IITA

Research to Nourish Africa

# Cassava in vitro processing and gene banking


International Institute of Tropical Agriculture – Institut international d' agriculture tropicale – www.iita.org

# Contents

# Introduction

This manual describes the cassava in vitro gene banking 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 gene banking.

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Intended use:

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

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

# A

# Explant production

# Explant production

Prior to starting in vitro gene banking, 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.

*Figure 1. Cassava cuttings planted in screen house*

*Figure 2. Cassava cuttings thermo-treated*


*Figure 3. Cassava cuttings 4 weeks after planting in screen house*

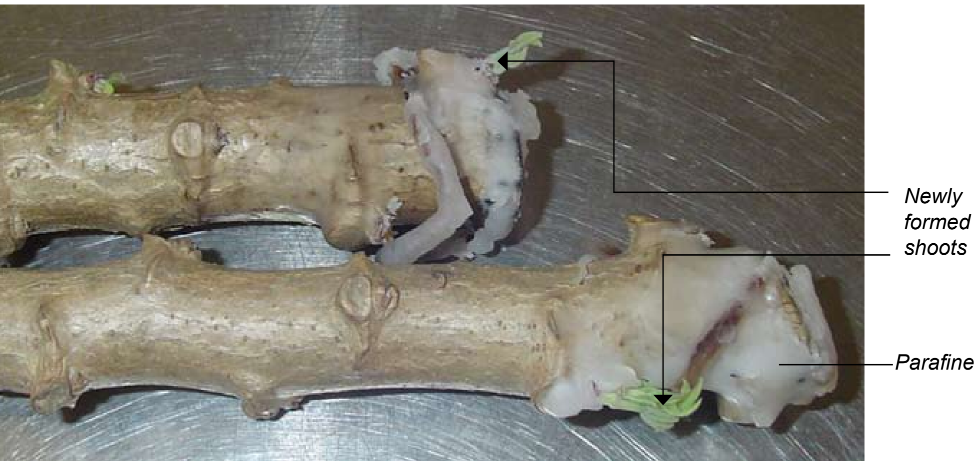


*Figure 4. Cassava cuttings after 4 weeks thermo-treatment*

# B

# Germplasm in vitro introduction

# Germplasm in vitro introduction

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.


*Figure 5. Nodal cuttings in Cleaning process*

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).


*Figure 6. Cassava cuttings after cleaning*


*Figure 7. Cuttings ready for in vitro planting*

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 °C, photoperiod 12/12).



*Figure 8. Cutting introduction in in vitro culture condition*
*Figure 9. Cutting introduction in in vitro culture condition*
*Figure 10. Newly sub-cultured seedling*

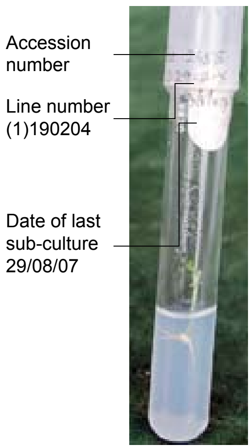


*Figure 10. Newly sub-cultured seedling*

[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).

## 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.


*Figure 11. Cassava seedlings at different stages of growth*

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).

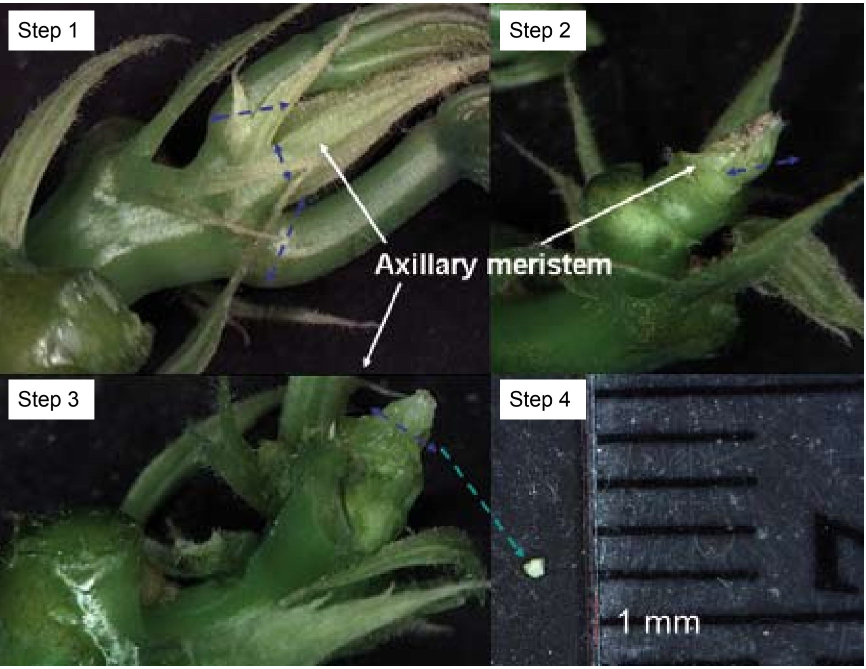


*Figure 12. Meristem excision.*

Step 1: Locate the meristem;
Step 2: First leave primordia removed
Step 3: Second leave primordia removed.
Blue arrows indicate where to cut.

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

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).

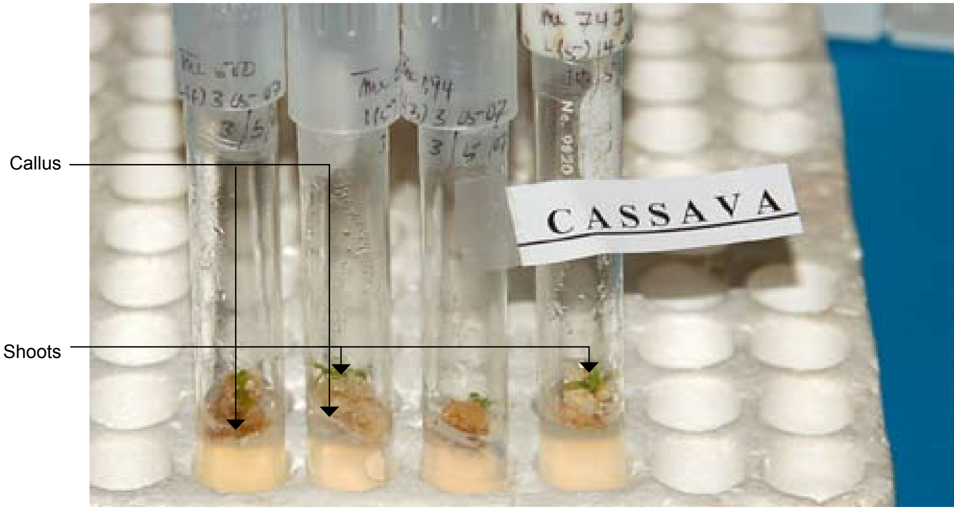


*Figure 13. Callus observation after meristem excision*

*Figure 14. Cassava seedlings at different stages of development*

# C

# Germplasm in vitro multiplication (propagation)

## C. 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-cut ting 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)

*Figure 15. Cassava micro-cuttings.*

# D

# Germplasm gene banking

# D1 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°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.




*Figure 16. Cassava  seedlings to be sent to genebank*
*Figure 17. Contaminated seedlings to discard*
*Figure 18. Necrose seedlings to discard*

## D2 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).

## D3 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 be cause 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.

## D4 Germplasm inventory:

An inventory of all germplasm is performed once a year.

# E

# Germplasm acclimatization (postflask management)

# Germplasm acclimatization (postflask management)

Only seedlings showing a well-developed root and shoot systems must be considered for acclimatization. In 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).

*Figure 19. Peat pellets*

*Figure 20. Peat pellet content mixed with vermiculite*

- 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 seed lings environment is kept humid by regularly adding water.




*Figure 21: Filling of inner bag*
*Figure 22: Gentle cleaning of seedling*
*Figure 23: Seedling after removal from test tube*
*Figure 24: Seedling planting*

- Three to 4 weeks later (once seedling start elongating), each seedling is removed from its inner bag and trans ferred 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 per formed 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).




*Figure 25: Seedlings ready for screen house growth conditions*
*Figure 26: Seedlings enclosed in plastic bags at early stage of post-flask treatment.*
*Photo 27: Cassava germplasm in screen house. Left: Plants ready for planting/indexing – Right: Seedlings at early stage of acclimatization*

# F

# Basic equipment/items/ chemicals required for cassava gene banking

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## Equipments and consumables

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

# G

# Media and stock solutions preparation and storage

# Media composition preparation and distribution

## Media composition

### 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 (0.666 µM) | 0.15mg | 0 (0.222 µM) | 0.05 mg |
| 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).

## Stock 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 | Room temperature | No storage | Varies with commercial bleach titration in NaClO | | 3–5% |

*Use mask to prepare the solution

# 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.

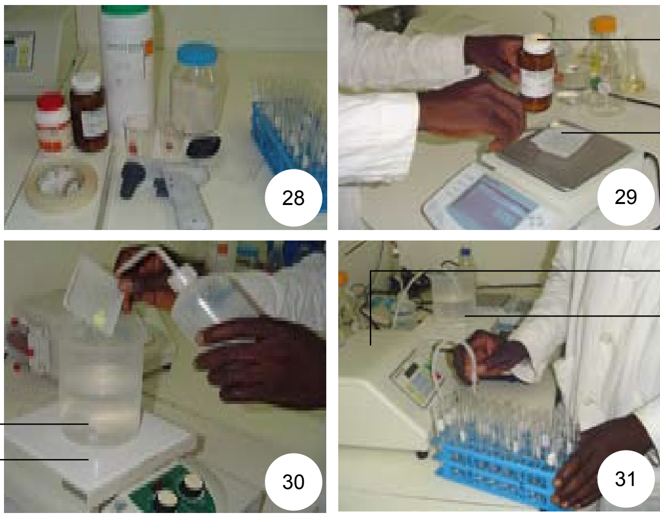


*Figure 28. Basic consumable used in plant tissue culture*
*Figure 29. Weighing MS powder*
*Figure 30. Dissolving chemical with magnetic stirrer*
*Figure 31. Medium distribution with automatic dispenser*

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 °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

4. 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 °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 (figure 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


*Figure 32. Alumium and paper foil packed for autoclave*

# H

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

# 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).

*Figure 33.*
*Sub cultures*

*Figure 34.*
*Global view*
*in vitro*
*genebank*