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Enhanced mass regeneration of pro-vitamin A cassava (*Manihot esculenta* Crantz) varieties through multiple shoot induction from enlarged axillary buds

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Abstract

An efficient mass propagation system for a rapid delivery of pro-vitamin A cassava varieties to farmers is essential for acquiring food sufficiency and reducing vitamin A deficiency in Africa. The study investigated multiple shoot inductions from axillary buds of three pro-vitamin A enriched cassava varieties using a combination of 10 mg/l 6-benzylaminopurine (BAP) and 0.1-0.5 mg/l naphthalene acetic acid (NAA). Growth, photosynthetic pigment content and molecular stability of the regenerated plants with random amplified polymorphic DNA (RAPD) markers are also assessed. Shootbud formation in TMS 30572 variety was found to be 8.9% higher than in the three pro-vitamin A varieties. But, the number of regenerated shoots was higher by 7.3% in pro-vitamin A varieties than in TMS 30572. The formation of roots was outstanding in UMUCASS 38. Root carotene contents of regenerated and mother plants were not different. The highest number of shootbuds per explant and frequency of regenerated shoots were obtained through a medium containing 10 mg/l BAP and 0.3 mg/l NAA. The RAPD analysis of regenerated plants showed a uniform profile of 1628 bands among the regenerants and mother plants. The study concluded that a combination of 10 mg/l BAP and 0.3 mg/l NAA enhances a multiple shoot induction with no genetic infidelity of regenerated plants.

Key words: cassava, pro-vitamin A, genetic fidelity, mass propagation, shoot culture

Abbreviations

BAP – 6-benzylaminopurine; IBA – indole butyric acid;
NAA – naphthalene acetic acid; RAPD – randomly amplified polymorphic DNA

Introduction

Food insecurity and vitamin A deficiency (VAD) are two major problems affecting the development, health and social welfare in developing countries (Maziya-Dixon et al., 2006; Bull et al., 2011).VAD lowers immunity, impairs vision and leads to blindness and death, particularly in children. It affects 20% of pregnant women and 30% of children under the age of 5 in West Africa (Maziya-Dixon et al., 2006). Cassava (*Manihot esculenta* Crantz) is identified as a staple crop to eliminate food insecurity and VAD because of the increasing daily intake of cassava products in developing countries (Maziya-Dixon et al., 2006; Bull et al., 2011). Reserved carbohydrates in cassava tubers are used to manufacture livestock feed and human foods in various forms. In addition, cassava starch is used for production of ethanol, food additives, agrochemicals and pharmaceuticals (Nweke et al., 2002). Young cassava leaves are an important component of local vegetables for poor people in developing countries (Nweke et al., 2002; El-Sharkawy, 2004). Furthermore, cassava production is compatible with low-external input crop farming practised in developing countries because the crop produces tuber yields under nutrients and moisture stress conditions (El-Sharkawy, 2004). The harvesting of cassava tubers can be delayed for a short period of time without deterioration, which makes the

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crop a reliable source of carbohydrates in periods of food scarcity (Nassar and Ortiz, 2010).

In 2011, the Nigerian Government approved the release of three new "yellow" varieties of cassava rich in beta-carotene, the precursor of vitamin A. The varieties, UMUCASS 36 (TMS 01/1368), UMUCASS 37 (TMS 01/1412) and UMUCASS 38 (TMS 01/1371), were selected from a pool of over 25,000 progenies, evaluated across agroecologies in Nigeria for 5 years (Ukenye et al., 2013; Oparinde et al., 2014). Pro-vitamin A cassava was developed through conventional breeding similar to most other improved varieties cultivated by farmers in Africa. The production and distribution of pro-vitamin A cassava varieties in developing countries has the potential to reduce food insecurity and VAD (Aniedu and Omodairo, 2012).

The consumer demand for food products from pro-vitamin A cassava is increasing in Nigeria and other African countries as a result of improved awareness of its health benefits (Oparinde et al., 2014). The consumers' acceptance has expanded the cultivation scale of pro-vitamin A cassava. This in turn has led to the shortage of stem cuttings during the critical planting season period because cassava is propagated vegetatively. Moreover, the scarcity of cassava cuttings is increasing by the low multiplication rate of this important crop. For example, from a mature cassava plant, only 10-25 cuttings can be obtained per year. Thus, inadequacy of stem cuttings is a constraint for rapid propagation of pro-vitamin A cassava varieties. Shoot culture is a technique that can accelerate rapid propagation of new cassava varieties (Kondamudi et al., 2009; Bull et al., 2011). A shoot culture propagated via multiple shoot induction allows generating about 100 plantlets from 1 nodal explant annually (Rostami et al., 2013). This method does not require a large area of cultivation and has no constraints imposed by growth seasons (Konan et al., 1997). Also, plants regenerated from a shoot culture are uniform and free from pathogens that cause devastating diseases in cassava crops. In addition, unlike cuttings, shoot cultures are not bulky, and therefore facilitate a rapid transfer of pro-vitamin A cassava varieties to farmers and other laboratories for research purposes across various regions in Africa. Moreover, shoot culture can also serve as a means for long- and short-term conservation of the varieties.

Application of shoot culture technique is routine for the production of disease-free propagules of elite cassava varieties since its first use about 43 years ago (Konan et al., 1997; Abdalla et al., 2013). Multiple shoots are induced from meristems and immature leaves cultured on media containing phytohormones (a cytokinin alone or in combination with auxin) that break down the apical dominance of the meristem and allow shootbuds to proliferate. Konan and coworkers (1997) described an axillary bud culture where the best cultivar TMS 30555 produced at least 25 shoots per explant from 63% of the explants on a medium with 10 mg/l BAP. Similarly, Mussio and coworkers (1998) successfully induced adventitious shoots from an immature leaf on a medium containing 2,4-D,which were further transferred on another medium containing 23 μM zeatin. Villaluz (2006) developed a method for rapid meristem development and mass propagation method in cassava using liquid and solid media containing 0.25 mg/l GA_3, 0.1 BAP or 0.2 mg/l NAA. Scientists have postulated that the most efficient multiple shoot induction protocol in cassava should produce 1.2×10^{20} shoots per year, which is practically difficult to achieve. Production of 400,000 plants per year was reported in China by propagating NanZhi 188, a high-yielding variety, using an efficient low-cost *in vitro* propagation system for the distribution to farmers (Villaluz, 2006; Kondamudi et al., 2009; Mapayi et al., 2013). In our previous study on shoot induction in a medium fortified with 10 mg/l BAP from the three pro-vitamin A varieties, the best result was recorded from UMUCASS 36 with 73.4% regenerated shoots and 7.2 shoots per explant (Opabode et al., 2015). The present study was designed to increase the number of regenerated plants per explant compared with the previous effort by including 10 mg/l of BAP and 0.1 mg/l NAA in the shoot induction medium. The objectives of this study were: 1) to induce a multiple shoot from an axillary bud using BAP and NAA, 2) to assess the molecular stability of the regenerated plants by RAPD markers and 3) to examine the growth and photosynthetic pigments of regenerated plants.

Materials and methods

Source and micropropagation of in vitro plantlets

Nodal segment explants of *in vitro* plantlets of three (UMUCASS 36, UMMUCASS 37 and UMUCASS 38) pro-vitamin A cassava varieties and a control (TMS 30572) used in this study were obtained from the Tissue

Culture Laboratory, Genetic Resources Center, International Institute of Tropical Agriculture (IITA), Ibadan. The carotenoid contents of UMUCASS 36, UMUCASS 37, UMUCASS 38 and TMS 30572 were 6.9, 7.4, 7.8 and 0.9 ppm, respectively (Agbaje et al., 2007).

Basal medium and culture conditions

The basal medium (BM) was used in all experiments unless otherwise stated. The BM consisted of 4.43 mg/l Murashige and Skoog (MS) mineral salt (Murashige and Skoog, 1962, Sigma-Aldrich, USA), 8 g/l agar and 30 g/l sucrose. The pH of the BM was adjusted to 5.8 by HCl (1 N) or NaOH (1 N) before autoclaving at 121°C for 15 min at 1.05 kg/cm^2 pressure. All cultures were kept at 26 ± 1°C and a 16-h photoperiod with 25 μmol/m^{2}s^{-1} irradiation provided by Philips 32-W cool white fluorescent lamps (Philips Electric Company, Hyderabad, India).

Enlargement of axillary buds and multiple shoot induction

Multiple shootbud induction from axillary buds was achieved using a procedure by Konan and coworkers (1997), with modifications. One hundred and eighty nodal segments (5-7 cm long) of explants from *in vitro* shoot cultures (3-5 weeks old) of each cassava variety were cut, placed horizontally and incubated on a BM supplemented with 10 mg/l of BAP for 7 days. In the experiment, to induce shootbuds, 120 enlarged axillary buds of each cassava variety were removed from the nodal explants with a scalpel and subsequently subcultured on the BM fortified with 10 mg/l BAP and 6 concentrations of NAA (0.0, 0.1, 0.2. 0.3, 0.4 and 0.5 mg/l) for 4weeks. Data on the survival of isolated axillary buds, the frequency of shootbud formation and the number of shootbuds per explant were recorded.

Shoot elongation and rooting

For shoot elongation, 180 shootbuds (1-2 mm) of each cassava variety were detached from the shootbud clumps and transferred individually to the BM supplemented with 1.0 mg/l BAP and 10.0 mg/l IBA. Observations were made on the survival of shootbuds and the number of regenerated (elongated) shoot 2 weeks after transfer. Thereafter, 120 regenerated shoots of each variety were transferred on a hormone-free basic medium for rooting as outlined by Li and coworkers (1998). Data on the frequency of root formation and the number of roots per plantlet 3 weeks after transfer were recorded.

Acclimatization and soil establishment of regenerated plants

One hundred and twenty regenerated plantlets (4-5 cm) with well-developed roots were carefully removed from the culture tubes and placed in plastic pots filled with peat and vermiculite (50 : 50, AS Jiffy Products Ltd, Norway). Plantlets were placed in a humidity chamber, grown at 22-26°C for 3 weeks and gradually exposed to a source of natural light for a period of 2 weeks. Thereafter, the regenerated plants were transferred to plastic pots filled with loamy soil (pH = 7.2; organic carbon = 4.3%) and grown for 12 weeks.

DNA extraction and RAPD analysis

To determine the genetic integrity of the regenerated plants, DNA was extracted from young leaves (0.5-0.8 g) of two plants per treatment as described by Dellaporta et al., (1983). The DNA of 44 plants comprising 40 regenerated and 4 mother plants were analyzed. Purification of crude DNA was achieved with RNase A followed by washing thrice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1 v/v/v) and subsequently with chloroform/isoamyl alcohol (24 : 1 v/v). Nanodrop spectrophotometer was used to quantify the amount of isolated DNA. The quality of the DNA was verified by running 2 μl of the DNA alongside a molecular weight λPst I (Bioline, USA) on 1% agarose gel in 1 × TAE (Tris Acetate EDTA) buffer at 500 V for 1 h. For RAPD analysis, DNA samples were diluted to 20 ng/μl.

Seven random 10-mer primers (Operon Tech, Alameda, USA) from B and C series (OPB06, OPB08, OPB12, OPC01, OPC02, OPC05, OPC06) were used for the RAPD analysis. The clarity of banding pattern from previous studies formed the basis of selection of the primers (Opabode et al., 2015). The RAPD analysis was performed as described by Williams and coworkers (1990). PCR was performed in a volume of 25 μl containing 25 ng of template DNA, 2.5 ml of 10X assay buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 0.1% gelatine), 1.5 mM $MgCl_2$, 200 μM each of dNTPs,15 ng of primer and 0.5 U Taq DNA polymerase (Bioline, USA). DNA amplification was performed in a thermal cycler (PTC 2000, MJ Research, India). In the first step, the samples were held at 94°C for 5 min for complete

denaturation of the template DNA. Second step consisted of 42 cycles with 3 ranges of temperature, i.e. at 92°C for 1 min denaturation of template DNA, at 37°C for 1 min for primer annealing and at 72°C for 2 min for primer extension followed by running the samples at 72°C for 7 min to complete the extension. The PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide [0.5 μg/ml of gel solution in TAE buffer (40 mM Tris, 20 mm acetic acid, 20 mM EDTA to pH 8)] for 3 h at 60 V. The size of the amplicons was determined using the size standard GeneRuler 100 bp DNA Ladder Plus (Bioline, USA). DNA fingerprints were visualized under UV light and photographed using a gel documenting system (Bio-Rad, CA, USA). The RAPD analysis using each primer was repeated at least twice to establish the reproducibility of the banding pattern of different DNA samples of cassava.

Growth rate and pigments extraction of the regenerated plants

The growth rate (GR) and the amount of photosynthetic pigments of the regenerated plants were measured to assess their growth vigor. GR was determined from the equation: GR = W2 – W1/t2 –t1,where W2 and W1 are dry weights at two successive harvests, t2 and t1, respectively. Pigments were extracted from fresh leaves (1 g) with 80% acetone following homogenization. The homogenized mixture was separated by centrifugation at 5000 × g for 10 min. The absorbances of the supernatant were measured using a spectrophotometer at the following wavelengths: 645, 653, 662 and 664 nm, for chlorophyll a and b and 470 nm for carotene as outlined by Lichtenthaler and Wellburn (1985). Measurements were performed in triplicates. The equations used for calculations are as follows:

Chlorophyll a = 11.75 A662 – 2.350 A645;

Chlorophyll b = 18.61 A645 – 3.960 A662; and

Carotenoids = 1000 A470 – 2.270 Chl a – 81.4 Chl b/227

Determination of root beta-carotene content

Cassava tuberous roots from each variety were washed with clean water, peeled and cut into flakes. The flakes were dried at 50°C and milled into cassava flour. The beta-carotene content of the tubers was determined by the AOAC method (1997). Two grams of the samples were hydrolyzed with 25 ml of 5% alcoholic KOH and extracted thrice with 50 ml petroleum ether. The petroleum ether fraction was evaporated to dryness and the pellet was dissolved in 10 ml chloroform. The absorbance was measured using a spectrophotometer at 440 nm. The concentrations of carotene in test samples were read from the standard curve.

Mass production of plantlets by nodal explant

The number of plantlets produced per explant per year was calculated for each cassava variety based on the results of the present study using the formulas of Pennell (1987): $Y = A^n \times B \times F1 \times F2 \times F3$, where Y is the number of plantlets/plants that could be produced; A is the number of shoots produced at each subculture period (multiplication factor); n is the number of subculture at a certain period (per year); B, is the number of initial explant which grow; F1 is the percentage of successful culture at the stage of shoot induction; F2 is the percentage of successful culture at the stage of shoot elongation and F3 is the percentage of successful acclimatization.

Experimental design and statistical analysis

All regeneration experiments were arranged in a completely randomized design with sixreplicates. About 30 explants represented a replicate in nodal segment enlargement and shoot elongation experiments while 20 explants constituted a replicate for the rest of the study. The percentage and count data were subjected to arcsine and square root transformations to normalize variances as described by Opabode et al. (2015). The data were further subjected to one-way analysis of variance to detect means differences. Means were separated by Tukey's test at 5% probability level.

Results

Multiple shoot induction, root formation, acclimatization and survival of regenerated plants

The increase in size of axillary buds (Fig. 1A) commenced soon after nodal explants were placed on basal media supplemented with 10 mg/l BAP. Within 7 days after culture initiation, axillary buds enlarged up to 2-3 mm. An average 90% of the axillary buds were enlarged and they retained the green color. Surviving isolated axillary buds (Fig. 1B) increased in size following transfer on basal media containing 10 mg/l BAP and 0.1 mg/l NAA and produced clumps of shootbuds (Fig. 1C) by the 4th week. Excised shootbuds became elongated (Fig. 1D),

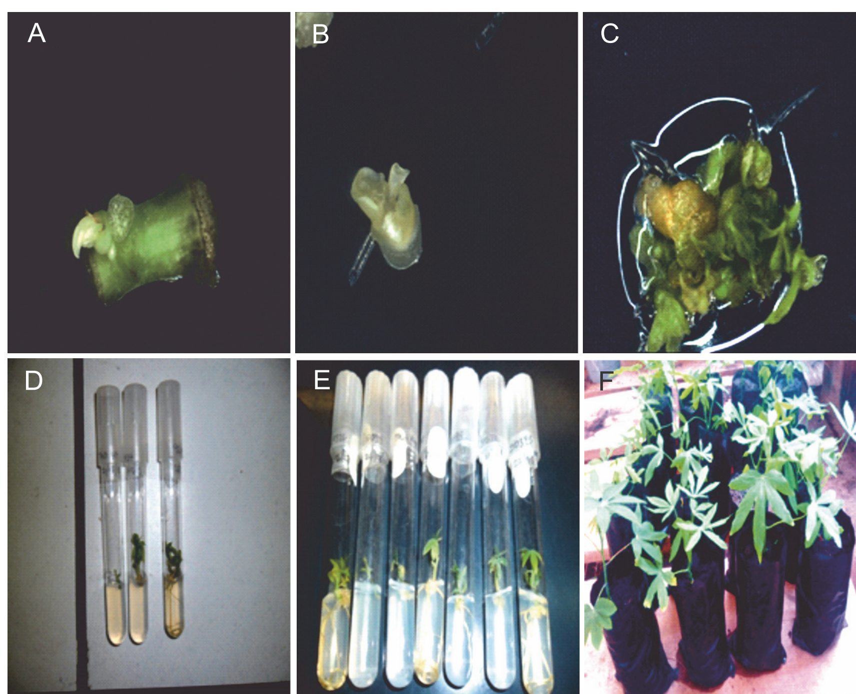

Fig. 1. Multiple shoot induction in UMUCASS 36: A) enlarged axillary bud; B) isolated axillary bud on induction medium; C) multiple shootbud emerging after 3 weeks of growth; D) elongated shoot on elongating medium; E) regenerated shoots producing roots and F) regenerated plants following acclimatization in a containment facility

produced roots (Fig. 1E) at the 6th and 8th weeks, respectively, and formed growing young plants (Fig. 1F) following acclimatization.

The influence of cassava A variety on frequencies of shootbud formation, regenerated shoots, root formation and number of roots per plantlet was significant ($P < 0.05$) – Table 1. No significant influence of the variety on other parameters was detected. The influence of NAA concentration on the survival of isolated axillary buds, the number of shootbuds per explant, survival of shootbuds, frequency of root formation, number of roots per plantlet, survival of regenerated shoots, leaf chlorophyll content and root carotene content at $P < 0.05$ and the frequency of shootbud formation, root formation and GR of the regenerated plants at $P < 0.01$ were significant (Table 1). The interaction of various cassava varieties with the concentration of NAA in growth media was not significant ($P > 0.01$) for any parameter tested.

Shootbud formation in TMS 30572 was higher by 8.9% than in the other three cassava pro-vitamin A varieties tested (Table 2). But, the number of regenerated shoots of cassava pro-vitamin A varieties was higher by 7.3% than TMS 30572. The formation of roots was outstanding in UMUCASS 36 (98.3%) and UMUCASS 38 (96.45) but worse in TMS 30572 (77.3%). However, root proliferation was best (10.2 roots per plantlet) in UMUCASS 38. Root carotene content of pro-vitamin A varieties was higher by 28.2%than that of TMS 30572 (Table 2). A culture medium free from NAA generated the highest losses of axillary explants, while on culture media containing 0.3-0.5 mg/l NAA no loss of isolated axillary buds was recorded. Similarly, all explants transferred on media containing 0.3-0.5 mg/l NAA gave the highest frequency of shootbud formation while the medium that was free of NAA had the lowest frequency of shootbud formation. The number of shootbuds per explant on the medium containing 0.3 mg/l NAA (37.6 shootbuds/explants) was outstanding and nearly doubled that of other NAA concentrations. The best survival of shootbuds (100%) on a shoot elongation medium was obtained from explants growing on media containing 0.3 and 0.4 mg/l NAA. Following shoot elongation, the best regenerated shoot frequency (95.7%) was obtained on media with 0.3 mg/l NAA and the lowest (68.7-69.5%) in media containing no NAA or 0.1 mg/l NAA. All the shoots obtained from media containing 0.2-0.5 mg/l NAA

Table 1. Means square of growth parameters of four cassava cultivars

SV	DF	SIAB	SF	NSE	SURV	RS	RF	NR	SURVR	GR	CHL
Replicate	5	25.7	32.1	18.7	28.4	18.4	16.2	33.2	15.8	45.7	35.8
Variety (V)	3	78.2^NS	56.6*	48.3^NS	78.4^NS	45.3	34.2*	47.8*	23.5^NS	89.4^NS	77.1^NS
Conc. (C)	5	98.7*	103.6^^	207.9*	234.5*	225.4***	105.8*	127.8*	236.1*	245.2**	236.7*
VX C	15	43.8^NS	51.9^NS	67.5^NS	72.4^NS	56.2^NS	58.4^NS	88.3^NS	64.6^NS	74.4^NS	83.5^NS
Error	115	25.7	30.8	31.9	37.4	36.2	29.6	34.8	28.4	43.4	33.7
R^2 [%]		67	74	68	72	70	68	47	42	57	52
CV [%]		8.9	15.8	18.6	15.8	11.9	5.7	19.5	21.6	17.6	18.5

* – significant at $P = 0.05$ probability level, ** – significant at $P = 0.01$ probability level, SV – source of variation, DF – degree of freedom, SIAB – survival of isolated axillary bud, SF – frequency of shootbud formation, NSE – number of shootbud per explant, SURV – survival of shootbud, RS – frequency of regenerated shoot, RF – frequency of root formation, NR – number of root per plantlet, SRVR – survival of regenerated plant, GR – growth rate of regenerated plant, CHL – total chlorophyll content

Table 2. Frequencies of shootbud formation, regenerated shoot, root formation and number of root per plantlet as affected by variety

Variety	Shootbud formation [%]	Regenerated shoot [%]	Root formation [%]	Number of root per plantlet	β -carotene [ppm]
UMUCASS 36	84.1 ± 5.6 c	90.4 ± 4.8 a	98.3 ± 3.5 a	8.2 ± 2.3 b	7.1 a
UMUCASS 37	88.2 ± 5.0 b	87.8 ± 3.8 a	85.2 ± 3.8 b	7.6 ± 2.7 b	7.4 a
UMUCASS 38	84.5 ± 4.3 c	83.4 ± 3.5 a	96.4 ± 3.7 a	10.2 ± 2.1 a	7.8 a
TMS 30572	93.2 ± 4.2 a	63.4 ± 3.7 c	77.3 ± 5.2 c	8.4 ± 1.8 b	0.9 b
Mean	87.5	81.3	89.3	8.6	5.8

Values are means (± standard error) of three replicates; means followed by different letters in same column are significantly different at 5% level of probability according to Tukey Test

Table 3. Effect of NAA on regeneration of pro-vitamin A cassava varieties

NAA concentration [mg/l]	SIAB	Shootbud formation [%]	Number of shootbud/explant	Survival of shootbud [%]	Regenerated shoot [%]	Root formation [%]	Number of root/plantlet
Control	66.3 ± 3.6 d	72.5 ± 8.4 d	8.1 ± 3.4 d	96.9 ± 8.3 b	68.7 ± 4.5 d	89.3 ± 7.6 c	6.6 ± 2.0 c
0.1	87.4 ± 5.6 c	83.5 ± 7.4 c	10.5 ± 3.2 c	94.5 ± 9.1 b	69.5 ± 6.7 d	95.6 ± 8.6 b	8.6 ± 2.3 b
0.2	92.9 ± 6.7 b	94.7 ± 6.4 b	12.5 ± 2.1 c	98.6 ± 8.8 b	75.8 ± 5.3 c	100.0 ± 0.0 a	10.9 ± 2.4 b
0.3	100.0 ± 0.0 a	100.0 ± 0.0 a	37.6 ± 2.8 a	100.0 ± 0.0 a	95.7 ± 8.7 a	100.0 ± 0.0 a	17.9 ± 3.4 a
0.4	100.0 ± 0.0 a	100.0 ± 0.0 a	25.0 ± 4.5 b	100.0 ± 0.0 a	83.5 ± 6.0 b	100.0 ± 0.0 a	15.0 ± 4.5 a
0.5	100.0 ± 0.0 a	100.0 ± 0.0 a	23.8 ± 4.2 b	85.7 ± 5.3 c	80.5 ± 8.8 b	100.0 ± 0.0 a	14.8 ± 3.4 a

Values are means (± standard error) of three replicates; means followed by different letters in samecolumn are significantly different at 5% level of probability according to Tukey Test; SIAB – survival of isolated axillary bud

produced roots when regenerated shoots of each variety were transferred on a hormone-free basic medium for rooting. Moreover, the number of roots per plantlet was the highest (14.8-17.9 roots/plantlet) in plantlets obtained from media containing 0.3-0.5 mg/l NAA.

Survival, growth and photosynthetic pigments of regenerated plants

The percentage of the survived regenerated plants, their GR, leaf chlorophyll a and b and carotenoid content improved as the concentration of NAA in the media was

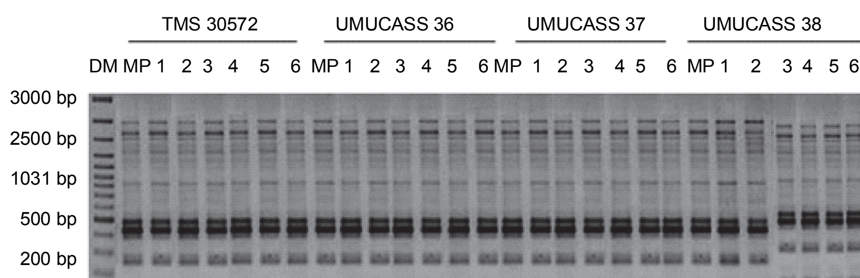

Fig. 2. Randomly amplified polymorphic DNA analysis by the primer OPB12 of 4 mother plants and 24 regenerated plants. DM – GeneRuler ladder; MP – mother plants; 1 – regenerated plants on medium containing 10 mg/l BAP alone; 2 – regenerated plants on medium containing 10 mg/l BAP and 0.1 mg/l NAA; 3 – regenerated plants on medium containing 10 mg/l BAP and 0.2 mg/l NAA; 4 – regenerated plants on medium containing 10 mg/l BAP and 0.3 mg/l NAA; 5 – regenerated plants on medium containing 10 mg/l BAP and 0.4 mg/l NAA; 6 – regenerated plants on medium containing 10 mg/l BAP and 0.5 mg/l NAA

increasing up to 0.3 mg/l NAA, before it started declining with a further increase in NAA concentration (Table 3). All plantlets obtained from media containing 0.3-0.5 mg/l NAA survived after acclimatization. The GR of young regenerated plants under greenhouse conditions ranged from 5.2 to 7.4 g/day with plants grown on media containing 0.2-0.5 mg/l NAA. The largest number of plantlets produced per year was obtained from explants grown on a medium containing 0.3 mg/l NAA and the smallest in control media and those containing 0.1-0.2 mg/l NAA (Table 3). Leaf chlorophyll a and b and carotenoid contents of plants grown on media containing 0-0.2 mg/l NAA were significantly ($P < 0.05$) higher than in those obtained from media containing 0.4-0.5 mg/NAA.

RAPD analysis

To establish the genetic integrity of regenerated plants, the RAPD analysis was conducted when plants were 6 months old. The DNA of 44 plants comprising 40 regenerated and 4 mother plants produced a total of 1628 bands amplified using 7 primers. The bands produced monomorphic patterns across all 44 plants analyzed. The 37 bands obtained from the 7 primers ranged in size from 150 bp to 3000 bp (Table 2). The number of bands for each primer varied from 4 to 7, with an average of 5.5 bands per RAPD primer. The number of bands was the highest (7) for OPB12 primer, ranging from 300 to 2700 bp and the lowest (4) for OPC02 primer, ranging from 500 to 750 bp. The RAPD banding pattern of UMUCASS 36 showing monomorphic bands obtained from OPB 12 is shown in Figure 2. Amplification of DNA isolated from the mother plant and regenerants of cassava variety UMUCASS 36 for polymorphic occurrence of OPB12 primer products identified 7 distinct locations as indicated by bands corresponding to 250, 500, 550, 1035, 2700 and 2800 base pairs (Fig. 2).

Discussion

A rapid production and delivery of disease-free pro-vitamin A enriched cassava plants to the farmers is essential for eradication of VAD and attainment of food security. To achieve these goals, *in vitro* multiple shoot induction from axillary bud using BAP and NAA was investigated with a view to establish an efficient mass propagation system for the new multipurpose varieties of cassava. Next, the genetic stability of the regenerated plants was assessed with RAPD markers to confirm their molecular integrity. In the present study, the addition of NAA in culture media increased the number of shootbuds per axillary bud by an average of 63%. The best response was observed on a medium containing 0.3 mg/l NAA which produced 37.6 shootbuds per axillary bud compared with 8.1 shootbuds per axillary bud in a medium with no NAA addition (control). In our previous attempt to induce multiple shoots from the same cassava varieties, with 10 mg/l BAP alone as inducing agent, an average of 5.6 shootbuds were produced per axillary bud (Opabode et al., 2015). Clearly, a combination of BAP and NAA in culture medium led to improved multiple shoots induction and development in pro-vitamin A cassava varieties. Development of multiple shoots from stem node explants has been described to be a function

Table 4. Effect of NAA on survival, growth and photosynthetic pigment of regenerated plant

Concentration of NAA [mg/l]	Survived regenerated plant [%]	Number of plantlet/year	Growth rate [g/day]	Chlorophyll a [mg/g]	Chlorophyll b [mg/g]	Carotenoid [mg/g]
Control	95.3 ± 8.3 c	56 573 ± 11 d	5.3 ± 2.3 b	53.3 ± 7.8 b	23.8 ± 7.8 b	45.8 ± 4.8 b
0.1	98.2 ± 5.6 b	83 324 ± 13 d	5.2 ± 2.5 b	54.2 ± 6.3 b	22.6 ± 5.4 b	52.8 ± 6.2 b
0.2	97.5 ± 7.8 b	97 345 ± 15 d	7.4 ± 3.1 a	56.6 ± 6.5 b	24.7 ± 6.7 a	50.5 ± 7.4 b
0.3	100.0 ± 0.0 a	169 719 ± 23 a	7.4 ± 2.6 a	83.9 ± 9.1 a	25.5 ± 4.5 a	63.2 ± 3.8 a
0.4	100.0 ± 0.0 a	143 120 ± 19 b	7.2 ± 2.4 a	80.4 ± 7.8 a	28.7 ± 8.9 a	64.5 ± 3.9 a
0.5	100.0 ± 0.0 a	120 234 ± 16 c	7.2 ± 2.5 a	81.3 ± 8.3 a	28.4 ± 9.2 a	64.7 ± 4.0 a

Values are means (± standard error) of three replicates; means followed by different letters in same column are significantly different at 5% level of probability according to Tukey Test

Table 5. Properties and amplification products of RAPD primers used for the study

S/N	Primer	Sequence	GC [%]	Number of bands	Range of applicon
1	OPB-06	TGCTCTGCCC	70	05	200-1000
2	OPB-08	GTCCACACGG	70	06	250-1050
3	OPB-12	CCTTGACGCA	60	07	300-2700
4	OPC-01	TTCGAGCCAG	60	05	250-2000
5	OPC-02	GTGAGGCGTC	70	04	500-750
6	OPC-05	GATGACCGCC	70	05	150-2800
7	OPC-06	GAACGGACTC	60	05	300-3000

of cytokinin activity. The role of BAP and NAA in bud breaking has been reported in many plants (Rasool et al., 2009). For example, in some non-pro-vitamin A cassava varieties, multiple shoots have been induced using BAP alone or in combination with auxins or other cytokinins from apical meristems, immature leaf lobes or nodal explants. Pounti-Kaerlas (1998) reported that BAP at concentrations between 0.5 and 10 mg/l (alone or in combination with low amounts of auxins) could be used to break down the apical dominance of cassava axillary or apical meristems, which allowed for a proliferation of multiple shoots that were harvested a few months after culture initiation. The frequency of shoot formation obtained in this study was comparable with the result of previous studies (Konan et al., 1997; Rasool et al., 2009). However, the number of shoots per explant observed in the present study was higher than in most reported studies (Konan et al., 1997; Opabode et al., 2015). Konan and coworkers (1997) reported that each of cassava explants grown on a medium with 10 mg/l BAP alone produced at least 25 shoots. In this study, explants grown on a medium containing 10 mg/l BAP and 0.3 mg/l NAA produced on average 36 shootbuds on a medium containing 10 mg/l BAP and 0.3 mg/l NAA. A combination of BAP and NAA in the culture medium has been reported to promote multiple inductions in *Prunella vulgaris* explants (Rasool et al., 2009).

Importantly, the influence of cassava variety on the frequencies of shootbud formation, regenerated shoots (elongation of shoot), root formation and the number of roots per plantlet was recorded in the present study. Genotypic variation among cassava varieties during *in vitro* shoot induction has been widely reported. Konan and coworkers (1997) noted differential *in vitro* responses in shoot induction, number of shoots, shoot elongation and root formation among 17 varieties of cassava from Africa, South America and India. In a study on multiple shoot induction of three superior Indonesian cassava varieties, Sukmadjaja and Widhiastuti (2011) observed differences in their responses to hormones' combinations. They reported that the highest number of shoots for Darul Hidayah, Malang-6 and Adira-4 varieties were 4.93,4.2 and 7.2, respectively, when growth media were supplemented with 1 mg/l BAP and 0.1 mg/l thi-

diazuron. Similarly, Demeke et al. (2014) reported varietal differences in shoot and root formation between two Ethiopian varieties of cassava, 44/72-NR and 44/72-NW, when cultured on MS medium with the addition of 0.75 mg/l BAP and 0.75 mg/l kinetin.

In vitro formation of roots in cassava can occur on a hormone-containing or hormone-free medium. In the present study, excised shoots transferred on hormone-free MS medium produced roots within 2 weeks. The proportion of shoots that produced roots (77.3-98.3%) and the number of roots per plantlet (7.6-10.2) observed in this study were higher than those from previous reports, regardless of whether roots were inducted on hormone-free or hormone-containing media. For instance, Dimeke and coworkers (2014) observed 6.14 roots per plantlet within 4 weeks when the shoots were cultured on a medium supplemented with 0.5 mg/l NAA. Also, Acedo (2006) reported 41% and 60% rooting on liquid and solid media, respectively, supplemented with 0.25 mg/l GA3, 0.1 mg/l BAP and 0.2 mg/l NAA. The differences in culture media composition, cassava varieties and culture conditions might be responsible for the changes in root formation.

RAPD is a technique efficient in identifying genetic variations in closely related plants (Williams et al., 1990). This is achieved by simultaneous amplification of different regions of the genome because the primers have a high multiplex ratio, making the polymorphism detection easy. These attributes confer power on RAPD to detect genetic stability of *in vitro* regenerants (Martins et al., 2004). As a result, the RAPD technique has been successfully applied to detect genetic similarities or dissimilarities in micropropagated plant material such as almonds, ginger, aerial yam, bamboo or banana (Martins et al., 2004; Mohanty et al., 2008; Dixit et al., 2003; Nadha et al., 2011; Lakshmana et al., 2007). A RAPD analysis performed in pro-vitamin A cassava varieties revealed 37 distinct bands from 7 random primers in regenerated plants from enlarged axillary buds. More importantly, RAPD markers showed genetic uniformity between the mother plants and the regenerants. These results suggest that *in vitro* manipulation of pro-vitamin A cassava does not induce mutations or genetic alterations which might result from the stress imposed during the plant tissue cultures. Such changes could be in the form of DNA methylation changes, chromosome rearrangements and point mutations. In this report, axillary buds of the mother plants were used to induce multiple shoots from which the regenerants were derived. A rapid and mass production of elite genotypes from axillary buds is a preferred method because of low risk of genetic instability. Furthermore, previous studies have established that shoots and meristems regenerated from somatic embryogenesis, grown in *in vitro* cultures of cassava plants exhibit genetic stability. Using a slow growth method based on RAPD and RFLP markers, Angel and co-workers (1996) established that the genetic stability is maintained during 10 years for *in vitro* cultures of cassava. Similarly, using flow cytometry, Hankoua and coworkers (2005) confirmed genetic stability of field-grown cassava plants derived from somatic embryos.

Conclusion

This work demonstrated multiple shoots induction from axillary buds of three pro-vitamin A cassava varieties using BAP and NAA, with no genetic infidelity of the regenerants. The results of this study suggest that a combination of 10 mg/l BAP and 0.3 mg/l NAA was the best for multiple shoot induction in terms of the number of shoots per explant and frequency of regenerated shoots. This work has demonstrated that a large number of virus-free and uniform plantlets could be regenerated within a short time and in limited space via multiple shoot induction for the production of stem cuttings for distribution to farmers at the beginning of the planting season. This is important because the availability of stem cuttings at planting seasons has the potential to increase production and consumption of pro-vitamin A cassava varieties, thus reducing diseases associated with VAD.

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