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Tissue culture, conservation biotechnology, virus indexing and seed systems of vegetative crops

Charles Mugoya | Yona Baguma | Robert Kawuki | Michael Otim | Francis Wachira | Clet Wandui Masiga

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Foreword

Tissue culture, conservation biotechnology, virus indexing, and seed systems of vegetative crops are interventions which have been proposed to improve access to healthy seed with the premise that they will catalyze and/or sustain optimal agricultural productivity. Plant tissue culture is a technique with which plant cells, tissues or organs are grown on artificial nutrient medium, either static or liquid, under aseptic and controlled conditions. The technique enables production of clones and propagation of multiple plants within a short period of time in limited space. Hence, tissue culture allows production of healthy plantlets even when climatic conditions are not favorable and/or when foundation seed is limited. Moreover, it also provides for conservation of germplasm for future use. Thus, it is an important tool for the survival of humanity. Conservation biotechnology refers to the application of the tools of modern biotechnologies for conservation of germplasm. Conservation of germplasm is critically important for management of genetic resources and species that are bound to be extinct. It is also useful for storage of germplasm for breeding and future purposes. Conservation biotechnology interfaces with molecular biology, tissue culture and cryopreservation, all of which are significantly important for offsite cryostorage, restoration and translocation agricultural projects. Virus indexing is a procedure used to test plant material to determine whether or not it has a virus. A number of virus indexing procedures have been established to avoid infected materials from getting to the farmers fields and/or being introduced into disease-free areas. The procedures currently being used for virus indexing include biological, serological and nucleic acid-based methods. These procedures depend on reliable diagnostic methods for pathogen detection.

An efficient and effective seed system is critically important for agricultural development, as witnessed in a majority of European and American countries. Seed systems are composed of organizations, individuals and institutions involved in different seed system functions which include the development, multiplication, processing, storage, distribution and marketing of seeds. Like other crops, vegetative crops are no exception. These characteristics therefore make seed system an important component of agricultural productivity. The aforementioned innovations in tissue culture, conservation biotechnology, virus indexing and seed systems, have by made major strides in cereal and grain crops as compared to vegetative crops like cassava and sweet potato. More worrying is the fact that the stated techniques are not widely practiced in Eastern and Central Africa, where these crops are considered staples. This is due to lack of or limited skilled personnel knowledgeable in these procedures. This unfortunate scenario translates to limited information available on these topics availed for scientists and/or technicians working on these crops in the region.

The compilation of this manual arises from the fact that knowledge on tissue culture, conservation biotechnology, virus indexing and seed systems for vegetative crops (in this case cassava and sweet potato) and associated techniques, are scattered and not readily available for use by practitioners or would-be-practitioners. This manual is a response to this gap and thus presents principles and practices of tissue culture, conservation biotechnology, virus indexing and seed systems. The manual will be used by research scientists and technicians working in tissue culture, conservation biotechnology, virus indexing and seed systems for vegetative crops. The manual could also be useful to students and lectures undertaking these modules as part of the curriculum at colleges or universities. To my knowledge, this is the first compilation on these diverse topics that are tailored to vegetative crops. Thus, this is both an opportunity and a challenge. It is our sincere hope that later issues will cover more case studies and future advances in these fields. Users are encouraged to adapt the references to their own working conditions and to add more materials as they deem fit.

I extend my heartfelt thanks to colleagues who have worked tirelessly to get this assignment done.

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Acronyms

2, 4-D: 2, 4-dichlorophenoxyacetic acid

ABA: Abscisic acid

ABA: Abscisic acid

AGT: Agro-Genetic Technologies Uganda Ltd

BA 6: Benzyl adenine

BAP 6**:** Benylaminopurine

CBO: Community based organization

CCC: Chloride chloride

CIP: International Potato Center

DNA: Deoxyribonucleic acid

DMSO: Dimethyl sulfoxide

DUS: Distinctness, uniformity and stability

EDTA: Ethylenediaminetetraacetic acid

GA_3: Gibberellic acid

GMO: Genetically Modified Organisms

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

IITA: International Institute of Tropical Agriculture

ISTA: International Seed Testing Association

ITPGRFA: International Treaty on Plant Genetic Resources for Food and Agriculture

MAAIF: Ministry of Agriculture, Animal Industry and Fisheries

MCB: Maintenance medium

MFPED: Ministry of Finance, Planning and Economic Development

MMB-I: Medium for *in vitro* introduction

MPB: Propagation medium

MS: Murashige and Skoog

NAA: Naphthalene acetic acid

NAADS: National Agricultural Advisory Services

NaCRRI: National Crops Resources Research Institute

NARO: National Agricultural Research Organization

NGOs: Non Governmental Organizations

NPTs: National Performance Trials

NSB: National Seed Board

NSCS: National Seed Certification Service

NVRC: National Variety Release Committee.

OECD: Organization for Economic Co-operation and Development

PBR: Plant Breeders' Rights

PCR: Polymerase chain reaction

PGR: Plant genetic resources

PQIS: Plant Quarantine and Inspection Services

PVP: Plant Variety Protection

R&D: Research and Development

RFLP: Restricted Fragment Length Polymorphism

TC: Tissue culture

TRIPS: Trade Related Aspects of Intellectual Property

UNADA: Uganda National Agro-input Dealers Association

UPOV: Union for the Protection of New Varieties of Plants

USDA-ARS PGRCU: United States Department of Agriculture - Agricultural Research Service Plant Genetic Resources Conservation Unit

VRC: Variety Release Committee

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Why the manual?

With the unrelenting increase in human population, particularly in sub-Saharan Africa, it is critically important that concerted efforts be made to increase crop productivity to match the expected increase in food demand. The enormity of this problem is well appreciated when one bears in mind that the increase in human population relative to constrained and/or diminishing natural resources notably arable land for agriculture. It is therefore imperative to respond to this challenge by developing and thereafter up-scaling technologies that are important so as to increase productivity per unit area and conserve the natural resource base. This will impact positively on many rural-based communities in the process increase their per capita income and most important provide food security to the farmers.

Plant tissue culture has, and continues to be of great interest within the realms of molecular biology, plant breeding and plant health. Indeed, tissue culture techniques have been employed to complement and/or aid conventional methods of plant breeding i.e., in germplasm exchange, embryo rescue and in the development of double haploids. Further, tissue culture has been used as a tool for: a) multiplication of superior clones, b) *ex-situ* conservation of valuable germplasm, and c) production of pathogen-free plants. Indeed, it is because of these diverse applications that plant tissue culture is now an integral part of curricula of many universities and/or research institutions.

There is a large body of literature on tissue culture spanning the period since its inception in the early 1800s to-date. This historical aspect is beautifully reflected in some of the references cited in this manual. Most of this valuable information is quite varied in presentation style and is largely limited to scientific journals, conference proceedings and text books, which unfortunately are not available to many scientists in sub-Saharan Africa. The compilation of this manual aims to fill this gap, providing the answer to the question, 'why the manual'? It would be pointless to present the same information, and thus, this manual is a compilation of four diverse and critically important topics for vegetative crops: tissue culture, conservation biotechnology, virus indexing and seed systems. Tissue culture, conservation biotechnology and seed systems are tailored for cassava and sweet potato, while virus indexing presents the case of banana, which has by far progressed in this area. These topics are specifically tailored for cassava, sweet potato and banana researchers working in the different National Agricultural Research Systems of sub-Saharan Africa. It is our hope that the knowledge acquired from the use of this manual will enable scientists make informed decisions in an effort to increase productivity of the stated crops.

The crops targeted in this manual are cassava and sweet potato which are largely grown by subsistence farmers in sub-Saharan Africa. Both crops are clonally propagated and are highly heterozygous. These natural characteristics present several challenges to researchers whose objective is to attain optimal yields in these crops. For instance clonal propagation perpetuates pathogen load in susceptible varieties; this is a key concern on germplasm exchange and/or movement. On the other hand, the heterozygous nature complicates the breeding process.

It suffices to note that because these crops are grown by many farmers, efforts made to increase their productivity will in return impact positively on the livelihoods of many communities that primarily depend on these crops. The selected topics (tissue culture, conservation biotechnology, seed systems and virus indexing) complement each other and are thus useful towards the attainment of optimal yields in both cassava and sweet potato. The authors hope that this manual will assist researchers and students working on cassava and sweet potato explain in simple language each of the highlighted topics, with the objective of understanding why those disciplines are important for crop improvement. The diversity of the topics presented suggests that the manual can be read at different levels, and will thus be useful to several readers including teachers. It will also be useful for those who just want to know a little of the background on tissue culture procedures, conservation and seed systems of clonally propagated crops.

CHAPTER 1

GENERAL INTRODUCTION TO TISSUE CULTURE

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1.1 A brief on tissue culture

Tissue Culture or *in vitro* culture words which can be used interchangeably, defines a technique through which small plant organs, seed, embryos, cells or protoplast are aseptically isolated and grown on artificial nutrient media under controlled environment. *In vitro*, a Latin word meaning "within the glass" refers to the first laboratory culture vessels to be used, such as test tubes, flasks and petri dishes that were made out of glass. The use of glass ware is waning and due to advancement in material science plastics and polypropylene laboratory wares are being used as a replacement. In plant, tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation. In vegetatively propagated plants such as cassava and sweet potatoes that accumulate microorganisms over time, shoot meristem is the explants of choice to try and get rid of the contaminants.

Several workers have contributed to the development of tissue culture and its application in botany and plant breeding. The idea of tissue culture had its origin in the first scientific attempts to explain the complexity of a multicellular organism. In 1838, two German biologists, Schwann and Schleiden , formulated the cell theory. Although they realized that complex interactions must occur between cells, tissues and organs, they suggested that each cell is an independent unit, capable of forming a complete organism, a status referred to as being totipotent. Several years later, Haberlandt began experiments to exploit this theory in botany. He tried to culture single cells into complete plants but was not successful with the monocotyledonous plant species he used. However, in 1922, Kotte, a student of Haberlandt, succeeded in culturing tomato root tips. Unfortunately, the cultures did not grow for long because the culture medium that consisted of only inorganic salts and sucrose was nutritionally inadequate. In addition, the cultures got contaminated because there was lack of knowledge about the need for asepsis.

The phenomenon of totipotency became the basis of tissue culture technology and practically, depending of the plant species, whole plants have been generated from almost any plant part such as leaf and root tissue, stem cylinders, axillary buds or even single cells by subjecting them to appropriate tissue culture growth conditions. *In vitro* culture of explants results in differentiated and undifferentiated growth. Differentiated growth results in the formation of shoots from the axils of the main shoot and these shoots are termed axillary shoots. When the shoots are induced anywhere on the cultured explant other than at the axils, the resultant shoots are termed adventitious shoots. On appropriate media, embryo-like structures can develop on cultured vegetative organs, cells or callus cultures. These are called somatic embryos or embryoids and they can be germinated into complete plants. Undifferentiated growth results in the formation of an amorphous mass of undifferentiated and actively dividing cells called callus.

1.2 The tissue culture laboratory

Tissue cultures has four basic *in vitro* stages (Murashige, 1974) through which an explants has to pass so as to generate a full plant. Each of these stages has special needs for culture medium composition and culture environment. Some of the physical needs include an environment with controlled light, temperature regimes and humidity which is called a growth chamber. In general, a laboratory for plant tissue culture requires a basic organization that comprises three areas: a) General laboratory (media preparation area) provided with spaces for common or independent work; b) transfer area for the aseptic manipulation of plant material and c) growth rooms for culture maintenance under controlled conditions for light, temperature and humidity. The set up should have two separate rooms; one room for washing, sterilization, storage and preparation of culture media, and the other for culture maintenance i.e. the growth room or chamber. The laminar airflow

or the transfer chamber can be located in the general laboratory or in an area specifically designed as a transfer room, depending on availability of space.

1.2.1 Micropropagation unit

One of the main applications of tissue culture is the rapid clonal propagation of disease-free plantlets within a short period of time. Micropropagation has been achieved using nodal and stem cuttings as described in detail in later chapters. An efficient micro propagation unit includes: a) a virus-free germplasm as explant source, b) tissue culture laboratory and, c) a well aerated and drained propagation screen-house. When planning a micropropagation unit, the following factors should be considered: availability of space, environmental factors (temperature and humidity), adequate financing, type of work to be developed and required production capacity. Thus, depending on production capacity three types of micro propagation units can be set up:

a) Small scale. In this set up, the facilities for *in vitro* work can be adapted to a house setting, using the available equipment and materials to carry out the basic micropropagation activities. This size facility could be used to micro-propagate plants for interested people, or mother plants for screen-houses. b) Medium scale. This set up provides a pilot set up where the micro-propagate is designed to meet the demands of a fairly large population. In this set up, the equipment are designed in a more efficient manner with an increased efficiency in ouput production. There is high uniformity in the produced product. c) Large scale. In this set up, the equipment are designed so as to provide maximum output. The equipments are large and the product is of commercial interest.

1.2.2 Washing and media preparation area

The area for washing should have an appropriate washbasin (stainless steel and be acid or alkali resistant), tap water, tables that allow stand-up work and shelves to dry and keep the washed materials. The media preparation area must be equipped with a refrigerator to keep heat labile chemicals and stock solutions used in the media preparation, weighing balance, heaters such as a hot plate with magnetic stirrer or a microwave oven, pH meter, oven for drying glassware, a trolley for carrying hot media flasks and containers, shelves along the walls for storage of chemicals, a water distiller/purifier, and pressure cooker or autoclave.

1.2.3 The culture area

This is the culture incubation area or the growth chamber where growth conditions are adjusted according to the species in culture. In this area, temperature variations, light intensity and quality, relative humidity and photoperiod should be taken into consideration.

Light requirement depends on the type of culture and intended growth pattern. For example, callus cultures and seed germination are conducted in the dark, while morphogenesis is induced by light. Light is provided in terms of quality, quantity and duration (day length). Different light spectra confer specific developmental responses but for the sake of consistency and reproducibility, white light that provides a full spectrum is used. The standard way of providing white light is by using white-cool fluorescent tubes. Although high light intensities promote plant growth, *in vitro* culture is usually conducted at light intensity lower than in the field because high intensities come with the disadvantage of generating heat.

High intensity is also undesirable because the growth of *in vitro* cultures is not dependent on photosynthesis. Light is provided at an intensity of 1000-3000 lux. This intensity can also be quantified in terms of photosynthetic active radiation (PAR) that ranges between3 to 15 W/m^2, depending on the quality of the source of light. Where possible, light intensity can be increased to 5000-10,000 lux during the rooting stage as this improves the post flask survival of the plantlets. Generally, the best day length for *in vitro* cultures would be the same as used for cuttings or intact plants. Thus, a photoperiod of 12 to 16 is widely used, the longer duration being reported to promote shoot proliferation. Temperature setting depends on the plant species. Thus, temperate-zone plants require moderate temperatures (22-25^{o}C) while tropical species will perform best at higher temperature (26-30^{o}C). Fluctuations between day and night temperatures occur when cooling is controlled by an air conditioning unit. However, the temperature should not fall outside 20- 35^{o}C.

Temperatures lower than the optimum will reduce the growth rate. Slow growing cultures increase the sub-culturing duration and this response is exploited in the *in vitro* germplasm conservation of banana and cassava.

There is little control over humidity because the relative humidity within the culture vessels is approximately 100%. However, a very dry atmosphere will cause moisture loss from the culture medium. This can be prevented by sealing the culture vessels with a layer of parafilm or cling film. Placing a container of water in the culture room can increase the ambient humidity but very humid conditions promote molds on the walls.

1.3 Tissue culture procedures

The aim of plant micropropagation is to produce clones (true copies of a plant in large numbers) and is usually divided into the following five stages according to Ahloowalia, B.S. *et al.*, 2002.

Stage 0- This is the pre-propagation step or selection and pre-treatment of suitable mother plants.
Stage I - Initiation of explants in culture media under aseptic conditions
Stage II - Subcultures for multiplication/proliferation of explants in appropriate media for shoot proliferation
Stage III –Sub-cultures of explants in appropriate media for rooting to produce complete plants
Stage IV - weaning/hardening of the *in vitro* propagated plants in readiness for transfer to the greenhouse.

1.3.1 *In vitro* culture establishment stage

Establishing cultures is one of the most challenging stages of tissue culture. This consists of taking plants from the field to the test-tube. For this, a clean lot (free from pathogens) that guarantees the quality, uniformity, and strength of the generated material is selected. The selected plants will be those with optimum growth, development, and good phytosanitary conditions. To establish virus-free planting materials, these plants can go through the process of thermotherapy followed by meristem culture. These pathogen-free stocks could be used as a source of explants for the large scale production of disease-free materials. There are diverse types of *in vitro* cultures based on the type of explant used as applied to different applications. These are presented in the Table 1.1 below.

Table 1.1: A checklist of different explants that can be subjected to tissue culture

Explant	Type of culture	Applications
Shoot tip	Shoot tip culture	Propagation, micrografting
Meristem domes	Meristem culture	Virus elimination
Ovaries and ovules	Ovary and ovule culture	*In vitro* fertilization
Mature or immature embryos	Embryo culture	Embryo rescue
Anthers	Anthers culture	Production of haploid plants
Immature pollen	Pollen culture	Production of haploid plants
Any plant part	Callus culture	Propagation, transformation
Friable callus	Cell suspension culture	Propagation, transformation
Cells with digested cell wall	Protoplast culture	Somatic hybridization, transformation

1.3.2 Propagation stage

The propagation stage consists of the rapid and massive multiplication of plantlets. The shoot multiplication and or proliferation rates depend on the variety. In the same crop, shoot multiplication rates may vary depending on the type and concentration of phyto-hormones in the nutrient medium. The time of each propagation cycle depends on the variety behavior, the nutrient media composition, and the environmental conditions to which it is subjected.

1.4 Aseptic conditions in the laboratory

Asepsis is a condition free of pathogens or contaminants. The air, plant and laboratory surfaces, clothing and our bodies carry microorganisms such as fungi, bacteria and yeasts. These microorganisms have fast growth rates and once in contact with the nutritive culture medium they will colonize it and cause contamination. These contaminants have a faster growth and will outgrow the cultured plant cells and tissues whose growth rates are much lower. In addition, microorganisms produce toxins and can also alter the pH in the culture medium that will subsequently affect culture growth. Therefore, aseptic conditions are a prerequisite to successfully grow explants on culture nutrient medium. Aseptic conditions can be obtained through sterilization. This is a process where all unwanted biological activity is stopped. In the case of plant tissue and cell cultured-based technologies, this process involves the total removal of or killing of the microorganisms and their spores. The tricky part is, however, that the ways used to kill microorganisms depend on the type of material to be sterilized. Whereas there is a wide option for sterilizing laboratory ware, a lot of caution is needed when handling plant materials; we need to remove microorganisms but at the same time keep the explant as viable as possible.

1.4.1 Asepsis in the facilities

The tissue culture laboratory is designed in such a way that the architectural and operations plans deliver convenience and reduce the incidences of contamination. Thus, laboratory has four basic environments: 1) an office, 2) a washing and media preparation room, 3) a transfer room, and 4) a growth room, which require a minor to major grade of cleaning, as well as a minor to major restriction on personnel access.

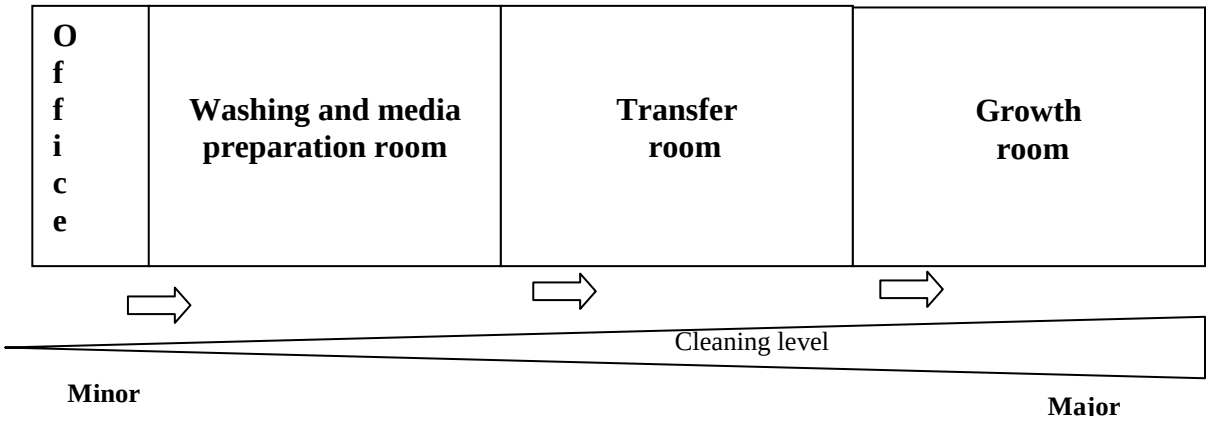

Figure 2.1: The four basic environments of a tissue culture laboratory

1.4.2 Sterilization of plant material

The most challenging stage in tissue culture is to establish a sterile culture from a plant growing in its natural environment loaded with all sorts of microbes. The surface pathogens and organisms can be removed by treating the explant source with disinfectants such as ethanol, calcium hypochlorite, sodium hypochlorite (now commercially available as household bleach or Jik) in a process termed as surface sterilization. Whereas seeds and mature tissue can withstand harsh sterilization, young tissues are more fragile and will require gentle treatment. Therefore, the sterilization procedure for a given explant needs to be optimized in terms of the type of disinfectant, the concentration and time of exposure. Disinfectants such as mercury

chloride and silver nitrate are more effective but require careful handling, as they are very toxic to the environment. As such, their use should be limited.

Deep-seated organisms such as bacteria require the application of antibiotics, such as cefotaxim or cabenicillin, in the culture medium. For fungi, fungicides such as benomyl can be applied to the explant donor plant or to the explant source during surface sterilization. The strategy for virus elimination is to use the meristematic dome (0.2-0.4 mm) with a few surrounding leaf primordia as explant. The principle underlying this approach is that the virus particles move through the conductive vessels and these vessels are not yet formed in the meristematic region. Heat treatment of the donor plant at 35-42°C to inactivate the virus is usually combined with meristem culture.
To increase on the efficiency of surface sterilization, special care is given to the plants that are used for starting *in vitro* culture. The general practice is to maintain these plants in a pest and disease free-state as diseased or pest damaged plants can increase the possibility of presence of endogenous bacteria. Therefore, the plants are grown in a glasshouse/screenhouse to reduce the number of microorganisms. This protection can further be reinforced by spraying the plants with fungicides. Watering should be done at the root level as water on the leaves will encourage build up of contaminants. Aerial watering can also wash the microorganism into the leaf axis that will make sterilization difficult.

To reduce the effect of disinfectants on the explants and increase culture survival, the turgidity of harvested plant parts (explant source) should be maintained up till the explant is inoculated into culture medium. The explant source should first be soaked in water or stored in a plastic bag prior to surface sterilization. The explant source should also be bigger than the final explant so as to prevent damage of the final explant by chemicals during surface sterilization. If the shoot tip is to be used, remove the old leaves and retain the petioles and a bit of the stem. The damaged parts are then cut off before culturing.

1.4.3 Sterilization of culture vessels and tools

Glassware, metallic tools and paper can be sterilized by pressurized steam at 121°C for 15-20 minutes in an autoclave or pressure cooker. Glassware can also be sterilized in an oven at 160°C for 2 hours. Plastic wares is sterilized by irradiation (Gama or U.V light), and place in microwave at high power for two to three minutes depending on the quality of plastic. Tools can also be cleaned with detergents and soaked in bleach, or dipped in ethanol and flamed. Methanol should be avoided, as it is toxic when inhaled or when it gets in contact with the skin! To avoid the fire risks that would result from working with a flame and ethanol, hot bead sterilizer have been designed that can heat up to 300°C. These sterilize the dissecting tools within 10 seconds.

1.4.4 Sterilization of solutions

Water and media are conveniently sterilized by pressure cooking (autoclaving) for 15-20 minutes at 121°C. The duration depends on the efficiency of the autoclave and the volume of the solution to be sterilized; bigger volumes will require more time to allow the heat to penetrate. The exceptions are the heat labile media components and antibiotics that are sterilized by ultra-filtrating their solutions through membranes of 0.22-0.45 µM pores. The sterile solutions are then added to bulk-autoclaved medium that has been cooled to 50°C and then dispensed into jars or test tubes

1.4.5 Sterilization of working surface

Air carries dust particles that are mostly loaded with spores and bacteria which are a source of contamination to the culture medium. Therefore, carefully carrying out *in vitro* manipulations under a still air cabinet will reduce the incidences of contamination. A more sophisticated clean working environment can be created by working under a laminar air flow bench. The machine pumps the air through a high particulate particle (HEPA) filter of 0.45 µM pore size before it reaches the working surface. The HEPA filter removes 99.9% of the particles from the air and creates a sterile environment ensuring that the operations are carried out without contamination. Working areas can further be sterilized by cleaning the surfaces with 95% ethanol or by running U.V light prior to working. Avoid working under UV light when it is on because prolonged exposure can cause skin cancer, induce cataracts and blindness.

1.5 Culture nutrient medium

The culture nutrient medium is a watery solution of all the substances that a plant requires for growth in the right proportions. The medium has chemical and physical properties that are determined by the plant species and intended purpose for the culture. Chemically, the salt composition of the medium is a simulation of the macro- and micro-elements required by plants *in vivo*. The commonly used micro and macronutrients are presented in Table 1.2 However, through optimization of nutrient requirements for the different plant species, a range of media compositions have been developed and these continue to be modified to cater for new species being handled. The detachment of the explant from the photosynthesizing mother plant requires the inclusion of vitamins. A carbon source is added to compensate for the inadequate photosynthesis by the cultures. In this case, sucrose is the common carbohydrate but glucose or fructose can be used though these are more expensive.

Where the medium composition is deficient, organic supplements, such as amino acids, casein hydrolysate, yeast extract, coconut milk and plant juices become necessary. However, these supplements have an undefined composition, but some have been found to be sources of nitrogen, vitamins and growth regulators. Growth regulators (wrongfully referred to as hormones) in form of cytokinins, auxins and gibberellins are incorporated into media to achieve the desired morphogenesis. Generally, a high ratio of cytokinins: auxins promote shoot proliferation, the reverse support rooting, while a 1:1 ratio gives rise to undifferentiated callus. However, the required amount of any of the growth regulators and the resultant growth response will depend on the explants' inherent capacity to synthesize these two growth regulators. Therefore, the optimum combination should be derived experimentally. Gibberellin, GA3 that promote shoot elongation is commonly used in meristem culture.

The media components are normally required in very small quantities. Thus, for accuracy, consistence and convenience, the components are first dissolved in stock solutions e.g. 10X, 100X or 1000X from which small volumes are aliquoted and diluted to prepare the final medium. Preparation of stock solutions improves accuracy for components that are required in milli/micrograms in the final solution and thus also help to reduce human and experimental errors. The stock solutions are prepared with sterile water and the solutions stored at 4^{o}C or frozen. This low temperature prevents microbial growth. Some of the medium components are not soluble in water and other solvents e.g. weak acids and alkaline solutions are used. For instance, cytokinins are dissolved in 1 M HCl while auxins dissolved in either1 M NaOH, absolute ethanol or DMSO.

Stock solutions should be labeled with the date of preparation, chemical name, concentration, sterile or non-sterile and name of the person who prepared the solution. Culture medium is definite in composition and pure water should be used for preparing the stock solutions and final media to avoid other additives. Water can be purified by distillation or reverse osmosis.

Table 1.2: Essential micro- and macronutrients used in tissue culture medium

Element	Function
Nitrogen	Component of proteins, nucleic acids and some coenzymes. Element required in greatest amount
Potassium	Regulates osmotic potential, principal inorganic cation
Calcium	Cell wall synthesis, membrane function, cell signaling
Magnesium	Enzyme cofactor, component of chlorophyll
Phosphorus	Component of nucleic acids, energy transfer, component of intermediates in respiration and photosynthesis
Sulphur	Component of some amino acids (methionine, cysteine) and some cofactors
Chlorine	Required for photosynthesis
Iron	Electron transfer as a component of cytochromes
Manganese	Enzyme cofactor
Cobalt	Component of some vitamins
Copper	Enzyme cofactor, electron-transfer reactions
Zinc	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum	Enzyme cofactor, component of nitrate reductase

CHAPTER 2

PREPARATION OF SOLUTIONS AND MEDIA FOR SWEETPOTATO TISSUE CULTURE

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2.1 Preparation of MS stock solutions:

The growth of *in vitro* plantlets depends mainly on the nutrient media utilized and, consequently, on the quality or accuracy of prepared stock solutions. The Murashige & Skoog basal medium (1962) is often used in sweet-potato plantlet production. Its concentrations of salts and vitamins are adequate for the normal growth of sweetpotato plantlets under *in vitro* conditions. Though basal media are easily obtained from commercial companies, use of stock solutions is cost effective. In addition plant growth regulators namely auxin and cytokinin are supplemented through media. Their preparation is made with high precision and preservation done at optimal working condition so that they have maximum bioactive effect. Therefore during media preparation, stock solutions can be aliquoted into smaller volumes for storage to minimize the need to thaw large amounts every time media is prepared. This also reduces the risk of contamination from repeated use and most important destruction of the bioactive ingredients within the media or hormone. All solutions must be properly labeled indicating initials of the person who prepared the stock/s, date, details of the stock and any other key remarks.

STOCK SOLUTION A: SALTS

	Component	Weight (g)
1	NH_4NO_3	35
2	KNO_3	40
3	$CaCl_2.2H_2O$	09
4	KH_2PO_4	3.5
5	H_3BO_3	0.1
6	$MnSO_4.4H_2O$	0.5
7	$ZnSO_4.7H_2O$	0.2
8	KI	0.02
9	$Na_2MoO_4.2H_2O$	0.005

Dissolve in 200 ml of distilled water. Keep the solution in a well labeled vial at 4°C

1. Weigh 5 mg of $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$.
2. Dissolve them in 10 ml of distilled water.
3. To 1 ml of the previous solution of ($CuSO_4.5H_2O$ and $CoCl_2.6H_2O$) add 200 ml of distilled water. Store the solution in a well labeled vial at 4°C.

STOCK SOLUTION B: $MgSO_4$:

Weigh 3.7 g of $MgSO_4.7H_2O$ in a100-ml reagent container. Add distilled water up to 50 ml, mix thoroughly and top up to the 100 ml mark. Keep the solution in a well labeled vial at 4°C.

STOCK SOLUTION C:
Weigh 0.75 g of Na EDTA. Dissolve while hot in 20 ml of distilled water. Let the solution cool.
Weigh 0.55 g of $FeSO_4$ $7H_2O$. Dissolve it in 20 ml of distilled water.
Mix both solutions and fill up to 100 ml with distilled water. Keep the solution in a well labeled vial at 4°C in the dark. Dark conditions can easily be achieved by wrapping the vial with aluminum foil.

STOCK SOLUTION D: Vitamins
Weigh the following reagents:

Reagent	Weight (mg
Thiamine HCL	20
Glycine	100
Nicotinic acid	25
Pyridoxine HCL	25

Dissolve the components in 500 ml distilled water. Stir well and aliquot the solution in 20 ml vials and store at 0°C. The vitamin solutions should be discarded after 2-3 months of preparation.

MS basal solution: To prepare 1 litre of basal medium mix the following components:

Stock Component	Volume (ml)/L
Solution A	100
Solution B	10
Solution C	05

Others:

Inositol	100mg	
Ca Nitrate		10mg
Putrecine HCl		2g
L-Arginine		10g

Vitamins:

Ca pantothenate	20mg	
GA_3		10mg
Ascorbic acid		10mg

Make up to 1 liter with distilled water.
Naphthaleneacetic acid (NAA): Stock solution of NAA 1,000 ppm

1. Weigh 0.2 g of NAA and dissolve well by adding some NaOH 1N drops.
2. Add 200 ml of distilled water.
3. Keep in a well labeled vial in a fridge at 0°C.

Note: One ml of NAA stock solution (1,000 ppm) contains 1 mg of NAA.

Benzylaminopurine (BAP): Stock solution of BAP: 1,000 ppm

1. Weigh 0. 2 g BAP and dissolve well with some drops of NaOH 1N.
2. Add 200 ml distilled water.
3. Keep in a well labeled vial at 0°C.

Note: BAP may be sterilized together with the culture medium. However, the loss of some activity may result. One ml of stock solution (1,000 ppm) contains 1 mg of BAP

Indoleacetic acid (IAA): Stock solution of IAA: 1,000 ppm

1. Weigh 0.2 mg of IAA and dissolve well with some alcohol drops.
2. Add 200 ml of distilled water.
3. Keep it in a well labeled vial at 0°C.

Note: IAA is heat labile, filter sterilization is recommended. One ml stock solution (1,000 ppm) contains 1 mg of IAA.

Kinetine (KIN): Stock solution of KIN: 1,000 ppm

1. Weigh 0.2 g KIN and dissolve well with some drops of NaOH 1N.
2. Add 200 ml of distilled water.
3. Keep in a well labeled vial at 0°C.

Note: KIN may be sterilized together with the culture medium. However, the loss of its activity may result. One ml of the stock solution (1,000 ppm) contains 1 mg of KIN.

2,4-D: Stock solution of 2,4-D: 1,000 ppm

1. Weigh 0.2 g of 2,4-D and dissolve well using alcohol drops.
2. Add 200 ml of distilled water.
3. Keep in a well labeled vial at 0°C.

Note: 2,4-D may be sterilized together with the culture medium. However, a loss of its activity is also possible. One ml of the stock solution (1 000 ppm) contains 1 mg of 2,4-D.

Antibiotic: Rifampicin (Rimactan 300)

1. Cut small squares of filter paper (1 0 mm x 1 0 mm).
2. Place them in a petri dish and sterilize
3. In a flow chamber, place the squares carefully on sterilized petri dishes, slightly separated one from the other.
4. Dissolve a capsule of Pimactan (300 mg) in 150 ml of distilled water. Sterilize with filters of 0.22 µm.
5. Place 3 drops of the antibiotic solution, approximately 0.09 ml, on each square.
6. Let the antibiotic dry in the flow chamber. Keep all the squares in petri dishes, covered and sealed with parafilm.
7. Keep the temperature at 4°C, until the petri dishes are ready to be used.

Note: When ready to use, with forceps, take a square containing antibiotic by one side, and introduce it in a tube. Press over the medium close to the place where the node will be planted. The antibiotic will diffuse and cover the planted area including the node.

Antibiotic: Sodic cefotaxim (Claforan)

1. Cut small squares of filter paper (5 mm x 5 mm).
2. Place them in a petri dish and sterilize
3. In a flow chamber, place the square very carefully with a forceps, on the surface of sterilized petri dishes, slightly separated from each other.
4. Prepare an antibiotic solution by dissolving 1 g of Claforan in 25 ml of sterile distilled water sterilize with 0.22 µm filters.
5. In a flow chamber, place a drop of approximately 0.03 ml on each square.
6. Let the antibiotic dry in the flow chamber. Keep all the squares in petri dishes, covered and sealed with parafilm.
7. Keep the temperature at 4°C, until the petri dishes are ready to be used.

Note: When ready to use, with forceps, take a square containing antibiotic by one side and put it into a tube. Press over the medium close to the point where the node will be planted. The antibiotic will diffuse and cover the planted area including the node.

Preparation of calcium hypochlorite

1. Weigh 50 g of calcium hypochlorite. Dissolve it in 1,000 ml of distilled water (5%).
2. Shake it for 3 to 4 hours and let it rest 6 to 8 hours, or overnight.
3. Filtrate the solution by using a filter paper and maintain it hermetically closed in a flask in a safe place.
4. Use 50 ml of solution and add 50 ml of distilled water

Preparation of an acaricide solution

1. Weigh 5 g of an acaricide and dissolve it in 1,000 ml of distilled water. Stir well.
2. Always use the solution while fresh.

1 N Hydrochloric acid (HCl)
1N HCL is used to adjust the pH downwards.
1. Pour 91.4 ml of distilled water into a beaker (Use a mask and gloves for protection from the acid vapors).
2. With a pipette measure 8.8 ml of hydrochloric acid (commercial concentrate, 36.5-38.0%]. Do not breathe when taking out the acid. Use a rubber-bulb pipette or pipette filler with disposable plastic pipettes.
3. Homogenize and keep in a broad-mouth vial, closed and at room temperature.

I N Potassium hydroxide (KOH)
1N KOH is used to adjust the pH upwards.
1. Place 50 ml of distilled water in a beaker
2. Add 5.6 g of KOH and dissolve well.
3. Bring to 100 ml with distilled water. Keep solution in a closed broad-mouth vial at room temperature.

Note: Depending on the pH of the medium being prepared, add the 1N HCL or 1 N KOH drop by drop until the required pH is attained.

2.2 Culture media for sweet potato

For sweet potato, the compounds used for the nutrient media preparation contain the basal medium, vitamins and other substances, whose proportions vary according to the explant to be cultured. One critically important component is gibberellic acid, which breaks bud dormancy and accelerates explant growth. For the conservation medium, sorbitol is used to induce osmotic stress, which retards nutrient absorption and, therefore, growth. Other substances added to the culture media are: a) vitamins, which participate in cell enzymatic functions, stimulating the growth of the explants and b) polyamines, nitrogenous compounds derived from the amino acids, which stimulate growth through cell division. Activated carbon absorbs the eliminated inhibitory substances through the explants' sap in the medium, and stimulates root morphogenesis and growth.

2.2.1 *In vitro* introduction medium

1. Dissolve a packet of MS (Murashige & Skoog basal medium) in 600 ml of distilled water.
2. Add 25 g of sucrose and 5 ml of vitamin solution and stir.
3. Add 1 ml of gibberellic acid.
4. Bring the volume up to 1,000 ml with distilled water.
5. Adjust the pH to 5.6 with NaOH /1N KOH, or 1 N HCl).
6. Add 3.0 g of Phytagel.
7. Dissolve the gelling agent with heat (microwave oven: 100% intensity for 12 minutes or heating block). Stir well until it dissolves, preventing it from boiling.
8. Dispense 2 ml of the medium in 13 x 100 mm tubes.
9. Sterilize at 121°C and 15 pounds pressure, for 20 minutes.
10. Take the culture tubes from the autoclave, cool and store at 4°C until used.

2.2.2 Sweet potato propagation medium

1. Dissolve a packet of MS (basal medium Murashige & Skoog) in 600 ml of distilled water
2. Add 25 g of sucrose and stir well.
3. Fill up to 1000 ml with distilled water.
4. Adjust the pH 5.6.
5. Add 3.0 g of Phytagel.

6. Dissolve the gelling agent with heat (microwave oven: 100% intensity, 12 minutes or heat on a heating block). Stir well until the gelling agent is dissolved, preventing it from boiling.
7. Dispense 12 ml in 25 x 150 mm tubes.
8. Sterilize at 121°C and 15 pounds pressure for 20 minutes.
9. Take the culture tubes from the autoclave, cool and store at 4°C until used.

2.2.3 Sweet potato meristems medium

1. Dissolve a packet of MS (Murashige & Skoog basal medium) in 600 ml of distilled water.
2. Add 25 g of sucrose and 5 ml of vitamin stock solution.
3. Add 2 ml of putrescine (stock solution: 10,000 ppm). and 0.5 ml of gibberellic acid
4. Fill it up to 1,000 ml with distilled water.
5. Adjust the pH to 5.6. Add 3.0 g of phytagel.
6. Dissolve the gelling agent with heat (microwave oven: 100% intensity, 1 2 minutes or heat on a heating block). Stir well until the gelling agent is dissolved, preventing it from boiling.
7. Dispense 2 ml in 13 x 100 mm tubes.
8. Sterilize at 121°C and 15 pounds pressure for 20 minutes.
9. Take the culture tubes from the autoclave, cool and store at 4°C until used.

2.2.4 Sweet potato conservation medium

1. Dissolve a packet of MS (Murashige & Skoog basal medium) in 600 ml of distilled water
2. Add 20 g of Sucrose, 40 g of Sorbitol and 5 ml of vitamin solution and stir.
3. Fill up to 1,000 ml with distilled water
4. Adjust the pH to 5.6.
5. Add 7. 5 g of agar.
6. Dissolve the agar with heat stirring until it is dissolved, preventing it from boiling
7. Dispense 12 ml of medium in 25 x 125 mm tubes.
8. Sterilize at 121°C and 15 pounds pressure for 20 minutes.
9. Take the culture tubes from the autoclave, cool and keep it at 4°C until used.

CHAPTER 3

SWEETPOTATO TISSUE CULTURE TECHNIQUES

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3.1 Micropropagation

In vitro plantlets, which are free of pathogens, are used as initial material for sweet potato seed programs (Figure 3.1). The methods used in sweetpotato micropropagation mainly depend on the production volume and the available infrastructure. The methods mentioned in this manual have been verified in many institutions and are based on the rapid growth of individual nodal cuttings, or stems with multiple nodal cuttings.

3.1.1 Node micropropagation

This method is based on the principle that the node of an *in vitro* plantlet placed in an appropriate culture medium will induce the development of the axillary bud, resulting in a new *in vitro* plantlet. It must be noted that propagation by nodes is based on the development of a pre-existing morphological structure. The nutritional and hormonal condition of the medium breaks the dormancy of the axillary bud and promotes its rapid development.

Callus formation and plant regeneration must be avoided because they tend to affect the genetic stability of the genotype. Under room-controlled conditions micropropagation is fast and each node planted in a propagation medium will produce a plantlet which will occupy the full length of the test tube, after approximately six weeks. The resultant *in vitro* plantlets may be transplanted to *in vitro* conditions in small pots in the screen house.

3.1.2 Micropropagation by node cuttings in a liquid medium

This technique is applied to produce a large number of nodes rapidly. Stem cuttings with 5 to 8 nodes are prepared by removing both the apex and the root of the *in vitro* plant to be propagated. The stems are placed in the corresponding propagation liquid medium. It is also possible to use isolated nodes. The nodes will sprout and new plantlets will develop over a period of 3 to 4 weeks.

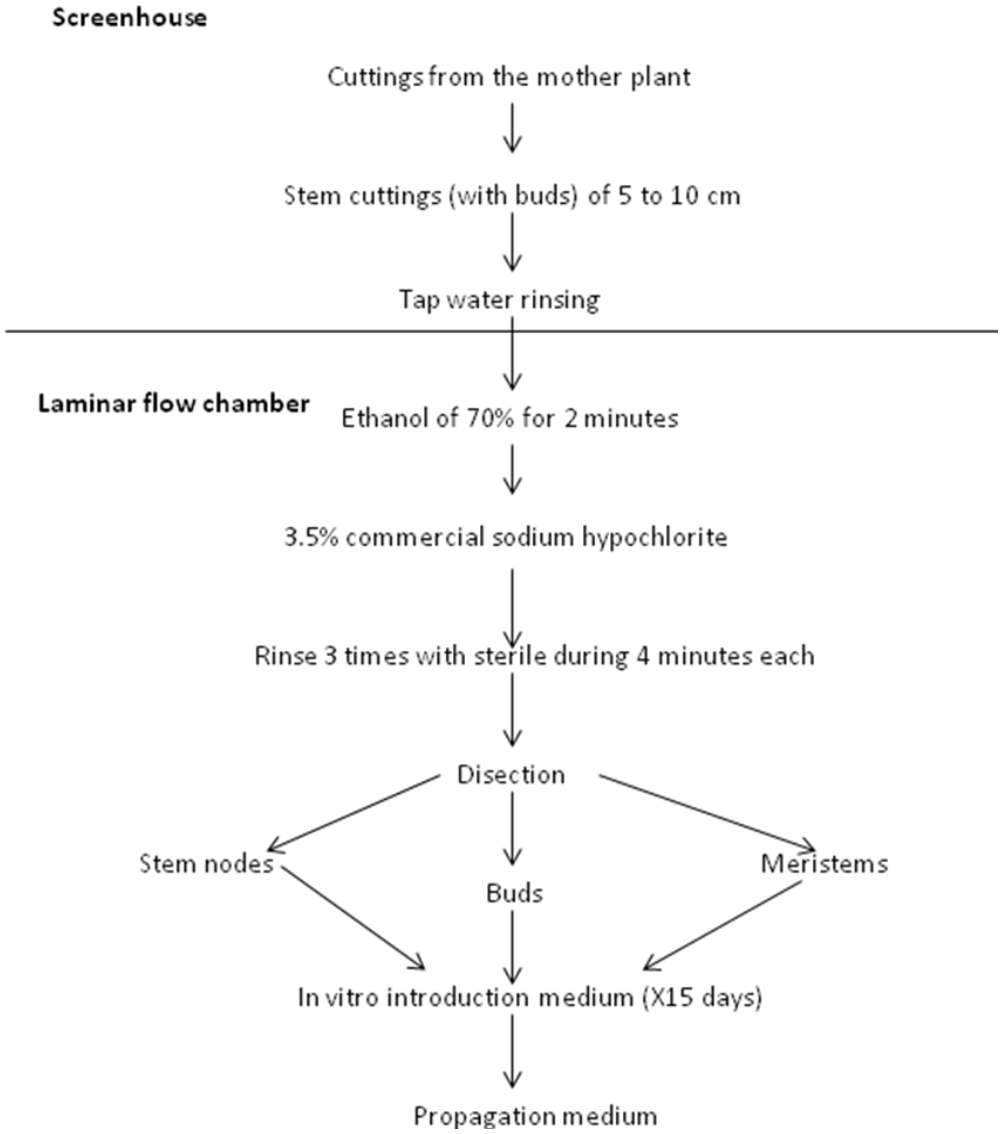

Figure 3.1: Procedure for *in vitro* introduction of sweet potato displaying the asepsis conditions

3.2 Micropropagation procedure

1. Sterilize petri dishes (placed in paper bags or comets) and prepare the laminar flow chamber by disinfecting the internal surfaces with 70% alcohol. The scheme for micropropagation is shown on fig 3.2 below.

Figure 3.2 Micropropagation process

2. Sterilize the tools with an instrument sterilizer and place them on a sterile petri dish.
3. Using a forcep, take off the plantlet from the tube and place it on a sterile petri dish.
4. Remove the leaves and cut the nodes.
5. Open a tube containing fresh sterile micropropagation medium and place a node inside. Plunge it slightly into the medium with the bud up and close the tube properly and seal the tube with a gas-permeable plastic tape (parafilm or saram wrap) and label it correctly.

Note: It is recommended to place two explants in 16 x 125 mm tubes, three in 18 x 150 mm tubes, five in 25 x 150 mm tubes, and 20-30 in magenta vessels.

3.3 Common problems in micropropagation

Some of the problems that may appear in tissue culture depend on the crop and or variety. To solve them it is necessary to apply one of the preventing/solving methods such as:

3.3.1 Phenolization

The explants frequently become brown or blackish shortly after isolation. When this occurs, growth is inhibited and the tissue generally dies. The young tissues are less susceptible to darkening than the more mature ones. Phenolisation may generally be prevented by several methods. Firstly, removing the phenolic compounds produced by dispersion through absorption using activated carbon or polyvinilpyrolidone (PVP). Secondly, by modifying the redox potential; reducing agents including ascorbic acid, citric acid, L-cysteine HCL, ditriotreitol, glutation and mercaptoethanol reduce the availability of active oxygen in stationary liquid or solid media. Thirdly, by inactivating the phenolase enzymes using chelating agents such asNa Fe EDTA, EDTA, diethyldithiocarbamate and dimethyl-dithiocarbamate. Fourthly, phenolisation can be resolved by reducing the phenolasic activity and the availability of substrate through either low pH or provision of a dark environment.

3.3.2 Absence of rooting

The explants can naturally form roots during propagation, without an additional rooting stage, as with the potato. However, some wild sweet potato species may show root production deficiency. Rooting may be induced by incorporating auxins, such as IAA, NAA, and IBA, or activated carbon to the culture medium.

3.4 Virus eradication through meristems culture and thermotherapy

If a healthy plant is sown in the field, it is exposed to infections caused by an array of pathogens which have a negative effect on yield, and in some cases may kill the plants. However, not all the plant cells may become infected. A group of cells (in the meristem) that are in a state of continuous non-differentiated multiplication are often virus-free. The *in vitro* meristems culture, together with growth at high temperatures produces sweet potato plantlets free from viruses in more than 90% of planted meristems. This routine method was established at the International Potato Center (CIP) to obtain virus-free plantlets for national and international distribution. Hereafter, the procedure to obtain virus-free plantlets is described.

Procedure for obtaining virus-free sweet potato plantlets

1. Approximately 18 to 20 plantlets (virus-infected) are propagated in magenta vessels
2. After a growth period of 20 to 25 days, or when the plantlets are 4-5 cm high, they are placed in the thermotherapy chamber where growth conditions are: 16 hours of light 34°C; 8 hours of darkness 32°C.
3. The vessels are maintained in the thermotherapy chamber for one month.
4. Afterwards, the vessels are removed from the chamber, and the exterior cleaned with 98% alcohol. Thereafter, they are introduced to the culture room.
5. To obtain meristems: Cut the apical portion and remove the leaves that cover the meristem (approximately 3 to 4 leaves); the meristem is observed with a prominent leaf primordium.
6. Remove the meristem with part of the leaf primordium; cut only the translucent portion using new sterile scalpel blades then place the meristem in the culture medium.
7. Evaluate meristem growth and transfer them to fresh media if necessary.
8. Each meristem that originates a plant is called 'line' which will be labeled according to the accession it belongs to. For example, Yellow Line 1, Yellow Line 2, Yellow Line 3, etc.
9. Five tubes with several plants are propagated to evaluate the potato virus (SPVd) to determine the host range, the morphological evaluation, and the *in vitro* maintenance.
10. The results of each evaluation are obtained, and the infected material is replaced with clean material.

Figure 3.3: Virus cleaning procedure

3.5 Initiation of callus from sweetpotato explants

When a totipotent explant is cultured on appropriate medium, usually with varying ratio of both auxin and cytokinin it give rise to an unorganized, growing and dividing mass of cells called callus. The production of callus from sweetpotato explants provides scientists with more room to use plant tissue culture in crop improvement. In culture, the proliferation of callus can be maintained more or less indefinitely, provided that it is subcultured onto fresh medium periodically. During callus formation there is some degree of dedifferentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology and metabolism.

One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesize. This has important consequences for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant. To counter this, vitamins and a carbon source are added to the culture medium. Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus. During long-term culture, the culture may lose the requirement for auxin and/or cytokinin. This process, known as 'habituation', is common in callus cultures from some plant species (such as sugar beet). Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of either shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are desirable in transformation.

3.6 Cell suspension cultures of sweet potato

Callus cultures are categorized as either compact or friable. For compact callus also known as non friable the cells are densely aggregated, whereas in friable callus the cells are only loosely associated with each other and the callus is soft and breaks apart easily. Thus, friable callus capability to break apart easily makes it good for cell-suspension cultures. Explants from some plant species or particular cell types tend not to form friable callus, making cell-suspension initiation a difficult task. The friability of callus can sometimes be improved by manipulating the medium components or by repeated subculturing. It's also possible to improve by culturing it on 'semi-solid' medium (medium with a low concentration of gelling agent).

When friable callus is placed into a liquid medium (usually the same composition as the solid medium used for the callus culture) and then agitated, single cells and/or small clumps of cells are released into the medium. Under appropriate conditions, these released cells continue to grow and divide, eventually producing a cell-suspension culture.

A relatively large inoculum should be used when initiating cell suspensions so that the released cell numbers build up quickly. The inoculum should not be too large as toxic products released from damaged or stressed cells can build up to lethal levels. Large cell clumps can be removed during subculture of the cell suspension. Cell suspensions can be maintained relatively simply as batch cultures in conical flasks. They are continually cultured by repeated subculturing into fresh medium. This results in dilution of the suspension and the initiation of another batch growth cycle. The degree of dilution during subculture should be determined empirically for each culture. However, drastic dilution will result into a greatly extended lag period or, in extreme cases, death of the transferred cells. After subculture, the cells divide and the biomass of the culture increases in a characteristic fashion, until nutrients in the medium are exhausted and/or toxic by-products build up to inhibitory levels. This is called the 'stationary phase'. If cells are left in the stationary phase for too long, they will die and the culture will be lost. Therefore, cells should be transferred as they enter the stationary phase. It is therefore important that the batch growth-cycle parameters are determined for each cell-suspension culture.

3.7 Somatic embryogenesis

Somatic embryogenesis is the process of initiation and development of embryos or embryo-like structures from somatic cells. In somatic embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a

cell or small group of cells without the production of an intervening callus. Direct somatic embryogenesis is rare for many plant tissues in comparison with indirect somatic embryogenesis.

For the indirect somatic embryogenesis, callus is first produced from the explants. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus. Somatic embryogenesis has been reported in calli derived from anther cultures, leaf, shoot tip, stem and root explants and lateral buds. However, the production of sweetpotato plants in culture is still genotype-dependent, ranging from 0 to 85% in tested cultivars. Somatic embryogenesis in sweetpotato is genotype dependant and auxin cytokinin ratio is important in the development of this type of embryos. In most crops, an intermediate ratio of auxins and cytokinins promote growth of callus without differentiation. High auxin to cytokinin ratio promotes root development while low ratios promote shoot development. All embryogenic competent cells of callus are capable of developing into fully functional embryos.

The genetic material of these cells often changes in culture giving rise to variant plants a process known as somaclonal variation. Somaclonal variation is a general phenomenon of all plant regeneration systems that involve a callus phase and it's an aberrant growth of plants as a result of stress factors absent/present in media, dedifferentiation as a result of repeated sub culturing, chromosomal and point mutations or simply genetic variation in source plant plus a combination of above factors. There are two general types of somaclonal variation; the heritable, genetic changes (alter the DNA), and stable, but non-heritable changes (alter gene expression, i.e. epigenetic).

3.8 Organogenesis

Organogenesis is the production of organs either directly from an explant or from a callus culture. Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either from a callus culture or directly from an explant. Alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. In particular, it is the auxin to cytokinin ratio of the medium that determines which developmental pathway the regenerating tissue will take. It is usual to induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium. These shoots can then be rooted relatively simply.

CHAPTER 4

PREPARATION OF SOLUTIONS AND MEDIA FOR CASSAVA TISSUE CULTURE

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4.1 Cassava tissue culture media

The composition of culture media determines the *in-vitro* growth of cassava. Like other tissue culture media, cassava culture media is based on basic Murashige and Skoog (MS) media, which supplies essential elements as a complex mixture of salts. Essential elements consist of macro and micro elements, iron source, organic supplements such as vitamins or amino acids and carbon source. These different components are mixed to constitute a complete culture medium. The essential micro and macro nutrients important for tissue culture are presented in Table 4.1. Stock solutions of media components are usually prepared and stored before subsequent use for convenience. Pre-mixed commercial standard media are also available for use in the large industrial plant tissue culture laboratories. Growth and development require large quantities of macro developments which consists nitrogen, phosphorous, potassium, magnesium, calcium, sulphur and carbon. Micro elements that comprise of manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc are required in trace amounts for growth, development and other diverse roles. Organic supplements contain thiamine and myonositol as essential vitamins while glycine is a form of amino acid that supplies a reduced form of nitrogen. Sucrose is the most preferred source of carbon for tissue culture however glucose can be used as a reliable alternative carbon source. Depending on the type of culture preferred, media can be prepared as liquid or solid. Cultures that necessitate growth of plant cell or tissue on medium surface are solidified by addition of a gelling agent such as agar, phytagel, agarose etc.

4.2 Preparation of stock solutions

Stock solutions are made in 10x, 25x, 100x or 1000x depending on solubility of the compound. Most of the components of the media are required in very small quantities (micrograms or milligrams) and thus are hard to weigh with precision. Prior preparation of stock solutions save time and reduce experimental and human error associated with routine media preparations. It is important to prepare concentrated stock solutions of some compounds because they are more stable and can therefore be stored for longer periods than the dilute solutions. Constitution of cassava stock solutions is presented in Table 4.2. The appropriate amount of each compound is weighed, put in a clean flask and dissolved using recommended solvent such as water, ethyl alcohol, 1 N NaOH, or 1 N HCL. Double distilled water is added to make up the required volume while stirring. Separate stock solutions for calcium salts may be required to prevent precipitation while iron stock solutions should be prepared separately from other micronutrients and heated in a microwave before use. It is important to make vitamin stock solutions each time media is prepared if a refrigerator or freezer is not available. It should also be noted that vitamin stock solutions can be stored safely in a refrigerator for 2-3 months and should be discarded after that period. The stock solutions can be sub-divided into aliquots in small vials and stored to reduce thawing time of large volume of frozen stock solution. Vitamins stock solutions for cassava are prepared in x1000 and made by dissolving 10 g myo-inositol, 100 mg nicotinic acid, 100 mg pyridoxine-HCL, and 1 g thiamine-HCl in 1 L distilled water.

Table 4.1: Essential macro and micro nutrients for cassava tissue culture

Essential elements	Concentration in stock solution	Comments	Amount per 1000mls of medium
25x MS Macroelements	**(g/L)**	Store at 2-4^{O} C	40mls
Ammonium Nitrate	41.25		
Potassium Nitrate	47.5		
Magnesium Sulfate. $7H_2O$	9.25		
Potassium Phosphate monobasic	4.25		
Calcium Chloride	11		
100X MS Microelements	**mg/L**	Store in fridge/freezer	10 mls
Boric Acid	620		
Manganese Sulphate	1690		
Zinc Sulphate .$7H_20$	860		
Potassium iodide	830mg/10mL; add1ml/L		
Di-Sodium molybdic acid. $2H_2O$	2.5mg/ml; add 1mL/L		
Cupric sulphate. $5H_2O$	2.5mg/mL;add 1mL/L		
Cobalt chloride. $6H_2O$	2.5mg/mL; add 1mL		
200x MS Iron sources	**g/L**	Wrap in aluminum foil	5 mls
Di-Sodium EDTA. $2H_2O$	7.44		
Iron Sulphate. $7H_2O$	5.56		
1000x Vitamins Stock	**mg/100 mol**	**Store at -20°C for 2-3 months**	**1 mls**
D-Biotin	20	Powdered vitamin mixtures are hygroscopic	
Glycine free base	400	Warm to re-dissolve incase of precipitation	
Myo-Inositol	1000		
Nicotinic acid free acid	10		
Pyridoxine HCL	10		
Thiamine HCL	100		
Sucrose	N/A		20g
MS basal salt mixture	N/A		4.3g

Table 4.2: Preparation of MS stock solutions for cassava tissue culture

Chemicals concentration	**10X g/L**	25X g/L	**50X g/L**
MS Macronutrients ml/L	**100 ml/L**	**40 ml/L**	**20 ml/L**
Ammonium nitrate	16.5	41.25	82.5
Potassium nitrate	19	47.5	95
Magnesium sulfate heptahydrate	3.7	9.25	18.5
Potassium phosphate monobasic	1.7	4.25	8.5
Calcium chloride dihydrate	4.4	11	22
MS Micronutrients (10 mL/L)	**100X mg/L**		
Boric acid	620		
Manganese sulfate monohydrate	1690		
Zinc sulfate heptahydrate	860		
Potassium iodide	83		
Sodium molybdate dihydrate	25(250 mg/ 10 mL)	1 mL of stock	
Copper sulfate pentahydrate	2.5(25 mg/10 mL)	1 mL of stock	
Cobalt chloride hexahydrate	2.5(25 mg/10 mL	1 mL of stock	

Chemical concentration	**100X g/L**	**200X g/L**
MS Fe-EDTA ml/L	**10 ml/L**	**5 ml/L**
Disodium EDTA 2H2O (dissolve first in dark bottle)	3.72	7.44
Ferrous sulfate heptahydrate	2.78	5.56

4.3 Types of cassava cultures

Cultures of cassava are initiated from sterile pieces of a whole plant called explants. Explants taken from a younger and vigorously growing tissue are most effective. Below are the different types of cultures used;

1. Node cultures: These are stem pieces carrying single or multiple nodes and are cultured on media
2. Shoot tip cultures: These are cultures containing apical meristem that are excised and cultured *in-vitro* to produce clumps of shoots from either axillary or adventitious buds. They are the most common types of cultures for clonal propagation.
3. Meristem cultures: These are small excised shoot apices each consisting of the apical meristematic dome with or without one or two leaf primodia.
4. Callus cultures: These are culture as a result of growth of an amorphous mass of cells arising from uncoordinated and disorganized growth of small plant organs.
5. Suspension cultures: Consist of a population of cells dispersed in an agitated liquid medium.

CHAPTER 5

CASSAVA TISSUE CULTURE TECHNIQUES

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5.1 Somatic embryogenesis and organogenesis

Somatic embryogenesis and organogenesis are the two ways through which complete plants can be produced from cultured cells. Somatic embryogenesis refers to formation of embryo-like structures called somatic embryos either directly or indirectly from somatic cells. These embryos may develop from a single or group of cells. Repeated cell division produces groups of cells that develop into organized structures referred to as organized embryogenic structures (OES) in cassava. The organized embryogenic structures further develop into heart and torpedo-stage embryos which can be regenerated into a whole plant. Somatic embryos are produced as adventitious structures directly on explants of zygotic embryos from callus or suspension cultures. The embryo initiation stage thrives on high concentration of 2, 4-D, while formation of somatic embryos is supported by medium with low levels of 2, 4-D. Embryogenesis in cassava forms the basis for genetic transformation and has facilitated the introduction of genes of agronomic interest into the crop. Organogenesis is the formation of organs either directly from an explant or from a callus culture. A groups of cells of the apical meristem in the shoot apex, axillary buds, root tips or floral buds are stimulated to differentiate and grow into shoots and ultimately into complete plants. The axillary buds formed in the culture undergo cyclic proliferation to produce large number of tiny plants. These plants are then cultured on relatively high amounts of auxin to form callus which can then be sub-cultured to multiply. A cell suspension is produced by introducing callus into liquid medium and shaken. The suspension can be sub-cultured and multiplied into more liquid cultures. The cell suspension forms cell clumps, which eventually form calli giving rise to plants through organogenesis or somatic embryogenesis. Auxin: Cytokinin ratio in the medium determines the regeneration capability of calli into plants. The description media components for the above mentioned techniques is presented in Appendix 5

5.2 Cassava micropropagation process

Micropropagation is a technique of growing plants from meristematic tissue or somatic cells of superior plants on suitable nutrient media under controlled aseptic physical conditions. The aim of micropropagation is to produce true copies (clones) of a plant in large numbers under natural conditions or that similar to those in which the plants will ultimately be grown. In cassava, the process is divided into the following stages:

5.2.1 Selection and pre-treatment of suitable mother plants

Collection, establishment and maintenance of sufficient pest and disease free mother plants preferably in the green house are the first steps towards successful tissue culture. Cultures initiated from healthy mother plants exhibit minimal contamination and improved growth and multiplication rates. Therefore mother plants and batches of micro-propagated plants should be routinely indexed, especially for viral disease using appropriate techniques. In cassava, young shoots are the preferred explants. Explants taken from healthy donor plants are surface sterilized with sodium hypochlorite and Tween 20. Surface sterilization eliminates fungal and bacterial contaminations but not viruses and viroids and can survive through successive multiplication of infected mother plants. Records of the explant, health status of the donor plant, varietal identity, vigour, conformity and elimination of somaclonal variants are critical for *in-vitro* plant quality.

5.2.2 Initiation of the culture

The sterilized cassava explants are inoculated on freshly prepared semi-solid MS medium (MS2A) to initiate mother culture. The cultures are sealed, labeled and incubated in growth room. Signs of growth are visible within 2 weeks after *in-vitro* introduction. The seedlings become suitable for multiplication after they have developed roots and show 4-5 nodes. Following the initiation, cultures should be monitored and any

contamination discarded as they appear while established cultures are maintained to be used for further sub-cultures.

5.2.3 Routine subculture and rooting of cultures

Established cultures are proliferated on fresh MS2A medium at three weeks intervals depending on the response of different varieties to *in-vitro* conditions. Repeated sub-cultures of a single established mother culture can give rise to a large number of propagules in a short time. The same MS medium used for initiation and micropropagation supports both root and shoot development in cassava without plant growth regulators. Sturdy well-rooted cassava plants are ideal for high survival during weaning and later transfer to soil.

5.2.4 Weaning of cassava

The final stage of the tissue culture operation is the hardening of the rooted cassava plantlets. It is the most delicate step constraining mass propagation because of the high mortality rate of cassava plantlets during transfer to soil. Transfer of plantlets from culture tubes to soil subjects them to dehydration, nutrient loss as well as root and stem damage. The extent of survival during acclimatization depends on the care taken to avoid these stress factors. To achieve best results, hardening of cassava plantlets is best done gradually from high to low humidity and from low to high light intensity conditions.
It is important to use sterile soil and distilled water during the transfer stage to increase plantlet survival rates. Plants should be allowed to fully harden for at least $2^1/_2$ months to increase the survival of planted material. The hardening enhances the confidence of stake holders in tissue culture technology. The uniformity and consistency in field performance of tissue-cultured plants is important in building the confidence of the farmers in adopting the technology in their production systems.

5.3 Challenges and possible remedies in cassava tissue culture work in Uganda

Challenges

- Unreliable supply of electricity: Power fluctuations interfering with the photosynthetic stages of tissue culture plantlets.
- Cost of the plantlets: Most of the rural farmers cannot afford the cost of the tissue culture materials.
- Cost of laboratory reagents: Laboratory consumables are very expensive and not easily available in the local markets.
- Unreliable source of water: Many laboratories do not have steady and reliable supply of water.
- Labour: Since tissue culture is a new field in most developing countries, there is lack of trained and skilled personnel.
- Perception and Attitude: Tissue culture technique and materials are always associated with GMO's which has not been well received by many communities.
- Lack of motivation: Poor attitudes by technicians towards tissue culture work have contributed to the slow progress in the field because fellow researchers consider the technology as a simple procedure that can be done by anybody.

Remedies

- An automatic stand by generator to minimize the power fluctuations.
- Subsidized costs of plantlets as an incentive to famers to enable them accept and market the technology.
- Affordable quality laboratory supplies to reduce the cost of production.
- Steady and reliable source of water such as harvesting of rainwater and sinking bore holes.
- Recruit and train enough personnel in tissue culture techniques
- Establish clean and disease free materials through thermotherapy and virus indexing.
- Capacity building: Sensitization of community on the use of tissue culture materials to control pest and diseases and improve crop production through workshops, seminars and trainings

CHAPTER 6

CONSERVATION BIOTECHNOLOGY FOR VEGETATIVELY PROPAGATED CROPS: THE CASE OF CASSAVA AND SWEET POTATO

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6.1 Introduction

Plant genetic resources (PGR) are the sum of all combinations of valuable genes resulting from evolution of plant species. The genetic material of plants determines their characteristics and hence their ability to adapt and survive. Conservation of PGR can simply be defined as the process of storing and/or keeping of PGR, with the objective of providing security for the material, maintaining genetic integrity and facilitating its diverse utilization.

Plant genetic resources comprise the following:

a) Cultivars in current use and newly developed varieties
b) Older (obsolete) cultivars
c) Farmers' traditional cultivars and landraces (local varieties)
d) Wild and weedy relatives of cultivated species and
e) Special genetic stocks including elite and current breeders' lines, aneuploids and mutants.

The history of plant evolution and domestication provides ample evidence that plant genetic diversity is humanity's best defense against poverty, food insecurity, alien pests, and threats to the natural resource base. Therefore, the use of PGR remains the best way of meeting future human needs. This is because PGR have great potential of driving the economic and social development of the rapidly growing human population. Unfortunately, valuable biodiversity components of both wild and cultivated species have been lost in the past 100 years. For instance, more than 15 million hectares of tropical forest are lost each year, besides experts estimating that as much as 8% of plant species could disappear in the next 25 years! Most of these are a result of intensive land use due to human population pressure, unsustainable industrialization and urbanization, which are being exclusively perpetuated by human activities.

Vegetatively propagated crops notably cassava and sweet potato are very important staple crops in the region. Furthermore, these starchy root crops have a high potential of being major sources of animal feed and raw materials for diverse industrial applications. These crops are high priority commodities in the research and development agenda of national agricultural research programs in the East African region, in ensuring increased and/or sustained production at farm level. Conservation of these crops as a component of PGR is one area that has received limited attention in the region. In this chapter, we appraise the conservation methods and discuss some of the challenges associated with conservation of such vegetatively propagated crops.

6.2 How can conservation be done?

For conservation strategies to operate there must be a place to store and/or maintain germplasm. Policies and protocols for their use must also be instituted. A place where germplasm is gathered is usually called a plant genetic resource centre, which includes the germplasm banks or gene banks. These can constitute seeds in cold storage, field collections, plants conserved *in vitro* or cloned DNA fragments from a single genome. Conservation biotechnology is one option that can be used in PGR conservation; this involves conservation of germplasm ex-situ using *in vitro* banks, DNA banks, pollen and spore banks. A rational conservation strategy will aim to conserve the existing crop diversity over a long term period. While implementing a conservation program, it is important to: (a) meet agreed upon standards of management, (b)

have minimum duplication of tasks, (c) have safety back-up arrangements, (d) ensure availability and access to the material, and (e) have an efficient information management system.

6.2.1 In-vitro banks

In-vitro banking is a process of germplasm conservation that follows the similar techniques as tissue culture, except that the growth speed is reduced by modifying the physical and/or chemical conditions of the culture. *In-vitro* banking is effective for both short and/or medium term period (in years) and can be achieved by manipulating the media composition, temperature, light intensity and sub-culturing as discussed below.

6.2.1.1 Growth restriction media

After years of research, media for vegetatively propagated crops have been developed and optimized for *in-vitro* growth, as already illustrated in earlier chapters. However, germplasm conservation requires minimal plant growth while maintaining culture viability. Use of growth restriction media maximizes the time interval between transfers (subcultures) of *in-vitro* plantlets. Laboratory experiments aimed at limiting *in-vitro* growth of vegetatively propagated crops include the use of hormonal growth retardants such as abscisic acid (ABA), growth inhibitors such as B995 or chloride chloride (CC), as well as osmotic regulators with addition of low assimilation sugars such as manitol or sorbitol.

Plantlets cultured in growth retardants have survival rates ranging between 70% and 90%. For example, plants grown in a medium containing abscisic acid at 5-20mg/L had survival rates ranging between 70 to 85% after 8 months, and showed strong genotypic effects (Jarret *et al*., 1991). On the other hand, plants grown with the retardant maleic hydrazide at 5mg/L had a survival rate of 70-90% after 6 months (Desamero, 1990). Plants grown with cycocel at 500mg/L had the same survival rate after one year (Guo *et al*., 1995). Related studies have indicated that some vegetative crops plantlets can remain in 10-40g/L sorbitol for 6-12 months without subculturing (Desamero, 1990).

Laboratories intending to adopt this method of germplasm conservation should optimize the concentration of the retardants in the culture media for the crop or variety in question using the above mentioned methods as reference protocols.

6.2.1.2 Restriction of storage temperature

In-vitro growth of plantlets may be restricted by reducing the incubation temperature. At 8°C, survival time is less than one month. Adequate *in vitro* growth of vegetatively propagated crops can be obtained with temperatures between 28°C and 30°C. As with other *in vitro* cultures, low temperature and growth retardants may be used simultaneously; the combined use of osmotic regulation and low temperature (15°C) appears to be a costly way of maintaining vegetatively propagated crops germplasm collections.

6.2.1.3 Reducing light intensity and Osmoticums effect

Reducing light intensity suppresses photosynthesis of organic compounds that are necessary for plant growth. A combination of osmoticums, low temperature, and low light intensity has been the most effective strategy in lengthening periods between subcultures. Programs can be designed for short or medium term storage of vegetatively propagated crops germplasm. The addition of osmoticums or growth retardants to the medium has proved efficient for reducing growth rates of different plant species. Osmoticums such as mannitol or sorbitol reduce mineral uptake by cells through differences in osmotic pressures thereby retarding plant growth (Dodds and Roberts, 1995).

6.2.1.4 Sub-culturing of the callus for conservation of germplasm

Explants, when cultured on appropriate medium, usually with both an auxin and a cytokinin, can give rise to an non-organised growing and dividing mass of cells called callus. In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is sub-cultured onto fresh medium periodically. This allows the application of callus cultures in germplasm conservation. As indicated earlier, callus culture is often performed in the dark as light induces photosynthesis that can encourage differentiation of the callus. However, during long-term culture, the tissues may lose the requirement for auxin and/or cytokinin, a process, known as 'habituation'. Manipulation of the auxin: cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, somatic embryogenesis and organogenesis for the regeneration of plants.

6.2.1.5 Steps for optimum in vitro germplasm conservation

In vitro conservation comes with the risk of losing material due to failure of cooling equipment, contamination of cultures, or mislabeling of accessions. Therefore, the following recommendations will help to maintain an *in vitro* collection more efficiently:

a) Frequently evaluate *in vitro* growth during the first month to detect plants with growth problems.
b) Maintain aseptic conditions in the culture growth room to avoid sources of contamination (dust, dirt, mites, or contaminated material). Treat the room as a restricted area.
c) Equip the *in vitro* laboratory with an electric alarm connected to a control panel that monitors environmental conditions.
d) Develop a database with unique codes for each accession in the collection. Each accession should have at least two code numbers for proper identification. Labels for the cultures should be generated directly from the database to prevent human errors while transcribing.
e) Closely monitor the cultures regularly during *in vitro* storage. Isolate contaminated cultures as soon as they are detected to prevent spreading the problem to clean plants. Include specific antibiotics in culture media to eliminate endogenous bacteria in the cultures.

Advantages of in vitro germplasm conservation

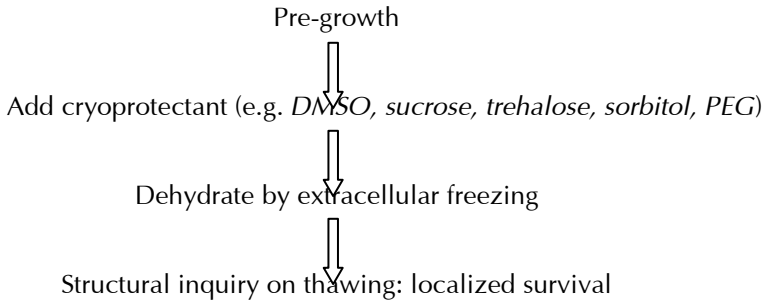
1. Tissue culture saves space and labour, which enables more germplasm to be conserved.
2. Tissue culture allows the conservation of special material, such as out breeding offspring and the plants produced by somatic hybridization (Xia & Zhu, 1987), or materials bearing resistant genes but not producing storage root.
3. *In vitro* conservation is generally more secure, less expensive and labour intensive than seed stem storage.
4. *In vitro* materials occupy less volume and are more convenient for transportation than field-grown propagules.
5. There is less restriction in quarantine when the materials are exchanged internationally (Engleman, 1991). This helps in exchanging healthy genetic resources as tissue cultured materials can be made free of insects and diseases; thus *in vitro* conservation is an excellent technology.

Disadvantages of in vitro germplasm conservation:

1. The major problem with *in vitro* conservation is genetic instability especially due to somatic mutations, since these are clonally propagated crops.
2. *In vitro* plantlets cannot be characterized and evaluated like field-grown plants.
3. Tissue culture requires electrical energy for the refrigeration of culture rooms, which can be a huge challenge and in terms of production costs in developing countries.
4. Requirements of hygiene in tissue culture are very strict; otherwise contamination by microbes will lead to loss of the materials.
5. In developing countries, the prices of some consumables, such as electric power, chemical reagents, etc are relatively high, so the cost of *in vitro* conservation is more expensive than that in field gene bank.

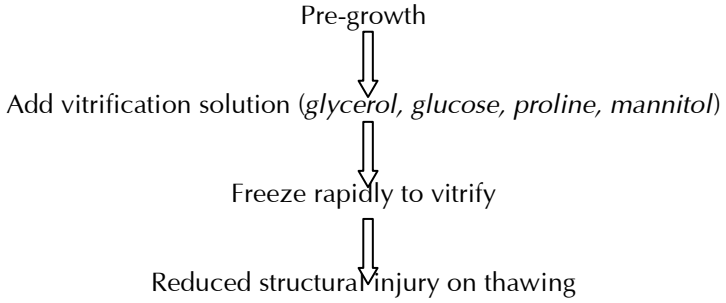
6.2.1.6 Protocol for cryoconservation

The genetic diversity of vegetatively propagated crops and the range of physiological characteristics that they possess preclude use of a single method for cryoconservation. Several methods have been examined to cryopreserve shoot tips and buds from less-hardy and non-cold acclimatable species. However, the two-step cooling and vitrification methods are commonly used (Towill, 1991). The two-step cooling method has been successfully applied to many species and is a feasible method for cryoconservation. Survival after two-step cooling is probably due to the vitrification of the cellular contents during the immersion step from about -35°C to liquid nitrogen. Below is an illustration of the typical two step cooling model:



The vitrification method of cryopreservation utilizes concentrated cryoprotectants that form a glass upon being rapidly cooled i.e., the system vitrifies (Steponkus *et al*., 1992). Vitrification may avoid some damaging events which occur during ice formation in the two-step cooling method. The advantage of vitrification to the user is simplicity of the technique, since no cooling rate apparatus are required and it has potential to use larger pieces of tissue. Tests have shown that survival can be obtained after vitrification in protoplasts (Steponkus *et al*., 1992), cells (Sakai *et al*., 1991), somatic embryos (Uragami *et al*., 1989) and shoot tips when these materials are treated appropriately. Other methods have been reported to give fairly high levels of survival after low temperature treatment. Some of these involve encapsulation of shoot tips in alginate beads with exposure to dehydrating conditions prior to using rapid cooling (Paulet *et al*., 1993). In these studies, pre-treatment of the shoot tips with elevated levels of sucrose is essential to maintaining viability. The combination of sugar, desiccation, and rapid cooling probably leads to vitrification in the shoot tips.

The standard vitrification procedure consists of applying the cryoprotectant solution to the shoot tips, exposing the samples to liquid nitrogen, warming, and removing the vitrification solution. The details of vitrification often vary with species. A key issue is the application of the cryoprotectant solution. This is usually done in two steps. Shoot tips are first exposed to a dilute solution of a permeating cryoprotectant, such as dimethylsulfoxide or ethylene glycol. Then they are exposed to a concentrated solution which serves mainly to dehydrate the shoot tip; this solution is of a composition that vitrifies during cooling. Steponkus *et al*. (1992) gave an excellent discussion of what strategy might be used to arrive at useful solution(s) which involves an analysis of permeation and dehydration characteristics. Permeation is not needed for many materials and may be detrimental. In these cases survival with vitrification is obtained using the desiccation phase alone, although pre-treatment with sugars is still required to obtain survival. A typical model of vitrification is highlighted below.



Advantages of cryopreservation

1. It can lead to safer, cheaper, and long-term conservation of genetic resources.
2. Thousands of vegetatively propagated plant clones can be maintained under controlled slow-growth conditions within the gene bank and grown out every 12 to 18 months to produce fresh clones.
3. Cryopreservation involves less work and is cheaper than maintaining either an active *in vitro* collection or outdoor conservation areas (*in situ* sites).
4. The prepared shoot tips take up very little space and can be stored indefinitely without being disturbed.
5. Cryopreservation can also be used to support transformation of vegetatively propagated plants where the development of friable embryogenic callus cell lines is time consuming, with the inherent risks of genetic instability and low plant recovery overtime.

Disadvantage: The major disadvantage of cryopreserved embryogenic tissue of shoot tips is that it shows less genetic stability than tissue culture product. Towill and Jarret (1992) reported the first successful case of conserving the vegetatively propagated sweetpotato shoot tips from plantlets *in vitro* in liquid nitrogen through vitrification. The highest viability of the cryopreserved shoot tips from two vegetatively propagated crops clones was 83%.

6.2.1.7 Temperature effects in cryopreservation

Cryogenic storage at very low temperatures is presumed to provide an indefinite, if not near infinite longevity to cells, although the actual "shelf life" is rather difficult to prove. In experiments with dried seeds, researchers found that there was noticeable variability in deterioration when samples were kept at different 'frozen' temperatures, even ultra cold ones. Temperatures below the glass transition point (Tg) of water (~ -136°C) appear to be accepted as the range where biological activity substantially slows down, and -196°C (liquid phase of liquid nitrogen) is the preferred temperature for the storage of important specimens. While fridges, deep freezers and extra cold deep freezers, all similar to domestic ones, are used for many items, generally the ultra cold of liquid nitrogen at -196°C is required for successful conservation of the more complex biological structures to virtually stop all biological activity.

6.2.1.8 Risks involved in cryopreservation

Phenomena which can cause damage to cells during cryopreservation mainly occur during the freezing stage, and include: solution effects, extracellular ice formation, dehydration and intracellular ice formation. When having reached the frozen stage, the preserved material is relatively safe from further damage. However, estimates based on the accumulation of radiation-induced DNA damage during cryogenic storage have suggested a maximum storage period of 1000 years. For solution effects, as ice crystals grow in freezing water, solutes are excluded, causing them to become concentrated in the remaining liquid water. High concentrations of some solutes can be very damaging. Extracellular ice formation occurs when tissues are cooled slowly, and in the process, water migrates out of cells and ice forms in the extracellular space. Too much extracellular ice can cause mechanical damage to the cell membrane due to crushing. For dehydration, the migration of water results into extracellular ice formation. The associated stresses on the cell can cause damage directly. And for intracellular ice formation, though some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells.

Prevention of risks can be achieved through controlled-rate and slow freezing. These are well established techniques pioneered in the early 1970s, which enabled the first human embryo frozen birth Louise Brown in 1978. Since then machines that freeze biological samples using programmable steps, or controlled rates, have been used all over the world for human, animal and cell biology this involves 'freezing down' a sample to better preserve it for eventual thawing, before it is deep frozen, or cryopreserved, in liquid nitrogen.

Such machines are used for freezing vegetatively propagated plant stem cells and general tissue conservation in research laboratories around the world. Lethal intracellular freezing can be avoided if cooling is slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid. However, that rate differs between cells of differing size and water permeability: a typical cooling rate around 1°C/minute is appropriate for many cell types after treatment with cryoprotectants.

6.2.2 DNA banking

Deoxyribonucleic acid (DNA) is the hereditary molecular material in most living organisms. In DNA banking, DNA is extracted from cells and purified. The DNA is then cut by enzymes, and the resulting fragments (finger printing or profiling) undergo procedures that permit them to be analyzed. The pattern of the fragments helps to reveal genetic differences and similarities between living organisms. DNA profiling can be used to determine whether the collected sample already exists in the collection or not, thus preventing duplication in the same gene bank. It can also help in identifying endangered species worth conserving.

6.2.3 Spore/pollen banking

Pollen banking supports work on allergenic responses, plant hybridization and fertility, haploid plant production for breeding and for genetic transformation systems. The optimum storage conditions for pollen banking are:

- Low moisture content like in seeds
- Ease of storage relates to physiological state e.g. some can tolerate 10% moisture content (potatoes) and others are more sensitive to desiccation
- Most work done on fruits which can be stored for 10 years at -20^0c

6.3 Collection and movement of material for conservation

6.3.1 Collection of germplasm

Collecting activities are aimed not only at providing germplasm for immediate use in breeding, but also at conserving genetic variation for future use. By using variation for characters during collection missions from different regions, it is possible to select certain characters for detecting variability in the populations and establishing the minimum number of genotypes to be sampled. A minimum number of 20 - 130 plants per crop is recommended in order to capture 95% of the total variation in the field. However, it has been emphasized that the number of different populations sampled rather than the sample size per population determines the overall efficiency of a collection. Accessions can be acquired by two main ways: a) germplasm collections through explorations and b) germplasm collections from those held in trust.

There are two types of collections:

Active collections: This refers to collections kept for medium term, which are immediately available for distribution, utilization and multiplication. Active collections are kept in conditions which ensure that the accession viability remains above 65% for 10–20 years. Ideally, these are maintained in sufficient quantity to be available on request. Different combinations of storage temperature and moisture content can provide this longevity (IPGRI, 1996).

Base collections: This refers to collections kept for long term, solely for 'posterity', and is not drawn upon except for viability testing and subsequent regeneration. The accessions in base collections should be distinct in terms of genetic integrity and as close as possible to the sample provided originally. Base collections are stored at (−20°C) ensuring long-term viability of material (more than 50 years) as a security to the active collection. It is advised that safety back-up for long-term conservation in countries outside the respective country should be considered.

6.3.2 Germplasm characterization and evaluation

Adequate characterization of germplasm is necessary to facilitate improved acquisition, maintenance and utilization of PGR by breeders and end-users. Initially, characterization for morphological and agronomic traits over years and locations was the norm. However, morphologic characterization has limitations. First,

highly heritable traits often show little variation. Second, some agronomic traits are strongly influenced by the environment, making their measurement difficult. This has resulted into the search into alternative methods of quantifying genetic variation. One such option is the use of molecular markers that analyze polymorphism at the DNA level. Molecular markers may therefore be used for establishing 1) identity:- the determination of whether an accession or individual is catalogued correctly, is true to type, maintained properly, and whether genetic change or erosion has occurred in an accession or population over time; 2) the degree of similarity among individuals in an accession or between accessions within a collection; 3) patterns of variation among individuals, accessions, populations, and species; genetic structure is influenced by *in situ* demographic factors such as population size, reproductive biology and migration, and 4) the presence of particular allele or nucleotide sequence in a taxon, genebank accession, *in situ* population, individual, chromosome or cloned DNA segment.

A number of techniques can be used for the detection of variations (polymorphisms) in the DNA. Some of these are based upon the initial digestion of the DNA with restriction enzymes, while others depend upon the use of the polymerase chain reaction. Examples of genetic markers include: Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP), Restriction Fragment Length Polymorphism (RFLP), Cleaved Amplified Polymorphic Sequence (CAPS), Simple Sequence Repeat (SSR) markers also known as microsatellites, Single Nucleotide Polymorphisms (SNP), and Sequence Characterized Amplified Regions (SCAR). The results of molecular or biochemical studies should be complementary to morphological characterization.

6.3.3 Development of core collections

Core collections consist of a limited set of accessions chosen to represent the widest genetic variation in a crop species and its wild relatives with minimum repetition (Johnson and Hodgkin, 1999). This concept was created to deal with the increasing number and size of germplasm collections. Their essential features are restricted size, structured sampling of species or collections, and diversity. The procedure of forming a core collection involves:

a) Defining the collection to be represented. This starts by assembling all the relevant data (e.g. morphological traits, genetic markers) on the accessions in that collection then deciding the size of the core. Usually a proportion (10%) of the entire collection is used since it is believed this percentage will contain at least 70% of the alleles in the original collection (Brown, 1989a).

b) Grouping the accessions into groups that reflect the major genetic and ecological groups within the collection.

c) Choosing the entries for the core.

d) Managing the core.

6.4 Management of collections using biotechnology

Conserved plants must be cleaned and stored properly, rejuvenated when aged, tested for health and viability, investigated for their value in improving the crop, safely duplicated and exchanged. Whether conserved as seed, *in vitro* or in the field, managers of *ex situ* collections need to maintain the integrity of the accessions conserved and to identify any duplicates.

6.4.1 Regeneration and multiplication work

Material stored *in vitro* has a high risk of losing genetic integrity perhaps due to somaclonal variation. Also seeds lose viability even under good storage conditions and it is necessary to regenerate accessions from time to time. The frequency of regeneration depends on the initial viability, the rate of loss of viability and the regeneration standard (i.e. the percentage viability at which it is decided to regenerate the accession) (Roberts, 1984). The aim of regeneration is to increase the quantity of germplasm of any accession where the number available has been depleted, or to restore maximum viability to the germplasm lot. Regeneration of

germplasm is one of the most crucial processes in genebank management. It is costly in terms of resources and time, and it involves the risk of compromised genetic integrity. The material is planted out in the field and allowed one cycle of regeneration before it is stored back (rejuvenation).

6.5 Health and security concerns of conservation biotechnology

Over the past twenty years, the major concern in laboratory safety was how to deal with chemical hazards. We also need appropriate safeguards or biosafety measures which must be put into place for the operation of these conservation laboratories. It is important that conservation programmes that use biotechnology operate under standard operating procedures that are internationally recognized. This will build public trust and confidence, thereby promoting the development of sustainable applications of biotechnology, and establishing appropriate enabling mechanisms.

6.5.1 Biotech industry hazards and risk assessment

The major hazards of biotechnology are those associated with exposure to large concentration of aerosolized micro-organisms or their products. The primary stage of occurrence is at the level of research laboratory where biotech applications are used resulting in hazards such as laboratory infections due to handling of microorganisms, working with infectious agents and recombinant DNA-based micro organisms. It must be noted that, many agents not ordinarily associated with disease processes in humans can be opportunistic pathogens. At the laboratory level, these risks arise due to flouted safety regulations, use of obsolete equipment, having a large number of manual operations and poor house sanitation.

In agricultural applications these risks may manifest as genotypic or phenotypic change in the production process, development of resistant strains of bacteria to human therapeutic use of antibiotics, establishment of non-indigenous / genetically engineered microorganisms/ plant pathogens in the environment, causing crop vulnerability to sudden collapse due to uncontrolled out breaks of pests and diseases. In extreme cases, heavy metal ions may be transformed by the resistance strain of micro-organisms into organic derivatives that are toxic to aquatic animals. To the populace, the hazardous micro-organisms can be a potential public health risk leading to spread of infectious bacteria..

6.5.2 Risk assessment and management

Every country should have policies and regulations for overseeing safe use of biotechnology products at every stage of development or service in the environment. For example, at the research stage, control exists to help ensure the safety and well being of laboratory workers, including controls on the manipulation and release of genetically modified organisms. Legislation covers good laboratory and manufacturing practices. The purpose of risk management in biotechnology is to assign appropriate control measures or containment measures commensurate with the estimated hazards of the micro-organisms. It begins with executing good laboratory practices as the first line of defense at institutional level where the work in the laboratory is confined depending on the risk level. Employees working with poisonous, infectious or potentially infectious materials must be aware of the possible hazards. In addition, they must be trained and skilled to safely handle biohazardous materials. The facility must contribute to laboratory protection by providing a barrier to protect people and animals in the community outside. Depending on the biosafety level of a facility, design features need to take care of chemical, electrical, glassware and fire safety.

6.6 Utilization and maintenance of genetic resource databases

Having PGR databases constitutes the keystone for a sustainable, rational, efficient, and effective global network of gene banks. Ultimately, the purpose of these databases is to permanently safeguard PGR information vital to global food security, and to encourage the use of PGR by researchers, breeders, and farmers. Types of data stored include: passport, characterization/ evaluation and seed inventory information. Documentation of passport data on all accessions in the collection, especially habitat collection data, must not be static, but should rather be continuous. The databases require regular improvements to meet changing requirements, e.g. intellectual property details, International Treaty details, Standard Material Transfer Agreements. Databases generally have a life of approximately ten years.

CHAPTER 7

VIRUS INDEXING AND CERTIFICATION IN CLONAL CROPS: THE CASE OF BANANA

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7.1 Introduction

Development of tissue culture (TC) and *in vitro* plant propagation techniques have made it possible to mass propagate high quality banana and plantain (here afterwards referred as banana) planting material. These advancements, termed as micropropagation, have led to the advent of commercial TC industries dedicated to the production of banana planting material for domestic as well as international trade. Such materials have replaced conventional vegetative sucker production in many banana growing regions around the world (Israeli *et al.,*1995; Smith *et al.,*2005). While micropropagation offers several advantages over conventional sucker production, it does not exclude viruses, viroids, phytoplasma and fastidious bacteria, which may be present in the mother stocks (Diekmann and Putter, 1996). Propagation material derived from the infected mother stocks results in perpetuation of pathogens leading to low yields and poor quality fruits. In addition, infected material serves as vehicles for spread of pathogens which is a major concern for domestic and international movement of the planting material. Infected planting materials established in the fields are not amenable to curative procedures and they act as sources for secondary spread of pathogens by natural vectors like aphids, beetles, mealy bugs as well as through agriculture implements. This risk of pathogen spread through planting material is of a high concern; this is because banana is affected by several important pathogens of high quarantine significance.

In order to manage these risks, phytosanitation procedures that exclude viruses and other pathogens from TC material have been established under the framework of 'certification programs'. Such certification programs define the risks and protocols to identify quality and healthy mother stocks for propagation and distribution. However, there are no universal standards or guidelines for health certification of micropropagated banana established at country level based on the international phytosanitary guidelines for safe exchange of *Musa* germplasm (Diekmann and Putter, 1996). To meet the international standards, several countries have established basic standards and best practices for technical competences, operative aspects of production and quality control procedures to ensure production and marketing of only high quality and pathogen-free material (DPI&F, 2006; NCP-TCP, 2008). In this chapter an overview of procedures involved in ensuring health of mother stocks (nucleus material) and health certification of micropropagated material is presented. These methods are being applied to contain the spread of major banana diseases caused by: Banana bunchy top virus (BBTV); Banana streak virus (BSV); banana mosaic or infectious chlorosis caused by Cucumber mosaic virus (CMV) and Banana bract mosaic virus (BBrMV). Banana Xanthomonas wilt (BXW), sigatoka leaf spot disease and panama disease just to name a few secondary and tertiary infection as a compounded effect of viral effects.

7.2 Virus indexing to eliminate infected sources

Sterile tissue culture procedures are using apical shoot tissues of banana to eliminate several fungal and bacteria in the explants but not viruses. Therefore, virus-free source materials carefully selected by virus indexing are used to avoid perpetuation of viruses in apical shoot tissue culture (Figure 7.1. An effective virus indexing depends on reliable diagnostic methods that can lead to correct identification of the pathogen (Table 7.1). Various virus indexing methods based on the biological, serological and nucleic acid properties of the viruses have been described (Kumar, 2009), which are continuously being improved and optimized. Specificity (accuracy to detect the target species/strain) and sensitivity (ability to detect lowest amount of target) are the two important attributes that determines the selection of a test for virus indexing. Choice of test also depends on the objective of the indexing. For instance, tests aimed at detecting BSV should be sensitive and broad-specific to capture all the BSV strains. Broad-specific diagnostic tools that can capture all known strains/variants of the virus species are most useful for indexing purpose to identify virus-free material.

Indexing for banana viruses is performed using bioassays (mechanical inoculation and/or grafting to indictor plants), enzyme-linked immunosorbent assay (ELISA), electron microscopy (EM), polymerase chain reaction (PCR) and nucleic acid spot hybridization (NASH). The choice of test depends on the virus species, stage at which plants are tested and the type of tissue material. Standard testing procedures have been described for most banana pathogens, particularly for differentiating episomal BSV infection from integrated BSV genome sequences (Diekmann and Putter, 1996). Maximum sensitivity is the primary goal of the virus indexing program. Often, virus indexing programs use widely accepted or officially approved (accredited) tests. Virus indexing for certification demands the application of accredited test or testing of samples in the accredited labs (NCP-TCP, 2008).

Bioassays, useful for the detection of CMV and BBrMV but not BSV or BBTV, are seldom used because of poor sensitivity and risk of failure to detect virus in the potentially infected plants. ELISA and its variants [Triple Antibody Sandwich (TAS)-ELISA and Protein A Sandwich (PAS)-ELISA] are useful assays for the detection of CMV, BBrMV, BBTV and BSV, although its use in indexing of foundation and mother stocks is limited. PCR and its variants (Reverse Transcription [RT]-PCR, Immunocapture [IC]-PCR and IC-RT-PCR) have gained wide acceptance for banana virus indexing. PCR assays are available for the detection of all the banana pathogens. However, PCR does not differentiate BSV episomal (infectious particles) from integrated BSV. IC-PCR, in which virus particles are first, captured using BSV antibodies followed by the PCR detection of the viral genome present inside the virus particles is the method of choice for indexing episomal BSV. *In planta* concentration of CMV and BSV are known to fluctuate depending on weather conditions (Dahal *et al.,* 2000); this situation necessitates multiple testing for these viruses at different times to avoid false negatives. Indexing foundation stock and mother blocks (for deriving apical shoots), requires application of all possible indexing methods to ascertain the virus-free status. It is important to test every mother plant and select only virus-free plants for deriving planting material.

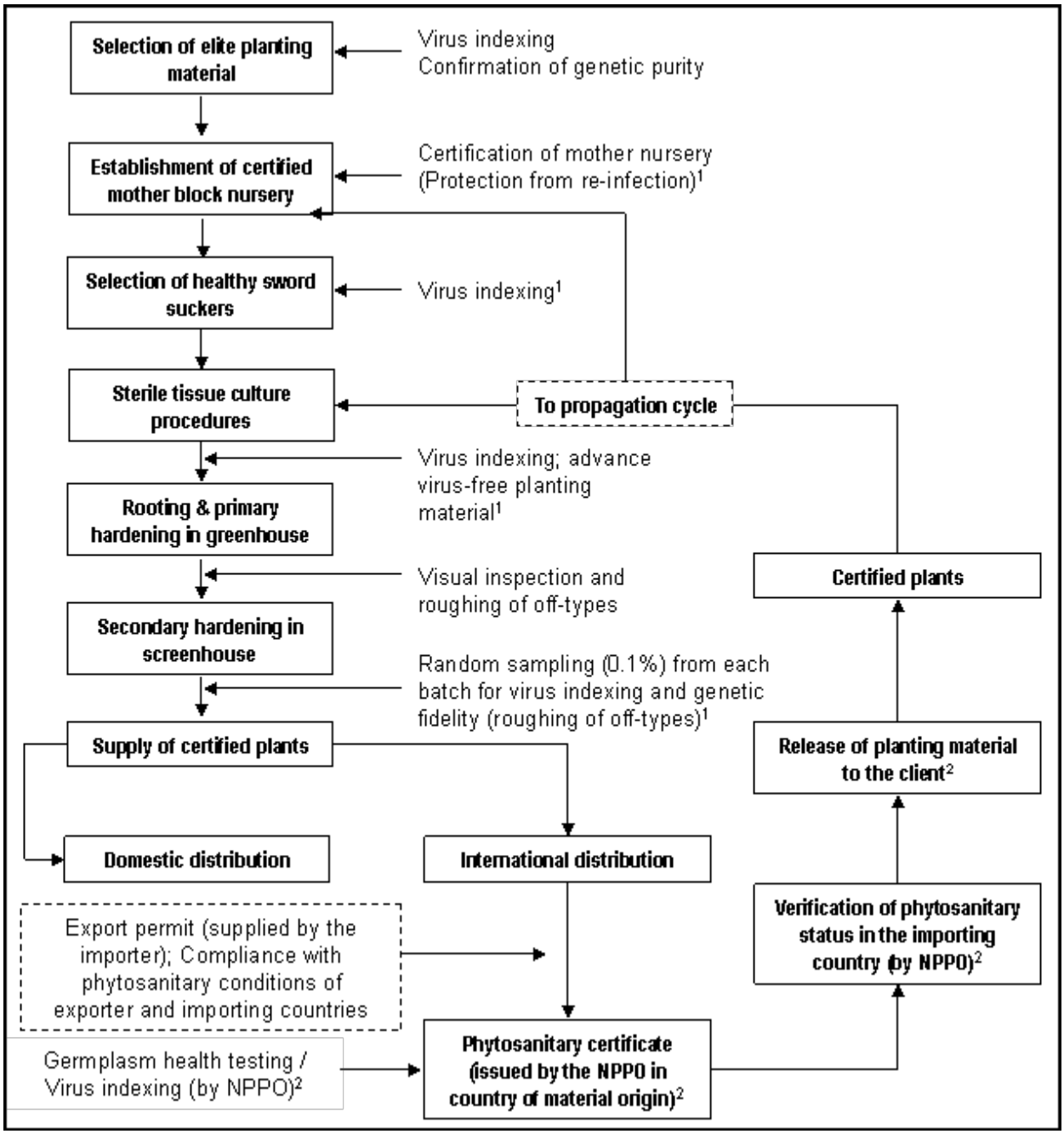

Figure 7.1: A generic scheme for the production of virus-free micro propagated banana in TC industry. **Note:** Actual procedure may vary depending on the capacity of the industry and country regulations. [1]Certification / virus indexing is performed in-house by the TC industry or in accredited labs. [2]Certification is mandatorily carried by the national plant protection organizations

Table 7.1: Diagnostic methods for indexing pathogens in banana

Pathogen / disease	Culture-based	EM/ISEM	ELISA	PCR	RT-PCR	IC-PCR/ IC-RT-PCR	NASH[4]
BSV (integrated)	na	na	-	+++[3]	na	Na	++
BSV (episomal)	-[1]	++[2]	+	na	na	+++[3]	++
CMV	-[1]	++[2]	+	-	+[3]	+++	++
BBrMV	-[1]	++[2]	+	-	+[3]	+++	++
BBTV	na	++[2]	-	+++[3]	na	-	++
AbMV	na	-	-	+++[3]	na	-	++
FoC Race 1 & Race 2	+	na	-	+++[3]	na	Na	-
FoC TR 4	+	na	-	+++[3]	na	Na	-
BXW	+	na	-	+++[3]	na	Na	-
M. fijiensis *M. musicola* *M. eumusae*	+	na	-	+++[3]	na	Na	-

na= not applicable; + = suitable method; - = not suitable / available; EM = electron microscopy; ISEM = immuno-electron microscopy; NASH = Nucleic acid spot hybridization; PCR = polymerase chain reaction; RT-PCR = reverse transcription PCR; IC-RT-PCR = immunocapture RT-PCR; IC-PCR = immunocapture PCR; [1]Detection by bioassays (mechanical inoculation to indicator plants) is possible; however, it is cumbersome for testing large scale testing and not sensitive; [2]Partially purified sample preparation enhances sensitivity of virus detection; [3]Method of choice; and [4]Complicated to perform.

7.3 Virus certification

Virus certification basically involves the same process as in virus indexing. In certification, the procedures for are performed strictly as per the method prescribed by monitoring authorities for virus indexed material to qualify for 'certification' as a guarantee to sanitary status of propagative material for virus-free (freedom from all viruses known to infect species tested) / virus-tested (only to the viruses for which diagnostics tests were employed). For micropropagated banana, freedom from BBTV, CMV, BSV, BBrMV and BanMMV is essential for the distribution of planting material.

7.4 Certification schemes

Certification schemes have been established to comply with official standards/regulations set by the national and international authorities. Such certification schemes guarantee the quality of propagative material to trueness-to-type (genetic purity) and absence of specified pathogens in the micropropagated plants (Golino and Savino, 2005). This process basically involves assessing the risks (pathogens and pests), selection of clean-planting material, virus testing, micropropagation and tests for genetic fidelity. A certificate is only provided to plants that are produced as per the directives of the scheme. Certification schemes depend on the objective of the program and vary considerably depending on crops and countries. Certification schemes originated as a method to control virus-diseases in seed-potatoes and tree crops (Anonymous, 1992). In the absence of effective control strategies and host resistance, cultivation of virus-free planting material has become the choice for growers and is vigorously promoted by the public and private agencies associated with welfare of agriculture sector. Certification schemes usually involve several components that ensure success of the program (Hollings, 1965; Meijneke, 1982; NCP-TCP, 2008).

Some of the key components are (i) clearly defined purpose of certification and protocols and standards for producing certified planting material; (ii) guidelines for selection of mother-stocks for micropropagation; (iii) availability of reliable indexing methods for the detection of regulated and unregulated quarantine pests and pathogens; (iv) protocols and procedures for production of virus-free planting material; (v) guidelines for maintaining the health of mother-stocks and propagated material; (vi) guidelines for labeling planting material for traceability; (vii) guidelines for national and international distribution of planting material; (viii) guidelines for infrastructure and technical competence; and (ix) guidelines for supervision and monitoring compliance by the producers.

Several banana growing countries are adopting certification schemes as a strategy to regulate the spread and propagation of viruses and other quarantine pathogens, and also guarantee the genetic purity of the variety and promote international export of planting material to earn foreign exchange. The banana certification scheme established in mid-1990s by the Queensland Department of Primary Industries in Australia is perhaps the earliest certification program established for banana in the world (DPI&F, 2006). It is used as the fundamental management tool for the control of BBTV in Australia, and has been subsequently adopted in India, Pakistan and several other countries where the disease is a major problem. Usually certification schemes are enforced by the appropriate national authorities. This body oversees the certification of mother block nurseries, indexing of source material and certification of hardened material for distribution. Several TC industries establish their own 'best practices' as per ISO/IEC 17025:2005 (standards management system for quality, administrative and technical operations). Several TC industries in Israel and South Africa produce plant material for international markets. These companies ensure compliance with regulations set by the importing countries. In India, the TC industry was initially unregulated; however, regulations were later enforced following the reports of spread of BBTV in micropropagated plants. As per the regulations in force since 2006, only TC industries that are accredited by the Government of India are qualified to produce banana planting material (NCP-TCP, 2008). Through this accreditation process the government agencies monitor quality of the plant material and assure supplier's guarantee to the product.

The certification procedures used in various countries may slightly differ, but the overarching goal is to protect farmers and national interest by ensuring the production of quality planting material that are free of dangerous banana diseases. Banana planting material meant for international distribution is monitored by the national plant protection organizations (NPPOs) as per the FAO-IPPC regulations on germplasm exchange (Diekmann and Putter, 1996).

7.5 Production of pathogen-free micropropagated material

This process has several technical steps as depicted in Figure 7.2. It begins with the selection of appropriate varieties depending on the market demand. In most countries, it is a legislation to multiply varieties that are approved for farmer cultivation for propagation by the TC industry.
Confirmation of genetic purity for the variety using appropriate DNA and/or morphological markers is required to eliminate off-types (Smith *et al*., 2005). Virus-infected material is eliminated by indexing procedures as detailed earlier, but elimination of fastidious bacteria is a problem (Thomas *et al.,* 2008). Most endophytic bacteria are not pathogenic, but they affect *in vitro* plants in storage. Quality control procedures ensure that appropriate initial material is used for micropropagation, culture and indexing conditions are satisfactory and the identity of indexing material (labeling and traceability) is maintained during the culture process.

Ensuring quality and virus-free status incurs additional production costs and a lag time during establishment of virus-free sources. In some countries foundation stocks of genetically pure and pathogen-free stocks of approved varieties are maintained by the government or grower associations for supply to the users. For example, the TC industry in Queensland and New South Wales is allowed to use only material grown in the Queensland Banana Accredited Nursery (QBAN) (DPI&F, 2006). In USA, Foundation Plant Services (FPS) of the College of Agricultural and Environmental Sciences, University of California at Davis maintains nuclear stock materials of deciduous crops, like grapes (Golino and Savino, 2005). In India, TC industries either establish and maintain their own foundation stock or procure it from another certified laboratory (NCP-TCP, 2008).

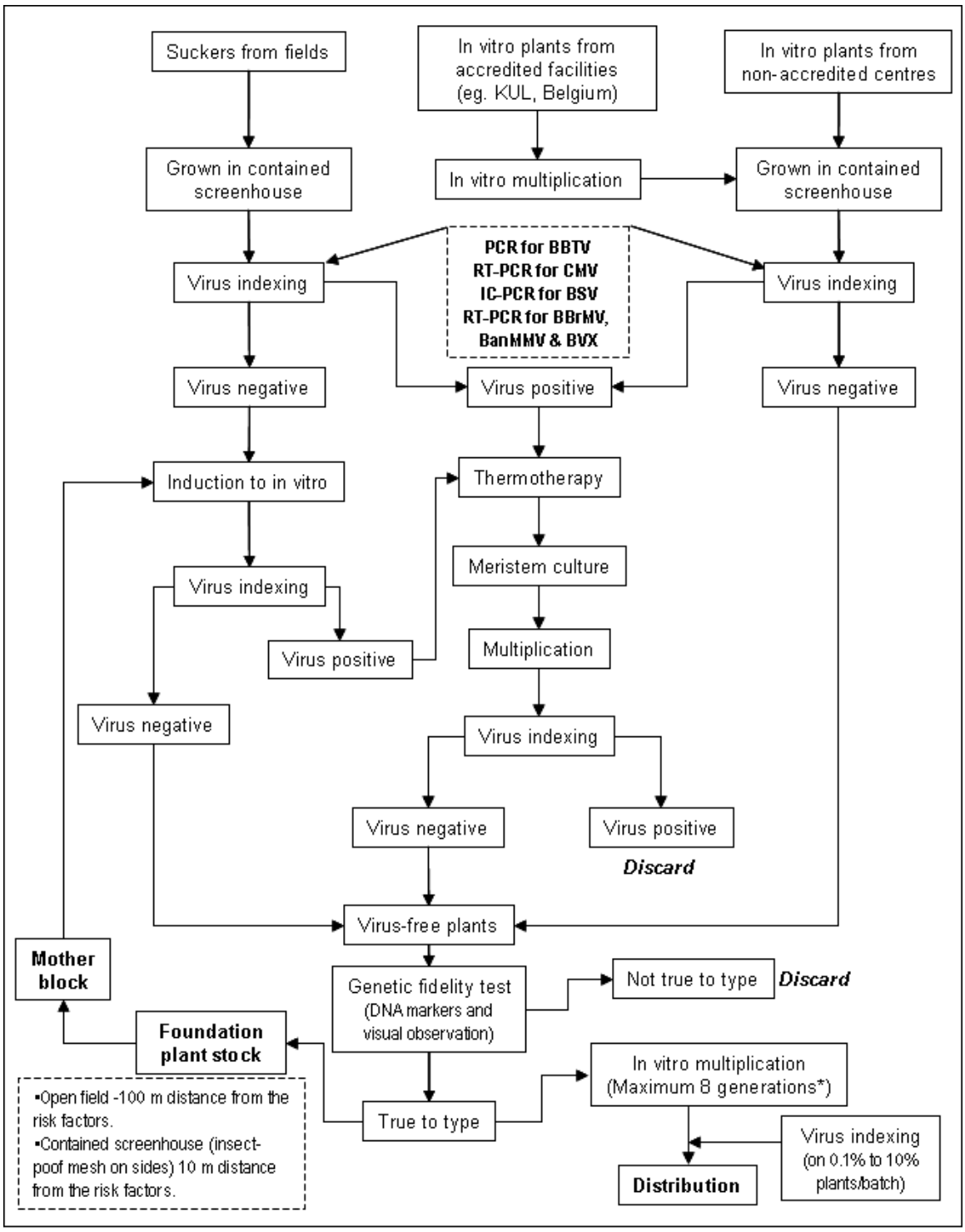

Figure 7.2: Scheme for production of virus-free banana for establishing a foundation plant stock, mother block nurseries and mass micropropagation for distribution. *Risk of somaclonal variation is after eight generations.

Centralized arrangements for maintenance and supply of foundation material and indexing services dramatically reduce the lag time, cost of production and the need for maintaining skills and capacities for diverse range of activities in every TC industry, and serve as an incentive for TC industries in developing countries to adopt quality standards.

7.6 Banana certification schemes in Africa

In Africa, international exchange of *Musa* germplasm is based on the FAO-IPPC and Inter-African Phytosanitary Council (IAPSC) enforced by the respective National Plant Protection Organizations (NPPOs) (Diekmann and Putter, 1996; Olembo 1999). As per the existing procedures, only *in vitro* plant material produced using aseptic procedures and certified as 'virus-free' are allowed to import through quarantine stations. An import permit issued by the NPPO of the importing country and a phytosanitary certificate with declarations on regulated and unregulated quarantine pests issued by the country of origin are required. There are no restrictions on importation of plant material containing endogenous BSV sequences. Exchange of suckers or rooted plant material in soil is prohibited and considered as dangerous.

Because of the dangers of pest and pathogen spread through suckers, micropropagation of banana is replacing conventional sucker planting in many countries around the world. Growers in several countries within Africa are also adopting micropropagated bananas that are produced locally or imported from other countries; the demand for micropropagated planting material is increasing. As per the FAO survey, 25 countries in Africa are using micropropagation techniques to produce planting material of about 35 crops, but commercial level production has been recorded in 12 countries (i.e. Cameroon, Gabon, Kenya, Madagascar, Mali, Mauritius, Morocco, Nigeria, South Africa, Tunisia, Uganda and Zimbabwe), and only four of these are producing (i.e. Cameroon, Gabon, South Africa and Uganda) banana (Dhlamini *et al.,* 2009).

Many countries in sub-Saharan Africa (SSA) lack specific guidelines or certification schemes for ensuring quality of micropropagated or macropropagated banana planting material produced for domestic distribution. This situation increases the risk of pathogen spread on the continent, as high volume of planting material is being generated through micropropagation programs of public and private sector and unregulated movement of planting material can contribute to spread of quarantine pathogens from one area to another. For instance BBTV and BXW are the two dangerous diseases that have restricted spread within the affected-countries. Without adequate regulation on quality of planting material produced for distribution, these pathogens can spread through contaminated sources to all the major banana producing regions in SSA (Arusha Report, 2009).

Some SSA countries stipulate commercial propagation of only virus-indexed banana planting material obtained from the International Transit Center, Belgium. Although, this procedure ensures quality of the initial planting material, there are no regulations to ensure that source material (foundation stocks) and mother-blocks used for propagation are safeguarded from reinfection risk in the field and assure quality of the planting material derived from these stocks. Virus infected plants are difficult to recognize in the field, and even TC material produced from such material do not produce symptoms and apparently look healthy leading to the unintentional distribution of infected material. History of intercontinental and intra-African spread of BBTV clearly indicates that this risk is real (Kumar *et al.,* 2009a).

Establishment of certification schemes to ensure safety and quality of planting material produced in SSA offers multiple benefits: it would increase grower confidence on micropropagated plants; reduce the risk of spread of quarantine pests; serve as an effective step to manage a number banana pests and diseases with a guaranteed success at affordable costs; rehabilitate banana regions severely ravished by BXW and BBTV; and increase the international trade of micropropagated banana. Certification schemes benefit the burgeoning micropropagation industry in SSA and foster production of clean and quality planting material which is an urgent need for millions of farmers ravished by BXW and BBTV and other biotic agents. Failures will result in multiplication of infected sources affecting the yield and quality of the fruit while contributing to widespread distribution of dangerous pathogens like BBTV and BXW. It has been well established that benefits of healthy planting material by far exceed the production costs (Raju and Olson, 1985; Sutula, 1996). Commercial producers, growers, crop improvement programs and governments will all derive benefit from cultivation of disease-free planting material.

CHAPTER 8

SEED SYSTEMS FOR VEGETATIVELY PROPAGATED CROPS

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8.1 Vegetative propagation

Vegetative reproduction is a type of asexual reproduction for plants, and is also called vegetative propagation, vegetative multiplication, or vegetative cloning. It is a process by which new plant "individuals" arise or are obtained without production of seeds or spores. It is a natural process in many plant species as well as non-plant organisms such as bacteria and fungi. Natural vegetative reproduction is mostly a process found in herbaceous and woody perennial plants, and typically involves structural modifications of the stem, although any horizontal, underground part of a plant (stem or a root) can contribute to vegetative reproduction of a plant. In a few species, leaves are also involved in vegetative reproduction.

Most plant species that survive and significantly expand by vegetative reproduction would be perennial almost by definition, since specialized organs of vegetative reproduction, like seeds for annuals, serve to survive seasonally harsh conditions. A plant that persists in a location through vegetative reproduction of individuals over a long period of time constitutes a clonal colony. In a sense, this process is not one of "reproduction" but one of survival and expansion of biomass of the individual. When an individual organism increases in size via cell multiplication and remains intact, the process is called "vegetative growth". However, in vegetative reproduction, the new plants that result are new individuals in almost every respect.

Advantages

- The offspring's are genetically identical and therefore advantageous traits can be preserved.
- Only one parent is required which eliminates the need for special mechanisms such as pollination.
- Many plants are able to tide over unfavorable conditions. This is because of the presence of organs of asexual reproduction like the tubers, storage roots, corm, bulbs, etc.
- Vegetative propagation is especially beneficial to the agriculturists. They can raise crops like bananas, sugarcane, potato, etc that do not produce viable seeds. The seedless varieties of fruits are as a result of vegetative propagation.
- The modern technique of tissue culture can be used to grow virus-free plants.

Disadvantages

- The plants gradually lose their vigour as there is no genetic variation.
- They are more prone to diseases that are specific to the species. This can result in the destruction of an entire crop.

8.2 What is a seed system?

A seed system is composed of individuals, organizations, and institutions involved in the development, multiplication, processing, storage, distribution and marketing of seeds (Maredia and Howard, 1998). A seed system includes both informal (and traditional) and formal sectors. The formal and informal seed sectors are the two major components of a seed supply system. They are essential and complementary to insure an effective seed security strategy. The informal sector is composed of individual farm households, each

carrying out most seed system functions on its own, with little or no specialization. By contrast, the formal sectoris composed of public (e.g NARS) and private organizations (e.g. seed companies) with specialized roles in supplying new varieties. Different types of seeds flow from organizations and individuals in one stage of the seed chain to the next through separate informal and formal seed supply channels. Rules and regulations such as variety release procedures, intellectual property rights, certification programs, seed standards, and contract laws influence the structure, coordination and performance of the seed system. A well-functioning seed system is defined as one that uses the appropriate combination of formal, informal, market and non-market channels to efficiently meet farmers' demand for quality seeds.

The informal seed sector is traditional and refers to the collective efforts of farmers and their local community. These farmer and community-based seed acquisition and distribution channels form the basis of a dynamic, but ill defined system. It is characterised by a seasonal crop cycle involving crop production, empirical selection of desirable types, farmers harvesting, cleaning and storing their own seed, exchange of seed between family members and relatives, trade or barter in the local market place, planting and cultivation. Much of the information concerning agronomic performance, yield, disease resistance, quality, cultural preference and diversity of end uses is undocumented. This sector relies largely on local resources and inputs. Therefore seed supply at this level can be very vulnerable to disaster and socio-political disruption. Ironically, this sector provides 85% of planting seeds to small holder farmer.

The formal seed sector is generally regarded as comprising public (national, regional and international agricultural research and policy organizations) and private (private sector companies and business associations) research and development on plant breeding and related aspects of seed physiology and plant disease, variety release, deliberate seed multiplication on seed farms, seed processing, storage, marketing and distribution. This sector drives the establishment of rules and regulations to manage variety release, quarantine, plant variety rights, seed certification, product labelling, marketing, pricing, consumer protection etc. It is this sector that provides the basis for effective regional negotiation and agreement to achieve regional seed security.

8.3 Informal Vs formal seed sectors

The informal and formal seed sectors should be complementary and highly interactive. This is, however, not the case in the sub-region. They operate as separate and non interacting entities. Seed regulatory reform for both the informal and formal sectors – variety testing and regulation and seed quality control – is unsatisfactory because seed regulation is poorly organised, inappropriate standards are used, there is little or no opportunity for farmer and seed producer involvement and the seed regulatory process is not transparent. This could be attributed to decreasing national budgets for public sector research and declining donor support for long term breeding and variety development, erosion and control of plant genetic diversity, pressure to establish plant variety rights, emergence of variety development and seed production at the local level and the collapse of a number of parastatal seed organizations. Moereover, the private sector of the formal seed system has focused on species and crops that show high monetary return. Indeed, along with parastatals they have tended to focus on breeding, production and sales of hybridcrops of commercial or industrial interest rather than those for food security for the poorest of the poor in the region.

In the general absence of Plant Breeders' Rights (PBR) or other forms of Intellectual Property protection, it is simply not economic for private companies to market self or open pollinated varieties. The informal sector on the other hand has concentrated on those crops and seed systems which are the bases for local food production. This includes crops that are predominantly self-pollinating, but also open pollinated crops. The informal sector is responsible for ensuring sustainable supply of propagating material of asexually propagated food crops such as cassava, plantain, yams, potato and sweet potato. This important role of the farmer and community based seed sector is often overlooked when seed systems are being considered from the perspective of commodity crop production. The main source of planting seed used by the small farmers is from self stored seed or is obtained by purchase or barter from local sources. In effect the proportion of seeds changing hands is high, whilst only a small percentage is obtained from the formal sector. Therefore, there is an overwhelming dependence on farmer and rural community based seed delivery system to sustain crop production.

8.4 How do seed systems evolve?

A seed system passes through several phases as it evolves from a traditional to an advanced system. In phase 1, the informal seed system predominates; most farmers save their own seed or obtain seed from nearby farmers or villages, and the rate of new varietal development and adoption of new seeds is low. During phase 2, seeds of improved varieties developed by the public sector begin to replace local varieties, use of complementary inputs (e.g. fertilizer) is limited but increasing, and an emerging private sector is involved in multiplication and distribution of public varieties. During phase 3, the private sector begins to play an active role in research and development (R&D), particularly in developing hybrids and seeds for specialized cash crops. Seed distribution systems become more organizationally varied and decentralized, and many components of the mature seed system exist but the supply of seed from the formal sector still ranges from fair to poor.

In phase 4, the agricultural sector as a whole and the seed system in particular are well developed. Commercial seed production and marketing are common, effective seed laws and regulations are in place, linkages with actors outside the seed sector are well established, and the use of improved seed is high. Correspondingly, the rules, regulations, and infrastructure coordinating the components of the seed system evolve to allow organizations to specialize in different functions of the seed system. The public sector may specialize in basic research and research on subsistence crops, and in regulating the seed system, for example. The national and international private sectors increasingly focus on research, production and marketing of seed for hybrids, specialty crops, vegetable crops, and commercial food and fiber crops. NGOs try to fill the gap by concentrating on multiplication and distribution of seed for crops and farmers not targeted by the private sector.

However, the transformation process described above should not normally be interpreted as a simple linear progression of a national seed system from an informal to a formal system. Seed systems for different commodities follow distinct development paths as they move from one phase to the next, for instance, the path for a hybrid maize seed system will be different from that for millet or cowpea, and those systems may never reach the technical, organizational and institutional complexity of a hybrid maize seed system in phase 4. The seed system for maize in the advanced phase (such as in the U.S.) may be composed only of formal seed channels, with the private sector meeting the market demand for hybrid seed each season. In Uganda, the maize seed system in Sironko district is close to phase 4, yet in many other districts it is still in phase 1 – 2. On the other hand, seed systems for beans and groundnuts, even in a mature phase, may have all the components of the seed system, with both formal and informal sectors playing important roles in meeting the demand for seed.

8.5 Traditional seed technologies

Traditional seed technologies refer to collective efforts of farmers who save and store part of their harvest for future planting and or exchange with other farmers. The majority of farmers in developing countries are rural subsistence farmers relying on the farmer seed exchange and saving system and traditional farming technologies and practices. Very few farmers are involved in commercial farming who commonly depend on the formal seed sector for supply of seed and information. The farmer seed selection and production system is one of the richest technologies still practiced by most traditional farmers and is based on practices passed from generation to generations. Most traditional methods of agriculture were developed empirically through millennia of trial and error, natural selection, and keen observation (Thurston, 2008). This seed system has simple technical aspects ranging from seed acquisition, production, storage and marketing. Some of the key techniques used include altering of plant and crop architecture, biological control, burning, adjusting crop density, depth or time of planting, planting diverse crops, fallowing, flooding, mulching, multiple cropping, planting without tillage, using organic amendments, planting in raised beds, rotation, sanitation, manipulating shade, and tillage.

Farmers do not buy seed but exchange it freely. Therefore in most traditional agricultural practices genetic material is often considered a common good, based on free exchange as people search for better seed. In this matrix there is division of labour and women play an important role in the seed system and they are in most cases the haven of knowledge. Landraces are often named on the basis of identifiable characteristics shared among communities (Hudson, 2004). As a result, farmers have developed an intimate relationship with their environment knowing what, how and when to plant. They have also consciously and unconsciously accumulated a number of crop landraces through domestication, selection and improvement. In fact, modern plant breeding has benefited a lot from the traditional farmer seed system in its search for and collection of material in farmers fields for use in crop improvement programmes. For equipment, farmers use light tools for farming such as sticks, hoes, spades, ox-drawn ploughs and carts.

However, the major challenge for the use of traditional seed technologies is how to make sure that this technology is re-oriented to address the problems of low yields, pests and diseases, soil fertility depletion, climate change, droughts and increasing poverty levels. The other impediment for traditional farming technologies is the threat from modern technologies such as genetic engineering. From the mechanical point of view, the question is whether traditional farming tools like the hoe, spade, ox drawn ploughs and carts can be replaced by heavy machinery such as tractors and combine harvesters? The other threat for the farmer-saved seed technologies is that there are political, national and institutional policies driven by the concept of economic liberalization to modernize agriculture. These modernization issues are pertinent to the continuity of traditional agricultural technologies that need to be preserved.

8.6 Legal and regulatory framework of the seed system: The case of Uganda

There are two Laws/Acts and their respective regulations that are directly related to seeds. They are the Seeds and Plant Act, 2006 and the Plant Protection Act of Uganda (Plant Protection and Health Bill, 2003).

8.6.1 The Seeds and Plant Act

The seed industry in Uganda is basically governed by the Agricultural Seed and Plant Statute that came into force on the 23rd September 1994 and was later revised as the Seeds and Plant Act, 2006 which came into force in June 2007. The Agricultural Seed and Plant Statute, 1994 was developed during the time when the Uganda Seed Project was the only Seed Company in Uganda. The Seeds and Plant Act, 2006, came into force when the seed sector was already liberalized and had a number of seed companies operating in the formal seed sector. The Agreements of the Harmonization of Seed Policies and Regulations in Eastern Africa were also already in place.

The Seeds and Plant Act, 2006, provides for the promotion, regulation and control of plant breeding and variety release, multiplication, conditioning, marketing, importing and quality assurance of seed and other planting material and related matters. Uganda is a member of the Organization for Economic Co-operation and Development (OECD) Seed Schemes. The Seeds and Plant Act, 2006 regulations are based on procedures of the OECD scheme, for example field inspection procedures and seed classes/generations nomenclature. Although the National Seed Testing Laboratory is not yet accredited to the International Seed Testing Association (ISTA), ISTA rules are adhered to.

The laws and regulations relating to the seeds sector fall under the mandate of Ministry of Agriculture, Animal Industry and Fisheries (MAAIF). The Seeds and Plant Act, 2006 established a body known as the National Seed Board (NSB), under MAAIF to administer the Act and with the following functions:

- Advising the minister on the National Seed Policy.
- Advising the minister on the informal seed sector and vegetatively propagated materials.
- Advising the minister on variety introductions, breeders' seed production and maintenance. Establishing a system of implementing seed policies through technical committees.

- Formulating and advising the minister on the regulations and standards controlling the development of the seed in distinctness, uniformity and stability.
- Coordinating and monitoring the public and private seed sector in order to achieve the national seed industry objective.
- Giving advice to plant breeding organizations on the market and farmers' requirements.

The bulk of the functions relating to the seed sector are implemented by the National Seed Certification Service (NSCS), under the Regulation and Certification Unit in the Seed Certification Section. According to the Seeds and Plant Act, 2006, the NSCS is responsible for the design, establishment and enforcement of certification standards, methods and procedures. The Seeds and Plant Act, 2006 outlines the responsibilities of the NSCS as follows:

- Reviewing, adjusting, maintaining and enforcing seed standards
- Revising the National Seed Board of modifications to seed standards and providing the Board with information on any technical aspects affecting seed quality
- Providing training to persons responsible for the implementation of the Seeds and Plants Act, 2006
- Registration and licensing of all seed merchants, seed conditioners and seed dealers
- Receiving and testing of all new varieties intended for release and multiplication, assisted by the Technical Committee
- Carrying out distinctness, uniformity and stability tests for candidate varieties
- Establishing standards for variety performance trials and trials and for distinctness, uniformity and stability trials
- Monitoring activities of the formal and informal seed sectors
- Accreditation and licensing, field inspection, seed sampling and laboratory seed testing
- Carrying out field inspection, testing, labeling, sealing and eventual certification
- Reviewing the history and performance records of selected varieties
- Determining the contribution of varieties for agricultural development
- Making recommendations for de-gazetting of obsolete varieties
- Determining the varieties to be fully released, partially (restricted) released, referred or rejected.

8.6.2 The Plant Protection Act

The current Plant Protection Act (Bill) is based on the Plant Protection Act: CAP 244 1962, but proposals to amend it have been made several times. A Plant Quarantine and Inspection Services (PQIS) unit was established in 1989 under the Department of Crop Protection of the MAAIF. Thus, there is a 1990 Bill/Proposal entitled, "The Plant Health Protection Act, 1990" which provides for the eradication of pests and diseases destructive to plants, prevention of introduction and spread of pests and diseases destructive to plants and for related matters. The 1990 Bill was revised in 1992. The 1992 Bill has been reviewed and is now the Plant Protection and Health Bill 2003 which is being discussed by the Parliament.

The objective of the Bill is to have "An Act" to consolidate and reform the law relating to protection of plants against destructive diseases, pests and weeds; to prevent the introduction and spread of harmful organisms that may adversely affect Uganda's agriculture, the natural environment and livelihood of the people; to ensure sustainable plant and environment protection, to regulate the export and import of plants and plant products and introduction of new plants in accordance with international commitments on plant protection so as to protect and enhance the international reputation of Ugandan agricultural imports and exports, to entrust all plant protection regulatory functions to the Government, and for other related matters. The Bill also provides for regulations of the "living modified organisms".

8.6.3 The Plant Variety Protection Bill

Uganda is not yet a member of the International Union for the Protection of New Varieties of Plants (UPOV) but is a signatory to the World Trade Organization (WTO) treaty which includes the Trade Related Aspects of Intellectual Property (TRIPS). A Plant Variety Protection (PVP) law has been drafted but is still a bill which is currently with the Solicitor General in the Ministry of Justice and Constitutional Affairs for legal interpretation. It suffices to note that the proposed PVP Bill largely caters for Plant Breeders Rights (PBR). Until the PVP law is in place, granting of PBR cannot operate. The PVP draft has provision of the Registrar of Plant Breeders who shall be granting free rights. According to the National Seed Certification Service (NSCS), the PVP Bill states that rights/grants shall last for 15 years for annual crops and 25 years for perennial crops.

8.6.4 Genetically Modified Organisms (GMOs)

Uganda is a party to the Cartagena Protocol on Biosafety. In 2002, the President of the Republic of Uganda commissioned the application of Biotechnology in Uganda. Currently, all living organisms (including seeds and planting materials) coming into the country must be cleared by the National Biosafety Committee of UNCST, based in the Ministry of Finance, Planning and Economic Development (MFPED). UNCST is the "Competent Authority" on Biotechnology and Biosafety. An application to introduce GMOs must be made to the UNCST and when accepted the material must be tried in restricted glass house then confined fields and evaluated through a special regulatory system. The Uganda National Biotechnology and Biosafety Policies (94) have been drafted by UNCST and the National Biosafety Committee.

8.7 Seed import and export

8.7.1 Import requirements

According to the Seeds and Plant Act, 2006, a person who intends to import seed must apply to the Certification Service for a license. Only approved seed varieties which meet the standards established by the NSCS for domestic seed trade accompanied by a declaration of minimum certification standards on an ISTA certificate or domestic certificate and a phytosanitary certificate shall be imported into Uganda.

The Regulations (Draft Statutory Instruments) to the Seeds and Plant Act, 2006 add that the person to import seed must be a licensed seed merchant or seed dealer; the ISTA certificate must be the Orange International Certificate and an East African Community Certificate will also be accepted. In addition, the Plant Protection and Health Bill, 2003 states that:

"no plant or plant product may be imported into Uganda except through a point of entry"

"with exemption by the Minister, plants or plant products, can/may be imported without a phytosanitary certificate"

"the Minister for Agriculture on recommendation of the Plant Protection and Health Bill Technical Committee may for the purpose of essential scientific research or experiment, permit (allow) the importation into Uganda, anything not otherwise eligible for importation under the act".

Imported seeds have to be inspected by an authorized/gazette Inspector of the Department of Crop Protection, MAAIF, for compliance with sanitary and phytosanitary requirements. The phytosanitary certificate is issued by the Phytosanitary and Inspection Service of the Department of Crop Protection of the MAAIF.

8.7.2 Export requirements

According to the Seeds and Plant Act, 2006, all seeds intended for export must comply with the requirements of the importing country. The draft Statutory Instruments to Seeds Act states that all seeds for export shall meet the minimum standards set out in the Fourth Schedule (standards for Uganda certified seed). The Plant Protection and Health Bill 2003 states that any person intending to export a consignment of

plants or plant products to another country (which requires the material to be accompanied by a phytosanitary certificate), must submit the consignment to an Inspector of the Department of Crop Protection, MAAIF, for examination within 14 days of the date of export or in accordance with the requirement of the importing country. If the Inspector is satisfied with the consignment, he/she issues the phytosanitary certificate; otherwise the consignment is appropriately treated to remove the risk of injurious pest/disease or is denied the phytosanitary certificate. The Seeds and Plant Act, 2006, states that genetically modified seeds will be regulated in accordance with the UNCST Act or any relevant law.

8.8 Seed supply systems: A case for Uganda

8.8.1 The formal seed system/sector

There are many players in the seed system, e.g. public sector and the private sector participants. They include MAAIF and its organs, namely, National Agricultural Research Organization (NARO), NSB, Variety Release Committee (VRC), NSCS, private seed companies (}13 companies), seed distributors and stockists, government and donor projects, Non Governmental Organizations (NGOs), Relief Agents and farmers.

All the players are linked together through MAAIF and NARO (a semi-autonomous body under MAAIF). Although the production of certified seed was started in 1969, it was formalized by enactment of the Agricultural Seeds and Plant Statute 1994 (revised in 2006).

MAAIF, through the NSB formulates seed policies and implements them. The NSCS in the Department of Crop Protection is responsible for implementing the Seeds and Plant Act, 2006 and handling all matters relating to seeds. NARO is the main new variety producer in Uganda and is involved in plant breeding, production and maintenance of breeder seed and germplasm conservation. In addition, Makerere University's Department of Crop Science is also involved in plant breeding of a few crops, like soya beans and cowpeas. Further, some seed companies are currently initiating research in variety production. The VRC conducts the release of varieties and the NSCS registers all those varieties released and are legible for multiplication and processing by the private seed companies.

The companies receive breeder's seed from NARO, multiply and process them on their Foundation Seed Farms and produce certified seed. However, in most cases, seed companies make arrangements with NARO institutes to have foundation seeds produced at the institutes. Through contract seed growers, seed companies multiply the foundation seed into certified seed, process it in their premises, while the NSCS provides the seed quality control service for all the seeds produced and processed. Some of the private seed companies import seeds (especially hybrid maize and sunflower and vegetable seeds) and distribute or sell to the farmers.

Seed companies market their seed through the network of seed distributors and stockists who in turn sell the seed to individual farmers or farmer groups. Government and donor projects as well as NGOs and relief agencies can purchase seeds from seed distributors or stockists and then distribute the seeds to farmers. In this case seed distribution can either be free of charge (as for relief organizations) or by use of some other methods, e.g. vouchers for seed, or diffusion and exchange method (farmer-to-farmer). The farmer-to-farmer method is commonly used by NGOs, community based organizations (CBOs) and government projects. The organization structure of the seed system is presented in Figure 8.1.

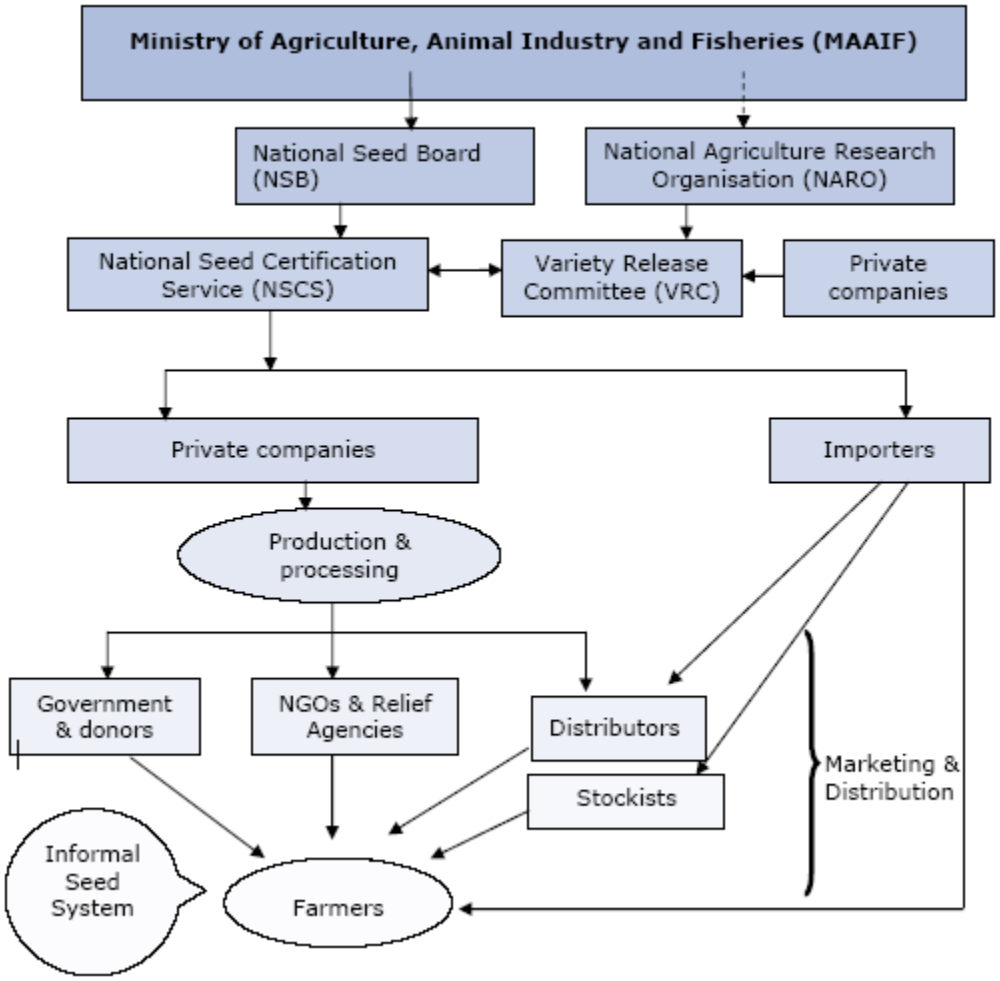

Figure 8.1: Organizational structure for the seed system's participant (Source: Muhhuku)

8.8.2 Informal seed system/sector

The organization of participants in the informal seed system is related to the activities of the formal seed system at the marketing or distribution level. In the farmer-based system, individual farmers/families use their own saved seed from what they harvested for food. The seed may also be obtained from relatives, friends, neighbors, local markets or shops. The informal seed sector is important in multiplying and disseminating planting materials of vegetatively propagated crops like cassava, banana and sweet potato. The farmer-saved seed is a collection of traditional varieties and modern varieties (being re-cycled) originating from the formal system or from on-farm testing activities by plant breeders. The varieties are usually mixed.

In the community-based seed production system, farmers organize themselves either in groups or communities to produce seed of selected crops and varieties. Unlike in the farmer-based system, attention in the current case is placed on improved varieties and some level of quality is maintained. Improved varieties of crops like beans, open-pollinated maize, rice, cowpea, soya bean among other crops are re-cycled. NGOs or donors help farmers or communities to produce seeds that are either given for free or at some cost. There are also cases where researchers have mobilized farmers to produce seeds at community level to enable them access germplasm of improved varieties.

8.9 Seed marketing and distribution

8.9.1 Seed marketing and distribution in the formal sector

Seed marketing is done through the private sector network of distributors/dealers and stockists, the NGO, government and donor agencies. Seeds are produced by seed companies, from whom seed distributors buy. Seed stockists buy the seeds from distributors, and sell them to farmers. Each seed company has a network of seed dealers who include retailers and stockists. However, it is common to find one seed dealer having seed of various varieties from different seed companies. In some instances, individual businessmen access seed from seed companies. By the year 2002, the number of agro-input dealers was 2,100 and up to 90% of them were not only selling seeds but other agricultural inputs too. Before the onset of private seed companies, the formal seed marketing and distribution was carried out through the government seed projects. The USP initially distributed seeds through the District Agricultural Officers; however, the system was also inadequate and forcing the government to liberalize the seed market.

Currently, the seed companies have seed stores at their processing bases from which they distribute to their stockists in various parts of the country. Seed dissemination is done in various ways, first by creating awareness through agricultural shows, establishing demonstration plots in key strategic places, making adverts in the printed media (including newspapers, posters and brochures); and in audio-visual media,. Occasionally, field days are organized and aimed at making the farming communities aware of the varieties available on the market. Seed companies also participate in government relief programmes.

8.9.2 Seed marketing and distribution in the informal sector

The informal seed system is unregulated without specified standards and has no organized seed production chain. It is mainly a community-based seed production arrangement using farm-saved seed. Farmers have different seed acquisition channels within and outside their farming communities. These include saving one's own seed, purchasing from local markets and shops or directly from fellow farmers, exchange and loan, gifts from various people like neighbors, friends, relatives or other farmers, as well as receiving seeds from projects by organizations operating in their area. Others again exchange labour for seed. NGOs and CBOs provide seed and other inputs and promote new technologies through demonstrations.

The informal sector is particularly important in multiplying and disseminating planting material of vegetatively propagated crops like cassava, banana and sweet potato. There is no organization for multiplication, marketing and distribution of these materials. Although some farmers specialize in producing sweet potato vines for sale in local markets, the majority of the planting material is obtained from one's own seed or from other farmers. NARO breeders have attempted to multiply and distribute planting materials of new varieties of these vegetatively propagated crops through mother gardens, e.g. bananas. Due to limited institutional capacity they are multiplied and distributed at a few sites. Commercial private nurseries produce and sell coffee seedlings of improved clonal materials and seedlings of fruit trees. The NARO tissue culture laboratory at NARL and a private entrepreneur with a laboratory in Buloba near Kampala (AGT) are able to supply Tissue Culture planting materials, especially of bananas.

There are also seed projects (community-based and farmer-to-farmer projects) operated by NARO, to produce crops like maize, upland rice and bean seeds by farmer-to-farmer system. For all the crops handled by the informal sector, new varieties originate from NARO and this increases new variety dissemination and adoption. In addition, the informal seed sector has the strong advantage of conserving biodiversity of landraces. Farmers are not in a position to purchase seed from the formal sector because a seed agent may not be present in the area, but where they exist, high prices are a constraint.

8.10 Seed quality control and technology adoption: a general outlook

Certification (inspection) of vegetatively propagated planting materials has been initiated in Uganda. There is already an activity by MAAIF to promote the informal seed sector (through training and capacity building of

farmers) to become a formal one, starting with potato producers. The farmers to benefit from this activity include Kapchorwa Seed Potato Producers Association (KASSPA), Uganda Seed Potato Producers Association (USPPA – based in Kabale), Soroti Sweet Potato Seed Producers and Processors Association (SSPSPA), and West Nile Region Seed Producers. A National Seed Policy to guide the development of the seed industry has been drafted and is being reviewed by the NAADS.

According to NARO and NGOs, the adoption rate for new varieties and improved varieties is recognized as a major challenge to the development of the seed industry. The National Seed Policy, NARO and the National Agricultural Advisory Services (NAADS) are preparing strategies to address this problem by planning some of their activities together. NARO is working out a new strategy to track technology adoption process right from the time of germplasm introduction through to the utilization of the product/technology in the rural areas and in the markets. MAAIF is promoting "Quality Declared Seed" in an attempt to increase the demand and the availability of improved seeds and planting materials (such as sweet potato and Irish potato, banana and other horticultural crops) on the market.

A policy brief in form of cabinet memorandum which seeks to promote 'quality declared seeds' to complement the formal seed supply system in Uganda has already been prepared and cleared by the Treasury. However, seed stockists are still limited to townships and the majority of rural farmers are not yet being served this is partly due to poor road networks. Uganda National Agro input Dealers Association argue that seed companies still to have a limited rural network of stockists, there is a low level of extension service (7% extension coverage), the price of seed is high for farmers, and there is a lack of a grain outlet market and these a matters need to be addressed so that equitable access to quality and certified seeds can be achieved.

According to MAAIF, the quality of seed as judged by farmers still has the challenge of improvement, especially in respect of packaging (pack size, labeling), product information, grading, uniformity of seed treatment chemicals, germination etc. According to NGOs and USTA, the problem of poor seed quality can be attributed to all the players in the private sector and the NSCS. Some of the possible causes of poor seed quality is:

- Corruption/lack of integrity by the seed companies/seed business people: they buy grain and sell it to farmers as seed without indication as to whether it is "standard seed" or not [grade 4 type of seed]

- Ignorance of the resource-poor farmers and the general public (even enlightened people have been victims of poor seed quality sales): seed buyers do not know their rights, e.g. they do not know the seed law and do not know that the law protects those who buy poor seed. However, even if seed buyers knew the law, the institutions mandated to handle seed quality complaints are not yet in place - for example, the Tribunal mentioned in the Seeds and Plant Act, 2006 is not yet in place

- Insufficient follow-up by NSCS: the regulatory mechanism is not effective due to limited human resource capacity. There is poor law enforcement. Anybody can sell "seeds" as long as they are treated (even colored with some form of dye).

- Wanting cheap seed: some farmers/clients go for cheap seeds, which subsequently turn out to be of disappointingly poor quality

- Need to meet big orders by the private seed companies: happens when there are big orders of seed and certified seed available on the market are small. For example, according to USTA, around September/October 2007, there was an order of about 1,200MT of groundnuts. To meet such orders standard seed were sold to meet the demand of the moment.

- Difficulty in ascertaining varietal purity of seeds on the market: this arises due to lack of proper records on seed crop acreage grown and source of breeder or basic seed for the crops. Post-control test results have shown that some varieties on the market no longer have resemblance with their authentic breeder seed/control

- Unwillingness of seed companies to invest in legume seed production: it is considered that legume seed crops have low seed multiplication rates. According to USTA, it may take up six seasons (three years) to multiply 5 kg of bean seed (variety not mentioned) to obtain 500,000 kg seed. This may not be economical. Thus, the short route is to purchase legume grain, process it and sell it as seed

8.11 Implications of agricultural policies on the seed system: The case of Uganda

There are many national and international policies and legal instruments with implications on seed systems. Agriculture being the mainstay of the Ugandan economy a number of policies have been put in place to promote production. The major policy framework on agriculture is the Plan for Modernization of Agriculture (PMA) of 2000. Its thrust is to transform agriculture from subsistence agriculture to market driven commercial agriculture. The PMA identifies non-availability of high yielding technological packages, efficient and cost effective cultivation technology, low adoption rates of appropriate technology due to weak research, extension and farmer linkages as some of the problems facing the agricultural sector. One of the key strategies identified for transforming agriculture in the PMA is supporting the dissemination and adoption of productivity-enhancing technologies.

Noteworthy, the PMA was developed as part of the broader initiative by government to eradicate poverty in line with the Poverty Eradication Action Plan, which recognizes the need to increase agricultural production and agriculture technology research. The government is also promoting another initiative, Prosperity for All (PFA). In dramatic language, the goal of the programme has been stated as the need to transform the peasantry and traditional, unplanned production operational methods and poor unhealthy living conditions of the people to commercialized, industrialized production and healthy living conditions. This policy position has great implications on traditional farm saved seed technologies. Ideally, this project will be a novel initiative to support rural farmers with credit facilities and technical support, but there are a number of institutional and systemic problems in implementing the PFA. Some of the problems include corruption, policy and institutional overlaps and the fact that the programme is driven by political rhetoric than being based on a comprehensive legal and policy framework as there is no clear framework to define roles, systems and procedures. In certain cases, there has been duplication of work between NAADS and the credibility of most programs have been questioned, since their mandates are almost similar.

The Seeds and Plants Act was passed in 2006 and its main purpose is to promote and regulate plant breeding and variety release, seed multiplication, conditioning, marketing and importation. In principle, the Act mainly deals with commercialization of improved seed varieties in Uganda. It does not apply to genetically modified seeds. Neither, does it deal with farm seed saving, selection and exchange. The Act only relates to the operations of breeders of seeds, seed conditioners, dealers and merchants. Nonetheless, the relevance of this piece of legislation to smallholder farmers is that it criminalizes the sale of seed that does not conform to the prescribed standards of germination and purity for that type of seed. Further, selling seeds under a false description than its varietal name and tampering with seed samples are criminal offences. Ideally, the criminalization of selling poor quality seed and falsifying seed information is a positive aspect to protect rural smallholder farmers from unscrupulous private seed companies and individuals by holding them liable for seed failure.

However, the inclusion of criminal sanctions in the Act is in itself not enough to ensure compliance with the law if there are no systematic and effective enforcement measures. Farmers' rights also find recognition in the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) which seeks to protect their traditional knowledge, the right to participate in benefit sharing, decision making and access to

technology transfer. It should be pointed out that traditional farmers are the foundation of all the seed material being the subject of modern technologies. In contrast to modern biotechnologies such as GMOs and plant breeding, the location of indigenous/traditional knowledge in communities and not individuals or firms present difficulties in the quest for protection of knowledge under existing IPR regimes. The legal and policy frameworks that work in developed countries on protecting PBR and intellectual property rights on GMOs might not work in African countries. This is because the majority of farmers do not know that there are rights to seed technologies as they mainly rely on farm saved systems and it does not seem that IPRs will deter traditional farmers from using native seeds.

Another technology that has not been given enough policy attention in Uganda is organic agriculture. So far, civil society and farmer groups' efforts to promote the finalization of the National Policy on Organic Agriculture have not achieved the desired result, as the process has been slow. This discourages investment as there will be no clear policy direction albeit there has been keen discussions in East Africa on the formulation of the East African Standards for Organic Products, which outlines procedures for production of organic products. Member countries are expected to domesticate the standards into national policy or law. The standards will be important for organic farmers as it profiles the technologies to be used for organic farming.

Without proper policies, the adoption of technologies like genetic engineering will be difficult in poor countries where farmers rely on farmer-saved seed and free exchange of seed. Yet, these technologies are protected by patents and driven by corporate interests. The option left for policy makers is to improving the investment in agriculture, infrastructure, marketing and policy gaps that exist in the use of improved varieties by farmers. The other major obstacle to farmers' participation in the development of agricultural technologies and policies is that they are not actively involved in the debate on agricultural improvement. For instance, some scientists advocate the benefits of new technology for the poor farmers; while on the other hand NGOs opposed to GM crops are purporting to speak for the rural poor. More often than not, the farmers find themselves between the contours of a debate whose concepts, effects, advantages and disadvantages they may not appreciate and comprehend but affect them directly. The worst case scenario is where they may not even know that there is such a debate because the arguments many be lab-based or confined to urban hotels and conference centers. From repeated studies, certain technologies could be straitjackets imposed on farmers which they have little knowledge of their origin and as a step towards synergistic approach to agriculture, farmers should be more actively involved as an important stake holder in agricultural policies

8.12 Recommendations

A flexible seed technology development system is crucial to effectively respond to the subsistence farmers' challenges. Combining the best of traditional agriculture methods with the best of modern agriculture should go a long way towards sustaining agriculture. The formal sector can continue producing hybrids and other improved seed along with the informal sector. Since informal farmer-to-farmer spread of seeds is the single most frequently used source of seed by farmers, it is necessary for government to strengthen the informal sector as an important low-cost source of quality seed, and to use it as a vehicle for providing resource-poor farmers with improved seed of modern varieties at affordable prices. Government can encourage the growth of such an informal sector by: providing it with access to improved seed and innovations, extension advice on seed production, processing, treatment and storage; and paying particular attention to quality control, maintenance of reserve stocks of seed.

Increasing farmers' participation in variety development and verification would help address the need for increasing varietal choices. This could significantly cut research costs, improve varietal diversification, and enhance local seed systems and adoption rates. Marketing of seed is an important concern for rural farmers. The public sector can act as a mediator in bringing together the farmers' seed producer groups and the seed distributors, such as private companies, local vendors and NGOs. Marketing issues can be better dealt with by establishing demand forecasting systems, whereby demand for any crop variety is known and is passed

on to the producers, and the clients are aware of the sources of different seed. Making use of information networks is also important.

In most communities, there are informal networks or relations for the flow of information and technologies. This can be strengthened by coming up with a Market Monitoring System, whereby farmers will be constantly advised of market commodity price fluctuations and to predict future and potential markets for seed. Further, civil society and farmer groups need to advocate for group marketing of agricultural products like seed. Farmer groups at local level allow farmers to take advantage of economies of scale through collective marketing. This is because buyers are far more willing to deal with associations rather than contact individual farmers. The associations provide training in the basics of organic productions and they help monitor compliance with national and international standards. Access to information on modern technologies and farmers' social networks will influence adoption of improved varieties. Furthermore, education has a positive impact on the speed of adoption of new technologies.

To strengthen farmers groups, civil society organizations especially, public interest law groups should give legal advice to the rural farmer groups when dealing with private seed companies to protect poor farmers from exploitation by private companies. The linkages and relationship between seed producers, dealers and farmers should be a subject of law; and research organizations should make efforts to understand the dynamics and protect farmer's rights.
Another strategy in promoting farmers rights is through legal education or taking the law to the people. The government can strengthen informal system through the provision of low-interest finance, technical assistance, and publicly bred parent material. The extension system should advise farmers on the characteristics and correct adaptation zones especially of newly released varieties. Breeding programs need to give proportionate weights to yield and non-yield characteristics when selecting the best varieties.

Considering the limited success of the public sector in delivering seed to small-scale farmers in remote areas, and the lack of commercial interest on the part of large seed companies, it seems that small and location-specific enterprises may be the best option for fulfilling this role. These can be in the form of community seed banks. However, the strategy in promoting such enterprises should differ from the usual 'top-down' measures characteristic of large seed projects and companies, because the conditions are different in terms of crops, resources and potential profitability. Small-scale enterprises require community-based interventions, since these businesses are meant to serve small-scale farmers in rural areas. Attracting investment in this form of seed supply, and creating sufficient interest in the community for the seed enterprises, are formidable challenges. They need a strong commitment on the part of the government in introducing favourable policies and providing adequate incentives that can encourage investment in agriculture.

Glossary

Adventitious: development of organs such as buds, leaves, roots, shoots and somatic embryos from non-zygotic structures such as shoot and root tissues and callus

Agar: a polysaccharide powder derived from algae and used for gelling a medium

Aseptic: Free from infection or contamination by fungi, bacteria, viruses, mycoplasma or other microorganisms

Aseptic Technique: Procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cultures

Autoclave: A machine capable of sterilizing wet or dry items using steam under pressure. Pressure cookers are a type of autoclaves.

Auxins: A group of plant growth regulators that promote callus growth, cell division, cell enlargement, adventitious buds, and lateral rooting. Endogenous auxins are auxins that occur naturally. Indole-3-acetic (IAA) is a naturally occurring auxin. Exogenous auxins are auxins that are man-made or synthetic. Examples of exogenous auxins included 2, 4-Dichlorophenoxyacetic acid (2, 4-D), Indole-3-Butyric acid (IBA), α-Naphthaleneacetic acid (NAA), and 4-Chlorophenoxyacetic acid (CPA).

Callus: An unorganized, proliferate mass of differentiated plant cells, a wound response.

Cell culture: culture of cells or their maintenance *in vitro* including the culture of single cells.

Cell line: cells that originate from a primary culture at the time of the first successful subculture.

Chemically Defined Medium: A nutritive solution for culturing cells in which each component is specifiable and ideally of known chemical structure.

Clone: Plants produced asexually from a single source plant. Clones are considered to be genetically uniform.

Clonal Propagation: Asexual reproduction of plants that are considered to be genetically uniform and originated from a single individual or explant.

Contamination: Being infested or infected with unwanted microorganisms such as bacteria or fungi.

Culture: A plant growing *in vitro*.

Cryopreservation: ultra-low temperature storage of cells, tissues, embryos and seeds.

Cytokinin: A group of plant growth regulators that control cell growth and morphogenesis and stimulate cell division. Endogenous cytokinins, cytokinins that occur naturally, include zeatin and 6-γ,γ-dimethylallylaminopurine (2iP). Exogenous cytokinins, cytokinins that are man-made or synthetic, include 6-furfurylaminopurine (kinetin) and 6-benzylaminopurine (BA or BAP).

Differentiated cells: Cells in culture that maintain all the specialized structure and function typical of the cell type *in vivo*. This is development of cells to form tissues or organs with a specific function.

Diploid: cells, tissues and organisms, which have two sets of all chromosomes, not including the sex chromosomes

Embryo culture: *In vitro* culture of isolated mature or immature embryos.

Explant: tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

Ex situ: outside the natural habitat

Extinction: an irreversible loss of a species or a genetic line

Gibberellins: plant growth regulators that influence cell enlargement. Endogenous growth forms of gibberellin include Gibberellic Acid (GA_3).

Growth chamber: a chamber used for the incubation of culture containers or plants under controlled environment

Hormones:Growth regulators, generally synthetic in occurrence, which strongly affect growth (i.e. cytokinins, auxins, and gibberellins).

In situ: in the original place

In vitro :(Latin '*To be grown in glass***'**). Propagation of plants in a controlled, artificial environment using plastic or glass culture vessels, aseptic techniques, and a defined growing medium.

In vivo:(Latin '*To be grown naturally***'**); opposite of *in vitro*, meaning outside e.g. in screenhouse or field

Medium:A nutritive solution, solid or liquid, for culturing cells.

Meristem: a group of undifferentiated cells situated at the tips of shoots, buds and roots, which divide actively and give rise to tissue and organs.

Micropropagation:*In vitro* Clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.

Node: A part of the plant stem from which a leaf, shoot or flower originates.

Plant Tissue Culture: The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.

Regeneration: In plant cultures, a morphogenetic response to a stimulus that results in the products of organs, embryos, or whole plants.

Shoot Apical Meristem: Undifferentiated tissue, located within the shoot tip, generally appearing as a shiny dome-like structure, distal to the youngest leaf primordium and measuring less that 0.1 mm in length when excised.

Somaclonal Variation: Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.

Somaclones: Plants derived from any form of cell culture involving the use of somatic plant cells.

Stage 0: The first step in micro-propagation also known as the pre-propagation stage in which the mother plants are maintained in the greenhouse under pest-free conditions. It also involves appropriate pre-treatment of the mother plants with pesticides to minimize contamination in the *in vitro* cultures.

Stage I-A step in *in vitro* propagation characterized by the establishment of an aseptic tissue culture of a plant.

Stage II-A step in *in vitro* propagation characterized by the rapid numerical increase of organs or other structures.

Stage III-A step in *in vitro* propagation characterized by preparation of propagules for successful transfer to soil, a process involving rooting of shoot cuttings, hardening of plants, and initiating the change from the heterotrophic to the autotropic state.

Stage IV-A step in *in vitro* plant propagation characterized by the establishment in soil of a tissue culture derived plant, either after undergoing a Stage III pretransplant treatment, or in certain species, after the direct transfer of plants from Stage II into soil.

Sterile : (A) Without life. (B) Inability of an organism to produce functional gametes. (C) A culture that is free of viable microorganisms.

Sterile Techniques: The practice of working with cultures in an environment free from microorganisms and other biological contaminants.

Somatic embryos: non-zygotic bipolar embryo-like structures obtained from somatic cells.

Subculture: With plant cultures, this is the process by which the tissue or explant is first subdivided, then transferred into fresh culture medium.

Tissue Culture: The maintenance or growth of tissue, *in vitro*, in a way that may allow differentiation and preservation of their function.

Totipotency: A cell characteristic in which the potential for forming all the cell types in the adult organism are retained.

Transgenic: plants that have a piece of foreign DNA.
Undifferentiated:-With plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo.

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Appendices

Appendix 1: General rules for working in a tissue culture laboratory

1. Do not eat or drink in the lab
2. Always wear a Lab coat while working
3. Restrict access to working staff only
4. Clean the floor and shelves daily
5. Regularly fumigate the room (every 3 to 6 months).
6. Turn on the laminar flow hood and bead sterilizer at least 15 minutes before use
7. Spray the bench with 70% alcohol and wipe using tissue paper or cotton wool before and after using laminar flow hood
8. Do not leave any items on the laminar flow bench after use
9. Keep items away from the grid protecting the filter
10. Only keep in the hood items to be used, additional items for use should be kept in trolley
11. Any item entering the hood should always be sprayed with 70% alcohol
12. Wrap all instruments in aluminum foil or enclose in autoclavable polyethylene containers before autoclaving
13. Ensure that the air conditioner is functioning normally and the temperature is maintained within the required range
14. Replace improperly functioning bulbs immediately
15. Remove any contaminated plants from growth room immediately, wash and dry the tubes for autoclaving

Appendix 2: General media preparation procedure

Requirements: Chemical reagents/stock solutions, distilled water, clean and dry culture vessels, check list, functioning equipments (weighing balance, autoclave, pH meter, media dispenser, laminar flow hood,

1. Prepare a check-list of all components needed to prepare media
2. Ensure that all media components and other requirements necessary for media preparation (glassware, spatula, tubes on racks, pen, etc.) are sufficiently available
3. Add 200 ml distilled water in a container, add a 'magnetic flea' and place on a magnetic stirrer
4. Weigh an appropriate quantity of the reagents (e.g. Sucrose, MS powder), add to the water and turn on the magnetic stirrer at low speed
5. Weigh/measure and add all remaining components one after another except agar. *Tick all listed products as they are added in the media*.
6. Make sure all components are properly dissolved
7. Adjust volume to 1 litre (if final volume= 1 litre) with distilled water
8. Adjust the pH to 5.8 ±0.1 (with 0.5M NaOH or 0.5M HCl)

Perform steps 9–12, if your laboratory is equipped with a media dispenser or steps 14–15, if media is dispensed manually

9. Add agar
10. Heat and stir media on a hot plate (or in microwave after removing the flea/stirrer) till agar is dissolved.
11. Once agar is fully dissolved, use a media dispenser to appropriate volume e.g. 5 mL of media per tube. Cover each tube with a plastic cap immediately
12. Autoclave at 121 °C for 15 minutes, allow slow cooling to 0°C.

In absence of automatic media dispenser*;*

13. Transfer the media into media 500mL/1L bottles and close the cap loosely for autoclaving
14. Take the media out of autoclave and dispense the appropriate volume into tubes under laminar flow hood. Manual dispensing can also be performed using sterile pipette
15. Allow media to cool or solidify (in case semi-solid media is required), preferably store in a cool environment (ideally at 8–10 °C).
16. Always label the media and stick autoclave tape during media autoclaving.
17. Use prepared media within 2 weeks

Appendix 3: Preparation 1mM Picloram Stock

1M of Picloram = 241.5g/1000mL
To prepare 1mM of Picloram, the following method is recommended: $C_1V_1 = C_2V_2$
C1=let it be Y, C2 = 241.5g, V1 = 1000ml and V2 = 500ml
1000mL = 241.5g
1ml = (241.5/1000)g/mL
500ml = (241.5/1000)g/mL Ч 500mL
= 120.75g/500mL
1000mM = 1M
1mM = (1/1000) Ч 120.75
= 0.12075g/500mL

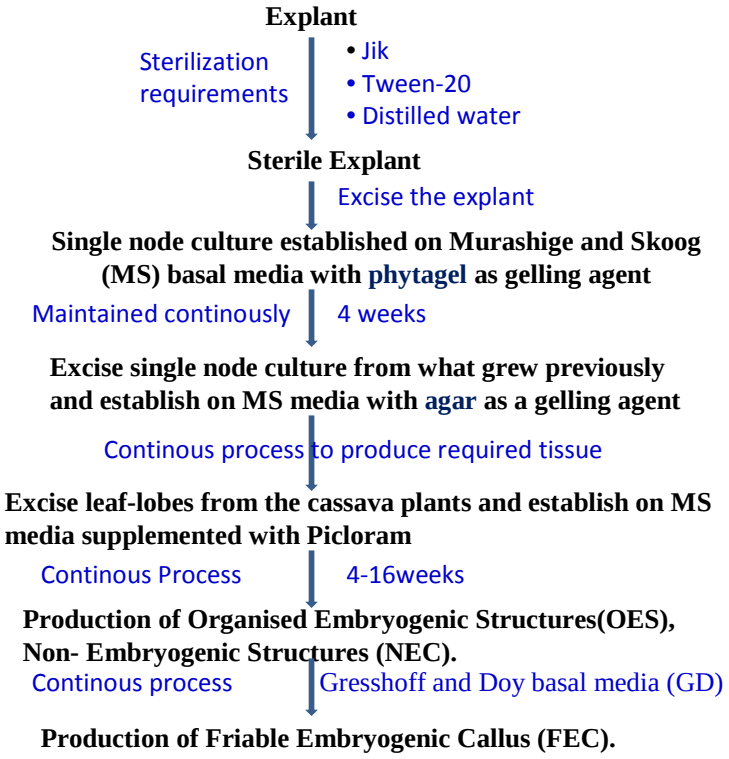
Procedure for preparation of 1mM Picloram Stock of 500mL
(1) Heat 250mL milliq-water or distilled water to near boiling in the microwave
(2) Add 0.12075g of Picloram powder
(3) Stir on hot plate until fully dissolved this may take an hour to achieve
(4) Make up to 500mL in a measuring cylinder with milliq-water or distilled water.

Note: 50ml of the 1mM Picloram Stock solution is added to the 1L of MS media to achieve the 50µM concentration

Appendix 4: Preparation of 1litre of MS2 50P for establishment of leaf-lobes

Item		specifics of the item
(1)	MS Salts	4.3g/L
(2)	MS vitamins	1ml
(3)	Sucrose	20g/L
(4)	Picloram	50mL
(5)	pH	6.13-6.15
(6)	Phytagel	3.0g/L
(7)	Or Agar	8.0g/L

Appendix 5: Steps in Somatic Embryogenesis



Appendix 6: Virus eradication through meristem culture and thermotherapy

If a healthy plant is sown in the field, it is exposed to infections caused by an array of pathogens which have a negative effect on yield, and in some cases may kill the plants. However, not all the plant cells may become infected. A group of cells (in the meristem) that are in a continuous non-differentiated multiplication are often virus-free. The *in vitro* meristems culture, together with growth at high temperatures produces sweet potato plantlets free from viruses in more than 90% of planted meristems. This routine method was established at the International Potato Center (CIP) to obtain virus-free plantlets for national and international distribution. Hereafter, the procedure to obtain virus-free plantlets is described:

Procedure

1. Approximately 18 to 20 plantlets (virus-infected) are propagated in magentas.
2. After a growth period of 20 to 25 days, or when the plantlets are 4-5 cm high, they are placed in the thermotherapy chamber where growth conditions are: 16 hours of light at 34°C; 8 hours of darkness at 32°C.
3. The magentas are maintained in the thermotherapy chamber for one month.
4. Afterwards, the magentas are taken out of the chamber, and the exterior is cleaned with 98% alcohol. Thereafter, they are introduced to the culture room.
5. Meristems are obtained as follows: Cut the apical portion and remove the leaves that cover the meristem (approximately 3 to 4 leaves); the meristem is observed with a prominent leaf primordium.
6. Using new scalpel blades, remove the meristem with part of the leaf primordium; cut only the translucent portion.. Place the meristem in the culture medium and ensure that the meristem is in the tube.
7. Evaluate meristem growth and transfer them to fresh media if necessary.
8. Each meristem that originates a plant is called 'line' which will be labeled according to the accession it belongs to. For example, Yellow Line 1, Yellow Line 2, Yellow Line 3, etc.
9. Five tubes with several plants are propagated to evaluate the potato virus (SPVd) to determine the host range, the morphologic evaluation, and the *in vitro* maintenance.
10. The results of each evaluation are obtained, and the infected material is replaced with clean material.

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