IITA
Research to Nourish Africa

Yam in vitro genebanking

Contents

Introduction

This manual describes the yam in vitro genebanking process set up by IITA. Within the last 3 years, this process has been successfully used to duplicate over 1000 accessions of yam from field to in vitro culture. Research is still going on to optimise yam in vitro genebanking, especially at meristeming level.

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

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Intended use:

- Best practice reference manual for genebank staff
- Provides the frame for further development of an ISO normalization (quality control of in vitro germplasm) for yam genebanking
- Support capacity building in in vitro culture and gene banking.

This 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 genebanking, it is necessary to select adequate plant parts that will be introduced in vitro, i.e. explants. In the case of yam, preferred explants are apical or axillary buds generally taken from field- or screen house-planted germplasm (fig. 1 and 2). IITA genebank is presently testing the use of dry tuber sprouts (non planted tubers) as source of apical and axillary buds (fig. 3). This approach is promising as the later explants show little bacterial contamination in comparison to the former ones.

Figure 1. Yam field bank

Figure 2. Yam in screen house

Figure 3. Direct shooting from dry tubers

B

Germplasm in vitro introduction

2.1 Micro-cutting

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 of the bud or apical cutting = apical shoot on 1 cm stem) (fig. 4). Cut them with scissors or scalpel and keep them dry in a clean container; 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 5–10% sodium hypochlorite (NaClO)[1] solution containing a few drops of a wetting agent e.g., Tween 20; and soak for 10–20 (fig.5) minutes (fig.5).Use only freshly made hypochlorite solution. When working with new explant type, it is better to run preliminary cleaning tests to determine the longest exposure time sustained by explants. It is sometimes necessary to repeat the sterilization.

[1]Commercial bleach titration ranges between 10 to 30% NaClO.

Figure 4. Dioscorea alata vines, axillary bud and micro-cutting (black arrows indicate where to cut the stem to obtain a nodal cutting)

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 (fig. 6).

5 With the help of a scalpel (with sterile blade), cut the whitening edge of the stems (hypochlorite effect) (fig. 7). 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 step is optional and is only applied when explants show fungus contaminations).

7 Remove from the solution and dry on filter paper.

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

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. 8).

11 Transfer tubes to a growth chamber (temperature 28±30°C, photoperiod 12/12).

Figure 5. Nodal cuttings in cleaning process

Figure 6. Nodal cutting rinse after exposure to sodium hypochlorite

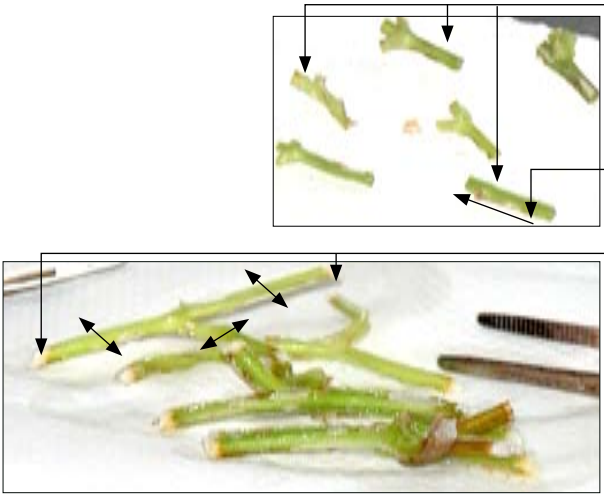
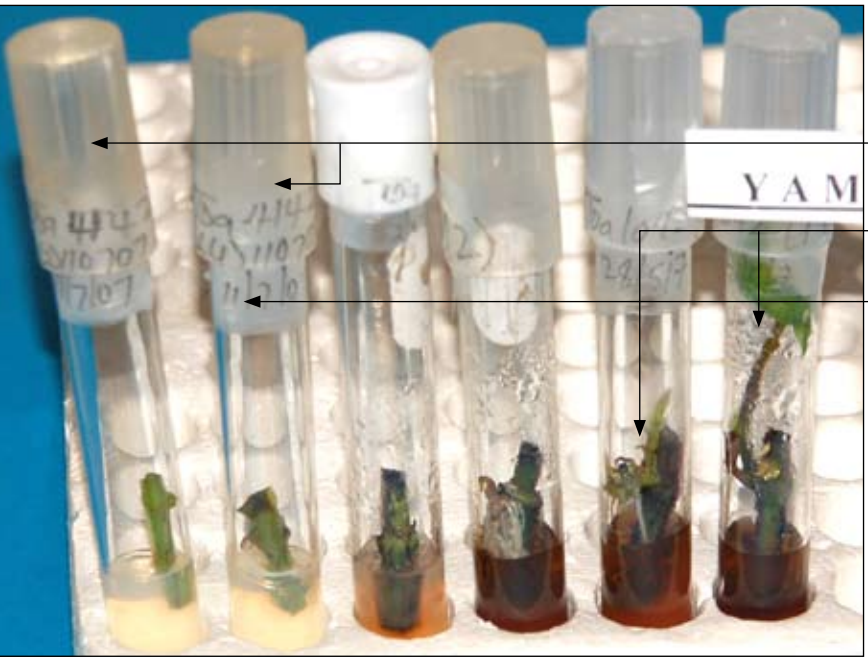


Figure 7. Nodal cutting after cleaning (double black arrow indicates where to cut to obtain a micro cutting)

Figure 8. Labelling



T: All IITA accessions start with T for Tropical
Da: Dioscorea alata
L(1): meristem 1 excised on 110707
110707: Date of last subculture
In the present case =date of in vitro introduction

[2]A line number is created as follows: Shoot number + 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. 9). Once seedlings have developed 2 to 6 buds, they are sent to multiplication (see below).

2.2 Meristem in vitro introduction

(First 3 steps below are as described above in 1–3).

1 For each accession, select 10 cuttings (either nodal cutting = 1 bud + 1 cm stem maximum on each side or apical cutting = 1 apical shoot on 1 cm stem) (Fig. 4). Cut them with scissors or scalpel and keep them dry in a clean container. Record the 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 5–10% sodium hypochlorite (NaClO)[1] solution containing a few drops of a wetting agent e.g., Tween 20; and soak for 10–20 minutes (Fig. 5).
Use only freshly made hypochlorite solution. When working with new explant type, it is better to run preliminary cleaning tests to determine the longest exposure time sustained by explants. It is sometimes necessary to repeat the sterilization.

Figure 9. Yam nodal cuttings 6 weeks after in vitro introduction

Figure 10. Meristem excision with stereo microscope under laminar flow

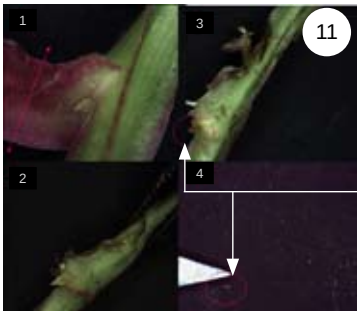
[1]Commercial bleach titration ranges between 10 to 30% NaClO.

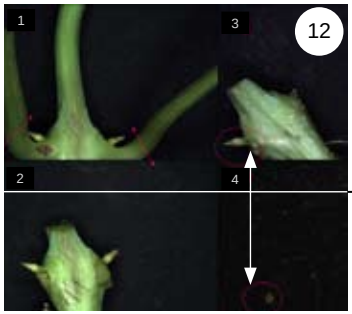
Steps 15 to 18 have to be performed under laminar flow (sterile conditions).

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

5 Place one shoot under stereomicroscope[2] (Fig.10) and locate meristem. Cut each leaf primordia one after another till the meristematic dome becomes visible (Figs. 11, 12, 13; steps 1, 2, and 3). To do so, either use sterile scalpel (blade number 11) or needle.

6 Cut the base of the dome (Fig. 11, 12, 13; step 4) and transfer to meristem media (Fig. 14, tube 1).





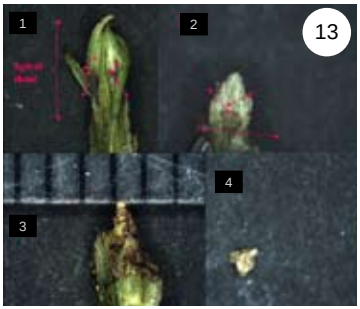
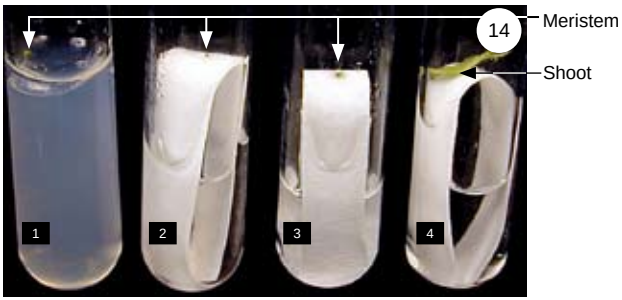


Figure 11. Dioscorea Alata axillary meristem excision
Figure 12. Dioscorea rotundata axillary meristem excision
Figure 13. Dioscorea alata apical meristem excision
Figure 14. Yam meristems at different stages of development



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

8 Label each tube with an accession number, date of introduction, and line number (record by writing on parafilm using long lasting marker).

9 Transfer tubes to growth chamber (temperature 28±30^{o}C, Fig. period 12/12).

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

Obvious signs of growth are visible within 2–3 weeks following meristem excision (greening of meristem) (Fig.14, 3 test tubes on the left). In many cases, the time required to induce shoot elongation (Fig. 14, right test tube 4) varies between 3 to 16 months. Moreover, direct morphogenesis is not always observed and shoots often rise from a clump of callus/somatic embryogenis tissues type (Fig. 15). Media optimization is still under investigation.

Figure 15. Shoot formation on callus/somatic embryo like clumps

C

Germplasm multiplication (propagation)

Germplasm multiplication (propagation)

Steps 1 to 6 of the following procedure are performed under laminar flow (sterile conditions) and all instruments used must be sterilized.

1 Open test tube.
2 With the help of forceps, pull the seedling out of the tube (Fig. 16, step1).
3 Transfer the seedling on sterile filter paper set on sterile aluminum paper foil (Fig. 16, step 2).
4 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 missorientation, allow the upper part of the stem to be shorter than the lower one (Fig. 16, step 3).
5 Open a test tube containing fresh multiplication medium and plant the longer part of the stem into it.
6 Close each tube with a plastic cap and seal with parafilm.
7 Label each tube with corresponding line number and date of subculture.
8 Transfer tubes to growth chamber (temperature 28±30°C, Fig. period 12/12). Nodal cuttings produce roots and shoots within 2 to 3 weeks (Fig. 17).

Figure 16. Yam in vitro multiplication

Figure 17. Yam micro-cuttings at different development stage

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) (Fig. 18) are transferred to the genebank (Fig. 19). Genebank culture conditions are as follows: 16±20^{o}C and 12/12 photoperiod.

Germplasm is observed weekly in order to eliminate and record any contaminated or necrosed seedlings (Fig. 20.)

Figure 18. Yam subculture ready to be transferred to genebank

Figure 19. Yam in vitro genebank

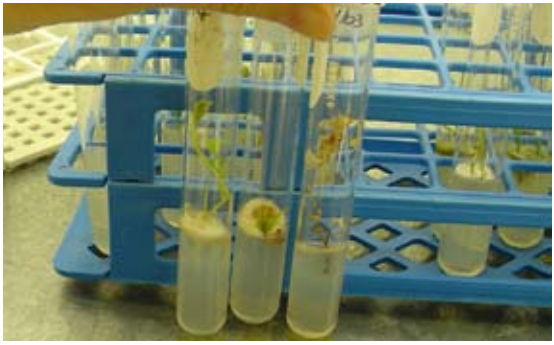


Figure 20. Contaminated (2 left) and necrosed (right) in vitro seedlings

D2 Germplasm regeneration.

Depending on accessions, yam germplasm maintained in the genebank conditions requires subculturing every 10 to 24 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)

- 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)
- 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

The computerization of this data allows us to know

1 Present number of seedlings in subculture
2 Seedlings potential (= in the bank + in subculture)
3 Present number of seedlings in subculture

Computerization of all data is advisable to facilitate germplasm management. The use of pocket PC speeds up data collection and reduces record mistakes (Fig. 21). Ultimately, bar coding in in vitro collections will further improve genebank management in terms of cost and data reliability. IITA is presently setting up such system.

Figure 21. In vitro culture monitoring with pocket PC

D4 Germplasm inventory.

An inventory of all germplasm is performed once a year.

E

Germplasm acclimatization (postflask management)

Germplasm acclimatization (postflask management)

In vitro seedlings need special attention prior to being sent back to field conditions. Only those showing a well-developed root and shoot systems are processed as follows for acclimatization:

- Gently remove in vitro seedling from its test tube and rinse its root system with water (to eliminate agar).
- Plant each seedling in one soaked peat pellet (allow 2 to 3 hours of soaking in water) and enclose in a plastic bag after sprinkling some water on seedling (Fig. 22). Maintain in a warm and lighted place.
- Three to 4 weeks later (once seedlings start elongating), fragment each peat pellet and transfer each seedling in a pot containing sterile soil. Enclose each seedling in a bigger plastic bag.
- Once seedlings reached 30–50 cm, plastic bags are open to allow further plant growth. This step is performed in an insect-proof room to avoid disease dissemination.
- Fully developed plants (50–100 cm high) can then be transferred in field conditions.

1. Jiffy pod after imbibition
2. Seedling freshly planted on jiffy pod
3. Seedling ready for transfer in the field

Figure 22. Yam seedling acclimatization in screen house

F

Basic equipments and items required for Yam gene banking

Basic equipment/items required for yam genebanking

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)	Meristems culture** $g.l^{-1}$	Cuttings from plants $g.l^{-1}$	Multiplication $g.l^{-1}$
Murashige and Skoog Mineral and vitamins (1962)	/	/	4.43	2.215–4.43	4.43
Inositol	$C_6H_{12}O_6$	180.2	0.1	0	0.1
Saccharose	$C_{12}H_{22}O_{11}$	342	30	15–30	30
Adenine sulfate	$C_{10}H_{12}N_{10}O_4S$	368.37	0.08	0	0
L-cystein	$C_3H_7NO_2S$	121.6	0.02	0	0.02
Agar	/	/	4–7	7	4–7
NAA (Naphthalene Acetic Acid)	$C_{12}H_{10}O_2$	186.2	0.0002 (1.07 µM)	0	0
BAP (Benzyl Amino Purine)	$C_{12}H_{11}N_5$	225.3	0.00015 (0.666 µM)	0	0
GA3 (Gibberellic acid)	$C_{19}H_{22}O_6$	346.4	0.00008) (0.2312 µM	0	0
Kinetin	$C_{10}H_9N_5O$	215.2	0	0	0.001 (0.476 µM)

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 bleach (NaClO)	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 checklist of all compounds needed for media preparation and sort them as well as all other necessary items (glassware, spatula, tubes on racks, pen, etc.) (Fig. 23).
2. Dispense number of tubes required on racks.
3. For one litre medium preparation, dispense approximately 200 ml distilled water in a glass container (such as becher, bottle, erlen …), add a 'magnetic flea' in water and place on a magnetic stirrer.
4. Weigh adequate quantity of MS powder.
5. Add MS powder to the water and turn on the magnetic stirrer at low speed.
6. Once MS powder is dissolved, 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.
7. Adjust volume to 900 ml (if final volume= 1 liter) with distilled water.
8. 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).

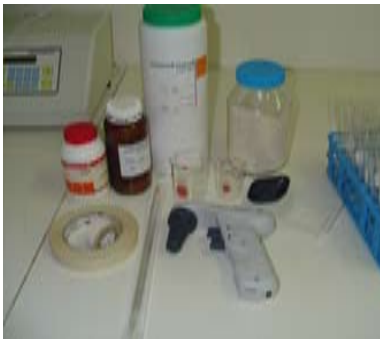


Figure 23. Basic equipment and products needed for media preparation

12. Once agar is fully dissolved, use a media dispenser to distribute 5 ml of media per tubes. Cover each tube with a plastic cap.

13. Autoclave at 121 ^{0}C for 15 minutes - allow slow cooling. Go to 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 plus 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 off and preferably store in a cool environment (ideally at 8–10 ^{o}C). Use within 1–2 weeks following preparation.

Miscellaneous recommendations for media culture 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 fails to confirm sterilization, media is discarded while other items can be re-autoclaved.
- Autoclave cycle: All items (forceps, filter papers, etc.) entering the autoclave are either wrapped in aluminium foil or enclosed in autoclavable container.
- Autoclave is cleaned at least once a week.
- Weighing: Use a clean spatula for each product. Clean the balance after use.

- Measuring: Do not directly pipette in stock solution. Dispense a small quantity of the solution in a clean container and pipette the exact amount needed. Discard the remaining stock.
- Each staff must clean the bench surface they used once media preparation is completed.

H

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

8. 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 disposable 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 alcohol (70%) on 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).