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Relative Efficiency of Positive Selection and Tissue Culture for Generating Pathogen-free Planting Materials of Yam (*Dioscorea* spp.)

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Abstract

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Yams are staples in West Africa. They are propagated from tubers in an informal seed system. This encourages a build-up of diseases, and necessitates the rapid development of a formal seed system where certified seeds are functional. Although few reports exist on the use of meristem culture to generate pathogen-free yam, the success rate for the most economically important species in the sub-region, *Dioscorea rotundata*, for the most prevalent viruses is inadequate. To generate pathogen-free yam planting materials, the relative efficiency of tissue culture and positive selection was compared. Twenty-one asymptomatic yam plants were positively selected from 8187 stands of five landraces. Five of these stands were tested virus-negative by multiplex polymerase chain reaction (PCR) for *Yam mosaic virus* (YMV), *Yam mild mosaic virus* (YMMV) and *Cucumber mosaic virus* (CMV), and by PCR for the genus *Badnavirus* (BV), giving 0.08% success. Single nodes of the positively selected stands were used to establish *in vitro* plantlets, which were screened onto bacteriological indexing medium. The same was done for meristem- and node-derived plantlets of the improved variety TDr 95/19158. Incidence of endophytes ranged from 18 to 32% in the nodal plantlets while it was 0% in the meristem-derived plantlets. The effect of meristem culture combined with thermotherapy on the virus infection status was determined using virus-tested, one week old *in vitro* plantlets of eight improved genotypes. These *in vitro* plantlets were incubated at 36 ± 0.5°C and 16 h photoperiod for 21 days, after which meristems were excised, regenerated into plantlets and re-tested for viruses. Seventy-three percent of the samples were recovered from YMV but the effect on BV was inconsistent. Positive selection can be used as a palliative in generating quality declared seed but meristem culture combined with thermotherapy is more efficient for generating certified seed tubers of yam.

Keywords: asymptomatic selections; endophytes; micropropagation; seed systems; thermotherapy; yam virus diseases

The yams (*Dioscorea* spp.) are starchy root staples and a primary source of income in West Africa (Aighewi *et al.* 2015). About 58 million tons are produced annually worldwide, 93% and 65% of which are produced in West Africa and Nigeria, respectively (FAO 2013). Although yam has zygotic seeds borne on female plants (Figure 1), it is used for propagation only for genetic improvement purposes. Traditionally, yams are propagated for consumption by whole tubers (Figure 1), or tubers cut into about 25 g setts. This means that few new plants are grown from one tuber each season. There is also a competition for the use of tubers for food and for seed production. Yam is affected by viral, fungal, nematode and bacterial diseases which cause yield losses (Hughes *et al.* 1998; Odu *et al.* 1999). Viruses are widespread

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Figure 1. Seed tubers of yam (left); female flowers bearing fruits (middle); zygotic seeds from fruits (right)

in yam fields in single and mixed infections (Oppong *et al.* 2007; Eni *et al.* 2010; Asala *et al*. 2012; Odedara *et al.* 2012). Poty-, potex-, badna- and cucumo-viruses have been reported in *Dioscorea* spp., but the most significant loss is caused by *Yam mosaic virus* (YMV) (Malaurie *et al.* 1998). Diseases accumulate over generations of the use of unhealthy seed tubers with threat of the extinction of valuable germplasm. Therefore, there is scarcity of virus-free seed yam. Up to 63% of the cost of production is spent on the purchase of seed (Ironkwe *et al*. 2007). This makes it necessary to improve the existing informal seed production and development of a formal yam seed system where regulatory rules are functional (Balogun *et al.* 2014).

In generating virus-free seed, positive selection (PS) and tissue culture (TC) have been reported to be successful for potatoes (Gildemacher *et al.* 2009). In PS, asymptomatic plants and tubers are selected for growing the next generation of the crop. The TC technique of meristem culture combined with heat, cold or chemotherapy is used when donor plant is infected with viral pathogens (Berg & Bustamante 1974). Culture of meristems from heat-treated white yam plants cleaned them from YMV (Ng & Hahn 1985) but meristem regeneration was limited by blackening and premature senescence. In addition, virus indexing was not carried out by PCR and the success rate was not reported. Filloux and Girard (2006) also proposed that efficiency of this technique should be confirmed for YMV as the sample size tested, especially in *D. rotundata*, was small. Recurring culture contaminations during micropropagation also cause high losses in micropropagation schemes and germplasm losses in *in-vitro* genebanks while products of such micropropagation cannot be certified to be pathogen-free (Thomas 2004). This study compared efficiencies of PS and TC techniques of meristem culture combined with thermotherapy in generating yam planting materials free from viruses and other pathogens of yam.

MATERIAL AND METHODS

Tuber portions (50 g) of five landraces (Table 1) were planted in April, 2013 in a serpentine layout at 1 and 0.5 m inter- and intra-row spacing, respectively, to generate 8187 stands at the International Institute of Tropical Agriculture, Ibadan, Nigeria (7°26'N, 3°54'E), a rainforest-savanna transition zone. The stands were visually assessed for mottling, leaf and vein chlorosis, leaf distortion and malformation. Asymptomatic stands (Figure 2), totalling 150, were tagged 16 weeks after planting and harvested in December, 2013. Tubers from twenty-one out of one hundred and fifty plants were selected against tuber crack and gall, dry rot and mealy bug, cut into 40 g setts and planted to 50 stands per landrace in April 2014 in 10 l pots filled with 7 l of sterile top soil inside an aphid-proof screenhouse. Thirty-four asymptomatic stands were further selected and tested for these viruses: *Yam mosaic virus* (YMV), *Yam mild mosaic virus* (YMMV), *Badnavirus* (BV) and *Cucumber mosaic viru*s (CMV) using polymerase chain reaction (PCR) (Nkere 2016) at two and four months after planting. Tubers were harvested eight months after planting from stands tested to be virus-free and planted in June, 2015 in an aphid-proof screenhouse. At three to four months after planting, single node cuttings were made from each virus-negative stand and planted in a mixture of sterile carbonized rice husks + top soil (50 : 50 by volume). The number of rooted vines per clean mother plant was recorded.

In order to determine the incidence of endophytes in the virus-free certified positively selected plants,

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Table 1. Progress to recover seed yam from infected stocks using positive selection

Genotype	No. of plants	Asymptomatic plants selected		PCR-clean plants		Efficiency of PS (%)
		(No.)	(%)	(No.)	(%)	
Hembakwase	930	11	1.18	2	0.22	18.2
Pepa	940	17	1.81	1	0.11	5.9
Alumaco	2250	6	0.27	0	0.00	0
Ekpe	1537	19	1.24	0	0.00	0
Amula	2530	5	0.20	2	0.08	20.0
Total	8187	58	4.70	5	0.41	8.62
Mean ± SE		11.6	0.94	1	0.08 ± 0.04	8.62 ± 4.3

SE – standard error; efficiency of positive selection (PS) – No. of PCR clean plants/No. of positive selection

single nodes from three genotypes (Hembakwase, Pepa and Amula) were surface-sterilized by immersing in 2 ml/l lambda-cyhalothrin + 7 g/l Team (4 g/l mancozeb + 0.8 g/l carbendazim) for 10 min, air-drying in the laminar flow box for 45 min, immersing in 70% ethanol, 2% and 1% sodium hypochlorite (NaOCl) for 5, 15 and 30 min, respectively, before rinsing in three changes of sterile distilled water. The nodes were cultured in agar-solidified, modified Murashige and Skoog (1962) medium (MS) containing 20 mg/l L-cysteine, 100 mg myo-inositol, 0.5 mg/l kinetin, 30 g/l sucrose and 7 g/l agar. Cultures were incubated at 25 ± 2 ℃, 4000 lux and 12 h photoperiod provided by white fluorescent tubes (Balogun *et al.* 2014). Roots, stems and leaves of 8-week-old plantlets were chopped onto bacteria indexing medium containing 10 g/l sucrose, 8 g/l casein hydrolysate, 4 g/l yeast extract, 2 g/l potassium hydrogen phosphate, 0.15 g/l magnesium sulphate heptahydrate and 10 g/l agar in sterile Petri dishes and incubated at 27 ± 2°C for 7 days in darkness (Thomas 2004). Incidence of endophytic bacteria was recorded. The same procedure was repeated using meristem- and node-derived plantlets of the improved genotype TDr 95/19158.

Another experiment was conducted to determine the effect of heat therapy on the virus status. Meristem-derived plantlets of improved genotypes of water yam (TDa 95/01166) and white yam (TDr 95/05575, TDr 95/19158, TDr 95/19177, TDr 95/18544, TDr 89/0070, TDr 89/02677, TDr 89/02565) were tested for YMV, YMMV, CMV and BV using PCR. Two-node cuttings from the virus-positive plantlets were subcultured onto the same multiplication medium and incubation conditions as described above for one week followed by transfer to a growth cabinet set at 36 ± 0.5°C and 16 h photoperiod for twenty-one days. Newly initiated meristems about 0.5–1.0 mm long were excised with the aid of a dissecting microscope and cultured on modified MS medium

Figure 2. Symptomatic (left) and asymptomatic (right) yam plants

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Figure 3. *In vitro* microtubers weighing 0.3–2.7 g (left) planted and sprouted (right)

containing 30g/l sucrose, 100 mg/l myo-inositol, 20 mg/l L-cysteine, 80 mg/l adenine hemisulphate, 0.2/l mg benzylaminopurine and 1 mg/l uniconazole-P. Plantlets were regenerated from the meristem for 16 weeks and transferred into yam multiplication medium described above for further growth, which took sixteen weeks. Leaf samples from the heat-treated meristems which regenerated into plantlets and those of the virus-positive controls were tested for viruses using PCR. The incidence of infected plants recovered from each virus or mixed infection was recorded and compared with descriptive statistics (%) using SAS (Version 9.4 (TS1M2) 2012).

RESULTS AND DISCUSSION

Incidence of asymptomatic stands from PS both at vegetative stage and on harvested tubers ranged from 0.2% to 1.8% among the landraces with a mean of 1% across genotypes (Table 1). Eleven stands were virus-free at two months after planting, while six out of these tested virus positive ones at four months of age. This suggests that even with PCR, expression of virus infection symptoms varied with plant age. Thus, a total of only five stands were virus-free out of the initial 8187 stands. This double PCR-test is therefore more reliable although it is at added cost. No virus-free stocks were retrieved in Alumaco and Ekpe genotypes.

In planting the positively selected tubers, minisetts were used so that more than one stand was planted from one tuber. Among plants raised from the same mother tuber, some stands were positive for viruses and others were negative. Aiheiboria (2015) reported that plants raised from the head portions of tuber had the highest mean value for incidence and severity of YMV infection while the tail portions were least infected and discussed that as photosynthates flow down to the tuber, they carry along the virus particles. Thus, virus concentration varied with tuber portion, and by extension, it suggests that the smaller the seed tuber portion, the higher the probability of uniformly virus-free progenies from it.

Consequently, small, whole seed tubers are quality-, time- and cost-effective as there will also be no need to cut into minisetts and this reduces labour costs and risk of disease transmission from knives used for cutting. In a different study, *in vitro* microtubers weighing up to 2.7 g (Figure 3) sprouted successfully when planted in pots (Pelemo 2015, personal communication). Meristem culture could therefore generate genetically more uniform and virus-free stands due to the small size of the explant (Villordon & LaBonte 1996) and slow down the process of cultivar decline that may accrue due to accumulation of viruses and mutations. However, it will be worthwhile to determine genetic variations among progenies from different types of planting materials (tuber, vine, meristems and bulbils).

The efficiency of PS, determined as the percentage of PS-clean to that of PCR-clean differed among genotypes, and ranged from 0% in Alumaco and Ekpe to 20% in Amula in the second selection cycle, suggesting that symptomatic expression of virus infection differed among genotypes. It will be necessary to confirm through breeding for virus resistance the differential expression of virus infection (absence of symptom, tolerance or resistance) among yam genotypes. Contrary to Kinyua *et al.* (2001) that PS is at no added cost to the farmer, this study shows

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Figure 4. Rooted vine cuttings of white yam

Figure 5. Uncontaminated yam plantlet (left) tested positive for bacterial endophytes in bacteria indexing medium (right)

that PS is at added cost to the farmer as fewer stands are reserved for planting, making it necessary for the farmer to buy more seeds to plant on his farm. Innovative, field-applicable diagnostic tools will be very useful for the efficient use of PS by seed yam producers. In addition, the rate of PS success depends on number of virus-free stocks in the initial population and is unpredictable.

The numbers of single node vines cut from five stands of Hembakwase were 34, 13, 8, 4, 50; from one stand of Pepa it was 23 while it was 7 from one stand of Amula. Based on the virus-negative result of the five positively selected stands, additional single nodes, not meristems, were cut from the stands for *in vitro* introduction in order to remove endophyte-contaminated stocks through bacteria indexing, with an average of 121.3 ± 59.3 vines per plant. Vine cuttings from positively selected plants (Figure 4) increased the rapidity of virus-free stock generation by a factor of 121, and could be more, depending on the vigour of the positively selected mother plant. An optimum fertilizer regime for maximum canopy development will significantly increase the number of vine cuttings.

Table 2 shows that the percentage incidence of endophyte-free stocks ranged from 67% in Pepa to 79% in Amula with a mean of 72.5%. Thus, about 27% of the nodal explants harboured endophytes (Figure 5). Mbah and Wakil (2012) reported that contaminations were due to endophytes harboured by yam plantlets intercellularly, which may pass latently into culture and spread horizontally and vertically in cultures. A mean of 30% endophytic cultures was also recorded among plantlets derived from nodes in TDr 95/19158 but none of the meristem-derived plantlets had endophytes although the sample size was smaller. These may be due to the smaller size of meristem relative to the nodes, and that the terminal region of the shoot meristem which is above the zone of vascular differentiation is unlikely to contain patho-

Table 2. Incidence of endophytes in plantlets of 4 yam genotypes regenerated from different explants

Genotype	Explant	No. of plantlets indexed	Endophyte negative		Endophyte positive (%)
			(No.)	(%)	
Hembakwase	single node	213	153	72	18
Pepa	single node	115	77	67	23
Amula	single node	33	26	79	21
Mean (%)				73	27
TDr 95/19158	meristem	3	3	100	0
TDr 95/19158	meristem	4	4	100	0
TDr 95/19158	single node	88	63	72	18
TDr 95/19158	single node	386	261	68	32

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Table 3. Effect of thermotherapy and meristem culture on some common viruses infecting yam

Virus eliminated	No. of plants	
	treated	cleaned (%)
Yam mosaic virus (YMV)	15	11 (73)
Yam mild mosaic virus (YMMV)	2	2 (100)
Badnavirus (BV)	25	6 (24)
YMV + BV	12	3 (25)

genic organisms due to the absence of differentiated conducting tissues. Although culturing the nodes *in vitro* gave cleaner seedlings due to the inclusion of an endophyte indexing step that reduced losses to contamination significantly, additional 3 weeks are needed for hardening.

Previous studies on contamination control in yam tissue cultures advocated the use of a combination of five antibiotics, with 33% success and this was specific to some genotypes (Mbah & Wakil 2012). However, endophytes may not be completely undesirable as various endophytic bacteria in association with plants were reported to increase the adaptive response of plants to stress through plant growth stimulation (nitrogen fixation, auxin and cytokinin production) and disease protective properties (Jasim *et al.* 2015). In black pepper, the endophyte *Pseudomonas fluorescens* increased production of defence enzymes against the pathogen *Phytophthora capsici* (Paul & Sarma 2005). It will therefore be necessary to test the relative adaptability of endophyte-containing and non-containing yam to stress conditions, and also their effects on propagation ratio *in vitro* or *in vivo.* This may also affect the rate of degeneration of otherwise 'virus-free' planting materials when grown in the field.

The use of meristem culture combined with thermotherapy recovered plantlets from YMV with 73% success (Table 3). Meristem culture was reported to clean *D. alata* from viruses (Mantell *et al.* 1980). Filloux and Girard (2006) reported the virus elimination success of 2%, 21%, 46% and 60% for BV, YMV, YMMV and Potex viruses using thermotherapy while Shin *et al.* (2013) reported that cold treatment combined with shoot tip culture cleaned

Table 4. Relative efficiency of positive selection and tissue culture for generating virus-free planting materials of yam

Positive selection (PS)		Tissue culture	
Planting, first cycle of positive selection using vegetative parts and tubers	32 weeks	planting, collection of explants	12 weeks
Tuber dormancy	16 weeks	*in vitro* culture of single nodes	8 weeks
Planting of PS tubers and second cycle of PS; PCR test 1 and 2	16 weeks	pre-culturing for thermotherapy	1 week
Vine cutting from PS stands, confirmatory PCR test, tuber production	20 weeks	thermotherapy	3 weeks
Planting, first cycle of positive selection using vegetative parts and tubers	32 weeks	meristem culture and plantlet regeneration, PCR test 1	16 weeks
		multiplication of plantlet (1 : 4 propagation ratio)	12 weeks
		hardening	3 weeks
		vine cutting from virus-free plant, confirmatory PCR, tuber production	32 weeks
Total duration to virus-free tuber production	84 weeks		87 weeks
Virus-free success rate (SR)	0.08	virus-free success rate	73.3
Total number of virus-free plants from one infected tuber (1 kg) cut to 20 (sett weight = 50 g)	NVC × SR × stands = 121 × 0.08% × 20 = 2	total number of virus-free plants from one infected (1 kg) cut to 20 (sett weight = 50g)	NM × SR × NVC × stands × propagation ratio = 10 × 73.3 × 121 × 20 × 4 = 70 954
Efficiency (output/input)	2/20 = 0.1		70 954/20 = 3 547

Incidence of sprouting for positive selection, personnel, percentage meristem regeneration, contamination and endophytes for tissue culture are factors that can disrupt the above schemes; SR – percentage virus-free success rate; PCR – polymerase chain reaction; NVC – No. of vine cuttings per plant; NM – minimum No. of meristems per plant

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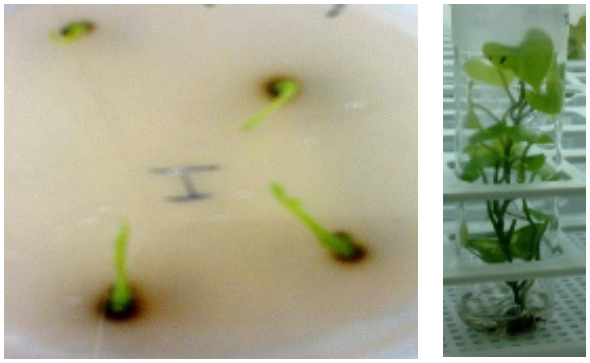

Figure 6. Regenerating heat-treated meristems (left) and meristem-derived plantlet (right)

D. opposita from YMV with 90% success. Cryo- and thermo-therapies were also reported to be effective for virus elimination (Houten *et al.* 1968; Gallet *et al.* 2007; respectively). In this study, meristem culture combined with thermotherapy had a success rate of 24% for elimination of BV and 25% for the mixed infection of YMV and BV. In addition, the effect on BV was inconsistent, as no BV was detected before treatment but it emerged after treatment in some of the samples of water yam tested. This also points to the recent report that BV is integrated into the yam genome (Seal *et al.* 2014). Considering that YMV is the most economically important virus affecting yam, the 73% elimination by thermotherapy is significantly higher than 0.08% achieved with positive selection. More than 3000 virus-free plants can be generated from 1 kg of infected tubers, compared to two plants from PS (Table 4). The longest phases of the recovering process are the canopy development after tuber planting (12 weeks) to generate explants and the regeneration of plantlets from meristems (16 weeks, Figure 6). It may be possible to reduce the duration of these 2 phases by inducing multiple sprouts in tubers as they undergo thermotherapy and culturing the meristems from the sprouts.

Positive selection is recommended as the first step against accumulation of bad quality seed yams where the seed system is informal while meristem culture is more efficient for sustainable generation of more uniformly virus-free stands. Production of certified seed yam should be initiated using tissue culture for the development of a formal seed system. The procedure should involve meristem culture and thermotherapy, regeneration of plantlets, PCR detection of viruses, tissue screening onto bacteria indexing medium, multiplication of clean stock, transplanting and acclimatization to produce pre-basic seed tubers. Vines can be cut from potted TC plants and rooted to produce basic seeds. These high quality virus-free stocks can be introduced into commercial propagation schemes like aeroponics (Maroya *et al.* 2014) and temporary immersion bioreactor systems (Balogun *et al.* 2014) to increase the quantity of certified seed yam available to seed value chain actors.

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