Virus Elimination and Testing in Cassava

A practical manual

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Contents

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This manual has been compiled from experience gained by Morag Ferguson and Abraham Choti of the International Institute of Tropical Agriculture (IITA) from conducting virus elimination in cassava in collaboration with Florence Munguti from the Kenya Plant Health Inspectorate Service (KEPHIS), Muguga, Kenya.

June 2020

Additional information can be found at:

Cassava *in vitro* processing and gene banking

https://www.iita.org/wp-content/uploads/2017/Cassava_in_vitro_processing_and_gene_banking.pdf

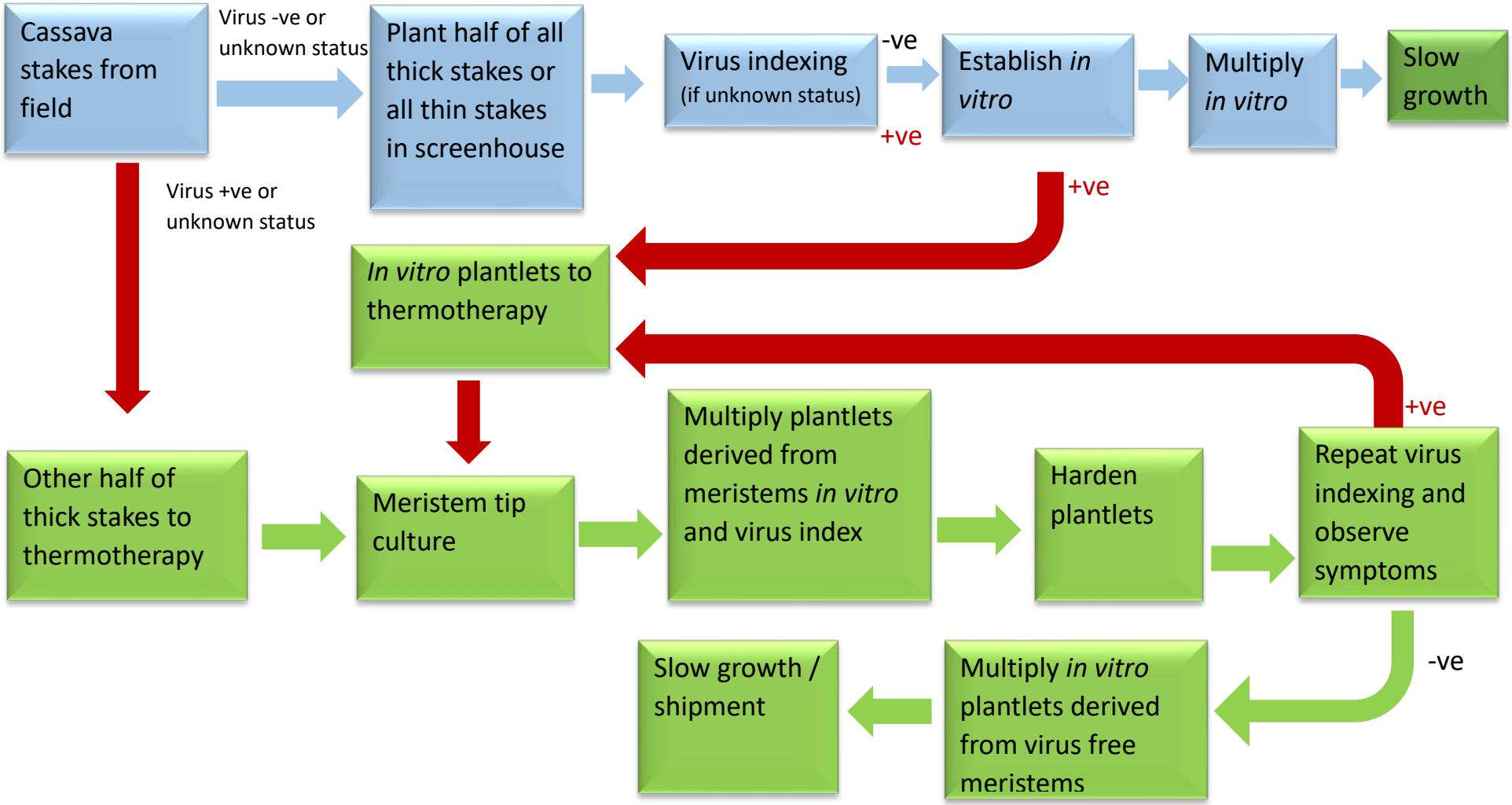

Fig 1: Workflow for virus elimination at IITA – Nairobi / KEPHIS

1. Collection of Cassava Stakes

It saves a huge amount of time, work and resources if virus free stems can be collected from the field. Collect stakes from plants that are not showing disease symptoms and FROM A SINGLE PLANT per clone. If possible, virus-test plants before taking cuttings to select plants that are virus free. If it is necessary to collect from more than one plant, make sure stakes from different plants can be distinguished, so label all stakes from plant 1 as (1), from plant 2 as (2) etc. Thick (>2cm diameter) stems should be collected if possible. Keep stems as long as possible to avoid drying during shipment and take sufficient stems to make 8 stakes of 20 -30 cm. Label carefully with masking tape and wrap in newspaper to prevent damage during transportation.

Collect leaf samples from all plants that have been sampled for stem collection. If the plant is showing symptoms, select leaves that are showing symptoms, otherwise sample from the top of the plant (leaf 3-5), mid and bottom of the canopy (lowest non-senescing leaf). For each selected leaf, the central lobe is picked. Different options for sampling are given in Section 10.1. Try to ensure that the midvein is sampled as viruses live in the phloem tissues.

Get import permits and phytosanitary certificates as required.

2. Arrival of cuttings

As soon as the cuttings arrive at the virus elimination laboratory, they are given a unique identity number (in the case of IITA/KEPHIS, this is an ICK (IITA Cassava @ KEPHIS) number and they are entered into a database on Clone Tracker or Microsoft Excel sheet. The date of arrival, place of origin and the name of the person supplying the stems are also recorded.

2.1. On reaching the station, stems are cut into 20 – 30 cm lengths including several nodes. Aim for eight stakes so that four “thick” stakes (>2cm diameter) can be placed in the plant growth chamber and four (possibly “thinner” stakes) in the screenhouse. Sterilize all stakes in buckets, leaving the masking tape labels on. This should be done sequentially, not together:

- 2.1.1. Foliar insecticide: 0.25 mL/L of Dynamec (18 g/L a.i. Abamectin). You can buy a sachet of this in Kenya which needs to be diluted into 20 L of water. Soak for 30 min or longer. You can also use ACTARA.
- 2.1.2. Systemic pesticide: Soak in Imidiacloprid for one hour (sometimes difficult to get in Kenya)
- 2.1.3. Fungicide: Ridomil, again this can be purchased in a sachet in Kenya that needs diluting in 20 L of water. Soak for 30min or longer.

2.2. After soaking, plant half of the stakes in pots to observe any symptom development and to act as a backup in case the clone is lost in tissue culture. Take the rest of the stakes for thermotherapy (see below). The stakes for thermotherapy should be relatively thick (> 2cm diameter) so they do not dry out in the plant growth chamber.

2.3. Perform virus-testing as soon as stakes in the screenhouse sprout if diagnostics were not conducted on samples brought from the field.

Fig. 2. Treating cassava stakes prior to introduction to the glasshouse

3. Thermotherapy using thick stakes

Thermotherapy is most efficiently applied to thick stakes collected from the field.

After sterilization (above), let the stakes dry for a while, and then dip ends of each stake into melted wax (Fig. 3) to stop them drying out during thermotherapy.

3.1. Place a plastic label around the stakes and tie them with a bit of wire.
3.2. Place in the plant growth chamber at 28 °C for 6h in the dark and 38 °C for 18 hours under light per day with 80% humidity for 4 weeks until they have sprouted (Fig. 2).
3.3. At four weeks excise tips (a larger portion of the shoot for cuttings which are already virus free) or meristems (top few cells of the apical meristem specifically for virus elimination).

Fig. 3. Sprouted cassava stakes after thermotherapy treatment, showing wax treatment.

4. Establishing plantlets *in vitro* followed by thermotherapy

Sometimes thick stems or stakes are not available so thin stakes (<1.5cm diameter) need to be established in pots in a glasshouse before introducing to thermotherapy where they would simply dry out. Once established, nodal cuttings or apical meristems (all classified as explants) are taken to establish the clone *in vitro* prior to thermotherapy. Once the clone is established *in vitro*, it can be taken for thermotherapy if necessary. If stems are virus negative thermotherapy is not required and clones can just be maintained *in vitro*.

4.1. Plant thinner stems in pots in the glasshouse. Wait until they have a few leaves and nodes begin to grow. Virus-test for CMD and CBSD as soon as possible.

4.2. Excise explants into a sterile jar (around 50 mL). The explant could be an apical tip with two or three nodes that can be cut into several nodal cuttings after sterilization, or a nodal cutting (Fig. 4). Surface sterilize using the procedures described below. The accession number or name is recorded carefully on each container. You must work quickly and start the surface sterilization within a few minutes of taking the explants, so they are not left to dry.

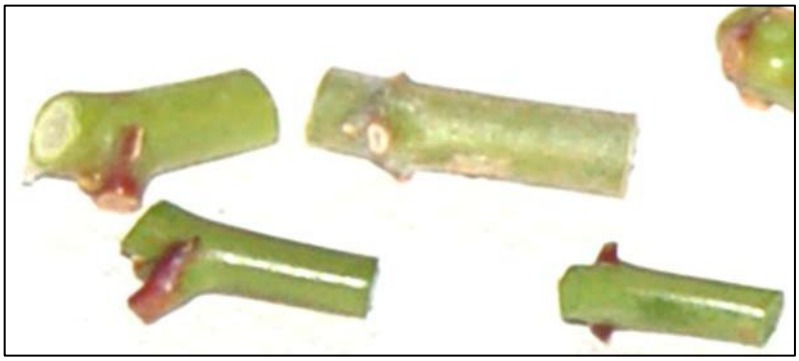

Fig. 4. Nodal cuttings. Note the upper end is shorter than the lower end. This helps to place the nodal bud facing upwards when placed in agar.

(Source: IITA)

4.3. Surface sterilization: Disinfection solutions are prepared as follows:
   4.3.1. sterile distilled water (SDW)
   4.3.2. alcohol solution (ethanol 70% v/v)
   4.3.3. Sodium hypochlorite solution (common bleach or jik). Jik often has approx. 3.75% or 2.6% active ingredient, but you need to dilute to 0.75% active agent with sterile distilled water (SDW). **Important:** Use only freshly made sodium hypochlorite solution.

4.4. Soak explants in sufficient amounts of the following solutions so explants are well covered. Soak in succession (occasionally shake for better contact) and under the laminar flow (sterile environment) as follows:
   4.4.1. Two drops of Tween20 in 20 mL SDW for 20 minutes with shaking every 3 minutes.
   4.4.2. Rinse three times in SDW.
   4.4.3. Alcohol solution (ethanol 70% v/v) for 5min.
   4.4.4. 0.75% v/v sodium hypochlorite solution for 20 - 25 minutes.
   4.4.5. Rinse three times in sterile water. You can keep in SDW for a while if necessary.

4.5. After sterilization, shoots are placed on a sterile surface (petri dish), using sterile techniques.

4.6. In a sterile laminar flow and using a scalpel and sterile blade, cut the white ends, damaged by hypochlorite, off the explant. Shoots can be cut into several nodal cuttings. Always make the upper end of a nodal cutting shorter than the lower end, to help orientate the bud upwards when placing in agar. Alternatively, for apical meristems, cut several sections (approx. 1cm length) each with one or two nodes avoiding the parts that have been damaged by bleach. The apical tip need not have a node.

4.7. With sterile forceps place each explant into a test tube with agar (one explant per tube) making sure the node bud is facing upwards (see Appendix 1 for agar media). The tubes are immediately sealed with aluminium foil and clingfilm and labelled.

4.8. Transfer tubes to a growth room at 27 ^{o}C (16 hours light and 8 hours dark).

4.9. After two weeks, remove any plants contaminated with bacterial and/or fungal infections.

4.10. For plantlets derived from nodal cuttings once the length has reached 3–4 cm, it can be taken for thermotherapy.

4.11. Place in a plant growth chamber at 28 °C for 6 hours in the dark and 38 °C for 18 hours under light per day with 80% humidity for three to four weeks.

4.12. During the fourth week, perform meristem excision and culture from apical or axillary buds.

5. Meristem tip culture

5.1. Under the laminar flow, using sterile forceps, place a shoot on a sterile surface (paper towel or petri dish) under the light of a stereomicroscope.

5.2. Gently cut and remove the white leaves, one by one from the outside without damaging the next internal one. To do this, use either a sterile scalpel (blade no. 24) or a needle (Fig. 5).

5.3. When the meristematic dome becomes visible, covered by 1 or 2 internal leaf primordia, the excision is complete, and the meristem can be cut at its base (Fig. 5)

5.4. Meristems are then transferred to meristem regeneration culture medium (Appendix 1) in test tubes.

5.5. Each culture vessel is labeled using a long-lasting marker or pencil (see Section 6 below).

5.6. Keep meristems in the dark for seven days at 27 ^{o}C, by wrapping the test tubes in aluminium foil.

5.7. The cultured meristems are then transferred to the growth chamber (T °27 ± 1 °C, 16 hours light/24 hours and light: 38 µmol/m²/s).

5.8. Obvious signs of growth are visible within 2–4 weeks: greening and elongation and/or callus formation.

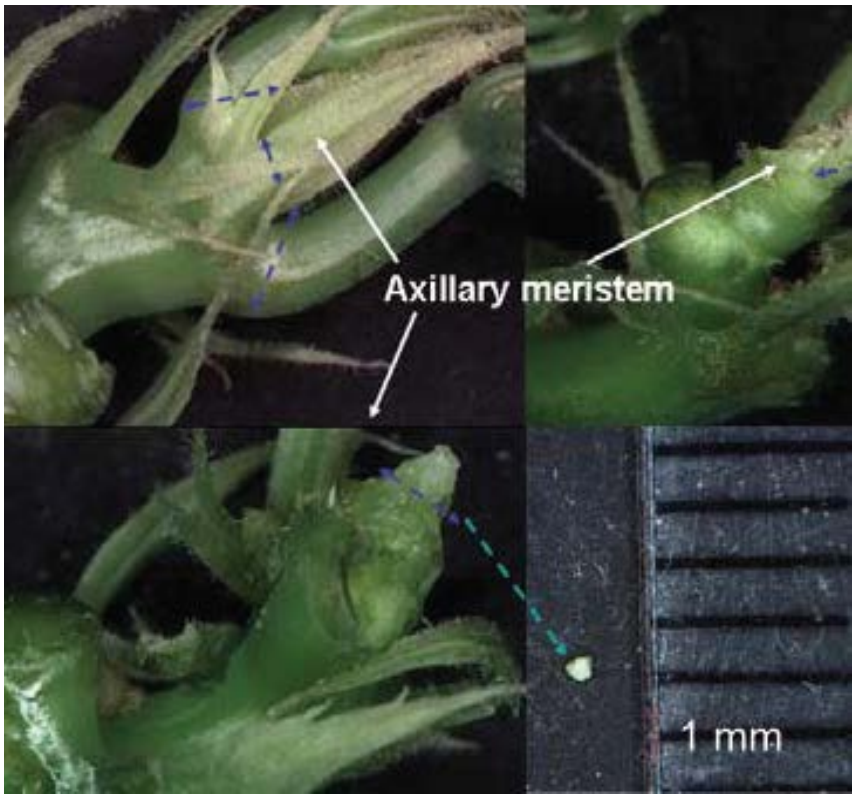

Fig. 5 Meristem tip culture

(Source: IITA)

6. Labelling of *in vitro* plantlets

6.1. Always work with one clone at a time to avoid problems of mislabeling.

6.2. It is important that each meristem and each tip excised is given a unique ID, as, due to the thermotherapy and meristem tip excision, one meristem might be virus free, while another may not be free from viruses. In addition, meristems are more likely to be free from viruses than tips.

6.3. Each meristem excised is given an M series ID such as M1, M2, M3 etc. This is the clone-meristem/tip combination.

6.4. Each tip or other explant (i.e. a meristematic shoot taken from a plant in the screenhouse) is given a T series ID: T1, T2, T3 etc. This is the clone-meristem/tip combination.

6.5. Excision from the mother plant, whether it is a field stake or plant in the screenhouse, when it is first taken *in vitro*, is labelled x0 (meaning that it has not been multiplied yet). The first multiplication is given a x1, second multiplication x2 etc.

6.6. Examples:
ICK23_M1x0
ICK88_T3x1
ICK203_M4x0

A full label on an *in vitro* tube would look like:

ICK786_M1x1
01/09/2018
Name of person

Key: ICK786_M1x1 means the clone ID (ICK786), the first meristem excised (M1), and the first multiplication (x1)
01/09/2018 is the date upon which the last multiplication or manipulation was done
Name of person is the name of the person performing the multiplication or manipulation; the operator

7. *In vitro* multiplication

If not contaminated, and once of a sufficient size, the *in vitro* plant material (whether derived from tip or meristem) can be multiplied and re-established on agar media (see Appendix 1 for multiplication media).

7.1. Work under a sterile laminar flow hood.

7.2. Gently remove the plantlet from the test tube and place on a sterile petri dish.

7.3. Cut sections of the plant with one or two nodes.

7.4. Place each cutting into a new test tube, or several cuttings into a baby jar containing approx. 2 cm multiplication agar media.

7.5. Return to the growth room at 27^{o}C.

Fig. 6 Sections of stem showing one or two nodes

(Source: IITA)

8. Hardening

Hardening is the process whereby tissue culture plantlets are gradually acclimatized to the more variable climatic conditions of growing outside of a test-tube. Tissue culture plantlets are gradually exposed to lower humidity, more variable temperatures and soil media. Hardening of cassava plants can be challenging, and different scientists have different ways of doing hardening. The National Crop Resources Research Institute (NaCRRI) have published an extensive manual on hardening of cassava plantlets entitled 'Handbook of Post-flask Management of Tissue Cultured Cassava Plantlets', Sept. 3-14th, 2013. Here we describe the methods used at IITA in Nairobi and KEPHIS.

Materials

- Small disposable plastic cups
- Larger snuggly fitting disposable cups for the top
- Gravel
- Sterile peatmoss : vermiculite (1:1)
- Plastic trays
- A polytunnel

Methodology:

8.1. Punch at least three holes in the bottom of each small disposable plastic cup.

8.2. Fill the base of the plastic cup to 1 cm depth with gravel (Fig. 7).

8.3. Prepare the peatmoss and vermiculite mix and put a small amount on top of the gravel in the disposable cup (Fig. 7). A variety of types of media can be used, a few examples are: Sterile soil: cocopeat (1:1) or Peatmoss: vermiculite (1:1) or Cocopeat alone.

8.3.1. We have found peatmoss : vermiculite (1:1) to work the best.

8.3.2. Cocopeat is bought in solid blocks and must be soaked overnight and washed through a couple of times to remove salt. If used alone it can dry out quite quickly.

8.3.3. Soil should not be used alone as it needs to be lightened with either cocopeat or even sawdust (woodshavings).

8.3.4. Fungus gnat infections, which are devastating, can occur if manure is used.
8.3.5. Apparently soil and vermiculite do not go well together.

8.4. To remove the cassava plantlet from the tube or baby jar, gently tap the tube to dislodge the plant, or use a spatula to loosen the plant from the media. The tube can also be dipped in water, and the media will gradually dissolve in the water. Carefully run water from a tap over the roots for about 1 minute, to ensure all the media has dissolved (Fig. 8).

8.5. Soak the plantlet in a basin of water for a couple of minutes. If media remains on the roots, it could cause infection.

8.6. Gently place the plantlet in the media and fill up around the plantlet (Fig. 8).

8.7. Do NOT push down hard around the plant.

8.8. Pour a very small amount of water around the plant and cover as soon as possible with the large plastic cup (Fig. 8).

8.9. Place on a plastic tray with 0.5 cm depth water. The water will be sucked up into the pot via osmosis (Fig. 8).

8.10. Place in a plastic tunnel between 14 ^{o}C minimum and 45 ^{o}C maximum. Ideal temperatures should be around 30 ^{o}C. A reasonably high temperature encourages humidity and growth (Fig. 8).

8.11. Humidity should be at least 80%.

8.12. Leave the large cups on for about one week and then start loosening them. You can leave them tilted on the plant for another few days before removing them completely. The time for removing the top cups will depend on the strength of the plant.

8.13. Leave the plants in the polytunnel without the top cups on for another two weeks. When they are strong, they can be removed from the polytunnel (Fig. 9). Try to remove them in the evening when it is a little cooler.

8.14. We have not used fungicide.

8.15. Some people use a plant food such as Easygrow starter 18:20:21 which is available in Nairobi. Higher phosphorous is generally beneficial for seedlings, although we have not found this necessary.

8.16. Once established, you can print out labels for each clone-meristem combination.

Challenges in hardening:

- Temperature: temperatures lower than 14 ^{o}C and higher than 45 ^{o}C should be avoided. A temperature of around 30 ^{o}C is ideal.
- Humidity: this must remain high, approx. 80% RH, which is difficult if temperatures are low.
- Young plantlets are very sensitive to waterlogging, so do not overwater.
- Never water from the top of the pot, always from the tray and water will rise up via osmosis.
- Start removing the larger cups and introducing the plants to lower humidity when temperatures are not too hot – better in the evening.
- We have had problems with fungus gnats (Fig. 10) that live in manure. Even if you sterilize the manure and soil, they will re-infest and colonise. The flies lay eggs in the soil. As the lava hatch, they feed on the seedling roots and young stem. They can cause huge destruction. We are now using peatmoss and vermiculite and hardening inside an insect-proof tunnel which we cover with plastic to make a polytunnel.

Fig. 7 Preparation of pots (A) with gravel (B) and substrate (C)

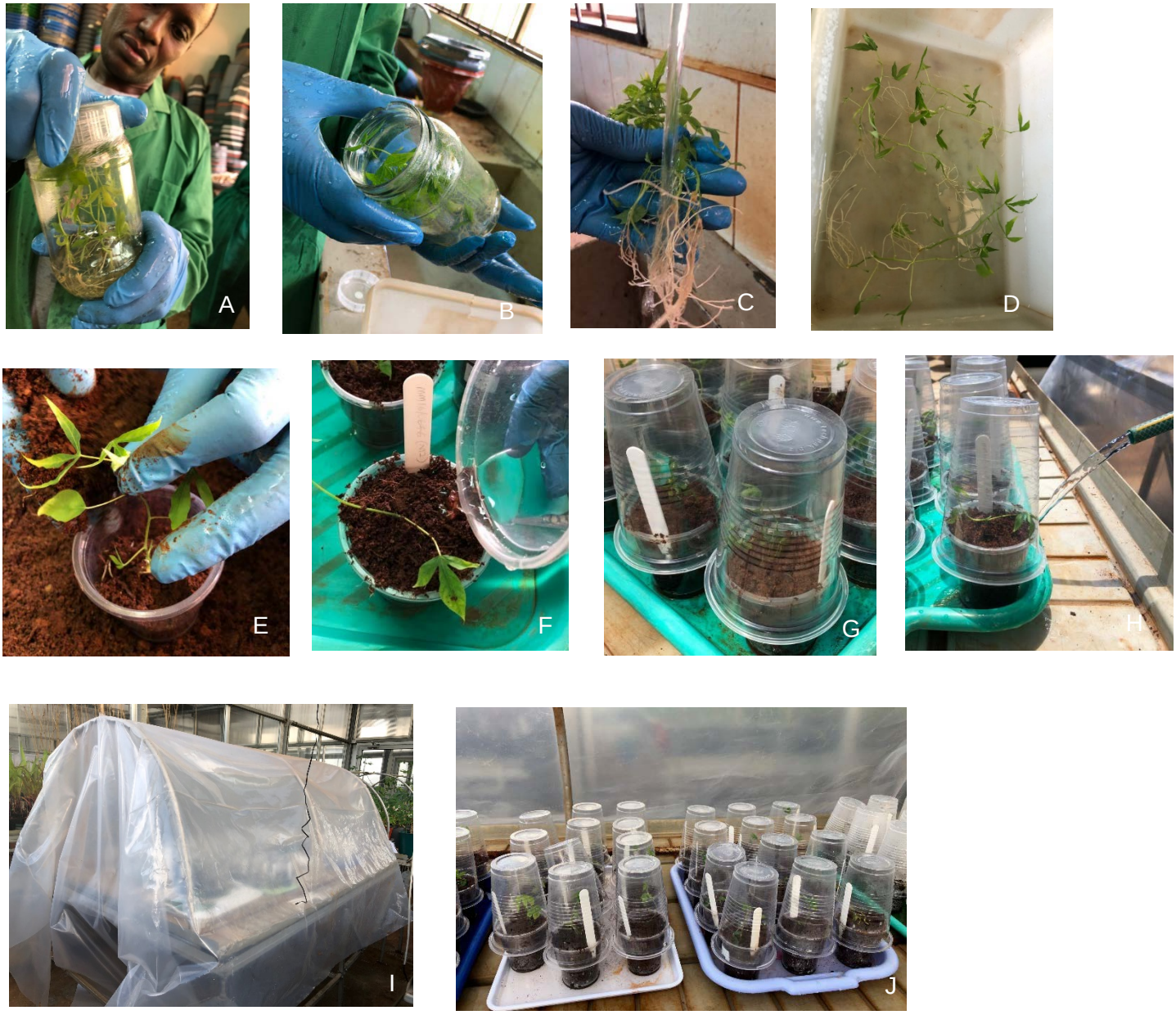

Fig. 8. Removal and washing of plantlets (A-D), planting (E-H) and covering of young seedlings (I,J)

Fig. 9. Removal of humidity chambers

Fig. 10. A fungus gnat, a major challenge in hardening of cassava in screenhouses

9. Slow growth

Once the plantlets or meristems are deemed virus free, and there is no immediate need for further multiplication, they can be transferred to *in vitro* slow growth conditions (i.e., a relatively lower storage temperature: 18 ± 1 ^{o}C), although some clones may lose their leaves. This will reduce the number of times that the plantlet needs to be regenerated.

10. Virus testing

Cassava brown streak disease (CBSD) [caused by cassava brown streak ipomoviruses; CBSIs] and cassava mosaic disease (CMD) [caused by cassava mosaic begomoviruses; CMBs] are the most important biotic constraints to cassava production in East Africa. It is therefore important to ensure that planting material propagated for delivery to farmers or conserved in genebanks or distributed among breeding programs is as far as possible free of these viruses.

10.1 Sampling and grinding for total nucleic acid extraction

- There are a number of options for how to sample, and grind tissue. A few of these are listed as options below.
- You can use both tissue culture plantlets, directly from tubes, or hardened plants.
- Complete a sample extraction sheet. Use consecutive numbers to facilitate easy location of samples.
- For CMD sample upper leaves as this is where the virus is concentrated
- For CBSD sample three leaves: one top (leaf number 3-5), one from mid-canopy and one from bottom (none senescing). The mid-vein should be sampled in each case as viruses reside in the phloem.
- Positive and negative controls must be included. It is recommended to collect controls from know plants and preserve in the same way test plants are preserved.

<u>Options:</u>

1. Collect in small plastic sample bags (approx. 7 x 5 cm) with one average leaflet per bag and extract fresh
2. Take eight 4 mm diameter punches, and place in 1.2 mL GenoGrinder tube in 96 well format box and lyophilize before grinding in a GenoGrinder
3. Take eight 4 mm diameter punches into 1.2 mL Genogrinder tube and use dry ice or liquid nitrogen to extract fresh and grind in Genogrinder
4. Sample one leaflet, and grind in pestle and mortar with warm extraction buffer
5. Take eight 4 mm diameter punches, collect in 2 mL Eppendorf tubes, dry in oven at 45 ^{o}C overnight, or lyophilize, and then grind using a Bullet Blender® (Next Advance) with beads.

10.2 DNA / RNA Extraction

This is a modification from Lodhi *et al.* (1994) for total nucleic acid extraction, optimized for cassava (Maruthi et al. 2002). This can be used for both CMD and CBSD.

Table 1: Extraction buffer

Reagent	Stock Conc.	Final Conc.	For 500 ml Extraction Buffer
CTAB	CTAB	2% CTAB	10 g
Tris-HCL (pH 8.0)	1M	100 mM	50 mL
EDTA	0.5 M	25 mM	20 mL

NaCl	NaCl	2.0M	58.4 g
Make up with SDW, then autoclave For 60 samples use approx. 50 mL extraction buffer. Just before use: Add 2% β-mercaptoethanol i.e. 200ul per 10ml EB (900ul for 45ml EM) Just before use: Add 1% PVP i.e. 100 uL per 10 mL EB (450 uL for 45 mL)			

Other requirements:
(5 M) KoAc ie 49 g in 100 mL, 245 g in 500 mL
20% SDS ie 20 g SDS in 100 mL water (never autoclave this)
Isopropanol
70% Ethanol i.e. 70 mL absolute ethanol, and make up to 100ml

1. Add 700 uL extraction buffer (Table 1) to the ground sample in a 2 mL Eppendorf tube and 50 µL 20% SDS, vortex and incubate at 65 °C in a water bath for 15 minutes. Place weight on top of rack to prevent caps popping off. Alternatively add 700 uL of warm extraction buffer and 50 uL 20% SDS to plastic sample bag and grind.

2. After incubation, leave at room temperature for 2 minutes. If using GenoGrinder strip tubes, cut off each tube from strip, undo cap and transfer all liquid into 1.5 mL Eppendorf tube by pouring.

3. Add 250 µL ice cold (5 M) KoAc and mix by inverting, incubate on ice for 20 minutes.

4. Spin for 10 minutes at high speed (14,000 rpm) at room temperature and transfer 700 µL of supernatant into a new tube.

5. Repeat steps 3 and 4.

6. Add 350 uL isopropanol, vortex incubate at -20 for 30 minutes and spin for 10 minutes at high speed.

7. Discard supernatant and wash pellet in 500 uL 70% ethanol by inverting 5 times.

8. Centrifuge at 13,000rpm for 5 minutes, pour off supernatant. Dry. To help drying you can either pipette out excess ethanol or centrifuge for a further 5 minutes – or both! Tap on bench to dislodge liquid. Use toothpick with towel wrapped to absorb ethanol (ensure that the toothpick and towel are nuclease-free).

9. Resuspend in 75 µL H_2O (by vortexing) or if pellet is small use 50 uL water

10. This protocol should give between 80 ng/uL and 200 ng/uL DNA, and half as much RNA.

Determine the quality of DNA/RNA using electrophoresis on an agarose gel:

- Make sure tank has been cleaned with 30% hydrogen peroxide, sprinkled liberally in the tank with water overnight

- Prepare a 1% agarose gel. Add 5 drops of Roth (Art. Nr. HP47.1, 15 mL, Ethidium bromide solution; 0.025% in dropper bottle) or GelRed (1uL in 100 mL gel)

- Load 5 uL of undiluted DNA, plus 5 uL loading dye (prepare on secured parafilm strip), include 5 uL 1 Kb ladder (this should be vortexed and spun down briefly before loading).
- Run at 100 V for 1 hr.
- Measure the quantity and quality of nucleic acids using a Nanodrop or Qubit and dilute DNA to approximately 25 ng/uL. You can do this using average readings. For 25 ng/uL use:

  300 – 250 ng/uL dilute by x10
  250 – 200 ng/uL dilute by x8
  200 – 150 ng/uL dilute by x6
  150 – 100 ng/uL dilute by x4
  50 – 100 ng/uL dilute by x2

10.3 End-point PCR for EACMV and ACMV

Target virus	Primer name	Sequence	TaOpt	Expected fragment size (bp)	Reference
ACMV	ACMV AL1/F	5'- GCGGAATCCCTAACATTATC -3'	53.7 ºC	1030bp	Stephan Winter
	ACMV ARO/ R	5'-GCTCGTATGTATCCTCTAAGGCCTG -3'			
EACMV incl. Ug variant	EACMVA2469s	5'- TGGAGATGAGGCACCCCATC -3'	56.7 ºC	722bp	Stephan Winter
	EACMVA391c	5'- TCCTCCGCACCTTGGATACG-3'			

Reaction conditions:

PCR reaction set up as follows on ice

Components	**Stock conc.**	**Final Conc.**	**1X RXN** (µl)
Nuclease-free water			9.5
One Taq Quick-load 2X master mix with standard buffer*	2X	1X	12.5
10 µM Forward primer	10 µM /µL	0.2 µM	0.5
10 µM Reverse primer	10 µM /µL	0.2 µM	0.5
Template DNA	25 ng/µL	50 ng	2.0
Total			**25**

*One Taq Quick-Load 2x master mix with standard buffer (Cat. No M0486L), New England BioLabs.

Thermal cycling profile

Steps	Temp (ºC)	Cycles	Time
Initial denaturation	95	1	3 minutes
Denaturation	95	35	30 seconds
Annealing	53 or 55	1	30 seconds
Extension	72	1	1 minutes
Final extension	72	1	10 minutes

Load 10 uL of the product onto gel.

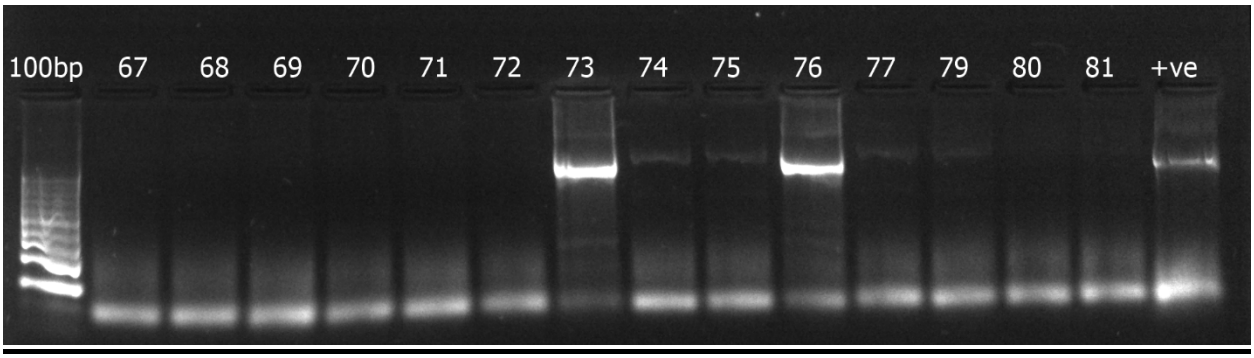

Fig. 11. ACMV indexing; positive samples 73 and 76.

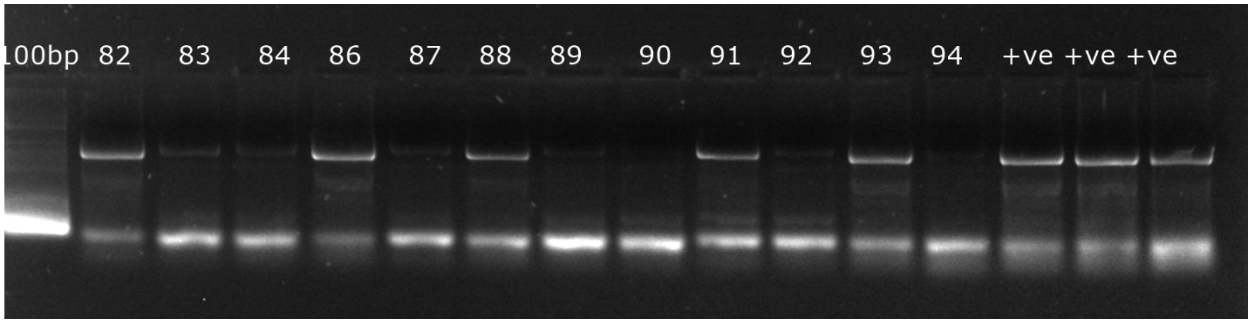

Fig. 12. EACMV indexing; positive samples 82, 86, 88, 91, 93

10.3. One-step qRT-PCR TaqMan assay for CBSV and UCBSV

Primers and probes

Primer description	Primer name	Primer sequence (5'-3')	Reference
Ugandan cassava brown streak virus	UCBSV-CP-F	AGATYAAGAARACDTTCAAGCCTCCAA	Shirima et al., 2017
	UCBSV-CP-R	AATTACATCAGGRGTTAGRTTRTCCCTT	Adams et al., 2013
	UCBSV-PROBE	FAM- TCAGCTTACATTTGGATTCCACGCTCTCA- BHQ1	Adams et al., 2013
Cassava brown streak virus	CBSVQ-F	GCCAACTARAACTCGAAGTCCATT	Adams et al., 2013
	CBSVQ-R	TTCAGTTGTTTAAGCAGTTCGTTCA	Adams et al., 2013
	CBSVQ-P	FAM- AGTCAAGGAGGCTTCGTGCYCCTC -BHQ1	Adams et al., 2013
Cytochrome oxidase	COX forward	CGTCGCATTCCAGATTATCCA	Weller et al., 2000
	COX reverse	CAACTACGGATATATAAGRRCCRRAACTG	Weller et al., 2000
	COX probe	FAM-AGGGCATTCCATCCAGCGTAAGCA-TAMRA	Weller et al., 2000

Kit: TaqMan kit Maxima Probe/ROX qPCR Master Mix, Thermo scientific, Germany
M-MLV Reverse Transcriptase, Invitrogen by Thermo scientific, Germany

CBSV assay

2x Fermentas MixMaxima	12.5μl
CBSV F primer	0.75μl
CBSV R primer	0.75μl
CBSV probe	0.75μl
M-MLV RT	0.15μl
H20	5.1μl
Total RNA[#]	5 ul
Total vol.	**25 ul**

[#]For detection only, undiluted RNA was used while for quantification RNA was diluted to final concentration 20 ng/μl.

UCBSV assay

2x Fermentas MixMaxima	12.5 μL
UCBSV F primer	1 μL
UCBSV R primer	1 μL
UCBSV probe	1 μL
M-MLV	0.15 μL
H_20	4.35 μL
Total RNA[#]	5 μL
Total vol.	**25 μL**

COX assay

2x Fermentas MixMaxima	12.5 μL
COX F primer	1 μL
COX R primer	1 μL
COX probe	1 μL
M-MLV	0.15 μL
H_20	5.1 μL
Total RNA[#]	5 μL
Total vol.	**25 μL**

Set up

- Run CBSV and UCBSV samples in duplicate
- Run one COX reaction for every sample
- Run positive, negative and non-template and no RT controls in every run

One-step reverse transcription qPCR thermal cycling program:

- 43 °C, 30 minutes, 1 cycle
- 95 °C, 2 minutes, 1 cycle
- 95 °C, 15 seconds; 60 °C, 30 seconds; 72 °C, 30 seconds, 40 cycles

Acknowledgements

The authors are grateful to Dr. Rudolf Shirima, IITA-Tanzania, for critical comments.

References

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Appendix 1

Agar media consists of MS basal media, sugar and purified agar. The MS media can either be purchased ready-made (eg. Sigma-Aldrich) or can be prepared from individual ingredients (see below).

Method:

1. If using MS basal media from individual ingredients, make this up by preparing 100 mL of solutions A to F according to Table 1. Add a further 500 mL of sterile distilled water and add 30 g sugar, dissolve and make up to 1000 mL (1 litre).
2. Gently heat, and at the same time add the appropriate amount of agar according to Table 2. Keep mixing and stirring as you add the agar and bring to boiling point to ensure a homogenous mixture.
3. Dispense into autoclaved test tubes or baby jars up 1-2 cm depth.
4. Seal with aluminium foil or cap.
5. Autoclave the media in a test tube or baby jar.
6. Leave at room temperature or fridge? to cool for approximately 4 hours to 1 day.
7. Don't be tempted to make too much media as if it is not stored in a sterile environment, it could lead to contamination. Use media within five days.

Table 1: MS Basal Media

Stock Solution		Salts / Vitamins	g/L (in stock solution)	Amount of stock solution per litre of MS media
A	1	NH_4NO_3	33 g	**50 mL**
	2	KNO_3	38 g	
B	3	$MgSO_4.7H_2O$	37 g	**10 mL**
	4	MnSO4.4H20	2.23 g	
	5	$ZnSO_4.7H_2O$	0.86 g	
	6	$CuSO_4.5H_2O$	0.0025 g	
C	7	$CaCl_2.2H_2O$	44 g	**10 mL**
	8	KI	0.083 g	
	9	$CoCl_2.6H_2O$	0.0025 g	
D	10	KH_2PO_4	17 g	**10 mL**
	11	H_3BO_3	0.62 g	
	12	$NaMoO_4.2H_20$	0.025 g	
E	13	$FeSO_4.7H_20$	2.785 g	**10 mL**
	14	Na_2-EDTA	3.725 g	
F	15	Inositol	10 g	**10 mL**
	16	Glycine	0.2 g	
	17	Nicotinic acid	0.05 g	
	18	Pyridoxine-HCL	0.05 g	
	19	Thiamine-HCL	0.01 g	

Table 2: Meristem media and multiplication media for cassava (PH = 5.7)

Component	**Meristem media (per liter)**	**Multiplication media (per liter)**
MS* basal media	4.43 g or 100 mL	4.43 g or 100 mL
Sugar (from supermarket)	30 g	30 g
Purified Agar	7 g	8 g

*Murashige and Skoog

Appendix 2

Sterile techniques

- Always sterilize the laminar flow before use with Dettol followed by 70% ethanol
- Laminar flow hoods can also be sterilized using UV
- The laminar flow should be kept in a clean room with limited accessibility
- Always sterilize scalpels in ethanol followed by flaming
- Autoclave test tubes and baby jars

Managing a growth room

It is important that the growth room is kept as clean as possible to minimize contamination.

- Accessibility should be limited
- Use double doors
- Change shoes before entering - shoes MUST be clean
- Wear a clean lab coat
- Minimize clothing such as sweaters or large coats
- Before touching anything spray your hands with 70% ethanol or Dettol
- Clean twice per week with Dettol, soap and/or bleach
- Always go to the lab first, then the glasshouse, not the other way around