MICRO-PROPAGATION OF CASSAVA PLANTS THROUGH THE TEMPORARY IMMERSION SYSTEM AND HARDENING OF MASSIVE NUMBERS OF CASSAVA VITROPLANTS

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ABSTRACT

Tissue culture is a technique widely used for propagation of plant material. This method has been used successfully to propagate species such us cassava, sweet potato, plantain and sugarcane. Recently, the development of techniques such us the Recipient for Automated Temporary Immersion (RATI) and Temporary Immersion System (TIS) have improved significantly the efficiency of tissue culture propagation methods.

The RATI and TIS techniques were originally developed in France and have been tested successfully in countries such us Cuba with cassava and other species. The use of these methods is helping research institutions to advance in the production of massive numbers of plants. However, one of the main limitations for a wider use of these techniques is the hardening period during which the explants have to be adapted to normal conditions, before its final transplanting to the production sites. This change of the conditions in which the plants are growing usually causes high death percentages in the plants produced with the *in vitro* multiplication systems.

CLAYUCA, in collaboration with CIAT, has done some work for the development of a methodology for the hardening phase of massive numbers of vitroplants. The present paper describes these experiences, discussing the different stages of a successful methodology developed for handling large numbers of *in vitro* plants produced with the help of biotechnology-based multiplication systems.

INTRODUCTION

Important advances have been obtained in recent years in the development of improved, higher yielding cassava varieties. This new cassava germplasm is helping farmers to obtain higher incomes and to improve their economic well-being. Despite the advances obtained in breeding efforts, one of the most important constraints for a more widespread use by farmers of the improved cassava varieties is the lack of planting material, in larger quantities, at the right moment and with the desired quality characteristics.

The use of biotechnology-based methods for rapid multiplication of improved varieties is one of the strategies that is helping to solve this limitation. One of the most important biotechnological methods available is the one known as Recipient for Automated Temporary Immersion, more popularly known as the RATI system. With the use of RATI systems for multiplication of cassava planting material, researchers at CIAT have been able to obtain very good multiplication rates, varying between 5 to 10, depending on the variety (**Table 1**).

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Table 1. Cassava multiplication rates with the RATI System.

Clones	Initial no. of explants	Total no. of explants	Multiplication rate
CM 3306	10	101	10.1
CM 523-7	10	106	10.6
MTai 8	10	61	6.1
MCol 1505	10	53	5.3

Source: *Escobar, 2001.*

The importance of these methods is the possibility of producing massive numbers of plants in very short periods of time. **Figure 1** illustrates the potential of these systems for producing millions of plants of a given crop. It can be seen that with a multiplication rate of 4 to 1, using cycles of 4 to 6 weeks, it is possible to produce up to 75,000 plants in one year.

Time (weeks)	0	6	12	18	24	30	36	42	48
Initial no. of plants	10	40	152	580	2,204	8,375	31,826	40,938	35,564
Final no. of plants (assumes 5% losses)		38	145	551	2,094	7,956	30,234	38,890	33,786
							1st exit of 20,000 plants	2nd exit of 30,000 plants	3rd exit of 25,000 plants
							↓	↓	↓
							——Hardening facilities——		

Figure 1. Multiplication rates with in vitro production systems.

The main limitation for a more widely disseminated use of these methodologies is the period of adaptation of the vitroplants after they have been multiplied. This phase, better known, as the "hardening period" is frequently associated with high percentages of losses from death of the vitroplants. The plantlets produced by such systems are like test-tube babies – weak, and fragile – after 6 to 11 months under artificial conditions of light, temperature, moisture, and nutrients in sterilized rooms. Consequently, they need to undergo a stage of acclimatization or hardening before they can be transferred to their final site. In cassava, this process is very delicate, becoming a bottleneck in the mass production of cassava planting materials through tissue culture. This paper describes some recent experiences of CLAYUCA and CIAT for the development of a methodology for post *in vitro* management of cassava planting material.

A. Hardening Process of Cassava Plantlets

Hardening large numbers of cassava plants that have been produced with the use of biotechnology-based methods such us RATI, is a process inevitably associated with losses. Most losses of plantlets occur during the transfer to the soil, that is, when the plantlets are moved from the artificial environment of test tubes to the natural soil, and must adapt to new microclimatic conditions. Where the transfer is not carried out with adequate management and handling, the percentage of losses can be very high (between 50% and 95%), thus limiting the adoption of the improved *in vitro* multiplication methods. It also discourages progressive farmers who may want to produce rapidly and safely disease-free planting materials, or to multiply massively a new promising variety over a short period. Other drawbacks of the hardening process are the cost and size of the installations needed, such as greenhouses and screenhouses.

Stages of the Hardening Process (HP)

A good, efficient hardening process includes at least six different stages:.

Stage 1: Pre-operational activities

The normal development of an HP demands prior planning that includes a detailed timetable of all activities involved, including:

a. Selecting and training personnel
b. Selecting and adapting installations
c. Laboratory tests
d. Acquiring equipment, materials and inputs
e. Requesting the biotechnology laboratory for the number of *in vitro* plants that can be "hardened" per week.

a. Selecting and training personnel

Labor should be qualified; if not, personnel should at least receive training on the basic aspects of HP. The number of workers needed depends on their experience and on the quantity of plants entering the process: a novice worker can handle about 200 plants per day and an expert up to 600.

b. Selecting and adapting installations

Two principal installations are used: the work area and a screenhouse or greenhouse (sometimes both).

The work area comprises the following spaces: a deposit for soil and sand, a mini-store for keeping materials and inputs, a soil surface for mixing, and a cool site for transplanting. This space should be protected from solar rays and strong winds, and should have, in addition, a washing area and a working table (**Photo 1**).

The screenhouse should have a roof, and be adapted for automatic climate control (**Photo 2**). It should have microsprays , suspended either over the tables or from the roof and installed along the floor to control the temperature and relative humidity, especially in the first days of the hardening process. Although bright lighting is needed to favor plantlet development, the morning, mid-day, or afternoon sun should not be allowed to reach

directly the plantlets during the first eight days of acclimatization. Accordingly, a protective screen can be installed. The best option for protective screen according to CIAT experience is the Polypropylene meshing ("Saram"), covered on the outside with several sheets of aluminum paper (each 30 cm wide), and separated at 5-cm intervals. The screen should be highly functional, installed on both sides of the screenhouse (to face sunrise and sunset), and reaching 1 or 2 m above the tops of the bags in which the plantlets sit. It should manually be withdrawn gradually, as the sun traces its path through the sky to let light enter the installation. The aluminum reflects the sun's rays and prevents heating of the area where the plantlets are being hardened. The maximum temperature within a screenhouse fluctuates between 33º and 38ºC, and the minimum between 18º and 22ºC.

The Greenhouse should have an automatic microspray irrigation system. Both in the greenhouse and screenhouse, the system should be controlled with a solenoid valve and control clock. The system saves 90% of the costs of labor needed to irrigate the plantlets.

Space for acclimatization. In both the screenhouse and greenhouse, the transplanted plantlets should occupy a space that needs to be increased by as much as three times as the plants grow for 2 or 3 months after transplanting, depending on the variety, and its stage of development. For example, to plant one hectare of cassava, using 10,000 plants, the greenhouse or screenhouse space needed initially is an area of 25 to 34 m^2. Two months later, these 10,000 plants will need an area of 50 to 68 m^2.

c. Laboratory tests

The soil, sand, and water to be used in the installations should first be analyzed chemically and biologically to correct potential problems.

d. Acquiring equipment, materials and inputs

To acclimatize the cassava plantlets, the following elements are needed:

- Soil mill, sieve and mixer; sterilizer; fumigator; protective equipment for fumigation of pesticides
- Test tubes, balance, flask washer, scissors, plastic or bamboo trays
- Broad container (e.g., tray) to receive plantlets with agar that have been extracted from their flasks
- Bucket, spade, wheelbarrow, and garden spades; hose and irrigator.
- Black plastic bags (7 × 14 cm) with perforations for drainage, and transparent plastic bags (1 × 1 m)
- Field book, registration forms, indelible marker, pencil; plastic mini-stakes for identification

To prevent possible contamination of plantlets, all implements to be used should be disinfected. For example, if roots or leaves are cut with scissors, these should be disinfected in a soapy solution every time a cut is made.

e. Requesting from the biotechnology laboratory the appropriate number of plants that can be hardened

There needs to be good coordination between the number of plantlets that are requested from the *in vitro* multiplication facility and the space available. The ideal

situation is to minimize as much as possible the period of time during which the vitroplants are kept before transplanting them to the soil. Experiences obtained by CLAYUCA and CIAT indicate that it is feasible to acclimatize approximately 302 cassava plantlets per square meter of useful area of greenhouse or screenhouse.

Stage 2: Operational or technical activities

The success of an HP depends on the comprehensive management of a series of operations, starting from the reception of vitroplants up to their transplanting to the field.

a. Receiving in vitro plants

The vitroplants coming from the biotechnology laboratory are usually delivered in boxes containing flasks. Upon arrival, the plantlets are quickly removed from the boxes and placed, at intervals, in a cool place with artificial lighting or indirect sunlight. They are then counted and their numbers recorded according to variety (**Photos 3** and **4**).

In this step, a **pre-selection** is also carried out, consisting of separating the flasks according to the *in vitro* plants' height and vigor and eliminating those observed as contaminated, broken, abused, or malformed.

b. Pre-adapting the plantlets

If the *in vitro* plants have spent several days being transported in closed boxes, the flasks are placed as indicated previously, but left until the plantlets recover. Or, the *in vitro* plants can be left for 1 or 2 days in the installations where they will be submitted to HP. This period also offers the opportunity of making a second pre-selection for vigorous *in vitro* plants.

c. Preparing the soil substrate

To prepare the substrate in which the plantlets will be grown, one part of black soil (i.e., from the non-clayey arable layer) that has been milled and sieved is mixed with three parts of coarse sand that has been washed and sieved (**Photo 5**). The substrate should be "sterilized" with steam where the presence of nematodes and fungi is suspected (**Photo 6**). If no sterilization equipment is available, then:

- The sand can be placed in a metal pipe or drum, with sufficient water added, and the whole heated to 100ºC.
- To sterilize the soil, a thin layer is spread over black plastic, covered with another, but transparent, plastic, and a hermetic seal made between them both. The soil is left for one week under full exposure to the sun.

d. Preparing for transplanting

Before transplanting, the installations should be disinfected, the small bags for the *in vitro* plants filled with substrate, the mixture of fertilizer and fungicide prepared, and the trays and large bags made available for further use in miniature humidity chambers.

Likewise, personnel should be re-trained in transplanting. This exercise will verify the personnel's productivity: usually, a skilled technician transplants about 600 plants on a working day and a beginner about 200 plants.

Disinfecting and cleaning the site. All the installations should be disinfected with sodium hypochlorite and rigorously disinfested. The equipment and implements should be organized in their places. Cleaning should be extended to the site where transplanting will be done and to the screenhouse or greenhouse where the plantlets will be hardened.

Preparing the bags. Either black or transparent plastic bags (7 × 14 cm) are filled with the previously prepared mixture of sand and soil (see above) to three quarters their volume. The mixture is firmly compressed into the bag to obtain a compact substrate. Such compaction will later stimulate root production, making the roots longer and thicker.

Preparing the trays. The bags containing the already compacted substrate are placed on the trays and the following solution is prepared:

- In 1 liter of deionized water (or rainwater) mixed with 1 g of a fungicide that controls soil fungi (e.g., Banrot) and 2 g of a fertilizer rich in phosphorus (e.g. formula 10-52-10).
- Immediately irrigate each bag with 10 cc of this mixture (first irrigation).

Preparing humidity chambers. The base of each tray is introduced into a bag of transparent plastic (1 × 1 m when folded) that has been rolled down, concertina style, to its base (**Photo 7**). Later, the bag can be quickly unfolded upwards, and its mouth tied firmly (**Photo 8**). The setup will then function as a "humidity chamber".

Stage 3: Transplanting

Transplanting is a very "traumatic" stage for the plantlets, especially when it is carried out by unqualified or inexperienced labor. Plantlets undergo microclimatic stress when moved from their flasks to the miniature humidity chambers, suffering dehydration and nutrient stress, as they change from a substrate rich in nutrients to one that is very poor (soil/sand mixture); and almost unavoidable mechanical damage to several parts of the plantlet (e.g., root cap, absorbent hairs, roots, stem, and leaves). The success of the plantlets' acclimatization and survival depends on the care with which transplanting is done.

Transplanting must be done immediately after the *in vitro* plants are extracted from their flasks. Transplanting activities include:

a. Selection

A first selection is carried out, choosing those flasks with the most vigorous plantlets, that is, colored an intense green, standing erect, and between 5 and 7 cm tall.

b. Extracting the in vitro plants

This operation consists of:

- Removing the plastic tape and flask covers. Adding deionized water or rainwater to the flask to moisten the agar substrate and facilitate extraction of the whole (plantlet + agar)
- Holding the flask in one hand while gently smacking the flask with the other to loosen the agar from the flask's walls. If it does not separate, a spatula is used, taking care not to damage the roots
- Carefully extracting the plantlet by inclining the flask; tweezers are not used because of the risk of damage to the stem
- Placing the plantlet in a broad container such as a deep tray containing de-ionized water or rainwater, which is gently moved by hand to dislodge the agar
- Gently removing the particles of agar that still adhere to the roots with the flask washer
- Carrying out a second selection of vigorous plantlets to eliminate small, poorly formed, or weak plantlets

c. Transplanting into bags

With one hand, a plantlet is taken to a bag, introducing the roots and lower part of the stem. This hand must be held rigid to prevent breaking the absorbent hairs and roots. With the other hand, the fourth part of the substrate is added, ensuring that the roots remain in their "normal position", as they were in the flask, thus preventing either physical or physiological damage caused by change of position.

Once transplanting has been achieved for all the bags in the tray, the plantlets receive a second irrigation with 10 cc of the mixture of fertilizer and fungicide used previously.

d. Humidity chambers and hardening

The following steps start the real process of hardening the plantlets:

- The tray is marked with a label on which appear the variety's name, number of bags, date and hour of transplanting, and the transplanter's name.
- The large transparent bag (1 × 1 m) in whose bottom the tray had been placed is unfolded, and its mouth closed by tying with a string. Each large bag then becomes a humidity chamber.
- The humidity chambers are then transferred to the site in the installation where HP will be carried out. To prevent the upper part of each chamber from lying on top of the plantlets and damaging them, the cord is tied to a wire strung over the chambers.

Stage 4: Maintaining the transplanted plantlets

In this stage, considerable attention must be given to the microclimatic changes occurring within the installations, the irrigation the plantlets require, their nutrition, and the presence of pests and diseases.

The bags containing the plantlets should not be moved during the first month after transplanting so as not to cause root damage, especially to the cap and absorbent hairs. These parts are particularly fragile in this early stage of development. Damage or breakage in radical tissues increases the probability of pathogen invasion and of slowed growth and development. Such care also assumes considerable importance in Stages 5 and 6 of the HP.

a. Microclimate and humidity chambers

Between days 8 and 12 after transplanting (DAT), the string closing the humidity chamber is removed—if possible in the afternoon —and the large transparent bag is opened up completely (**Photo 9**).

- The goal of this operation is to enable the plantlets to adapt to the microenvironment of the installations.
- If a tendency to wilting is observed, the bag should be re-closed and the humidity chamber treatment continued.
- If the plantlets have adapted well to the microenvironment by the second or third day after opening the large bag , the bag is rolled down to the tray's base or removed altogether, leaving the tray with its plantlets. During this step, the plantlets must be protected from strong dehydrating winds (**Photo 10**).

b. Irrigation

If the plantlets have been irrigated with the correct quantity of nutrient solution and the environment within the miniature humidity chamber properly formed, the plantlets will not need further irrigation.

However, if, and only if, the first symptoms of physiological wilting are presented in plantlets after being removed from the humidity chamber, the substrate receives the third irrigation. To reduce risk of attack from pathogens, care is taken not to wet the leaves. Each plantlet is irrigated with 10 cc of a nutrient solution consisting of a mixture of 2 g of fertilizer rich in phosphorus to promote root formation (e.g., formula 10-52-10) and 1 g of Agrimins (a fertilizer rich in micronutrients) per liter of deionized water (or rainwater).

According to the microclimatic conditions of the installations and the state of appearance of the plantlets, one or two daily irrigations can be programmed, each with 10 cc of water normally used to irrigate other plants. Between 21 and 25 DAT, a microspray irrigation (MSI) system should be implemented in the screenhouses, thus significantly reducing labor costs. At CIAT, the plantlets are given from 2 to 3 minutes of MSI in the morning and, if necessary, another 2 or 3 minutes in the afternoon (**Photo 11**).

- When MSI is applied, rigorous inspections must be made to detect any phytopathological problem in the plantlets.
- The "secret" of this operation, which is crucial to the success of HP, is in applying irrigation when the first symptoms of physiological wilting are observed. Thus, the substrate will not remain too moist and thus promoting possible pathogen attack in the roots. It is important to remember that, at this stage, cassava plantlets are highly susceptible to excess moisture in the substrate.

c. Fertilizer applications

The substrate used (1 part of soil and 3 parts of sand) is of low fertility, and a program for fertilizer applications is thus indispensable. Every eight days, the plantlets will receive applications of macro- and micro-nutrients so they may develop normally.

A compound rich in phosphorus is first applied to favor root development (e.g., formula 10-52-10). This application is alternated (at 8-day intervals) with a complete fertilizer containing macro- and micro-nutrients. If formula 10-52-10 is not available on the market, it can be replaced by combining formula 10-30-10 and Agrimins. Fertilizer

application is suspended when the plantlets' color is normal for the varieties to which they belong (**Photo 12**).

If symptoms of a nutrient deficiency are observed, the affected plantlets can be given a foliar fertilizer application containing simple or complete fertilizers. One deficiency that tends to appear in plantlets during the first month is that of zinc, which can be corrected by adding the element to the soil in one of the irrigations, at a rate of 3 g of Zn SO_4 dissolved in 1 liter of water and the solution applied at 10 cc per plant.

Stage 5: Separating the plantlets

Between 30 and 34 DAT, the plantlets need more light and higher temperatures for growth and development. Accordingly, the plants are placed more widely apart in an area that is double or triple the one occupied initially (**Photo 13**).

Stage 6: Transplanting to the field

The plants remain in the mesh house or greenhouse for 70 to 90 days before being taken to the field.

Transfer

On transporting the bags from the greenhouse (or mesh house) to the field, the plantlets must be protected from strong air currents that could cause abrasion or dehydration (**Photo 14**).

b. Adaptation and final transplanting

The plants should be brought together in a large group within the site chosen for planting and left for 3 to 6 days so that they may adapt to the new environment. The plants are then transplanted to their final sites in the field (**Photo 15**). For the next few days, the farmer should watch out for the appearance of any nutritional deficiency or presence of pests or diseases, and apply the corresponding integrated management. In cases when the transplanting is done in the dry season, the plants need to be irrigated. Some weeks after the transplanting operation, the plants are adapted to their new conditions and will continue to grow until they reach maturity (**Photo 16**).

CONCLUSIONS

1. Hardening of the plants that have been produced through *in vitro* methods is a very delicate phase during which plants need to receive special care and maintenance.
2. Handling large numbers of plants that are coming from a biotechnology laboratory is a process that demands time, specialized labor and resources. This process ends when the plants are already established. Up to this moment, the unit costs of producing each plant could be considered relatively high.
3. The main advantage of the hardening method is that it gives farmers the opportunity to obtain a first harvest of a given cassava variety which the farmer wants to plant in large areas. With the vegetative planting material obtained in this first harvest, the farmer can start to establish his commercial fields.
4. Another advantage of the availability of a safe method for hardening cassava vitroplants is that it allows research and technology transfer institutions to multiply

varieties that have desired traits, in massive amounts, to facilitate the dissemination of improved varieties.

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Photo 1. Work area.

Photo 2. A screenhouse useful for hardening vitroplants.

Photo 3. A box containing 50 flasks and 200 cassava plantlets

Photo 4. Plantlets just removed from box and kept in a cool place with indirect sunlight.

Photo 5. Preparation of the substrate for transplanting the plantlets.

Photo 6. Steam sterilization of the substrate to avoid the presence of nematodes.

Photo 7. The tray placed inside the plastic bag.

Photo 8. The tray and the plastic bag acting as "humidity chambers".

Photo 9. Opening the humidity chamber.

Photo 10. Transplanted plants after the plastic bag is completely removed.

Photo 11. Microspray irrigation helps to maintain good quality plants during hardening phase.

Photo 12. A fertilization programe is essential to obtain healthy plants.

Photo 13. Plants in final stages of hardening process demand wider spaces, more light and higher temperatures.

Photo 14. Hardened plants ready for transfer to the field.

Photo 15. Transplanting hardened plants.

Photo 16. An established field with hardened plants.