

Novelty, rapidity and quality in seed yam production: the case of Temporary Immersion Bioreactors

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Cover photo: Bioscience technician monitoring airflow within the bioreactor system.

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Acronyms:

YIIFSWA: Yam Improvement for Income and Food Security in West Africa project

TIBs: Temporary Immersion Bioreactor System

CPTC: Conventional Plant Tissue Culture

RITA: Recipient for Automated Temporary Immersion

BIB: Bioreactor of Immersion by Bubbles

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Summary

As part of the strategic goal of improving yam seed systems at a fast rate, YIFSWA is developing protocols for the production of quality seed yam using Temporary Immersion Bioreactors (TIBs). The latter is new generation tissue culture technology which, for a long time, had found application in the scale-up of the production of secondary metabolites from cell and suspension cultures; different types now exist which are adapted to whole plant cultures. The system immerses plants intermittently in a liquid nutrient in a sterile environment, in contrast with conventional tissue culture where there is continuous immersion, so as to increase propagation rates. The SETIS, twin-flask types of 128 units, were installed by YIFSWA in September 2013 at IITA's Ibadan station. Prior to YIFSWA, most of the work on the application of TIBs in yam was on *D. alata* (water yam). At IITA, emphasis is on *D. rotundata* (white yam) – the preferred species in the West African yam belt. Our research is exploring the potentials for both plantlet and yam microtuber production in TIBs to facilitate the production of high-quality pre-basic seed yam from which healthy basic and certified seed yam will be produced. We are optimizing performance, based on medium composition, immersion frequency, genotype differences, light intensity and quality, and post-flask management while also building capacity in the public and private sectors. Relative to CPTC, the quality of seeds produced was significantly improved due to the elimination of endophytes; the propagation ratio was doubled and post-flask losses were reduced by 200%. The TIBs technology is a viable business venture for seed yam production and a strategic objective to fast-track yam genetic improvement, both of which will facilitate the evolution of the formal yam seed system in the shortest possible time.

Introduction

Conventional Plant Tissue Culture (CPTC) technology involves the culture/growth of small plant parts in laboratory containers such as test tubes in a nutrient mix (medium) to regenerate the complete plant (called plantlets). The technology is ideal for crops with a long growth cycle, those with hard-to-germinate seeds (dormant), those with low propagation rates or those that lose viability easily (recalcitrant). The technology itself has the advantages of a controlled laboratory environment, not susceptible to changing weather conditions, so that production cycles can be planned. Clean, high-quality and uniform plants are produced (Yam and Arditti 2009) from otherwise infected mother plants because small uninfected plant parts are cultured.

The CPTC technology has found application in being capable of producing disease-free plantlets. This is because yam propagation is slow (Balogun and Gueye 2013) and vegetative (less than 1:10 compared with 1:300 in some cereals) which also encourages a build-up of diseases, especially within the existing informal seed system, causing significant yield losses. The slow rate of propagation also does not facilitate genetic improvement owing to the limited number of plants produced per year on which selection is based.

However, although CPTC was rapid, meristem cultures took between 6 and 24 months (Adeyemi, personal communication) in this system. In addition, a long time is required for acclimatization and losses at transplanting are also high when environmental control is minimal. Other tissue culture technologies which will fast-track genetic improvement in yam, such as embryo rescue and somatic embryogenesis, are yet to be perfected for economically important yam genotypes. This slow response of yam in CPTC, relative to other crops such as cassava and potato limits the use of *in vitro*-produced, virus-tested plantlets. There is also a limited application of biotechnological techniques such as genetic transformation, haploid plant production and embryo rescue in yam breeding (Balogun and Gueye 2013). In addition, frequent subculturing in conventional tissue culture increases labor costs while the small size of the culture container (hence the amount of nutrients) and insufficient aeration (Ziv 1991) result in fragile plantlets (Ziv *et al.* 1998) and high losses at transplanting. The need to improve on these systems witnessed the emergence of the bioreactor technology.

Temporary Immersion Bioreactor Systems

Temporary Immersion Bioreactor (TIB) technology (Adelberg and Simpson 2002) is a propagation system that grows plants rapidly by immersing them intermittently in liquid nutrients in sterile laboratory containers (bioreactors). The system is propelled by air flow under pressure. In temporary immersion, the cultures are immersed in the medium for a pre-set duration at specified intervals. Their construction and operation are very simple, which has made them attractive low-cost alternatives. A typical design uses two vessels (plastic or glass), one of which holds the liquid medium and the other the cultures. The TIB system is new generation tissue culture technology, and the timed immersion of plant tissues in liquid medium allows for the aeration of cultures. Each unit is a bioreactor – an enclosed sterile laboratory environment – provided with inlets and outlets for air flow under pressure. This circumvents the limitations associated with conventional tissue culture. In most crops tested (pineapple, cocoa, potato) TIBs increased propagation rates. Another version of

TIBs includes a system where a single vessel with a reservoir on one side is mechanically tilted at pre-set intervals (Adelberg and Simpson 2002). In this manner, the medium periodically bathes the cultures in the vessel and maintains the propagules in a vertical position.

For a long time, bioreactors had been used in scaling up the production of plant secondary metabolites, including those that are of medicinal or health value to humans, using cell suspension cultures. These include flavonoids, phenolic acids, digitoxin and the anticancer substance Taxol, from the Pacific yew tree (*Taxus* sp.). Such suspension cultures were grown in stirred tank bioreactors (Srinivasan *et al.* 1995). Later, a diversity of bioreactors was developed to accommodate the culture of whole plants which are sensitive to shear stress. These are airlifts and bubble columns, the rocking bioreactor for the cultivation of differentiated plants *in vitro* systems (Steingroewer *et al.* 2013), including liquid-phase (stirred tank, airlift and connective flow bioreactors), gas-phase, hybrid bioreactors, and TIBs. The gaseous phase bioreactors are composed of cultures mechanically supported on a porous base and intermittently sprayed with medium (Ushiyama 1988) or exposed to a nutrient mist (Weathers *et al.* 1988). Excess medium is directed in the vessel and re-circulated. These bioreactors can provide excellent growth and development for most tissue and organ cultures.

In the liquid layer bioreactors, only the base of cultures is exposed to the medium. The control of illumination, temperature and the gaseous environment is much the same as in standard tissue culture vessels. Stationary support systems for liquid layer bioreactors have been also developed from sealed clear plastic film with a wire frame (Takayama *et al.*, 1991). Small, plastic films are also being used instead of vessels in commercial laboratories.

Other TIBs use different designs in vessels and rotation. As the vessel turns, the culture is intermittently dipped in the medium. TIBs are simple and cost-effective to run. They are uniquely able to provide a lower level of shear stress and significantly reduce shoot hyperhydricity, culminating in increased productivity. The recipient for automated temporary immersion (RITA) (Alvard *et al.* 1993) is another type of TIBs in which the upper container containing the plant is linked to the lower compartment containing the medium and internal pressure regulates the movement of medium up or down in such a way that the immersion of cultures can be timed. There is also the bioreactor of immersion by bubbles (BIB) (Soccol *et al.* 2008) where the nutrient and air are provided to cultures by bubbling. Others are the glass jar TIB and the Box-in-Bag bioreactor which were successfully applied for the cultivation of *Coffea arabica* L. (Ducos *et al.* 2008). In all these cases, the cultures are immersed in the medium in a timed manner, in terms of frequency and duration of immersion, to allow for aeration.

As part of its objective to develop novel technologies for the high ratio propagation of high quality seed yam, the Gates-funded YIIFSWA project is developing protocols for producing seed yam using conventional tissue culture, aeroponics and TIB technologies. The use of aeroponics – growing yam in soil-free, mist nutrient – has been demonstrated (Maroya *et al.* 2014a, 2014b).

Advantages and disadvantages

The advantages of bioreactors include an increased culture multiplication rate, faster culture growth, a reduction in medium cost and also in energy, labor and laboratory space. The increased rate of multiplication and growth primarily reflects the effect of a liquid medium (Levin *et al.* 1997). The elimination of gelling agents (e.g., agar) reduces medium cost. In bioreactors, the culture density in

liquid media is much higher than in the conventional vessels with semisolid media. The conventional tissue culture vessels are typically kept on shelves with a large space between the shelves. The use of bioreactors requires a much smaller space in the growth room, fewer clean work stations (laminar flow hood), and less space for media preparation, vessel storage and washing than in the CPTC. The smaller size of the laboratory and the lower number of people reduce airconditioning needs, hence energy costs. Reduced requirements for lighting and labor, the simplification of medium preparation, reduced washing of vessels and easier handling of the cultures all lead to cost reduction.

There are, however, many disadvantages because of a number of problems associated with the use of bioreactors in micro-propagation. These include contamination, a lack of protocols and production procedures, problems of foaming, shear stress and the release of growth-inhibiting compounds by the cultures. Unfortunately, culture contamination, which is a major problem in conventional commercial micro-propagation (Leifert and Waites 1990; Leifert and Woodward 1998; Leifert 2000), is even more acute in bioreactors. In conventional micro-propagation, discarding a small number of contaminated vessels is an acceptable loss; in bioreactors, even a single contaminated unit is a huge loss. However, despite these difficulties, commercial laboratories have developed effective procedures to control contamination in bioreactors.

Control of contamination

Prevention of contamination in bioreactors requires the proper handling of the plant materials, of equipment during transfers, and of cultures during production. Only the surface-sterilized explants, multiplied in small vessels and indexed for freedom from endophytes, are used to initiate cultures in bioreactors. If the bioreactor is small, it is sterilized in an autoclavable plastic bag, sealed with a cotton wool plug, and opened only under the laminar flow cabinet. If the bioreactor is large, other methods must be provided to protect the vessel after autoclaving and during its transport to the sterile area. The vessel is assembled under laminar flow while care is taken that non-sterile objects do not enter the sterile air stream and the vicinity of open ports. After inoculation, all the ports are sealed and the bioreactor is brought to the growth room and connected to an air supply. For harvesting, the bioreactor is returned to the sterile area and the ports are opened so that the air stream cannot carry contamination from the outside. If proper procedures are followed, the bioreactor can be reused without re-sterilization.

Despite the precautions taken in initiating cultures, bioreactors can become contaminated from the environment or from microbes latent in the culture. The contamination can be controlled with the use of one or a combination of antimicrobial compounds, acidification of the media, and micro-filtration of the medium. Acidification of the medium (pH 3) has been used to control contamination in the multiplication of banana and in media for other crops (Leifert *et al.* 1994). Contamination can also be controlled by circulation of the medium through a 0.2 μ microporous filter (Levin *et al.* 1996). However, the removal of contaminants needs frequent changes of filter which is too expensive. Chilling the filter significantly retards filter clogging.

The formal seed system

The formal seed system is about quality control and certification at all stages of seed yam production (Balogun *et al.* 2014), and a gradual increase in the quantity of pre-basic to basic seeds and then

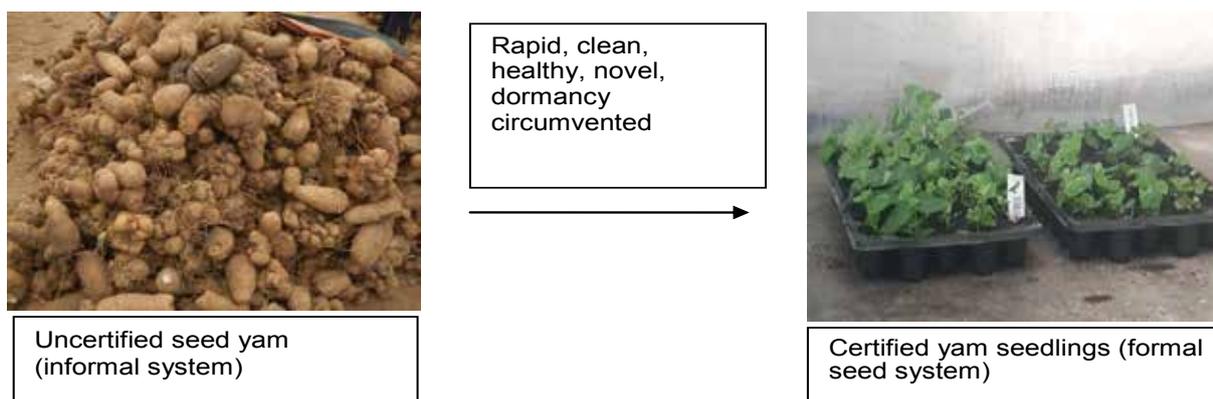


Fig. 1. Left: Seed yam in the informal seed system; Right: Seed yam in the formal seed system.

certified seeds that can be commercialized to farmers. The introduction of the formal seed system will require novel technologies, especially in terms of rapidity of production, uniformity, quality control and certification of CLEAN (healthy) pre-basic/basic and commercial seed yam in large quantities. Such rapidity is offered by the automation in the TIB technology.

The choice of SETIS™ bioreactors

The goal of producing the highest quality seed yam in the shortest possible time prompted the YIIFSWA team to consider a number of bioreactor types. The aim is to utilize bioreactors to produce plantlets at any time of the year in addition to the production of microtubers; this circumvents acclimatization and has a storage advantage. In September 2012, with a back-up of 28 units, 128 units of the SETIS™ bioreactors were installed. This type of bioreactor utilizes compressed air to move the liquid medium from the medium reservoir up to the plant vessel but employs the force of gravity to return the medium into the medium vessel; this saves a lot of energy and ensures complete drainage. In addition, the large size of the SETIS™ type TIBs will accommodate reasonable production of good-sized minitubers when protocols are successful.

One of the most beautiful components of the SETIS type bioreactor is the provision for ventilation, i.e., the air in the head space is changed at regular intervals, simulating a field or screenhouse environment. Also, the facility has a programmable logic control which is internet-ready for feeding and ventilation but which can also be made manual or semi-manual and so take care of the private sector interest in low-cost options.



Fig. 2. Left: The Director-General of IITA, Dr.N. Sanginga, visiting the IITA Bioreactor System; Right: Cross-section of 128 units of SETIS bioreactors at IITA.

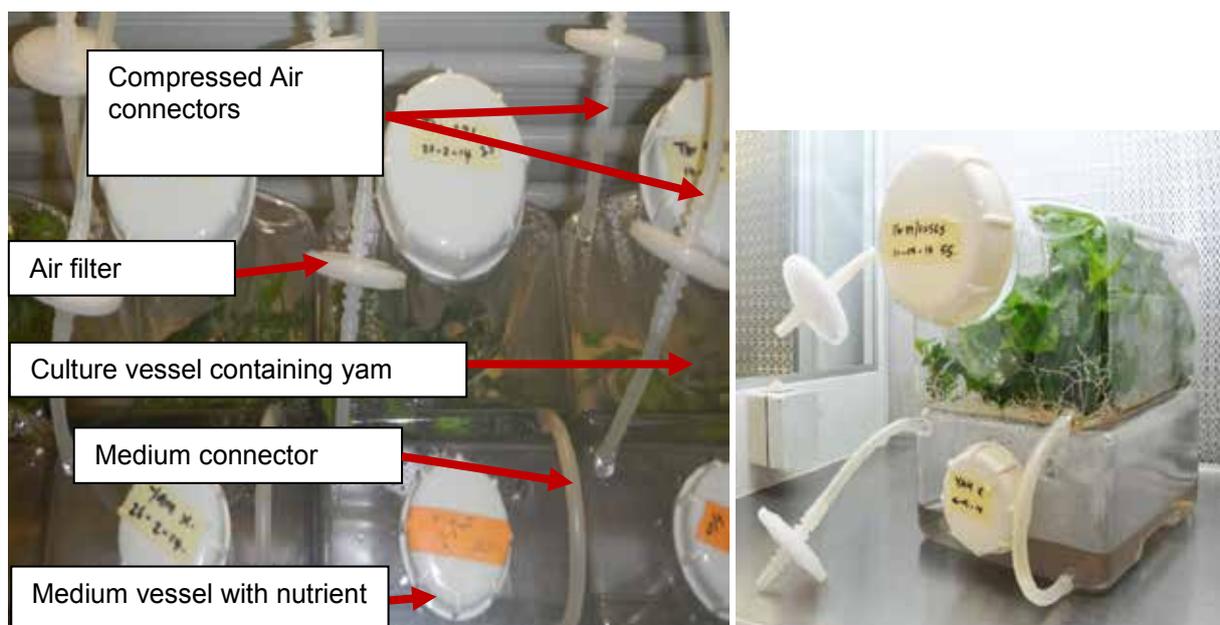


Fig. 3. Different components of SETIS™ TIBs at left and one unit with plants at right.

Initial experience

In vitro plantlets of the variety Pona were used in a first trial run and shoot vigor at 6 hourly immersions in medium increased relative to conventional medium in 4 weeks. However, contamination was a great challenge due to the reemergence of microbial endophytes and all 89 plantlets of Pona first introduced were lost to contamination. We therefore focused on in-depth indexing for endophytes before introduction into bioreactors and this improved the quality of the seed yam produced as they are free of fungi and bacteria in addition to viruses. Our findings suggest that the landraces harbor more endophytes than the improved varieties although research is ongoing to confirm this.



Fig. 4. Left: Apparently clean plantlet in CPTC; Right: The same plantlet positive at bacteria endophyte indexing step.

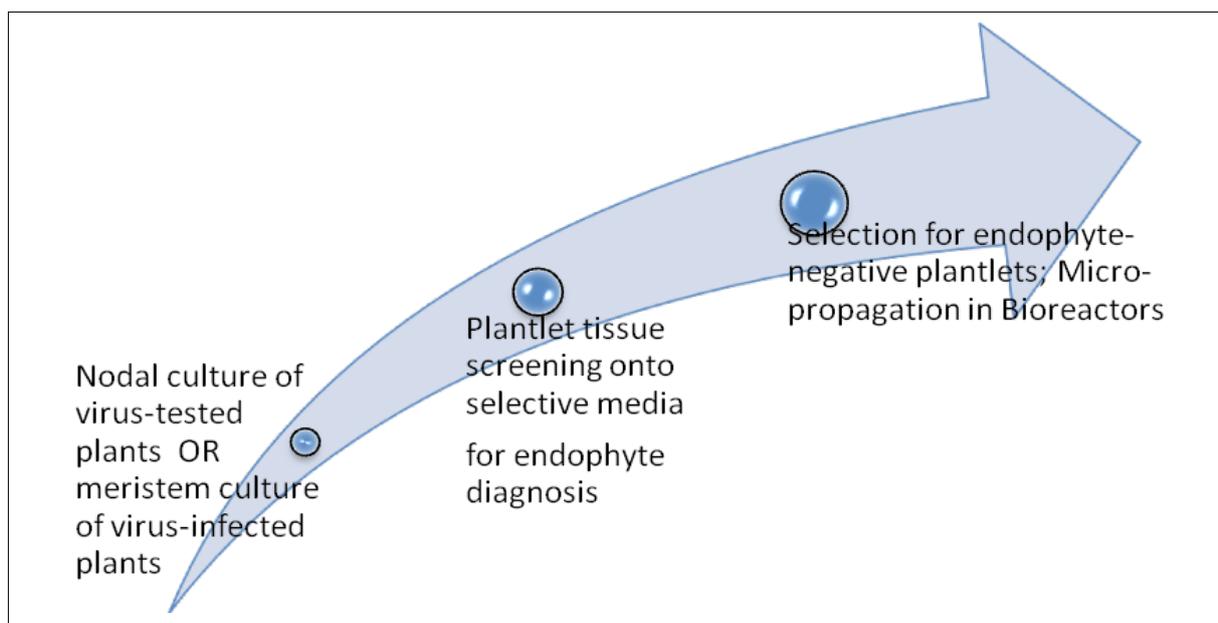


Fig. 5. Current healthy seed yam production procedure using TIBs.

Current scenario

The procedure in current use for the generation of clean seeds is shown in Figure 5. Mother plants growing in an aphid-proof screen house are virus-tested and free stocks are introduced in CPTC. Thereafter, tissues are screened on to endophyte-indexing medium after which clean tissues are introduced into TIBs.

So far, 3 genotypes of *D. alata* and 8 of *D. rotundata* have been successfully grown in the TIBs. In a trial involving 6 genotypes at a population density of 50 nodes per bioreactor and within an 8-week period, the highest number of nodes obtained from 1 initial *in vitro* node ranged from 8 in *D. rotundata* clone TDr 95/19158 to 14 in *D. alata* clone TDa 98/01167 with a mean of 8.7 across genotypes. Values that are higher or lower can be obtained depending on genotype, population density and nutrient immersion frequency. This means that if contamination is efficiently controlled and labor is available, up to 31,000 seedlings can be produced from 1 node per year in comparison with 194 in CPTC. YIIFSWA has now gone up to 100 plantlets per bioreactor, and research finding is showing that this can be increased.



Fig. 6. Left: 1 bioreactor of 50 plantlets; Middle: one plantlet from the bioreactor; Right: 13 nodes from 1 plantlet. One grown bioreactor can make 6 new bioreactors.

Savings on space requirement

In terms of the laboratory space required, CPTC requires ten times more than the bioreactors (Table 1). This is because in the TIBs, with dimensions 30.8 × 18.0 × 15.5 cm³, we have reached 100 plantlets per bioreactor with higher vigor.

In the CPTC in a rack (40 test tubes) with dimensions 30.0 × 12.0 × 15.5 cm³ 40 plantlets are accommodated.

Table 1. Some advantages of producing seed yam using TIBs technology.

Factor	CPTC	SETIS™ TIB technology
Laboratory space occupied by one plantlet	139.0 cm ³	13.8 cm ³
Average number of nodes in 8 weeks (multiplication ratio)	4	8
Time required from 1 <i>in vitro</i> node to acclimatized plantlet	70 days (<i>in vitro</i> growth) + 28 days (hardening) = 98 days	56 days (<i>in vitro</i> growth) + 7-14 days (hardening) = 70 days
Number of cycles per year of 365 days	3.7 (approx. 4)	5.2 (approx. 5)
Number of seedlings per year (365 days) =ratio ^{cycle}	4 ⁴ = 256	8 ⁵ =32,768
Percentage survival at direct transplanting	24.1	94.6
Actual number of seedlings (less losses)	194	31,000

More rugged post-flask management

One of the most important advantages of TIBs is in post-flask management of plantlets. In CPTC, hardening takes a minimum of 3 weeks in a temperature- and humidity-controlled environment. In TIBs, the plantlets are so vigorous that they survived direct transplanting in the screen house and in aeroponics without air conditioners and humidifiers. A 200% increase in survival at transplanting was recorded and a time-saving of 3 weeks per production cycle, depending on genotype.

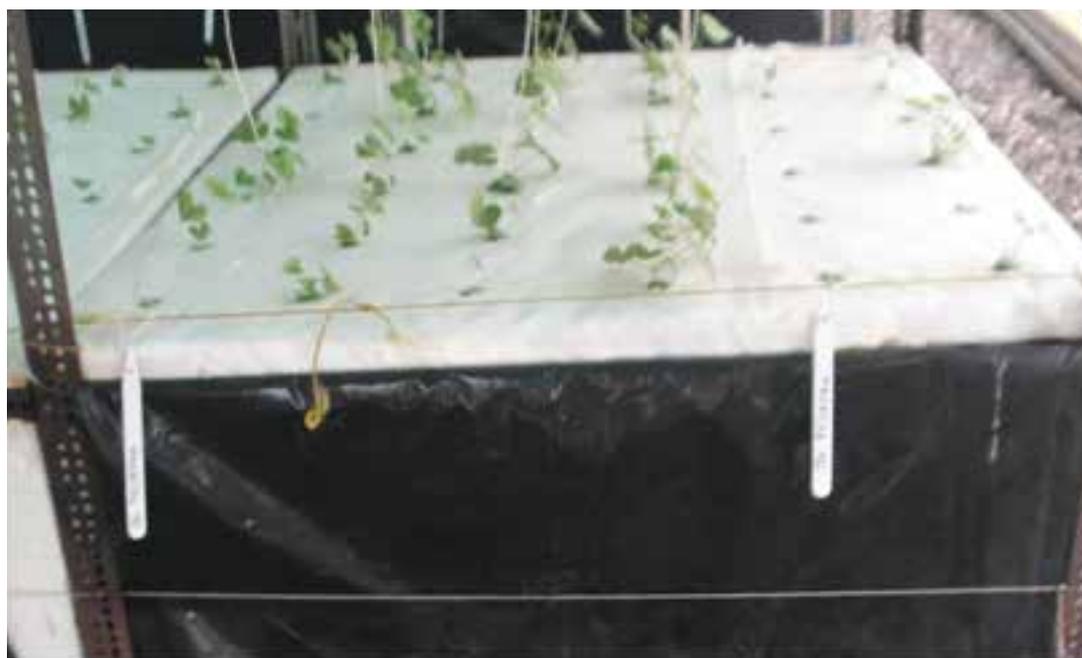


Fig. 7. Comparison of direct planting of plants from bioreactor and from CPTC in aeroponics. Two rows on the far right: CPTC plantlets; Other rows: TIB plantlets.

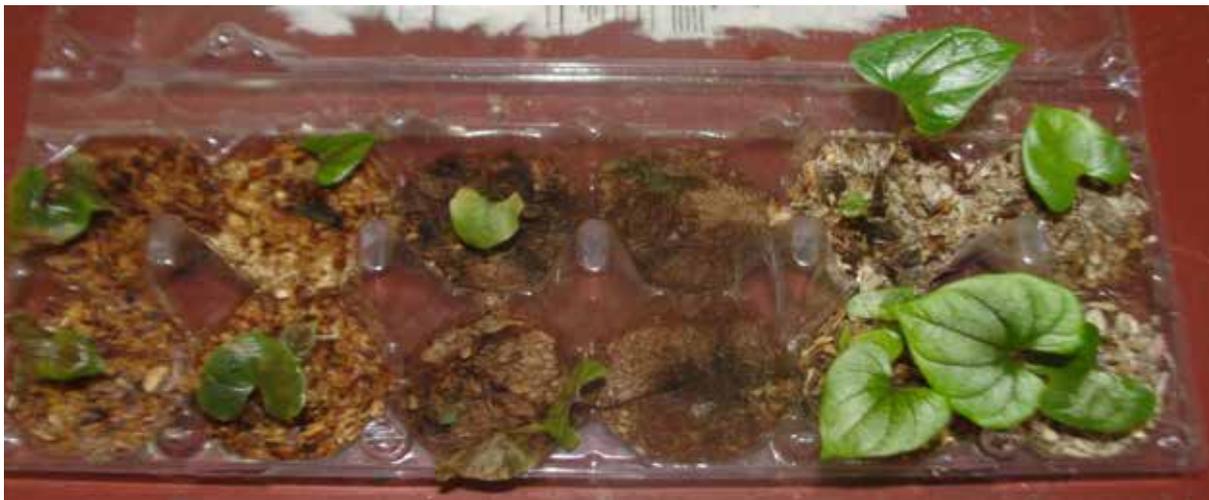


Fig. 8. Top: Plantlets at 2-node stage in kenaf substrate 1 week after transplanting. Middle and down: Acclimatized plantlets 3 weeks later. Left: Kenaf c"ore; Middle: kenaf mix, Right: kenaf bast.

YIIFSWA is also looking at alternative (better aerated, water retaining and nutrient-rich) substrates that are organic and can support transplanting. We tried milled stems (Balogun and Raji 2014) of the kenaf plant (core, bast and a mixture of core and bast) which are not carbonized.

Challenges

The initial challenge was that of contamination control and this is being managed. The current challenges include the provision of manual immersion control, not only as back-up to the programmable logic control but also as a cheaper alternative. In addition, it will be necessary to put in place strategies that will reduce costs and improve yields in TIBs and this is research-demanding. Combining meeting the target of seed production within the YIIFSWA time frame with researching for optimization will reduce the rate of one or the other. This will make it necessary to involve dedicated public and private TIBs laboratories in seed production. The most critical aspect will be fitting the post-flask management into the timing of field production cycles to meet the seasonal demands for yam when irrigation farming is not yet adapted to yam production in Africa.

Conclusion

As YIIFSWA continues working on the science of optimizing the TIBs technology for seed yam production, the provision of technical backstopping to stakeholders is also progressing. The quality of seed yam produced has increased while protocols are being improved for higher rates of propagation. Farmers' use of high-quality seed yam will increase yield and profit margins and improve their livelihoods. Increased propagation rates will make more yam available per year.

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