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Cassava in vitro processing and genebanking

This manual describes the cassava in vitro genebanking process set up by IITA. Within the last 3 years, this standardized process has been successfully used to duplicate over 2000 accessions of cassava from field to in vitro culture.

The manual also describes minimum requirements in terms of equipment and consumables for cassava in vitro propagation and genebanking.

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Intended use:
- Best practice reference manual for genebank staff.
- Provide the frame for further development of an ISO normalization (quality control of in vitro germplasm) for cassava genebanking.
- Support capacity building in in vitro culture and genebanking. It is accessible on IITA website and available in hard copy (upon request).

Intended users: Genebank managers, supervisors, and staff and trainees.

1. Explant production

Prior to starting in vitro genebanking, it is necessary to produce adequate plant parts that will be introduced in vitro, i.e., explants. In the case of cassava, preferred explants are young shoots. They are either obtained from cuttings grown in screenhouse (Fig. 1) or from thermo-treated cuttings (Fig. 2). In the latter case, both ends of cutting (20–30 cm long) are covered with wax prior to transfer into a hot environment (28–38 ^{o} for respectively 6 hours dark, and 18 hours light. Thermo-treatment is highly advisable when germplasm show obvious signs of virus contaminations[1]). Whether transfer in screen house or in growth chamber, cuttings produce new shoots within 3–5 weeks (Figs 3 and 4).

[1] The benefit of thermo-treatment is still under investigation.

2. Germplasm in vitro introduction

2.1 Cutting in vitro introduction.

Each technician is advised to treat 10 accessions maximum at a time, 10 cuttings per accession.

1 For each accession, select 10 cuttings (either nodal cutting = 1 bud + 1 cm stem maximum on each side or apical cutting or 1 apical shoot on 1 cm stem). Cut them with scissors or scalpel and keep them in a clean container of distilled water. Record accession (number/name) on each container.

Explants are then sterilized as follows:

2 Back in the laboratory; soak the shoots in 70% alcohol for 5 minutes.

3 Replace alcohol with 3–5% sodium hypochlorite (NaClO) [2] solution containing a few drops of a wetting agent e.g., Tween 20; and soak for 10–15 minutes.

Use only freshly made hypoclorite solution (Fig. 5). When working with new explant type, it is better to run preliminary cleaning tests to determine the longest exposure time sustained by explants. [2]Commercial bleach titration is generally between 10 and 30% NaClO.

Steps 4 to 9 have to be performed under laminar flow (sterile conditions).

4 Using sterilized forceps, transfer cuttings into a sterile container and rinse them 3 times with sterile distilled water.

5 With the help of a scalpel (with sterile blade), cut the whitening edge of the stems (hypochloride effect) (Fig. 6). In order to avoid explant miss-orientation in vitro, allow the upper part of the stem to be shorter than the lower one (Fig. 7).

6 Transfer into a sterile container and soak in 1% fungicide solution (benlate or mencozeb) for 5 minutes (this is optional; only apply when explants show fungus contaminations).

7 Remove from the solution and dry on filter paper (Fig. 7).

8 With sterile forceps, plant the longer part of each explant into agar (one shoot per tube) (Figs 8 and 9).

9 Cover each tube with a plastic cap and seal with parafilm.

10 Label each tube with an accession number, the date of introduction and line number[2] (record by writing on parafilm using long lasting marker) (Fig. 10).
11 Transfer tubes to a growth chamber (temperature 28–30 ^{o}C, photoperiod 12/12).

[2]A line number is created as follows: Shoot number + a date of first in vitro introduction; Example: (1) 1/12/2006 = First shoot introduced on the 1 December 2006.

Obvious signs of growth are visible within 2 weeks following in vitro introduction (Fig. 11). Once seedlings have developed roots and show several nodes (4–6 nodes) they are sent to multiplication (see below).

2.2 Meristem in vitro introduction
(First 3 steps below are as described above in 1–3).

12 For each accession, select 10 cuttings. Cut them with scissors or scalpel and keep in a clean container containing distilled water. Record accession (number/name) on each container.

Explants are then sterilized as follows:

13 Back in the laboratory; soak the shoots in 70% alcohol for 5 minutes.

14 Replace alcohol with 3–5% sodium hypochlorite (NaClO)[2] containing a few drops of a wetting agent e.g., Tween 20; and soak for 10–15 minutes. Only use freshly made hypoclorite solution (Fig. 5). When working with new explant type, it is advised to run preliminary cleaning tests to determine the longest exposure time sustained by explants.

[1]Commercial bleach titration is generally between 10 and 30% NaClO.

Steps 15 to 18 have to be performed under laminar flow (sterile conditions).
15 Using sterilized forceps, transfer shoots into a sterile container and rinse them 3 times with sterile distilled water.

16 Place one shoot under stereomicroscope[3] and locate meristem. Cut each leaf primordia one after another till the meristematic dome becomes visible (Fig. 12, step 1, 2, and 3). To do so, either use sterile scalpel (blade number 11) or needle.

17 Cut the base of the dome and transfer to meristem media (Fig. 12, Step 4).

18 Cover each tube with a plastic cap and seal with parafilm.

19 Label each tube with an Accession number, Date of introduction and Line number (record by writing on parafilm using long lasting marker).

20 Transfer tubes to growth chamber (temperature 28–30 ^{o}C, photoperiod 12/12).

Obvious signs of growth are visible within 2–4 weeks (greening and elongation and/or callus formation). Calluses are occasionally observed at the base of the shoot (Fig. 13). 2 to 4 cm long shoots with buds are then sent to multiplication (Fig. 14).

[3] The stereomicroscope must be sprayed and clean with 70% alcohol prior placing it on the laminar flow bench.

3. Germplasm in vitro multiplication (propagation)

Steps 21 to 26 of the following procedure are performed under laminar flow (sterile conditions) and all instrument used must be sterilized.

21 Open test tube

22 With the help of forceps, pull the seedling out of the tube.

23 Transfer the seedling on sterile filter paper set on sterile aluminum paper foil.

24 Holding seedling with forceps cut the stem in micro-cutting with the help of a scalpel (blade number 10). Each micro-cutting must carry at least one bud. To avoid miss-orientation, allow the upper part of the stem to be shorter than the lower one (Fig. 15).

25 Open a test tube containing fresh multiplication medium and plant the longer part of the stem into it.

26 Close each tube with a plastic cap and seal with parafilm.

27 Label each tube with corresponding line number and date of subculture.

28 Transfer tubes to growth chamber (temperature 28–30^{o}C, photoperiod 12/12).

4. Germplasm genebanking

4.1 Transfer to genebank. Four to six weeks following multiplication, fully developed seedlings (i.e., seedling showing some roots and at least 2 nodes) are transferred to the genebank (Fig. 16). Genebank culture conditions are as follow: 18–20 ^{o}C and a 12/12 photoperiod.

Germplasm is observed weekly in order to eliminate and record any contaminated (Fig. 17) or necrosed (Fig. 18) seedlings.

4.2 Germplasm regeneration. Depending on accessions, cassava germplasm maintained in the genebank requires subculturing every 6 to 18 months. Ideally, 10 seedlings of each accession are maintained in the genebank. All in vitro stored accessions are screened every 6 weeks. Accessions showing obvious sign of deterioration (drying leaves, dry medium, etc.) and/or which stock is low (less than 4 seedlings) are sent for multiplication (see section 3).

4.3 Germplasm monitoring

Germplasm in introduction phase. Newly introduced meristem or nodal cuttings are processed in batches. For each batch, a record table with the fields listed below is created. At that stage, data are recorded manually. Only data related to successful introductions are then computerized:

- Batch number
- Accession number
- Date of in vitro introduction
- Number of explants introduced
- Contamination
- Necrosis
- Operator
- Send to multiplication 1
- Contamination while in multi 1
- Necrosis while in multi 1 etc.
- Number of seedlings sent to the bank

Germplasm in genebank. Once an accession is newly introduced in the bank, its number is added to the database. For each accession the following data is recorded:

- Accession number
- Date of introduction in vitro (in case of replacement all previous entries are discarded)
- Type of explant (meristem/nodal cutting)
- Virus-free lines certified (yes/no)
- In the bank at the time of last inventory (Insert date of last inventory)
- In subculture at the time of last inventory (insert date of last inventory)
- Present number of seedlings in subculture
- Seedlings potential (= in the bank + in subculture)
- Contamination in bank (number of tubes eliminated because of contamination)
- Necrosis in bank (number of tube eliminated because of necrosis)
- Out 1 (number of tubes sent to subculture 1)
- Date out 1 (date of subculture 1)
- Obtained 1 (number of micro-cuttings obtained after subculture 1)
- Out 2 (number of tubes sent to subculture 2)
- Date out 2 (date of subculture 2)
- Obtained 2 (number of micro-cuttings obtained after subculture 2)
- Present number of seedlings in subculture
- Subcontamination (number of tubes eliminated from subculture due to contamination)
- Subnecrosis (number of tubes eliminated from subculture due to necrosis)
- Back 1 to bank (number of tubes sent back to the bank from subculture 1)
- Date back 1 (date when subculture 1 are sent back to the bank)
- Back 2 to bank (number of tubes sent back to the bank from subculture 2)
- Date back 2 (date when subculture 2 are sent back to the bank)
- Old cuttings discard (number of cuttings in the bank discarded during replacement)
- Extra subculture discard (tubes from subculture discarded)
- Nodal cutting from the bank sent to acclimatization
- Nodal cutting from the bank sent for other experimentation
- Nodal cutting from subculture sent for safe duplication in Cotonou
- Nodal cutting from the bank sent for multiplication for distribution

Computerization of all data is advisable to facilitate germplasm management. The use of pocket PC speeds up data collection and reduces record mistakes. Ultimately, bar coding in vitro collections will further improve genebank management in terms of cost and data reliability. IITA is presently setting up such system.

Germplasm inventory. An inventory of all germplasm is performed once a year.

5. Germplasm acclimatization (postflask management)

Only seedlings showing a well-developed root and shoot systems must be considered for acclimatization. *I*n vitro seedlings need special attention prior to be sent back to field conditions and should be treated as follow:

- For 100 seedlings, soak 67 peat pellets (Jiffy pods) (Fig. 19) in water for 2–3 hours.
- Mix content of pet pellets with 650 g of vermiculite (Fig. 20).
- Fill up ¾ of small plastic bags (inner bag) with the mixture (Fig. 21).
- Gently remove in vitro seedling from its test tube and rinse its root system with water (to eliminate agar) (Figs 22 and 23).
- Plant one seedling per inner bag and sprinkle with water (Fig. 24).
- Transfer each inner bag in a big plastic pot (18 cm diameter) and enclose in a bigger plastic bag (outer plastic bag, 22 x 90 cm) (Fig. 25).
- Fold the upper part of the outer bag and hang it in a warm and lighted place (Fig. 26). Ensure seedlings environment is kept humid by regularly adding water.
- Three to 4 weeks later (once seedling start elongating), each seedling is removed from its inner bag and transferred into its bigger pot after filling it with sterile soil. Each pot is then re-enclosed in outer bag.
- Once seedlings reached 30–50 cm, outer bags are open to allow further plant growth. This step must be performed in an insect-proof room to avoid disease dissemination.
- Once seedlings has fully developed (50–100 cm high), it can be transferred in field conditions (Fig. 27).

6. Basic equipment/items/chemical required for cassava genebanking

6.1 Equipment and consumable

Aluminium foil
Autoclavable plastic containers for sterilization (optional)
Autoclavable recipient (bottle, erlen) and closure system
Autoclave
Autoclave control tape
Balance (for g and mg)
Beads sterilizer or burner
Becher
Computer + excel/access software + pocket PC (optional)
Erlens
Filter papers
Forceps (long and short)
Fridge/freezer
Hot plate
Instrument holder
Laminar flow cabinet
Light fitted stereomicroscope
Long lasting markers
Magnetic stirrer and flea (optional)
Measuring cylinder
Media dispenser (optional)
Paper towel
Parafilm
PH meter
Pipette
Spoon, spatula, and weighing boats

Scalpels and surgical blades (number 10 and 11)
Scissors
Spray bottle
Test tubes (16 x 125 mm) and cap
Thermometer
Tube racks
Water distiller/deionizer/water tank

6.2 Media preparation and distribution

Media composition

Table 2. Mineral and organic compounds of culture media.

Product	Chemical formula	Molecular weight (g)	Meristem culture	Cuttings from plants	Cuttings from in vitro seedlings
Murashige and Skoog Mineral and vitamins (1962)	/	/	4.43g	4.43g	4.43g
Inositol	$C_6H_{12}O_6$		0.1g	0.1g	0
Saccharose	$C_{12}H_{22}O_{11}$	342	30g	30g	15-30g
Adenine sulfate	$C_{10}H_{12}N_{10}O_4S$	368.37	0.08g	0	0
Agar	/		4	5	5
NAA (Naphthalene Acetic Acid)	$C_{12}H_{10}O_2$,	186.2	0.2mg (1.07 µM)	0	0.01mg (0.0537µM)
BAP (Benzyl Amino Purine)	$C_{12}H_{11}N_5$	225.3	0.15mg (0.666 µM)	0	0.05 mg (0.222 µM)
GA3 (Gibberellic acid)	$C_{19}H_{22}O_6$	346.4	0.08mg (0.2312 µM)	0	0

Murashige and Skoog medium powder: Purchased from Duchefa Biochemie, product number (M0222).

Media preparation

Media preparation steps:

1. Prepare a check-list of all compounds needed to prepare media.
2. Sort all products and other items necessary to prepare media (glassware, spatula, tubes on racks, pen, etc.) (Fig. 28).
3. Dispense number of tubes required on racks.
4. Add 200 ml distilled water in a glass container (such as becher, bottle, erlen …). Add a ‘magnetic flea’ and place on a magnetic stirrer.
5. Weight adequate quantity of MS powder (Fig. 29).
6. Add it to the water (Fig. 30) and turn on the magnetic stirrer at low speed.
7. Weight/measure and add all remaining products one after another except agar. **Tick all listed products** as they are added in the media. Make sure all products are properly dissolved.
8. Adjust volume to 900 ml (if final volume= 1 litre) with distilled water.
9. Adjust the pH at 5, 7±0, 1 (with 0.5M NaOH or 0.5M HCl) and adjust final volume to 1 liter.

After adjusting the pH, perform steps 10–13, if your laboratory is equipped with a media dispenser or steps 14–15, if media is dispensed manually.

10. Add agar.
11. Heat and stir media on a hot plate (or in microwave after removing the flea/stirrer) till agar is dissolved. Make sure not to overheat/boil the media (heating should be stopped before boiling point).
12. Once agar is fully dissolved, use a media dispenser to distribute 5 ml of media per tubes (Fig. 31). Cover each tube with a plastic cap.

13. Autoclave at 121 ^{0}C for 15 minutes - allow slow cooling. See step16.

In a case where your laboratory is not equipped with an automatic media dispenser, step 14–15 is a processing alternative.

14. Transfer the media to an autoclavable recipient (bottle or erlen) with a capacity of at least 200 ml larger than the quantity of liquid it contains. This will prevent media loss/splash in the autoclave chamber during the autoclave cycle. When using a bottle, close the cap loosely (to avoid pressurization).
15. Once the autoclave cycle is completed, take out the recipient and dispense the media into tubes while still hot (still liquid). Note that adequate number of tubes + caps must be autoclaved prior media distribution. Distribution is performed under laminar flow (sterile conditions) using sterile pipette. This step can be semi automatized by using an automatic pipetter. Distribute 5 ml per tube.
16. Allow media to cool and preferably store in a cool environment (ideally at 8–10 ^{o}C). Use within 1–2 weeks following preparation.

Miscellaneous recommendations for media preparation:

- Autoclave control: It is advisable to stick autoclave-control tape to all items entering a sterilization cycle (Test tubes, bottle containing media, dissection instruments, etc...). If tape color does not confirm the autoclave was successful, media is discarded while other items can be newly autoclaved. Note that media cannot be autoclave twice (Photo 32).
- Instrument sterilization is performed by wrapping instrument in aluminum foil or
- Use a clean spoon or stick (preferably in glass) to dilute products if magnetic stirrer is not available. Make sure the flea used with magnetic stirrer is clean.
- Autoclave cleaning: Clean the autoclave chamber regularly (at least once a week).
- Product weighing/measuring. For powder product, use a clean spatula for each product. When using a stock solution, do not directly pipette from the main stock. Dispense a small quantity of the solution in a clean container and pipette the exact amount needed.
- Keep working areas as clean as possible. This includes bench surfaces and balances.
- Always clean the bench after use.

Solution preparation and storage

Growth regulators storage

	Storage		Solvent	Range of concentrations used ($mg.ml^{-1}$)
	Powder	Liquid		
Auxins				
NAA	Room temperature	Fridge	1N NaOH	1 - 0.1 -0.01
Cytokinins				
BAP	Room temperature	Fridge	1N NaOH	1- 0.1 - 0.01
Adenine Hemisulfate*	Room temperature	Fridge	Water	5
Others				
GA3	Room temperature	Fridge	Ethanol	1 / 0.1 / 0.01

***** For Adenine hemisulfate preparation: Dissolve 1 g in 200 ml with heat.

Growth regulation solutions preparation

Target concentrations	Quantity of growth regulator	Volume of water
1 $mg.ml^{-1}$= Solution A	50 mg	50 ml
0.1 $mg.ml^{-1}$ = Solution B	5 ml solution A	45 ml
0.01 $mg.ml^{-1}$ = Solution C	5 ml solution B	45 ml

Other solutions storage and preparation

	Storage conditions		Quantity		
	Powder	Liquid	Water (ml)	Product	Concentration
Ascorbic acid	Room temperature	Fridge	100	1g	0.01 g. ml^{-1}
Ethanol solution	Room temperature		70	30 ml	30%
Fungicide* powder	Room temperature	No storage	100 (hot)	1g	1%
Commercial bleach (NaClO)	Room temperature	No storage	Varies with commercial bleach titration in NaClO		3–5%

*Use mask to prepare the solution

7. General recommendations for laminar flow room, growth room and genebank maintenance and use

- Laminar flow room access and use
- Food and beverage not allowed
- Lab coat compulsory
- Only staff working under laminar flow allowed
- Floor and shelves must be vacuum-clean and wipe daily.
- Regularly fumigate the room (every 3 to 6 months).

Use of Laminar flow cabinet

- Switch on at least 15 minutes prior to use
- Turn on bead sterilizer at least 10 minutes prior to start and only use when the “sterilizing” indicator is on.
- Spray the bench with alcohol (70%) and wipe prior and after using the laminar flow. Do not spray the filter area.
- Do not leave any items on laminar flow bench after use.
- Keep away any item from the grid protecting the filter.
- Only keep items in use in the sterile space. Use the trolley to keep extra items such as rack, bottle etc.
- Spray with alcohol (70%) any item entering the sterile space.
- Autoclave all dissecting instruments regularly (ideally every day). Either wrap Instrument in aluminum foil or enclose in autoclavable polyethylene containers) prior autoclave.
- Clean all dissecting instruments with liquid soap every day.

Genebank and growth rooms’ access and use (Figs 33 and 34)

- Only authorized person allowed
- Only working staff allowed
- Fumigate all in vitro culture rooms regularly (every 3 to 6 months).
- Regularly clean the shelves with bleach.
- Regularly check room temperature (use temperature data logger when possible to detect fluctuations).