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Effect of Scopoletin and Carotenoids on Postharvest Physiological Deterioration (PPD) of Transgenic High Beta Carotene Cassava

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Abstract

Cassava tubers suffer from postharvest physiological deterioration (PPD) which normally sets in within 72 hours of harvest. This study examines the role of scopoletin and carotenoids in the onset or delay in PPD in two transgenic varieties EC20-7 and EC20-8 compared to a wild variety TME-7. Scopoletin and carotenoids were quantified by liquid chromatography-mass spectrometry. The scopoletin content (0.10 – 0.20 nmol/g) in the fresh varieties was not significantly ($P>0.05$) different from the amount in stored cassava roots (12.58 – 14.90 nmol/g). The carotenoid content values in EC20-7 variety were 6.66 µg/g (α-carotene), 80.45 µg/g (β-carotene) and 5.98 µg/g (lutein). As for EC20-8, α-carotene, β-carotene and lutein values were 6.19 µg/g, 69.11 µg/g and 3.12 µg/g, respectively. There was no significant ($P>0.05$) difference between the varieties in α-carotene content but in their lutein content. The results indicate that carotenoids are more relevant in the delay of PPD and scopoletin content is not a major factor in PPD vascular streaking or discolouration. Hence scopoletin content of cassava varieties may not be considered as a chemical marker for determining the potential of PPD in cassava tubers.

Keywords: postharvest physiological deterioration (PPD), metabolites, vascular discoloration, cassava, carotenoid

1. Introduction

Cassava (*Manihot esculenta* Crantz) is one of the major staple crops in Africa. The plant is highly cultivated because of its resistance to drought and pests as well as being a rich source of starch and energy (Edoh *et al*., 2014). However, the tubers undergo post-harvest physiological deterioration (PPD), an active physiological response triggered by harvesting, and this poses a greatest hurdle in the utilization of cassava. PPD is characterized by a rapid discolouration of the cassava tubers and occurs within 24-48 hours after harvest. It is triggered by wounds on the tubers which occur during harvesting and handling. The discolouration first appears in the vascular system around the wounds (Reilly *et al*., 2007) from where it spreads to the rest of the tuber. In just a few days after harvest, a blue-black coloration known as vascular streaking will accumulate in the vascular bundles in the parenchyma (Reilly *et al*., 2003). The deteriorated cassava roots have an unfavourable appearance and taste, which makes them commercially unacceptable. Some conventionally bred high beta-carotene varieties known as yellow cassava which were envisaged to assist in combating both vitamin A deficiency (VAD) and postharvest physiological deterioration have been introduced (Sanchez *et al.,* 2006). Another strategy is a transgenic modification of cassava varieties to increase their carotenoid content through genetic engineering as β-carotene content is associated with a reduction in post-harvest physiological deterioration and is thought to be due to oxidative nature of carotenoids (Rudi *et al*.,2010; Sanchez *et al*., 2006). Therefore, it is important to have information on the chemical and biochemical properties of cassava varieties with pronounced delay in postharvest physiological deterioration. The accumulation of stress response metabolites is an important indicator in PPD development as the process involves a wide range of compounds including coumarins and phenolic compounds and also other compound classes such as phytosterols and fatty acids such as palmitic, linoleic, and oleic acids and their derivatives (Sakai *et al*., 1986). The accumulation of 22 diterpenes was also confirmed in wounded cassava roots (Sakai and Nakagawa, 1988), and this is an unusual plant stress response. However, it is the hydroxycoumarins that accumulate most 'dramatically' during PPD (Bayoumi *et al*., 2010).

The most significant one of these hydroxycoumarins is scopoletin and its glucoside scopolin, while esculetin and its glucoside esculin are also accumulated but in less significant quantities. These secondary metabolites may act as anti-oxidants or antimicrobial agents (Buschmann *et al*., 2000, Sakai and Nakagawa, 1988). Scopoletin (7-hydroxy-6-methoxychromen-2-one) is synthesized as part of the phenylpropanoid metabolism. There is evidence that scopoletin is involved in plant defensive response in cassava, tobacco, tomato, and other plants (Sudha and Ravishankar, 2002, Sun *et al*., 2014). At the onset of PPD, the level of scopoletin in cassava roots rises rapidly, increasing from less than 1 ng/mg to 60-80 ng/mg in fresh roots in 48 hours, and remains at a high concentration during further development of PPD (Buschmann *et al*., 2000). The dramatic accumulation of scopoletin in PPD indicates that it plays an important role in the deterioration process. Supporting this is a pruning treatment that delays PPD and also lowers scopoletin synthesis in cassava roots (Van Oirschot *et al*., 2000). Scopoletin may scavenge ROS with its hydroxyl group; to support this, scopolin, the glycoside of scopoletin whose hydroxyl group is bound to a glucose residue, does not show ROS scavenging activity (Reilly *et al*., 2003). Wheatley *et al*. (1985) applied a range of phenolic compounds to fresh cassava root samples, and found that scopoletin was the only one that induced a rapid PPD discoloration, implying that scopoletin might act as a signaling molecule to trigger PPD (Wheatley and Schwabe, 1985). However, there is no evidence that scopoletin might have a signaling function in PPD, while there is considerable evidence for its anti-oxidant and anti-microbial activities. This study examines the role of scopoletin and carotenoids in the onset or delay in PPD in two transgenic varieties EC20-7 and EC20-8 compared to a wild variety TME-7.

2. Materials and Methods

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. Scopoletin was quantified by liquid chromatography-mass spectrometry using a Sciex 6500 QTRAP while an Agilent HPLC coupled to Thermo Finnigan LCQ Advantage ion trap mass spectrometer was used to quantify the carotenoids. 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 with the assistance of the Genetic Resource Unit and Cassava Programme of NRCRI. Collection of two transgenic bred (EC-20) high beta carotene cassava stakes and wild type (TME-7) were carried out with the assistance of the International Institute for Crop Improvement (IICI) Department of Donald Danforth Plant Science Center. The cassava stakes were planted in the greenhouse at the Center, and harvested four months after planting (4MAP).

2.2 Postharvest Physiological Deterioration (PPD) Set-up for Experimental Cassava Varieties

At 12 weeks the experimental cassava roots were carefully harvested for PPD experiment. The unbroken cassava roots from each of the experimental genotype (TME 7 (wild type), EC20-8 and EC20-7 (transgenic type)) were stored in a growth chamber at Donald Danforth Plant Science Center for five days. The oxidative vascular streaking and discoloration associated with postharvest physiological deterioration (PPD) was observed at 48 hours after harvesting of the roots. The ambient room conditions during the storage period was 27 ^{o}C and 60 % relative humidity (Ukpabi *et al*., 2014). The deterioration was imaged through a digital camera and the results were analyzed by visual inspection of the images.

2.3 Sample Preparation

This was carried out as described by Gamez-Meza *et al*. (1999). Fifty (50) mg of dried and ground roots was weighed. 100 µL of 0.1% formic acid was added to the powder and vortexed for one minute. The samples were homogenized in a TissueLyser II at 15 Hz for 10 minutes, and then allowed to extract overnight at a concentration of 100 mg/mL with 100 % LC-MS grade methanol in a 4 ℃ cold room. The samples were centrifuged at 13.2 rpm for 10 minutes at 4 ℃, and the supernatant collected. Samples were extracted two more times for 30 minutes and the supernatant was collected as previously described; the pooled supernatants were dried using a speed-vac. Samples were clarified using 0.8 µm PES spin-filters prior to separation.

2.4 Carotenoid Profiling and Quantification

Separation of the carotenoids was achieved by injecting 40 µL of sample onto the Agilent HPLC using a 250 x 2.0 mm YMC RP-C30 column. Ninety six (96) % methanol with 4 % water (aq) was used as mobile phase (A) and 90 % methyl-t-butyl ether, 7 % methanol with 3 % water (aq) as mobile phase (B). The gradient initiated at

100 % A with a hold for four minutes, then increased linearly to 85% B over 30 minutes, then a ramp back to 100 % A. The column was re-equilibrated for ten minutes before the next injection. Carotenoid quantification was accomplished by a combination of HPLC retention times and absorption spectra on an Agilent HPLC coupled to a Thermo Finnigan LCQ Advantage ion trap mass spectrometer by comparing peak areas of carotenoid standards of an external calibration curve prepared using analytical grade standards (PMSF-DDPSC protocol, 2016).

2.5 Determination of Scopoletin

Separation of scopoletin was achieved by injecting 2 µL of sample onto the Eksigent micro LC 200 using a 100 x 0.5 mm Targa C18 column. 0.1% formic acid (aq) was used as mobile phase (A) and acetonitrile with 0.1% formic acid as mobile phase (B). The gradient initiated at 95% A for three minutes, then increased linearly to 95% B over 5 minutes with a hold for 5 minutes, then a ramp back to 95% A. The column was re-equilibrated for ten minutes before starting the next injection. Quantitation was accomplished on a Sciex 6500 QTRAP by comparing peak areas of endogenous target analytes to that of an external calibration curve prepared using analytical grade standards (Gamez Meza *et al*., 1999).

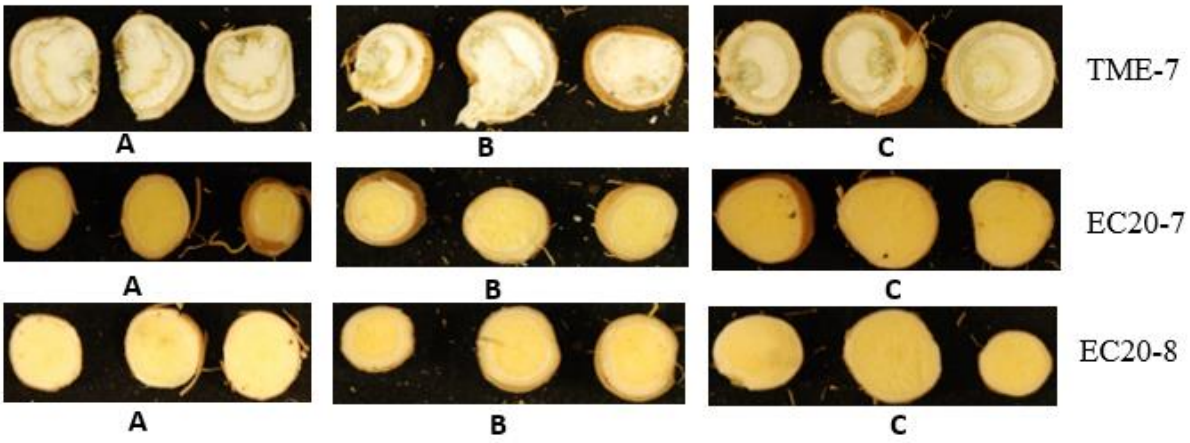
2.6 Statistical Analysis

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

3. Results and Discussions

3.1 Postharvest Physiological Deterioration (PPD)

The results of the PPD set-up for 120 h are presented Figure 1 and 2.



Figuer 1. Photographs of postharvest physiological deterioration (PPD) in replicates of the cut experimental cassava lines after 48 hours

EC20-7 = Transgenic type 1

EC20-8 = Transgenic type 2

TME-7 =Wild Type

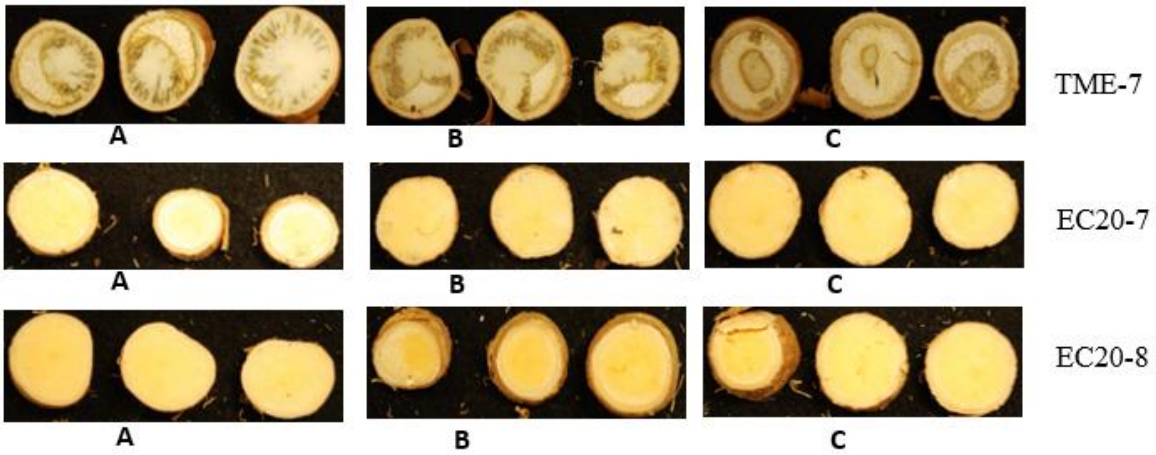


Figure 2. Photographs of postharvest physiological deterioration (PPD) in replicates of the cut experimental cassava lines after 120 hours

EC20-7 = Transgenic type 1

EC20-8 = Transgenic type 2

TME-7 = Wild Type

Figure 1 and 2 show the colour changes in cassava at 48 h and 120 h of storage. While the wild variety TME-7 (white) showed symptomatic appearance of PPD from the onset of harvest, the transgenic EC20-7 and EC20-8 varieties exhibited non-symptomatic appearance or delayed PPD even after 120 hours of harvest.

The result indicates that only EC20 varieties which had delayed PPD have appreciable quantities of carotenoids. Therefore, it is possible that the presence of carotenoids in the cassava roots contributed significantly to the delay of PPD vascular discolouration in these varieties as earlier suggested by Sanchez *et al.* (200) and Morante *et al*, (2010). Postharvest physiological deterioration in cassava is known to be linked to oxidative bursts (Sayer, *et al*, (2012) and carotenoids are also known to effectively scavenge reactive oxygen species (ROS) and other free radicals from different origins. This scavenging property has the potential to deliver protection against oxidative damage to plants and other organisms that undergo photosynthesis at all levels of complexity (Edge, *et al*, (2010).

3.2 Scopoletin Levels

Table 1 shows the result of scopoletin levels in fresh and stored un-deteriorated experimental cassava varieties.

Table 1: Scopoletin levels (nmol/g) of experimental cassava roots on dry matter basis

Lines	Fresh Roots	Stored Roots
TME-7	0.15 ± 0.08^{a}	14.66 ± 11.18^{a}
EC20-7	0.10 ± 0.04^{a}	12.58 ± 3.52^{a}
EC20-8	0.20 ± 0.61^{a}	14.90 ± 8.27^{a}

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

EC20-7 = Transgenic type 1

EC20-8 = Transgenic type 2

TME-7 =Wild Type

Previous studies indicated that scopoletin content of cassava roots increased with PPD up to 72 hours after harvest (Okeke *et al*., 2017). Hence, PPD vascular discolouration was linked to scopoletin content of the roots. In the fresh cassava roots, the scopoletin content in the varieties ranged from 0.10 – 0.20 nmol/g. Though there were observed differences in PPD rates among the cassava roots, no significant (P>0.05) differences were observed in the scopoletin content of the fresh cassava varieties used in this study which agreed with previous study by Aristizabal *et al.* (2007) which reported that scopoletin content in freshly harvested cassava roots are usually low but increased within hours of harvest. The scopoletin content of the stored cassava roots ranged from

12.58 – 14.90 nmol/g which indicated a remarkable increase. From this study, there is an indication that the scopoletin content of both the TME-7 (white) and EC20 (yellow) varieties increased during storage. Nevertheless, the transgenic EC20-7 and EC20-8 varieties with scopoletin content of 12.58 nmol/g and 14.90 nmol/g exhibited non-symptomatic appearance or delayed PPD (Plate 2) after 120 hours of harvest. These results suggest that scopoletin is not the major cause of PPD as previously considered (Wheatley, *et al*. 1985). Thus, scopoletin content of cassava varieties may not be used as a chemical marker for determining the PPD potential of cassava varieties.

3.3 Carotenoids

The results in Table 2 show the carotenoids identified in the study.

Table 2. Quantity of identified carotenoids of the experimental cassava roots (µg/g) on dry matter basis

Carotenoids	Alpha carotene	Beta carotene	Lutein
TME-7	ND	ND	ND
EC20-7	6.66 ± 2.05^{b}	80.45 ± 10.86^{b}	5.98 ± 1.60^{c}
EC20-8	6.19 ± 4.34^{b}	69.11 ± 45.80^{b}	3.12 ± 2.17^{b}

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

EC20-7 = Transgenic type 1; EC20-8 = Transgenic type 2; TME-7 =Wild Type

The detected and quantified carotenoids were alpha (α)-carotene, beta (β)-carotene and lutein. The variety EC20-7 had the highest α-carotene (6.66 µg/g), β-carotene (80.45 µg/g) and lutein (5.98 µg/g) content followed by EC20-8 with α-carotene (6.19 µg/g), β-carotene (69.11 µg/g) and lutein (3.12 µg/g). There were no significant ($P>0.05$) differences between EC20-7 α-carotene (6.66 µg/g); β-carotene (80.45 µg/g) and EC20-8 α-carotene (6.19 µg/g); β-carotene (69.11 µg/g) but a significant ($P<0.05$) difference was observed in the lutein content of EC20-7 (5.98 µg/g) and EC20-8 (3.12 µg/g). The three carotenoids -alpha (α)-carotene, beta (β)-carotene and lutein detected and quantified in this study were not found or detected in TME-7 variety. It is evident that the lutein content of EC20-7 was comparatively higher than the wild variety. The experimental result show that only EC20 varieties have appreciable amount of α-carotene therefore it is possible that this type of carotenoid is what is relevant in postharvest physiological deterioration (PPD) not necessarily beta-carotene or lutein. Alpha-carotene is known to impart yellow, orange or red colouration to many fresh foods (Cazzonelli, 2011) and Rodriguez-Amaya, 2001).

5. Conclusion

The transgenic high β-carotene varieties (EC20) which had delayed PPD had appreciable quantities of α-carotene, therefore, it is possible that this carotenoid is more relevant in the delay of PPD discolouration. Also, scopoletin content of the roots tremendously increased in the first three days of storage, and microscopic images showed that enhanced scopoletin content is not a major cause of PPD vascular streaking or discolouration in the roots. Hence scopoletin content of cassava varieties may not be considered as a chemical marker for determining the PPD potential of cassava varieties as previously suggested.

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