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Clean Breeder Seed Yam Tuber Production using Temporary Immersion Bioreactors

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Published by the International Institute of Tropical Agriculture (IITA)
Ibadan, Nigeria

IITA is the lead research partner facilitating agricultural solutions for hunger and poverty in the tropics. It is a member of the CGIAR Consortium, a global research partnership that unites organizations engaged in research for sustainable development for a food secure future.

International address:
IITA, Grosvenor House,
125 High Street
Croydon CR0 9XP, UK

Headquarters:
PMB 5320, Oyo Road
Ibadan, Oyo State

ISBN 978-978-8444-89-3

Correct citation: Morufat Balogun, Norbert Maroya, Julius Taiwo, Ossai Chukwunalu, Adeola Ajayi, P. Lava Kumar, Olugboyega Pelemo, Beatrice Aighewi and Robert Asiedu. 2017. Clean Breeder Seed Yam Tuber Production using Temporary Immersion Bioreactors. IITA, Ibadan, Nigeria. 66 pp.

Cover photo: Clean Breeder Seed Yam Seedlings growing in Temporary Immersion Bioreactor system at IITA. Inset: Staff of National Centre for Genetic Resources and Biotechnology being shown hardened bioreactor plantlets by Dr. Morufat Balogun (third from left) during a technical backstopping workshop on TIBs.

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Acknowledgement

The authors are grateful to the Bill & Melinda Gates Foundation for providing funds for the second phase of YIIFSWA project. They are also grateful to the Genetic Resources Centre for their assistance in housing the initial thermotherapy work, the IITA Bioscience Unit for providing bench space for the SETISTM Temporary Immersion Bioreactor System work and the IITA Germplasm Health Unit for phytosanitation and virus indexing support.

Acronyms

PB	Pre-basic
BS	Breeder seed
MS	Murashige and Skoog medium
YMV	Yam mosaic virus
BV	Badnavirus
TIBS	Temporary Immersion Bioreactor System
PF	Plant form
CTC	Conventional Tissue Culture

The importance of yam and constraints of production

Yams (*Dioscorea* spp) are a primary source of income and starchy staples in West Africa from where 93% of global yam production emanates (FAO 2013). Nigeria alone produces 65% of global yam production, equivalent to 37 million tonnes.

In traditional yam propagation, the seed system is informal. Farmers save planting materials from the previous year's harvest and plant whole tubers, or tubers cut into about 25 g setts (Aighewi et al. 2015). Few new plants are therefore grown from one tuber per season. This traditional vegetative propagation using unclean, farmer-saved planting materials leads to further build-up of diseases such as viruses, anthracnose, and nematodes either singly or in combination, causing up to 25% yield reduction (Degras 1993). The slow rate of propagation (less than 1:10 each season) and the conflict between the use of tubers for consumption and for planting causes scarcity of seed yam, such that planting materials alone account for up to 63% of production costs (Agbaje et al. 2005; Ironkwe 2007).

In addition, farmers can scarcely plant the whole of their field due to non-availability of seed yam in sufficient quantities. Use of poor quality seed yam (Plate 1) causes a steady decline in yield and loss of valuable varieties over time (Balogun and Gueye 2013). Quality control and certification is only assured in a formal seed yam production system where regulatory rules are functional.

The need for a formal seed system for yam

Overcoming the menace of poor quality seed yam requires an efficient technology for cleaning the existing seed yam, confirming its health status, rapidly multiplying the cleaned materials in the system, and disseminating

Plate 1. Symptomatic leaves and tubers of yam.

it efficiently to the farmers. This necessitates the development of a formal seed yam system where regulatory rules are functional, including genetic purity and physiological quality (Balogun et al. 2014) of all categories of seed yam, from breeder (Pre-basic) through foundation (basic) to commercial (certified) seed. In a formal seed system, breeder seeds are of highest quality (Balogun et al. 2017) and purity, foundation and commercial seeds being produced from them.

The project "Yam Improvement for Income and Food Security in West Africa (YIIFSWA)", funded by the Bill & Melinda Gates Foundation (BMGF), was envisioned to increase yam productivity by 40% for smallholder yam farmers in Ghana and Nigeria by delivering key global good research products towards a 10-year vision of doubling incomes from yams in West Africa and contribute to food security for producers and consumers. In achieving this goal, YIIFSWA has developed novel technologies for high ratio propagation of high quality pre-basic (PB) and basic seed yam, among which is the use of the Temporary Immersion Bioreactor System (TIBS). TIBS is an advanced tissue culture (TC) technology, where plants are intermittently immersed in nutrient solution in contrast to conventional TC with continuous immersion. Technologies for using the 128 units of the SETIS™ type TIBs installed by YIIFSWA were developed, ranging from the establishment of in vitro cultures through the generation of clean pathogen-free stocks and rapid propagation to post-flask management.

Tissue culture as a method of healthy seed yam production

2

Conventional Plant TC is the growth of plant tissues in a closed, static sterile and laboratory environment. Conventional TC is static in that there is no flow of air or medium, which is mostly semi-solid or liquid. The technology has been used to rapidly propagate disease-free plantlets of yam, cassava, potatoes bananas, plantain, cocoa and oil palm, among others, in a procedure known as micropropagation (Ovono et al. 2007; Balogun et al. 2014). The technique is independent of climate variations, saves time and space and has greater output of disease free and elite propagules in addition to safer and quarantined movements of germplasm across nations. Thousands and up to millions of uniform plants are produced using this technique. Micropropagation is simply explained as rapid, asexual in vitro propagation. Parts cultured, which are called explants, include apical meristems, axillary buds, nodes, immature leaves, and roots among others.

Initial stock from tissue culture is the singular preferred starting material for breeder (or pre-basic (PB)) seed yam production. This is because the quality is assured due to production in a controlled environment using the smallest size of explants with no risk of reinfection. Producing breeder seed yam involves establishment of in vitro cultures from previously unclean materials; elimination of viruses; identification of pathogen-free stock plantlets; multiplication in conventional tissue culture (CTC) or Temporary Immersion Bioreactors (TIBs); hardening of plantlets, and post-laboratory management of hardened plantlets to produce pre-basic tubers (PBT) or vines (PBV).

The use of tissue culture takes some time initially to generate clean stocks, but the numbers are more and the quality is high. Plantlets generated through meristem culture combined with heat therapy, and indexed as free from pathogens, including viruses, constitute stocks for rapid multiplication

(Balogun et al. 2017). This conventional tissue culture system ensures that the viral agents are not passed onto subsequent clonal generations. In YIIFSWA, virus-free plantlets of 20 popular yam genotypes in Nigeria have been established this way.

The tissue culture building should contain a cloak room for wearing laboratory overalls and foot wears, medium preparation room (general washing area), transfer room, quality control room, culture rooms, general office area, rest rooms, emergency exit, and store (Plate 2). There should be stable electricity and water supply. The facility can be part of a building with well-sealed partitions as done by YIIFSWA or stand-alone to prevent cross-contaminations from non-tissue culture work. The sizes of the different rooms can be scaled up or down depending on the planned production capacity.

Equipment needed (Appendix 1) include Laminar Flow hood, autoclave, dryers, sterilizers, water distiller or deionizer, weighing balance, pH meter, magnetic/hot plate stirrer, dispensers, refrigerators, microwave oven. Supplies include medium reagents, clean room supplies, dissecting tools, culture vessels (i.e., test tubes, baby food jars, petri dishes, plastic vessels), pipette, spatula, weighing boats, forceps, wash bottles, gloves, laboratory coat, nose mask, and refrigerator. These items are listed in Appendix 1, and can be sourced from some companies listed in Appendix 11. Other reagents and supplies include medium constituents like paper and autoclave tapes and marker for labelling, gloves, laboratory and screen house coats, nose masks and head covers.

Steps to producing initial stock of pre-basic seed yam

Establishment of in vitro cultures

This step usually starts with an uncertified or unclean tuber (Plate 1). A tuber that is showing signs of sprouting should be cut into minisetts of 30–50 g each (Plate 3) and treated (Plate 4). The infected tuber should be planted using good top soil preferably in pots in a screenhouse so as not to further accumulate or spread disease if planted on the

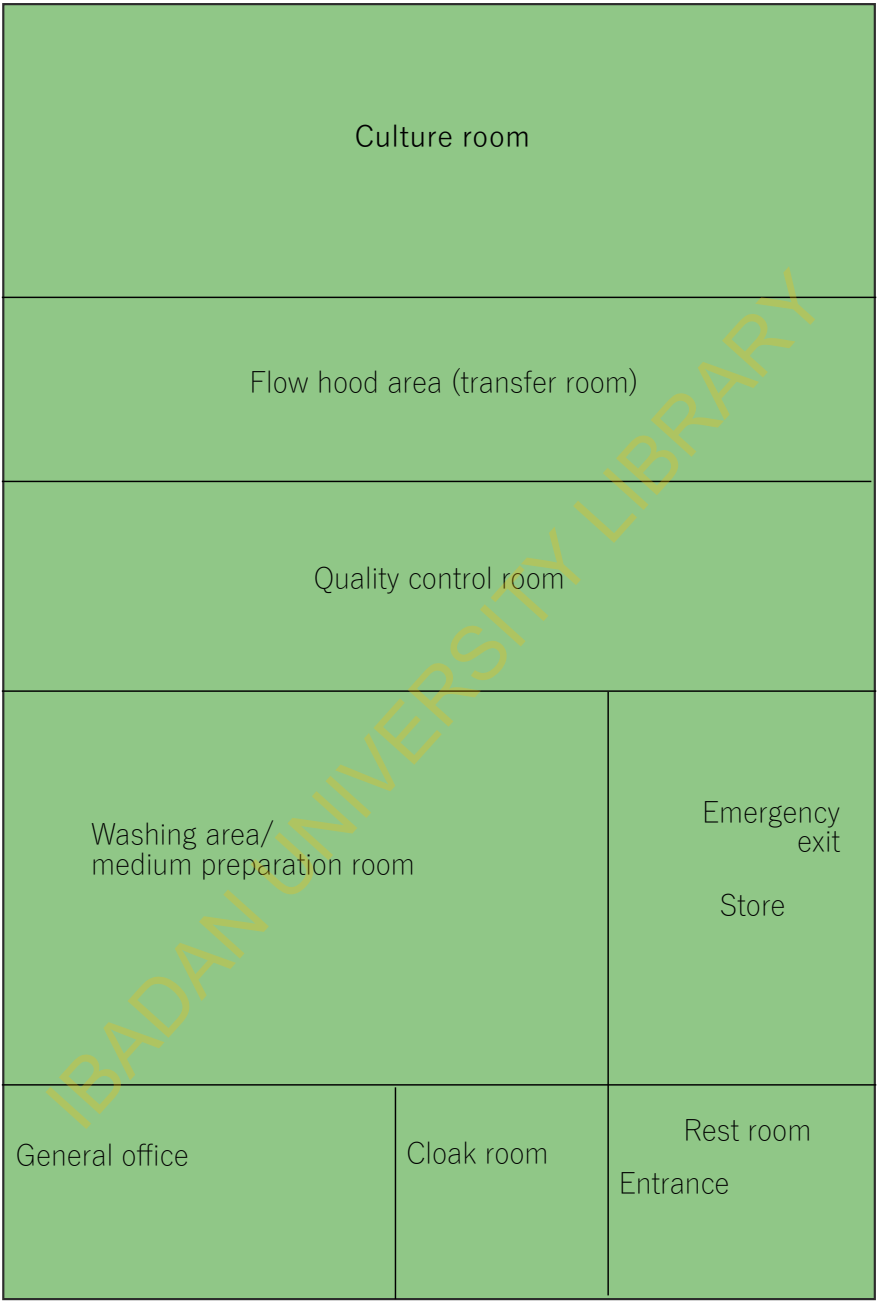

Plate 2. Layout of a Tissue culture facility.

Plate 3. Preparation of mother plants for tissue culture. Up: Sprouted yam tuber; Bottom: Minisetts cut from one tuber.

field. The soil should be sterilized to avoid introducing more soil-borne diseases to the tubers. Soil can be sterilized by solar heating, oven drying, dry heat, cobalt-60 irradiation, microwave, chloroform, antibiotics, steam sterilization among others (Wolf et al. 1989). However, steam sterilization (e.g., autoclaving) gets rid of both living microorganisms and resting fungal spores in the soil. The sterilized soil should be left in the pot for one week before planting (Plate 5) so as to check for weed or fungal growth. This will confirm soil sterility before planting. Prior to planting, the screenhouse should be sprayed with a fungicide/insecticide mixture while the plants should also be sprayed (Plate 5) every two weeks, especially in highly humid environments to reduce the surface microbes that can cause contamination in tissue culture.

Leaves of 2–3 month-old plants should be indexed for viruses. This will serve as baseline reference to confirm disease status of the plants. Single node cuttings (1–2 cm long) from the mother plant (Plate 6) should be surface-sterilized using insecticide and fungicide solution followed by sodium hypochlorite (Appendix 2). Our findings showed that contamination was up to 100% when sodium hypochlorite treatment was not preceded with treatment in fungicide + insecticide solution.

Plate 4. Yam minisetts being treated in insecticide and fungicide solution.

Plate 5. Planting of mother plants to generate vines for establishment of in vitro cultures of yam. Top: Spraying of screenhouse with insecticide and fungicide mix; Bottom : Planting in sterile top soil in pots.

Plate 6. Disinfection of mother vines for in vitro culture. Top (left): Growing yam plants. Top (right): Cutting of single nodes; Bottom: disinfection of single nodes from potted plants.

The nodes are cultured in agar-solidified, modified Murashige and Skoog ((MS)1962) medium as described by Ng (1992) (Plates 7–8). Constituents of the medium and steps involved are shown in Appendix 2 and plates 9–10. Our studies also showed that addition of 1 g of activated charcoal per litre of medium reduced oxidation in the cultures and enhanced plantlet vigor.

Plate 7. Preparation of medium for in vitro culture nodes. Top: Weighing of medium constituents and pouring into distilled water in a beaker (Down).

Plate 8. Preparation of medium for in vitro culture of explants. Top: Stirring of medium constituents and reading of pH; Bottom (Left): Dispensing of medium into culture vessels; Right: Autoclave for medium sterilization.

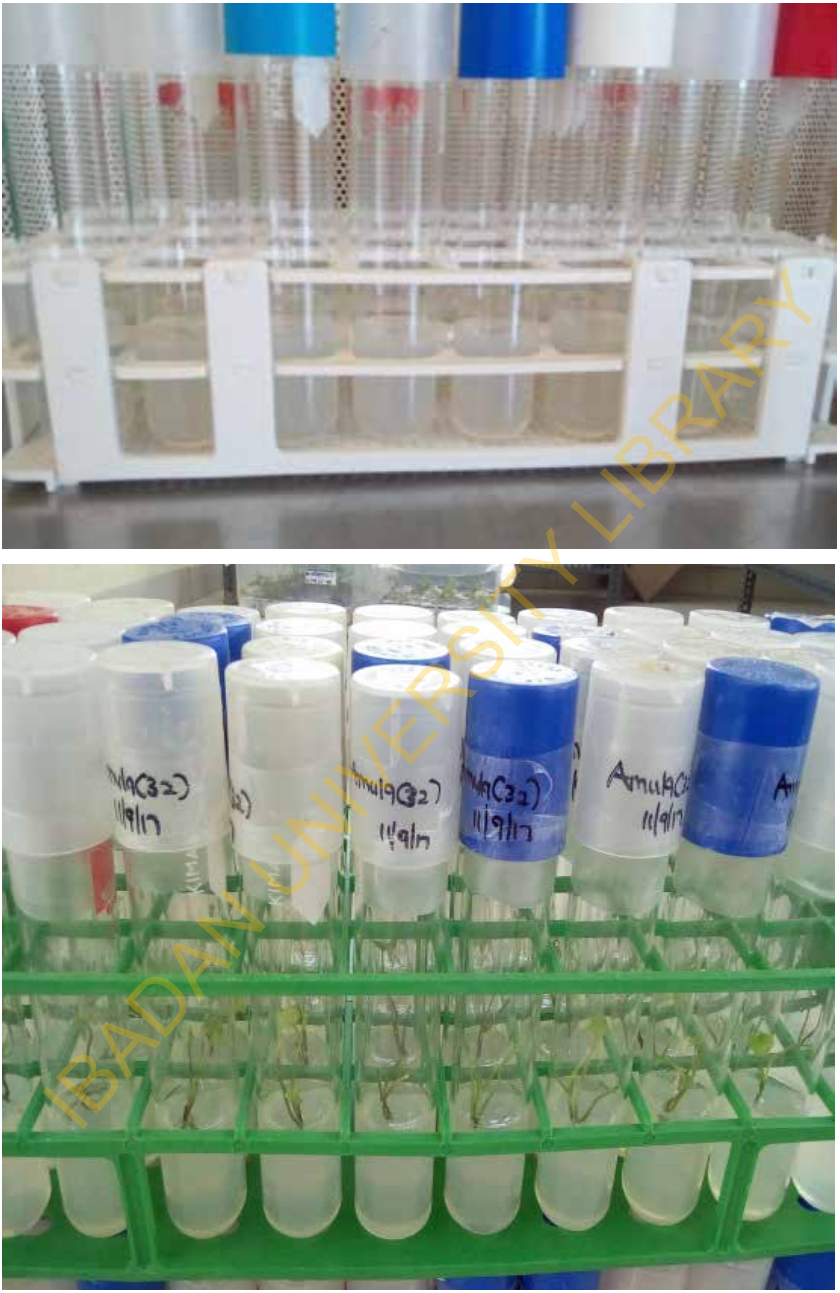

Plate 9. Establishment of in vitro cultures of yam. Top: Freshly prepared medium; Bottom: Medium into which nodes have been introduced.

Elimination of virus and other pathogens

After eight weeks, plantlets should be sub-cultured into two-node cuttings onto the same multiplication medium and incubation condition as described earlier (Ng, 1992) for one week. They should then be transferred to a growth cabinet (Plate 11) set at 36 $\pm$ 0.5°C and 16 h photoperiod for 21 days. Using a dissecting microscope, the meristems (about 0.5–1.0 mm long) should be excised and cultured on modified MS medium containing reagents shown in Appendix 3.

Petri plates are easier to handle for meristem culture due to ease of introduction of meristem, observation and transfer into other vessels for multiplication. However, test tubes can also be used. Plantlets are regenerated from the meristem (Plate 12) and transferred into the yam multiplication medium described above, preferably agar-free, liquid medium for further growth. Regeneration from meristem takes up to

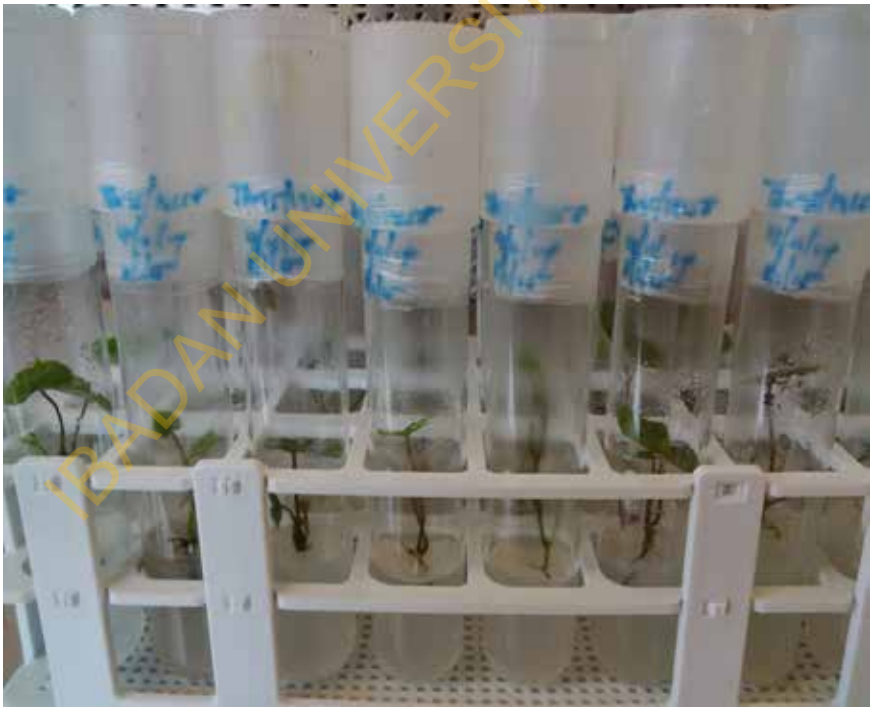

Plate 10. Yam plantlets sub-cultured into two-node cuttings.

16 weeks depending on the genotype, significantly lower than the six months to two years recorded before YIIFSWA (Balogun et al. 2014).

Using this method, yam mosaic virus (YMV) was eliminated in 73% of the plantlets (Balogun et al. 2017). In some samples of water yam, no BV was detected before treatment but detected after treatment, suggesting that BV is integrated into the yam genome (Seal et al. 2014). As YMV is most economically important for yam,establishment of about 73% of YMV-free plants is assuring. More than 3000 virus-free plants can be generated from 1 kg infected tubers. This is the longest phase of the Breeder seed stock plantlet production process as shown in Appendix 10.

Plate 11. Temperature and humidity-controlled growth cabinet for heat treatment to clean plantlets from viruses.

There have also been reports of successful cleaning by immersing the shoot tips in hot water for 45 minutes in the laminar flow hood before meristem excision. Cryotherapy (cold treatment, Shin et al. (2013)) was also reported to clean plantlets of viruses.

Plate 12. Regeneration of clean stock of plantlets. Top: Excision of meristem under the microscope; Bottom left: Cultured meristem regenerating shoots; Bottom right: Regenerated plantlet in liquid medium.

Identification of pathogen-free plantlets

Leaf samples from the regenerated heat-treated plantlets and those of the virus-positive mother plants (controls) should be retested for YMV, *Yam mild mosaic virus* (YMMV), BV and *Cucumber mosaic virus* (CMV) using polymerase chain reaction (PCR) (Nkere 2016) to confirm their health status. Regenerated plantlets should be checked for endophytes before micropropagation to avoid build-up of contaminants.

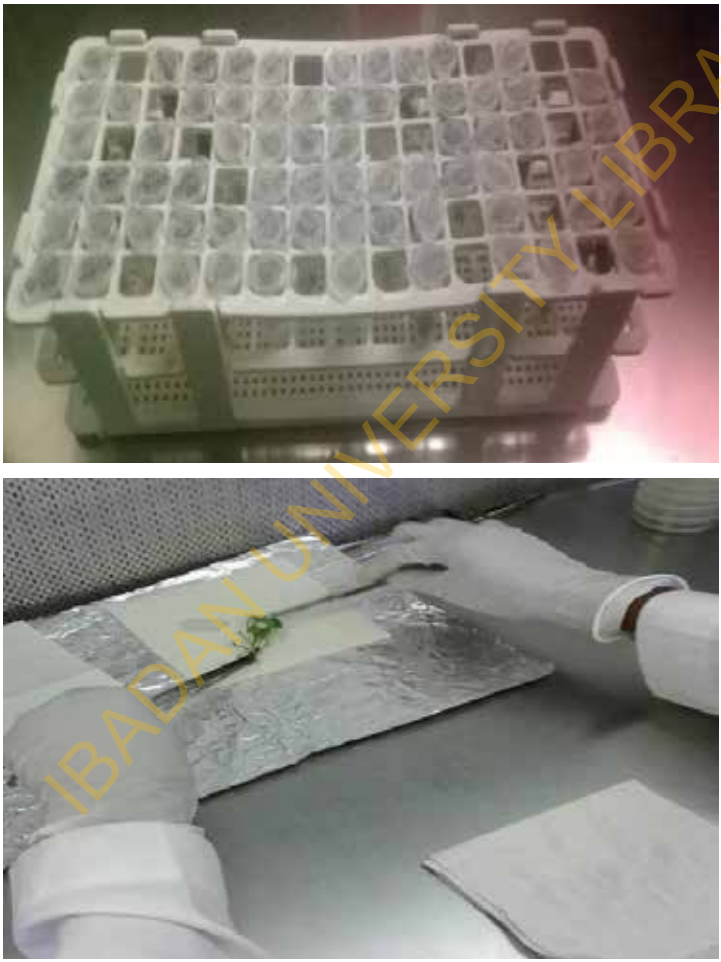

Plate 13. Sampling for pathogen indexing. Top: Excised leaf samples of meristem-derived, heat-treated plantlets in eppendorf tubes for polymerase chain reaction-based virus indexing; Down: Chopping of plantlets for bacteria indexing.

Some roots, stems, and leaves of 8-week-old meristem-derived plantlets are chopped onto a bacteria indexing medium containing (per liter) 10 g sucrose, 8 g casein hydrolysate, 4 g yeast extract, 2 g potassium hydrogen phosphate, 0.15 g magnesium sulphate heptahydrate, and 10 g agar in sterile petri plates and incubated at 27 + 2 °C for seven days in darkness (Thomas 2004) Plate 14. Some companies however sell already prepared bacteriological indexing medium.

Plate 14. Indexing for endophytes. Top: Bacteria-indexed plantlet returned to liquid medium (Left); Chopped plantlet tissues in petri plate (Right); Bottom: Tissues under incubation for Bacteria indexing.

The incidence of microbial growth around the chopped explants should be observed daily. Only non-endophytic stocks (Plate15, top) should be used for rapid propagation. Use of clean stocks reduces losses from culture contamination in large, commercial micropropagation schemes. We found up to 32% endophytic cultures from plantlets derived from nodes while it was 0% in the meristem-derived plantlets.

Plate 15. Identification of pathogen-free stock plantlets for pre-basic seed yam production. Non-endophytic (Top) and endophytic (Bottom) plantlets of yam.

This endophyte indexing system is more efficient, as the use of a combination of five antibiotics had only 33% success in getting rid of the endophytes, being also genotype-dependent (Mbah & Wakil 2012). Although some endophytic associations were reported to increase adaptive response of plants to stress through plant growth stimulation (nitrogen fixation, and auxin and cytokinin production) and disease protective properties (Jasim et al. 2015), it is safer to avoid them in micropropagtion schemes pending determination of the effect of specific endophytes on the field performance of yam.

Scale-up propagation of clean stocks

This can be done using conventional solid/liquid culture medium or automated systems like Temporary Immersion Bioreactors. The same medium as described for nodal culture is used, but this can be modified by each laboratory, especially plant growth regulator combinations, to suit the genotype and prevailing conditions.

Multiplication in conventional tissue culture

The components for one liter of medium are dissolved in about 1000 ml of distilled water in a beaker and stirred. The pH is adjusted to 5.7 by adding 1 M NaOH (to increase) or 1 M HCl (to reduce) the pH. Agar is added (if solid medium) and the solution is heated gently on a hot plate while stirring until all the agar has dissolved. Alternatively, a microwave oven can be used to melt the medium for 10 minutes. The medium is then dispensed into culture vessels (test tubes, plastics, baby food jars, etc.). and autoclaved at 103.4 KPa and 121 $^{\circ}$C for 15–20 minutes. Single node cuttings from pathogen-free plantlets (Plate 16) are introduced into the medium and incubated in culture rooms. The steps are repeated after about eight weeks to multiply the plantlets (Appendix 4).

Using six genotypes and plantlets from nodal explants, we found that the propagation ratio decreased from 7.2 + 0.4 at first introduction (cycle 1) to 3.9 + 0.2 in the 5th cycle when sub cultured at 10 weekly intervals. Regularly replenishing tissue cultures with new mother stocks

is necessary to maintain vigorous plantlets. Aeration was also found to increase plantlet growth as propagation ratio increased in vented plastic vessels having lids with 4 mm air filters relative to non-vented vessels.

Multiplication in Temporary Immersion Bioreactor Systems (TIBS)

The need for frequent subculturing which increases labor costs, in addition to restriction of the plantlets in terms of access to nutrients and air due to small size of the culture container causes hyperhydricity and vitrification, which is a stress condition in tissue-cultured plants, manifested mainly as abnormal leaf functioning. Protein and photo synthesis, gas exchange, cellulose and lignin synthesis, and ethylene

Plate 16. Yam plantlets in agar-solidified conventional tissue culture in test tubes.

Plate 17. A cross section of 23 out of the 128 units of IITA/YIIFSWA's SETIS Type bioreactor visited by IITA's Director-General, Dr N. Sanginga (middle) and Dr M. Gedil (left) received by YIIFSWA Tissue Culture Specialist, Dr Morufat Balogun (right).

production are affected, resulting in fragile plantlets and suboptimal propagation rates. Consequently, plant propagation ratios in conventional tissues are suboptimal (for yam, four new plantlets are obtained from one grown plantlet every 8–10 weeks). These limitations led to the development of TIBS (Paek *et al.*, 2005).

A bioreactor is an enclosed, sterile environment which utilizes liquid medium and which is provided with inlets and outlets for airflow under pressure. Successful yam organogenesis has been achieved in TIBS (Adelberg and Simpson 2002; Balogun et al. 2014). TIBS is an advanced tissue culture (TC) technology, where plants are intermittently immersed in nutrient solution in contrast to CTC with continuous immersion. Different designs of TIBS exist, some using compressed air pressure and others using gravitational pressure or a combination of both to save costs. Improved culture aeration and gas contacting combined with automation are associated with bioreactor systems causing increased productivity and reduced labor cost associated with conventional

tissue cultures. Simple activities like labelling and sealing the vessels take significantly less time when using TIBS than CTC.

The SETIS™ Type Temporary Immersion Bioreactor System

YIIFSWA installed 128 units of the SETIS™ type twin flask TIBS (Plate 17), each TIBS having one container for the nutrient and another for the plant (Plate 18).The number of plantlets per TIBs tested so far is up to 150.

The number of new plantlets obtained from one plantlet sub-cultured from plantlets grown in TIBS using single node cuttings in a 60-day cycle ranged from 3 to 12. This is more than double that obtained in CTC but varies with genotype. Plant materials produced in TIBS are also more vigorous.

Plate 18. Inset: One unit of SETIS™ TIBs with plant and medium vessel. Top: Plant vessel containing well grown plantlets ready for hardening or subculturing.

The SETIS TIBS can take three liters of medium, implying that 300–600 plantlets can be cultured at a time. It therefore occupies 10-fold less the space occupied by CTC in test tubes while hardening is more successful because the plantlets are healthier. Additional advantages of SETIS TIBS include automation, ability to combine research with production due to facility for a minimum of four unique immersion and ventilation frequencies and internet compatibility. There are 4 control points in IITA/YIIFSWA's SETIS™ system. This implies that four different immersion and ventilation settings can be made to run at the same time. All these enhance process control and management while the larger size of vessel allows better canopy development and large batches can be handled with lower risks of mix-ups.

Culture starts in CTC for 1–3 cycles before introduction into TIBS. Figure 1 shows the number of plantlets produced in TIBS and CTC when introduced into TIBS in the 3rd cycle (assumes a 1:4 and a 1:6 propagation ratio in CTC and TIBS, respectively, every 8-week cycle). Microtubers were also produced in SETIS. The advantage is that they can be planted directly in soil, not requiring hardening. Microtuber formation was up to 40% and 2.7g per plantlet.

The components of SETIS™ TIBS (http://www.setis-systems.be/) include an air compressor to provide air under pressure for immersion in nutrients, air dryer to remove moisture from compressed air, air filters to remove particles from dried air, programable logic control (or manual timers) to program the immersion and ventilation times, and tubing to connect the systems and the bioreactor units (Plates 19 and 20).

The compressor is located in a clean corridor or covered roof top and connected to an air dryer to reduce the weight of air followed by connection to filters that remove particulate impurities. Next is the programmable logic control (PLC) and the bioreactor units. The PLC allows automated, online programing of the immersion and ventilation time. Although the light controls of the culture rooms can also be built into the PLC, digital or manual timers (Plate 21) can be used for the lighting. It must be ensured that the pressure reaching the PLC is 0.1–0.2 bar as required for SETIS™ so as not to break the containers.

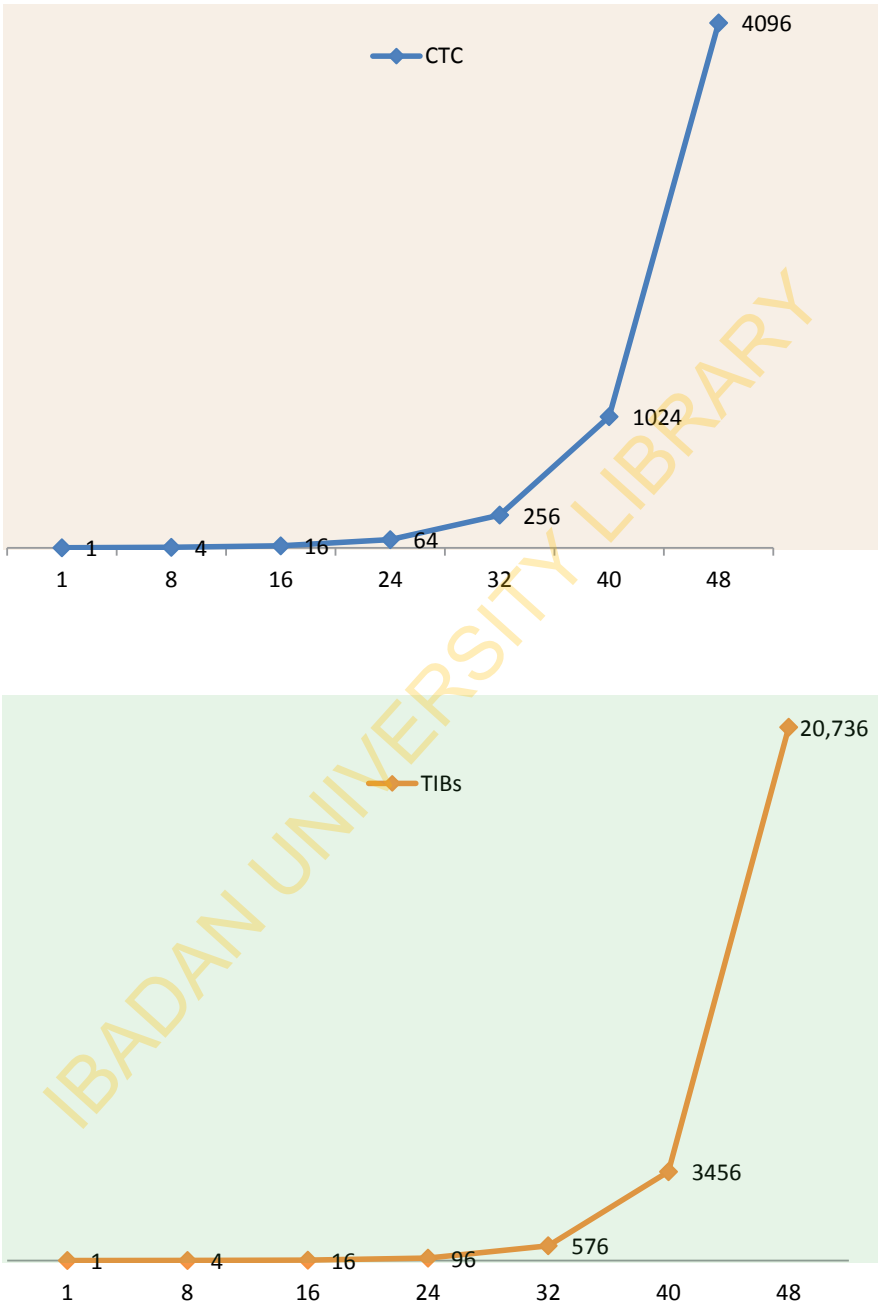

Figure 1. Number of plantlets produced in CTC (Top) and TIBS (Bottom).

Plate 19. Air compressor (Top) and air dryer (Bottom) for the SETIS™ Type Temporary Immersion Bioreactor system.

Plate 20. Filters (top) and programmable logic control (down) for SETIS™ type TIBs.

The PLC unit, having a specific IP address, is connected to the computer via an ethernet port, followed by a manual ethernet connection. The SETIS web configuration page is reached at IP address/SETIS/MainMenu.php. The manufacturer's guide should be followed to set time, immersion and ventilation frequencies for the bioreactors. Each programmable logic control has a minimum of four control points. One control point is a unique ventilation and immersion frequency setting which can range through four, six, eight, ten or twelve hourly immersions for duration of 1–3 minutes and 1-3 minutes ventilation interval.

The steps involved (Appendices 5 and 6) in the setting up of the medium and culture vessels are shown in plates 22 and 23 respectively. The medium and culture vessels should be placed vertically in the autoclave (Plate 24). Two filters, their attached respective silicon tube and the media transfer silicon tubes are packed in plastic container or high density foil for autoclaving.

Plate 21. Light timers.

Plate 22 . Handling TIBs vessels. Top: Pouring of 1-3 litres of medium in vertical position. Bottom: Insertion of silicon gasket in screw cap

Plate 23. Top: Tight closing of medium vessel; Bottom: Covering of air and media connectors with foil

Plate 24. Top: Dissecting instruments and connecting tubes packed for autoclaving; Bottom: Medium to be autoclaved for 15-20 minutes in a vertical position.

To avoid contamination (Plate 25) when subculturing, open the culture vessel facing the direction of airflow in the laminar flow hood to introduce single nodes or other plant materials and close the cap tightly with a SETIS cap holder.

The medium and culture vessel containing the explants should be brought to stand vertically side by side followed by removal of the aluminium foil from the air connectors and attachment of the air filters through the silicone tube. The long silicon tubing is used to connect the medium connector of the two vessels. To clean the used or new vessels, the stages are shown on Table 1.

Plate 25. Sub culturing into SETIS™ Type TIBs

Table 1. Cleaning the SETIS™ Type bioreactor vessels.

Steps	New vessels	Used vessels
	• Rinse	Autoclave
	• Dry	Rinse
	• Autoclave for 30 minutes	Soak in 70% commercial (bleach) for three hours
		Wash with neutral detergent
		Rinse
		Dry

The medium should be changed every 2–3 weeks. This is because the pH reduces over time and the plants stop growing vigorously. Twin bioreactor vessels whose medium is to be changed should be brought to the flow hood, the medium connector should be disconnected from the spent medium. The aluminium foil from the air connector of the new media vessel should be removed, silicon tubing attached without touching its edge followed by placing a clean, autoclaved air filter via the silicone tube. The following link is a video clip that explains the TIBS www.youtube.com/watch?v=bxzlzltmr4s

The Plant form Type Temporary Immersion Bioreactor system (PF TIBS)

In order to enhance out-scaling of the bioreactor technology, YIIFSWA tested the Plant Form type bioreactor system (Plate 26). The PF TIBS has been designed for easy handling, while the air exchange can be controlled using air pumps and timers. One full setup is made of 12 units of bioreactors working like a kit that can be disseminated to partners as one package. It does not require compressors, dryers, etc. needed for big production facilities with SETIS. As more plantlets are needed, more units of PF TIBS can be purchased to increase production volume. It requires lower electricity while the timers, filters and tubing can be locally sourced. Plantlets have been successfully hardened from PF TIBS.

Plate 26. The Plant-form bioreactor system.

Maintaining sterile conditions

Sterile conditions must be maintained within the culture vessel and in the laboratory environment (Appendix 7). This is because a single fungal spore or bacteria cell in the growth media will reproduce rapidly, outgrow, and eventually kill the plantlet. The contamination will then spread to all the plantlets within a culture container. Therefore, regular checks for functionality of the laminar flow hood and autoclave, regular cleaning of the laboratory floor with disinfectant solutions, and fumigation when the microbial load is high should be adhered to. Fumigating solutions are commercially available and the regularity will depend on air cleanliness in each environment. In some cases, simple household insecticide is sprayed every week in addition to wiping of surfaces with sodium hypochlorite. It can be weekly, monthly, quarterly, or biennially. Laboratory overalls and indoor footwear should be used by workers always in the laboratory while nose masks, head covers, and hand gloves should be used when working at the laminar flow hood.

The cultures should be sealed with parafilm or kitchen cling film, labelled with the date and name of sample, and incubated in the culture room set at 25 + 2°C, 14–16 hours photoperiod, and 4000 lux of light. These days, energy saving, light-emitting diode (LED) bulbs are available to save on power. The cultures should be routinely checked daily to discard contaminated ones. At about 6–10 weeks after culturing, depending on variety, the plantlet vigor is high and there are no signs of senescence. At this time, they should either be subcultured into new vessels using about 1 cm single node cuttings or hardened for pre-basic seed yam production.

Post-flask management of plantlets

Hardening of plantlets

Good root development is fundamental to successful hardening. Old plantlets should not be acclimatized as they die in large numbers, while also having a short time to express good vigor before completing their growth cycle.

Well controlled relative humidity (RH) is key in reducing wilting due to water stress in hardening tissue culture (TC) plantlets. In TC, RH is higher than 95%, and the waxy cuticle on the leaves of plantlets is not as high as screenhouse plants (Gilly et al., 1997; Zaid and Hughes, 1995). In outside environment however, humidity varies with season especially in the tropics where yam is mostly grown and temperature can be as high as 40°C in February in the West African yam belt. Consequently, reducing RH in TC vessel before transplanting will reduce the shock due to water stress that causes wilting in the TC plants at hardening. Use of vented vessels or using loosely fitting closures or even bottom cooling of culture can be used to achieve this, causing higher transpiration and development of more functional stomata to control water loss while also improving nutrient uptake (Cassells and Walsh, 1994). Hardening is less demanding of technician's time in bioreactor grown plantlets because gas contacting is improved in bioreactor systems while medium change (nutrient replenishment) is possible without plantlet manipulation (otherwise impossible in CTC).

The optimum growth rate of de-flasked plantlets frequently does not occur until new leaves and roots develop in the greenhouse environment. However, as species differ greatly in their requirements, so also do yam genotypes due to evapotranspiration rates and tolerance to water stress such that the acclimatizing protocol will have to be customized for specific genotypes. In hardening, plantlets should not be exposed to direct sunlight. Nets with 75% shading or reflective sheets or Aluminium shade nets (Appendix 11) should be used to achieve this. Optimum temperature is between 25 and 28°C.

The system can be adapted in different ways. The plantlets can be arranged on wooden or plastic trays and enclosed in plastic sheets (Appendix 8). When condensation is visible (usually about two weeks after de-flasking depending on ambient weather), the sheets are punctured at a few points. The number of punctures should be increased with time.

The location also affects the success of transplanting because of varying weather (temperature and humidity) conditions. It is very important that the pre-basic seed producer understands these fluctuations to adjust post-flask management strategies. As an example, February is usually hot about (34°C) in Ibadan, Nigeria while June/July is coldest (28/26°C). Relative humidity is about 73% and 93/94%, respectively, in the same months. As June/July is closer to the in vitro temperature and relative humidity in tissue-culture, a higher success rate is expected. In hot situations, foggers are preferred as they control the relative humidity around the plants being hardened. Consequently, handling and management will differ at the two periods. Options for hardening TIBS plantlets are detailed below.

Multiplication medium to hardening

Plantlets with good root development should be transferred into containers with perforated bottom, or customized transplanting trays, containing sterile top soil (30-60 minutes of steam sterilization) mixed with carbonized rice husk in equal volumes or other commercial substrates, Plate 27).

Plate 27. Substrate for hardening of plantlets from Tissue culture. Top: Carbonized rice husk (CRH); Bottom: Mixture of CRH with top soil being filled into seedling bags.

Plantlets should be immersed into a solution of 7 g/l Team (4 g/l mancozeb + 0.8 g/l carbendazim) followed by transfer into prepared substrate and covering with transparent plastic sheet (Plate 28). Vessels that are vented (from Zeshine, Appendix 11) also perform well for hardening well rooted plantlets (Plate 29). After 14–21 days (depending on prevailing humidity and temperature) when using plastic sheets, the sheet is punctured or opened slightly. The number of punctures should be increased every 2–3 days until the sheets are torn or removed completely (about 3rd-4th week after transplanting). In the case of vented vessels, there are five vents on the cover at the top. The first vent

Plate 28. Hardening of TIBs plantlets in basket humidity chambers.

Plate 29. Plantlets being hardened in vented vessels.

is opened on the third day after deflasking while other vents are opened at one day intervals until all the vents are completely opened. However, the cover is completely removed after about 3 weeks.

In some cases when plantlets are planned for planting in aeroponics, the height is not enough in the 3rd week. The plantlets can be subcultured using two-node cuttings to obtain taller plants within a shorter time before hardening.

Microtubers weighing up to 2.7g were produced in the SETIS Temporary immersion bioreactor system and planted directly in pots. In addition to being alternative propagules, they also do not require acclimatisation as with tissue culture plantlets (Plate 30).

Plate 30. Microtubers harvested from TIBs (top) and sprouted after directing planting in pots (bottom).

Use of Vivipak

Another successful approach is the use of customized Vivipak (Plate 31), Appendix 9) vessel (ViviTECH, Appendix 11) It is a gamma-sterilized disposable vessel containing tray with ready-to-use, sterile substrate (cocopeat) inserts. The plantlets are rinsed with sterile distilled water in the flow hood and subcultured using two-node cuttings, into the ViVipak trays. It is then sealed with ViFoil, a semi permeable barrier that controls the gas exchange between the ViTray and the climate of the growth environment. This not only ensures good root development and hardening but also propagates the plantlets in one step. After two to three weeks, the plantlets are transferred into trays (Plate 32) or pots (Plate 33).

Plate 31. Yam plantlets from TIBs in Vivipak (Vifoil removed) ready for potting.

Management of hardened plantlets to produce pre-basic (Breeder) seed tubers

Successfully hardened plantlets will retain turgid leaves and bring out new shoots. They should be transferred into pots containing sterile topsoil or into aeroponics (Maroya et al. 2014a, b) in an aphid-proof screenhouse. The topsoil can be amended with organic (30 g poultry manure per 5 kg topsoil) or inorganic fertilizer (0.1 g $MgSO_4$; 0.2 g K_2SO_4; 0.12 g NH_4NO_3; 0.2 g triple superphosphate; 0.4 g agrolyzer micronutrient per 5 kg topsoil), especially when leaves are showing yellowness. The plants should be watered as necessary. Pre-basic tubers weighing 50 – 200 g were harvested from hardened, potted plants 4–5 months after potting. Single node vine cuttings (Plate 33) were also generated from potted plantlets from TIBs and rooted (Plate 34) for further clean seed yam tuber production (Plate 34). Harvested tubers should be treated with a mixture of insecticide and fungicide solution as described previously and stored in baskets, wooden boxes, or shelves in a well-ventilated container

Plate 32. Successfully hardened plantlets in trays.

Plate 33. Potted plantlets from TIBs (Top); Vine cuttings from potted plantlets (Down).

Plate 34. Rooted single node vine cuttings from potted TIBs plantlets (Top); Breeder seed yam tuber from potted TIBs plantlets (Down).

Documentation in Pre-basic seed yam tuber production

3

Inadequate documentation can cause serious problems between pre-basic seed suppliers and their clients. A variety name may be wrong, or the quality may not be as claimed. These errors quickly bulk up in conventional tissue culture or the bioreactor system.

The basic thing is to have a documentation gadget for each staff. This can be notebooks or tabs. Each plant should be traceable to a staff responsible for its micropropagation, hardening, potting or storage. There should be a locational reference on the shelf (e.g TIBs 3, Shelf 1 row 2 is handled by Mr. 'X' The number of subcultures from each plantlet should be recorded so as to know if and when the propagation ratio starts reducing so as to replace stocks.

Within each genotype, each plantlet regenerated from a particular meristem should have a unique number. This is because different meristems from the same mother plant can differ in vigour, which can also affect response to thermotherapy and consequently the health of the plantlets.

Labelling should be as automated as possible (e.g use of bar codes that are linked to pre-basic stock databases). It should include date cultured, unique stock number, and variety name, in the minimum. When changing vessels, labelling should be done before putting plants to be double sure. Records should be backed up and saved online where there is lower risk of data losses.

Conclusion

4

There has been major progress in terms of use of Tissue culture in general and Temporary Immersion Bioreactor system in generating high quality breeder (pre-basic) seed yam tubers. This is evident in the scale of production achieved over four years of standardization by YIIFSWA. Efforts are further required in reduction of incidence of contamination, local availability of TIBs components, fine-tuning for specific varieties and increased application to somatic embryogenesis and microtuber production. The use of TIBs in genetic improvement still remains to be exploited in yam breeding such as investigating metabolic and genomic studies under controlled conditions. The CTC and TIBs is potentially able to drive future research and seed production activities of yam.

Appendix

Appendix 1. Items needed for production of Breeder seed yam using Temporary Immersion Bioreactors.

Yam tubers	Culture establishment	Elimination of pathogen from plantlets/ multiplication in conventional tissue culture	Multiplication of pathogen free plantlets in bioreactors	Hardening of plantlets	Ex-vitro handling
Yam tubers and Pots for planting	√				√
Top soil	√			√	√
Soil sterilizer	√			√	√
Karate (lambda-cyhalothrin)	√				
Team (Mancozeb + carbendazim)	√				
Watering hose	√				
Culture vessels (test tubes and racks/petri dishes/other vessels)	√	√			
Vessel dryer	√	√	√		
Refrigerator with fridge and freezer compartments	√	√	√		
Weighing balances (0.1 and 0.0001 g)	√	√	√		
Spatula and weigh boats	√	√	√		
Water distiller/reverse osmosis equipment/ deionizer	√	√	√	√	
Beakers/measuring cylinders/pipettes	√	√	√	√	
Magnetic stirrer with hot plate	√	√	√		
Pipette filter and pipettes	√	√	√		
pH meter	√	√	√		
Wash bottles	√	√	√	√	
Microwave oven	√	√	√		
Medium dispenser	√	√	√		

Appendix 1 contd. Items needed for production of Breeder seed yam using Temporary Immersion Bioreactors.

	Culture establishment	Elimination of pathogen from plantlets/ multiplication in conventional tissue culture	Multiplication of pathogen free plantlets in bioreactors	Hardening of plantlets	Ex-vitro handling
Autoclave/Pressure cooker	√	√	√		
Hand sprayers	√	√	√	√	
Laminar flow hood	√	√	√	√	
Scissors	√	√	√	√	
Detergent (Tween 20 or Teepol)	√				
Sodium hypochloride solution (Jik, Hypo, etc.)	√		√		
Forceps, blade, scalpel	√	√	√	√	
Growth cabinet		√			
Stereo binocular microscope		√			
Culture shelves	√	√	√	√	
Cool white fluorescent lights (LED)	√	√	√		
Timers	√	√	√	√	
Data loggers	√	√	√	√	
Air conditioners	√	√	√		
Compressor			√		
Air filters			√		
Backup power (Inverter/ generator/solar)	√	√	√		
Programable logic control			√		
Air dryer			√		
Bioreactor vessels			√		
Membrane filters			√		
Hardening substrate				√	
Poultry manure or synthetic fertilizer*					√
Hardening vessels and/or misters (or white plastic sheets and baskets)				√	

Appendix 2. Step by step description of establishment of in vitro cultures from tubers.

1. Cut tubers that have broken dormancy into minisetts of 30-50g each;
2. Immerse minisetts in a mixture of 2 ml/l lambda-cyhalothrin + 7 g/l Team (4 g/l mancozeb + 0.8 g/l carbendazim) and allow to air-dry for 2 days;
3. Steam-sterilize top soil for 2 hours and allow to cool;
4. Put 7 l soil in 10 l pots inside an aphid-proof screenhouse;
5. Confirm soil sterility by lack of weed or fungal growth after one week;
6. Plant minisetts in pots.
7. Every 2 weeks, spray with fungicide/insecticide (team/karate) to reduce surface contaminants.
8. Prepare yam multiplication medium containing 7g agar, 4.43g MS with vitamins, Myo-inositol 0.1g, Sucrose 30g, 0.5mg kinetin.
9. Adjust pH to 5.7 $\pm$ 0.1
10. Dispense 10ml per plantlet into test tubes or other vessels.
11. Autoclave at 103.4kPa and 121^{o}C for 15 minutes.
12. Cut 1-2cm long, single nodes from 2-3 months old plants.

Surface-sterilize in:

13. 2 ml/l lambda-cyhalothrin + 7g/l Team (4g/l mancozeb + 0.8g/l carbendazim) for 10 minutes;
14. 70% ethanol 5 minutes;
15. 2% sodium hypochlorite solution for 15.
16. 1% sodium hypoclorite solution for 30 minutes
17. Rinse in 3 changes of sterile distilled water.
18. Introduce into the prepared yam medium in the laminar flow hood;
19. Place on shelves in the culture room at 25$\pm$ 2^{o}C, 4,000 lux of light from cool white fluorescent tubes or LED bulbs at 16-h photoperiod.

Appendix 3. Preparation of meristem culture medium (500ml) containing Uniconazole-P.

1. 15g Sucrose,
2. 50mg Myo-Inositol,
3. 10mg L-Cysteine,
4. 40mg Adenine hemisulfate,
5. 0.1mg Benzylaminopurine (dissolved in few drops of 1N NaOH). Prepare 1mg/ml by weighing 100mg, dissolve in NaOH and make up to 100ml. Label as 1mg/ml. Take 10ml of this solution and make up to 100ml to give 0.1mg/ml stock solution. Use 1ml of the 0.1mg/ml stock solution in 500ml of medium
6. 0.5mg Uniconazole-P (dissolve in some drops of acetone). Prepare 1mg/ml by weighing 100mg, dissolve in acetone and make up to 100ml. Label as 1mg/ml. Take 10ml of this solution and make up to 100ml to give 0.1mg/ml stock solution. Use 5ml of the 0.1mg/ml stock solution in 500ml of medium
7. Plant Preservative Mixture (PPM: 0.5ml) This is optional

Appendix 4. Step by step procedure for multiplication in Conventional Tissue Culture

1. Dissolve 4.43g of MS powder in about 800 ml of distilled water in a beaker and stir.
2. Add 30 g sugar and stir to dissolve.
3. Add 0.1g of Myo-Inositol.
4. Add 1g of activated Charcoal. Add 10mg L-Cysteine.
5. Prepare 50 ml of 0.1mg/ml Kinetin solution by dissolving in HCl. Add 5ml of the stock solution to the medium.
6. Add distilled water to make the total volume up to 1 L.
7. Adjust pH to 5.7 by adding 1M NaOH or 1M HCl as necessary. Stir.
8. Weigh out 7g of agar and add it to the medium solution (not necessary if liquid medium).
9. Heat the solution gently on a hot plate while stirring until all the agar has dissolved. Alternatively, a microwave oven can be used to melt the medium for 10 minutes.
10. Dispense in to vessel (test tubes, plastics, baby food jars, e.t.c) at rate of 10ml per explant.
11. Place the vessels (with lids sitting on the tubes but not completely tightened) in an autoclave and sterilize at 103.4KPa and 121^{o}C for 15-20 minutes. When the autoclave has cooled, remove the tubes and tighten the lids.
12. Introduce explants into medium and incubate in culture rooms.
13. Repeat the steps after about 6-8 weeks to multiply the plantlets.

Appendix 5. Setting up SETIS Bioreactor units.

1. Prepare medium and culture vessels as described on pages 28 and 29.
2. At the laminar flow hood, open the culture vessel facing the direction of the airflow, introduce single nodes or other plant materials and close cap tightly with SETIS cap holder;
3. Bring media vessel to stand vertically beside culture vessel and remove aluminium foil from the air connectors of both vessels;
4. Attach the air filters through the silicone tube (hands must not touch the connectors and edges of the silicon tube;
5. Remove aluminium foil from media connectors of the 2 vessels and connect the two vessels with long silicon tube;
6. On the shelf, stack culture vessel on media vessel via the key-in positions to and avoid sliding;
7. Connect both filters with their respective compressed air circuit on the shelf.

Appendix 6. Changing medium in Bioreactor.

1. Prepare new, autoclaved medium vessel as described above and bring to the flow hood;
2. Bring old twin bioreactors with plants to hood and place vertically (slowly);
3. Disconnect the media transfer silicone tube at the side of media connector of the media vessel and discard old media vessel;
4. Bring the new media vessel and remove the foil covering its media connector;
5. Reconnect the media transfer silicone tube to media connector of new vessel;
6. Remove aluminum foil from air connector of new media vessel;
7. Without touching the connectors and edge of the silicone tube with your hand, place new air filter via the silicone tube;
8. Attach the culture to new medium on the shelf immediately.

Appendix 7. Maintaining aseptic conditions.

1. Fumigate the laboratory as required;
2. Frequency of fumigation will depend on air quality in each environment. It can be weekly, monthly, quarterly or biennially;
3. Regularly service the laminar flow hood and autoclave following manufacturer's manual;
4. Clean the laboratory floor with disinfectant solutions (e.g 2.5% sodium hypochlorite);
5. Wear laboratory overalls, indoor foot wears and head covers;
6. Nose masks should be used when working at the laminar flow hood and no verbal communication in the laminar flow hood.
7. Vessels containing explants should be sealed with parafilm or kitchen cling films.
8. Check for contaminated cultures and discard daily.
9. At highly humid environments or seasons, desiccants can be placed at strategic places to absorb moisture.
10. The influx of visitors to the culture rooms must be reduced if completely unavoidable.

Appendix 8. Step by step description of acclimatization of plantlets (Conventional Tissue Culture or TIBs).

1. Observe that there is good root development in plantlets;
2. Steam-sterilize top soil wrapped in sac bags for 30-60 minutes and bring out to cool;
3. Mix equal volume of top soil with carbonized rice husk (or washed river sand or other commercial substrates);
4. Put in containers with perforated bottom, basket, customized transplanting trays etc.,);
5. With gloves in hand, prepare solution of 7g/l Team (4g/l mancozeb + 0.8g/l carbendazim);
6. Remove plantlets from TIBs into fungicide solution;
7. Slowly and carefully, pick each plantlet and plant in prepared substrate;
8. Cover completely with transparent plastic sheet without delay;
9. After 7–10 days (depending on prevailing humidity and temperature), puncture plastic sheet at about 3 points or open slightly. The number of punctures should be increased every 2–3 days until the sheets are torn or opened completely (2nd or 3rd week after transplanting);

Appendix 9. Stepwise description of post-flask management using Vivipak.

1. Make 2-node cuttings from grown plantlets in flow hood;
2. Rinse in sterile distilled water to remove medium residues;
3. Introduce into commercial pre-mixed substrate (Vivipak) in the laminar flow hood using sterile dissecting instruments;
4. Incubate in screenhouse (cool season) or growth room (hot weather);
5. After 3 weeks, transplant into pots;
6. Where ambient temperature is above 29^{o}C high, misters should be used during the first 12 days of hardening.

Appendix 10. Summary of duration of the different steps to producing Breeder seed yam tuber using tissue culture.

• Planting, collection of explants	• 12 weeks
• In vitro culture of single nodes	• 8 weeks
• Pre-culturing for thermotherapy	• 1 week
• Thermotherapy*	• 3 weeks
• Meristem culture and plantlet regeneration, PCR test 1*	• 16 weeks
• Scale up propagation (TIBs or CTC)	• 12 weeks
• Hardening	• 3 (to 4) weeks
• Tuber production	• 32 weeks
• Total	• 87 weeks

*Average virus-free success rate is 73.3%

Appendix 11. Some contacts to points of sale for materials for Breeder (Pre-basic) seed tuber production.

Equipment	https://phytotechlab.com/ www.labassociates.org http://www.thomassci.com/ www.hortamericas.com
Reagents	www.labassociates.org www.millaboratories.org www.sigma.org www.duchefabiochemie
Setis Bioreactors plant form	http://www.setis-systems.be/; www.plantform.se
Post flask management materials	http://www.fertiplus.eu/Fertiplus/index.xhtml www.hortamericas.org http://www.greenhousemegastore.com/category/flats-trays-inserts http://www.duboisag.com/ https://www.viewpointtw.com/aluminium-shade-net.htm www.vivi.nu
PTFE filters and culture vessels	http://www.zeshine.cn/B052TC-R500_en.htm

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