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Molecular Fingerprinting and Selection of Appropriate Media for Rapid *In Vitro* Multiplication of Three Yam Varieties

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Additional information is available at the end of the chapter

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1. Introduction

Yam is the colloquial name for some species in the genus *Dioscorea*, members of the family *Dioscoreaceae*. Yams are perennial monocotyledonous plants with a vine and underground tubers. There are about 700 species within the family, nine of which are medicinal plants that accumulate steroid saponins in their rhizomes and six species namely *D. bulbifera*, *D. cayenensis*, *D. dumentorum*, *D. prahensilis*, *D. alata* and *D. rotundata* are edible. The cultivation of the edible tuber is mostly in Africa, Asia, Latin America and the Oceania regions. In West and Central Africa, especially Ghana the underground yam edible tuber is very important as a staple providing food security and income for some 26.2% of the population [6]. The daily average yam consumption is approximately 300 kcal per capita [4]. Being the third most important energy source in the Ghanaian diet, yam accounts for 20% of total caloric intake [4]. It is a versatile root crop which has various derivative products after process as it can be barbecued, roasted, fried, grilled, boiled, smoked and when grated it is processed into a dessert recipe. At present farmers are only getting about 20% of the potential yield of yams. In Ghana, the consumer has developed preference for a particular *Dioscorea rotundata* variety locally called “Pona” because of its peculiar taste and texture. It is with this in mind that the CSIR-CRI Yam breeding program evaluated and selected for release three new yam varieties (CRI-Pona, CRI-Kukrupa, and Mankrong Pona). These varieties were officially released in May 2005 and are all high and stable yielding, pest and

disease tolerant and have good culinary characteristics [14]. These released yam varieties are in high demand by farmers and all possibilities are being explored to enhance the production of clean planting materials which could be achieved using tissue culture techniques.

1.1. Yam production and associated challenges

Traditionally, yams are grown from the edible tuber in the form of whole tuber or sections, or setts on mounds and as they produce vines, they are staked with sticks to permit the vines to climb up the stake (Figure 1). The growth period is between 6 and 10 months, depending on the variety, after which they produce the edible underground tubers (Figure 2). Annually production of yam stands at about 48 million tonnes in the sub-saharan West African region and this represents 93% of the global production indicating that the region is the hub for production of the tuber. The countries involved in the production are Benin, Cote d'Ivoire, Ghana, Nigeria and Togo. Globally, Ghana is the leading yam exporter, having exported 20,841 metric tons of yams in 2008, but with the increasing global demand for yam from Europe, the U.S and neighbouring African countries, there is a potential for higher production and export volumes [10]. Research conducted in Council for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI) showed there is a potential to increase the yields of yam from the average of 12 tons/ha to between 65-70 tons/ha. The major challenge in meeting the production targets are inadequate access and high cost of seed yam, hence despite the availability of fertile land and demand for yam domestically and abroad cultivation is low. Typically, cost of seed covers about 58% of production cost. This challenge is due to the fact that yams are vegetatively propagated from the edible tuber, and there is the general lack of high quality disease-free seed yam as the planting materials are usually infested with Fungi, Bacteria and Viruses. In order for farmers to maintain the clonal materials yams have been propagated vegetatively. This means of propagation has the potential of contributing immensely to the spread of diseases and pathogens endemic in the planting materials since same planting materials are used for propagation year after year which leads to accumulation when clean materials are not used. Currently the use of tissue culture techniques along with cryotherapy [7], chemotherapy and thermotherapy are the sure means of producing clean virus-free planting materials of vegetatively propagated crops. The presence of several viruses in West African yam was reported in 1992 [20] and these are supposed to have led to significant losses in crop production [9]. *Yam mosaic virus*, a *Potyvirus* is the most important infection in West African yams. It was first detected in *D. cayenensis* in Côte d'Ivoire [21]. The symptoms include mosaic, shoestring, green vein banding and stunting of the yam plant [17; 5]. Other viruses reported in yams include Badnavirus and Cucumber mosaic virus. It has been reported that sequences of badnaviruses have been integrated into the yam genome [18]. This has culminated from years of cropping yam with infected planting material. It is therefore important that tissue culture techniques be developed to facilitate the production and mass propagation of clean planting material. Other pathogens commonly associated with yams are nematodes and anthracnose.



Figure 1. Yam plant vines growing on a stake in the field

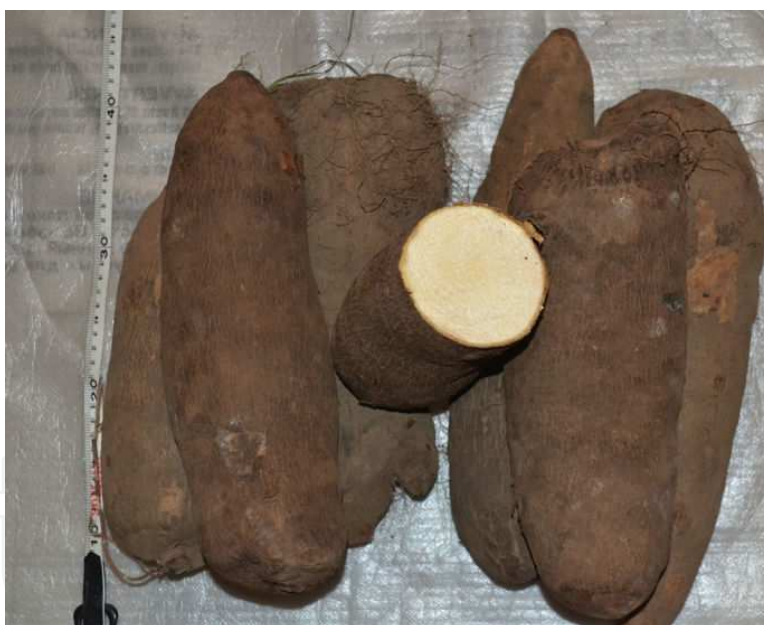


Figure 2. Edible yam tubers harvested from underground

1.2. Tissue culture production

Crop propagation through *in vitro* approaches offers a scope for improving root and tuber crops with desirable traits in larger quantities. The technique allows for rapid mass propagation of clean planting materials all year round in a limited space and is ideal for the conservation of germplasm. This notwithstanding, the widespread application of tissue culture has few

limitations such as high initial cost of production, choice of crops restricted to species with acceptable propagation protocols and reproducibility of protocols.

Propagation of plants through tissue culture has become an important and popular technique to reproduce crops that are otherwise difficult to propagate conventionally by seed and/ or vegetative means. Specialised and matured cells are manipulated to give rise to multiple copies of the parent plant under optimum aseptic environmental conditions and appropriate stimuli. It offers many unique advantages over conventional propagation methods such as rapid clonal multiplication of valuable genotypes, expedition release of improved varieties, production of disease free plantlets, non-seasonal production, germ-plasm conservation and facilitating their easy international exchange. The application of tissue culture techniques towards the production of clean planting material is critical for vegetatively propagated crops. On the field, tissue culture produced plants were found to establish more quickly, grow more vigorously and produce higher yields than conventional propagules with approximately 30% higher yield [15].

A number of factors come into play when establishing crops *in vitro*, and these include the plant part used (explant), its developmental stage, conditions under which it was grown, and the choice of growth conditions. Success of most tissue culture works depend much on the levels and kinds of plant growth regulators included in the medium. Root and shoot initiation, callus formation and differentiation are closely regulated by the relative concentration of auxin and cytokinin in the medium [3]. Plant growth regulators are critical media component in determining the developmental pathway of the plant cell. When establishing yam cultures *in vitro*, it has been shown that the age of the explant is critical [2] and also there seem to be a good reserve of endogenous auxins hence cultures do not require the supplementation of auxins [2]. Cytokinins such as benzylaminopurine (BAP) and kinetin are generally known to reduce apical meristem dominance as well as enhance both axillary and adventitious shoots formation from meristematic explants [8]. BAP has a marked effect in stimulating the growth of axillary and adventitious buds, and foliar development of shoot tip cultures [1]. In *Dioscorea* it has been shown to enhance the development of multiple buds and shoots under high concentrations [2]. Efficient growth and development is achieved when *in vitro* growth media and conditions are determined for the various growth stages namely culture establishment, mass propagation and plantlet development prior to transferring crops to the field. In yams, nodal culture establishment requires the addition of NAA and BAP to the medium, whereas meristem establishment requires the inclusion of GA₃ and adenine sulphate to the medium. The mass propagation state requires only cytokinin be it Kinetin or BAP, whereas the rooting and plantlet establishment stage does not require any growth regulator in the medium [2]. *In vitro* manipulations when established are very important for germplasm maintenance.

1.3. DNA fingerprinting

In Ghana, crops developed, evaluated and selected for utilisation are given to the farmers through the agriculture extension agents. Most of the time as much as each crop variety has a

name, they are all referred to as agriculture varieties. There are even incidents where different settlements give different names to the same crop variety, although the selection of the name at the variety developmental stages is done with the farmer. This makes it very difficult for the researcher after releasing a variety to track the extent of spread and adoption. Fingerprinting and documentation of genetic make up at the DNA level is thus vital to facilitate the researchers efforts. Fingerprinting can also be referred to as genotyping; which is the process of determining the genetic constitution – the genotype – of an individual by examining their DNA sequence. This provides information necessary to characterise germplasm and is a vital tool for identification of germplasm, as well as ensuring the genetic integrity with time. Genotyping can be applied to a broad range of organisms, including microorganisms. The genotype of an individual provides the fingerprint and comparing fingerprints allows you to determine the similarity between two individual, to find matches. This kind of information is vital to document the identity of crop varieties released to facilitate the ability to trace individual at any point in time.

Currently, methods of characterization used by breeders include morphological, agronomic, and biochemical systems. Characterization based on morphologic characteristics alone may be limited since the expression of quantitative traits is subjective to strong environmental influence. Alternatively, molecular characterization techniques are capable of identifying polymorphism represented by differences in DNA sequences. This has the ability of analyzing variation at the DNA level during any stage of the development of the plant, where environmental influences are excluded. The PCR-based methods constituted a new milestone in the field of DNA fingerprinting that has to be included in the requirements for varietal release in Ghana.

1.4. Study objective

The need for clean planting materials on mass production scale is crucial to complement limitations of seed production in yam industry. *In vitro* rapid multiplication offers the best system to be used in efforts to meet seed yam targets in the dissemination of clean planting material of released root and tuber varieties. This system is not in existence in Ghana, therefore first objective of this paper is to establish appropriate medium for each of the three released yam varieties to enhance *in vitro* rapid multiplication, and document *in vitro* production scheme for the released yam varieties.

Following the evaluation and selection of a crop variety for dissemination, it is vital to have a system that will permit the ability to trace the product. Genomic fingerprinting is one such tools that when used can facilitate variety identification. This study uses SSR Microsatellites to establish the molecular identity of three released yam varieties alongside 21 other yam accessions comprising species *Dioscorea rotundata*, *D. cayenensis*, *D. bulbifera*, *D. alata*, *D. dumentorum*, and *D. esculenta*. The documentation of this information will enable the researcher to identify their samples at any point in time and also provide genetic relatedness information that is vital for breeding.

2. Materials and methods

2.1. *In vitro* manipulations

2.1.1. Establishment of Mother Plant under greenhouse conditions

Three yam varieties released by CSIR-CRI namely CRI-Pona, Mankrong Pona and Kukrupa were used in the study. Samples of yam tubers were obtained from the CSIR-CRI yam breeding program, sectioned into minisetts and treated with ash and benlate prior to planting in pots at the Screenhouse. Mini setts sprouted after approximately six weeks and vines older than three weeks were harvested for *in vitro* manipulations.

2.1.2. Preparation of explants

Yam vines were harvested from plants growing in the screenhouse, labelled appropriately and the cut end was dipped in deionised water and sent to the Tissue Culture laboratory. Vine was thoroughly washed under running tap water and the nodal cuttings and shoot tips were excised (Figures 3 and 4) into autoclaved deionised water in labelled beakers for surface sterilisation.



Figure 3. Yam Nodal cutting explant freshly harvested from the field for initiation *in vitro*



Figure 4. Yam Shoot tips explant freshly harvested from the field for meristem excision and initiation *in vitro*

Surface sterilization was carried out under sterile conditions in the laminar flow cabinet as follows: Explants were transferred into beakers containing 70% ethanol for five minutes and then surface sterilized with 20% sodium hypochlorite solution (with 6% active chlorine) containing 2-3 drops of tween 20 for 15 minutes. They were washed with sterile distilled water three times after which edges of explants were trimmed. Explants were then further surface sterilized with 10% sodium hypochlorite solution containing 2-3 drops of tween 20 for 10 minutes and the edges of the explant trimmed, rinsed three times in autoclaved distilled water and kept in autoclaved water prior to culturing. After sterilization, meristems (approximately 1x1 mm) were excised from the shoot tips using a dissecting microscope. The meristems and nodal cuttings were then labelled appropriately and cultured on appropriate media and labelled accordingly. The individual cultured explants were code labelled for ease of tracing the material used for initiation. This was vital because once a culture is screened for the presence of a virus and it test negative or positive, the implication is that all clonal materials generated from that particular explant are either cleaned or infected respectively. Infected explants can therefore removed from the mass propagation system.

2.1.3. Nutrient media preparation

2.1.3.1. Initiation medium for Yam

Murashige and Skoog [11] basal salts complimented with growth hormones and vitamins were used. The growth regulators used for meristem cultures were BAP, NAA and GA3. Where nodal cuttings were used, GA3 was not included in the medium. The medium was further supplemented with Adenine Sulphate (AdSO₄) (80 mg/l) as a cytokinin additive and L-cysteine (20 mg/l) as an antioxidant, 30 g sucrose and 7 g agar were used as carbon source and gelling agent respectively. The details of the media composition are as in the table 1 below. The following vitamins Myo- inositol, Nicotinic acid amide, Pyridoxine, Thiamine- HCl and Glycine were used as documented by Murashige and Skoog [11]. The pH of the medium was set at 5.7 ± 1 and sterilised in an autoclave at a temperature of 121°C at 15 psi for 15 minutes. Culture vessels used were pyrex test tubes with dimension 16 X 125mm. Medium to be used for meristem cultures were slated after removal from the autoclave prior to allowing them to cool down. This provided a broad surface for the excised meristem to be placed on the upper part as represented in the Figure 5. Meristem cultures were transferred every eight weeks onto fresh medium till shoots differentiated from the explants after ten months. Shoots differentiating from nodal buds were excised onto the same medium till actively growing shoots were obtained.

2.1.3.2. Rapid multiplication medium for Yams

Actively growing shoots (Figure 6) from both meristem and nodal bud cultures were subcultured onto complete MS medium with vitamins supplemented with 2.5 µM kinetin referred to as yam multiplication medium. During subculture, the shoot tips (Figure 7) and nodal cuttings (Figure 8) were excised from a growing shoot and grown on freshly prepared medium. Kinetin concentration in the rapid multiplication medium was manipulated to optimize yam

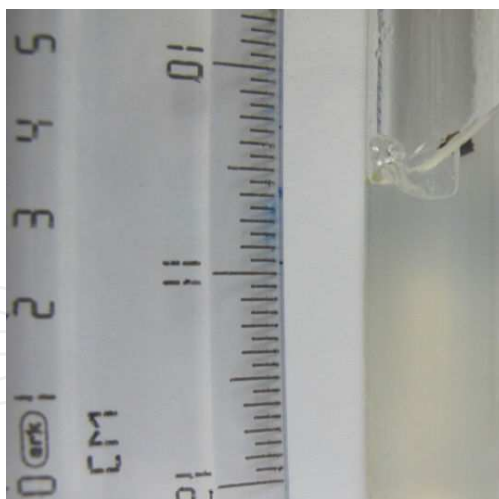


Figure 5. Growing Yam meristem placed on slanted medium

multiplication medium. The complete MS medium with vitamins was supplemented with kinetin at 0, 2.5, 5, and 10 μM concentration. Another medium that was used was the complete MS medium, supplemented with the following vitamins Myo-inositol, Nicotinic acid amide, Pyridoxine, Thiamine-HCl and Glycine and labelled as “mm”. The pH of all the media was set at 5.7 ± 1 using 0.1 M NaOH for adjusting it from a lower pH. The media were sterilised at a temperature of 121°C at 15 psi for 15 minutes in an autoclave. The various media used in these experiments are presented in the Table 1.



Figure 6. Actively growing yam cultures

2.1.4. Incubation

All cultures were incubated at a temperature of $26 \pm 2^\circ\text{C}$ with a photoperiod of 16 hours of light and 8 hours of darkness.

Initiation/Meristem Medium	Quantity / Final Concentration in Media		
Component	Meristem Initiation medium	Nodal Cutting Initiation medium	Rapid Multiplication medium
MS Basal Salts*	4.33 g/l	4.33 g/l	4.33 g/l
MS Vitamins*	Manufacture's instruction	Manufacture's instruction	Manufacture's instruction
AdSO ₄	80 mg/l	80 mg/l	80 mg/l
BAP	5 X 10 ⁻⁶ M	5X10 ⁻⁶ M	-
NAA	1 X 10 ⁻⁷ M	5X10 ⁻⁷ M	-
GA ₃	2.25 X 10 ⁻⁷ M	-	-
Kinetin	-	-	0 – 10 X 10 ⁻⁶ M
Sucrose	30 g/l	30 g/l	30 g/l
L-Cysteine	20 mg/l	20 mg/l	20 mg/l
Agar	7-7.5 g/l	7-7.5 g/l	7-7.5 g/l
pH	5.7 ± 0.1	pH	5.7 ± 0.1

*Murashige and Skoog basal salts and vitamins [11]

Table 1. Medium composition for yam (1 litre)



Figure 7. Nodal cutting excised from actively Growing cultures for rapid multiplication

2.1.5. Biomass determination

Dry matter was also estimated as a measure of growth using 5 plantlets per treatment. Fresh weight was estimated by weighing the plantlets while dry weight was estimated by drying the plantlets in an oven at 80°C for 48 hours. Each treatment was replicated thrice.



Figure 8. Yam Shoot tips excised from actively Growing cultures for rapid multiplication

2.1.6. Data collection and analysis

Data was taken after 8 weeks of initiation and subsequent data were taken every 8 weeks by counting the number of leaves, shoots, multiple buds as well as root development and the general performance of the cultures was also noted. Statistical package used to analyse data was SAS 9.1.

2.2. Fingerprinting

2.2.1. Plant materials

Sampling of leaves for DNA isolation towards genomic studies was made from the field germplasm holding of the CSIR – Plant Genetic Resources Research Institute, located at Bunso in the Eastern Region of Ghana. For this study, a total of 21 samples were selected at random from six different species of yam grown in Ghana namely *Dioscorea alata*, *D. dumenterom*, *D. rotundata*, *D. cayenensis*, *D. bulbifera* and *D. esculenta* (Table 2). There were at least three different samples of each species and these were screened alongside the three released yam varieties.

2.2.2. Extraction of genomic DNA

Genomic DNA was isolated from 100 mg of young tender leaves. They were weighed into 2 ml eppendorf tubes and grounded with liquid nitrogen into fine powder. The genomic DNA was extracted using the following manufacture's instructions of the Qiagen protocol from the DNeasy plant mini kit.

2.2.3. DNA quantification and gel electrophoresis

The quality of DNA was checked on 0.8% agarose in 1x TAE (Tris-acetic EDTA) buffer by gel electrophoresis with Ethidium bromide (0.5 ug/ml). Electrophoresis of the DNA was carried out at 120 V for 40mins and then visualized with a UV transilluminator. The quality of DNA was ascertained and the concentration was projected by the intensity and comparison to 1 kb lambda DNA mass ladder (1 kb invitrogen). Quantification of DNA was

Accession	Lab Code	Specie	Population Identity
KT/01/015	Dr 013	<i>D. rotundata</i>	Pop1
UWR/97/101	Dr 026	<i>D. rotundata</i>	
UWR/97/059	Dr 039	<i>D. rotundata</i>	
UWR/97/085	Dr 052	<i>D. rotundata</i>	
FA/89/039	Da 110	<i>D. alata</i>	Pop 2
82/326	Da 130	<i>D. alata</i>	
FA/89/026	Da 137	<i>D. alata</i>	
AGA/97/173	Da 143	<i>D. alata</i>	
82/430	Dd 178	<i>D. dumeterom</i>	Pop 3
AGA/97/202	Dd 183	<i>D. dumeterom</i>	
BD/96/023	Dd 186	<i>D. dumeterom</i>	
TA/97/013	Db 187	<i>D. bulbifera</i>	Pop 4
TA/97/093	Db 193	<i>D. bulbifera</i>	
TA/97/141	Db 201	<i>D. bulbifera</i>	
SO/89/093	De 202	<i>D. esculenta</i>	Pop 5
TA/97/071	De 208	<i>D. esculenta</i>	
82/407	De 215	<i>D. esculenta</i>	
TA/97/057	Dc 109	<i>D. cayenesis</i>	Pop 6
SCJ/89/001	Dc 221	<i>D. cayenesis</i>	
BD/96/026	Dc 249	<i>D. cayenesis</i>	
82/129	Dc 251	<i>D. cayenesis</i>	
CRI Pona	CRI-Pona	<i>D. rotundata</i>	Pop 7
Mankrong Pona	Mankrong Pona	<i>D. rotundata</i>	
Cri-Kukrupa	CRI-Kukrupa	<i>D. rotundata</i>	

Table 2. List of accessions used for the study

evaluated by reading absorbance at 260 nm and 280 nm with the spectrophotometer. The DNA was diluted to 10 ng/μl for PCR amplifications.

2.2.4. PCR amplification

A set of 16 set of primer pairs [19] were used in the experiment (Table 3). DNA amplification was carried out with a 96 well plate Bio-Rad™ Thermocycler from BIO-RAD. The PCR conditions were optimized for cycling number, concentrations of the primer, MgCl₂ and DNA template. The reaction mixture (10 ul) contained 6.075 ul of Nuclease free sterile water (DNA

grade water), 1 ul of 10x PCR Buffer, 0.9 ul of MgCl₂ (25 mM), 0.4 ul dNTPs (10 mM), 0.25 ul primer (50 ug/ml) of each forward and reverse, 0.125 ul of SuperthermTaq Polymerase (1unit) and 1 ul of 10 ng DNA template. The cycling conditions were as follows: an initial denaturing step of 94°C for 5 mins, 35 cycles of 94°C for 30 secs, 51°C for 1min, 72°C for 1 min and a final elongation step of 72°C for 1 min. In every experiment, a negative control was included where the template DNA was replaced with PCR grade water. Amplification products were examined on a 6% polyacrylamide gel (Water, 10x TBE, 4% acryl amide (19:1), 10% APS and TEMED) and stained with silver nitrate. A 100 bp ladder (Gene Ruler™, Fermentas) was used as a size marker.

Primers	Sequence	Tm/°C
Da1F08R	5'CTATAAGGAATTGGTGCC	54.4
Da1F08F	5'AATGCTTCGTAATCCAAC	54.9
Da1D08F	5'GATGCTATGAACACAACAACTAA	52.5
Da1D08R	5'TTTGACAGTGAGAATGGA	54.6
Da1C12R	5'AATCGGCTACACTCATCT	54.4
Da1c12F	5'GCCTTTGTGCGTATCT	54.2
Da1A01F	5'TATAATCGGCCAGAGG	54.1
Da1A01R	5'TGTTGGAAGCATAGAGAA	53.9
Dpr3F10R	5'ACGCACATAGGGATTG	54.9
Dpr3F10F	5'TCAAAGGAATGTTGGG	54.8
Dpr3F12R	5'TCAAGCAAGAGAAGGTG	54.4
Dpr3F12F	5'TCCCCATAGAAACAAAGT	54.2
Dab2E07F	5'TTGAACCTTGACTTTGGT	55.3
Dab2E07R	5'GAGTTCCTGTCCTTGGT	54.5
Dpr3F04R	5'GCCTTGTTACTTTATTC	46.2
Dpr3F04F	5'AGACTCTTGCTCATGT	46.7
Dpr3D06R	5'ACCCATCGTCTTACCC	55.3
Dpr3D06F	5'ATAGGAAGGCAATCAGG	54.8
Dpr3B12R	5'CCATCACACAATCCATC	54.9
Dpr3B12F	5'CATCAATCTTTCTGCTT	54.3
Dab2D08R	5'GATTTGCTTTGAGTCCTT	54.1
Dab2D08F	5'ACAAGAGAACCGACATAGT	53.4
Dab2E09F	5'AACATATAAAGAGAGATCA	45.3
Dab2E09R	5'ATAACCCTTAACTCCA	46.3

Primers	Sequence	Tm/°C
Dab2D06F	5'TGTAAGATGCCACATT	54.4
Dab2D06R	5'TCTCAGGCTTCAGGG	55.1
Dab2C12R	5'CGAACGATCCAATAAAA	54.2
Dab2C12F	5'AGGCATCTTGGGAAA	54.3
Da3G04F	5'CACGGCTTGACCTATC	54.5
Da3G04R	5'TTATTCAGGGCTGGTG	55
Dab2C05F	5'CCCATGCTTGTAAGTTGT	53.9
Dab2C05R	5'TGCTCACCTCTTTACTTG	53.5

Source: Tostain *et al.*, [19]

Table 3. List of primers, their sequences and melting temperatures

2.2.5. Silver staining

The gels were placed on a shaker with a minimal shaking to allow solution flow over gel swiftly. Fixation was done with 10% Glacial Acetic Acid (100 ml acetic acid, 900 ml water) for 15 mins. This was washed off with distilled water and 1.5% Nitric acid (15 ml Nitric acid, 985ml water) solution was added for 5 mins. The silver stain solution (1.0 g Silver nitrate, 1.5 ml 37% formaldehyde, topped it up with water up to 1000 ml) was preceded after washing off the nitric acid solution for 20mins. Finally, the developer (30 g Sodium carbonate, 1.5 ml 37% formaldehyde and 0.25 ml Sodium thiosulphate 10 mg/ml, water up to 1000 ml) was added and allowed to develop the photographic stains/ bands for visualization. This was stopped with 10% acetic acid and then stored in distilled water for photographic capturing and scoring.

2.2.6. Gel scoring and data analysis

Bands were scored manually as present (1) or absent (0) from the gels. Similarity matrix was calculated using NYSTS software while cluster analyses were also carried out using Genstat and dendrograms were constructed. Similarity matrices from each primer were compared pair wise using a randomization test. POPGENE32 [22] was used for genetic population analysis as well as to test the effectiveness of loci used.

3. Results and discussion

3.1. *In vitro* manipulations

3.1.1. Explant response on initiation medium

The growth and development of different crop species vary considerably. The data obtained following *In vitro* initiation of nodal cuttings and meristems are presented in Tables 4 and

5 respectively. The nodal cuttings for Kukrupa and Mankrong-Pona when grown in yam initiation medium, had higher success rate than CRI-Pona (Table 4). The success rate was higher in the nodal cuttings (52.9 – 86.6%) than in the apical meristems (46.4 – 53.33%). In both situations, Mankrong-Pona had a higher success rate than CRI-Pona. The variety Kukrupa was however not included in the apical meristem experiment. The measure of percentage success was based on explants that developed to the extent of producing shoots. Well-developed shoots were obtained in all the successful nodal cutting explants after eight weeks in culture although by two weeks, some explants had already started producing shoots (Figure 9). Meristem development was however very slow since it took six months for shoot differentiation to occur and following that, by ten months, multiple shoots had started developing (Figure 10 a&b). Following culture initiation, successful meristems initially expanded due to cell division, turned dark with green clusters of cells, which later differentiated into buds and then further into shoots. Shoot differentiating from most of the Makrong-Pona meristem cultures had more than ten leaves and up to eight shoots per culture however, on the average, there were 4.43 shoots per culture, whereas CRI-Pona had 1.25 shoots per culture (Table 5). Shoots differentiated from nodal bud explants 12 days after culture (Table 4). The extent of success on meristem cultures was expected to be low, mainly due to the minute size of the explant used in initiation. Culture development is slow as it takes up to ten months for shoot to develop. However multiple shoots develop from the meristems and this facilitates rapid *in vitro* development. Hence if only a few meristems are successful *in vitro*, mass propagation is achieved. Development of shoots from nodal cutting explants is notably high and reliable. However, this method cannot be reliable if pathogens especially viruses have to be eliminated from the crop variety.

Variety	Total number of explants initiated	Number successful	% Success	Average Shoot formation
Cri-Pona	51	27	52.9	1.3
Kukrupa	69	54	78.3	2.1
Mankrong-Pona	45	39	86.7	1.5

Table 4. Yam Nodal Cutting initiation Success Rate of the three released varieties

Variety	Introduction number	Number successful	% Success	Mean No. of leaves	Mean No. of shoots
Mankrong Pona	90	48	53.3	7.4	4.4
Cri-Pona	84	39	46.4	3.3	1.3

Table 5. Yam Meristem initiation success Rate of the three released varieties

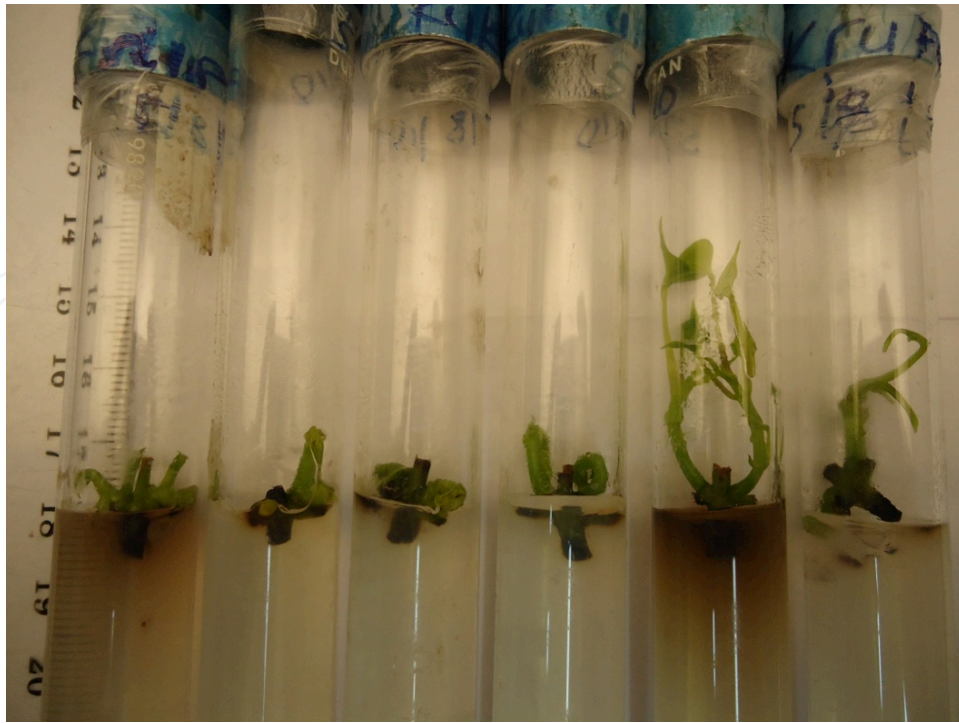


Figure 9. Yam nodal cultures sprouting *in vitro* after two weeks in culture



Figure 10. Four months old yam meristem (A) differentiating into multiple shoots after 10 months in culture Yam meristem explant forming multiple shoots after ten months (B) in culture.

3.1.2. Selection of appropriate rapid multiplication medium

Media supplemented with kinetin at different concentrations as well as MS medium enriched with vitamins and growth additives were used in this study. The responses of the three

released varieties on different media are presented in Figures 11 and 12 below. The performance of the three released varieties varied on the four different media. Considering shoot development, the mean was highest on medium containing 10 μM kinetin for CRI-Pona at 4 ± 1.39 , 8.6 ± 1.08 for CRI-Kukrupa on 5 μM kinetin and 8.29 ± 0.7 for Mankrong-Pona on 2.5 μM kinetin. With the exception of CRI-Kukrupa where highest number of leaves (9.33 ± 1.8) occurred on the control medium (no kinetin), CRI-Pona had 4.67 ± 1.8 leaves on the same medium as medium with highest number of shoots (10 μM kinetin), and Mankrong-Pona also had 13 ± 1.18 leaves on medium containing 2.5 μM kinetin. Notably this attempt is to maximise *in vitro* rapid multiplication. In tissue culture, nodal cutting are used to generate shoots, and the number of leaves generating is the determining factor for the multiplication rates that can be attained, since within each leaf axil is a bud that can develop into a whole shoot. Hence the higher the number of leaves the higher the multiplication rate. Shoots differentiate from buds and within each bud are clusters of meristematic cells which are capable of differentiating in shoots when the growth conditions are appropriate. Reports in previous research where BAP was the cytokinin used had a maximum of four shoots developing on the average [2]. Later efforts on generating somatic embryos reported 7-9 shoots developing per culture where kinetin was used [16]. This present study reported 3 to 8 shoots per culture, it is therefore indicative that Kinetin is an appropriate growth regulator to be used for rapid multiplication on yam.

Comparing the effect of different media on rapid multiplication of yam nodal culture in terms of shoots and leave (Table 6), medium supplemented with 2.5 μM Kinetin was the best although the difference was not significant. In tissue culture since a lot of clonal materials can be generated from one culture differences in terms of number are significant although statistically it may not be significant. The performance of cultures on the medium labelled "mm" which was not supplemented with Kinetin was very poor and significantly low numbers of leaves and shoots were recorded. This confirms that to achieve rapid multiplication of yam *in vitro*, the inclusion of kinetin is critical. Although the data obtained indicated that to maximize rapid multiplication *in vitro*, different media have to be used for the different varieties released, medium supplemented with 2.5 μM kinetin may be appropriate for all the varieties.

Comparing effect of the yam different varieties, nodal cultures during rapid multiplication (Table 7) in terms of shoots response of Mankrong Pona and CRI-Kukrupa were similar. The variety CRI-Pona had significantly low number of shoots. In terms of leaf development, CRI-Kukrupa had a significantly higher (8.3) number of leaves whereas CRI-Pona had the lowest (3.7). This response was due to varietal differences and Mankrong Pona will be recommended over CRI-Pona where high numbers are needed to be produced within a limited time. There was significantly very high positive correlation between leave and shoot development during rapid multiplication (Table 9). This indicates that the concentration of Kinetin used promote both leaf and shoot development as high concentrations of some cytokinin (BAP) can inhibit leaf development will occur [2] and this will not favour mass propagation.

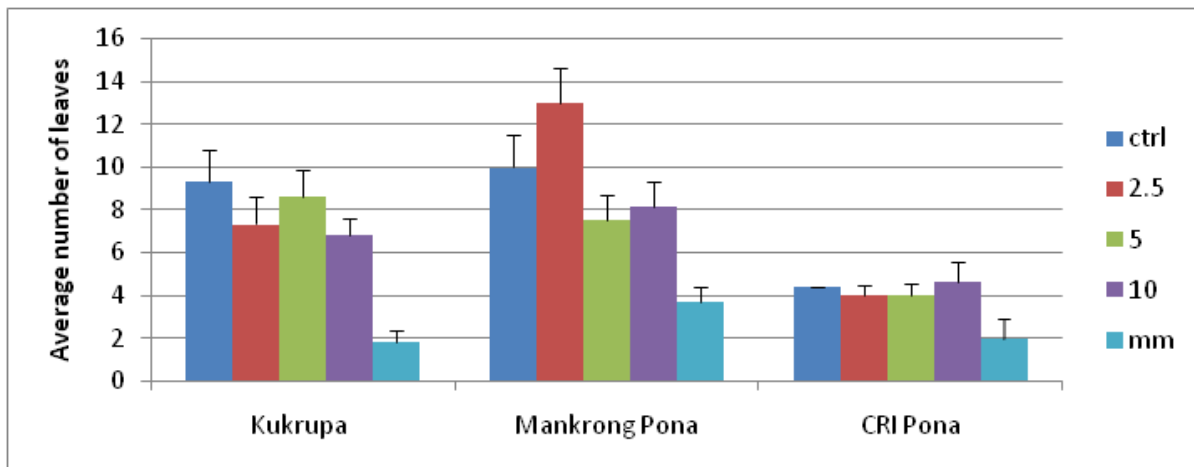


Figure 11. Development of Leaves in cultures of the three released varieties during rapid multiplication

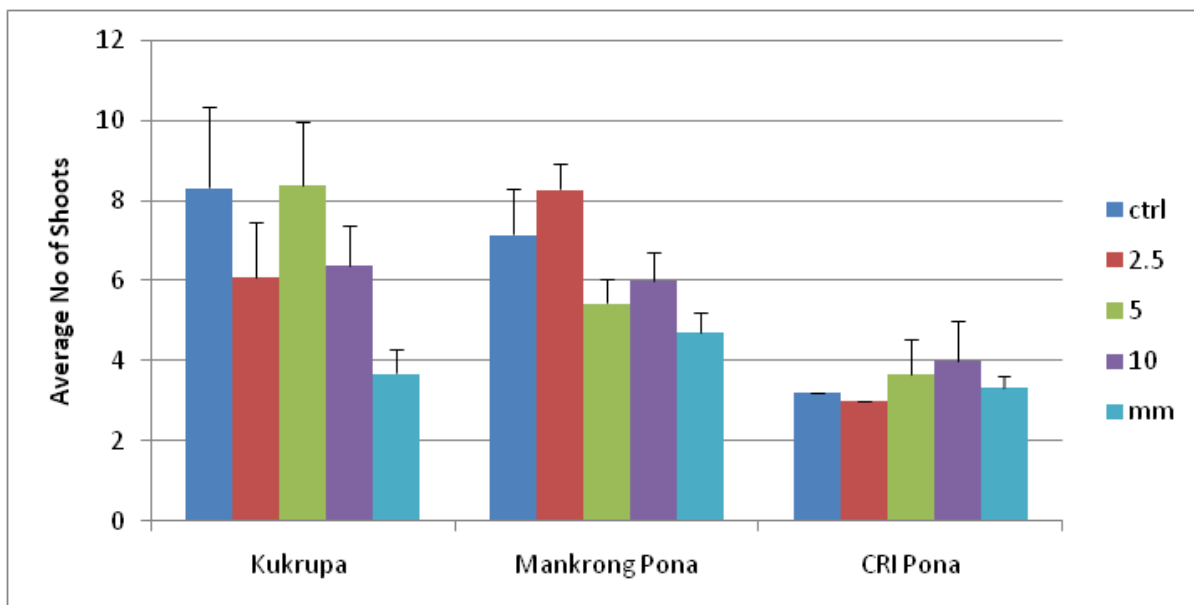


Figure 12. Development of Shoots in cultures of the three released varieties during rapid multiplication

The study considered biomass as an additional measure of growth. The data, as shown in Figure 13 below revealed that plant biomass was consistently low and on the medium labelled “mm”. The variety Kukrupa had the highest biomass on the control medium. Considering Mankrong Pona high biomass was on the control medium as well as 2.5 and 5 μ M Kinetin supplemented medium. Significantly high biomass was recorded for CRI-Pona cultures growing on 10 μ M Kinetin supplemented medium, it is the same medium on which highest number of leaves and shoots were observed for that variety. It is therefore possible that CRI-Pona accumulated biomass at the expense of sacrificing organ differentiation.

Media	Mean(\pm Stder)	Shoots	Leaves
0	Mean	6.000ab	7.857a
	Stder	0.839	0.988
2.5	Mean	6.421a	8.895a
	Stder	0.788	0.1.149
5	Mean	6.000ab	7.211a
	Stder	0.658	0.801
10	Mean	5.809ab	7.333a
	Stder	0.519	0.773
mm	Mean	3.952b	2.476b
	Stder	0.355	0.423

Similar letters are not significant according to Tukey test ($p < 0.05$); Stder – Standard error

Table 6. Comparison of effect of different media on nodal culture of Yam (Number of Shoots and Number of Leaves)

Media	Mean(\pm Stder)	Shoots	Leaves
CRI Pona	Mean	3.389b	3.778c
	Stder	0.617	0.919
Kukrupa	Mean	6.000a	5.875b
	Stder	0.462	0.689
Mankrong	Mean	6.182a	8.318a
	Stder	0.394	0.588

Similar letters are not significant according to Tukey test ($p < 0.05$); Stder – Standard error

Table 7. Comparison of effect of different varieties on nodal culture of Yam (Number of Shoots and Number of Leaves)

		Leaves	Shoot
Leaves	Pearson Correlation	1	.816**
	Sig. (2-tailed)		.000
	N	94	94
Shoot	Pearson Correlation	.816**	1
	Sig. (2-tailed)	.000	
	N	94	94

** . Correlation is significant at the 0.01 level (2-tailed).

Table 8. Correlations

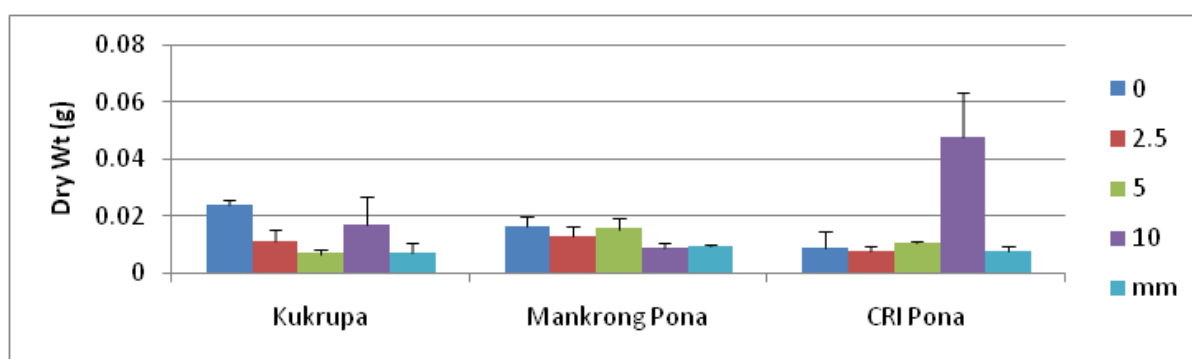


Figure 13. Dry weight and moisture content among the different treatments of the three released varieties

3.2. Fingerprinting results and discussion

Microsatellite in DNA represents repetitive DNA based on very short repeats such as dinucleotides, trinucleotides or tetranucleotides, consisting of repeats of a motif, and is otherwise referred to as Simple Sequence Repeats (SSR). These repeats serve as molecular markers by which genetic identity can be documented. Although there are several molecular marker systems namely: Amplified fragment length polymorphism (AFLP), DNA amplification fingerprinting (DAF), Inter-simple sequence repeat (ISSR), and Random amplified polymorphic DNA (RAPD) just to name a few, microsatellites or SSRs (Simple Sequence Repeats) has been reported as being useful for genotyping due to its high polymorphic information content (PIC). It is a codominantly inherited marker with locus specificity and genomic coverage is extensive. Also, systems provide simple PCR amplification detection methods. This present study used a set of yam simple sequence repeat (SSR) markers developed in different species of yam (*Dioscorea* sp.), where microsatellite-enriched bank was created from *Dioscorea alata*, *Dioscorea abyssinica* and *Dioscorea praehensilis*. That study identified and characterized 16 polymorphic loci, which were found to be transferable to species of other *Dioscorea* sections [19].

In this present study, when the 16 SSR primers were screened, 15 produced scorable bands. These 15 primers were used to screen a total of 25 yam accessions (including the three released varieties). Following amplification of PCR products, there were a total of 94 alleles and an average of 6.26 alleles per loci. Similarly when used to screen 22 *D. rotundata* accessions from Benin, 117 alleles were observed with an average of 7.3 alleles per loci [19]. The data obtained in this study was subjected to NTSYS analysis and this revealed the three released varieties clustering into one group (Figure 14). One *D. rotundata* accession from the CSIR-PGRRI collections was grouped with the released varieties. A similarity matrix (Table 9) established the percentage similarity between Mankrong-Pona and CRI-Pona to be 97%. The data obtained indicated that the released yam varieties are distinct from other *D. rotundata* accessions being conserved at the CSIR-PGRRI. It is argued that similarity above 95% is indicative that the two samples are duplicates. This has to be investigated further since *in vitro* growth rates of the two released varieties (Mankrong Pona and CRI Pona) are distinctively different in this current study. It is possible that the set of loci used to conduct molecular diversity assessment are unable to detect much differences in the released *Dioscorea* varieties as the SSR libraries used

to design the primers were generated from *Dioscorea alata*, *Dioscorea abyssinica* and *Dioscorea praehensilis* and not *D. rotundata*. The investigator who developed the primers however demonstrated that they are transferable to *D. rotundata*, although only 3 accessions of rotundata were used in that particular study [19].

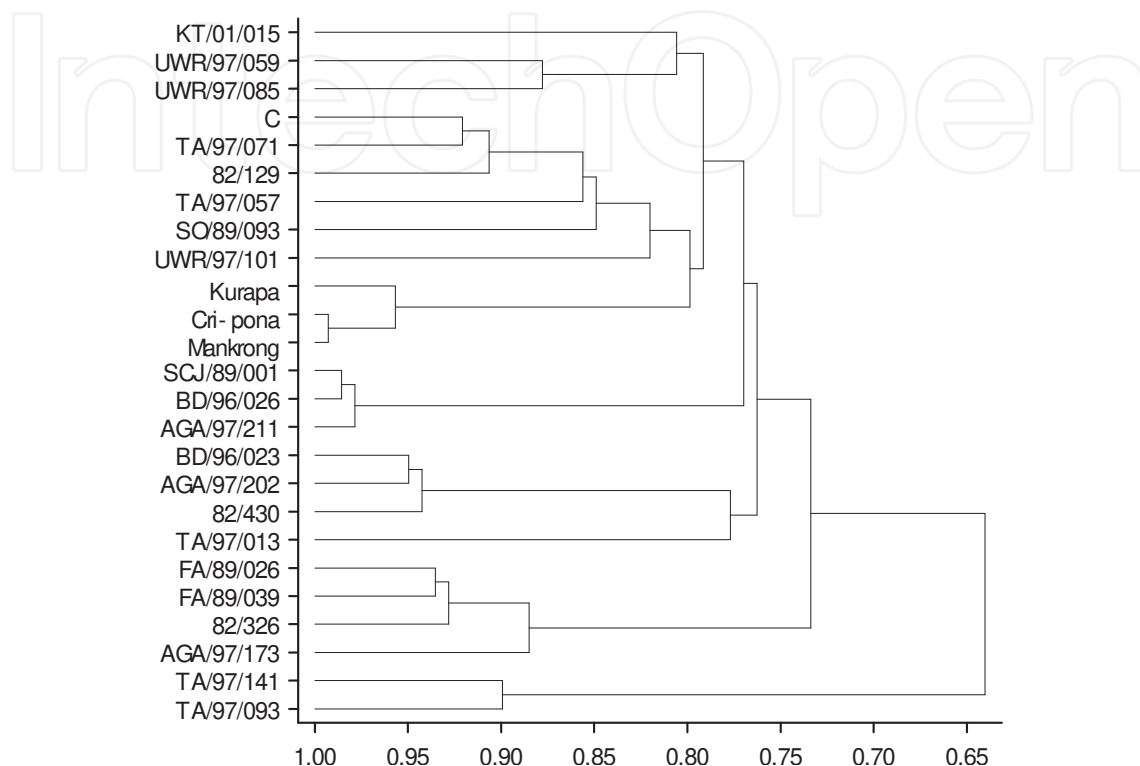


Figure 14. Dendrogram of 25 accessions of yam based on unweighted neighbour joining cluster analysis

	Dr 013	Dr 026	Dr 089	Dr 052	Da 110	Da 130	Da 137	Da 143	Dd 187	Dd 183	Dd 186	Db 187	Db 193	Db 201	De 202	De 208	De 215	Dc 109	Dc 221	Dc 249	Dc 251	Qi-pona	Mankrong	Kurapa	
Dr 013	1																								
Dr 026	0.10	1																							
Dr 089	0.66	0.21	1																						
Dr 052	0.40	0.42	0.69	1																					
Da 110	0.24	0.16	0.27	0.46	1																				
Da 130	0.16	0.14	0.23	0.47	0.85	1																			
Da 137	0.16	0.20	0.28	0.47	0.85	0.85	1																		
Da 143	0.23	0.27	0.25	0.44	0.72	0.58	0.68	1																	
Dd 187	0.02	0.21	-0.13	0.04	-0.01	-0.03	0.07	0.24	1																
Dd 183	-0.01	0.24	-0.10	0.08	0.02	0.00	0.11	0.23	0.89	1															
Dd 186	0.02	0.27	-0.07	0.12	0.00	-0.02	0.09	0.16	0.89	0.94	1														
Db 187	-0.02	-0.02	-0.02	0.04	0.15	0.12	0.22	0.14	0.28	0.37	0.36	1													
Db 193	-0.11	-0.01	-0.09	-0.10	0.02	-0.02	0.03	-0.09	-0.05	0.04	0.04	0.37	1												
Db 201	-0.11	-0.01	0.00	-0.06	-0.03	-0.07	-0.07	-0.14	-0.15	-0.06	-0.07	0.23	0.78	1											
De 202	0.08	-0.08	0.10	0.12	0.17	0.20	0.20	0.05	-0.07	0.02	-0.02	0.24	0.20	0.20	1										
De 208	0.05	-0.15	0.14	0.04	-0.07	-0.09	-0.09	-0.16	-0.04	-0.03	-0.01	0.09	-0.01	0.06	0.33	1									
De 215	0.23	0.03	0.23	0.13	-0.03	0.00	0.00	-0.10	-0.09	-0.07	-0.04	0.04	0.04	-0.06	0.20	0.38	1								
Dc 109	-0.01	0.33	0.20	0.24	0.17	0.18	0.23	0.06	-0.05	-0.01	0.03	0.05	0.07	-0.02	0.08	0.16	0.56	1							
Dc 221	0.02	0.36	0.22	0.25	0.14	0.15	0.20	0.03	-0.07	-0.03	0.01	0.02	0.07	-0.02	0.06	0.15	0.63	0.96	1						
Dc 249	0.04	0.33	0.20	0.19	0.12	0.13	0.18	0.01	-0.10	-0.06	-0.02	0.00	0.07	-0.07	0.03	0.09	0.56	0.91	0.96	1					
Dc 251	0.03	0.10	0.16	0.31	0.08	0.07	0.07	-0.01	0.11	0.13	0.15	0.27	0.17	0.09	0.15	0.43	0.32	0.28	0.27	0.12	1				
Qi-pona	0.02	0.34	0.19	0.20	-0.06	-0.03	-0.03	-0.02	-0.01	-0.04	-0.01	-0.05	0.00	0.05	-0.01	-0.04	0.25	0.31	0.38	0.31	0.30	1			
Mankrong	0.04	0.36	0.21	0.22	-0.05	-0.02	-0.02	-0.01	0.01	-0.02	0.01	-0.03	0.02	0.07	0.01	-0.04	0.27	0.33	0.40	0.33	0.31	0.97	1		
Kurapa	-0.01	0.31	0.07	0.08	-0.09	-0.06	-0.06	-0.04	-0.04	-0.07	-0.04	-0.02	0.10	0.10	0.08	-0.11	0.17	0.25	0.33	0.30	0.13	0.89	0.85	1	

Table 9. Jaccard's coefficient similarity matrix of 25 yam genotypes using 15 SSR primers

Data analysis using population genetic analysis software PopGen32 [22], revealed 100% overall polymorphic loci overall the populations. According to the genetic variation statistics at all

loci [12] presented in Table 10, the mean number of effective alleles (ne) was 4.05 ± 1.43 , loci DaIA01 and Dab2C05 had the highest value at 6.82 ± 1.2 , however, locus Dab2E07 had the lowest number at 1.73 ± 0.7 . The mean observed number of alleles was 5.33 ± 1.45 . Again, loci DaIA01 and Dab2C05 recorded the highest number of observed alleles (8), however, locus Dab2E07 had the lowest number of alleles (3). With an average allele sample size (n) of 38, locus Dpr3F04 had a high sample size of 42 whereas a value of 26 was recorded in loci Dpr3F10 and Dab2E07. The mean, highest (loci-DaIA01) and lowest (locus Dab2E07) Shannon index (I) were 1.46 ± 0.37 , 1.99, and 0.74 overall respectively, indicating that locus DaIA01 estimated the highest level of genetic diversity among the samples used in this study. The overall allele frequency (Table 11) revealed that Allele A of locus Dpr3F04 was the least frequent allele (0.022) among the samples as this allele was unique to only one sample in the *D. alata* population. However allele A in locus Dab2E07 was the most frequent allele (0.73) as this allele was present in *D. rotundata*, *alata*, *cayenensis* and the released varieties, however it was absent in *D. alata* sample, *D. bulbifera*, *D. dumentorum* and *D. esculenta*. The Nei's original measures of genetic identity and genetic distance [13] as shown in Table 12 revealed that the released varieties population are closer to *D. cayenensis* accessions (0.57) than *D. rotundata* population (0.89) used in this study. However they were very distantly related to *D. bulbifera* (2.39).

Locus	Sample Size	na*	ne*	I*
DaIA01	44	8.0000	6.8169	1.9937
DaIC12	40	6.0000	4.0609	1.5552
DaID08	42	5.0000	4.3881	1.5384
DaIF08	44	4.0000	3.6391	1.3278
Da3G04	42	5.0000	4.0091	1.4837
Dab2CO5	44	8.0000	6.8169	1.9904
Dab2C12	44	5.0000	3.3379	1.3307
Dpr3D06	30	4.0000	3.1915	1.2609
Dab2D06	32	6.0000	5.1717	1.7020
Dab2D08	34	5.0000	2.8614	1.2822
Dpr3F04	46	5.0000	3.5986	1.3764
Dpr3F10	26	7.0000	5.2000	1.7756
Dpr3F12	42	5.0000	3.0625	1.3108
Dab2E07	26	3.0000	1.7333	0.7436
Dab2E09	32	4.0000	2.9767	1.2342
Mean	38	5.3333	4.0576	1.4604
St. Dev		1.4475	1.4276	0.3206

* na = Observed number of alleles

* ne = Effective number of alleles

* I = Shannon's Information index

Table 10. Summary of Genetic Variation Statistics for All Loci

Allele \ Locus	DaIAO1	DaIC12	DaID08	DaIF08	Da3G04	Dab2CO5	Dab2C12	Dpr3D0
Allele A	0.1818	0.0500	0.2857	0.2955	0.1429	0.0455	0.3409	0.4333
Allele B	0.2273	0.3750	0.1190		0.1667	0.1136	0.3864	0.3000
Allele C	0.1136	0.2000	0.1905		0.2619	0.1591	0.0909	0.1333
Allele D	0.0909	0.1000	0.2857	0.2955	0.3571	0.1364	0.1591	0.1333
Allele E	0.1136		0.1190	0.2955	0.0714	0.1136		
Allele F	0.1136	0.0500		0.1136		0.2273	0.0227	
Allele G	0.0455	0.2250						
Allele H	0.1136					0.1364		
Allele I						0.0682		

Allele \ Locus	Dab2D06	Dab2D08	Dpr3F04	Dpr3F10	Dpr3F12	Dab2E07	Dab2E09
Allele A	0.0625	0.0588	0.0217	0.0385	0.1667	0.7308	0.1875
Allele B	0.1875	0.0588	0.1957	0.0769	0.1429	0.1923	0.5000
Allele C	0.2188	0.5294	0.3478	0.3077	0.5000	0.0769	0.1250
Allele D	0.2500	0.1765	0.3261	0.0769	0.1667		
Allele E	0.0938	0.1765		0.1538	0.0238		0.1875
Allele F	0.1875			0.1923			
Allele G				0.1087	0.1538		
Allele H							
Allele I							

Table 11. Overall Allele Frequency :

pop ID	1	2	3	4	5	6	7
1	***						
2	0.4794	***					
3	1.4823	1.4745	***				
4	0.9579	0.9490	1.6502	***			
5	0.7289	1.1958	1.6637	0.7025	***		
6	0.5685	0.9745	2.1447	0.9580	0.3903	***	
7	0.8903	1.4844	2.3915	1.3699	0.9354	0.5665	***

Table 12. Nei's original measures of genetic identity and genetic distance

4. Conclusion

In this study, *in vitro* performance of the three released yam varieties revealed that they respond differently to the same medium. Maximising the used of tissue culture for mass

propagation of clean healthy planting materials is crucial and to achieve this individual varieties have to be micropropagated in appropriate medium. Other growth regulators may have to be screened to further optimise the performance of CRI-Pona. The study has made fingerprint information available to monitor the integrity of the released varieties.

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