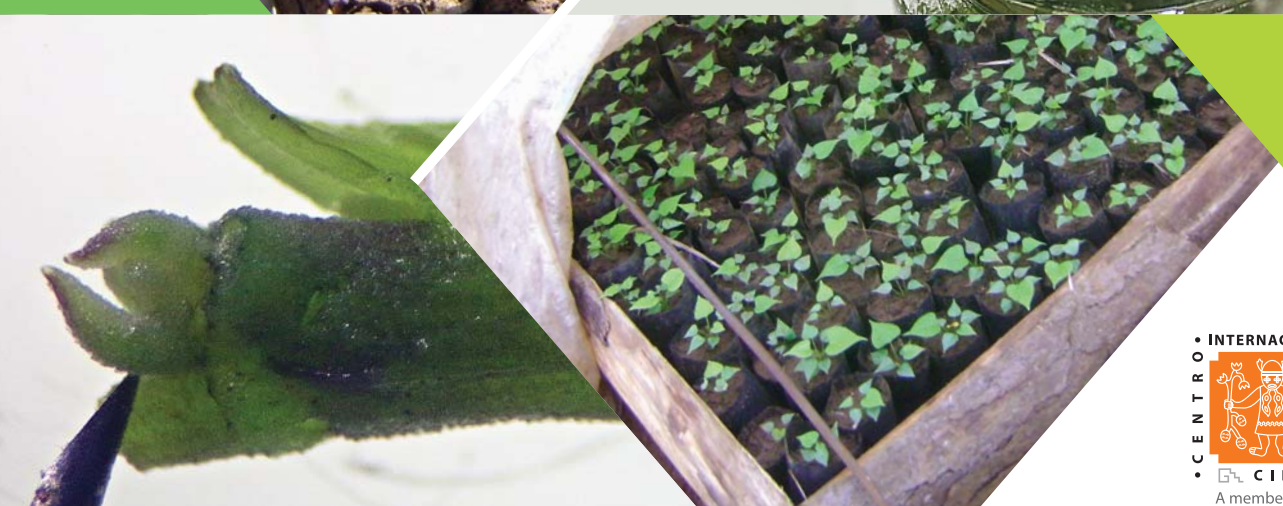


CENTRO INTERNACIONAL DE LA PAPA
CIP
A member of the CGIAR Consortium

Micropropagation and hardening sweetpotato tissue culture plantlets

A manual developed from the SASHA Project's experience in Tanzania

S. Namanda, R. Gatimu, S. Agili, S. Khisa,
I. Ndyetabula, and C. Bagambisa

KEPHIS

SASHA
Sweetpotato Action for Security and Health in Africa

LZARDI
Lake Zone Agricultural Research & Development Institute

Micropropagation and hardening sweetpotato tissue culture plantlets
A manual developed from the SASHA Project's experience in Tanzania

ISBN: 978-92-9060-469-3
DOI: 10.4160/9789290604693

International Potato Center
P.O. Box 1558, Lima 12, Peru
cip@cgiar.org • www.cipotato.org
Produced by CIP-Sub-Saharan Africa Regional Office (SSA), Nairobi

Correct citation for the entire manual:
Namanda, S., Gatimu, R., Agili, S., Khisa, S., Ndyetabula, I., Bagambisa, C., (2015): Micropropagation and hardening sweetpotato tissue culture plantlets. A manual developed from the SASHA Project's experience in Tanzania. International Potato Center (CIP), Lima, Peru. vii, 39 pp.

Production Coordinators and Editors
Margaret McEwan, Christine Bukania and Bramwel Wanjala

Design and Layout
Christine Bukania
Communications Department, Covers

Printing
Straight Jacket Media Limited

Press run: 100
September 2015

PREFACE

Sweetpotato is an important food and cash crop in sub-Saharan Africa (SSA), because it is tolerant to adverse growing conditions and has a short growing period. However, sweetpotato farmers in Africa have problems accessing healthy planting material. Often, they rely on sprouts from un-harvested roots left in the ground from the previous season. Such planting material may have high levels of sweetpotato virus infection, hence leading to considerable reduction in yields. Providing adequate supplies of improved quality of planting material at the right time is vital for the increased production and utilization of sweetpotato. Notably, in the Lake Zone of Tanzania, sweetpotato virus diseases exert enormous pressure on the growing crop. The use of pathogen tested tissue culture plantlets as source material is one approach to improving the quality of planting material available to farmers. Tissue culture is a promising technology, however it is costly and some critical steps are required for the successful transfer of *in vitro* plantlets to field conditions for further multiplication. There had been limited efforts to test the use of this method at large scale as part of a sweetpotato seed system intervention.

Between 2010 and 2012 the International Potato Center (CIP) through the Sweetpotato Action for Security and Health in Africa (SASHA) project, partnered with the Lake Zone Agricultural Research and Development Institute (LZARDI), the Kenya Plant Health and Inspectorate Service (KEPHIS), Catholic Relief Services (CRS) and CRS-affiliated community development organizations to initiate an intervention for the promotion of timely dissemination of quality planting material. The intervention, popularly referred to as 'Marando Bora' involved the successful cross border transfer of 35,000 sweetpotato tissue culture plantlets by road from Nairobi (Kenya) to Bukoba (Tanzania), a distance of about 1,500km, and the subsequent acclimatization (or hardening)process.

This manual is based on the experiences and lessons from the implementation of the SASHA Marando Bora project in Tanzania. It seeks not only to bridge the gap in information and capacity, but also to generate interest and guide similar efforts elsewhere in SSA. It shares the hands-on practices for: the initiation and mass propagation of tissue culture plantlets, transportation, hardening and field establishment. Factors that increase the survival and growth of plantlets into field plants are highlighted. This manual is intended for scientists and technicians who are responsible for the successful transfer of sweetpotato tissue culture plantlets for field multiplication and who are working in limited resource settings in SSA. The manual will also provide seed system practitioners in general, an overview of the tissue culture based procedures required for germplasm management and exchange so as to ensure that the quality of seed available to farmers is improved.

ACKNOWLEDGMENT

Successful consultations were held between Margaret McEwan, CIP Research Leader for the Marando Bora project and Jan Low, team leader for the Sweetpotato Action for Security and Health in Africa (SASHA) project for financial and logistical support.

The document was compiled by a team of researchers and technicians from CIP, LZARDI and KEPHIS who participated in the transfer of plantlets and wanted to share their experiences and lessons. The team was led by Sam Namanda (CIP Mwanza), assisted by Sammy Agili, Rosemary Gatimu (CIP Nairobi), Stephen Khisa (KEPHIS Nairobi), Innocent Ndyetabula and Concessa Bagambisa (LZARDI Maruku). Technical comments were provided by: Ted Carey (CIP-Ghana), Robert Mwanga (CIP-Uganda), Zakayo Kinyua (Kenyatta University, Kenya), and Settumba Mukasa (Makerere University, Uganda). The document was edited by Christine Bukania, Bramwel W. Wanjala and Margaret McEwan (CIP-SSA),

LZARDI proposed the varieties to be included in the project. KEPHIS cleaned-up and provided virus indexed material to Genetic Technologies International Limited (GTIL), Kenya for micro-propagation. The tissue culture plantlets were then transferred to LZARDI for hardening at Maruku Agricultural Research Institute. Primary (or pre-basic) multiplication was carried out at Agricultural Research Institute and Ukiriguru Research Station. Catholic Relief Services (CRS) coordinated secondary multiplication with implementing partners to generate adequate quantities for distribution to Decentralized Vine Multipliers (DVMs). The implementing partners were Community Based Organizations (CBOs): Diocese of Shinyanga, Mwanza Rural Housing Project (MHRP), Rulenge Diocesan Development Office (RUDDO), Tanzania Home Economics Association (TAHEA), Buhemba Rural Agriculture Centre (BRAC), and Kituo cha Mafunzo ya Kuboresha Mazingira na Kilimo Adilifu (KIMKUMAKA). These CBOs supervised multiplication in preparation for subsequent dissemination to farmer beneficiaries.

This manual should be cited as follows:

Namanda, S., Gatimu, R., Agili, S., Khisa, S., Ndyetabula, I., Bagambisa, C., (2015): Micropropagation and Hardening Sweetpotato Tissue Culture Plantlets. A Manual developed from the SASHA Project's experience in Tanzania. International Potato Center (CIP), Lima, Peru. vii, 39 pp.

Technical comments provided by: Carey, E., Mwanga, R., Kinyua, Z.M. and Mukasa, S.B.

Edited by: Bukania, C., Wanjala, B.W. and McEwan, M.A.

ACRONYMS

BAC	Biotechnology Action Council
BETCEN	Biotechnology Education and Training Center
BRAC	Buhemba Rural Agriculture Centre
CBOs	Community-based organizations
CIP	International Potato Center
CRS	Catholic Relief Services
DVM	Decentralized Vine Multiplier
KEPHIS	Kenya Plant Health Inspectorate Service
KIMKUMAKA	Kituo cha Mafunzo ya Kuboresha Mazingira na Kilimo Adilifu
LZARDI	Lake Zone Agricultural Research and Development Institute
MHRP	Mwanza Housing and Rural Project
NB	Nutritive Broth
RUDDO	Rulenge Diocesans Development Office
SASHA	Sweetpotato Action for Security and Health in Africa
SMTA	Standard Material Transfer Agreement
SSA	Sub Saharan Africa
TAHEA	Tanzania Home Economics Association
UNESCO	United Nations Educational Scientific and Cultural Organization

Table of Contents

PREFACE I
ACKNOWLEDGMENT II
ACRONYMS III
GLOSSARY VI
PART ONE: INTRODUCTION 1
1.1. About the manual 1
1.2. Importance of sweetpotato 2
1.3. Status of sweetpotato seed systems in East Africa 2
1.4. Acclimatizing plantlets for open field conditions 3
1.5. Hardening Sweetpotato tissue culture in Tanzania – The experience of the SASHA Project 4
2. PART TWO: SOURCING EXPLANTS AND MICROPROPAGATION OF PLANTLETS 6
2.1. Introduction to general laboratory procedures and equipment requirements 6
2.1.1. Facilities, equipment and supplies 6
2.1.2. Preparatory work: media and labels 8
2.2. Sourcing healthy material for micropropagation 8
2.3. Care of mother plants as source of explants 8
2.4. Preparation of mother plants as source of explants 8
2.5. Excising and introducing explants to *in vitro* culture 9
2.6. Bacteria detection test of *in vitro* culture 12
2.7. Incubating plantlets in the laboratory 13
2.8. Sub-culturing node cuttings in the laboratory 16
3. PART THREE: HARDENING-OFF OF PLANTLETS IN PREPARATION FOR FIELD MULTIPLICATION 17
3.1. Introduction 17
3.1.1. Facilities, equipment and supplies 18
3.1.2. Preparation of reception area and shade structure 19
3.2. Stage 1*: In vitro* hardening-off of plantlets in the laboratory 19
3.2.1. Preparation and packaging for transport 20
3.3. Stage 2: *In vitro* hardening-off of plantlets in the reception area 21
3.3.1. Handling more stressed *in vitro* plantlets 21
3.3.2. Handling less stressed *in vitro* plantlets in the reception area 22
3.4. Stage 3: Transfer of plantlets from *in vitro* to *in vivo* conditions in screen house 23
3.4.1. Preparing plantlets for transfer from *in vitro* to *in vivo* in sterile compost soil 23
3.4.2. Transplanting into polytubes and adaptation under high relative humidity tents . 24
3.5. Management of potted plants outside the high relative humidity tents 27
3.6. Establishment of net protected beds 27
4. Transfer to open multiplication beds 28
ANNEXES 30
Annex 1: Tissue culture media for sweetpotato micropropagation and conservation 30
Annex 2: Overview of thermotherapy and meristem culture techniques 32
REFERENCES 35

List of Figures

Figure 1: Flowchart: Sourcing explants and micropropagation of plantlets 7
Figure 2: Flowchart: Hardening-off of plantlets in preparation for field multiplication 19
Figure 3: Flowchart: Transfer to open field multiplication 28

List of Tables

Table 1: Facilities, equipment and supplies for sourcing explants and micropropagation of plantlets . 6
Table 2: Data record sheet on progress of growth and reproduction of explants during incubation .. 15

List of Photos

Photo 1: Sweetpotato mother plants for sourcing explants 10
Photo 2: Excising, trimming storing and labeling explants from the screen house to carry to the laboratory 11
Photo 3: Preparing shoot tips and node cuttings for tissue culture (adapted from Dennien et al.) 12
Photo 4: Contaminated cultures showing fungal and bacterial infections 14
Photo 5: Mass propagation and packing for transit 20
Photo 6: Examples of stressed (e.g. etiolated) plantlets received at Maruku Agricultural Research Institute 21
Photo 7: Plantletsstored on tiered shelves in the reception area at Maruku Agricultural Research Institute: Partially opened containers gradually reduces relative humidity and allows in more carbon dioxide 22
Photo 8: Preparing the plantlets for transfer from *in vitro* to vivo in sterile compost soil 25
Photo 9: Transplanting into polytubes and maintaining high relative humidity in polytents 26
Photo 10: Polythene cover fully removed after 8 days of gradual opening 27
Photo 11: Plantlets transferred to net protected beds 29
Photo 12: Field beds (1.2 m wide x 6 m long) for establishment of sweetpotato primary beds at Maruku Agricultural Research Institute, Tanzania 29
Photo 13: Cutting out the meristem 34

GLOSSARY

Acclimatization The process through which a plantlet adjusts to a gradual change in its environment (such as a change in temperature, humidity, photoperiod, or pH), allowing it to maintain performance across a range of environmental conditions (see also weaning; hardening-off).

Explant Tissue aseptically obtained and prepared from the donor plant for culture (flower stalk, leaves, roots)

Hardening-off Adapting plants to outdoor conditions by gradually withholding water, lowering the temperature, increasing light intensity, or reducing the nutrient supply. The hardening-off process conditions plants for survival when transplanted outdoors. The term is also used for gradual acclimatization to *in vivo* conditions of plants grown *in vitro*, e.g., gradual decrease in humidity. See also: acclimatization; free-living conditions (see also weaning; acclimatization).

In vitro Living in test tubes, outside the organism or in an artificial environment, typically in glass vessels in which cultured cells, tissues, or whole plants may reside.

In vivo The natural conditions in which organisms reside. Refers to biological processes that take place within a living organism or cell under normal conditions.

Micropropagation The practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods.

Tissue culture A general term used to describe the culture of cells, tissues or organs in a nutrient medium under sterile conditions.

Virus indexing Testing of plants for the presence or absence of viruses.

Weaning The process by which the plantlets are moved from a high-humidity, low light, warm environment to what would be considered a normal growth environment for the species in question (see also acclimatization; hardening-off).

PART ONE: INTRODUCTION

1.1. About the manual

The experience of moving the largest ever consignment of sweetpotato tissue culture plantlets in SSA, and the entire process of generation, transportation, hardening and transfer of tissue culture plantlets to establish field multiplication has provided lessons that are worth sharing. A review of existing literature has shown that there is limited documentation of the experiences of applying this technology in low resource settings such as Tanzania, which is typical of other countries in SSA. Although tissue culture technology is a promising tool that can provide clean planting material, farmers are yet to fully benefit from the technology. This is due to the high costs of initial investment, lack of capacity to produce and maintain clean planting material, and multipliers' lack of access to appropriate information on how to manage the transfer and hardening of *in vitro* plantlets to open field conditions (Baguma 2008). Sharing documentation on this pilot initiative can guide similar interventions elsewhere in SSA.

The manual presents step by step guidance for the successful micropropagation, hardening and transfer of tissue culture plantlets for subsequent field multiplication. The guidance in the manual draws from practical lessons learned during the Marando Bora project, supported by references from literature. The aim is to contribute to more efficient handling and management of sweetpotato tissue culture in resource limited contexts.

The manual is divided into three parts:

PART ONE: Introduction, general background and field experiences from the SASHA Marando Bora project.

PART TWO: Sourcing explants and micropropagation of plantlets. This covers procedures for: using mother plants of **known health status** as a source of explants; excising and introducing explants to *in vitro* culture; and sub-culturing plants in the laboratory. Procedures for dealing with plants of **unknown health status** such as thermotherapy and meristem culture are well covered elsewhere in literature, so this topic is only briefly discussed in this manual.

PART THREE: The hardening or weaning process. Preparatory steps for the construction of the hardening shade and reception of *in vitro* plantlets are explained: The first stage of hardening which is an initial adaptation period of *in vitro* plantlets in the **laboratory** prior to shipment; the second stage where the *in vitro* plantlets are transferred into a **reception area** and gradually exposed to higher light and lower humidity conditions and; a third stage where the *in vitro* plantlets have been transferred *in vivo* in **the screen house**. Part three includes recommendations for the establishment of net tunnels for conservation and transfer to open field beds for multiplication.

1.2. Importance of sweetpotato

Sweetpotato is the sixth most important food crop in the world after rice, wheat, potatoes, maize, and cassava; and the fifth most important food crop in developing countries after rice, wheat, maize, and cassava (FAOSTAT, 2010). Sweetpotato is one of the most widely grown root crops in Sub-Saharan Africa (SSA), covering around 2.9 million hectares with an estimated production of 12.6 million tonnes of roots in 2007 and expanding faster than any other major food crop (Low et al., 2009).

The crop is particularly important in East and Central Africa. It is a staple in Rwanda, Burundi, and Uganda, and plays a major role in food security in Kenya, Tanzania and the Democratic Republic of Congo. Elsewhere in SSA, sweetpotato has traditionally been grown on a small scale as a secondary food crop that is critical for food security during periods of prolonged drought or when catastrophes lead to food shortages. In parts of West Africa, sweetpotato is becoming an important commercial crop.

Sweetpotato combines a number of advantages which gives it a potential role in combating food shortages and malnutrition that may increasingly occur, especially as population growth exerts pressure on available land (Gibson 2009). Sweetpotato is typically a smallholder's crop, often grown on marginal soils with limited inputs (Namanda et al., 2011). It takes a short time to reach maturity and can thrive in areas with short rainy periods and in prolonged dry conditions. It also responds favourably to inputs such as fertilizer and irrigation (Namanda et al., 2011). An average yield of 7 t/ha at 30 percent dry matter for sweetpotato is relatively better than that achieved by some other major crops in developing tropical countries including maize (1 t/ha), rice (2 t/ha), sorghum and millet (<1 t/ha each) (UBOS 2010). Total crop failure due to adverse weather conditions is rare, so many farmers plant sweetpotato as a fall back to provide food for their families in case of emergency (Namanda et al., 2011).

Sweetpotato provides significant amounts of energy and protein. When compared to other crops in the developing world, its production efficiency of edible energy is outstanding (Woolfe, 1992). A number of varieties that are good sources of beta carotene, the precursor of vitamin A (Yanggen and Nagujja 2006, Pfeiffer and McLafferty 2007), have been released into the seed systems pipeline (Mwanga et al., 2004, Mwanga et al., 2009, Ssemakula et al., 2013).

1.3. Status of sweetpotato seed systems in East Africa

One of the key constraints to sweetpotato productivity in SSA is lack of sustainable seed systems - including improved disease management, seed quality and supply (Low et al., 2009). In some production areas, planting material completely desiccates, resulting in lack of planting material at the beginning of the subsequent rainy season (Low et al., 2009). The traditional seed sourcing methods fail to provide adequate planting material at the right time for planting (Gibson 2009, Namanda et al., 2011). In addition, with traditional seed sourcing methods, there is a higher risk of occurrence of disease and weevil infestation (Stathers et al., 2005), causing food insecurity due to crop losses (FAO 2012).

The crop is vegetatively propagated using vine cuttings as planting material sourced from standing crops or volunteer plants (Gibson 2009). Re-cycling the planting material accumulates systemic pathogens due to exposure to virus vectors such as aphids and whiteflies. Single and complex infection of sweetpotato virus diseases can contribute to subsequent yield decline (Adikini *et al*., 2015) of up to 99 percent (CIP 2005). This has also been noted by a number of authors including Bryan *et al*., 2003; Clark & Hoy 2006; Mukasa *et al*. 2006, Clark 2007; Gasura *et al*. 2009, Lewthwaite *et al*., 2011. In Uganda for example, varieties such as Ejumula released in 2004 (Mwanga *et al*., 2004) have been abandoned by farmers due to poor yield (CIP, 2014).

Improved seed systems have a proven track record in raising the productivity of clonal crops (Murashige 1978); for example, the adoption of virus free planting material increased average yield by 30 percent in Ghana (FAO 2013) and by more than five times in Uganda (CIP 2014). The likelihood of farmers to consume sweetpotato increases with their participation in projects promoting conservation and use of quality sweetpotato planting material (Okello *et al*., 2015).

1.4. Acclimatizing plantlets for open field conditions

Tissue culture micropropagation technology has a number of advantages over traditional plant vegetative propagation techniques. Key among them is the fact that it results in production of disease free planting material, leading to improved yield and quality (Vuylsteke and Swennen 1992; FAO, 2003). Unfortunately, the increased use and efficiency of micropropagation is still restricted by the high percentage of plants which are lost or damaged when transferred to *ex vitro* conditions (Pospíšilová *et al*. 1992). Several protocols for micropropagation have been developed, but only a few have been demonstrated at field level. Each of these protocols involves several steps, among which rooting and acclimatization are critical for successful micropropagation (Chabukswar and Deodhar, 2005).

During *in vitro* culture, plantlets grow in closed containers under controlled humidity, light, nutrient and aseptic conditions. The closed vessels limit the inflow of carbon dioxide and outflow of gaseous plant products. The enriched propagation media decreases the water potential of the plant medium. These conditions, together with the use of growth regulators, result in plantlets with abnormal morphology, anatomy and physiology (Kozai 1991, Pospíšilová *et al*. 1992, 1999, Buddendorf-Joosten and Woltering 1994, Desjardins 1995, Kozai and Smith 1995). The plantlets have to correct these abnormalities as they are transferred from *in vitro* to *in vivo* conditions in the greenhouse or in the field where there is much higher irradiance or light intensity, low relative humidity, higher water potential in the substrate and unrestricted water loss from the leaves (Pospíšilová *et al*., 1999). Thus, the plantlets need a transitional period of acclimatization during which temperature, relative humidity, light intensity, carbon dioxide concentration and air flow rates are adjusted (Hayashi *et al*., 1988).Van Huylenbroeck and De Riek (1995) described two stages: an adaptation period of slow shoot growth and root formation; and a period of fast growth of roots and shoots.

1.5. Hardening sweetpotato tissue culture in Tanzania – The experience of the SASHA Project

There was no facility in Tanzania to provide sufficient quantities of virus indexed tissue culture plantlets; so these had to be sourced from KEPHIS Nairobi and transported to Maruku Agricultural Research Institute in Bukoba, Tanzania. More than 35,000 plantlets of different varieties including Ejumula, Kabode, Polista, Ukerewe, Jewel, and Mataya were transferred by road from Kenya to Tanzania, in four different batches, with an interval of about 45 days between successive transfers. Each transfer of materials consisted of paper boxes packed with a total of 10,000 plantlets in plastic containers (holding ten plantlets each) loaded into a station wagon. It took at least three days to cover the 1,500 km to Bukoba. Road transport was selected because it cost significantly less than airfreight, and the care of the plantlets could be more closely supervised. The vehicle had to be cleared by customs, and the plantlets were inspected by plant health services at the border crossing. These mandatory clearances contributed to extended travel days, which in turn resulted in the delicate plantlets being subjected to longer stress periods.

Ordinarily, tissue culture plantlets have an initial three to seven day period of acclimatization under controlled laboratory conditions at 30-40% relative humidity, so that the leaves regain normal stomatal functioning (Conner and Thomas 1982). The recipient research institute was beginning with plantlets that had spent about three days in transit by road. Therefore, on arrival the *in vitro* plantlets were directly transferred into the hardening process.

Maruku Agricultural Research Institute did not have established facilities for hardening the large numbers of plantlets other than an old small screen house nursery where coffee seedlings used to be handled.

During implementation the following factors were found to be of vital importance:

- The cost effectiveness of the structure: Ahloowalia and Prakash (2004); Ahloowalia and Savangikar (2004) indicated that the high cost of establishing facilities, the unit cost of production of micropropagated plants, and often, the return on investment is not proportional to the potential economic advantages of the technology. Hence, alternatives to expensive inputs and infrastructure have been sought and developed to reduce the costs of plant micropropagation. It is however important to ensure that the low cost options lower the cost of production without compromising the quality of plantlets.
- The light intensity is adequately moderated and the plantlets are protected from rain and heavy wind. Plantlets are transferred to an appropriate substrate (e.g. sand, peat, compost), and gradually hardened (Ahloowalia and Prakash 2004). Low cost options such as plastic domes or tunnels, which reduce the natural light intensity and maintain high relative humidity during the hardening process, can be used.
- Pest-free conditions are maintained, especially to avoid cutworms, fungal contaminants and plant infections. Fungicides have also frequently been used to guard against pathogenic attack when transplanting tissue cultured plants (Conner and Thomas, 1982).

Thus, an advance technical team had earlier been dispatched from CIP in Nairobi, to work with colleagues at Maruku Agricultural Research Institute to rapidly establish procedures and structures for receiving and hardening the plantlets before transfer to open field multiplication. In view of the need to immediately start delivering plantlets and considering the lack of adequate facilities, a decision was made to adapt the existing coffee screen house nursery for the initial reception and handling; and construct an additional temporary structure for hardening the plantlets.

Since the coffee screen house which was to act as the initial reception room had very limited space inside, wooden tier shelves were made to increase the capacity of the plantlets that could be accommodated. *In vitro* plantlets in their original transit containers were placed in this coffee screen house for initial recovery from stress which was mainly as a result of the long distance trip. Thereafter, they were moved to the newly constructed temporary shade structure for final hardening before subsequent transfer to the net tunnels for conservation and open field multiplication.

Wooden box frames using wood off-cuts, (measuring 1 m wide and 6 m long), were constructed. A metal frame was also erected over each box in order to tightly fix a polythene cover and thus create tents with high relative humidity. The plantlets were transferred from the *in vitro* media to *in vivo* polytubes filled with sterile soil. The polytubes were then placed in the wooden boxes, in the polythene tents. This process facilitated the hardening of the plantlets before transfer to protected net tunnels for conservation, and then to open beds for field multiplication.

The low-cost, hardening shade structure was constructed using locally available materials including papyrus mats for walls, and palm fronds for roofing. The perimeter walls were reinforced with papyrus mats to ensure adequate protection of the plantlets from strong blowing side winds. The top was covered with palm fronds and bamboo leaves to minimize possible direct sunlight and heavy rain drops. The inside roof and side walls of the temporary shade were reinforced using transparent polythene sheeting to minimize insect entry and protect plantlets against direct raindrops. The ground surface was made level and firm, and pesticide dust was sprinkled on the side of the floor to ward off possible pests including millipedes that would cause plant destruction.

2. PART TWO: SOURCING EXPLANTS AND MICROPROPAGATION OF PLANTLETS

2.1. Introduction to general laboratory procedures and equipment requirements

Plant tissue culture procedures require aseptic techniques; the laboratory technician must perform all media and tissue preparation operations using sterile conditions to avoid introduction of foreign pathogens. Should aseptic conditions not be strictly maintained, the nutrient enriched media could favour the growth and multiplication of bacteria pathogens that would over-run the cultured plants.

Good laboratory techniques include:

- Wearing clean protective clothing and covering head to keep hair away from the cultures
- Cleaning laminar flow chambers prior to and after use; if possible keep separate laminar flow chambers for “dirty” and “clean” material use
- Keeping fingers or other body parts down-stream and away from cultures
- Keeping tools, such as scalpels and forceps, clean and sterile through frequent dips in 70% ethanol, followed by heat treatments

2.1.1. Facilities, equipment and supplies

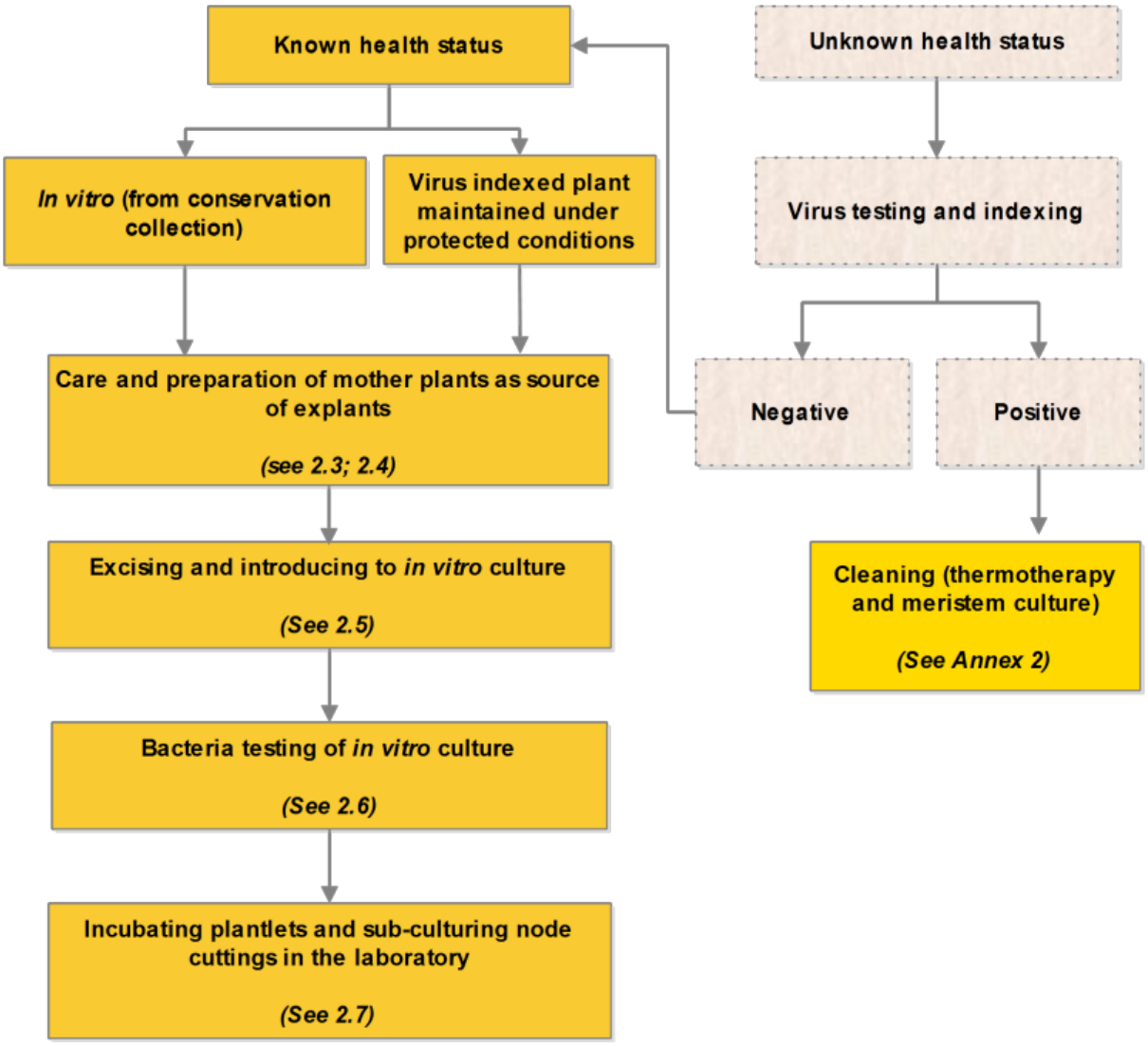
Make sure you have the standard list of facilities, equipment and supplies outlined in Table 1.

Table 1: Facilities, equipment and supplies for sourcing explants and micropropagation of plantlets

Facilities
▶ Tissue culture laboratory with growth room. Reliable electricity and water supply ▶ Screen house or temporary shade
Equipment
▶ Laminar flow chamber ▶ Autoclave ▶ Refrigerator ▶ Microwave ▶ Oven ▶ PH-meter ▶ Weighing balance ▶ Weighing bowls ▶ Bacticinerator, bead sterilizer, spirit lamp, Bunsen burner or candle and matches
Supplies
▶ Sterilized forceps and scalpel with blades ▶ Micro-pipettes ▶ Petri-dishes ▶ Jars or test-tubes

- Sterile 150ml bottles
- Measuring cylinders
- Plastic plant tags or sticky labels and pencil, or barcode system
- Laboratory-grade ethanol (70%) or isopropyl alcohol
- Sterile distilled water in 450-600 ml screw-capped bottles
- Tween 20 ® (a common detergent used in biology)
- Paper towels/cotton wool for swabbing benches and other surfaces with ethanol
- Saran wrap and Para film
- Ingredients for propagation media

Figure 1: Flowchart: Sourcing explants and micropropagation of plantlets



2.1.2. Preparatory work: media and labels

The small pieces of plant in tissue culture may not be able to manufacture all food requirements through the natural process. Therefore, the technician should ensure that the artificial growth medium contains plant regulators that will produce desired growth, including rapid shoot multiplication. The technician should also ensure that media pH of 5.7 is used to enable effective growth of plants and the activity of the plant growth regulators. Annex 1 shows tissue culture media for sweetpotato micropropagation and conservation.

Advance preparation of labels using barcodes is preferable, but if this is not possible, you should use a pencil or permanent marker and plastic plant tags or sticky labels.

2.2. Sourcing healthy material for micropropagation

This manual follows the micropropagation process using virus indexed source material (i.e. of known "clean" health status). This includes tissue culture plantlets which have been virus-indexed and conserved following established protocols (CIP [OP58], [OP21],[OP22], and [OP23],) or *in vivo* virus indexed "mother plants" which have been maintained in screen house conditions following established protocols. If the health status of the *in vitro* or *in vivo* material is unknown, it must be tested for sweetpotato viruses and if found positive, undergo virus cleaning through thermotherapy, meristem culture (see Annex 2) or other method, and then re-tested and virus indexed.

2.3. Care of mother plants as source of explants

Mother plants must be of known health status, i.e. pathogen tested and virus indexed. Mother plants must be kept in a screen house (preferably also in a screen box for double protection) to prevent infestations of insects and to minimize growth of microorganisms such as fungi and bacteria on the plants (CIP [OP58], 2010). Systems must be in place to regularly test and verify the health status.

Provide mother plants with adequate nutrition for vigorous growth. Water them from the bottom (by adding water to a saucer or tray in which the pots or polythene bags are standing), to prevent soil splashing onto the shoots (Baguma *et al*., 2010). Watering plants in this way reduces contamination of the shoots by bacteria and fungi and increases their chances of successfully being cultured in the laboratory.

2.4. Preparation of mother plants as source of explants

Seven days before initiating the process of sourcing explants, spray the plants with Ridomil ((R)-2-[(2, 6-dimethylphenyl)-methoxyacetylamino]-propionic acid methyl ester), Phenylamide fungicide, Mefenoxam (47.6%) at 2 g/litre of water against fungal infections (CIP [OP58] 2010).[1]

Select healthy mother plants grown for two months. The explants should be young, healthy looking and vigorous shoots on plants with green (not brown) nodes.

[1] The type of broad spectrum chemical approved for use will vary from country to country and it will also determine the amount of water used.

2.5. Excising and introducing explants to *in vitro* culture

Refer to CIP [OP58], 2010 and Dennien *et al*., 2003

1. Disinfect the scissors or scalpel blade by dipping it in ethanol/alcohol and flaming.
2. In the screen house, remove the leaves from a vine stem containing at least six buds, leaving only the first fully open leaf and the developing leaves (Baguma *et al*., 2010).
3. Cut stem segments containing one bud each and place them in a paper bag or plastic container. Take measures to prevent the explants wilting after removal.
4. Move the explants to the laboratory. Rinse the explants with running tap water three times to remove the surface dust.
5. Cut off the shoot tip, together with 100-150 mm of stem. Cut single nodes from the same vine, leaving 5–10 mm of stem above and below the bud.
6. Remove any remaining petioles, cutting them about 5 mm from the stem.
7. Place a maximum of 10 shoot tips and/or trimmed nodes, of the same variety, in a clean, sterilized 150 ml bottle or container.
8. Wipe the top of each container with the explants with ethanol or alcohol and then transfer to the laminar flow chamber.
9. Flush with 150 ml of 70% alcohol for 30-60 seconds. This removes the wax from the explant. Discard the alcohol and then rinse with sterilized water (CIP, 2010). Add 150 ml of 2.5% sodium hypochlorite solution; then wait 5-15 minutes.
10. Remove the explants from the hypochlorite solution and rinse with 150 ml sterilized water five times, shaking the container between each cycle. The first and second rinses are done immediately after discarding the hypochlorite solution, the third and fourth rinses are done after five minutes and the fifth rinse is done 10 minutes later. In the last cycle do not discard the water, so that the explants do not dry out.
11. Remove the explants from the water, shake off excess water and then rest them in a sterilized Petri dish, on a tile, or on a paper towel.
12. Using a blade, remove tissue bleached by the hypochlorite treatment (the tissue turns white). Place the explant on propagation medium MPB in an 18 x150 mm test tube. Culture one explant per vessel. In between each vine, disinfect the scissors or scalpel blade by dipping in ethanol/alcohol and flaming; allow instruments to cool before continuing. Repeat this procedure with the shoot tips and nodes of the other varieties.
13. Label test tubes with name of variety, date of initiation and name of technician. Use a pencil to write the variety name on a thin plastic plant tag, cut to the appropriate size and stick it on the outside of the bottle. Use a pencil when writing on tags because it does not drop ink[2].
14. Incubate cultures in a growth room at 25°C (+/- 2 °C) with a photo period of 16 hours of light and 8 hours dark.

[2] If available a barcode system can also be used for labelling.

NOTE:

- Before using the laminar flow chamber, you should place the equipment inside and then turn on the UV lights for 20–30 minutes. After this, wipe the inside of the cabinet with ethanol/alcohol. Do not forget to turn off the UV lights before you start work.
- Sterilize all the vines of one variety at a time.
- Double check all labels to ensure that they are correct; a mistake made here will be difficult to correct later.

Photo 1: Sweetpotato mother plants for sourcing explants

Photo: S. Namanda

Photo 2: Excising, trimming storing and labelling explants from the screen house to carry to the laboratory

Photo: C. Bukania

Photo 3: Preparing shoot tips and node cuttings for tissue culture (adapted from Dennien et al.)

Photo: C. Bukania

2.6. Bacteria detection test of *in vitro* culture

The following steps are used to carry out *in vitro* culture bacteria testing **CIP [OP53], 2010:**

1. After one week, evaluate cultures visually in order to detect bacteria and fungi contamination. Incinerate all vessels that are contaminated with bacteria or fungi.
2. For remaining cultures, select and submit clones to the bacteria detection test using nutritive broth (NB) after 4 to 5 weeks of culture.
3. Select one test tube per accession and under aseptic conditions, take out plantlet and place on a Petri dish. Remove the leaves and roots and cut the stem in 2-3 nodal segments.
4. Transfer isolated explants to 18 x 150 mm test tubes containing propagation medium MPB.
5. Cut residual material (basal part of plantlet) finely until a mince of plantlet leaves, stems and roots is obtained.
6. Inoculate this mince of residual material and a little portion of culture medium into 16 x 150 mm test tubes containing 3 m of NB.

7. Incubate the cultures at 30°C for 72 hours. Evaluate bacteria presence at 24, 48 and 72 hours. Negative cultures follow additional culture at room temperature. When results of the NB bacteria detection test are obtained, select a vigorous and bacteria negative clone and discard the other tubes.
8. If the health status of the selected plantlet is already known (i.e. virus indexed), then sub-culturing for micropropagation can be started. If it is of unknown status then testing for virus identification, thermotherapy and meristem culture techniques should be undertaken before micropropagation (See Annex 2).

2.7. Incubating plantlets in the laboratory

The following steps are based on Dennien *et al*., 2003, CIP [OP59], 2010

1. When putting lids on vessels, screw them on firmly and then turn them back a quarter turn. This will allow gas exchange with the outside, and prevent the build-up of ethylene, which is produced by the plantlets. Ethylene build-up can be lethal to plants.
2. Incubate the cultures on a bench, on shelves or in a growth room at 25 ± 2 ºC under lights of 5,000–10,000 lux. The following setup has been found to be satisfactory:
    a. Six fluorescent lights: a mixture of three 'grow' lights (at least one blue and one red) and three white lights, providing a light intensity of about 5,000–10,000 lux a day/night;
    b. Setting of 16 hours light and 8 hours dark lights placed vertically to give the plants even lighting, or lights placed horizontally above each of the shelves.
3. When the lights go off at night, temperatures cool slightly, and culture vessels and lids shrink; when lights come on during the day, culture vessels and lids swell. As a result of these changes, air enters the culture vessels. The culture room, benches or shelves where the cultures are kept must therefore be kept as clean as possible. To reduce the chance of contamination, access to the culture room should be limited to essential inspections.
4. Check the cultures after 3-5 days, and then regularly check them for contamination. If present, bacterial contamination may be seen after a few days. Colonies of bacteria often form at the base of the stems inside the culture medium. They appear as opaque haloes that develop rapidly into a mass of various colours, often orange-pink, green-black or black. Although fungal contamination may be seen after a few days, it usually is not seen for a week or so. It appears as furry, fuzzy colonies with no clearly defined edges growing on the surface of the culture medium. Discard any cultures that are contaminated; do not try to save them by attempting to remove the infection with bleach or fungicide.

Photo 4: Contaminated cultures showing fungal and bacterial infections

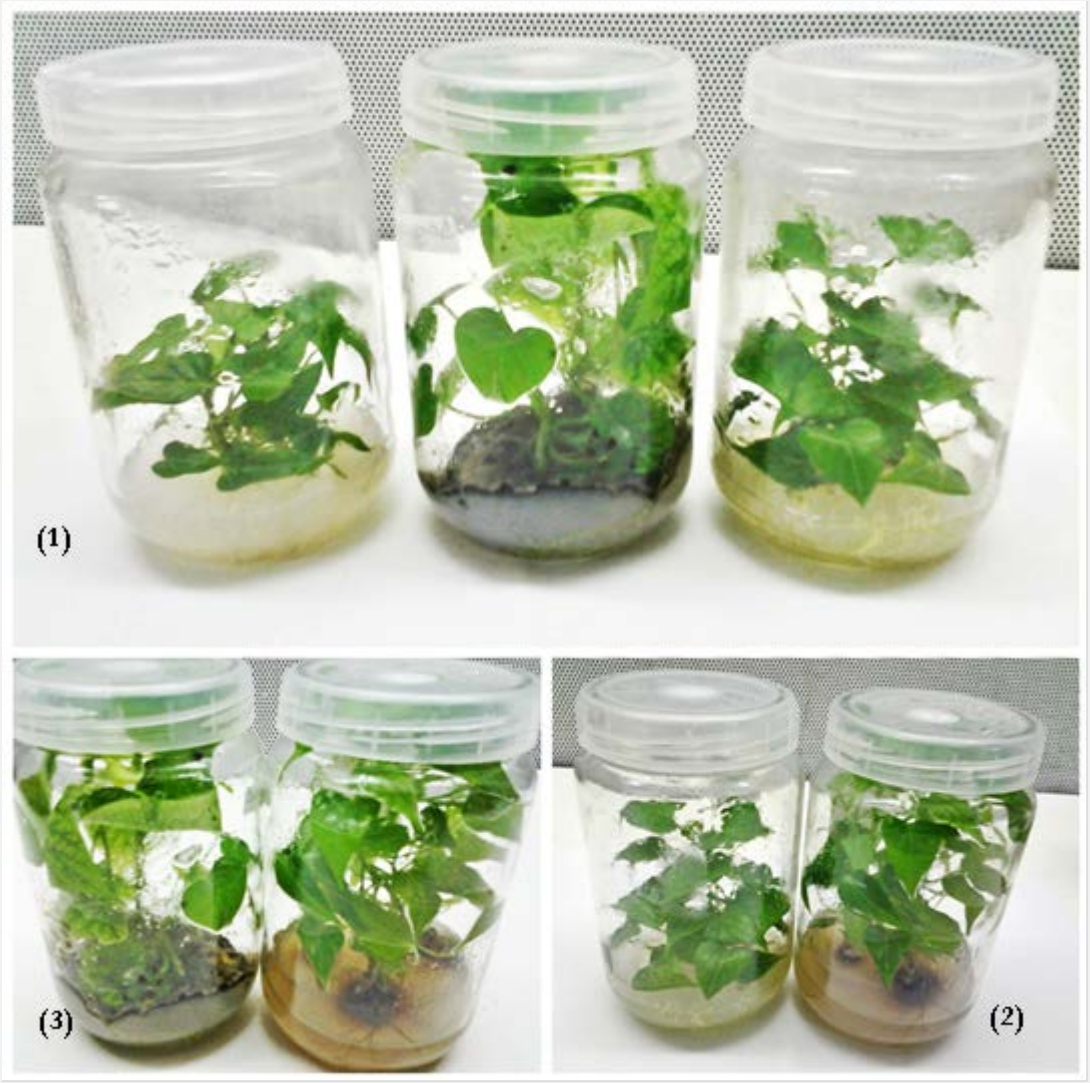


Photo: R. Gatimu

(1) Healthy culture (left and right) and contaminated cultures showing black-coloured fungi (centre)

(2) Healthy culture (left) and orange-coloured bacterial infections (right)

(3) Black-coloured fungal infection (left) and orange-coloured bacterial infection (right)

The record sheet below helps the technician keep records on progress of growth and reproduction of explants during incubation.

Table 2: Data record sheet on progress of growth and reproduction of explants during incubation

Week	Cultures/explants					
	# clean cultures	# contaminated (discarded)	# with new shoots	Mean # of shoots	# with roots	Mean # roots
1						
2						
3						
4						
5						
6						
Additional notes						

2.8. Sub-culturing node cuttings in the laboratory

Single node cuttings are used for rapid generation of new plantlets. Each of these nodes has buds, which can be cultured. The technician can repeat the process over and over again to produce a large number of buds, each capable of producing a whole plant. During sub-culturing, leaves and roots are removed, and each stem is cut into single nodes. The pieces are then transferred into fresh culture medium. The following procedures are based on (CIP [OP59] 2010).

1. Under aseptic conditions, take plantlets out of *in vitro* vessels, and place them on a Petri dish using sterilized forceps. It is recommended that you use 4-5 week-old plantlets which have been grown in propagation medium MPB.
2. Use a sterilized knife handle with a No. 10 blade to remove the leaves and roots, and cut the stem cleanly in several segments of approximately 1.5 cm containing 1-2 buds.
3. Place one explant per 13 x 100 mm tube, two explants per 18 x 150 mm and 25 x 150 mm tubes, or 5 per magenta vessel on the surface of the medium, with the bud facing upwards[3].
4. Seal the tubes with a gas-permeable plastic tape (e.g. Saran wrap) and label correctly. Use Saran wrap to seal culture tubes for multiplication; for the last sub-culture before distribution, seal the containers with air-tight Para film.
5. Incubate cultures in a growth room at 25°C +/- 2 °C, with a photoperiod of 16 hours of light and 8 hours of dark.
6. After 4-5 weeks of *in vitro* culture, visually evaluate plantlets for growth development and bacteria/fungi contamination. Incinerate all plantlets with abnormal growth or with contamination.
7. Distribute plantlets with normal development or use them in a new *in vitro* multiplication cycle.

[3]NB: for micropropagation two explants can be placed per 18 x 150 mm test tube; unlike during initiation where one explant per test tube is recommended to avoid cross contamination.

3. PART THREE: HARDENING-OFF OF PLANTLETS IN PREPARATION FOR FIELD MULTIPLICATION

3.1. Introduction

Sweetpotato tissue culture plantlets are usually much smaller and held in more precisely controlled environments than nursery seedlings or cuttings. They are cultured on nutrient media under aseptic conditions, and grown in high humidity [about 100%] (Conner and Thomas 1982). Under such conditions the plantlets have: i) fewer palisade cells – i.e. not able to produce their own food; ii) poor vascular connection between roots and shoots and thus reduced water conduction; and iii) poorly developed cuticle or waxy layer, which results in great water loss through evaporation when the plantlet is transferred to a less humid *in vivo* environment (Thiant, 2003). Since the plantlets are highly vulnerable to environmental stress, carefully controlled hardening-off procedures are necessary for their survival. Stress could be caused by prolonged hours in darkness, which causes etiolation and yellowing; shocks due to erratic actions during transportation; and possible depletion of the nutrient medium.

Hardening or acclimatization is weaning the plants from the protective, sterile, humid climate through a transitional phase to enable them to survive the harsh, dry climate (Thiart, 2004, Baguma *et al*, 2010). It involves slowly weaning the plantlets from a consistent high humidity, low light, warm environment and prepares them for a natural growth environment, thus to the environment with variable humidity (Baguma *et al*, 2010). It is the most delicate stage because it subjects the plantlets to dehydration, nutrient loss and root or stem damage (Pospíšilová *et al*., 1999; Pospíšilová *et al*., 1992; Baguma *et al*, 2010).

According to (Diaz-Perez *et al*., 1995a), *In vitro* root development usually enhances transplanting success because functioning roots can create a favourable plant water balance. In order to effectively reduce mortality rates, Conner and Thomas (1982) recommended that culture vessels should be placed in a greenhouse for up to 10 days before plantlet removal, to allow for some acclimatization to greenhouse light and temperature regimes prior to transplanting. It has also been found that by leaving the plantlets in shade for 3-6 days, they can be conditioned to their new environment through exposure to diffused natural light (Ahloowalia *et al*., 2004). Oggema et al., (2007) reported that if plantlets are planted in plastic polythene bags and kept under high relative humidity polythene tents for 7 days, and watering is maintained, the plants will have developed an efficient root system, produced new leaves and become photo synthetically active (Thiant, 2003).Thus, hardening phases are intended to minimize water loss, facilitate regaining of normal stomatal functioning to regulate transpiration, facilitate rooting. These minimize rapid wilting during transplantation and enhance survival rates at establishment (Conner and Thomas 1982).

Commercial application of micropropagation of plants is still limited by high production costs and the often high mortality rate during the *in vivo* hardening phase, when micropropagated plants adapt to normal photoautotrophic conditions (Vyas *et al*., 2008, Zobayed *et al*., 2000, Dhawan and Bhojwani, 1986).

Some protocols for micropropagation - each involving several steps - have been developed, but only a few have been demonstrated at field level (Chabukswar and Deodhar 2005). Van Huylenbroeck and De Riek (1995) described two stages: an adaptation period of slow shoot growth and root formation; and fast growth of roots and shoots.

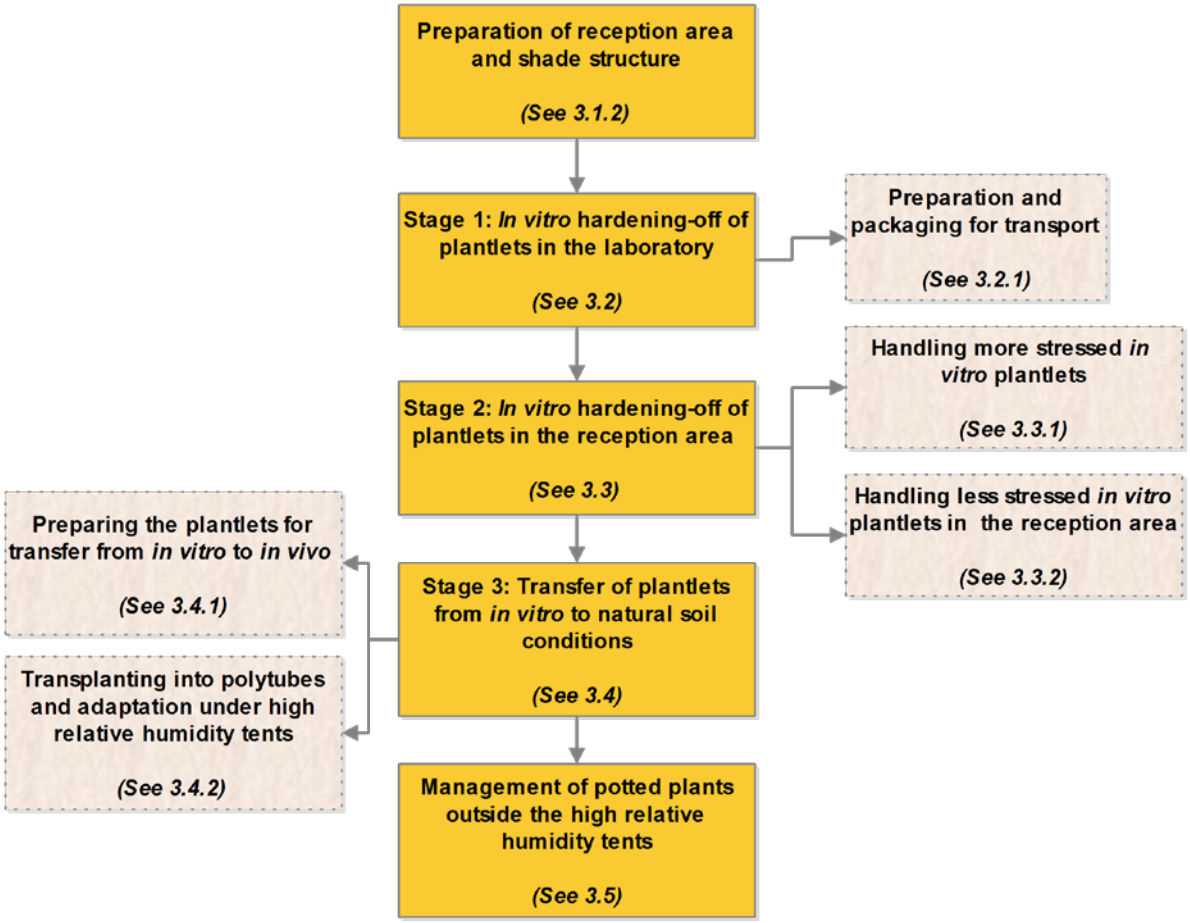
The following sections of the manual presents three stages of the hardening or weaning process: an initial adaptation period of *in vitro* plantlets in the **laboratory** prior to shipment; the second stage where the *in vitro* plantlets are transferred into a **reception area** and gradually exposed to higher light and lower humidity conditions and when there is slow shoot growth and root formation; a third stage where the *in vitro* plantlets have been transferred *in vivo* in **the screen house or temporary shade** and where there is faster growth of roots and shoots. After this, cuttings can be taken for multiplication in open beds; or conservation in net protected beds.

3.1.1. Facilities, equipment and supplies

Ensure that you have the equipment, materials and supplies listed in the table below for clean and successful handling during transfer of plantlets to sterilized compost medium.

Facilities
▶ Tissue culture laboratory ▶ Reception room ▶ Screen house or temporary shade ▶ Wooden box frames for arranging potted plants after transplanting. Each frame should measure 2 m long by 1.2 m wide and accommodate 400 to 500 polytubes.
Equipment and supplies
▶ Thermometer ▶ Transparent polythene covering sheets ▶ Semi-circular bent iron bars ▶ Polytubes (5cm diameter and 10 cm long) ▶ 3 basins ▶ Soap ▶ Laboratory-grade ethanol (70%) or isopropyl alcohol ▶ Sterile soil compost mix (3:2:1 loam, manure, gravel) or agromix to serve as potting medium ▶ Cotton wool moistened with 70% alcohol ▶ Nationally approved fungicide e.g. Benlate or Benomyl in recommended quantities ▶ Detergent ▶ Gloves ▶ Jars or test-tubes ▶ Stick of pencil thickness ▶ Clean water ▶ Spray bottle for misting inside of tunnel chambers

Figure 2: Flowchart: Hardening-off of plantlets in preparation for field multiplication



3.1.2. Preparation of reception area and shade structure

A week prior to receiving the plantlets clean and disinfect the reception area to reduce the possibility of contamination. Check to ensure that the light (e.g. translucent roof) and temperature conditions (e.g. 22-24°C) are adequate to facilitate the initial recovery process. If necessary, construct wooden shelves to increase the number of plantlets that can be handled in the reception area. When using a temporary shade structure, check and repair any damages to the polythene sheeting or netting. Place foot baths at the entrance of the reception area and shade structure.

Equipment and supplies (e.g. sterilized potting compost, polytubes, insecticide, protective clothing) should be sufficient for the expected number of plantlets.

Procure the necessary handling materials for use during transfer, such as gloves and alcohol, and ensure that there is running, sterile tap water.

3.2. Stage 1: *In vitro* hardening-off of plantlets in the laboratory

Tissue culture plantlets do not rely on photosynthesis; instead, they use sucrose as a source of energy. Therefore, an early hardening-off process could start while the plants are still *in vitro*. This process could gradually adjust the plantlets to rely on photosynthesis, and activate the stomata so that loss of water through the cuticle is minimized (Zobayed *et al*., 2000). In the laboratory, plantlets can be hardened through the following measures either singly or in combination:

a) Lowering the mineral salts (e.g. half strength MS media);
b) Increasing the concentration of the gelling agent. This has the effect of hardening the root structure;
c) Changing the concentration of sucrose (higher or lower) in the growth media (Pospíšilová *et al.*, 1999). By increasing the sucrose content the plantlet stabilizes, does not grow so fast and becomes more robust. By reducing the sucrose content the plantlet struggles to locate nutrients, with the effect of hardening the root structure;
d) Lowering the relative humidity in the vessel to stimulate the formation of the wax layer on the leaves limits the loss of water through evaporation.

3.2.1. Preparation and packaging for transport

When preparing plantlets for transportation, transfer them into propagation media MPB of the same composition but a firmer consistency. Place between five to seven plantlets in each plastic container and seal with air-tight Para film. Pack the plantlets in well-labelled carton boxes.

Plantlets are often transported by road. Therefore, it is important to take measures to protect them from damage e.g. by labelling which side of the carton should face upwards.

Prepare all the necessary transportation documentation in good time. When transporting plantlets within the country, ensure that you have obtained a phytosanitary statement. However, when transporting them across a national border, in addition to the statement, you will require a phytosanitary certificate, an import permit from the importing country, and a Standard Material Transfer Agreement (SMTA). Ensuring that you have the right documentation could significantly reduce the amount of time required to get customs clearance, and hence, reduce the time that the delicate plantlets will be subjected to stress.

Photo 5: Mass propagation and packing for transit

Photo: R. Gatimu

3.3. Stage 2: *In vitro* hardening-off of plantlets in the reception area

Upon arrival, offload the packing boxes in the reception area, which has already been prepared. Sterilize the outside of the boxes and the surface to be used with mist spray of alcohol before opening and removing the culture vessels from the packing boxes. Sort the culture vessels into those with plantlets which are visually more stressed and less stressed categories before arranging them on the tiered shelves.

Place the culture vessels in an upright position, which exposes them to light and temperatures under the translucent roofing. The reception area facilitates the urgent recovery of the plantlets that are not in good physiological state, after transportation.

3.3.1. Handling more stressed *in vitro* plantlets

This category includes visually etiolated, yellowed and weakened, as well as overgrown plantlets (Photo 6); or those that have spent 56-72 hours being transported by road.

Leave the containers with such plantlets sealed for at least 5-7 days after arrival, under high temperatures (25-30 °C) and as high relative humidity as possible. Maintaining the vessels closed influences the growth of the *in vitro* plants and keeps out microorganisms (Pospíšilová *et al*. 1999).

After about a week, proceed to handle the plantlets the same way that unstressed plantlets would be handled.

Photo 6: Examples of stressed (e.g. etiolated) plantlets received at Maruku Agricultural Research Institute

Photo: S. Namanda

3.3.2. Handling less stressed *in vitro* plantlets in the reception area

Less stressed cultures, especially those with little or no visual shoot browning can be kept at 22-24^{o}C room temperature and 1000 lux of illumination provided by the translucent roofing for two weeks. Keep the light in the room evenly distributed during the whole period to attain uniform growth of plantlets. These conditions allow the plantlets to recover from detrimental effects of darkness (etiolation, chlorosis and tissue necrosis due to phenolic oxidation), and vehicle shocks after a long journey.

After 2-7 days of storage, open the lids gradually to reduce the relative humidity towards the ambient environment that exists outside the containers. To allow in more air circulation, the lid covers should be systematically opened by loosening partially to a quarter opened; half opened (Photo 7) on the fourth day and fully opened on the fifth day after reception.

The partial opening of the containers containing plantlets gradually lowers the relative humidity, in turn stimulating growth of the wax layer (protective) coating on the leaves. This minimizes water loss through evaporation from the plantlets, and maintains a sterile condition until transfer to the final hardening shade.

Photo 7: Plantlets stored on tiered shelves in the reception area at Maruku Agricultural Research Institute: Partially opened containers gradually reduces relative humidity and allows in more carbon dioxide

Photo: S. Namanda

3.4. Stage 3: Transfer of plantlets from *in vitro* to *in vivo* conditions in screen house

The plantlets are transferred from the tissue culture medium into polytubes with potting medium, in the shade structure. The nature of soil mixes used for transplantation can influence both survival rates and subsequent growth. The soil mix (3:2:1 loam, manure, gravel) should be porous enough to prevent water-logging. The soil mix should be disinfected and sanitized e.g. by steam. Fungicides (e.g. nationally approved formulations, applied in recommended amounts) have frequently been used to guard against pathogenic attack when transplanting tissue cultured plants. The polytubes with the transplanted plants should be placed in wooden frames covered in closed tent-like structures made from polythene, designed to maintain high relative humidity. Intermittent mist can be used to maintain the relative humidity and moderate temperatures of between 20^{o}C and 27^{o}C.

In case you are planning to transplant a large number of plantlets, ensure that there is adequate labour available to complete the activity quickly, so as to avoid long exposure of plantlets to possible infection.

3.4.1. Preparing plantlets for transfer from *in vitro* to *in vivo* in sterile compost soil

1. Wearing protective gloves disinfected with alcohol spirit to avoid possible immediate contamination of the plantlets, remove healthy rooted plantlets from the vessel and gelling agent by gently pulling them out.
2. Use a basin of clean water to carefully wash the plantlets. Do this by dipping them in and out of the clean water to remove gelling agent adhered onto roots.
3. Wash the plantlets in a second basin of clean water to ensure that the entire gelling agent is thoroughly washed off and the roots are not broken. The sucrose-containing culture medium needs to be thoroughly washed off from around the roots to avoid its colonization by micro-organisms associated with damping off.
4. Trim the very long roots (Thiant, 2004). These would be damaged during planting or would die off because they are not functional, and frequently lack root hairs. Trimming will also allow new roots to form once the plant has established.
5. Pre-treat the plantlet by lightly dipping the root base in a nationally recommended fungicide or a short time. This will prevent any possible fungal infection (Van Vuuren, 1996; Thiant, 2004).
6. Avoid wetting the whole plantlet during the washing process and dipping in the fungicide solution.

3.4.2. Transplanting into polytubes and adaptation under high relative humidity tents

See ***Photo 9***

1. Put sterile and free draining compost soil mix (Thiant 2004) into polythene bags (5 cm diameter and 10 cm long) and moisten with water.
2. Make a pencil thickness hole in the compost soil mix.
3. Holding by the stem, insert each plantlet into the hole and then press lightly around the base for the plantlet to hold firm and upright.
4. Carefully arrange the planted polytubes in wooden box frames constructed on the ground. Each frame should accommodate 400-500 polytubes.
5. Insert waterproof labels indicating variety, source and date of transfer against each bed.
6. Apply mist water and then close the tent roof tightly using the polythene sheeting to encourage build-up of high relative humidity, and to minimize loss of water from the plants through evaporation.
7. Inspect daily to ensure that the polythene cover is tightly sealed and that there is adequate moisture and relative humidity build-up in the polytents. In case the relative humidity build-up is not sufficient, it could be an indication that either the tightness of the roof covering is not adequate, or the polythene has holes, or the initial mist watering was not adequate.
8. The screen house or temporary shade structure (palm frond, mat, and grass thatch) moderates the sunlight and temperatures for the polytents.
9. Gradually open the polytent by initially removing a quarter cover after three days, half on the fourth day, three-quarter on the sixth day and then fully on the eighth day.
10. Regular watering of the plants in the polytubes should start once the polytent has been fully opened.

Photo 8: Preparing the plantlets for transfer from *in vitro* to vivo in sterile compost soil

Photo: R. Gatimu

Step 1: Gently pulling the plantlets from the agar

Step 2: Washing off artificial media: First washing basin showing more intensity of agar removed from plantlets (*NB: The contents are disposed of and replaced with clean water in case of washing other plantlets continues)*

Step 3: Rinsing off artificial (structural) culture media: Second washing basin with light intensity of agar removed from plantlets (NB: Also replaced when it gets dense with agar in case more plantlets are to be washed)

Step 4: Fungicide solution in which washed plantlets are dipped before planting in compost soil

Photo 9: Transplanting into polytubes and maintaining high relative humidity in polytents

Photo: S. Namanda

(1) Plantlets are transferred into polytubes

(2) Planted transplants are ready to be covered

(3) Polytents in a low-cost screen house at Maruku Agricultural Research Institute are being covered

(4) Plantlets in polytents under high relative humidity

3.5. Management of potted plants outside the high relative humidity tents

After seven to eight days, the polytents will be fully opened. Regular watering should start and be maintained at three times a week for a month. Apply nationally approved foliar fertilizers when the plants are well established to stimulate faster plant growth. Apply approved insecticides, such as Fenitrothion (O, O-Dimethyl O-(3-methyl-4-nitrophenyl to control cutworms, aphids, and other pests that would invade the plants. The humidity is gradually reduced and sunlight is increased until the plant is hardened off fully. When the vigorously growing healthy plants have attained at least four to six new leaves, it is ready to be transplanted (as either the whole plant or cuttings) to the open field for multiplication or as cuttings to net tunnels for conservation.

Photo 10: Polythene cover fully removed after 8 days of gradual opening

Photo: S. Namanda

3.6. Establishment of net protected beds

Basic (foundation) planting material can be conserved under net tunnels (see brochure) for up to six seasons. Each season the material can be planted out in open field multiplication to provide seed for sale to farmers.

4. Transfer to open multiplication beds

Sites for establishment of the primary field beds (for basic seed production) should be located at a distance of not less than 50 m for basic seed class or 20 m for quality declared seed class from existing sweetpotato production fields. A 5m wide barrier crop of maize or sugar cane can also be planted to minimize potential transfer of sweetpotato virus infection by whiteflies or aphids.

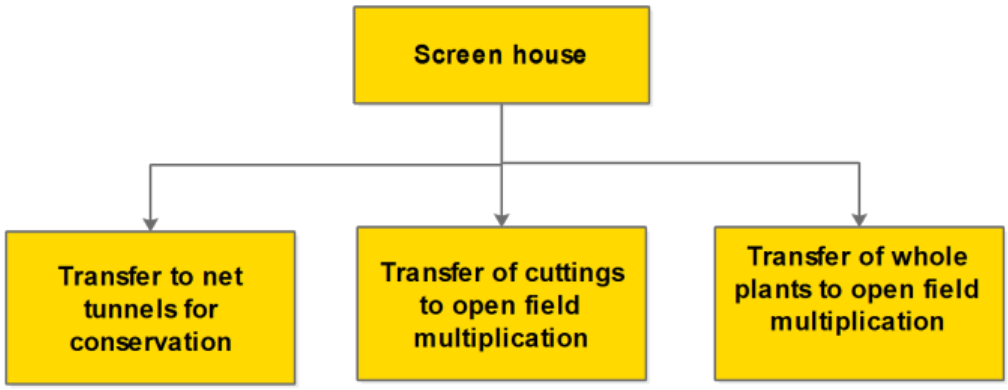
For basic seed and quality declared seed the rotation period should be six and two seasons respectively. The site should be well drained, located near a reliable source of water for irrigation in case of a dry spell, fertile, and free from noxious weeds such as couch and nut grass (Cyperaceae), which are difficult to weed.

A pre-planting compound fertilizer (NPK 25:5:5)[4] at the rate of 100 gm per m^2 (or as recommended after soil analysis) could be incorporated in to the soil to stimulate vigour, especially in sites with marginal soil fertility. If organic compost is used it should be sterilized to minimize the risk of being a source of pests and diseases.

The following procedure should be followed when transferring to field beds:

1. Plant five rows per bed at the spacing of 20 cm between lines and 10cm within the row or a total of 300 plants per bed of 1.2 m wide x 5 m long.
2. In case of dry spells, water the plants either early in the morning or late in the evening (depending on the weather condition) two to three times a week.
3. Ensure that the beds are weed free. Diseased plants, especially sweetpotato virus infected plants should be rogued out as soon as they are identified.

Figure 3: Flowchart: Transfer to open field multiplication



[4] In-country composition of NPK will vary. Seek local expertise

Photo 11: Plantlets transferred to net protected beds

Mrs. Tabitha Sumira next to her net tunnel, which is closed by tying the two ends with a manila string, Kasenya/Katwe village, Sengerema district, Tanzania.

Photo: K. Ogero

Photo 12: Field beds (1.2 m wide x 6 m long) for establishment of sweetpotato primary beds at Maruku Agricultural Research Institute, Tanzania

Photo: S. Namanda

ANNEXES

Annex 1: Tissue culture media for sweetpotato micropropagation and conservation

	MM3	MCB	MPB
MS salts (g/L)	4.3	4.3	4.3
Ascorbic acid (g/L)	0.1	0.2	0.2
Calcium nitrate (g/L)	0.1	0.1	0.1
Calcium panthotenate (mg/L)	2	2	2
Gibberellic acid (mg/L)	10	-	10
L-Arginine (g/L)	0.1	0.1	0.1
Putrescine-HCl (mg/L)	20	20	20
Sucrose (g/L)	30	30	30
Coconut milk (mL/L)	10	-	-
Agar (g/L)	-	-	-
Phytagel (g/L)[5]	2.8	3	3
pH	5.7	5.7	5.7

Ingredients for preparation of nutritive broth media

Peptone (g/L) 5, beef extract (g/L) 1, yeast extract (g/L) 2, glucose (g/L) 10, sodium chloride (g/L) 5 and pH 7.0. These are mixed in 1 litre of distilled water, stirred until dissolution is complete, the pH measured and the medium is distributed; 3 ml in 16x150 mm test tubes.

[5] There are alternatives for Phytagel, which could vary from lab to lab.

Annex 2: Tissue culture media for sweetpotato micropropagation and conservation

STOCK SOLUTION		SALTS/VITAMINS	gms/lit in stock solution	amount stock/L
Stock 1	1	NH_4NO_3	33.0g	50ml/l
	2	KNO_3	38.0gms	
Stock 2	3	MgSO4.7H2O	37.0gms	10ml/l
	4	MnSO4.H2O	2.23gms	
	5	$ZnSO_4.7H_2O$	0.86gms	
	6	$CuSO_4.5H_2O$	0.0025gms	
Stock 3	7	$CaCL_2.H_2O$	44.0gms	10ml/l
	8	KI	0.083gms	
	9	$CoCl_2.6H_2O$	0.0025gms	
Stock 4	10	KH_2PO_4	17.0gms	10ml/l
	11	H_3BO_3	0.62gms	
	12	$NaMoO_4.2H_2O$	0.025gms	
Stock 5	13	$FeSO_4.7H_2O$	2.785gms	10ml/l
	14	Na_2-EDTA	3.725gms	
Stock 6	15	Inositol	10.0gms	10ml/l
	16	Glycine	0.2gms	
	17	Nicotinic acid	0.05gms	
	18	Pyrodoxine- HCL	0.05gms	
	19	Thiamine-HCL	0.01gms	
Calcium Pantothenate		2mg/l		
Calcium nitrate		100mg/l		
L-Argenine		100mg/l		
Ascorbic acid		200mg/l		
PutrescineHCL		20mg/l		
Gibberelic acid		10mg/l		
Sucrose		30g/l		
Agar		8g/l		

Annex 3: Overview of thermotherapy and meristem culture techniques

Thermotherapy is a heat treatment given to heal a disorder, such as disease resulting from infection by internally borne viruses or virus-like micro-organisms. The logic is to increase the temperature to a level at which the plant meristem is still able to grow, but replication of the virus is inhibited. CIP uses a thermotherapy regime of 37^0C for 16 hours in continuous light (Dodds and Ng. 1987).

Since sweetpotato plants are more tolerant to heat than the micro-organisms within them, nearly all plants treated with thermotherapy survive (El Far and Ashoub, 2009, Walkey, D.G.A. and V.C. Cooper, 1975). There are two ways of using thermotherapy to free plants from virus infection: heating plants growing in pots, or heating sterile plants growing in tissue culture. Usually plants are subjected to thermotherapy when they are 4 – 6 weeks old. When thermotherapy is complete, the meristems are removed aseptically from the shoot tips and placed in sterile tissue culture medium. The sterile tissue culture medium is then placed under lights in the laboratory. The meristem is at the tip of the shoot; it is composed of actively dividing cells, which form the tissues that become the leaves and stems.

Once the plants have regrown from meristems, the next task is to grow them in pots in the screen house, glasshouse or screened compartment, and to test them for known viruses.

There are several advantages of using meristems.

Heat accelerates the growth of the plant, and at the same time reduces multiplication of viruses. Furthermore, meristems have no vascular tissues, making it difficult for viruses to infect the cells. It is postulated that the meristem and even the young leaf primordia have not been in contact with the main vascular system; therefore, should the vascular system have virus particles, they would not easily reach the meristem. In addition, the meristematic cells can produce virus-inhibitory substances. Furthermore, the presence of hormones in the culture media may contribute to virus elimination (Dodds and Ng. 1987).

It has been reported that sweetpotato virus diseases such as Sweetpotato Feathery Mottle Virus (SPFMV) and sweetpotato virus diseases (SPVD) were eliminated from sweetpotato plants by meristem culture alone, or in combination with thermotherapy (Dodds and Ng. 1987). Whereas Karesove, R., *et al*., 2002 reported that meristem tip culture alone obtained a few virus-free plants, El Far and Ashoub, (2009); Cooper, V.C. and. Walkey, D.J.A (1978); Mink, G.I., Wample, R., and Howell, W.E. (1998), indicated that thermotherapy followed by meristem culture resulted in plants free from SPFMV.

The guidelines outlined below highlight the successive steps and precautions for the generation of aseptic plantlets, hardening and transfer to tunnels and field beds.

What equipment and supplies do you need for thermotherapy and meristem culture?

- Laboratory-grade ethanol (70%) or isopropyl alcohol
- Spirit lamp or candle, and matches
- Sharp scissors or scalpel with blades
- New plastic bags, or clean bottles soaked in household bleach (diluted to a concentration of 1.5%; see Appendix 1) for 20 minutes and rinsed well in sterile distilled water

- Permanent marker pens
- Personal protective equipment

Procedure:

1. In the high-temperature cabinet, choose a potted plant of one variety.
2. Disinfect the scissors or scalpel blade by dipping in ethanol/alcohol and flaming; allow it to cool before use.
3. Wearing gloves, and using scissors or a scalpel blade, cut off vines about 150 mm long.
4. Remove most of the petioles, cutting them 5–6 mm from the stem.
5. Place the vines in a bottle and label it.

> NOTE:
>
> - Do not add water, facial tissues or paper towels to the bags or bottles because this increases the risk of spreading contamination.
> - Repeat the same procedures on other plants of the same variety, and then all other varieties in the cabinet. Continue to ensure that scissors or scalpels are disinfected between plants.
> - Be careful to maintain a free flow of air over the plant material - make sure that bottles and other equipment are not blocking the movement of sterile air over the work surface where the meristem is being extracted.
> - Ensure that all the following steps in cutting out the meristem are followed.

The following steps are used to cut out the meristem:

1. Thoroughly clean and sterilize all equipment.
2. Remove the shoot tips approximately 10 mm from the plants using a sterilized scalpel blade, and place them in a sterile Petri dish. Since the constant air flow in the laminar air-flow cabinet will rapidly dry out the shoot tips, it is best to have only three at a time in the Petri dish.
3. Do not discard the plants in tissue culture from which the shoot tips have been taken. Place the containers back on the shelves of the growth room. If the meristems do not survive for some reason, or are found to contain virus infections, these plants can be used again.
4. Remove the meristems from the shoot tips. Do this as quickly as possible to avoid having the meristems dry out under the heat of the microscope lamp. If possible, use fibre optic ‘cold light’ illumination.
5. Use a scalpel blade to slice away the leaves around the terminal bud, leaving all but the last 2 or 3 leaf primordia surrounding the meristem.
6. Use a sterile hypodermic needle to cut off the leaf primordia, until the meristematic dome is seen; this appears as a shiny opaque dome.

7. Use a new sterile needle to cut out the meristem, which might include the youngest leaf primordium, and transfer it to the surface of the meristem medium.
8. Repeat the same procedures for each tip, making sure that you use sterilized instruments and cutting surfaces; wipe down the inside of the laminar flow cabinet with ethanol/alcohol after 2 hours' work.
9. Label the culture vessel with the collection number, date of culture, variety name, initials of the person establishing the culture, total number of cultures for each variety, date for sub-culturing (at intervals of approximately 8–12 weeks).
10. Incubate the cultures.
11. Look at the cultures after 5 days, and then check regularly for contamination. If present, bacterial contamination may be seen after a few days. Colonies of bacteria often form at the base of the stems inside the culture medium. They appear as opaque haloes that develop rapidly into a mass of various colours, often orange-pink, green-black or black. Although fungal contamination may be seen after a few days, it is usually not seen for a week or so. It appears as furry, fuzzy colonies with no clearly defined edges, growing on the surface of the culture medium. Discard any cultures that are contaminated.
12. Continue the incubation of cultures that are free from contamination. The meristems will take 8–12 weeks to develop into a plant.
13. Discard meristems that have not developed into developed into a callus instead of shoots by 12 weeks.

Photo 13: Cutting out the meristem

Photo: C. Bukania

REFERENCES

Adikini, S., Settumba, B.M., Mwanga, R.O.M., Gibson, R.W., 2015. Sweetpotato cultivar degeneration rate under high and low potato virus disease pressure zones in Uganda. *Canadian Journal of Plant* Pathology, 37: 1, 136-147.

Ahloowalia, B.S., Prakash, J., Savangikar, V.A., and Savangikar, C., 2004. Plant tissue culture. In: Low cost options for tissue culture technology in developing countries. International Atomic Energy Agency (IAEA), Vienna, Austria 2003, IAEA-TECDOC-1384, pp. 3 – 10.

Ahloowalia, B.S., and Prakash, J., 2004. Physical components of tissue culture technology. In: Low cost options for tissue culture technology in developing countries. Proceedings of technical meeting organized by FAO and International Atomic Energy Agency (IAEA) Division of Nuclear Techniques in Food and Agriculture, held in Vienna, 26 – 30 August 2002, pp. 17-28.

Ahloowalia, B.S., and Savangikar, V.A., 2004. Low cost options for energy and labour. In: Low cost options for tissue culture technology in developing countries. Proceedings of technical meeting organized by FAO and International Atomic Energy Agency (IAEA) Division of Nuclear Techniques in Food and Agriculture, held in Vienna, 26 – 30 August 2002, pp. 41-46.

Baguma, Y., Kawuki, R., Otim, M., Wandui, M.C., Mugoya, C. (eds.), 2010. Tissue culture, conservation biotechnology, virus indexing, and seed systems for vegetative crops. A training manual.

Bryan, A.D., Pesic-vanesbroeck, Z., Schultheis, J.R., Pecota, K.V., Swallow, W.H., Yencho, G.C., 2003. Cultivar decline in sweet potato: I. Impact of micropropagation on yield, storage root quality, and virus incidence in 'Beauregard'. *Journal of the American Society for Horticultural Science,* 128:846–855.

Buddendorf-Joosten, J.M.C., and Woltering, E.J., 1994. Components of the gaseous environment and their effects on plant growth and development *in vitro*. *Plant Growth Regulation*. 15: 1-16.

Capellades, M., Fontarnau, R., Carulla, C., Debergh P. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured Rosa multiflora. *Journal of the American Society for Horticultural Science,* 115: 141-145.

Cassells, A.C. and Walsh, C., 1994. The influence of gas permeability of the culture lid on calcium uptake and stomatal function in *Dianthus* microplants. *Plant Cell Tissue Organ Culture,* 37:171-178.

Chabukswar, M.M., and Deodhar, M.A., 2005. Rooting and hardening of *in vitro* plantlets of garcinia indica Chois. *Indian Journal of Biotechnology*, 4: 409 – 413.

CIP [OP26], (International Potato Center, Standard Operational Procedure), 2015. *In vitro* conservation of sweetpotato - OP026. International Potato Center, Lima, Peru. 30 pp.

CIP [OP53], (International Potato Center, Standard Operational Procedure), 2010. Elimination of bacterial contaminants from sweetpotato in *in vitro* cultures – OP53. International Potato Center, Lima, Peru 6 pp.

CIP [OP58], (International Potato Center, Standard Operational Procedure), 2015. Introduction of sweetpotato to *in vitro* culture – OP 58. International Potato Center, Lima, Peru 22 pp.

CIP [OP59], (International Potato Center, Standard Operational Procedure), 2015. *In vitro* multiplication of sweetpotato – OP59. International Potato Center, Lima, Peru 23 pp.

Clark, C.A, and Hoy, M.W., 2006. Effects of common viruses on yield and quality of Beauregard sweet potato in Louisiana. *Plant Disease*, 90:83–88.

Clark, C.A., 2007. Encyclopedia of pest management. Volume II. Pimentel D. (ed.), New York: CRC press.

Conner, A. J. and Thomas, M. B., 1982. Re-establishing plantlets from tissue culture: a review. *The International Plant Propagators' Society Combined Proceedings*, 31, 1981. pp. 342-35).

Cooper, V.C. and Walkey, D.J.A., 1978. Thermal inactivation of cherry leaf roll virus in tissue cultures of Nicotiana rustica raised from seeds and meristem tips. *Annals of Applied Biology*, 88: 273-278.

Dennien, S., Homare, D., Hughes, M., Lovatt, J., Coleman, E., and Jackson, G., 2003. Growing healthy sweetpotato: Best practices for producing planting material. Australian Centre for International Agricultural research (ACIAR), Canberra.

Desjardins, Y., 1995. Photosynthesis *in vitro* - on the factors regulating CO^2 assimilation in micropropagation systems*. Acta Horticulturae,* 393: 45-61.

Dhawan,V. and Bhojwani, S.S., 1986. Micropropagation in crop plants. *Glimpses in Plant Research,* 7: 1- 75.

Díaz-Pérez, J.C., K.A. Shakel, and Sutter, E.G., 1995a. Effects of *in vitro* formed roots and acclimatization on water status and gas exchange of tissue cultured apple shoots. *Journal of the American Society for Horticultural Science,* 120:435-440.

Díaz-Pérez, J.C., Sutter, E.G., Shackel, K.A., 1995b: Acclimatization and subsequent gas exchange, water relations, survival and growth of microcultured apple plantlets after transplanting them in soil. *Physiologia Plantarum,* 95: 225-232.

Dodds, J.H. and Ng, S.Y.C., 1987. *In vitro* methods for pathogen elimination and international distribution of sweetpotato germplasm. In: *Exploration, maintenance, and utilisation of sweetpotato genetic resources.* International Potato Center (CIP), Lima, Peru, pp.323 – 329.

EL Far, M. M.M., Ashoub, A., 2009. Utility of thermotherapy and meristem tip for freeing sweetpotato from viral infection. *Australian Journal of Basic and Applied Sciences*, 3(1): 153-159.

Food and Agriculture Organization of the United Nations (FAO). 2003. FAO statistical databases (FAOSTAT). http://apps.fao.org/default.htm. Accessed March 2004.

International Potato Center (CIP), 2014. Delivering and Disseminating Biofortified Crops (DDBC) project in Uganda. Annual report submitted to HarvestPlus. International Potato Center, Lima.

International Potato Center (CIP), 2005. Current situation of sweetpotato viruses in the world, their detection and control. Report submitted by Salazar, L.F., International Potato Center, Lima, Peru.

Ruane, J., Dargie, J.D., Mba, C., Boettcher, P., Makkar, H.P.S., Bartley, D.M., and Sonnino, A. (eds), 2013. *Biotechnologies at work for smallholders: Case studies from developing countries in crops, livestock and fish*. Food and Agriculture Organization of the United Nations (FAO).

Food and Agriculture Organization of the United Nations (FAO), 2012. Food and Agriculture Organisation of the United Nations for a world without hunger. Global information and early warning system on food and agriculture (GIEWS).

FAOSTAT, 2010. Production and area harvested statistics for sweetpotato for 2010. http://www.faostat.fao.org/site/567/default.aspx#ancor. Accessed 15 May 2012.

Gasura, E., Mashingaidze, A.B., Mukasa, S.B., 2009. Occurrence, prevalence and implications of sweet potato recovery from sweet potato virus disease in Uganda. *African Crop Science Conference Proceedings,* 9:601–608.

Gibson, R.W., 2009. Review of sweetpotato seed systems in Africa, especially East Africa. Seed Systems Working Paper 2009. International Potato Center (CIP).

Hayashi, M., Nakayama, M., Kozai, T., 1988. An application of the acclimatization unit for growth of carnation explants, and for rooting and acclimatization of the plantlets. *Acta Horticulturae,* 230: 189-194.

Hussain, A., Qarshi, I.A., Nazir, H., and Ullah, I., 2012. Plant Tissue Culture: Current Status and Opportunities. Open access chapter. http://dx.doi.org/10.5772/50568, (http:///creativecommons.org/lincenses/by/3-0).

Kanechi, M., Ochi, M., Abe, M., Inagaki, N., Maekawa, S., 1998. The effects of carbon dioxide enrichment, natural ventilation, and light intensity on growth, photosynthesis, and transpiration of cauliflower plantlets cultured *in vitro* photoautotrophically and photomixotrophically. *Journal of the American Society for Horticultural Science*, 123: 176-181. 47pp.

Kapinga, R.E., Ewell, P.T., Jeremiah, S.C. and Kileo, R., 1995. Sweetpotato in Tanzanian farming and food systems: implications for research. International Potato Center (CIP) / Ministry of Agriculture, Tanzania.

Karesova, R., Janeckova, M., and Paprstein, F., 2002. Elimination of raspberry bushy dwarf virus from raspberry cv. "Gatineau." *Acta Horticulturae,* 585: 359-362.

Kozai, T., and Smith, M.A.L., 1995. Environmental control in plant tissue culture - general introduction and overview. In: Aitken-Christie, J., Kozai, T., Smith, M.L., eds. Automation and Environmental Control in Plant Tissue Culture, pp. 301-318.

Kozai, T., 1991. Micropropagation under photoautotrophic conditions. In: Debergh, P.C., Zimmerman, R.H. (eds.), Micropropagation. Technology and application, pp. 447-469.

Lewthwaite S.L., Fletcher P.J., Fletcher J.D. and Triggs C.M., 2011. Cultivar decline in sweet potato (Ipomoea batatas). *New Zealand Plant Protection,* 64:160–167.

Low, J., Lynam, J., Lemaga, B., Crissman, I., Barker, I., Thiele, G., Namanda, S., Wheatley, C. and Andrade, M., 2009. Sweetpotato in Sub-Saharan Africa. In: Loebenstein, G. and Thottappilly, G. (eds.), The Sweetpotato, 16: 359 – 390.

Mink, G.I., Wample R., and Howell, W.E. 1998. Heat treatment of perennial plants to eliminate phytoplasms, viruses, and viroids while maintaining plant survival. In: Hadid, A., Khetarpal, R.K. and Koganezawa, H., eds. Plant Virus Disease Control, pp: 332-345. St. Paul, MN: APS Press. The American Phytopathological Society.

Mukasa, S.B., Rubaihayo P.R., Valkonen, J.P.T. 2006. Interactions between a crinivirus, an ipomovirus and a potyvirus in coinfected sweet potato plants. *Plant Pathology*. 55: 458-468.

Murashige, T., 1978. Principles of rapid propagation. In*:* Hughes, K.W.; Henke, R. and Constantin, M. (eds.), Propagation *of* Higher *Plants* through tissue culture - A bridge *between* research and application, pp 14-24, Technical Information Center, U.S. Department of Energy, Oak Ridge, Tennesse. 305 pp.

Mwanga, R, O.M., Odongo, B., Niringiye, C., Alajo, A., Kigozi, B., Makumbi, R., Lugwana, E., Namakula, J., and Mpembe, I., 2009. Release of two sweetpotato varieties (NASPOT 9 and NASPOT 10) in Uganda. *HortScience,* 44(3): 828-832.

Mwanga, R. O.M., Turyamureeba, G., Alajo, A., Kigozi, B., Carey, E.E., Niringiye, C., Kapinga, R., Makumbi, R., Zhang, D., Tumwegamire, S., Lugwana, E., Namakula, J., Abidin, P.E., Lemaga, B., Nsumba, J. and Odongo, B., 2004. Submission to variety committee for release of sweetpotato varieties. National Agricultural Research Organisation (NARO).

Namanda, S., Gibson, R.W. and Kirimi, S., 2011. Sweetpotato seed systems in Uganda, Tanzania and Rwanda. *Journal of Sustainable Agriculture* 35: 870-884.

Oggem, J.N., Kinyua, M.G., Ouma, J.P. and Owuoche, J.O., 2007. Agronomic performance of locally adapted sweetpotato (Ipomoea batatas (L) Lam) cultivars derived from tissue culture regenerated plants. *African Journal of Biotechnology,* 6 (12): 1418 – 1425.

Okello, J.J., Sindi, K., Shikuku, K., Low, J., McEwan, M., Nakazi, F., Namanda, S., Babu, A. and Mafuru, J., 2015. Effect of technology awareness and access on the conservation of clean planting materials of vegetatively produced crops: The case of sweetpotato. *Agroecology and Sustainable Food Systems,* 39 (9): 955-977.

Pfeiffer, W.H. and Mclafferty, B., 2007. Harvest-Plus: Breeding crops for better nutrition. *Crop Science,* 47 (supplement): 88 – 105.

Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D., and Plzáková, Š., 1999. Acclimatization of micropropagated plants to *ex vitro* conditions. *Biologia Plantarum,* 42 (4): 481-497.

Pospíšilová, J., Solárová, J., Čatský, J., 1992. Photosynthetic responses to stresses during *in vitro* cultivation. *Photosynthetica,* 26: 3-18.

Prakash, S., Hoque, M.I., and Brinks, T., 2004. Culture media and containers. In: Low cost options for tissue culture technology in developing countries. International Atomic Energy Agency (IAEA), Vienna, Austria 2003, IAEA-TECDOC-1384, pp. 29 – 38.

Roberts, A.V., Smith, E.F., Mottley, J., 1990. The preparation of micropropagated plantlets for transfer to soil without acclimatization. In: Pollard, J.W. and Walker, J.M. (eds.), Methods in Molecular Biology 494. Vol. 6. Plant Cell and Tissue Culture, pp. 227-236.

Savangikar, V.A., 2004. Role of low cost options in tissue culture. In: Low cost options for tissue culture technology in developing countries. International Atomic Energy Agency (IAEA), IAEA, Vienna, Austria 2003, IAEA-TECDOC-1384, pp. 29 – 38, pp. 11 – 15.

Seelye, J.F., Burge, G.K., and Morgan, R., 2003. Acclimitising tissue culture plants: Reducing the shock. In: Combined Proceedings International Plant Propagators' Society, Volume 53, pp. 85 – 90.

Short, K.C., Warburton, J., Roberts, A.V., 1987. In vitro hardening of cultured cauliflower and Chrysanthemum plantlets to humidity. *Acta Horticulturae,* 212: 329-334.

Smith, E.F., Gribaudo, I., Roberts A.V., Mottley J., 1992. Paclobutrazol and reduced humidity improve resistance to wilting of micropropagated grapevine. *HortScience,* 27: 111-113.

Ssemakula, G., Niringiye, C., Otema, M., Kyalo, G., Namakula, J., Alajo, A., Kigozi, B., Makumbi, R., Yencho, C., and Mwanga, R.M.O., 2013. Submission to variety committee for release of sweetpotato varieties. National Agricultural Research Organisation (NARO).

Stathers, T., Namanda, S., Mwanga, R.O.M., Khisa, G. and Kapinga, R., 2005. Manual for sweetpotato integrated production and pest management farmer field schools in sub-Saharan Africa. International Potato Center, Kampala, Uganda. 168pp, xxxi.

Thiart, S., 2004. Manipulation of growth by using tissue culture techniques. *Combined proceedings International Plant propagators Society*, 53:61– 67.

Uganda Bureau of Statistics (UBOS), 2010. *Uganda Census of Agriculture 2008/2009, Volume III.* Agricultural Household and Holding Characteristics report. Ministry of Agriculture Animal Resources and Fisheries (MAAIF), Entebbe Uganda.

Uganda National Bureau of Statistics, 2010. *Uganda National Census of Agriculture* (UCA).

Van Huylenbroeck, J.M., De Riek, J., 1995. Sugar and starch metabolism during *ex vitro* rooting and acclimatization of micropropagated Spathiphyllum "Petite" plantlets. *Plant Science*, 111: 19-25.

Van Vuuren, R.J., 1996. Aseptic techniques for plant tissue culture. In: *Advanced tissue culture course proceedings*.

Vuylsteke, D., and Swennen, R., 1992. Biotechnological approaches to plantain and banana improvement at IITA. p. 143–150. In: G. Thottappilly, L. M. Monti, D. R. Mohan Raj, and A. W. Moore (eds.), Biotechnology: Enhancing research on tropical crops in Africa. IITA, Ibadan, Nigeria.

Vyas, S., Nagori, R., Purohit, S.D., 2008. Root colonisation and growth enhancement of micropropagated Feronia limonia (L.) swinggle by Piriformospora indica - A root cultivable endophte. In: International Journal of Plant development Biology, Global Science Books, pp. 128 – 132.

Walkey, D.G.A., and Cooper, V.C., 1975. Effect of temperature on virus eradication and growth of infected tissue culture. *Annals of Applied Biology*, 80: 185-190.

Wardle, K., Dobbs, E.B., and Short, K.C., 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *Journal of the American Society for Horticultural Science,* 108: 386-389.

Yanggen, D. and Nagujja, S., 2006. The use of orange-fleshed sweetpotato to combat Vitamin A deficiency in Uganda: A study of varietal preferences, extension strategies and post-harvest utilization. Social Sciences Working Paper No. 2006.

Ziv, M., 1986. *In vitro* hardening and acclimatization of tissue culture plants. In: Withers, L.A. and Alderson, P.G. (eds.), Plant tissue culture and its agricultural applications, pp. 187-196. Butterworths, London - Boston - Durban - Singapore - Sydney - Toronto – Wellington.

Zobayed, S. M. A., Afreen, F., Kubota, C. and Kozai, T., 2000. Water control and survival of Ipomoea batatas grown photoautotrophically under forced ventilation and photomixotrophically under natural ventilation. *Annals of Botany* 86: 603 – 610.

INTERNATIONAL POTATO CENTER