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Original Research Article

Shoot Induction from Axillary Bud of β-Carotene Enriched *Manihot esculenta* Crantz and Molecular Stability of Regenerants

Jelili Titilola Opabode, Olufemi Victor Ajibola, Oluyemisi Amos Akinyemiju

Department of Crop Production and Protection, Obafemi Awolowo University, Ile-Ife, Nigeria

Abstract

Inadequacy of planting materials is limiting the distribution of three high-β-carotene (pro-vitamin A) cassava varieties released in Nigeria to increase public vitamin A intake. However, studies have established the suitability of *in vitro* shoot culture for mass propagation of elite crop varieties for quick distribution to farmers.We investigated multiple shoot induction from axillary bud of three β-carotene enriched cassava varieties using BAP and assessed the genetic stability of the regenerated plants to establish mass propagation system. Multiple shoots were induced from BAP-enlarged axillary bud on basal medium supplemented with 10 mg/l BAP. Shootbuds were elongated on basal medium supplemented with 1.0 mg/l BAP and 10.0 IBA mg/l. Genetic stability of regenerated plants was assessed byeight RAPD markers. There were significant ($P < 0.05$) differences in survival of isolated axillary bud, shootbud formation, survival of shootbud, regenerated shoots, root formationand survived regenerated plants among cassava varieties. The order of shootbud formation among the cassava varieties was UMUCASS 37 > UMUCASS 36 >TMS 30555 > UMUCASS 38. The best (83.4%) shoot regeneration was recorded in TMS 30572 and lowest (47.8%) in UMUCASS 37, however, UMUCASS 36 recorded the best (98.5%) survival of regenerated shoot after hardening. Eight RAPD primers produced 56 bands, ranging from 200 to 3000 bp in size. RAPD analysis showed a uniform profile among regenerants and between mother plant and regenerants. The study concluded that multiple shoot formation could be induced in pro-vitamin A cassava varieties using BAP from axillary bud with no genetic infidelity of the regenerants.
Abbreviations: BAP – benzylamino purine; IBA – indole butyric acid; RAPD – random amplified polymorphic DNA

Keywords: Cassava varieties**;** genetic stability; shootbud survival; root formation.

INTRODUCTION

An estimated 600 million people in tropical and subtropical regions depend on cassava (*Manihot esculenta* Crantz) as their source of energy (Defloor et al., 1998). However, vitamin A deficiency (VAD) is a major public health problem in these regions. The VAD afflicts almost 20% of pregnant women and about 30% of children in these regions (Maziya-Dixon et al., 2006). To alleviate this health problem, Nigerian Government released three varieties of pro-vitamin A cassava, developed through conventional breeding, for cultivation in Nigeria and West African Region (Ukenye et al., 2013; Oparinde et al., 2014). It was envisaged that development and dissemination of pro-vitamin A cassava varieties will complement current efforts to address VAD by delivering vitamin A through a staple crop (Aniedu and Omodairo, 2012).

The nutritional benefits of pro-vitamin A varieties has significantly increased the cultivation of the new varieties due to rising demand and acceptability of their products in Nigeria and West African region (Oparinde et al., 2014). Traditionally, stem cuttings production is the main technique of multiplying and distributing the new varieties to farmers. Stem cuttings production by this method could not meet the demand for cuttings of the pro-vitamin A varieties since cassava has a relatively low multiplication ratio compare with crops like maize, tomato and potato (Puonti-Kaerlas, 1998; Sukmadjaja and Widhiastuti 2011). From a mature cassava plant, only 10-30 cuttings can be obtained per year. Thus the propagation rate is a limiting factor in the distribution of planting material of pro-vitamin A varieties for which there is a high demand. There is a need to increase the propagation rate by orders of magnitude. In addition, since cassava is propagated vegetatively, it is susceptible to diseases that are carried from one generation to the next through infected planting material (Bull et al., 2011). Similarly, international exchange of the new pro-vitamin A varieties and maintenance of germplasm require the use of disease-free stock. One technique that can accelerate a rapid propagation and diffusion of the new cassava varieties that is free of diseases to African farmers is plant tissue culture technique (Kondamudi et al., 2009; Bull et al., 2011). Through shoot culture, one nodal explant can produce one million plantlets with four nodes each in one year (Konan et al., 1997). Therefore, *in vitro* shoot culture for the purpose multiple shoot induction, has the potential for the production of a large number of virus-free plantlets for distribution to farmers within a short period.

Since the development of shoot meristem culture techniques 41 years ago for cassava, the technique has been widely investigated and applied for the production of virus-free plants of elite genotypes (Konan et al., 1997; Abdalla et al., 2013). Studies have revealed 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 axillary or apical meristem, which allowed the proliferation of multiple shoots few weeks after culture initiation (Konan et al., 1997; Kondamudi et al., 2009). The potential of the most efficient multiple shoot induction protocols has been estimated to be up to 1.2×10^{20} new shoots in one year. For example, in China, 8.0×10^{6} transplantable plantlets of a high-yielding genotype Nan Zhi 188 had been produced for distribution to farmers in two years using an efficient low-cost *in vitro* propagation system (Kondamudi et al., 2009; Mapayi et al., 2013). The objectives of this study were to assess multiple shoot induction from axillary bud using BAP and examine the genetic stability of the regenerated plants with RAPD markers with a view to establish an efficient mass propagation system for rapid delivery of the new varieties to farmers.

MATERIALS AND METHODS

Source and micropropagation of *in vitro* plantlets

In vitro plantlets of three (UMUCASS 36, UMMUCASS 37 and UMUCASS 38) beta-carotene enriched cassava varieties and a control (TMS 30572) used for the study were collected from the Tissue Culture Section of GeneBank, International Institute of Tropical Agriculture (IITA), Ibadan. To increase the number of plantlets available for the study, nodal explants were subcultured on basal medium.

Basal medium and culture conditions

Basal medium (BM), which consisted of full-strength MS (Murashige and Skoog 1962) salt (Sigma, USA) along with 0.8% (w/v) agar (Oxoid Ltd., England) and 30 g/l sucrose, was used in all experiments unless otherwise stated. The pH of the medium was adjusted to 5.8 by HCl (1N) or NaOH (1N) prior to autoclaving at 121 °C for 15 min at 1.05 kg cm^{-2} pressure. Growth regulators were filter-sterilized through 0.22-µM Millipore filters and added to media after autoclaving. For all experiments except otherwise stated, cultures were maintained in a 16 h photoperiod with 40 µmol m^{-2} s^{-1} light intensity provided by cool-white fluorescent tubes at 25 ± 2 °C.

Enlargement of axillary buds and multiple shoot induction

In this study, the procedure for multiple shoot induction from axillary bud as described by Konan et al. (1997) was followed. Two hundred nodal segments (5-7 cm long) of each cassava variety from *in vitro* shoot cultures (3-5-weeks-old) were cut, placed horizontally and incubated on basal medium supplemented with 10mg/l of BAP for seven days. Enlarged axillary buds were removed from the nodal segments with a scalpel and were subcultured on the fresh same medium as before to induce shoot formation for 4 weeks. One hundred and fifty axillary buds per cassava variety were cultivated. Data were recorded on survival of isolated axillary bud, shootbud formation and number of shootbud per explant.

Shoot elongation, rooting, hardening of plantlets and soil establishment

One hundred and fifty shootbuds of each cassava variety were detached from the shootbuds clumps and transferred to basal medium supplemented with 1.0 mg/l BAP and 10.0 IBA mg/l for shoot elongation. Observations were made on survival and number of regenerated (elongated) shoots two weeks after transfer. Thereafter, 120 regenerated shoots of each variety were transferred on hormone-free basic medium for rooting as outlined by Li et al. (1998). Data were recorded on root formation three weeks after transfer. About three weeks after successful root formation by the regenerated shoots, plantlets were carefully removed from the culture tubes and placed in plastic bags half-filled with peat and vermiculite (50:50). Immediately after transplanting, the plantlets were placed in a humidity chamber and gradually exposed to a source of natural light for hardening. At six weeks after transplanting, the plants were transferred to polybags filled with a rich loamy soil. Number of surviving plants was recorded.

DNA Extraction and RAPD analysis

DNA was extracted from young leaves (0.5-1.0 g) as described by Dellaporta et al. (1983). DNA samples were diluted to 25 ng/µl for RAPD-PCR analysis. A total of eight random decamer primers (Operon Tech, Alameda, USA) from B, C, D, and E series (OPB06, OPB08, OPB12, OPC01, OPC02, OPC05, OPC06, OPE19) were selected for RAPD analysis based on the results from previous studies (Angel et al., 1996; Herzberg et al., 2004). The RAPD analysis was performed as described by Williams et al. (1990). A 25µl volume PCR reaction was performed in a thermal cycler (PTC 2000, MJ Research, India) under conditions previously described by Herzberg et al. (2004). DNA fingerprints were visualized under UV light and photographed using a gel documenting system (Bio-Rad, CA, USA).

Experimental design and statistical analysis

All experiments were arranged in a completely randomized design with four replications, and experiments were repeated at least thrice. Percent and count data were subjected to arcsine and square root transformation to

normalize variances. Data were further subjected to analysis of variance to detect differences among treatments using PROC GLM procedure of the Statistical Analysis Systems (SAS, 2002). Means were separated by Tukey's Test at 5% level of probability.

RESULTS AND DISCUSSION

Enlargement of axillary bud (Fig. 1A) occurred within 4 days after the incubation of nodal explant on basal medium supplemented with 10 mg/l BAP. An average of 80% of the axillary buds was enlarged. Surviving isolated axillary buds (Fig. 1B) increased in size following transferred on basal medium containing 10 mg/l BAP and produced clump of shootbuds (Fig. 1C) by the fourth week. Excised shootbuds became elongated, produced root (Fig. 1D) at the 6th and 8thweeks, respectively, and formed growing young plants (Fig. 1E) following acclimatization. There were significant ($P < 0.05$) differences in survival of isolated axillary bud, shootbud formation, number of shoot per explant among the four cassava varieties (Table 1). The mean values of survival of isolated axillary bud, shootbud formation and number of shoot per explant were 70.2%, 67.9% and 5.6, respectively. The best (90.0%) survival of isolated axillary bud on regenerating medium was recorded in TMS 30555 while the lowest (56.2%) was observed in UMUCASS 36. The order of shootbud formation among the four cassava varieties was UMUCASS 37 > UMUCASS 36 > TMS 30555 > UMUCASS 38. Induced shootbuds were green and normal in all varieties. The percent shootbud formation obtained in this study agrees with the results of previous studies. However, the number of shoot per explant observed in the present study was lower than in most reported studies. Konan et al. (1997) reported that 63% of the explants each produced at least 25 shoots on medium with 10 mg/l BAP. In this study, an average of 68% of the explants produced a mean of 5.6 shoot per explant. Differences in culture environment and cassava varieties might be responsible for the observed results. Similarly, varietal difference significantly ($P<0.05$) influenced survival of shootbud on shoot elongation medium, percent regenerated shoot,

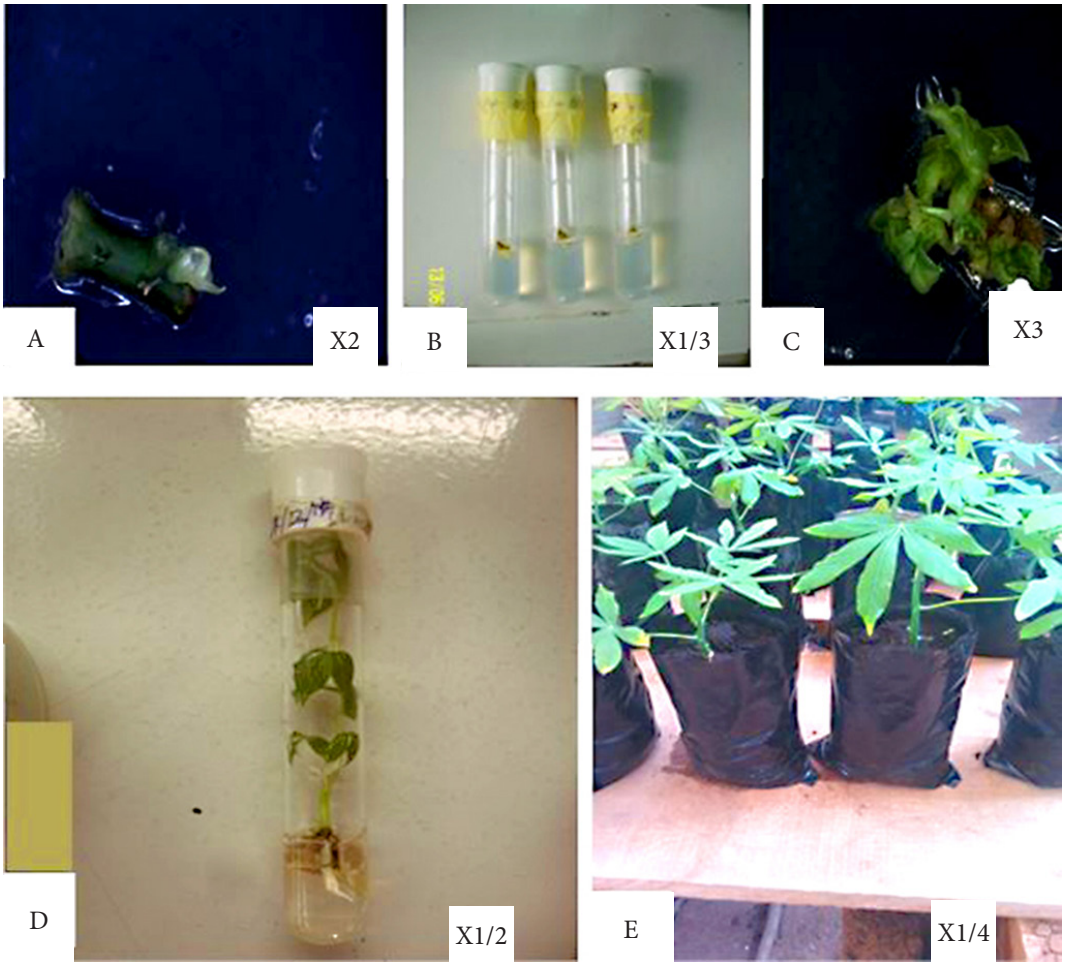

Figure 1: Multiple shoot induction in UMUCASS 36. A - Enlarged axillary bud; B- isolated axillary bud on induction medium; C - emerging multiple shootbud after 3 weeks; D- elongated shoot on elongating medium; E- regenerated plants following acclimatization in a containment facility.

Table 1. Survival of isolated axillary bud, shootbud formation, number of shoot per explant and appearance of shoot in four cassava varieties

Variety	SIAB (%)	Shootbud formation (%) per explant	Numberof shoot of shoot	Appearance
UMUCASS 36	56.2±3.6c	72.1±5.6a (108)	4.3±1.2c	Normal, green
UMUCASS 37	73.4±5.2b	73.4±5.0a (110)	5.2±0.9b	Normal, green
UMUCASS 38	60.9±4.8c	58.1±4.3c (87)	4.2±1.3c	Normal, green
TMS 30572 (control)	90.0±4.2a	68.1±4.2b (102)	8.8±1.4a	Normal, green
Mean	70.2	67.9	5.6	

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 SIAB – survival of isolated axillary bud

Table 2. Survival of shootbud, regenerated shoot, root formation and survived plants of *in vitro* regenerated plants

Variety	Survival of shootbud (%)	Regenerated shoot (%)	Root formation (%)	Number of root/plantlet	Survived plants (%)
UMUCASS 36	96.6±7.2b	73.4±4.8b (110)	98.9±3.5a (119)	7.2±2.3a	98.5±4.5a (118 /120)
UMUCASS 37	92.5±6.8c	47.8±3.8d(72)	87.2±3.8b (105)	5.6±2.7c	93.2±5.3b (84/90)
UMUCASS 38	98.2±6.2a	53.4±3.5c (80)	86.4±3.7b (104)	6.2±2.1b	97.8±5.7a (108/110)
TMS 30572 (control)	97.3±4.7a	82.4±3.7a (125)	97.3±5.2a (117)	7.4±1.8a	96.3±4.1a (111/115)
Mean	96.2	64.5	85.0	6.6	96.5

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

root formation, number of root per plantlet and survived regenerated plants following hardening (Table 2).The mean values of shootbud survival, percent elongated shootbud, root formation and number of root per plantlet were 96.2%, 64.5%, 85.0% and 6.6%, respectively. Survival of shootbud in shoot elongation medium was encouraging as all varieties recorded > 90% survival. The best percent regenerated shoot (83.4%) and number of root per explant (7.4/plant) were recorded in TMS 30572. However, root formation (98.9%) and surviving regenerated plants (98.5%) after hardening were greatest in UMUCASS 36. It is noteworthy that the three pro-vitamin A responded differently to shoot induction in the present study, indicating genotypic differences in most parameters measured. Genotypic variation has been widely reported among cassava varieties during *in vitro* shoot induction. Earlier, Konan et al. (1997) had noted differential *in vitro* responses in shoot induction, number of shoot, 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 response to hormonal combinations. It

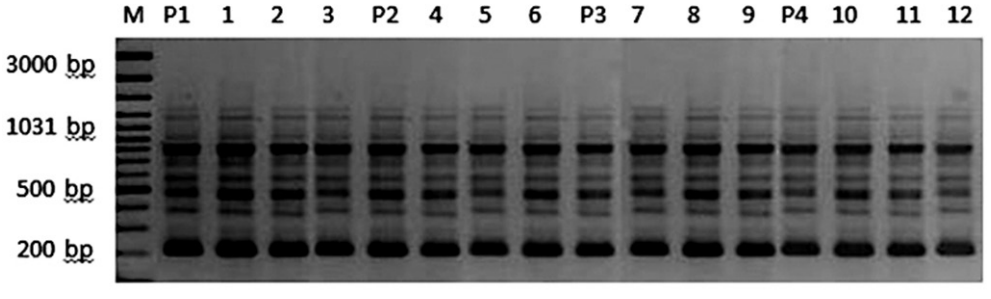

Figure 2: Randomly amplified polymorphic DNA analysis for the primer OPC05 from four mother plants and 12 regenerated plants of UMUCASS 36.M– GeneRuler ladder, P1-P4– Mother plant, 1-3 – randomly selected samples from regenerated plants

Table 3. Properties and amplification products of RAPD primers used for the study

s/n	Primer	Sequence	GC %	No. of bands	Range of applicon
1.	OPB-06	TGCTCTGCCC	70	05	200-1000
2.	OPB-08	GTCCACACGG	70	07	250-1050
3.	OPB-12	CCTTGACGCA	60	12	450-3000
4.	OPC-01	TTCGAGCCAG	60	07	250-2000
5.	OPC-02	GTGAGGCGTC	70	07	500-750
6.	OPC-05	GATGACCGCC	70	05	200-2800
7.	OPC-06	GAACGGACTC	60	07	300-3000
8.	OPE-19	ACGGCGTATG	60	06	450-2000

is encouraging that limited numbers of regenerated plants were lost at hardening in all varieties.

The monomorphic RAPD banding pattern of OPC05 primer from four mother plants and 12 regenerated plants of UMUCASS 36 is shown in Fig. 2. The RAPD technique produced monomorphic banding patterns across all 64 plants analysed. Eight selected RAPD primers utilized in this study gave rise to a total of 56 distinct and scorable bands, ranging from 250 bp to 3000 bp in size (Table 3). The number of bands for each primer varied from 4 to 12, with an average of 7 bands per RAPD primer. The number of monomorphic bands was highest (12) in case of primer OPB12, ranging from 450 to 3000 bp in size and lowest (5) in case of primer OPC05 and OPB06, ranging from 200 to 2800bp in size (Table 3). RAPD analysis of regenerated plants showed a uniform profile among themselves and a similar banding profiles to that of the mother plant indicating that no genetic variation among the regenerants and between the regenerants and the mother plant. Earlier, Angel et al. (1996) havedemonstrated the genetic stability of *in vitro* culture cassava germplasm when they reported the stability of cassava plants at the DNA level after retrieval from 10 years of *in-vitro* storage by slow growth method using combination of RAPD and RFLP primers. Similarly, Hankoua et al. (2005) established the genetic stability of field-grown cassava plants derived from somatic embryo by flow cytometry by confirming that DNA content of regenerants was homogeneous and similar to that of mother plants and ploidy level was unchanged (2n = 36).

CONCLUSIONS

The study concluded that multiple shoot could be induced in pro-vitamin A cassava varieties using BAP from axillary bud with no genetic infidelity of the regenerants.Among pro-vitamin A cassava varieties, UMUCASS 36 recorded the best performance in terms of shootbud induction, regenerated shoot, number of shoot per explant, root formation and survived regenerated plants. However, combination of auxins and or cytokinins should be exploited for multiple shoot induction in pro vitamin A to obtain higher numbers of shoot per explant.

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Corresponding author:

Jelili Titilola Opabode
Department of Crop Production and Protection
Obafemi Awolowo Unversity, Ile-Ife
Nigeria.
E-mail: jopabode@yahoo.com
Phone: +234 803 417 2865