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BIOCHEMICAL CHARACTERISTICS OF FRESH ROOTS OF TRANSGENIC HIGH BETA-CAROTENE CASSAVA

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ABSTRACT
Recent research efforts have developed high beta carotene or yellow cassava roots through genetic engineering and conventional breeding. Beta-carotene pigments are biosynthesized through a cellular isoprenoid pathway. In this study, biochemical techniques and microscopy were employed in determining beta-carotene distribution and metabolic isoprenoid pathway in fresh transgenic and conventionally bred high beta-carotene cassava roots. Transgenic high beta-carotene cassava varieties (EC20-7 and EC20-8) were compared with two conventionally bred high beta-carotene cassava varieties (UMUCASS 38 and UMUCASS 45) and an indigenous white fleshed cassava variety (TME-7). These five cassava varieties were planted in environmentally controlled greenhouse and harvested after four months. Results showed that the dry matter content of UMUCASS 38 (35.61 %) and UMUCASS 45 (36.96 %) were significantly ($P<0.05$) different from EC20-7 (24.23 %), and EC20-8 (23.18 %). β-carotene content on dry matter basis of the fresh yellow cassava roots (50.93 - 80.45µg/g) was not significantly ($P>0.05$) different between the transgenic and conventional varieties The observed carotenoids in this study were biosynthesized from isopentenyl diphosphate and its isomer dimethylallyl diphosphate. All the varieties, especially the EC20-8 transgenic variety, had appreciable levels of methyl-D-erythritol 4-phosphate. Microscopic analysis showed the distribution of carotenoids in the cortex, parenchyma and the xylem bundles. Carotenoids were not observed in the sclerenchyma, therefore, peeling of yellow cassava varieties during processing will not necessarily cause any loss in the carotenoid contents

Keywords: Cassava, Beta-carotene, fresh roots

Introduction

Cassava (*Manihot esculenta* Crantz.*)* is an important staple crop in much of the tropical world. The cassava edible root is specifically a starchy tuber, with many attributes. It can survive droughts; it is inexpensive, resistant to pests and easy to grow. Although it is a valuable source of energy, the nutritional composition is deficient in some other essential nutrients (Edoh *et al*, 2014). Conventional breeding and genetic modification techniques are presently being used to improve the beta-carotene content of cassava for a sustainable solution to vitamin A deficiency (VAD) in Africa. Carotenoids are natural isoprenoid pigments that provide leaves, fruits, vegetables and flowers with distinctive yellow, orange and some reddish colours as well as several aromas in plants. Carotenoids are terpenoids, a class of biosynthesized hydrocarbons that participate in various biological processes in plants, such as photosynthesis, photo-morphogenesis, photo-protection, physical development (Nisar *et al*, 2015) and the production of carotenoid-derived phytohormones, including ABA and strigolactone. The major plant carotenoids are antheraxanthin, capsanthin, α-carotene, β-carotene, ε-carotene, γ-carotene, α-cryptoxanthin, lutein, lycopene, neoxanthin, and zeaxanthin (Eldahshan and Singab, 2013). Beta-carotene is a major precursor of vitamin A (retinol) in humans (Grune *et al*, 2010) and an important micronutrient that affects human health (Burri, 1997). Diets containing carotenoid-rich vegetables, fruits and roots are useful in protection against some diseases such as cancer, heart disease, cataracts and ultraviolet-induced skin damage (Carvalho *et al*, 2016). The development of high yielding β-carotene crops such as golden rice, yellow cassava and orange sweet potato could provide close

to the recommended daily allowance of vitamin A for malnourished children and help combat vitamin A deficiency-induced mortality and morbidity. The beta-carotene content trait is also associated with a reduction in post-harvest physiological deterioration (PPD) of the harvested roots due to oxidative nature of carotenoids (Sanchez *et al*, 2006). PPD is gradually becoming important as urbanization in producing countries and has increased the distance and time between farm and market or processing centres. There are several estimates of the economic impact of PPD but estimated losses due to PPD in cassava range from 5–25% of the total expected value of the crop (Zidenga, 2012). There is therefore a need to use relevant scientific techniques to compare biochemical characteristics of yellow cassava developed through genetic engineering and conventional breeding.

Materials and Methods

Identification and selection of two conventionally bred (UMUCASS) high beta-carotene cassava or yellow cassava stakes were in collaboration with the Genetic Resource Unit and Cassava Programme of NRCRI, Umudike, Nigeria. Collection of two transgenic bred (EC 20) high beta carotene cassava stakes and wild type (TME 7) were with the assistance of the International Institute for Crop Improvement (IICI) Department of Donald Danforth Plant Science Center (DDPSC) St. Louis, Missouri USA. These cassava stakes were planted in a greenhouse and harvested four months after planting.

Dry matter content of the cassava varieties: The dry matter content of the cassava varieties was determined using a lypholyser modified method of Asare, 2004. The dry matter content was determined from the difference between the weight of the fresh and the dry sample of the cassava varieties.

Identification and quantification of beta-carotene content of the cassava varieties: An Agilent HPLC system equipped with a diode array detector (DAD) operating between 400 and 700 nm was used. Separations were performed on YMC RP-C30 column (250 x 2.0 mm i.d) and a Thermo Finnigan LCQ Advantage ion trap mass spectrometer equipped with an Atmospheric Pressure Ionization (API) interface, LC pump, syringe pump, PDA detector and surveyor auto-sampler. The instrument was operated in a selected ion monitoring (SIM) and positive mode. HPLC separations were achieved using 40 µL of sample, a 250 x 2.0 mm YMC RP-C30 column. A mixture of 96 % methanol in water was used as mobile phase (A) and 90 % methyl-t-butyl ether, 7 % methanol and 3 % water as mobile phase (B). Carotenoids were quantified by a combination of HPLC retention times and UV absorption spectra by comparing peak areas from calibration curves prepared using analytical grade carotenoid standards.

Identification and quantification of isoprenoid metabolites: Separation was achieved by injecting 10 µL of sample onto the Shimadzu Prominence HPLC using a 150 x 2.0 mm Phenomenex LC Synergi^{TM} Hydro-Rp 80A column. 10mM tributylamine with acetic acid in water was used as mobile phase (A) and 100% methanol as mobile phase (B). Quantitation was accomplished on a Sciex 6500 QTRAP® mass spectrometer by comparing peak areas of endogenous target analytes to those on an external calibration curve prepared using analytical grade standards.

Microscopy analysis: Cassava roots harvested at 12 weeks were used for microscopy analysis using a protocol from microscopy unit of DDPSC. The fresh cassava tubers were cut and rinsed with distilled water. The samples were then plated onto microscope slides to observe modes of carotenoid deposition. An optical microscope (BX61, Olympus) equipped with a DP70 camera was used in tandem with differential interference contrast technique. Images were obtained at various magnifications.

Results and Discussion

The results of this study with the 12week old experimental cassava roots as shown in Table 1 indicated that the percentage dry matter content of TME-7 was the highest (42.60 %) among the cassava varieties which was significantly ($P<0.05$) different from the EC20-7 (24.23 %) and EC20-8 (23.18 %) respectively. The percentage DMC of UMUCASS 38 and UMUCASS 45 varieties are 35.61 % and 36.96%, respectively which are significantly ($P<0.05$) different from EC20-7 (24.23 %), and EC20-8 (23.18 %) varieties. There was no significant ($P>0.05$) difference observed in the DMC of TME-7 (42.60 %), UMUCASS 45 (35.61 %) and UMUCASS 38 (36.96 %) varieties. Table 2 shows the identification and quantification of β-carotene in the cassava varieties used in this study. The β-carotene content ranged from 50.93 – 80.45 µg/g. EC20-7 had the highest β-carotene (80.45 µg/g) while UMUCASS 38 had the least (50.93 µg/g). Beta-carotene was not detected in the white variety (TME-7). However, there was no significant ($P>0.05$) difference between the β-carotene content of UMUCASS and that of EC20 varieties. Beta-carotene as reported by Cazzonelli, 2011 and Rodriguez-Amaya, 2001 are known to impart yellow, orange or red colour to fresh plant foods. Ingested plant carotenoid compounds enhance immune response and reduce the risk of degenerative diseases such as cancer, cardiovascular diseases and several age-related conditions (Siepelmeyer and Bernhardt, 2016) and these have

been attributed to their antioxidant and free radical scavenging activities (Eleazu and Eleazu, 2012). The EC20 and UMUCASS (yellow) varieties had significantly higher quantities of β-carotene and this may confer antioxidant and vitamin A reducing properties on these yellow cassava varieties.

The detected carotenoids in this study (Table 3) are those biosynthesized from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two independent pathways namely the cytosolic mevalonic acid (MVA) pathway and the plastidic methylerythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat, 2002) are present in plant cells for the production of these prenyl diphosphate precursors. However, carotenoids in higher plants are mainly synthesized from IPP and DMAPP produced by the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion, 2010). In this study, metabolites identified with LC-MS/MS were used to confirm the primary isoprenoid biosynthetic pathway of the carotenes in the cassava roots. The identified metabolites in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway are given in Table 3. The initial reaction of the pathway is the condensation of glyceraldehyde-3-phosphate from pyruvate to produce 1-deoxy-D-xylulose-5-phosphate (DXP) catalyzed by DXP synthase. The intramolecular rearrangement and reduction of DXP catalyzed by DXP reductoisomerase yields MEP in the next step of the pathway. MEP is afterwards converted, via 4-2-*C*-methyl-D-erythritol (CDP-ME) and CDP-ME 2-phosphate (CDP-ME2P) into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP) by the enzymes MEP cytidylytransferase, CDP-ME kinase and ME-cPP synthase, respectively. In the last two steps of the pathway, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) synthase transforms ME-cPP into HMBPP, whereas HMBPP reductase converts HMBPP into a mixture of IPP and DMAPP. Águila *et al*, 2012 reported an identical pathway in Arabidopsis. The results in Table 3 indicate that all the varieties, especially EC20-8 transgenic variety, had appreciable levels of MEP. It is possible that pyruvate and glyceraldehyde can be derived from the carbohydrate glycolytic pathway (Wang *et al*, 2016).

Plates 1 and 2 show the distribution of carotenoids in the 12week old fresh cassava tubers of EC20, UMUCASS and TME-7 varieties. Plate 2 shows the distribution of carotenoids in the parenchyma and the xylem bundles. Carotenoids were not observed in the sclerenchyma. This result shows that peeling of yellow cassava varieties during processing will not necessarily cause any loss in the carotenoid content.

Conclusion

The results of this study with the 12-week old cassava roots showed that the DMC of conventionally bred UMUCASS varieties were significantly higher than the EC20 transgenic yellow β-carotene cassava varieties but beta-carotene content may not be significantly different among the transgenic and conventional bred cassava varieties. This study also indicates that all the experimental varieties, especially EC20-8 transgenic variety, had appreciable level of MEP which is an important intermediary metabolite in the plastidal isoprenoid pathway for carotenes. Microscopically, carotenoids were not observed in the cortex of the root tubers.

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Table 1: Dry Matter Content of the Cassava Roots (%)

Variety	Mean
UMUCASS 38	35.61±1.96[b]
UMUCASS45	36.96±5.65[b]
TME-7	42.60±0.41[b]
EC20-7	24.23±1.90[a]
EC20-8	23.18±0.67[a]

Values are mean of triplicate determinations, values with the same letter are not significantly *(P>0.05*) different using Duncan Multiple Range Test

Table 2: Beta-carotene content (µg/g) on Dry Matter Basis

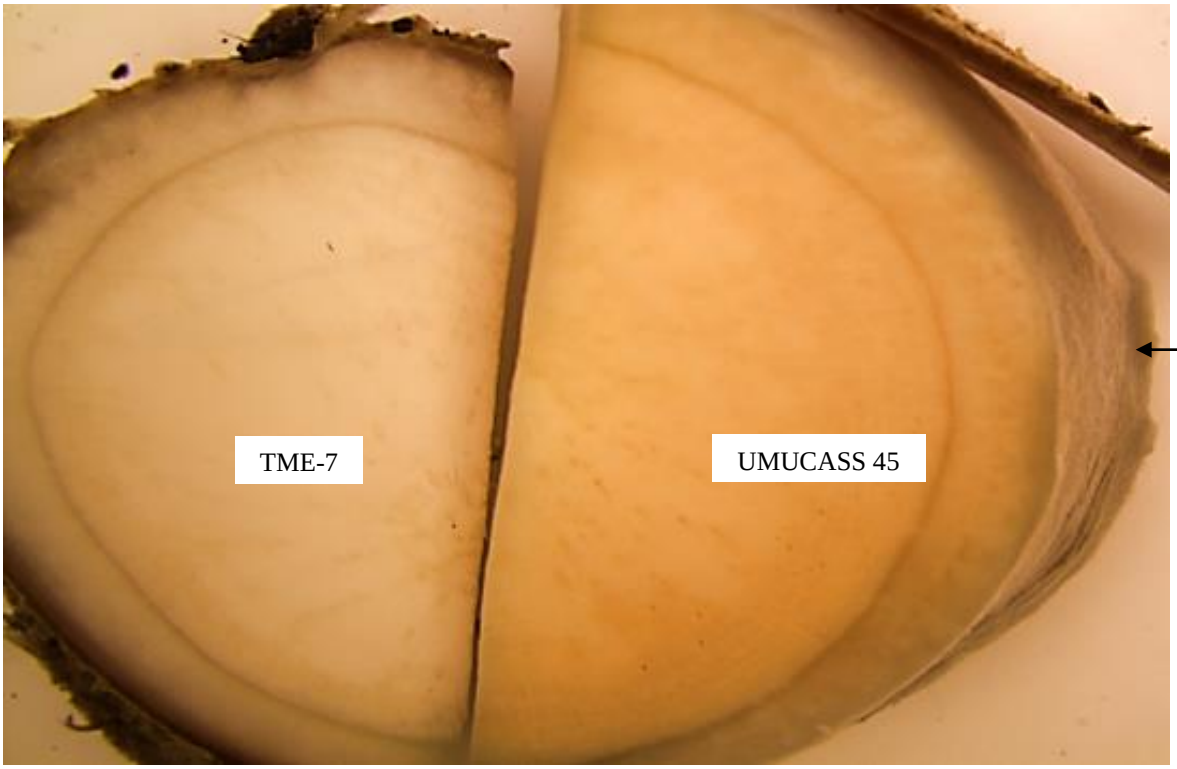
Variety	Beta carotene
UMUCASS 38	50.93±1.28[b]
UMUCASS 45	69.19±5.84[b]
EC20-7	80.45±10.86[b]
EC20-8	69.11±45.80[b]
TME-7	ND

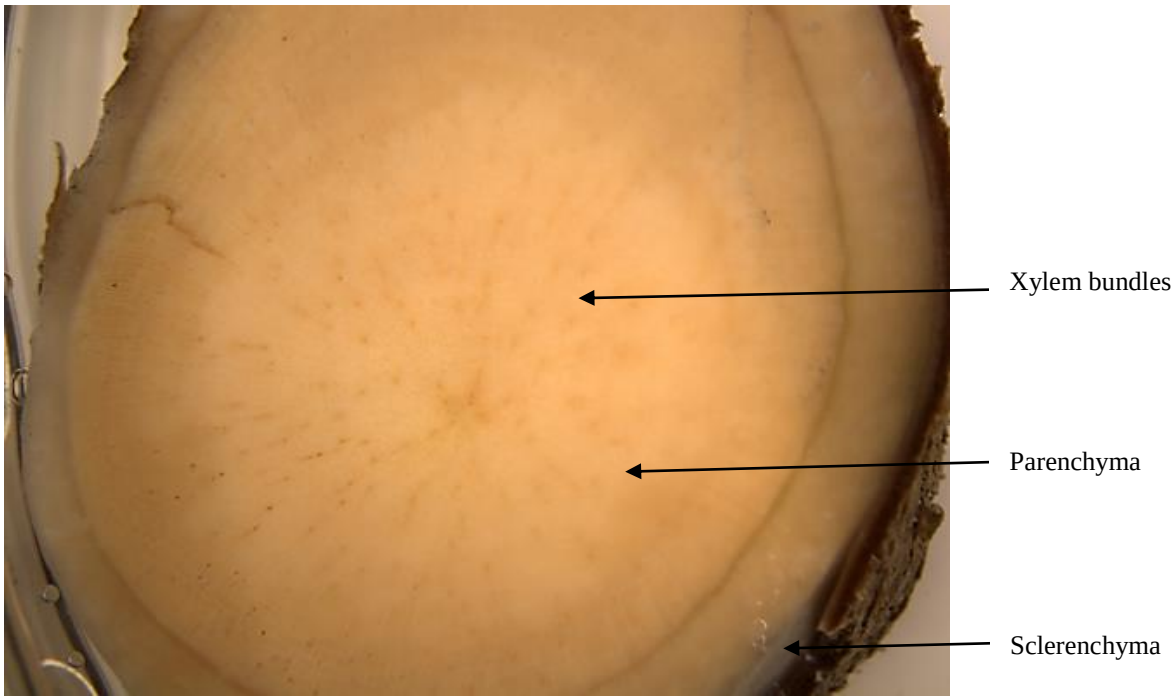
Values are mean of triplicate determinations, values with the same letter are not significantly *(P>0.05*) different using Duncan Multiple Range Test; ND= Not detected

Table 3: Intermediary metabolites of the cassava roots

Variety	Metabolites				
	CDP-ME2	CMEPP	DMAPP	HMBPP	MEP
UMUCASS 38	848.838	1.019	0.351	114.885	0.360
UMUCASS 45	476.728	ND	0.275	5.763	0.208
TME-7	635.552	0.141	0.280	35.556	0.308
EC20-7	104.630	0.620	0.375	3.278	0.453
EC20-8	ND	0.347	0.324	1.304	1.050
Linear range	0.4-100	0.4-5	0.4-100	0.4-5	0.4-50
Intercept	35818.3	66792.6	200393	140488	-235315
Slope	-201.083	3841850	943895	1102890	5266320
Correlation coefficient	0.253	0.996	0.970	0.997	0.999

Values are mean of triplicate determinations; ND = Not detected


TME-7
UMUCASS 45
Sclerenchyma


Xylem bundles
Parenchyma
Sclerenchyma