

Untargeted Metabolomics Profiling of High Beta Carotene Cassava with respect to Postharvest Physiological Deterioration

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Abstract

Cassava roots undergo postharvest physiological deterioration (PPD), and for most varieties it sets in within 72 hours of harvest. An untargeted metabolomics approach combined with a data-driven approach for statistical analysis was used to characterize and profile high beta-carotene cassava varieties with the aim of identifying any relevant metabolite changes that occur during PPD. Sixteen cassava root samples from four cassava lines were planted in a greenhouse and harvested after four months. The samples included four of 2 conventionally bred beta carotene cassava varieties – UMUCASS 38, UMUCASS 45 and four of 2 transgenic high beta carotene cultivars - EC20-7 and EC20-8 cassava lines. Extracts of fresh cassava roots from 20-100 mg tissues were used for the analyses and data were processed using Elements for Metabolomics software. Starch and lipid metabolites were the major constituents which may help explain the observed differences in starch and dry matter content among the varieties. The results provide further insight in the understanding of PPD and suggestions on controlling this deterioration in cassava are made.

Keywords: metabolomics, cassava, postharvest physiological deterioration, carotenoids, transgenic cassava

1. Introduction

Cassava (*Manihot esculenta* Crantz) is an important staple crop in the tropical parts or countries of the world. The roots are starchy tubers, with several attributes such as withstanding droughts, cheap, available and affordable, resistant to pests and easy to cultivate. Although it is a valuable source of energy, the nutritional composition is deficient in some essential nutrients vitamins and minerals (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 (Bayoumi *et al.*, 2010). Carotenoids are natural terpenoids responsible for the distinctive yellow, orange and some reddish colours (as well as several aromas) in leaves, fruits, vegetables and flowers of plants (Fraser *et al.*, 2004). Carotenoids are biosynthesized and they participate in various biological processes in plants, such as photosynthesis, photo-morphogenesis, photo-protection, physical development (Nisar *et al.*, 2015; Cao *et al.*, 2015) and the production of carotenoid-derived phytohormones, including abscisic acid and strigolactone (Fraser *et al.*, 2004). The major plant carotenoids are antheraxanthin, capsanthin, α -carotene, β -carotene, ϵ -carotene, γ -carotene, α -cryptoxanthin, lutein, lycopene, neoxanthin, and zeaxanthin (Howitt *et al.*, 2006). Beta-carotene is a major precursor of vitamin A (retinol) in humans (Grune *et al.*, 2010) and it is an important micronutrient in humans (Burri, 1997). Diets containing carotene-rich vegetables, fruits and roots are useful in protection against cancer, heart diseases, cataracts and ultraviolet-induced skin damage (Carvalho *et al.*, 2016). The development of high yield β -carotene crops such as golden rice, yellow cassava and orange sweet potato could provide the recommended daily intake of vitamin A for malnourished children and help combat vitamin A deficiency-induced mortality and morbidity. Beta-carotene content is also associated with reduction in post-harvest physiological deterioration of cassava roots due to the oxidative nature of carotenoids (Sanchez *et al.*, 2006). Postharvest physiological deterioration (PPD) is increasingly important due to urbanization in producing countries and the resultant increase in distance and time between farm and market or processing centers. There have been several estimates of the economic impact of PPD. Estimated losses due to PPD in cassava range from 5–25% of the total expected value of the crop

(Zidenga, 2012). The physiological changes that occur during PPD are due to the oxidation of phenolic compounds and involve the formation of reactive oxygen species (ROS). Alterations in gene expression, protein synthesis and the accumulation and oxidation of a range of secondary metabolites (Beeching et al., 2009). Metabolites represent the outcome of gene expressions and define the biochemical phenotype of a cell, tissue, organ or organism. Small metabolites include the intermediates and end products of metabolism, and they include primary (sugars, amino acids, fatty acids and organic acids) and secondary metabolites (phenyl propanoids, terpenes, flavonoids and alkaloids) (Commisso et al., 2013). Recent advances in metabolomics have enabled untargeted profiling of thousands of metabolites. Metabolomics can characterize the dynamic metabolome, showing changes in the abundance of small molecules during development and in response to external stresses (Vinayavekhin & Saghatelian, 2010). In this study, an untargeted metabolomics combined with a data-driven approach for statistical analysis was used to profile conventionally bred and transgenic high beta-carotene cassava varieties to identify the relevant metabolite changes that take place in cassava varieties.

2. Method

All reagents were of analytical grade unless otherwise stated. HPLC grade solvents were obtained from J.T. Baker and Sigma-Aldrich USA. LC-MS grade water was obtained from Honeywell part of Thermo Fisher Scientific, USA. Cassava samples were analyzed for their metabolite content using liquid chromatography-high resolution mass spectrophotometer (LC-HRMS) and hydrophilic interaction liquid chromatography (*HILIC*) Q-Exactive. All experiments were carried out at Donald Danforth Plant Science Center (DDPSC) St. Louis, Missouri USA in collaboration with the National Root Crops Research Institute (NRCRI), Umudike, Nigeria.

2.1 Sample Collection

Identification and selection of two conventionally bred (UMUCASS) high beta-carotene cassava or yellow cassava stakes were carried out with the assistance of 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 at the Donald Danforth Plant Science Center, St. Louis Missouri, USA and harvested four months after planting.

2.2 Sample Preparation

Twenty (20) mg of ground freshly harvested cassava root samples were weighed for each variety. Two hundred (200) μL of 80% methanol was added to the samples. The samples were mixed thoroughly using vortex for 1 minute before centrifuge at 13.2 rpm for 5 min at 4 °C. The supernatants were carefully removed and filtered with polyether-sulfone (PES) spin-filters before loading onto the LC-HRMS.

2.3 Dry Matter Content

The dry matter content of the cassava varieties was determined by a modified method of Asare, (2004) using a freeze drier. The dry matter content was determined from the difference between the fresh and dry weights of the samples. Dry matter was expressed as a percentage of dry weight relative to fresh weight (Morante *et al.* 2010).

2.4 Starch and Sugar Analysis

The starch content of the experimental fresh cassava roots were determined using methods for Carbohydrates in Handbook of Chromatography (Churms, 1982) while the iodine-starch test was determined following the protocol in the Advanced Bio imaging Laboratory of Donald Danforth Plant Science Center St. Louis, USA.

2.5 Chromatographic Analyses

High performance chromatographic (Fig. 1) analyses were performed with Zic-HILIC (Higgins Analytical INC USA) using a 150 x 0.5 mm column and a reverse phase (PLRPS) 100 x 0.5 mm C_{18} column on a Thermo Scientific Q-Exactive liquid chromatography coupled to a high-resolution mass spectrometer (LC-HRMS) respectively. Eluent A was 10mM NH_4HCO_3 (v/v) and 95 % ACN and eluent B was 10mM NH_4HCO_3 (v/v). Under gradient conditions with initial condition of 100% B and 0% A in 30 min, and back to the initial condition for re-equilibration, the analysis was carried out at a flow rate of 0.15 mL/min at room temperature. The raw data from Q-Exactive were processed using Elements for Metabolomics software.

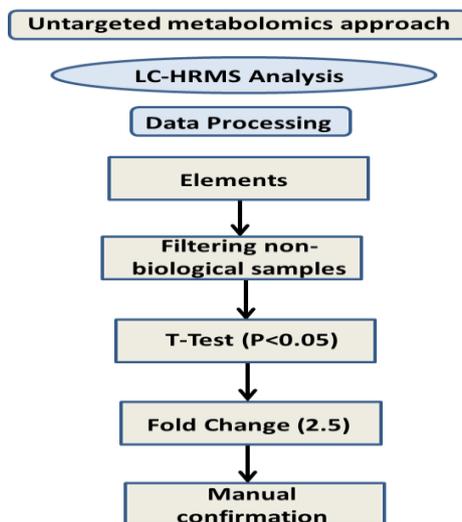


Figure 1. Flowchart of untargeted metabolomics approach used in this study

2.6 Statistics and Data Analysis

Statistical significance for all analysis carried out in triplicate was established using one-way analysis of variance (ANOVA), and data were reported as mean \pm standard deviation. Mean comparison and separation was established using Duncan Multiple Range Test ($P < 0.05$)

3. Results

3.1 Dry Matter Content (DMC)

The dry matter content of the cassava roots is presented in Table 1

Table 1. Dry matter content of the experimental cassava roots

Variety	Mean (%)
UMUCASS 38	35.76 \pm 2.37 ^b
UMUCASS 45	39.75 \pm 1.05 ^b
TME-7	39.35 \pm 5.64 ^b
EC20-7	24.55 \pm 2.19 ^a
EC20-8	23.90 \pm 0.75 ^a

Values are mean of triplicate determination, values with the same letter are not significantly different ($P=0.05$) using Duncan Multiple Range Test.

UMUCASS 38 = Conventional 1; UMUCASS 45 = Conventional 2

EC20-7 = Transgenic 1; EC20-8 =Transgenic 2

TME-7 =Wild Type

3.2 Starch and Sugar Content

The starch and sugar contents of the cassava roots on dry matter basis is presented in Table 2.

Table 2. Starch and sugar content of the cassava roots on dry matter basis

Variety	Starch (%)	Glucose (%)	Fructose (%)
UMUCASS 38	72.65 \pm 3.01 ^b	1.07 \pm 0.10 ^b	0.55 \pm 0.24 ^a
UMUCASS 45	75.75 \pm 1.9 ^{bc}	0.65 \pm 0.08 ^a	0.47 \pm 0.28 ^a
TME-7	77.09 \pm 0.09 ^c	1.06 \pm 0.13 ^b	0.44 \pm 0.23 ^a
EC20-7	54.10 \pm 2.55 ^a	1.05 \pm 0.20 ^b	0.47 \pm 0.25 ^a
EC20-8	52.25 \pm 1.01 ^a	1.19 \pm 0.05 ^b	0.76 \pm 0.13 ^a

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

UMUCASS 38 = Conventional 1; UMUCASS 45 = Conventional 2

EC20-7 = Transgenic 1; EC20-8 = Transgenic 2

3.3 Microscopic Distribution of Starch

Figure 2 show the distribution of starch granules in EC-20 and TME-7 roots



Figure 2. Microscopic view of stained EC20-7 transgenic yellow cassava variety



Figure 3. Microscopic view of stained TME-7 (Wild Type) cassava variety

3.4 Untargeted Metabolites of EC-20 and TME-7 using HILIC

The result of untargeted metabolites of EC-20 and TME-7 using HILIC is presented Table 3.

Table 3. Untargeted metabolites in fresh cassava roots comparing EC-20 and TME-7 roots using HILIC

Metabolite	Fold Change	Molecular Formula
Monogalactosyldiacylglycerol	719.00	$C_{35}H_{66}O_{10}$
Maltotriose	4.11	$C_{18}H_{32}O_{16}$
Digalactosyl diacylglycerol (DGDG)	3.30	$C_{43}H_{80}O_{15}$
Inosine	-3.49	$C_{10}H_{12}N_4O_5$
Phosphatidylcholine	-4.08	$C_{44}H_{84}NO_8P$

3.5 Untargeted Metabolites of UMUCASS and TME-7

The result of untargeted metabolites of UMUCASS and TME-7 using HILIC is presented in Table 4.

Table 4. Untargeted metabolites in fresh cassava roots comparing UMUCASS and TME-7 roots using HILIC

Metabolite	Fold Change	Molecular Formula
Lysophosphatidylcholine	4.20	$C_{26}H_{50}NO_7P$
Diacylglyceryl trimethylhomoserine	3.83	$C_{40}H_{67}NO_7$
Celastrol	-3.10	$C_{29}H_{38}O_4$
Inosine	-3.32	$C_{10}H_{12}N_4O_5$

3.6 Untargeted Metabolites of EC-20 and TME-7 RPLC

The result of untargeted metabolites of EC-20 and TME-7 using RPLC is presented in Table 5.

Table 5. Untargeted metabolites in fresh cassava roots comparing EC-20 and TME-7 roots using RPLC

Metabolite	Fold Change	Molecular Formula
Digalactosyl diglyceride	664.33	C ₅₃ H ₈₄ O ₁₅
Digalactosyl diglyceride	654.00	C ₅₃ H ₈₆ O ₁₅
Digalactosyl diglyceride	598.00	C ₅₃ H ₈₈ O ₁₅
Diacylglyceryl trimethylhomoserine	590.00	C ₄₆ H ₇₃ N ₇ O ₇
linoleic acid	3.14	C ₁₈ H ₃₂ O ₂
Dihydro-kaempferol	3.03	C ₁₅ H ₁₂ O ₆
Salicylic acid	2.99	C ₇ H ₆ O ₃
Aspartate	-3.00	C ₉ H ₁₆ N ₂ O ₅

4. Discussion

The percentage dry matter content (DMC) of TME-7 was the highest (42.60 %) among the cassava varieties and this was significantly ($P < 0.05$) different from the EC20-7 (24.23 %) and EC20-8 (23.18 %) respectively. The percentage DMC of UMUCASS 38 (35.61 %) and UMUCASS 45 (36.96 %) varieties were 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. Dry matter content relates to good cooking quality. Higher dry matter content suggests better cooking quality of the flour that would be produced from these cassava varieties in addition to their longer shelf life (Eleazu *et al.*, 2012). A positive correlation between dry matter and PPD was reported by Uarrotta *et al.*, (2015), and implies that varieties with high level of dry matter are more prone to suffer from PPD.

The starch content of the experimental cassava varieties indicate that EC20-8 (52.25 %) variety had the least percentage followed by EC20-7 (54.10 %) which were significantly ($P < 0.05$) different from the starch content of UMUCASS 38 (72.65 %), UMUCASS 45 (75.75 %) and TME-7 (77.09 %) varieties. A significant ($P < 0.05$) difference was observed in the glucose content of UMUCASS 45 (0.65 %) and UMUCASS 38 (1.07 %), EC20-7 (1.05 %), EC20-8 (1.19 %) and TME-7 (1.06 %). The fructose content of the experimental cassava varieties used in this study showed no significant ($P > 0.05$) difference among the varieties. The starch and sugar content show that TME-7 had the highest starch content which corroborates the findings of Aristizabal and Sánchez, (2007). Also, the distribution of starch granules in the transgenic roots (Fig. 2) seem to be confined between the phloem and xylem bundles while starch distribution in the whitish TME-7 roots Fig.3) were more widely distributed up to the sclerenchyma region of the root cortex. Postharvest physiological deterioration is associated with vascular streaking or discolouration and Sánchez *et al.*, (2006) observed a positive correlation between high starch content and the degree of PPD.

4.1 Untargeted Metabolites of EC-20 and TME-7

The untargeted metabolites of the fresh cassava roots for EC-20, TME-7 and EC-20 roots using HILIC are given in Table 3. Monogalactosyl-diacylglycerol (MGDG), maltotriose, and diagalactosyl-diacylglycerol (DGDG) were identified as the upregulated (> 2.0 folds) metabolites in TME-7 varieties. Monogalactosyl-diacylglycerol and diagalactosyl-diacylglycerol are galactolipids which have been recognized as principal lipid components in photosynthesis (Block *et al.*, 1983). Their quantity is mostly elevated in thylakoid membranes, in which MGDG exemplify 50% of the polar lipids. MGDG is well-maintained in practically all photosynthetic organisms, and it is one of the most abundant natural polar lipid in plants (Dormann and Benning, 2002). It is highly accumulated in the chloroplast membrane and also an important constituent of the thylakoid membranes (Masuda, 2011). Studies suggest its connectivity with chlorosome, a light-harvesting compound; and MGDG biosynthesis has been recognized in each photosynthetic organism to achieve photosynthesis under different environmental conditions (Masuda, 2011). Digalactosyldiacylglycerol is a typical membrane lipid of oxygenic photosynthetic organisms. DGDG is also important for the assembly and function of photosynthetic complexes in the thylakoid membranes (Dormann and Benning, 2002, Sakurai *et al.*, 2007). There are reports on the utilization of maltotriose and the possible relationship between maltotriose uptake and metabolism. Although maltotriose is considered a fermentable sugar, and this has been recently demonstrated for several brewer and baker strains (Londesborough, 2001). Maltotriose is mainly respired, which might explain its incomplete consumption at the final stage of oxygen-limited fermentation processes (Zastrow, 2000).

4.2 Untargeted Metabolites of UMUCASS and TME-7

The untargeted metabolites of the fresh cassava roots for UMUCASS, TME-7 and UMUCASS varieties using HILIC are given in Table 4. Lysophospholipids and diacylglyceroltrimethyl homoserine (DGTS) were identified as the upregulated (>2.0 folds) metabolites in TME-7 varieties. Lysophospholipids are polyunsaturated fatty acids synthesized from precursor fatty acids that are esterified to a complex glycerolipid (Ida Lager et al., 2013). The phospholipids of plant plasma membranes are produced in the endoplasmic reticulum. Most of these lipids get to the plasma membrane without passing through the secretory vesicular pathway. The transfer of phospholipids to the mitochondria and chloroplasts of plant cells also circumvents the secretory pathway and it has been suggested that lysophospholipids are transported via interaction sites between specific regions of the endoplasmic reticulum and the individual organelle, trailed by lysophospholipid acylation in the target organelle (Larsson et al., 2007).

4.3 Untargeted Metabolomics of EC20 and TME-7 Cassava Roots using RPLC Detection

The untargeted metabolites of EC-20 and TME-7 cassava roots in untargeted metabolomics relative to EC-20 varieties using RPLC is given in Table 5. It identified digalactosyl diglyceride (DGDG), DGTS, linoleic, dihydrokaempferol and salicylic acid as the secondary metabolites that were upregulated (>2.0 folds) in the transgenic varieties. Dihydrokaempferol is a flavonoid and one of the secondary metabolites that was upregulated in EC-20 cassava varieties. Flavonoids are widely distributed in plants and are an important part of our diet due to their health-promoting benefits, including reduced risk of cancer and cardiovascular diseases (Price and Rhodes, 1997; Lin and Harnly, 2008). Flavonoids are a large group of phytochemicals that are derived from multiple branches of the shikimic acid pathway, one of the most-characterized secondary metabolic routes in plant systems (Khanam et al., 2012). The metabolic pathway of flavonoids has been generally acknowledged to be involved in the regulation mechanism of plants to several stress conditions (Agati *et al.*, 2011]. The leading regulators of plant growth and defense are flavonoids, which are made and biosynthesized as a result of continuing natural selection and acclimatization processes (Jay-Allemand, 2015; Agati, 2012). Scavenging reactive oxygen species and growing tolerance to acclimatize to environmental variations are the predominant physiological functions of flavonoids as observed in tea plant, e.g., as antioxidants in photoprotection and this antioxidant activity is attributed to their reactions with free radicals as hydrogen donors (Zhang et al., 2017). Salicylic acid is an important signaling molecule in plants, it is a phenolic compound and has been found to be involved in the remediation of heavy metal toxicity, heat, osmotic and abiotic stress and drought response in plants (Vincent, 2011). According to Lu, 2009, salicylic acid act as an active calming agent for plants; also plays a very important role in the biochemical processes and regulations of the entire lifespan of the plant aside their biotic and abiotic functions. By understanding the objectives and its molecular modes of action in physiological processes could aid in the separation of the complex salicylic acid signaling network, favoring its vital function in plant health. Salicylic acid could also be responsible for the regulation of processes such as seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, flower formation, seed production, senescence, and a form of cell death that is not linked to the hypersensitive response (Mariana, 2011). Molecular genetic tools could not unravel the mechanism underlying the ability of cassava to survive and produce under drought conditions (Pingjuan et al., 2015). Untargeted metabolomics approach used in this study has identified salicylic acid as one of the compounds responsible for the management of drought and other abiotic stresses in cassava (Lu, 2009).

5. Conclusion

This study showed that cassava varieties with high level of dry matter and starch are more prone to suffer postharvest physiological deterioration. This is associated with vascular streaking which will be more distributed up to the sclerenchyma region of the root cortex. The intermediary metabolites and non-nutrient metabolites identified with HILIC and RPLC are of biochemical importance to the plant and nutritionally relevant but need further assessment as required by law for genetically modified (GM) foods.

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