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Acronyms

BMGF	Bill & Melinda Gates Foundation
BS	Breeder seed
BST	Breeder seed tubers
BSV	Breeder seed vines
BV	Badnavirus
CTC	Conventional Tissue Culture
MS	Murashige and Skoog medium
PF	Plant Form
SETIS	Trade Mark of TIBS
TIBS	Temporary Immersion Bioreactor System
YIIFSWA	Yam Improvement for Income and Food Security in West Africa
YMV	Yam mosaic virus

The importance of yam and constraints of production

Yams (Dioscorea spp) are a primary source of income and starchy staples in West Africa, where 92% of global yam production emanates (FAO 2020). Nigeria alone produces 68% of global yam, equivalent to 48 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 250 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 planting causes scarcity of seed yam, such that planting materials alone account for up to 63% of production costs (Agbaje et al. 2005; Ironkwe et al. 2007).

In addition, farmers can scarcely plant the whole of their field due to the non-availability of seed yam in sufficient quantities. Using 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 are 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 it efficiently to 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 (breeder) through foundation (basic) to commercial (certified) seed. In a formal seed system, breeder seeds are of the highest quality (Balogun et al. 2017), and purity, foundation, and commercial seeds are produced from them.

Plate 1. Symptomatic leaves (top) and tubers (bottom) of yam.

The project "Yam Improvement for Income and Food Security in West Africa (YIIFSWA)," funded by the Bill & Melinda Gates Foundation (BMGF), was envisioned in the first phase to increase yam productivity by 40% for 200,000 smallholder yam farmers in Ghana and Nigeria. It was to deliver key global good research products towards a 10-year vision to sustainably double incomes from yams for 3 million smallholder yam farming families 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 breeder (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 compared to the conventional TC with continuous immersion. Technologies for using the 128 units of the SETIS type TIBS installed by YIIFSWA were developed, ranging from establishing in vitro cultures through the generation of pathogen-free stocks and rapid propagation to post- flask management.

Tissue culture as a method of healthy seed yam production

Conventional plant TC grows plant tissues in a closed, static, sterile, and laboratory environment. Conventional Plant 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 a). The technique is independent of climate variations, saves time and space, and has greater outputs 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 the rapid, asexual in vitro propagation. Plant parts cultured, called explants, include apical meristems, axillary buds, nodes, immature leaves, and roots.

Initial stock from tissue culture is the singular preferred starting material for breeder or breeder (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 the 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 (TIB); hardening of plantlets, and post-laboratory management of hardened plantlets to produce breeder tubers (BT) or vines (BV).

The use of tissue culture takes some time initially to generate clean stocks, but more can be potentially nu produced 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. The YIIFSWA project has established virus-free plantlets of 20 popular yam genotypes in Nigeria by this means.

The tissue culture building should contain a cloakroom for laboratory overalls and footwear, medium preparation room (general washing area), transfer room, quality control room, culture rooms, general office area, rest rooms, emergency exit, and store. There should be stable electricity and a good water supply. The facility can be part of a building with well-sealed partitions as done by YIIFSWA or stand-alone to prevent cross-contamination 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 listed in Appendix 1, can be sourced from some companies listed in Appendix 2.

Steps to producing the initial stock of breeder seed yam

Establishment of in vitro cultures

This step usually starts with an uncertified or unclean tuber (Plate 1). A tuber showing signs of sprouting should be cut into minisetts of 30–50 g each (Plate 2) and treated (Plate 4) with a mixture of insecticide and fungicide. The infected tuber should be planted using good and sterilized topsoil, preferably in pots in a screenhouse so as not to further accumulate or spread disease if planted on the field.

Plate 2. Preparing mother plants for tissue culture.
Top: Sprouted yam tuber; Bottom: Minisetts cut from one tuber.

The soil should be sterilized to avoid introducing more soilborne 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., by autoclaving) removes both living microorganisms and resting fungal spores in the soil. The sterilized soil should be left in the pot for one week before planting to check for weed or fungal growth. This will confirm soil sterility before planting. Before planting, the screenhouse should be sprayed with a fungicide/insecticide mixture (Plate 4). The plants that emerge should be sprayed with the same mixture 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 a baseline reference to confirm the 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 3). Our findings showed that contamination was up to 100% when sodium hypochlorite treatment was not preceded with treatment in fungicide + insecticide solution.

Plate 3. Yam minisetts being treated in insecticide and fungicide solution.

Plate 4. Planting yam minisetts to generate mother plants with vines to establish in vitro cultures. Top: Spraying to disinfect the screenhouse with insecticide and fungicide mix; Bottom: Planting minisetts in sterile topsoil in pots.

Plate 5. Disinfecting mother vines for in vitro culture. Top (left): Growing yam mother plants. Top (right): Cutting single nodes; Bottom: Disinfecting 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 6–8). Constituents of the medium and steps involved are shown in Appendix 3. Appendix 4 shows how to prepare stock solutions of different growth regulators. Adding 1 g of activated charcoal per liter of medium reduced the production of phenolics in the cultures of white yam and enhanced plantlet vigor but was detrimental in water yam.

Plate 6. Preparing medium for in vitro culture of single nodes. Weighing medium constituents and pouring into distilled water in a beaker (bottom).

Plate 7. Preparing medium for in vitro culture of explants. Left): Dispensing the medium into culture vessels; Right: Autoclave for medium sterilization.

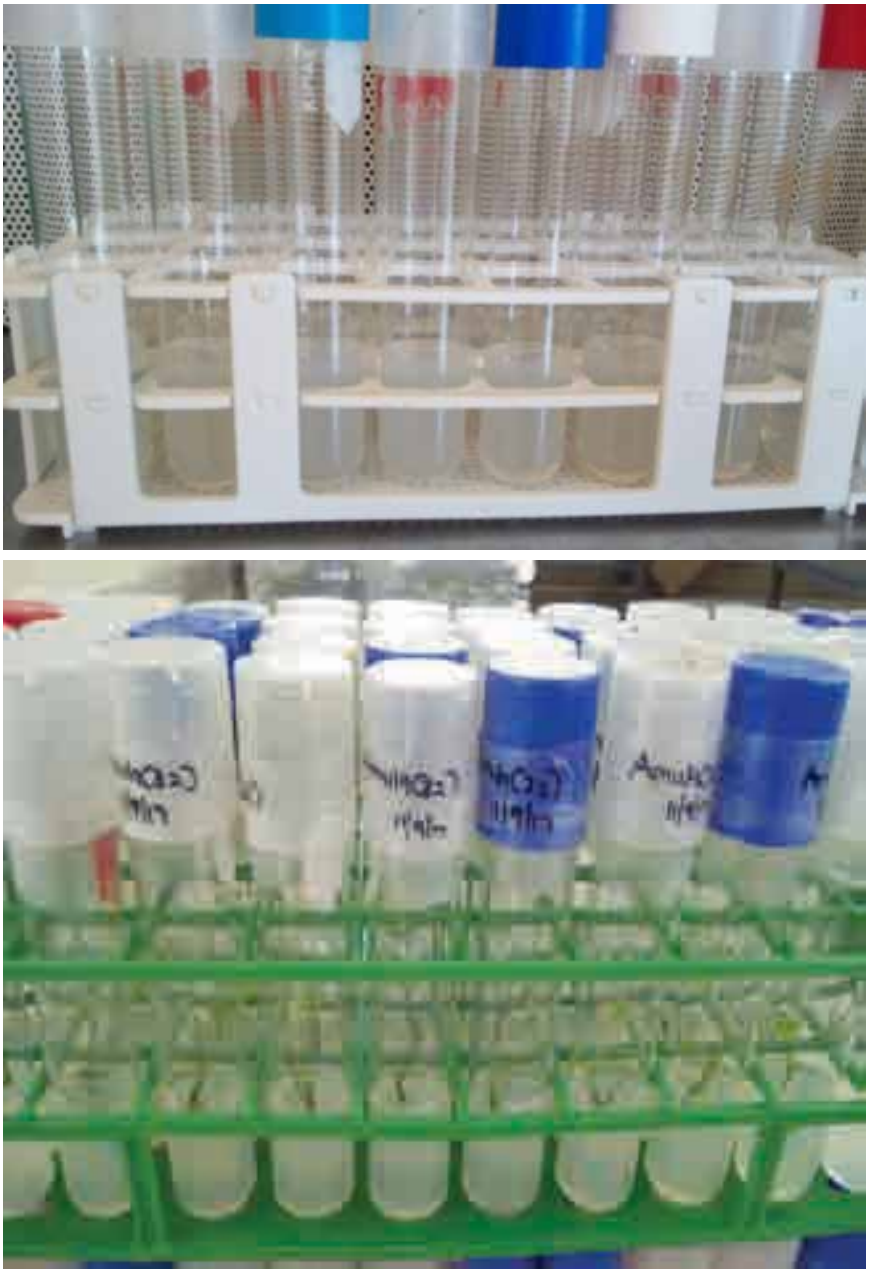

Plate 8. Establishing in vitro cultures of yam. Top: Freshly prepared medium; Bottom: Medium into which single nodes have been introduced.

Elimination of virus and other pathogens

After eight weeks, plantlets should be subcultured into two-node cuttings (Plate 9) 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 10) set at 36 + 0.5 ℃ 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. Petri plates are easier to handle for meristem culture due to the ease of introduction, observation, and transfer into other vessels. However, test tubes can also be used. Plantlets are regenerated from the meristem (Plate 11) and transferred into the yam multiplication medium described above, preferably agar-free, liquid medium for further growth. Regeneration from meristem takes up to 16 weeks depending on the genotype, and significantly lower than the six months to two years recorded before YIIFSWA (Balogun et al. 2014).

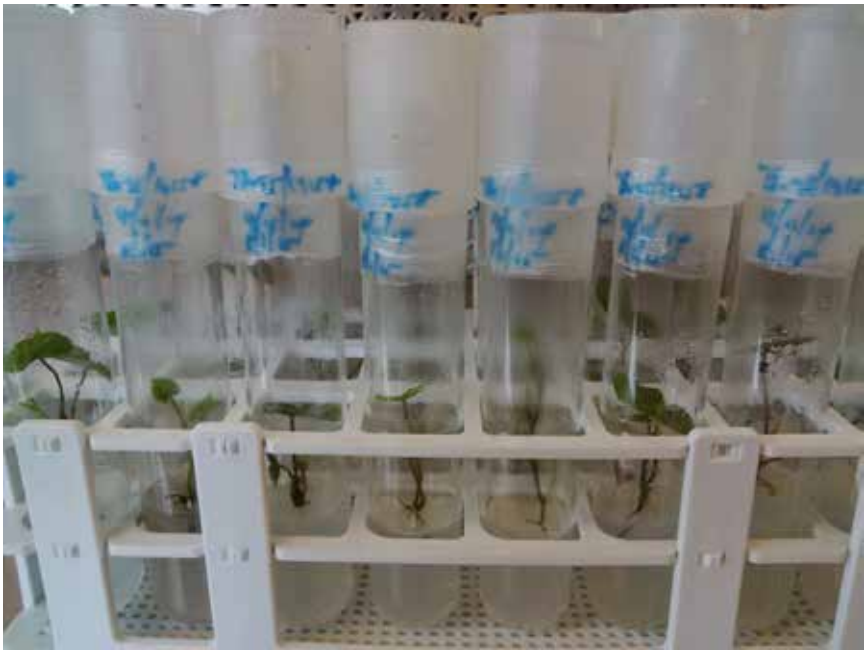

Plate 9. Yam plantlets subcultured into two-node cuttings.

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 Badnavirus (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, establishing about 73% of YMV-free plants is assuring. More than 3000 virus-free plants can be generated from 1 kg of infected tubers.

Plate 10. Temperature and humidity-controlled growth cabinet for heat treatment to clean plantlets from viruses.

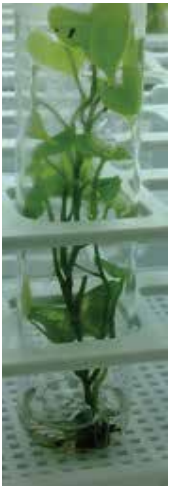

Plate 11. Regeneration of clean stock of plantlets. Top: Excising meristem under the microscope; Bottom left: Cultured meristem regenerating shoots; Bottom right: Fully regenerated plantlet.

Identifying pathogen-free plantlets

Leaf samples from the regenerated, heat-treated plantlets and those of the virus-positive mother plants (controls) should be retested for Yam mosaic virus (YMV), Yam mild mosaic virus (YMMV), Badnavirus (BV), and Cucumber mosaic virus (CMV) using polymerase chain reaction (PCR) (Nkere 2016; Plate 12) to confirm their health status. Regenerated plantlets should be checked for endophytes before micropropagation to avoid the build-up of contaminants.

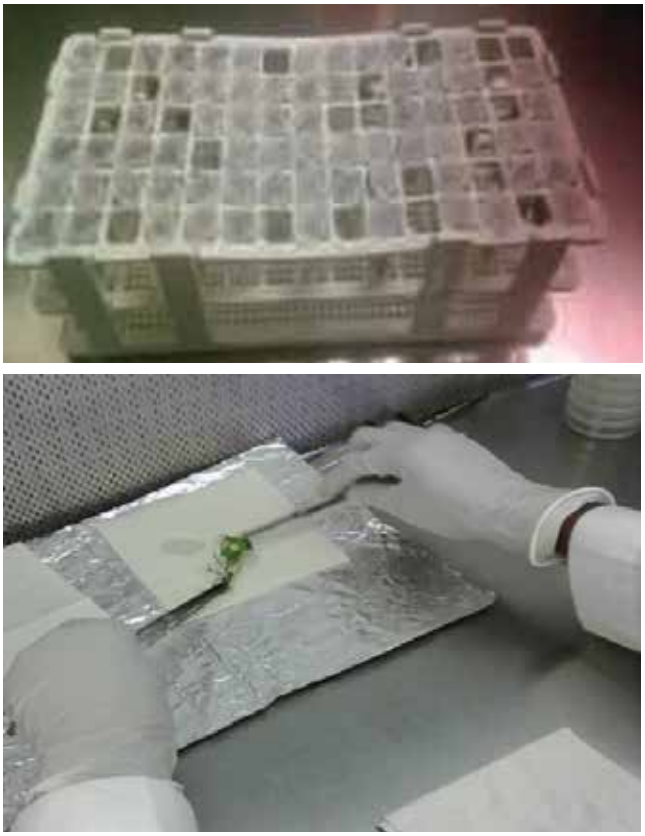

Plate 12. Sampling for pathogen indexing. Top: Excised leaf samples of meristem-derived, heat-treated plantlets in eppendorf tubes for polymerase chain reaction-based virus indexing. Bottom: Chopping plantlets for bacteria indexing.

Some roots, stems, and leaves of 8-week-old meristem-derived plantlets are chopped onto a bacteria indexing medium (Plate 13) 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℃ for seven days in darkness (Thomas 2004) (Plate 13). Some companies, however, sell already prepared bacteriological indexing media.

Plate 13. Indexing for endophytes. Top-Right: Bacteria-indexed plantlet returned to liquid medium (Top-Left); Chopped plantlet tissues in petri plate (Right); Bottom: Tissues incubated for bacteria indexing.

The incidence of microbial growth around the chopped explants should be observed daily. Only non-endophytic stocks (Plate 14, top) should be used for rapid propagation. The 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 14. Identifying pathogen-free stock plantlets for breeder seed yam production. Non-endophytic (Top) and endophytic (Bottom) plantlets of yam.

This endophyte indexing system is more efficient, as using 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 the 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 micropropagation schemes pending determination of the effect of specific endophytes on the field performance of yam.

Deciding which plantlets to retain as clean is the most critical step in the breeder seed production chain, and proper documentation is a major prerequisite. An internal record sheet (Appendix 5) of materials to be sent for virus indexing should be maintained by the laboratory staff. In contrast, an external record sheet (Appendix 6) should be given to the Plant Health Diagnostics Unit when sending the plant for indexing. Samples should be replicated on the internal record sheet, unknown to the virus indexing laboratory to serve as checks when results are received. The serial numbers on the internal record sheet should be used to track the serial number on the outbound sheet and the two must match. Appendix 7 shows a decision-making sheet that enhances the selection of clean stock plantlets upon receipt of the plant health results.

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 each laboratory can modify this, especially plant growth regulator combinations, to suit the genotype and prevailing conditions.

Multiplication in conventional tissue culture

The components for one liter of the medium are dissolved in 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 ℃ for 15–20 minutes. Single node cuttings from pathogen-free plantlets are introduced into medium repeatedly using full-grown plantlets (Plate 15) after about ten weeks to multiply them further using autoclaved dissecting instruments (Plate 16) and incubated in culture rooms.

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 also increased plantlet growth as the propagation ratio increased in vented plastic vessels with lids with 4 mm air filters compared to non-vented vessels.

Plate 15. Yam plantlets in test tubes with agar-solidified conventional tissue culture.

Plate 16. Dissecting instruments packed for autoclaving.

Multiplication in Temporary Immersion Bioreactor Systems (TIBS)

Generally, in Tissue culture, there is a need for frequent subculturing, which increases labor costs and restricts the plantlets in terms of access to nutrients and air due to the small size of the culture container. This causes hyperhydricity and vitrification, a stress condition manifested mainly as abnormal leaf functioning. Protein and photosynthesis, gas exchange, cellulose, and lignin synthesis, and ethylene 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 standardization of TIBS for yam (Paek et al. 2005).

A bioreactor is an enclosed, sterile environment, which utilizes liquid medium and 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 TC technology, where plants are intermittently immersed in nutrient solution compared 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 CTC.

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

Culture starts in CTC for 1 to 3 cycles before introduction into TIBS. Figure 1 shows that triple the number of plantlets produced in CTC is produced in TIBS assuming a 1 : 2.3 and a 1 : 3 propagation ratio every 14- and 8-week cycle, respectively). This, however, varies with genotype.

Plant materials produced in TIBS are also more vigorous. YIIFSWA installed 128 units of the SETISTM type twin flask TIBS with each TIBS having one container for the nutrient and another for the plant in the first phase of the project. Details on this can be found in the 2017 edition of this manual. However, due to lower electricity requirements, the Plant Form TIBS was standardized and outscaled to the NARIS.

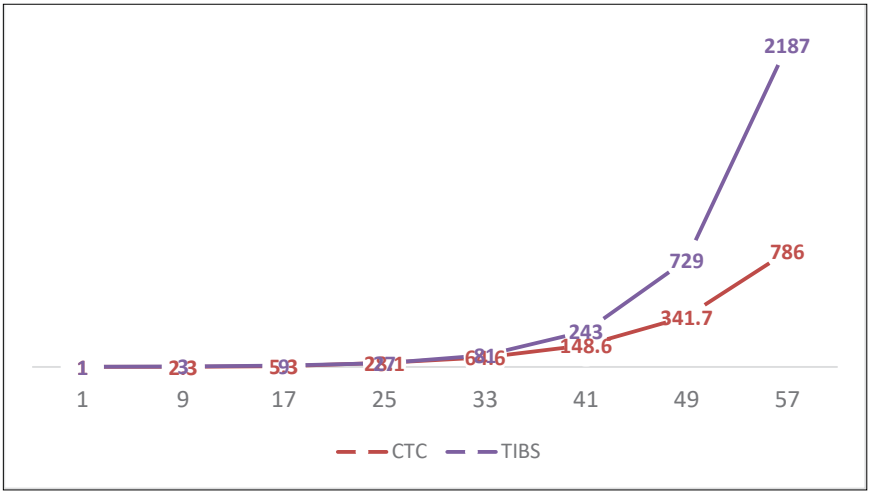

Figure 1. Up to 786 plantlets produced in Conventional Tissue Culture (CTC) and 2187 in the Plant Form Temporary Immersion Bioreactor System (TIBS) in 57 weeks.

The Plant Form Temporary Immersion Bioreactor system (PF TIBS)

To enhance the outscaling of the bioreactor technology, YIIFSWA tested the Plant Form type bioreactor system (Plate 17). 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. The parts are shown in Plate 18. It does not require compressors, dryers, etc needed for big production facilities using 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 17. The Plant Form Type Temporary Immersion Bioreactor system.

The steps involved in the setting up the medium and culture vessels for PF TIBS are shown in plates 18–24. Three filters are attached to vessels and all are autoclaved together.

1: Assembled Plant Form TIBS vessel; 2: Outer container having three inlets for gas exchange. The middle outlet is connected to a plastic tube on the inner chamber; 3: Filters, plastic tubes, nuts, clamps, and silicon rings for connection to the three inlets on the outer container,

4 (A): Inner chamber with 3 grooves and connection to middle filter;

5 (B): Basket with 3 rows of small holes for inner plant chamber; 6 (C): Frame with 4 legs, also part of the inner chamber; 7: Lid with 4 flaps and an inner silicon ring.

Plate 18: Culture Vessel Components of Plant Form Temporary Immersion Bioreactor.

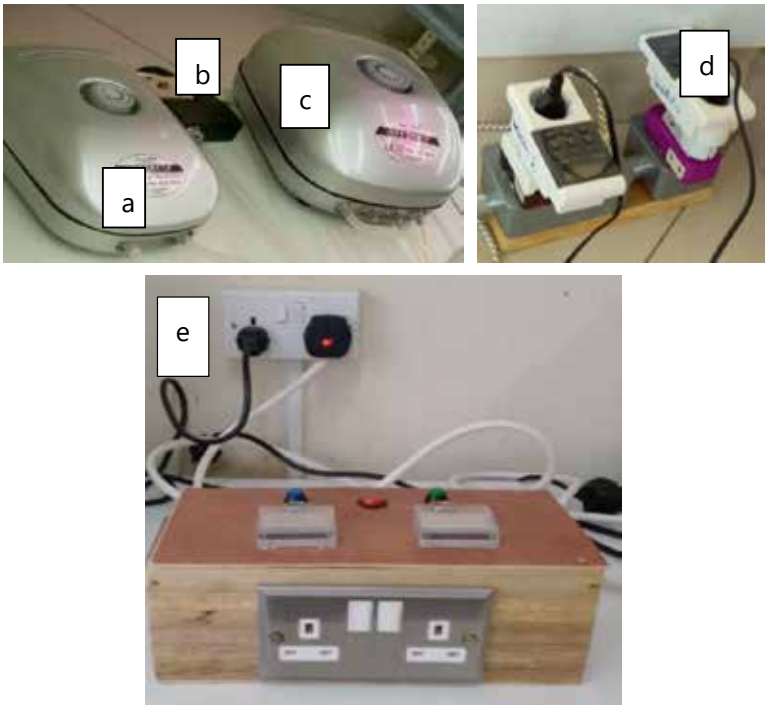

Plate 19. Automation Components of Plant Form Temporary Immersion Bioreactor.
a: Pump for nutrient immersion; b: Electric volt; c: Pump for ventilation; d: Timers for setting immersion and ventilation frequencies; e: Locally designed perpetual timer also used for the Plant Form TIBS

Plate 20. Connecting the parts. Inserting the silicon ring into the lid to ensure air-tightness (Top Left); Silicon tube on the inner chamber to be placed on the bottom of the outer container (Top Right); Bottom left: Fixed connectors into the inlets by inserting clamp on the outer container, putting silicon ring on the clamp from the other side and screwing the nut on the clamp. Bottom Right: Inserting four-legged frame above the basket

Plate 21. Setting up PF TIBS for plant introduction. Top, left: Closure of only two sides of the vessel before autoclaving; Top, right: Placement the PF TIBS into the autoclave for sterilization; Bottom right: Sterilized vessel showing “autoclaved’ indicator. Bottom right: sterilized filters connected to the tubing in the flow hood

Plate 22. Culture of plant into Plant Form TIBS. Top left: Introduction of yam nodes into autoclaved vessel in the laminar flow hood; Top right: Sealing; Bottom: Labeled vessel containing plants.

Plate 23. Addition of new cultures to existing ones on the shelf. Top left: Removal of stopper or binding rubber; Top right: Connecting white tubing to middle filter for immersion and green for ventilation. Bottom left: set up showing air and medium pump connected to tubes; Bottom right: Pumps connected to set timers.

Plate 24. One Plant Form vessel containing full-grown yam plantlets

Table 1. Cleaning the Plant Form bioreactor vessels.

Steps	Non-contaminatedvessels	Contaminated vessels
1	• Rinse in hot water	Soak in 2% sodium hypochlorite (Equivalent to 72% commercial bleach) solution for one hour
2	Dry in oven	Dry in oven
3	Autoclave TIBS vessel only with medium	Autoclave TIBS vessel only with medium
4	For autoclaving, wrap filters in foil before putting in plastic	For autoclaving, wrap filters in foil before putting in plastic
5	Allow to dry	Allow to dry
6		Dry

Note: The best is not to use any detergent for the bioreactor, only hot water. If you must, then use a neutral pH detergent.

The medium should be changed every 4 weeks and the vessel cleaned before the next use (Table 1). This is because the pH reduces over time and the plants stop growing vigorously. The basket containing the plantlets should be lifted and transferred into a freshly prepared medium in TIBS inside the laminar flow hood. Mark each air filter with a single stroke using a permanent marker after each use. Change the filters after six uses to prevent clogging and contamination outbreak.

Maintaining sterile conditions

Sterile conditions must be maintained within the culture vessel and in the laboratory environment (Appendix 11). 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 regular use will depend on air cleanliness in each environment. In some cases, simple household insecticide is sprayed every week and surfaces wiped 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 gloves should be used when working at the laminar flow hood.

The cultures should be sealed with parafilm or kitchen cling film, labeled with the date and name of sample, and incubated in the culture room set at 25 + 2 ℃, 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 checked daily to discard contaminated ones. At about 7–9 weeks after culturing, depending on variety, if the plantlet vigor is high and there are no signs of senescence, they should either be subcultured into new vessels using about 1 cm single node cuttings or hardened for breeder seed yam production. Plate 25 summarizes how decisons are made on breeder seed production.

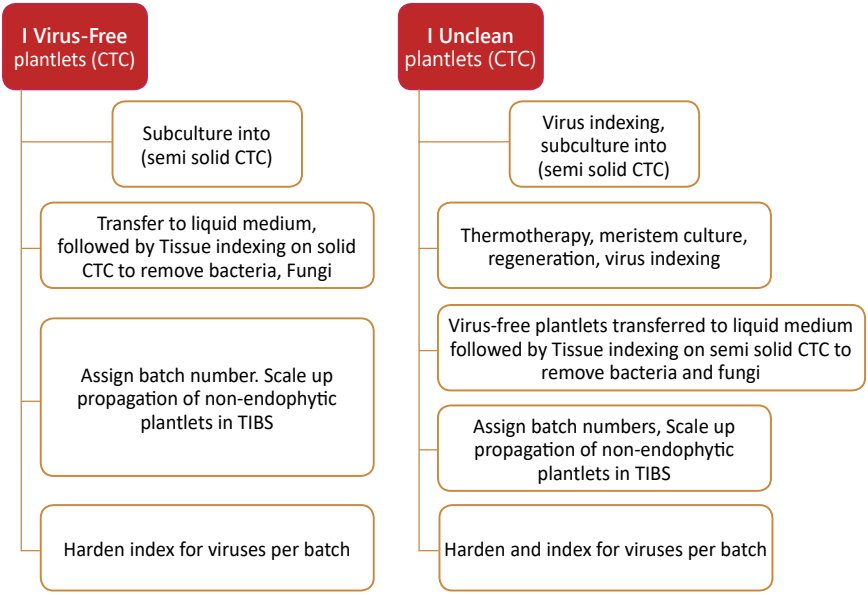

Plate 25. Decision making guide for generation and propagation of clean stock of Breeder yam planting materials

Post-flask management of plantlets

Hardening 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 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 an outside environment, however, humidity varies with season, especially in the tropics where yam is mostly grown; the temperature can be as high as 40℃ in February in the West African yam belt. Consequently, reducing RH in TC vessels before transplanting will reduce the shock due to water stress that causes wilting in the TC plants at hardening.

Using vented vessels or 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 improving nutrient uptake (Cassells and Walsh, 1994). Hardening is less demanding of a technician's time in bioreactor-grown plantlets because gas contacting is improved in bioreactor systems. At the same time, medium change (nutrient replenishment) is possible without plantlet manipulation (otherwise impossible in CTC).

Standard procedures should be followed (Appendix 12). 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 do yam genotypes due to evapotranspiration rates and tolerance to water stress. 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 aluminum shade nets should be used to achieve this. The optimum temperature is between 25 and 28 ℃.

The system can be adapted in different ways. The plantlets can be arranged on wooden or plastic trays and enclosed in plastic sheets. 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 transplanting success because of varying weather (temperature and humidity) conditions. It is very important that the breeder seed producer understands these fluctuations to adjust post-flask management strategies. For example, February is usually hottest (34 ℃) in Ibadan, Nigeria, while June/July is coldest (28/26 ℃). 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 bottoms or customized transplanting trays containing sterile topsoil (after 60 minutes of steam sterilization at 100 degrees Celsius) mixed with carbonized rice husk (Plates 26–27) in equal volumes or other commercial substrates.).

Plate 26. Making carbonized rice husk. Top row: Make a fire on a metal sheet using charcoal and allow the fire to go out but keep the glowing hot coals. Down, left: Cover with carbonizer; pour rice husk and allow to burn. Down, right: Incomplete burning will give black carbonized husk while complete burning will give whitish ash rice husk. The hotter the carbonizer, the closer to becoming ash. Both are sterile and work well for yam.

Plate 27. Mixture of CRH with sterilized topsoil is filled into plastic seedling bags to harden plantlets from tissue culture.

Plantlets should be immersed in a solution of 7 g/l Team (4 g/l mancozeb + 0.8 g/l carbendazim) followed by transfer into the prepared substrate and covering with a transparent plastic sheet (Plate 28). Vessels that are vented 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.

Plate 28. Hardening TIBS plantlets in basket humidity chambers.

The first vent is opened on the third day after de-flasking while other vents are opened at one-day intervals until all the five 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 third week. The plantlets can be subcultured using two-node cuttings to obtain taller plants within a shorter time before hardening.

Plate 29. Plantlets being hardened in vented vessels.

Use of Vivipak

Another successful approach is using a customized ViVipak (Plate 30, Appendix 13) vessel (ViviTECH, Appendix 11). It is a gamma-sterilized disposable vessel containing a tray with ready-to-use, patented sterile substrate 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 while making packaging and transportation easier.

However, due to border issues, Covid-19, and increased importation costs, the YIIFSWA II project developed an alternative substrate mix for the ViVipak trays and this has been used effectively for post-flask handling. The steps involved are shown in Plates 31–34.

After two to three weeks, the plantlets are transferred into trays (Plate 35) or pots (Plate 36).

Plate 30. Yam plantlets from PF TIBS in ViVipak (ViFoil removed) ready for potting.

Plate 31. Left: Filling the ViVipak trays with slightly wet cocopeat substrate; right: stacking the trays for autoclaving.

Plate 32. Stacked ViVipak trays for autoclaving (left). Two bottles are autoclaved along with the substrate (right). The first bottle contains 5.43 g of Murashige and Skoog basal medium and 1 liter of distilled water set to pH of 5.7. The second bottle contains 5.4 3g of MS + 7 g of Agar + 1 liter of distilled water, pH of 5.7.

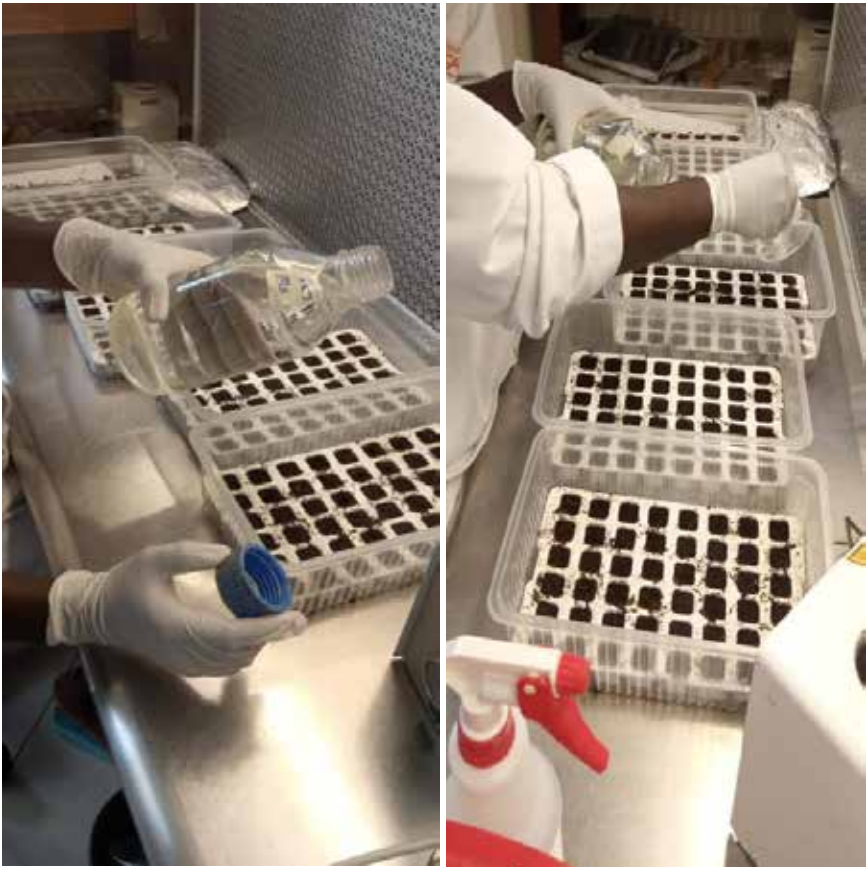

Plate 33. Addition of autoclaved MS_Agar (Left) and MS_distilled water (right) to the autoclaved substrate in the Laminar flow hood before it solidifies. The substrate to which MS_Agar water has been added is allowed to cool, then the substrates bind together due to the presence of agar in the mixture. One liter of the mixture is used for four ViVipak trays. After cooling and binding of the substrate, MS + distilled water is added (100 ml for each tray).

Plate 34. Subculturing into ViVipaks.

Management of hardened plantlets to produce 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 or hydroponics (Maroya et al. 2014b, Balogun et al. 2020) 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; and a source of micronutrients, especially when leaves are showing yellowness. The plants should be watered as necessary.

Breeder tubers weighing 50–200 g were harvested from hardened, potted plants 4–5 months after potting. Single node vine cuttings (Plate 35) were also generated from vigorously growing hardened plantlets at 1:4 multiplication ratio and rooted (Plate 36) for further clean seed yam tuber production. Harvested tubers (Plate 37) 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 35. Successfully hardened plantlets in trays.

Plate 36. Potted plantlets from PF TIBS (Top); single node vine cuttings from plantlets potted in PF TIBS (Bottom).

Plate 37. Rooted single node vine cuttings from potted PF TIBS plantlets (Top); Breeder seed yam tuber of PF TIBS plantlets from hydroponics (Bottom).

Documentation in Breeder seed yam tuber production

Production and labeling

Inadequate documentation can cause serious problems between breeder 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 CTC or the bioreactor system.

The best is to have a documentation gadget for each staff member. These can be notebooks or tabs. Each plant should be traceable on the Excel sheet (Plate 38) to a member of 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 to know if and when the propagation ratio starts reducing 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 vigor, which can also affect response to thermotherapy, and consequently, the health of the plantlets.

Labeling should be as automated as possible. The use of bar codes linked to Breeder stock mother plants in databases is encouraged. Bar codes (Plate 39) are machine-readable optical labels that contain information about the item to which it is attached. It should include date cultured, unique stock number, and variety name at the minimum. When changing vessels, labeling should be done before putting in plants to be doubly sure. Records should be backed up and saved online where there is less risk of data losses.

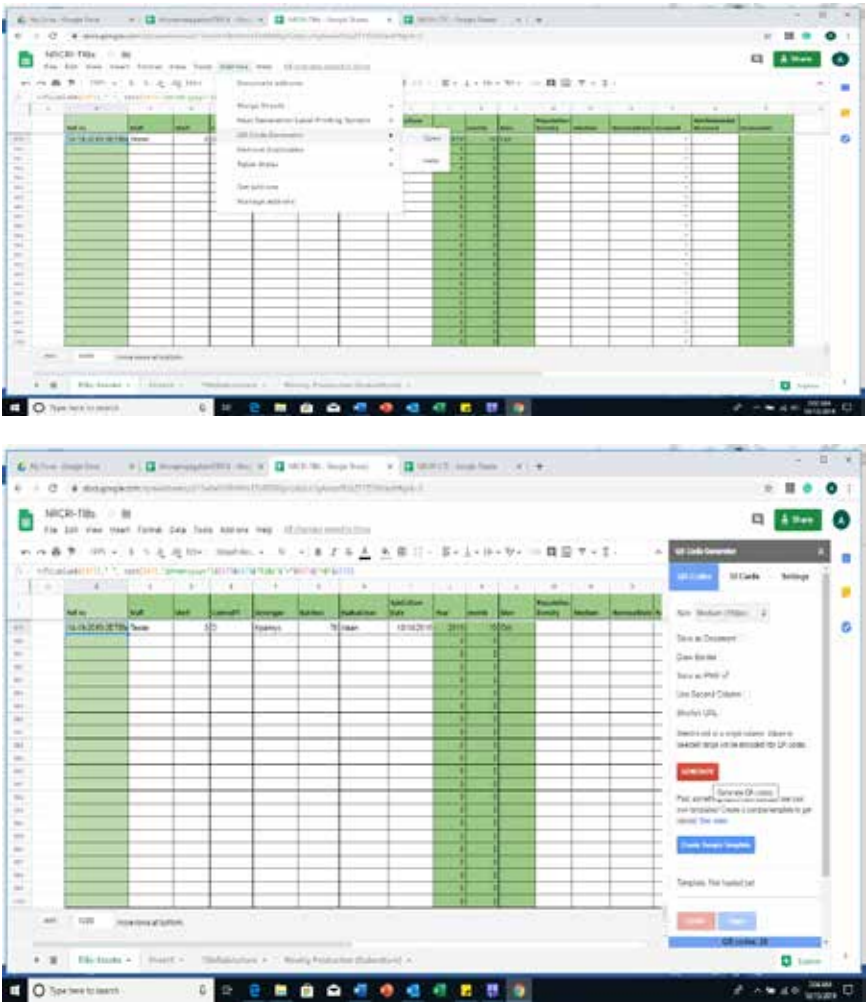

Plate 38. Generating bar code. Top: Selection of the Add On Menu, QR code generator, "open" and intended "Ref No", respectively on the Google documentation sheet; Bottom: Checked "save as PNG option", "Generate QR code" with new, blue button (Open Folder) leading to the location of the generated code in Google Drive.

Plate 39. Downloadable, printable bar code

Tracking cost of breeder seed production

Getting breeder planting materials into the hands of end-users usually involves setting prices. Consequently, all costs of production must be tracked and documented. The following aspects are considered:

1. The cost of the initial stock of plantlets: In the case of nucleus stock plantlet production in vitro, the plantlets are in either CTC or the PF TIBS. Hence the cost per plantlet is required as an input factor.
2. Cost of the nutrient medium used in the production: The number of plantlets produced is directly proportional to the volume of media consumed by the plantlets at production. Therefore, it is useful to know the cost of the medium per liter to derive the total cost of media for a production cycle or batch.
3. Equipment & supplies/consumables: The items in this category may require splitting over time because much equipment has a lifespan beyond the production cycle. The cost of such items will be based on the expected duration (depreciation value). Otherwise, the cost of the items may spike the production cost of the plantlets. Also, the cost of electricity, water, and laboratory bench space should be added in this category.
4. Human Resources: The staff involved in the production chain should be included, considering the different cadres and number of hours spent in production.

Challenges and Mitigation in breeder seed production

A major issue with breeder seed production in TC and TIBS is the control of contamination, especially in nucleus stock production, which reduces the quality. To mitigate this, documentation at the point of decision making with respect to clean/unclean stock plants and aseptic techniques in handling them should be strictly adhered to.

The second type of contamination is the presence of off-types, which reduce the genetic integrity as the varieties become mixed-up and are not true-to-type. To avoid this, proper labeling must be done at all stages. The handling of specific varieties should be separated by time and location. For example, if affordable and feasible, a particular day, personnel, culture room or screenhouse should be assigned to a particular variety.

Another challenge is the mismatch in demand and supply, as production is continuous in the laboratory while field planting by users is mostly seasonal. One way to handle this is to maintain only stock plantlets in the laboratory at off-peak demand periods. However, personnel retention is not guaranteed. Breeder seed producers should therefore develop facilities within their systems where microtubers produced from hardened plantlets can be stored until demanded in the cropping season. Research is still needed in seed yam tuber dormancy control beyond the normal 3-4 months.

Sometimes, large volumes are requested by clients within a short period. In this situation, breeder seed producers should establish partnerships to increase their overall capacity such that the responsible laboratory can sub-contract to others to accommodate large orders. YIIFSWA-II project is facilitating the exchange of information on demand for planting materials among NARIS to meet the demands of private seed companies.

Conclusion

There has been major progress in the use of Tissue Culture in general and Temporary Immersion Bioreactors in generating high-quality breeder seed yam, both seedlings and tubers. This is evident in the scale of production achieved over ten years of standardization and commercialization by YIIFSWA and YIIFSWA II projects, respectively. Further efforts are required in improving packaging and transportation of breeder planting materials locally and internationally at a reduced cost, local availability of TIBS components and extending storage time of breeder planting materials. Conventional Tissue Culture and Temporary Immersion Bioreactor system will further drive future seed research and production activities towards sustainable formal yam seed systems.

Appendix

Appendix 1. Items needed to produce 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	√				√
Topsoil	√			√	√
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 to produce 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	√	√	√		
Aquarium pumps			√		
Air filters			√		
Backup power (Inverter/ generator/solar)	√	√	√		
Plant Form Bioreactor vessels			√		
Hardening substrate				√	
Poultry manure or synthetic fertilizer*					√
Hardening vessels and/or misters (or white plastic sheets and baskets)				√	

Appendix 2. Some contacts to points of sale for materials for Breeder ((Breeder) 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 https://phytotechlab.com/
Plantform Bioreactors	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

Appendix 3. Step-by-step description to establish in vitro cultures from tubers.

1. Cut tubers that have broken dormancy into minisetts of 30–50 g 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 topsoil 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 7 g agar, 4.43 g MS with vitamins, Myo-inositol 0.1 g, Sucrose 30 g, 0.5 mg kinetin
9. Adjust pH to 5.7 $\pm$ 0.1
10. Dispense 10 ml per plantlet into test tubes or other vessels.
11. Autoclave at 103.4 kPa and 121 °C for 15 minutes.
12. Cut 1–2 cm long, single nodes from 2–3-month-old plants.

Surface-sterilize in:

13. 2 ml/l lambda-cyhalothrin + 7 g/l Team (4 g/l mancozeb + 0.8 g/l carbendazim) for 10 minutes
14. 70% ethanol 5 minutes
15. 2% sodium hypochlorite solution for 15.
16. 1% sodium hypochlorite 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 + 2 ℃, 4,000 lux of light
20. from cool white fluorescent tubes or LED bulbs at 16-h photoperiod.

Appendix 4. Handling of Plant Growth Regulators

	Mol. Wt	Equivalent of µM for 1mg/L	Solvent	Powder ST	Liquid ST	Sterilization method
Indole-3-butyric acid (IBA)	203.2	4.90	EtOH/1N NaOH	2–8℃	–0℃	CA/F
Naphthalene-acetic acid (NAA)	186.2	5.37	1N NaOH	RT	2–8℃	CA
2,4-dichlorophe-noxy-acetic acid (2,4-D)	221	4.53	EtOH	RT	2–8℃	CA
3,6-dichloro-2-meth-oxy-benzoic acid (Dicamba)	221.0	4.52	EtOH/ Water	2–8℃	2–8℃	F
Indole-3-acetic acid (IAA)	175.2	5.71	EtOH/1N NaOH	–0℃	–0℃	CA/F
Kinetin	215.2	4.65	1N NaOH	–0℃	–0℃	CA/F
6-Benzylaminopurine	225.3	4.44	1N NaOH	RT	2–8℃	CA/F
6-(gamma,gam-ma-Dimethylallylami-no) purine (2iP)	203.2	4.92	1N NaOH	-0℃	-0℃	CA/F
Giberellic acid	346.4	2.89	EtOH	RT	2–8℃	CA/F
Jasmonic acid	210.3	4.76	EtOH	2–8℃	–0℃	F
Abscisic acid	264.3	3.78	1N NaOH	–0℃	–0℃	CA/F
Uniconazole-p*	291.78		*	RT	2–8℃	CA/F

*For Uniconazole-p, prepare 1 mg/ml by weighing 100 mg, dissolve (Water, methanol, DMSO, Chloroform, Acetone) and make up to 100 ml. Label as 1 mg/ml. Take 10 ml of this solution and make up to 100 ml to give 0.1 mg/ml stock solution. Use required volume as determined by the final concentration desired in 1 liter of medium; RT: Room Temperature. Diluent is water for all.

Appendix 5. Internal tracking record sheet for stock plantlets.
Responsible Breeder seed production staff name:
Mobile phone no. Date submitted for indexing:
Received at Germplasm Health staff by:

SN on Out-bound sheet	Variety	Location of collection	Replicate	Label on vessel	Source (TC, AS, HTM, TUB, VC, HP)
			1		
			2		
			1		
			2		
			1		
			2		
			1		
			2		

TC: Tissue culture; AS: Aeroponics; TUB: Tuber; HTM: Heat-treated meristem; VC: Vine cutting; HP: Hardened plantlets.

Appendix 6. Out-bound virus indexing sheet.

Responsible HRP staff name: Mobile phone no.:

Date submitted for indexing: Received at GHU by:

S/ No.	Variety	In vitro/Ex vitro	Virus indexing Result

Appendix 7. Decision sheet for stock plantlets for TIBS.

Responsible staff name: Mobile phone no.:

Name of variety: Start date of indexing:

Date scored:

Test tube No.	Label on vessel	Petri dish No.	Bacteria	Fungi	Virus	Virus type	Recommend for TIBS?	New Batch no
1		1	-	-	-	-	Yes	1
		2	+	+	+	YMV	No	NA
2		1	-	+	+	YMMV	No	NA
		2	-	-	+	BADNA	Yes*	2
3		1	-	+	-	-	No	
		2	+	-	-	-	No	
4		1	+	+	-	-	No	

*Only If there is no other stock of the variety without Badna, otherwise, conserve as a backup.

NA: Not applicable

Appendix 8. Preparation of meristem culture medium (500 ml) containing Uniconazole-P.

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

Appendix 9. Preparation of stock solutions of Plant Growth Regulators
To prepare 1 mg/ml stock solution:

1. Add 100 mg of the plant growth regulator to a 100 ml glass container.
2. Add 2-5 ml of solvent to dissolve the powder.
3. When completely dissolved, make up to 100 ml volume with double distilled water.
4. Stir the solution while adding water to keep the material in the solution. Store the stock solution as recommended in the tables.
5. Use 1.0 ml of the stock solution to 1 liter of medium to obtain a final concentration of 1.0 mg of the plant growth regulator per liter of culture medium. (See Appendix 4)

$$\text{Volume of Stock Solution} = \frac{(\text{Desired Hormone Conc.} \times \text{Medium Volume})}{\text{Stock Solution Conc.}}$$

Appendix 10. Step-by-step procedure for multiplication in Conventional Tissue Culture.

1. Dissolve 4.43 g 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 0.1 g of Myo-Inositol
4. Add 1 g of activated charcoal
5. Prepare 50 ml of 0.1 mg/ml Kinetin solution by dissolving in HCl. Add 5 ml 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 7 g 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 into vessel (test tubes, plastics, baby food jars, etc) at rate of 10 ml per explant
11. Place the vessels (with lids sitting on the tubes but not completely tightened) in an autoclave and sterilize at 103.4 KPa and 121 ℃ 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 11. 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
4. following manufacturer's manual
5. Clean the laboratory floor with disinfectant solutions (e.g.,
6. 2.5% sodium hypochlorite)
7. Wear laboratory overalls, indoor footwear, and head coverings
8. Nose masks should be used when working at the laminar flow hood and no verbal communication in the laminar flow hood.
9. Vessels containing explants should be sealed with parafilm or kitchen cling films.
10. Check for contaminated cultures and discard daily.
11. At highly humid environments or seasons, desiccants can be placed at strategic places to absorb moisture.
12. The influx of visitors to the culture rooms must be reduced if completely unavoidable.

Appendix 12. 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 topsoil wrapped in sacks for 30–60 minutes and bring out to cool
3. Mix equal volume of topsoil 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 a solution of 7 g/l Team (4 g/l mancozeb + 0.8 g/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 the 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 13. 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 pre-mixed substrate in the laminar flow hood using sterile dissecting instruments
4. Incubate in screenhouse (cool season) or laboratory growth room (hot weather)
5. After 3 weeks, transplant into pots containing sterile soil or increase substrate volume by adding under the ViVipak trays.
6. Where ambient temperature is above 29 ℃, misters should be used during the first 12 days of hardening.

Appendix 14. Summary of the 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%

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