CHAPTER 19

Methodology for Hardening Large Numbers of *In Vitro* Cassava Plants

Roberto J. Segovia[1], *Armando Bedoya*[2], *William Triviño*[3], *Hernán Ceballos*[4], *Martin Fregene*[5], *Guillermo Gálvez*[6], *and Bernardo Ospina*[7]

Introduction

Tissue culture, a technique used to micropropagate plants, has been successfully applied in cassava (*Manihot esculenta* Crantz) for the massive production of disease-free *in vitro* plants, increasing productivity and, in certain cases, improving longevity. *In vitro* micropropagation is successfully used to produce cassava plantlets free of pathogens associated to diseases such as frogskin, cassava mosaic, and bacterial blight. Traditional micropropagation has low multiplication rates, which can be improved by using more efficient multiplication systems such as the automated temporary immersion device known as RITA® or other automated temporary immersion systems (ATIS)[8].

After 6 to 11 months in sterilized rooms under artificial conditions of light, temperature, moisture, and nutrients, the plantlets produced by such systems are like test-tube babies—weak and unadapted. As a result, they need to undergo a stage of acclimatization or hardening before they can be transferred to their final site in the field. In cassava, this process is very delicate, constituting a bottleneck in the massive production of cassava planting materials by tissue culture techniques.

1. Agronomist, formerly of Greenhouse Management, CIAT, Cali, Colombia. E-mail: rjsssegovia@gmail.com
2. Field technician, formerly of CLAYUCA, Cali, Colombia.
3. Field technician, CLAYUCA.
4. Breeder, Cassava Program, CIAT. E-mail: h.ceballos@cgiar.org
5. Plant geneticist and molecular breeder. Director, Bio Cassava Plus Program. Danforth Center, St. Louis, USA. E-mail: MFregene@danforthcenter.org
6. Virologist, formerly of CIAT-Biotecol, Cali, Colombia.
7. Executive Director, CLAYUCA. E-mail: b.ospina@cgiar.org
8. For an explanation of this and other abbreviations and acronyms, see *Appendix 1: Acronyms, Abbreviations, and Technical Terminology*, this volume.

Hardening massive numbers of *in vitro* cassava plants inevitably incurs in **loss** of plantlets, mainly when these are moved from the artificial to the natural environment (soil) and must adapt to new microclimatic conditions. Where the transfer is not carried out using the appropriate technology, the percentage of loss is very high (between 50%–95%), which affects the crop's agronomic progress while increasing the costs of implementing this alternative technology. It also discourages progressive farmers who would otherwise rapidly and safely produce disease-free planting materials or massively produce a new promising variety over a short period of time.

Other drawbacks of the acclimatization process are the cost and size of the facilities needed, such as greenhouses and screenhouses. These two factors reduce the feasibility of applying this new technology—and other similar ones—which could have a significant impact on agricultural production.

Researchers from the Latin American and Caribbean Consortium to Support Cassava Research and Development (CLAYUCA, its Spanish acronym), in association with others of Biotechnology of Colombia Ltd. (Biotecol, its Spanish acronym) and CIAT, have developed a methodology that enables the massive production of cassava planting materials, also known as plantlets. In 2001, a large number of plants were produced using ATIS. Through efforts described below, a sustainable and economic technology for functional hardening was achieved, significantly minimizing the percentage of plantlet loss during the hardening process (HP).

Stages of the Hardening Process

The six stages required for an efficient and successful HP are as follows:

Stage 1: Pre-operational activities

A successful HP demands prior planning, which includes preparing a detailed timetable of all activities integrating the process: definition of who will carry it out; selection and adaptation of facilities; laboratory tests; purchase of equipment, materials, and inputs; and confirmation from the biotechnology laboratory of the number of *in vitro* plants that can be "hardened" per week. A good rule of thumb is that approximately 300 cassava plantlets can be hardened per square meter of useful area of greenhouse or screenhouse.

Human resources. Labor should be qualified; if not, personnel should at least receive training in basic aspects of the HP methodology. The number of workers required will depend on their experience and the number of plants entering the process. A novice worker can handle about 200 plants per day while an expert can handle up to 600 (see below, "Preparing for Transplanting").

Facilities. Facilities usually consist of a work area and either a screenhouse or a greenhouse, sometimes both. Select the best of what is available, then make any necessary adaptations.

The ***work area*** comprises a depot for soil and sand, a small storage shed to keep materials and inputs, a "soil patio" for mixing, and a cool site for transplanting; the latter should be protected from direct sunlight and strong winds and should also have a washing area and table.

The ***screenhouse*** should be roofed and adapted for automatic climate control. Microsprays should be suspended either over the tables or from the roof as well as installed along the floor to control temperature and relative humidity, especially during the first days of the HP. Although plantlet development is favored by good light, this should not come directly from the morning, noon, or afternoon sun during the first 8 days of acclimatization.

A protective screen can be installed, using one of the following options: (1) sheets of polystyrene foam covered with aluminum foil; (2) venetian blinds externally covered with aluminum foil; and (3) polypropylene meshing externally covered with several sheets of aluminum foil (each 30 cm wide) and separated at 5-cm intervals. So far, CIAT has found the third option to be the best.

The protective screen should be highly functional, installed on the sides of the ***screenhouse*** facing East (sunrise) and West (sunset), at least 1 or 2 m above the tops of the bags containing the plantlets. The screen should be withdrawn gradually as the sun traces its path through the sky to let light enter the facility. The aluminum foil reflects the sun's rays and prevents the heating of the area where the plantlets are being hardened.

The maximum temperature within a screenhouse fluctuates between 33 and 38 °C, and the minimum between 18 and 22 °C. For details on the design and construction of a CIAT screenhouse type II, consult Roca and Mroginski (1991).

The ***greenhouse*** should have an automatic microspray irrigation system installed, which is controlled by a solenoid valve and control clock. This type of system reduces the cost of labor needed to irrigate the plantlets by 90%.

Both the screenhouse and greenhouse should have a space set aside to acclimatize transplanted plantlets, which can be increased by as much as three times as the plants grow for 2 or 3 months after transplanting. The degree of increase will depend on the cassava variety, its growth rate, and plant development.

For example, 10,000 plants are needed to plant 1 hectare of cassava. CLAYUCA's HP methodology initially places 10,000 plantlets in an area of 25 to 35 m^2 in the screenhouse or greenhouse, depending on the size and type of bags used for transplanting. Two months after transplanting, these 10,000 plants will need an area of 50 to 70 m^2.

Laboratory tests. To correct any potential problem, all soil, sand, and water to be used should be first submitted to chemical and biological analyses.

Equipment, materials, and inputs. The following elements are needed to acclimatize the cassava plantlets:

- Soil mill, sieve and mixer; sterilizer; fumigator; protective equipment for fumigation and pesticides
- Test tubes, balance, flask washer, scissors, plastic or bamboo trays
- Wide container (e.g., tray) with agar to place plantlets removed from their flasks
- Bucket, spade, wheelbarrow, and garden

spades as well as a hose and irrigator
- Black plastic bags (7 x 14 cm) with perforations for drainage as well as transparent plastic bags (1 x 1 m)
- Field book, registration forms, indelible marker, pencil, and plastic mini-stakes for identification of plantlets

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

Stage 2: Operational or technical activities

The success of the HP depends on the comprehensive management of a series of operations that range from receiving the *in vitro* plants to their transplanting in the field.

Receiving* in vitro *plants. Boxes containing flasks with *in vitro* plants are received from the biotechnology laboratory. The flasks with plantlets are quickly removed from the boxes, placed at intervals in a cool place with artificial lighting or indirect sunlight, then counted and numbers recorded according to variety.

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

Pre-adapting the plantlets. If the transportation of the *in vitro* plants in closed boxes has taken several days, the flasks are placed as indicated in the previous step but left until the plantlets recover. Other option is to leave the *in vitro* plants for 1 or 2 days at the facilities where they will undergo the HP. This time can be used to make a second pre-selection of vigorous *in vitro* plants.

Preparing the substrate. To prepare the substrate in which plantlets will be grown, one part of previously pulverized and sieved black soil (i.e., from the non-clay arable layer) is mixed with three parts of washed and sieved coarse sand. The substrate should be steam-sterilized if the presence of nematodes and fungi is suspected. If no sterilization equipment is available, then:

- Place the sand in a metallic pipe or drum, add sufficient water and heat to 100 °C.
- Spread a thin layer of soil over black plastic, cover with a piece of transparent plastic, forming a hermetic seal between both plastics and leave for 1 week under direct sunlight.

Preparing for transplanting. Before transplanting, make sure the facilities are fully disinfected. Fill the small black plastic bags for the *in vitro* plants with substrate, prepare the mixture of fertilizer and fungicide, and arrange trays and large bags for use in miniature humidity chambers.

Likewise, retrain personnel in transplanting procedures. This exercise will determine the personnel's productive capacity. Skilled technicians can transplant about 600 plants per working day, while beginners can only handle about 200 plants.

Disinfecting and cleaning the site. Rigorously disinfect the entire facility with sodium hypochlorite and organize equipment and implements to facilitate their use. Cleaning should also extend to the transplanting site and the screenhouse or greenhouse where the plantlets will be hardened.

Preparing the bags. Fill either black or transparent plastic bags (7 x 14 cm) with the previously prepared mixture of sand and soil (see above) to three quarters their volume. Firmly press the mixture into the bag to obtain a compact substrate. Such compaction will later stimulate root growth, making them longer and thicker.

Preparing the trays. Place the bags containing the already compacted substrate on the trays and prepare the following solution: mix 1 g of a soil fungicide (e.g., Banrot) and 2 g of a phosphorus-rich fertilizer (e.g., formula 10-52-10) in 1 liter of deionized water (or rainwater). Immediately irrigate each bag with 10 cc of this mixture (first irrigation).

Preparing miniature humidity chambers. Introduce the base of each tray into a transparent plastic bag (1 x 1 m when folded) that has been rolled down, concertina style, to its base in such a way that the bag can later be quickly unfolded upwards and its opening firmly tied shut. This will function as a "humidity chamber".

Stage 3: Transplanting

Transplanting is traumatic for the plantlets, especially when carried out by unqualified or inexperienced personnel. Plantlets undergo microclimatic stress when

moved from their flasks to the miniature humidity chambers, suffering dehydration; nutrient stress, as they change from a nutrient-rich substrate to one very poor in nutrients (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 mainly depends on the care with which transplanting is done.

Transplanting must be performed immediately after the *in vitro* plants are extracted from their flasks. When this process is carried out for the first time and the environmental conditions of the facilities are not well known, then transplanting should be carried out on a daily basis at 17:00 to prevent the plantlets from **dehydrating**.

Transplanting activities include:

Selection. A **first selection** is carried out, choosing those flasks with the most vigorous plantlets (intense green color, erect, and between 5 and 7 cm tall).

Extracting the* in vitro *plants. This operation consists of the following steps:

- Remove the plastic tape and flask covers.
- Add deionized water or rainwater to the flask to moisten the agar substrate and facilitate extraction of both plantlet and agar.
- Hold 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, use a spatula, taking care not to damage the roots.
- Carefully remove the plantlet by inclining the flask; do not use tweezers because the stem may suffer damage.
- Place the plantlet in a wide container, such as a deep tray containing deionized water or rainwater. Use your hand to gently move the water to dislodge the agar.
- Gently remove particles of agar still adhered to the roots with the flask washer.
- Conduct a **second selection** of vigorous plantlets to eliminate small, poorly formed, or weak plantlets.

Transplanting into bags. With one hand, place the plantlet in 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, add a fourth of the substrate, ensuring that the roots remain in their "normal position" that is, as they were in the flask, thus preventing physical or physiological damage that could be caused by a 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 previously used fertilizer and fungicide mixture.

Humidity chambers and hardening. Now the real process of hardening the plantlets begins:

- Label the tray indicating the name of the variety, the number of bags, the date and hour of transplanting, and the transplanter's name.
- Place each tray at the bottom of the large transparent bag (1 x 1 m) and tie the opening shut with a piece of rope, converting it into a miniature humidity chamber.
- Transfer the humidity chambers to the facility where the HP will be carried out. Tie the string to a wire strung over the chambers to prevent the upper part of each chamber from folding over on top of the plantlets and damaging them.

Stage 4: Maintaining the transplanted plantlets

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

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

Microclimate and humidity chambers. Between 8 and 12 days after transplanting (DAT), remove the string closing the humidity chamber—preferably in the afternoon—and completely open the large transparent bag to allow plantlets to adapt to the microenvironment of the facility.

If a tendency to wilting is observed, then reclose the bag and continue the humidity chamber treatment.

If plantlets have adapted well to the

microenvironment by the second or third day after opening the large bag (i.e., 10 to 15 DAT), the bag is rolled down to the tray's base or removed altogether, leaving the tray exposed with its plantlets.

During this step, plantlets must be protected from strong dehydrating winds.

Irrigation. If the plantlets have been irrigated with the correct amount of nutrient solution (see above) and the environment within the miniature humidity chamber is appropriate, plantlets will not need irrigation.

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

Depending on the microclimatic conditions of the facility and the turgor of the plantlets, schedule one or two irrigations per day, each with 10 cc of water normally used to irrigate other plants.

Between 21 and 25 DAT, install a microspray irrigation (MSI) system in the screenhouse, which significantly reduces labor costs. At CIAT, plantlets receive from 2 to 3 minutes of MSI in the morning and, if necessary, another 2 or 3 minutes in the afternoon.

- The use of MSI requires that plantlets be rigorously inspected to detect any pathological problems.

- The "secret" of this operation, which is crucial to the success of the HP, is to apply irrigation when the first symptoms of physiological wilting are observed. This ensures an adequate moisture level of the substratum, thus preventing possible pathogen attack in the root area. It is important to remember that, at this stage, cassava plantlets are highly susceptible to excess moisture in the substrate.

Fertilizer applications. The substrate used (1 part of soil to 3 parts of sand) is of low fertility, and the application of fertilizers is therefore indispensable. Every 8 days the plantlets will receive applications of macro- and micronutrients to ensure their normal development.

A phosphorus-rich compound (e.g., formula 10-52-10) is first applied to enhance root development. This application is alternated at 8-day intervals with a complete fertilizer containing macro and minor elements. If the formula 10-52-10 is not available on the market, it can be replaced by a combined formula including 10-30-10 and Agrimins. Fertilizer application is suspended once the color of the plantlets is normal for the varieties to which they belong.

If symptoms of deficiency of any element appear, affected plantlets can be given an application of foliar fertilizer containing simple or complete fertilizers. Zinc deficiency tends to appear in plantlets during the first month and can be corrected by adding Zn to the soil in one of the irrigations at a rate of 3 g dissolved in 1 liter irrigation water and applied at 10 cc per plant.

Stage 5: Separating the plantlets

Between 30 and 34 DAT, the plantlets have now become plants and therefore need more light as well as higher temperatures to grow and develop. Plants are spaced at a greater distance, in an area double or triple that initially occupied.

Stage 6: Transplanting plants to the field

The plants remain in the screenhouse or greenhouse for 60 to 90 days before being taken to the field. In case of restricted space or labor, plants can be taken to the field 30-40 days after transplanting.

Transfer. When transporting the bags from the greenhouse (or screenhouse) to the field, protect plants from strong air currents that could cause abrasion or dehydration.

Adaptation and final transplanting. The plants should be grouped together and placed in the site chosen for planting and left for 3 to 6 days so that they can adapt to the new environment. Plants are then transplanted to their final field sites. For the next few days, the farmer should closely monitor the site for the appearance of any nutritional deficiency or presence of pests or diseases to apply the corresponding integrated management practice as required.

Reference

Roca WM; Mroginski LA, eds. 1991. Cultivo de tejidos en la agricultura: Fundamentos y aplicaciones. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia. 970 p.