagation process. The pictures a), b) and c) show the cutting process for obtaining explants (apical buds and axillary buds), d) shows the selection and seeding of explants in the media, e) final labeled and sealed tube, f) and g) growth and conservation rooms where tubes are finally taken, respectively.

3.5 Indexing

Established plants that have been obtained after being submitted to thermotherapy treatment either from cuttings or *in vitro* thermotherapy should be evaluated for different viruses of quarantine importance such as the Cassava Common Mosaic CSCMV), Cassava Virus X (CsXV) and frogskin disease (FSD) (Frison & Feliu 1991).

In vitro plants, which have been submitted to thermotherapy treatments, should be propagated as follows:

- The apical buds from *in vitro* plant with thermotherapy treatment is planted in the culture media 17N (see Annex) and then placed for a period of 4-5 weeks in the growth room. The nodes of the same plant are grown in a culture media 4E and stay in the growth room until results are obtained.
- The plant has been planted in the culture medium 17N and after 4 weeks when the growth and development period is accomplished is delivered to the Germplasm Health Laboratory (see Handbook of Procedures for Germplasm Health Laboratory) to perform the respective indexation tests.

3.6 In-vitro Conservation

The need to maintain selected clones and promising hybrids in healthy conditions for distribution to national agricultural research programs stimulated some of the earliest studies on the maintenance of cassava *in vitro* germplasm (Roca et al. 1989).

The method of *in vitro* conservation consists of maintaining physical and chemical conditions of cultures that allow to extend to the maximum the range of transfer to fresh media without affecting the viability and stability of crops (CIAT 1984) and develop further the methodology to reduce their maintenance cost (Koo et al. 2004).

The growth rate of *in vitro* cultures can be controlled mainly by using factors such as temperature, organic and inorganic nutrients, light intensity and photoperiod, growth regulators, osmotic regulators and inhibitors of ethylene.

The process of slow growth developed at CIAT for cassava (SN media) has allowed the material to remain viable for a period between 18-24 months, while the traditional method (8S media) has allowed it to remain at an average of 11 months.

The established procedure for the handling of *in vitro* collection is as follows:

3.6.1 Introduction procedures

- Micropropagated material is planted in the conservation 8S media (3 tubes) and in the slow growth SN media (2 tubes) (see Annex). Apical buds and nodes are taken from the used explant, placing 2 or 3 new explants per tube. The dimensions of the glass tube used for conservation are 25 x 150 mm and foil paper is used to cover the tubes and seal them immediately with extensible tape.

Three of the five tubes kept for each clone have been filled with the 8S media in order to have material available at the time to prepare a shipment, and the remaining two have been filled with SN media where the growth rate has been lower and therefore constitute the reserve of material.

The minimum number of cassava *in vitro* tubes that can be conserved under minimum slow growth is obviously one, but as a safety and support and to enable a rapid recovery and availability for distribution and practical tests, it is 3-5 tubes per material (IPGRI/CIAT, 1994).

- Subsequently, a cardboard ring to which the clone identification with bar codes is stuck is placed on the test tube, and the tubes are put in a way-tray. The use of bar code helps us to minimize the problem of incorrect identification of materials and to facilitate the entry of information into the database (Figure 24).



Figure 24. Identification System for the accessions using bar codes.

- Information recorded in the data collector through a bar code consists of data related to the date of entrance of material in the conservation conditions, the growing media in which the material has been sown, and the person responsible for the subculture; then this information is transferred at the end of the day to the database (Figure 25).



Figure 25. Documentation process in the database of the Genetic Resources Program.

- Way-trays are located in the growth room for a period of 2-3 weeks, at the end of this period, state of development and health of plants is evaluated (noting if there is presence of fungi and bacteria).
- The materials are then placed in the conservation room under conditions described before for this room (Figure 26).



Figure 26. Layout of the shelves in *Manihot* conservation room.

3.6.2 Maintenance work and replacement of materials

The success of *in vitro* conservation lies in the quality of everyday tasks that are undertaken for its good operation. One of the main tasks is the supervision of the physical conditions of the conservation room (temperature, light, humidity, asepsis, etc.) as well as the performance of periodic revision of the physiological and phytosanitary state of the material being conserved.

Maintenance

Factors such as temperature, relative humidity and lighting must be controlled repeatedly because they are an essential part of the scheme of conservation. This monitoring is done every day, taking into account:

- Temperature and humidity

A hygromograph is located in the conservation room (Figure 27a) to monitor day and night temperatures and relative humidity of the room as previously set in order to keep reduced growth rates. A change in the temperature leads to a revision of the refrigeration unit. In case the relative humidity is greater than 70%, this condition that can lead to the development of microorganisms and increase the risk of contamination, the use of dehumidifiers to regulate this factor is therefore necessary (Figure 27b).

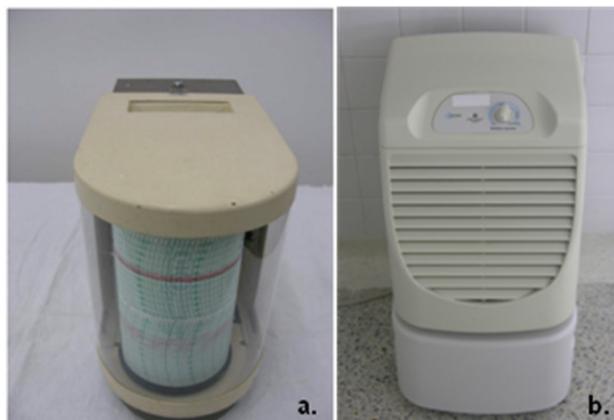


Figure 27. Equipment for monitoring environmental conditions in the Conservation room: a) hygrothermograph b) dehumidifiers.

- Lighting

Having an adequate light intensity depends on the smooth operation of fluorescent lamps. Therefore, damaged lamps must be replaced timely. The photoperiod is controlled with a clock that is battery operated, which replaces the main power in case it is interrupted (Figure 28).

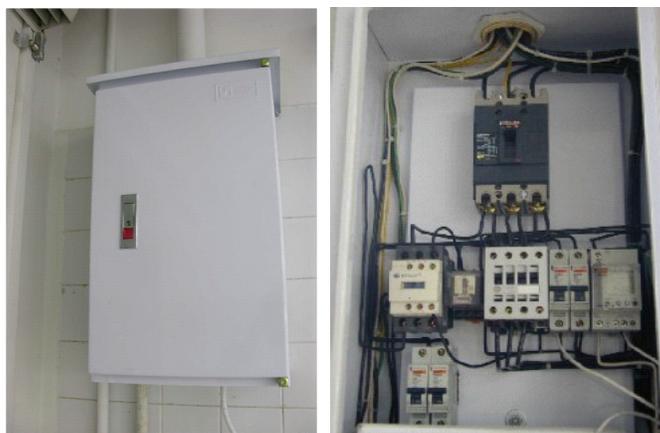


Figure 28. Required equipment for the photoperiod control in the respective rooms.

- Growing plants

One should keep in mind that growing plants have leaves and green stems and that roots show normal growth. Plants must be renewed before they present a total defoliation or show a necrotic zone between the root and stem. A yellowing color in the plants indicates that is necessary to carry out the subculturing and to plant them again in a fresh culture medium (Figure 29).

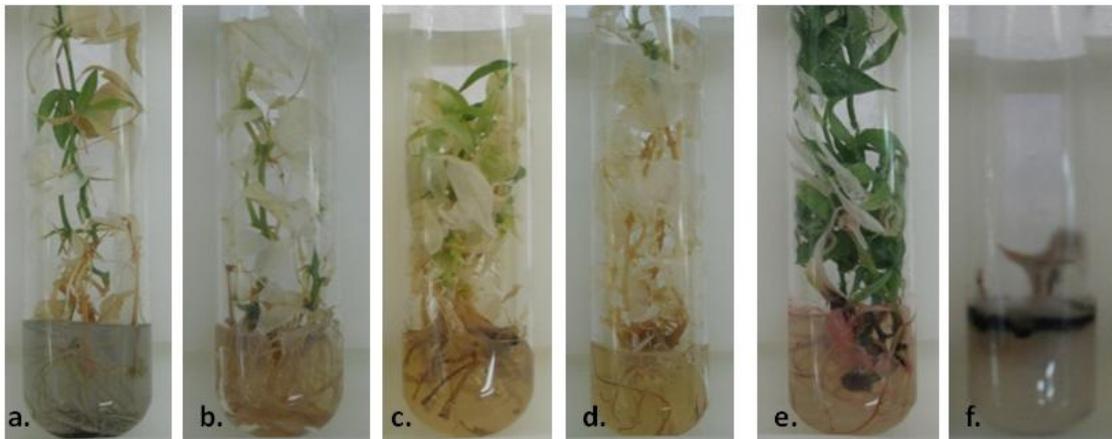


Figure 29. Physiological status of plants to exit to subculturing process. The pictures a), b), c) and d) show different states of damage and defoliation. In e) and f) shows bacterial and fungal contamination, respectively.

- Nutrient medium

When the culture medium is fresh and in good conditions, it is crystalline, but with the passing of time, it becomes darker. This may be due to the secretion of metabolites of the roots, especially of phenolic type. The use of activated charcoal significantly reduces this effect. When the medium shows these characteristics linked to the plant deterioration, it is necessary to proceed with the subculturing in a new media.

- Test tubes

It is important to review the status of the test tubes and to note that the glass has no cracks; one should also check that the cover and the plastic are in good conditions, in order to prevent the entry of contaminants and avoid dehydration of the culture media.

- Asepsis

Access of staff working in both field and greenhouses into the area of transfer, as well as the growth and conservation rooms should be restricted. It is important to use unique laboratory clothing to work in these areas.

Renewal Subculturing

In addition to the work described above for the maintenance of the conservation room efforts must be made for the renewal subculturing of the material that has completed its cycle of conservation. The procedure is as follows:

- Every week a review is done on each of the varieties that are in the *in vitro* germplasm bank. The phytosanitary and physiological status of the material is checked. There are different causes that require the exit of a variety towards subculturing:
 - a) In case a fungal contamination is present, in the case of a single tube being infected, it is removed, and if for some reason the 5 tubes exhibit this type of contamination the methodology of disinfection with sodium hypochlorite applies.
 - b) If the cause of contamination is bacterial, antibiotics (vancomycin) to a concentration of 20 - 40 mg/l (see Annex), or liquid media such as 8S with a pH of 3.5 or ampicillin at a concentration of 100 mg/l can be used.
 - c) In presence of defoliation, if the culture media begins to turn yellowish, or if the death of the stem is observed, it is necessary to make a subculturing (Figure 30).



Figure 30. Weekly review of plants to determine the opportunity of renewal or subculturing.

- The materials that go for subculture are registered in the data collector. The recorded information is as follows: Name of the variety, date of subculture, and name of the person responsible for the transfer and cause of the subculture.
- The materials are taken to the room where the subculture is done and its renewal is done again in the 8S media (3 tubes) and SN (2 tubes). The procedure is the same as in the introduction to conservation management (Figure 31).

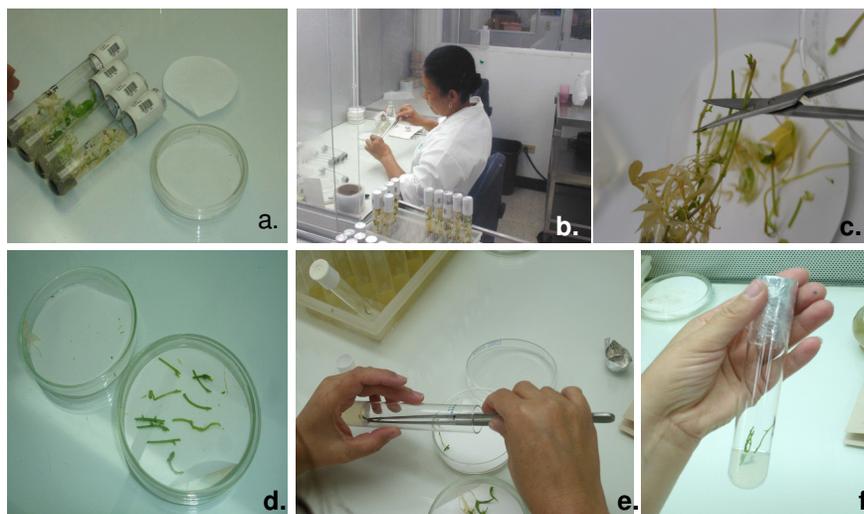


Figure 31. Subculturing process or renewal of materials in the subculture room. Photographs show: a) state at time of subculturing, b) and c) selection process and cut to obtain explants (apical buds and axillary buds), d) and e) show explants and planting of explants in the media, and f) the sealed tube at the end of the process.

- Once planted in the culture media (8S, SN, or 12A) data are recorded into the data collector and a record is made of the following parameters: name of variety, date of entry, culture media and person responsible.
- Tubes are placed back into the growth room (2-3 weeks) and after that time physiological and phytosanitary states are reviewed, they are then located in the conservation room staying in that place until the subculturing is required again.
- At the end of the day, the records accumulated in the data collector are registered in the central database.

The entry and exit record of a variety allow us to determine for how long a variety may be kept *in vitro* and with this information we can also determine the time when a variety must go to subculturing (Figure 32).

Accesión: BRA 65 Género: MANIHOT Especie: ESCULENTA

Conservación Ubicación Indexación Cambio de Med

Variedad: BRA 65

	Fecha Entrada	Medio Cultivo	Fecha Salida	Causa Salida
1	12/12/2006	8S MEDIO DE CONSERVACIO	15/08/2007	S SUBCULTIVO
2	12/12/2006	8S MEDIO DE CONSERVACIO	15/08/2007	S SUBCULTIVO
3	12/12/2006	8S MEDIO DE CONSERVACIO	15/08/2007	S SUBCULTIVO
4	12/12/2006	8S MEDIO DE CONSERVACIO	15/08/2007	S SUBCULTIVO
5	12/12/2006	8S MEDIO DE CONSERVACIO	15/08/2007	S SUBCULTIVO

Responsable_Entrada: A.MONTOYA Responsable_Salida: N.P.ESCOBAR

Bonsai: 1 Designada: 1 Core: 0 Disponible: 1 Enter

Accesión CIAT
Record: 2/?

Figure 32. Register in the database of entry and exit information of the accessions to subculturing.

3.7 Characterization

It is important for an *ex situ* collection that materials are well characterized, this can be achieved using a variety of techniques such as: a) morphological and agronomic descriptors, b) biochemical markers, including isozyme analysis, and c) molecular markers including RFLPs, RAPDs and microsatellites.

For the effective management of the cassava collection those methodologies have been used for the following purposes:

- 1) Verify the genetic integrity of materials kept under the *in vitro* conservation technique (after several years of conservation).
- 2) Detect mixtures of materials.
- 3) Identify genetic duplication (redundancy of materials).

The details of this characterization activity are presented in the Handbook of Procedures of the Genetic Quality Laboratory.

3.8 Distribution

Another important role of *in vitro* germplasm bank is the distribution of germplasm to different users. This activity is run under the rules established by the International Treaty on Plant Genetic Resources for Food and Agriculture approved by the FAO Conference at its 31st period of sessions on November 3, 2001, and entered into force since June 29, 2004. Article 12.4 of the Treaty establishes that access should be facilitated with the use of a standard material transfer agreement (SMTA) for materials of *Manihot*.

The *in vitro* germplasm distribution process comprises the steps described below:

FIRST STEP	→	Request of Germplasm Acceptance of SMTA
LOGISTICS	→	Selection of materials Micropropagation Preparation of shipment
REQUIREMENTS	→	Phytosanitary Certificate Import Permit
DOCUMENTATION	→	List of Materials Handbook of procedures PGR Database

Request of germplasm. Different users can make requests by various means: physical mail, electronic mail or through the website of the Genetic Resources Program at: <http://www.ciat.cgiar.org/urg>).

The Standard Material Transfer Agreement (SMTA) should have been accepted. Three methods of acceptance can be chosen:

- Physical signature on the acceptance document of the Standard Material Transfer Agreement.
- Standard Material Transfer Agreements in sealed shipments. The material is provided under previous acceptance of the terms of the Agreement. The supply of material and its retention by the receptor implies the acceptance of the terms of the agreement.
- Electronic acceptance of Standard Material Transfer Agreement. Through e-mail the established conditions are accepted. If this form is chosen, the material must also be accompanied by a written copy of Standard Material Transfer Agreement.

Selection of accessions and micropropagation. Materials for distribution are chosen by users according to the objectives and research needs they are developing. Once the accessions requested by the user are identified, micropropagation is initiated from the material kept in the *in vitro* bank. The materials are transferred to the multiplication medium or rooting (17N) which promotes the growth of the aerial part as well as of the roots, facilitating the process of transfer and acclimatization to the greenhouse or the subsequent *in vitro* multiplication by the user of the material.

After 4-6 weeks, rooted plantlets are fully developed, which is the most appropriate way, from the standpoint of management, for the exchange of clonal germplasm (CIAT 1982).

Preparation of shipment. The *in vitro* micropropagated plantlets are carefully reviewed prior to packaging. One notes the overall condition of the plant, the growth media, the absence of bacterial contamination and/or fungus, the sealing and proper labeling of material, which is made using a permanent ink black marker, indicating in clear and legible letter and numbers the respective identification of materials. All this with the purpose of ensuring that the shipping conditions are adequate.

The tubes are placed in grooved cardboard trays or preferably polystyrene, attached to them with tape. The trays are placed one above the other and the set is secured with tape, then packed in cardboard boxes, labeled with information about content, careful handling, recipient and sender.

The distribution package includes the relevant phytosanitary certificate issued by the plant quarantine authority of Colombia ICA, which explains the treatments and tests of diseases detection applied to plant material. Previously, the applicant must submit the relevant import permit issued by the competent authority in the place of destination of material or the relevant written communication in which it informs about the absence of this requirement for entry of plant material into the country of destination (Figure 33).



Figure 33. Documentation and packaging used for the distribution of *in vitro* germplasm. The documentation includes: a) List of material sent, b) Transfer Agreement SMTA, Phytosanitary certificate c) and d) procedures guide.

Moreover, a list of the material supplied is joined under the agreement accepted by the recipient. This list includes the passport data available and a booklet with instructions about further handling of the materials once they have arrived (Roca et al. 1984). It states that the information can be downloaded from the Web site: www.ciat.cgiar.org/urg.

Database. Once the shipment is prepared, the information is documented in the database, which includes data such as: number of shipments, number of transfer agreement, number of phytosanitary certificate, number of import permit, the recipient institution, recipient name, recipient country, type of user (there are 8 categories), identification of shipped materials, date of shipment and the purpose for which the material was requested (7 categories) (Figure 34) (Table 3).

Table 3. Different kind of users and purposes used in the distribution of *in vitro* materials.

CATEGORIES OF DISTRIBUTION	
TYPE OF USERS	PURPOSES
1. CGIAR Centers	1. Breeding
2. Commercial companies	2. Agronomic
3. Farmers	3. Applied Research
4. Germplasm banks	4. Basic Research
5. National institutions	5. Training
6. No governmental institutions NGOs	6. Others
7. Regional organizations	7. Conservation
8. Universities	

The screenshot shows a software application window titled "Developer/2000 Forms Runtime for Windows 95 / NT - [DISTRIBUCION YUCA]". The window has a menu bar with options: Acción, Edición, Consulta, Bloque, Registro, Campo, Ayuda. Below the menu bar is a toolbar with icons for file operations and navigation. The main area contains a form with the following fields and values:

- Accesión:
- Género:
- Especie:
- Nuevo Envío:
- Composicion del envío:
- Destinatarios:
- Material_enviado:
- Material Por Institucion:
- Datos Básicos**
 - Npedido:
 - F Solicitud:
 - F Envío:
 - N Acciones:
 - Tipo Material:
 - Cooperador:
 - Institución:
 - Sigla:
 - País ISO:
 - CHINA
 - Estación:
 - Apellido:
 - Nombre:
 - T Usuario:
 - Propósito:
 - Responsable:
 - Mta Número:
 - Certificado Fitosanitario:
- Más Información:
- Enter:

At the bottom of the window, there is a status bar with the text: "Identificador del pedido: inicial del cultivo [C] cuatro números del año y un consecutivo" and "Record: 1/1".

Npedido	Accesion	Procedencia	Cant Plantas	Cant Sem	Gramos	F Actualización
C20070024	CG 354- 2	PAL1999	3			26/09/2007
	CG 1231- 3	PAL1999	2			26/09/2007
	CM 6173- 8	PAL1999	3			26/09/2007
	SG 427- 87	PAL1999	2			26/09/2007
	SG 629- 4	PAL1999	2			26/09/2007
	SG 638- 6	PAL1999	3			26/09/2007
	SG 702- 11	PAL1999	2			26/09/2007
	SG 702- 19	PAL1999	3			26/09/2007
	SM 523- 2	PAL1999	3			26/09/2007
	SM 707- 17	PAL1999	2			26/09/2007
	SM 853- 21	PAL1999	2			26/09/2007
	ARG 6	PAL1999	2			26/09/2007

Figure 34. Recording of basic information and shipment composition in the database.

3.9 Safety backup

If the *in vitro* collection is the only source of the material, it must be duplicated in at least two sites as a security measure. In 2005, an agreement was signed with the International Potato Center (CIP) in Peru to keep a cassava “Black box” (a non-active duplicate). A total of 3 tubes per material are sent (in the minimal growth media, SN) (Figure 35).

The cassava core collection (630 accessions) is also conserved as a safety duplicate in liquid nitrogen.



Figure 35. Form of packaging of cassava duplicate security to be sent to the CIP.

4. QUALITY CONTROL

All the activities carried out in the tissue culture laboratory for the maintenance of the *in vitro* cassava collection require a careful management. Some of the possible risks and their control mechanisms are as follows:

MECHANICAL MIXING. There is a possibility of mechanical mixing. This risk has been reduced by labeling the accessions with their respective bar code that is applied to each of the five tubes kept in the bank. Possible doubts are cleared with the help of the information recorded in the database at the time of exit and entrance to the bank before and after the subculturing, respectively. Some morphological features developed *in vitro* can provide additional information, in the best of the cases. When it has not been possible to determine with clarity and certainty the name of the accession, it is recommended to discard the material and make the propagation using the tube that is kept in reserve in the bank.

Subculturing activities are carried out individually (by accession). Once the subculturing of one material has fully completed, the next one is started. The personnel who performs the subculture work has been instructed on the careful handling to avoid confusions.

PREPARATION OF STOCK SOLUTIONS AND CULTURE MEDIA. For proper preparation of stock solutions is important:

- The use of an analytical balance for weighing the components.
- To ensure that glassware materials used in the preparation and storage are clean and dry. This glassware must be washed and rinsed with distilled water to remove traces of salt of regular water.
- The mixture of components must be done using a magnetic stirrer.
- Once the preparation is finished the container where the solution is stored must be labeled indicating the name of the solution, concentration, preparation date and the responsible for it.

The proper marking and labeling of glass recipients allows the identification, facilitates the handling and location at the time of use for the preparation of culture media.

To facilitate and reduce risks in the preparation of media forms have been established with the formulations stating the quantities and the order in which the stock solutions should be added. The following must also be taken into account:

- Place on the benches the stock solutions in the established order in the formulation. Once added to the media these must be removed to avoid confusions or doubts when continuing with the preparation.
- Each solution must be added using a clean pipette. The pipettes should not be reused in the addition of another solution because the solutions could be contaminated and mixed.

- Once the preparation of culture media is done, these should be quickly dispensed in tubes or containers where they will be ready, then they are closed. It should be noted that the tubes, containers and lids must be clean.
- Subsequently, the way-trays are covered with paper, which is labeled with the name of the media and the date of preparation.

STERILIZATION PROCEDURE. Sterilization is the process by which any material, site or area is completely free of any living microorganism or spore. It is stated that such materials or sites are sterile or have been sterilized. In medical terminology, the word generally used is asepsis to designate the condition in which the pathogens microorganisms are absent. Those working in the plant tissue culture use the word *aseptic* as a synonym for sterile.

Disinfection is generally confined to the destruction process of microorganisms through chemical methods; sterilization often refers to the physical method for destroying microorganisms.

There are three methods of sterilization: sterilization with dry heat (electric oven), chemical sterilization (in cold) and sterilization by pressure steam (autoclave or moist heat with pressure) the latter is the method used in the tissue culture laboratory for sterilization of culture media, water and dry material. Some aspects to consider in the process of sterilization are:

- It is important to know the handbook of specific instructions for operating the autoclave. Follow the manufacturer's instructions whenever possible to ensure a proper use and operation of it.
- Wrap items (tools, including petri dishes among others) and culture media before sterilization, it helps to prevent and to reduce the probability of contamination before the use of these items.
- The elements and way-trays should fit in such a way as to allow the free flow of steam.
- In general, sterilize for 45 minutes dry material (petri dishes, water bottles, cardboards, erlenmeyers and tools). For culture media sterilize for 15 minutes at 121°C (250°F) and at a pressure of 15 pounds per square inch. The time begins to run when the autoclave reaches the desired temperature and pressure.
- If the autoclave is automatic, the heat is stopped and the pressure will begin to drop once the sterilization cycle is completed. If the autoclave is not automatic, the heat source should be turned off and wait until the manometer dials "zero" to open the autoclave, then slowly open the lid or door to allow the exit of the left steam - over a period of at least 10 minutes, finally get the packages out (Figure 36).



Figure 36. Process for the sterilization of culture media.

- Take into account that the time required to sterilize liquids in autoclave depends on many factors. The most important is the volume of fluid that is being sterilized. In general, the time is the following:

Range de volume (ml)	Time (minutes)
< 75	15
75- 100	20
250-500	25
1000	30
1500	35
2000	40

- Once the sterilization with autoclave is done, the items should be stored in optimal conditions. The culture media once they are cold are packed in plastic bags and refrigerated. Dry materials (petri dishes, cardboards and tools) are maintained in the oven dryer, erlenmeyers and sterile double-distilled water are placed on the respective shelf. When there is doubt about the sterility of any element to use, this should be considered contaminated and the process should be redone.

- There are three ways to monitor the effectiveness of sterilization, these are: mechanical indicators, chemical and biological. The most used in our laboratory are the indicating tapes with lines that change color when the expected combination of temperature, steam pressure and time is reached. Each package or item to sterilize should have a small piece of this indicating tape.
- It should be noted that certain chemical substances (hormones and antibiotics) are destroyed at sterilization temperatures with autoclave. In such cases, it is necessary to use other sterilization systems, such as filtering.

ENVIRONMENTAL CONDITIONS. The conditions of the growth and conservation rooms are monitored daily. Once changes are detected by the safety systems, corrective actions are taken to prevent that such disruptions could cause significant damage in the materials. Regular maintenance of refrigeration systems and lighting is done by staff specialized in this matter.

CONTAMINATION AND LOSS OF MATERIALS. The periodic review of the conservation status of the material in the bank prevents losses or damage to materials. The database and visual inspection are the main tools at the time of determining compliance of the conservation period.

The evaluation for the presence of bacterial or fungal contamination is performed by visual inspection. Once the number of contaminated tubes and the causative agent is determined, the process to follow is decided. If the contamination is bacterial an antibiotic is used or media with pH of 3.5. If contamination is fungal, disinfection is carried out using different concentrations of sodium hypochlorite. If it is possible, the recovery of material from non-contaminated tubes can be made and subsequent return to the conservation conditions.

The description of critical control points that have been identified to prevent contamination follows:

When you start the workday, it is important to wash hands with soap and water, and then rinse them with 70% alcohol before you start any planting session. It is essential to use the lab clothing, which should only be used within the laboratory facilities.

When you start the workday in the laminar flow chamber, the first step is the power of flow and light, then the cleaning is done using cotton and 96% alcohol. The outside of the containers that come from outside the working area must also clean with 70% alcohol and flame the mouth of each tube before and after sowing the explant.

The work of planting and dissection should be performed as close as possible to the flame of the burner to avoid prolonged exposure of the explants and culture media to the atmosphere. The manipulation of the explants must be from the center to the interior of the laminar flow chamber.

Every time the hands come out of the flow chamber, they should be sprayed with 70% alcohol before placing them back into the chamber. Avoid talking during the performance of the activities of subculture.

It is recommended to use several sets of the same sterilized tools to avoid overexposure of the explants to heat at the time of cutting and planting them.

Cleaning and cleanliness of the laboratory is performed daily. This includes vacuuming and mopping of floors with a solution of sodium hypochlorite at 5.25%. Similarly, a monthly cleaning and general disinfection is programmed that includes cleaning floors, benches and walls with detergent, suction surface and shelves, disinfection and cleaning of chairs, laminar flow chambers, laboratory carts, windows in all areas of work using 70% alcohol, followed by a 0.5% solution of sodium hypochlorite. In some cases, essence of mint is used for benches cleaning.

5. ANNEX

Annex 1. Stock Solutions (MS) for media preparation

Stock ^a	Substance	Quantity	Volume of Stock per liter
1	NH ₄ NO ₃	82.5 g	in 1000ml of H ₂ O double-distilled VS _{1L} = 20 ml
	KNO ₃	95.0	
	MgSO ₄ ·H ₂ O	18.5	
	KH ₂ PO ₄	8.5	
2	H ₃ BO ₃	620 mg	in 100ml of H ₂ O double-distilled VS _{1L} = 1 ml
	MnSO ₄ ·H ₂ O	2176 mg	
	ZnSO ₄ ·7H ₂ O	860 mg	
	Na ₂ MO ₄ ·2H ₂ O	25 mg	
	CuSO ₄ ·5H ₂ O	2.5 mg	
	CoCl ₂ ·6H ₂ O	2.5 mg	
3	KI	75 mg	in 100ml of H ₂ O double-distilled VS _{1L} = 1 ml
4	CaCl ₂ ·2H ₂ O	15000 mg	in 100ml of H ₂ O double-distilled VS _{1L} = 3 ml
5 ^b	a) Na ₂ EDTA	1492 mg	in 200ml of H ₂ O double-distilled VS _{1L} = 5ml
	b) FeSO ₄ ·7H ₂ O	1114 mg	
6	Thiamin-HCl	20 mg	in 200ml of H ₂ O double-distilled VS _{1L} = 5ml
7	M-inositol	1600 mg	in 200ml of H ₂ O double-distilled VS _{1L} = 6.25 ml

^a. Stock solutions 2 and 6 should be kept in the freezer; the rest at 8-10°C. Stock 5 should be stored protected from light.

^b. Separately dissolve *a* and *b* in 50 ml of water each, the *b* is heated in a water bath, both solutions are mixed thoroughly, let it cool and is completed with water to 200 ml.

Source: Roca, W.M., Rodríguez, J.A., Mafla, G., & Roa, J.C. 1984. Procedures for recovering cassava clones distributed *in vitro*. Centro Internacional de Agricultura Tropical (CIAT). Cali, Colombia. 8 p.

ANNEX 2. PROPAGATION MEDIA (4E)

MS (2% sucrose) + 0.04 mg/l BAP + 0.05 mg/l GA + 0.02 mg/l ANA
AGAR 0.7%.

Preparation:

In a volume of aprox. 200 ml of H₂O double-distilled add:

- | | | |
|-----|---|---------|
| 1. | Sol. stock 1 (MS) | 20 ml |
| 2. | Sol. stock 2 (MS) | 1 ml |
| 3. | Sol. stock 3 (MS) | 1 ml |
| 4. | Sol. stock 4 (MS) | 2.9 ml |
| 5. | Sol. stock 5 (MS) | 5 ml |
| 6. | Thiamine HCl (stock 100 ppm) | 10 ml |
| 7. | Inositol (stock 8000 ppm) | 12.5 ml |
| 8. | Sucrose | 20 g |
| 9. | BAP (stock 10 ppm) | 4 ml |
| 10. | GA (stock 10 ppm) | 5 ml |
| 11. | ANA (stock 10 ppm) | 2 ml |
| 12. | Activated charcoal | 0.5 g |
| 13. | Complete to 500 ml with H ₂ O double-distilled | |
| 14. | Adjust pm 5.7-5.8 | |
| 15. | Dissolve by heating 7 g AGAR in 500 ml of H ₂ O double-distilled | |
| 16. | Mix well the medium with AGAR Solution | |
| 17. | Final volume 1000 ml | |

* To prepare the media of Murashige and Skoog from stock solutions (see Annex 1).

Source: Roca, W.M., Nolt, B., Mafla, G., Roa, J.C., & Reyes, R. 1991. Eliminación de virus y propagación de clones en la yuca (*Manihot esculenta* Crantz) In: Roca, W.M., Mroginski, L.A., Fundamentos y Aplicaciones. pp 403-421.

ANNEX 3. ROOTING MEDIA (17N)

1/3 MS (2% sucrose) + 0.01 mg/l ANA + 0.01 mg/l GA + 25 mg/L. (10-52-10 Fertilizer)

Preparation:

In a volume approx. of 200 ml of H₂O double-distilled add:

- | | | |
|-----|---|---------|
| 1. | Sol. stock 1 (MS) | 6.7 ml |
| 2. | Sol. stock 2 (MS) | 0.3 ml |
| 3. | Sol. stock 3 (MS) | 0.3 ml |
| 4. | Sol. stock 4 (MS) | 1.0 ml |
| 5. | Sol. stock 5 (MS) | 1.7 ml |
| 6. | Thiamine HCl (stock 100 ppm) | 10.0 ml |
| 7. | Inositol (stock 8000 ppm) | 12.5 ml |
| 8. | Sucrose | 20.0 g |
| 9. | ANA (stock 1 ppm) | 10.0 ml |
| 10. | GA (stock 1 ppm) | 10.0 ml |
| 11. | (10-52-10 Fertilizer) | 25.0 mg |
| 12. | Complete to 500 ml with H ₂ O double-distilled | |
| 13. | Adjust pH 5.7-5.8 | |
| 14. | Dissolve by heating 7 g agar in 500 ml of H ₂ O double-distilled | |
| 15. | Mix well the medium with AGAR solution | |
| 16. | Final volume 1000 ml | |

Source: CIAT. 1982. El cultivo de meristemas para el saneamiento de clones de yuca. Guía de estudio. Centro Internacional de Agricultura Tropical, Cali, Colombia. 45pp.

ANNEX 4. CONSERVATION MEDIA (8S)

MS (2% Sucrose) + 0.02 mg/l BAP + 0.1 mg/l GA + 0.01 mg/l ANA.

AGAR 0.7%: 7 g in 500 ml of H₂O double-distilled

Preparation:

In 200 ml of H₂O double-distilled add:

- | | | |
|-----|---|---------|
| 1. | Sol. stock 1 (MS) | 20 ml |
| 2. | Sol. stock 2 (MS) | 1 ml |
| 3. | Sol. stock 3 (MS) | 1 ml |
| 4. | Sol. stock 4 (MS) | 2.9 ml |
| 5. | Sol. stock 5 (MS) | 5 ml |
| 6. | Thiamine HCl (stock 100 ppm) | 10 ml |
| 7. | Inositol (stock 8000 ppm) | 12.5 ml |
| 8. | Sucrose | 20 g |
| 9. | BAP (stock 10 ppm) | 2 ml |
| 10. | GA (stock 10 ppm) | 10 ml |
| 11. | ANA (stock 10 ppm) | 1 ml |
| 12. | Activated charcoal | 0.5 g |
| 13. | Complete with 500 ml of H ₂ O double-distilled | |
| 14. | Adjust the pH 5.7-5.8 | |
| 15. | Dissolve by heating 7 g AGAR in 500 ml of H ₂ O double-distilled | |
| 16. | Mix well the medium with AGAR solution | |
| 17. | Final volume 1000 ml | |

Source: CIAT.1984. El cultivo de meristemas para la conservación de germoplasma de yuca *in vitro*. Guía de estudio. Centro Internacional de Agricultura Tropical, Cali, Colombia. 41pp.

ANNEX 5. SLOW GROWTH MEDIA (SN)

MS (2% Sucrose) + 0.02 mg/l BAP + 0.1 mg/l GA + 0.01 mg/l ANA + 10 mg/l silver nitrate.
AGAR 0.7%: 7 g in 500 ml of H₂O double-distilled

Preparation:

in 200 ml of H₂O double-distilled add:

- | | | |
|-----|---|---------|
| 1. | Sol. stock 1 (MS) | 20 ml |
| 2. | Sol. stock 2 (MS) | 1 ml |
| 3. | Sol. stock 3 (MS) | 1 ml |
| 4. | Sol. stock 4 (MS) | 2.9 ml |
| 5. | Sol. stock 5 (MS) | 5 ml |
| 6. | Thiamine HCl (stock 100 ppm) | 10 ml |
| 7. | Inositol (stock 8000 ppm) | 12.5 ml |
| 8. | Sucrose | 20 g |
| 9. | BAP (stock 10 ppm) | 2 ml |
| 10. | GA (stock 10 ppm) | 10 ml |
| 11. | ANA (stock 10 ppm) | 1 ml |
| 12. | Silver nitrate (SN) | 10 mg |
| 13. | Complete with 500 ml of H ₂ O double-distilled | |
| 14. | Adjust the pH 5.7-5.8 | |
| 15. | Dissolve by heating 7 g AGAR in 500 ml of H ₂ O double-distilled | |
| 16. | Mix well the medium with AGAR solution | |
| 17. | Final volume 1000 ml | |

Source: Mafla, G., Roa J.C., & Guevara C.L. 2000. Advances on the in vitro growth control of cassava, using silver nitrate. *In: 'Cassava Biotechnology',* Carvalho, L., Thro, A.M., Vilarinhos, A.D. (eds.), Empresas Brasileiras de Pesquisa Agropecuaria, Brasilia, Brasil, pp. 439-446.

**ANNEX 6. MEDIA FOR WILD SPECIES
(12A₃)**

MS (3% sucrose) + 0.2 mg/lit Kinetin + Vitamins + 1.0 g/l activated charcoal

Preparation:

in 200 ml of H₂O double-distilled add:

- | | | |
|-----|---|---------|
| 1. | Sol. stock 1 (MS) | 20.0 ml |
| 2. | Sol. stock 2 (MS) | 1.0 ml |
| 3. | Sol. stock 3 (MS) | 1.0 ml |
| 4. | Sol. stock 4 (MS) | 3.0 ml |
| 5. | Sol. stock 5 (MS) | 5.0 ml |
| 6. | Thiamine HCl (stock 100 ppm) | 10.0 ml |
| 7. | Inositol (stock 8000 ppm) | 12.5 ml |
| 8. | Sucrose | 30.0 g |
| 9. | Kinetin (100ppm) | 2.0 ml |
| 10. | CuSO ₄ (100ppm) | 4.8 ml |
| 11. | Activated charcoal | 1.0 g |
| 12. | Complete to 500 ml with H ₂ O double-distilled | |
| 13. | Adjust pH 5.7-5.8 | |
| 14. | Dissolve by heating 7 g AGAR in 500 ml of H ₂ O double-distilled | |
| 15. | Mix well the medium with solution | |
| 16. | Final volume 1000 ml | |

Source: Velásquez, E. & Mafla, G. 1999. Conservación in vitro: Una alternativa segura para preservar especies silvestres de *Manihot* spp (Euphorbiaceae). *In*: Congreso Nacional de Conservación de la Biodiversidad (2, 1999, Bogotá, Colombia). [Memorias]. Pontificia Universidad Javeriana, Bogotá, DC, CO. p. 1-14.

ANNEX 7. ANTIBIOTIC PREPARATION

1. Cut little squares of filter paper (0.5 cm X 0.5 cm).
2. Put them in a petri dish, wrap it with paper and sterilize them for a period of 45 minutes.
3. In the laminar flow chamber separate each paper from one another.
4. Weight 75 mgs of Vancomycin directly in the laminar flow chamber and dissolve it in 15 ml of distilled sterile water.
5. Put 1 or 2 drops of antibiotic with a micropipette in each square.
6. Let the antibiotic dry.
7. With a sterilize tweezers take a paper containing the antibiotic and introduce it in the test tube.

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