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Carotenoid profiling of yams: Clarity, comparisons and diversity

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ABSTRACT

Screening carotenoids of elite accessions of yam (*Dioscorea* spp.) used in the global yam breeding program has been conducted to quantitatively determine the carotenoid composition of the crop. Comparisons to previous data reporting cerotenoid levels in yam has been made, in order to deduce greater perspectives across multiple studies. Characterisation of complex species and accession -specific profiles have shown a rich base of diversity that can inform breeding strategies. Key findings include; (i) the identification of accessions rich in β-carotene which can aid provitamin A biofortification, (ii) Data disputing the commonly held belief that yellow Guinea yam (*D. cayennensis*) has higher β-carotene content than that of white Guinea yam (*D. rotundata*), and (iii) the tentative identification of C_{25}-epoxy-apocarotenoid persicaxanthin with potential implications for tuber dormancy.

1. Introduction

Yams (*Dioscorea* spp.) are a staple starchy tuber for 60–100 million people (Mignouna, Abang, & Asiedu, 2003). Whilst production costs are greater, in low technology farming systems yams have a higher yield and/or production value than other starchy tropical staples (Oke, 1990). Yams have preferred organoleptic qualities to other carbohydrate sources (Bhattacharjee et al., 2011). The relatively long dormancy period ensures yam tubers have a longer shelf life, even without refrigeration (Knoth, 1993), and so yams are vital for year-round food-security in growing regions. Favourable sensorial traits, better storage qualities and socio-economic importance have led yam to being considered as an agricultural commodity of superior economic value to alternative starchy crops (Osunde, 2008). In the growing regions, demand outstrips supply and it has been noted that, as income increases, consumers shift from cassava to yam (Sanginga, 2015). Despite these consumer preferences, yam is understudied when compared to other tropical root and tuber crops. Recent research on tropical crops has involved biofortification efforts, including enhancing provitamin A through increasing amounts/biosynthesis of β-carotene. Approaches have involved capturing natural diversity and targeted breeding, e.g., the HarvestPlus program (www.harvestplus.org), the CGIAR research program on roots, tubers and bananas (CGIAR-RTB) (www.rtb.cgiar.org) and for taro; or through genetic modification such as for GoldenRice (www.goldenrice.org), BioCassava Plus (Sayre et al., 2011), Bananas21 (www.banana21.org) and the Next Generation Biogreen21 Program (sweet potato) (Park et al., 2015).

Vitamin A deficiency is prevalent in yam-growing regions. Increasing the provitamin A carotenoid content of yams has been cited as a key nutritional improvement and especially desired by females, offering a gender-equal breeding option (Abdoulaye et al., 2015). However, improvements in the provitamin A content of *Dioscorea* are in early stages and behind that of cassava and sweet potato and not even included in the HarvestPlus Program. As incomes in low-income food-deficit countries (LIFDC) rise, consumers may switch from the provitamin A enriched sweet potato or cassava to yams, which is currently inadequately consumed. In addition to provitamin A activity, other carotenoids have key roles in nutrition and health, such as lutein and

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zeaxanthin for eye-health, antioxidant activities and potential chemo-preventative effects (Fraser & Bramley, 2004).

Understanding carotenoid biosynthesis is vital to understand plant development, due to their essential role in photosynthesis and as precursors of various signalling molecules and hormones (Hou, Rivers, León, McQuinn, & Pogson, 2016), such as abscisic acid (ABA), strigolactones and apocarotenoids, which regulate many cellular processes, including fruit ripening, environmental interactions and especially may be important for yam tuber dormancy, which is less understood and contrasts with the model tuberous crops, such as potato and cassava.

Contributing to the delay/absence in biofortification efforts on yams could be the lack of literature regarding carotenoid compositions of different species and where available, reports are conflicting. Historically, yams have been considered to be low in β-carotene. The most widely cultivated species are the Guinea yams, comprising the white variety *D. rotundata* and yellow variety *D. cayennensis*. *D. cayennensis* is reported to have higher carotenoid content (Bhattacharjee et al., 2011; Gedil & Sartie, 2010; Lebot, 2008), whilst *D. rotundata* is preferred by farmers and consumers. A few studies have showed that β-carotene is the major carotenoid of *D. cayennensis* (Ukom, Ojimelukwe, & Alamu, 2016; Ukom, Ojimelukwe, Ezeama, Ortiz, & Aragon, 2014), yet, other reports show β-carotene is of minor presence in the species (Champagne et al., 2010) and that the major carotenoids are xanthophyll esters (Martin & Ruberte, 1975). Recent work has shown varieties of *D. dumetorum* with estimated vitamin A activity at levels similar to that of enhanced cassava genotypes (Ferede, Maziya-Dixon, Alamu, & Asiedu, 2010). However, studies on other varieties of the species have found low carotenoid content (Ukom et al., 2014). The same holds true for the species *D. alata* and *D. bulbifera*, where reports conflict regarding carotenoid compositions and quantities of β-carotene (Inocent, Ejoh, Issa, Schweigert, & Tchouanguep, 2007; Lako et al., 2007; Martin, Telek, & Ruberte, 1974; Ukom et al., 2016).

Comprehensive and comparative studies between different *Dioscorea* species could potentially be improved by following Metabolomic Society Initiatives (MSI), (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013) and community recommendations (Fernie et al., 2011) on reported parameters regarding analytical methods, such as sample storage conditions, e.g., frozen, fresh or freeze-dried materials; saponification or non-saponification of extracts and compound identification parameters. Champagne et al. (2010) published the only broad species study of carotenoid composition in *Dioscorea* to date. The work highlighted genotype diversity and emphasised the importance of future study and potential for biofortification in yams. However, the authors noted the study was exploratory and due to the complex nature of profiles, identification of major carotenoids was lacking for species including *D. cayennensis*, *D. bulbifera* and *D. alata*.

In the present study, detailed comparative cross-species carotenoid profiling has been undertaken on tuber of elite accessions routinely used in yam breeding, to provide clarity regarding the carotenoid compositions of different *Dioscorea* spp. The technique also allowed simultaneous fingerprint profiling of other isoprenoids, including tocopherols and quinones. Results showed diverse species-specific profiles and the analysis of elite accessions has identified those with high provitamin A content, putative blocks in the carotenoid biosynthetic pathway and tentative identification of the C_{25}-epoxy-apocarotenoid persicaxanthin, which may play a role in tuber dormancy. The investigation serves as a foundation to develop breeding strategies towards nutritional improvement of yams and a potential approach to elucidate mechanisms of dormancy.

2. Materials and methods

2.1. Reagents

All reagents were of analytical standard supplied as follows: $CHCl_3$, EtOAc, HCl, MeOH and MTBE (Fluka, Loughborough, UK); DCM (VWR International, Leighton Buzzard, UK); *meta*-chloroperoxybenzoic acid (mCPBA), NaOH, Tris, NaCl, KOH, water, ammonium acetate and formic acid (Sigma-Aldrich, Gillingham, Dorset, UK).

2.2. Plant material

Accessions covering four species of *Dioscorea* were grown in field conditions of the Yam Breeding Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The field plot design was controlled and plants grown between June 2013 and Feb. 2014. Tubers were harvested and shipped to the Royal Holloway University of London (RHUL), United Kingdom for further analysis. For standard extractions tubers from three biological replicates per accession were sectioned laterally and longitudinally into 12; and 6 representative sections per tuber frozen in liquid nitrogen (Price, Bhattacharjee, Lopez-Montes, & Fraser, 2017). Sections were freeze-dried (Lyovac GT2, Leybold-Heraeus, Chessington, UK); skin peeled and ground (via a cryogenic mill; SPEX CertiPrep Freezer/Mill 6750, Stanmore, UK) to a homogenous powder prior to extraction. All samples were stored at −80 °C prior to further processing.

2.3. Preparation of standards

Standards were prepared using treatment with mCPBA (Rodriguez & Rodriguez-Amaya, 2007) and/or dilute HCl (Meléndez-Martínez et al., 2009). For epoxidation 10 mg of carotenoid stock and 1 mg of mCPBA were stirred at room temperature (RT) for 2 h in DCM and washed with NaOH (5%) followed by washing twice with water and dried using a centrifugal evaporator (Genevac EZ-2 Plus, SP Scientific, Suffolk, UK). Standards were re-suspended in EtOAc, volume adjusted to ensure well resolved spectra and aliquots (100 μL) taken. For furanoid rearrangement, 1 μL of HCl (0.1 mM) was added to the aliquots. Compounds were identified with comparison to reported retention times, spectra and elution orders (Meléndez-Martínez et al., 2009; Rodriguez & Rodriguez-Amaya, 2007; de Rosso & Mercadante, 2007). Tangerine tomato (*Solanum lycopersicum* var. Tangella) was extracted to provide reference for ζ-carotene, phytoene and phytofluene. Additionally, β-zeacarotene, ζ-carotene, phytoene and phytofluene were extracted from *Phycomyces blakesleeanus* mutant S442. In brief, lyophilised mycelia from 7-day-old cultures of S442 grown on Sutter agar were ground in a tissue lyser II (Qiagen, Manchester, UK) at 30 rpm for 8 min and 10 mg extracted thrice using MeOH: $CHCl_3$ (1:1), incubated for 1 h on ice and phase separated *via* addition of water. Organic phases were pooled, dried under centrifugal evaporation and stored at −80 °C prior to further processing.

2.4. Extraction of carotenoids

Carotenoids were extracted following a modified protocol (Fraser, Pinto, Holloway, & Bramley, 2000), whereby 200 mg of lyophilised tissue were extracted in 15-mL borosilicate glass test tubes (Fisherbrand, Loughborough, UK). To each sample, 6 mL of chilled (−20 °C) MeOH:$CHCl_3$ (1:2) were added, vortexed (VELP Scientifica ZX3 Advanced Vortex Mixer, Usmate (MB), Italy) and incubated for 15 min at −20 °C. Subsequently, 2 mL of ice cold 100 mM Tris-HCl buffer containing 1 M NaCl (pH 7.5) were added. Samples were vortexed, centrifuged at 3250*g* for 5 min at 4 °C to facilitate phase separation, and the organic phase removed using a glass Pasteur pipette. Repeated extractions were undertaken until material was exhausted of visible colour (typically 1–2 extractions). Organic phases were dried using a centrifugal evaporator (no lamp) and stored at −80 °C before further processing. Saponification involved the addition of (2 mL) methanolic KOH (10%), vortexing and incubation at 40 °C (Techne Dri-Block DB-2A; Cole-Parmer, Stone, UK) for 15 min, followed by addition of chilled (−20 °C) $CHCl_3$ (4 mL) and phase separation by addition of water (2 mL). Saponified samples were then processed as per normal

extractions however; the organic phase was further washed with water (1 vol) twice before drying.

For increased identification of carotenoids, larger extractions were carried out on selected samples, using 1 g of material with extended centrifugation times of 10 min.

2.5. HPLC-PDA for photosynthetic isoprenoids

Samples were analysed using a high-performance liquid chromatography (HPLC) (Waters Alliance 2695 model, Elstree, UK) with a photodiode array detector (PDA) (Waters 966; wavelength range 200–600 nm).

Samples were reconstituted in EtOAc (50 µL) *via* sonication for 5 min at RT, centrifugation at 18407*g* (Eppendorf Centrifuge 5224, Stevenage, UK) for 5 min and removal of the top 40 µL into newly labelled glass vials with insert (Supelco, Bellefonte, PA) and capped. Prior to injection re-suspended samples were kept at 8 °C in the dark. Samples (20 µL) were injected onto a reverse-phase (RP) column (4.6 × 250 mm, C_{30}, 5 µm particle size; YMC Inc., Kyoto, Japan) coupled to a RP guard column (4.6 × 20 mm, C_{30}, 5 µm particle size; YMC Inc.) at 25 °C. The mobile phase was comprised of (**A**) MeOH, (**B**) MeOH/water (80:20, *v/v*) containing 0.2% (*w/v*) ammonium acetate and (**C**) MTBE. Elution from the column with a flow rate of 1 mL/min was carried out from 95% **A** and 5% **B** for 12 min, followed by a step to 80% **A**, 5% **B** and 15% **C** and a linear gradient to 30% **A**, 5% **B** and 65% **C** for 18 min. The column was then returned to initial conditions over the next 30 min (Fraser et al., 2000).

Peaks were automatically integrated using Waters Empower software with a minimum peak height of 1000. Samples were analysed continuously from 200 to 600 nm and peak areas extracted from recordings at 450 nm, 350 nm and 286 nm. Identification of isoprenoids was performed by the comparison of spectral and chromatographic characteristics to standards and literature references (Britton, 2004) in addition to elution order, as shown in Supplementary Table 2. Only peaks present in all three replicates of any individual sample were retained and solvent blanks subtracted. Sterol and sterol esters were excluded from data analysis, due to inability of precise identification *via* UV spectra.

2.6. LC-MS for mutatochrome (β-carotene 5,8-epoxide) identification

Concentrated samples were analysed using a maXis UHR Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), on-line with a UPLC UltiMate 3000 with PDA detector (200–600 nm; Dionex Softron, Gemering, Germany). Chromatographic procedures followed that of Perez-Fons et al. (2011). Briefly, separations were made on an RP C_{30} 3 µm column (150 × 2.1 mm i.d.; YMC Inc.) coupled to a 20 × 4.6 mm C_{30} guard column (YMC Inc.). The mobile phase comprised solvent **A**, MeOH containing 0.1% formic acid (by volume), and **B**, MTBE containing 0.1% formic acid (by volume). A gradient elution was used, starting at 100% **A** for 5 min, stepped to 95% **A** for 4 min and followed by a linear gradient over 30 min to 25% **A**. Later this gradient was stepped down to 10% **A** over 10 min. Initial conditions (100% **A**) were restored for 10 min to re-equilibrate the system. The flow rate was 0.2 mL/min.

Detection was *via* atmospheric pressure chemical ionisation (APCI) in positive mode with an ionisation temperature of 450 °C, dry gas (nitrogen) at 1.3 L/min and nebuliser at 2 bar. The APCI source settings for detection were: corona discharge voltage at 6000 nA and a capillary voltage of 1.5 kV, with the end plate set at 500 V. A full MS scan was performed from *m/z* 100–1600 and MS/MS spectra were recorded at an isolation width of 0.5 amu, operating in "auto MS" mode with a collision energy ramp from 35 to 70 eV. Instrument calibration was performed externally prior to each sequence with ESI-TOF tuning mix (Agilent Technologies, Cheadle, UK) and automated post-run internal calibration was performed by injecting the same calibrant solution at the end of each sample run *via* a six-port divert valve equipped with a 20-µL loop. Spectra were processed using Compass Data Analysis 4.0 (Bruker Daltonics). Base-peak UV chromatograms were extracted at 450 nm wavelength and aligned to the base-peak chromatogram (~8s shift). Compounds were targeted *via* extracted ion-chromatograms of the [M + H] ion.

2.7. Statistical analyses

All data analyses were performed using XLSTAT add-ins (Addinsoft, Paris, France) within Microsoft Excel (Redmond, WA). Provitamin A activity was calculated using estimates relative to β-carotene (100%), whereby α-carotene (53%), β-cryptoxanthin (57%), β-zeacarotene (30%), β-carotene 5,6-epoxide (21%), mutatochrome (β-carotene 5,8-epoxide; 50%), luteochrome (β-carotene 5,6,5′8′-diepoxide; 14%) (Bauernfeind, 1972) and 13-*cis*-β-carotene (62%) (Deming, Baker, & Erdman, 2002) were used. Furthermore, carotenoids with a likely reported activity were estimated as aurochrome (β-carotene 5,8,5′8′-diepoxide; 14%, similar to luteochrome), β-cryptoxanthin 5,6-epoxide (11%, a fifth of β-cryptoxanthin activity), β-cryptoxanthin 5,8-epoxide (27.5%, half of β-cryptoxanthin activity), yet with α-cryptoxanthin excluded due to lack of a β-ring structure. Principal component analysis (PCA) was performed on the Spearman correlation matrix. Agglomerative hierarchical clustering (AHC) was performed using complete linkage on the Spearman dissimilarity matrix. Kruskal-Wallis' one-way analysis of variance were performed using Monte Carlo permutations (10,000) for *p*-value calculation. Conover-Iman post hoc tests ($\alpha = 0.05$) were Bonferroni-corrected. All univariate tests were two-tailed. Spearman correlations were performed on replicate-averaged data for the associations between β-carotene, β-carotene epoxides and provitamin A activity, and comparisons made *via* Steiger's *Z*-test for dependent samples using Quantpsy (Preacher, 2013).

3. Results & discussion

3.1. Scaled-down extraction method

The extraction method utilised up to 20-fold less raw material and less than half the solvent of the commonly applied modified HarvestPlus approach (Champagne et al., 2010; Ferede et al., 2010; Ukom et al., 2014), whilst attaining profiles which matched some of those previously recorded, with many xanthophyll esters, e.g., *D. alata* (Champagne et al., 2010), *D. bulbifera* (Martin et al., 1974) and *D. cayennensis* (Martin & Ruberte, 1975) and stereoisomers present. The scaled-down extraction allowed increased throughput with easier sample handling and, consequently, the relatively long run-time of the HPLC was the limiting factor for the speed of screening. However, the complexity of isoprenoid compositions meant that poor resolution was achieved, e.g., when testing UPLC methodologies (Supplementary Fig. 1); and long run times were required, an issue previously reported (Champagne et al., 2010; Ukom et al., 2014). Similar to previous modification of the typical HarvestPlus procedure (Rodriguez-Amaya & Kimura, 2004), freeze-dried material was utilised to limit differential extraction by standardising the solvent:moisture content ratio and enabling precise comparisons.

A limitation encountered in this study was the presence of sterols in the samples, which hindered absorbance within the 200–300 nm range. Due to co-elution across the whole range of the chromatographic gradient, absolute quantification of tocopherols, quinones and phytoene was not possible. Despite this, these compounds were easily distinguished from sterols because of their fine spectra (Supplementary Fig. 2) and so allowed relative comparisons to be drawn.

3.2. Species-specific carotenoid profiles

Isoprenoid fingerprint profiles recorded from tubers of elite

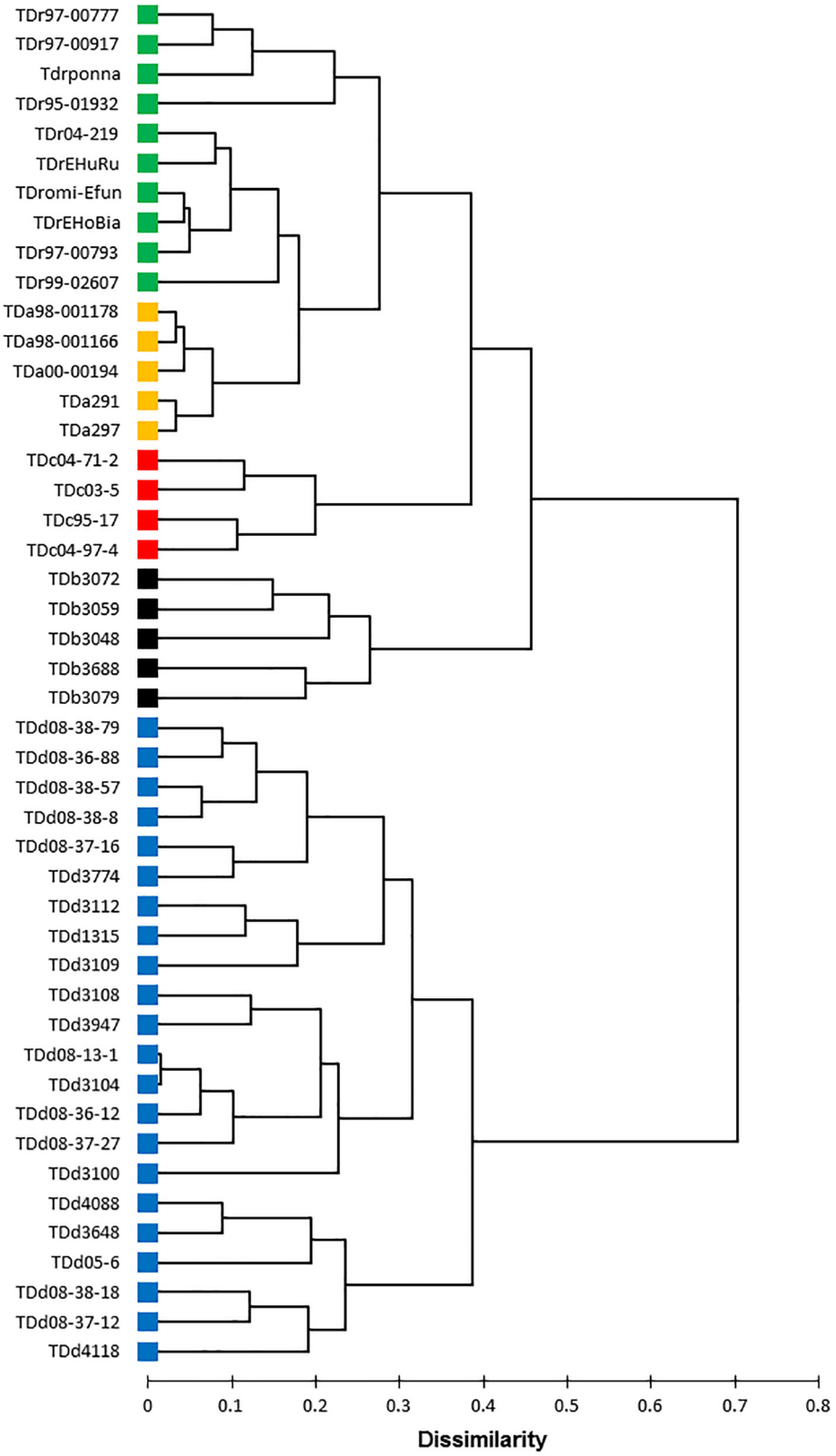

Fig. 1. Dendrogram of tuber samples based on mean-averaged (n = 3) isoprenoid composition fingerprints, recorded *via* HPLC-DAD at three wavelengths (450 nm, 350 nm and 286 nm).

accessions from the yam breeding unit at IITA using HPLC-DAD were species-specific and each species clustered in distinct groups (Fig. 1). The profiles of *D. dumetorum* were most divergent, forming a separate class to the other species and, except for *D. rotundata*, all species showed monophyletic grouping. At a species level, clustering largely matches the findings of primary metabolite profiles (Price et al. 2017). However, clustering of these accessions is not well matched between carotenoid profiles and primary metabolite composition. This is not surprising because the extensive biosynthetic pathway of carotenoids and many precursors were not measured on the primary metabolite

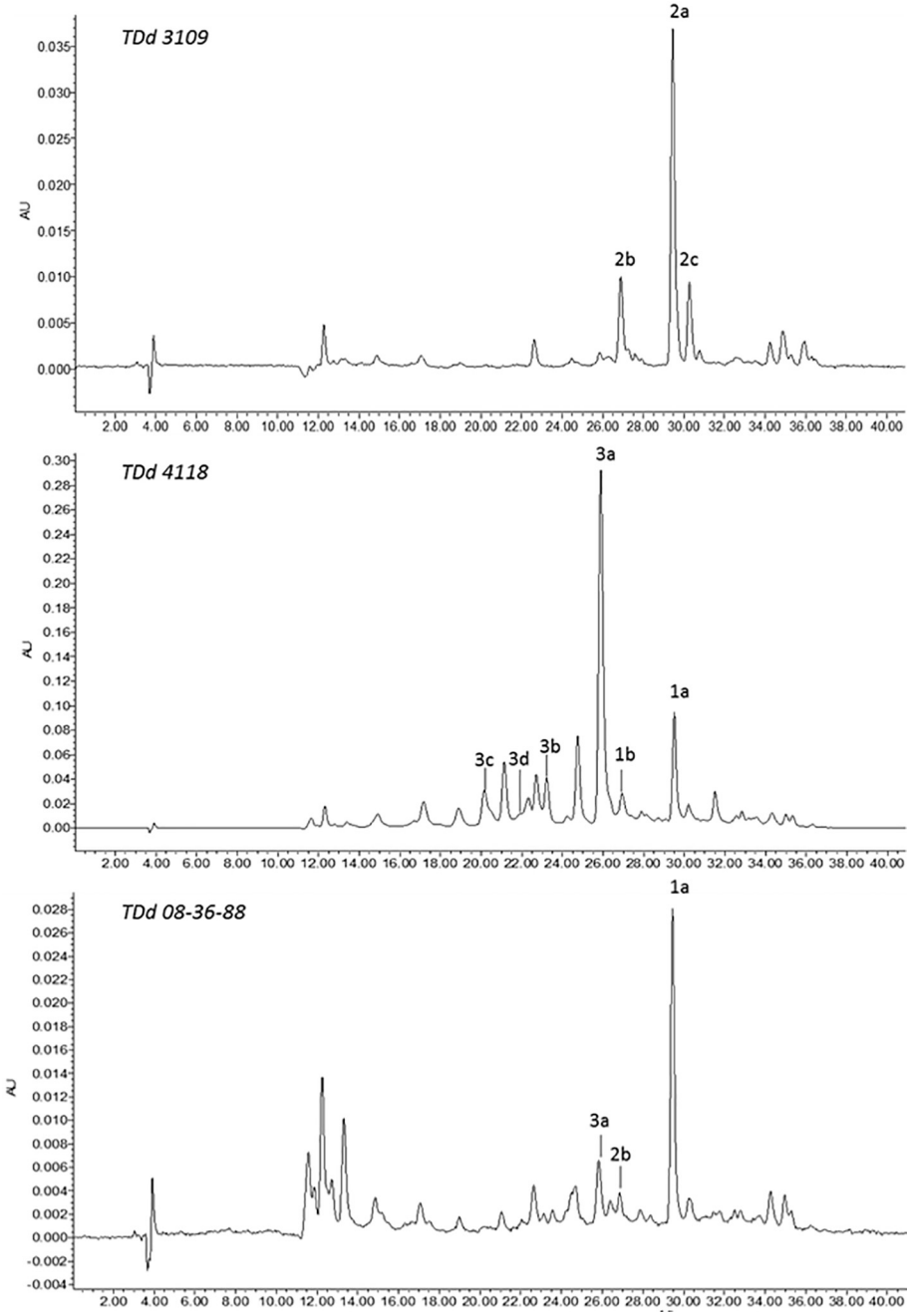

Fig. 2. Accessions of *D. dumetorum* showed diversity in carotenoid profiles including accessions accumulating various mixtures of (1) β-carotene (2) ζ-carotene and (3) β -carotene epoxides. Example HPLC-DAD chromatograms (recorded at 450 nm) for *TDd 3109*, *TDd 4118* & *TDd 08–36-88* (top to bottom). Peak identification: (1a) all-*trans*- β-carotene, (1b) 13-*cis*-β-carotene, (2a) ζ-carotene (putative 9,15,9′-*cis* isomer), (2b) ζ-carotene (putative tri-*cis* isomer), (2c) ζ-carotene (putative tri-*cis* isomer), (3a) mutatochrome [β-carotene 5,8-epoxide], (3b) β-carotene 5,6-epoxide, (3c) luteochrome [β-carotene 5,6,5′,8′-diepoxide] and (3d) aurochrome [β-carotene 5,8,5′,8′-diepoxide]. Identification was via co-chromatography and mass spectral comparison with authentic standards, detailed in Supplementary Table 2.

screening platform.

Many accessions of *D. dumetorum* were dominated by β-carotene epoxides, similar to those found by Ferede et al. (2010). Whilst Ferede et al. (2010) identified the abundant mutatochrome (9.4 ± 3.9 μg/g FW) and other major epoxides through co-chromatography, numerous chromatographic peaks remained unidentified. In this study, further co-chromatography with authentic standards increased identification of xanthophylls and additional LC-MS analysis was implemented to provide confirmatory detection of the major carotenoid epoxide mutatochrome (Supplementary Fig. 3). However, other epoxides, such as luteochrome, could not be confirmed on the LC-MS system, due to their low abundance and limited material availability.

Notably, accessions of *D. dumetorum* predominantly accumulating β-carotene have also been identified, e.g., TDd 08-36-88 (Fig. 2), TDd 3947 and TDd 3112. Despite this, the total β -carotene content was often less than in those which co-accumulated β-carotene epoxides (Table 1). It was previously reported that for *D. dumetorum*, mutatochrome was a significant predictor of provitamin A activity whilst β-carotene was not (Oladeji et al., 2016). Whilst similar correlation coefficients for β-carotene and mutatchrome with provitamin A content were reached in this work ($r = 0.788$ and 0.910; respectively, both $p < 0.001$), following Steiger's *Z*-test they were deemed equivalent.

However, there is the assumption that mutatochrome possesses half the provitamin A activity of β-carotene and until this is ascertained for humans, reports on provitamin A activity can only be considered estimates. The formation, stability and bioavailability of these epoxides over the storage of yam tubers will be an important factor to be addressed for considering biofortification. Both accessions TDd 08-38-18 and TDd 08-37-12 had similar β-carotene epoxide contents and provitamin A activities, rivalling those of the highest lines previously screened by Ferede et al. (2010). Whilst that group did not report intra-accession variation, in this work variation amongst accessions of TDd

Table 1
Provitamin A activity/carotenoids across all accessions.

species [abbreviation]	accession	carotenoid (μg/100 g DW)						
		all-*trans*-β-carotene		β-carotene epoxides[1]		provitamin A (PVA) activity[2]		PVA Intraspecies groups
D. alata L. [TDa]	00–00194	**AB**	164 ± 22	**A**	n.d.	**BC**	260 ± 24	C
	98–001166		153 ± 0.39		n.d.		251 ± 1.5	C
	98–001176		152 ± 7.3		n.d.		215 ± 8.6	BC
	297		107 ± 1.8		n.d.		168 ± 2.1	A
	291		110 ± 1.2		n.d.		170 ± 1.4	AB
D. bulbifera L.[3] [TDb]	3048	**B**	212	**B**	103 ± 8.8	**BC**	226 ± 1.2	n.s.
	3059		257		n.d.		257	n.s.
	3072		194		113 ± 8.3		210 ± 1.2	n.s.
	3079		175		111 ± 5.7		191 ± 0.79	n.s.
	3688		190		105 ± 2.0		205 ± 0.28	n.s.
D. cayennensis Lam. subsp. *cayennensis*[4] [TDc]	03–5	**A**	96.3 ± 2.3	**A**	n.d.	**AB**	124 ± 3.2	AB
	04–71-2		101 ± 2.0		n.d.		101 ± 2.0	A
	04–97-4		115 ± 7.0		n.d.		143 ± 7.2	B
	95–17		139 ± 11		n.d.		139 ± 11	B
D. dumetorum (Kunth) Pax [TDd]	4118	**AB**	253 ± 7.0	**C**	1280 ± 42	**C**	927 ± 36	JK
	3947		152 ± 17		217 ± 12		229 ± 21	EFG
	3109		n.d.		96.8 ± 0.96		19.4 ± 0.19	A
	08–37-12		215 ± 6.1		1640 ± 49		992 ± 29	K
	08–36-88		140 ± 5.4		207 ± 4.2		272 ± 7.7	HI
	1315		114 ± 3.1		107 ± 2.5		135 ± 3.6	AB
	3100		189 ± 23		112 ± 4.0		211 ± 24	EF
	3104		127 ± 5.6		101 ± 2.1		147 ± 6.0	ABC
	3108		144 ± 33		220 ± 18		313 ± 37	HI
	3648		241 ± 23		420 ± 35		489 ± 43	IJK
	08–13-1		130 ± 1.8		102 ± 0.56		150 ± 1.7	ABC
	08–36-12		138 ± 14		207 ± 13		210 ± 18	DEF
	08–38-8		120 ± 2.5		204 ± 4.4		246 ± 4.6	FGH
	08–37-27		138 ± 8.8		213 ± 1.8		211 ± 8.1	EFG
	08–38-57		135 ± 3.0		116 ± 3.6		159 ± 3.5	BCD
	3112		129 ± 10		101 ± 2.1		150 ± 10	ABC
	4088		203 ± 10		342 ± 13		345 ± 15	HIJ
	3774		129 ± 6.3		199 ± 2.0		256 ± 7.3	GH
	08–38-79		128 ± 1.2		200 ± 2.5		198 ± 1.2	CDE
	08–37-16		127 ± 1.0		99.1 ± 0.40		203 ± 1.2	DEF
	08–38-18		238 ± 33		1670 ± 280		993 ± 160	K
	05–6		332 ± 9.7		595 ± 28		695 ± 23	IJK
D. cayennensis Lam. subsp. *rotundata* (Poir) J. Miège [TDr]	95–01932	**A**	169 ± 21	**A**	n.d.	**A**	169 ± 21	E
	97–00917		134 ± 10		n.d.		134 ± 10	CDE
	99–02607[2]		132 ± 5.0		n.d.		132 ± 5.0	CDE
	EHoBia		109 ± 0.97		n.d.		109 ± 0.97	ABC
	EHuRu		104 ± 0.92		n.d.		104 ± 0.92	AB
	ponna		111 ± 0.71		n.d.		111 ± 0.71	BCD
	97–00777		129 ± 1.9		98.3 ± 0.97		142 ± 2.0	DE
	97–00793		102 ± 5.7		n.d.		102 ± 5.7	A
	04–219		102 ± 6.2		n.d.		102 ± 6.2	A
	omi-Efun		104 ± 1.7		n.d.		104 ± 1.7	A

n.d. = not detected, n.s. = not significant, amounts reported to 3 significant figures ± 1 standard deviation.
Letters represent resultant groups following comparisons via Bonferroni-corrected Conover-Iman post hoc tests following Kruskal-Wallis' one-way analysis of variance (α = 0.05); inter-species comparisons in bold (column-wise) with intra-species comparisons for PVA underlined.

[1] Quantified relative to standard dose curve for β-carotene; totals of β-carotene 5,6-epoxide, mutatochrome, luteochrome and aurochrome.

[2] Provitamin A activity calculated based on relative activity to β-carotene as described in *Materials and Methods*.

[3] β -carotene only quantified following saponification on one biological replicate, due to limited sample.

[4] β-carotene estimated following saponification of extract.

08-38-18 is over five times larger than that of TDd 08-37-12. As such, TDd 08-38-18 would make a better candidate for stable provitamin A biofortification.

Interestingly, despite all accessions of *D. dumetorum* clustering into one divergent group (Fig. 1), following principal component analysis (PCA), accession TDd 3109 was identified as an outlier distinct from all other samples (Supplementary Fig. 4). TDd 3109 presented a unique profile abundant in ζ-carotene, though other accessions of *D. dumetorum* also contained ζ-carotene but as a minor component (Fig. 2). Following analyses of the ζ-carotene accumulating *Phycomyces blakeseeanus* mutant S442 and tangerine tomato (*Solanum lycopersicum* var. 'Tangella'), TDd 3109 likely signified a mutant deficient in ζ-carotene isomerase (Z-ISO) or ζ-carotene desaturase (ZDS) and thus accumulated 9,15,9′-tri-*cis*-ζ-carotene. In line with this finding, TDd 3109 also showed increased phytoene and phytofluene content, as seen in ζ-carotene accumulating tomatoes, following virus-induced gene silencing of Z-ISO (Fantini, Falcone, Frusciante, Giliberto, & Giuliano, 2013). Consequently, TDd 3109 is a potential breeding resource for redirecting precursor biosynthesis, such as to generate lines with enhanced antioxidant activity or for the manipulation of tuber dormancy, since subsequent carotenoids act as precursors to many signalling molecules (Hou et al., 2016; Walter & Strack, 2011).

Accessions of *D. bulbifera* and *D. cayennensis* were dominated by xanthophylls including xanthophyll esters which masked the presence

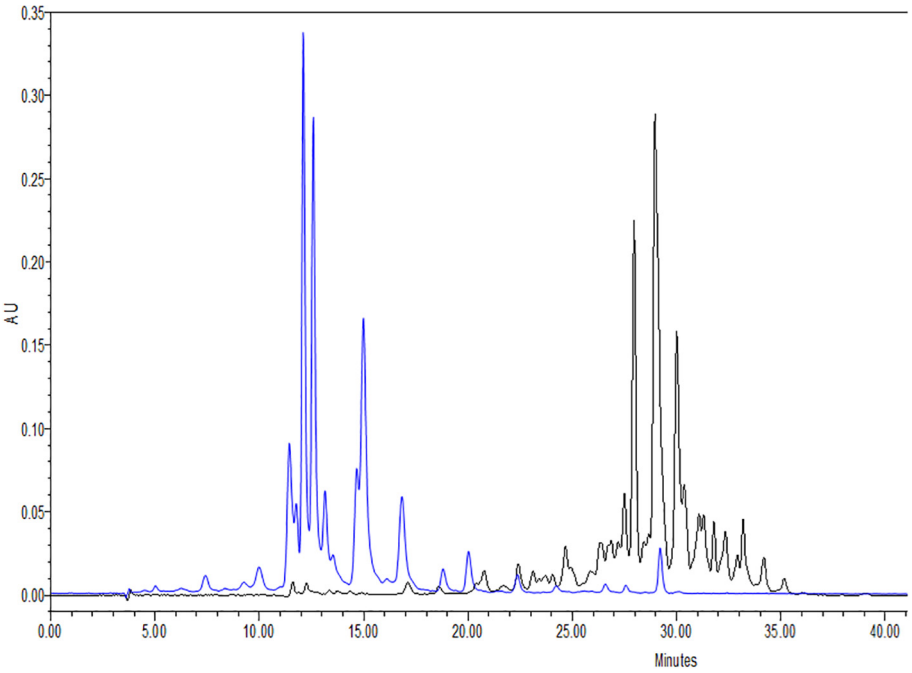

Fig. 3. HPLC-DAD chromatograms of accession TDc04-71-2 recorded at 450 nm, for non-saponified (black) and saponified (blue) samples. The abundance of xanthophyll esters in non-saponified samples masked the presence of β-carotene (retention time ~ 29.3 min), which was only measured following saponification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of β-carotene and required sample saponification (Fig. 3). Though overnight room-temperature saponification is typically recommended and conducted on the extract, a relatively fast (15 min) mildly-heated (40 °C) saponification step was implemented in this work. The procedure provided a faster process conducted directly on the tuber, rather than as an additional step. Following saponification, stereo-isomerisation of carotenoids occurred and was likely more pronounced due to heat; however, overnight room-temperature methods reported degradation (Rodriguez-Amaya & Kimura, 2004). Further methods could be developed using enzymatic saponification on tubers, e.g., *via* cholesterol esterase, to simplify the resultant mixture of cleaved products (Jacobs, LeBoeuf, McCommas, & Tauber, 1982).

With regards to other species, *D. bulbifera* showed higher abundance of free xanthophylls, xanthophyll epoxides and α-tocopherol. This is to be expected as the perennating organ used for this study (and often also termed tuber) was the aerial bulbils and thus photosynthetic tissue, evidenced as they harbour more chlorophyll. However, chlorophyll *b* and pheophytin *b* were more abundant than chlorophyll *a*, suggesting low light levels possibly caused by shading of bulbils by leaves. The β-carotene content of *D. bulbifera* was similar to that of *D. dumetorum* (Table 1), but quantification on this subset must be deemed preliminary, given the need for saponification and use of limited tissue, which prevented replication. That said, given the fact that the tuber of *D. bulbifera* is aerial and photosynthetic, a profile more similar to green tissues would be expected and thus would also tend to show greater quantities of β-carotene. In addition, *D. bulbifera* and *D. rotundata* contain higher levels of lutein, a carotenoid associated with the prevention of age-related macular degeneration (Fraser & Bramley, 2004).

Furthermore, all accessions of *D. bulbifera* showed peaks which have been tentatively identified as esters of the apocarotenoid persicaxanthin, given their spectra and retention shift following saponification (Supplementary Table 2). These esters were also tentatively identified in some accessions of each of the other species, except *D. alata* (Supplementary Table 1, Supplementary Fig. 5). The biological role of and biosynthetic pathway towards persicaxanthin is unknown yet, although persicaxanthin is reported in ABA-rich ripe fruits, such as peaches and plums (Gross & Eckhardt, 1981). A putative schematic diagram is shown (Fig. 4) where persicaxanthin is formed from the xanthophyll violaxanthin *via* an apo-12′-violaxanthal intermediate with the concurrent formation of the ABA precursor xanthoxin. Whilst apo-12′-violaxanthal was not detected following HPLC-UV analysis, persicaxanthin could be indicative of ABA levels or have a regulatory role in tuber dormancy.

Dormancy of yam is considered a double-edged sword; whilst the relatively long dormancy allows yam tubers to be stored for longer periods (up to 6 months) than other root and tuber crops, even at ambient temperature, it also extends the growing cycle and means only one annual crop can be produced.

D. cayennensis differed from *D. bulbifera* as it largely lacked any free xanthophylls with all being in esterified form. Though genetically closely related to *D. rotundata,* the two species were discriminated (Fig. 1). Discrimination between *D. rotundata* and *D. cayennensis* was not evidenced in measurements of central tuber metabolism (Price et al., 2017) or *via* genotyping-by-sequencing (GBS) (Girma et al., 2014) and results here support that carotenoid composition can be utilised to provide complementary chemotaxonomic classification. The approach is potentially faster than the detailed morphological classifications or scoring of numerous molecular markers typically required to assess yam diversity (IPGRI & IITA, 1997).

Carotenoid composition of the white *D. rotundata* has never been reported, with carotenoid content simply being deemed lower than yellow *D. cayennensis.* This reduction is commonly attributed to reduced β-carotene content (Martin & Ruberte, 1975; Martin & Sadik, 1977). In this study, *D. rotundata* typically had more lutein and less xanthophyll esters than *D. cayennensis*. However, the β-carotene content and provitamin A activity were not significantly different between the species (Table 1). As has been noted for *D. dumetorum*, tuber colour does not directly reflect provitamin A content and increased carotenoid content can have a negative correlation with proportion of provitamin A activity. Therefore, breeding towards vitamin A biofortification will require detailed compositional analysis, in contrast to the simplistic selection based on tuber colour, as has been applied for other crops, e.g., cassava (Oladeji et al., 2016).

Samples of *D. rotundata* were least dissimilar to those of *D. alata*, rather than the genetically closer cultigen *D. cayennensis.* That said, *D. alata* had greater α-tocopherol (Supplementary Table 1), averaged a greater β-carotene content and had significantly more provitamin A activity than *D. rotundata* (Table 1). Additionally, accessions of *D. alata* had noticeable quantities of the suspected 13-*cis*-β-carotene. Combined with the finding of epoxides within *D. dumetorum*, it indicates that

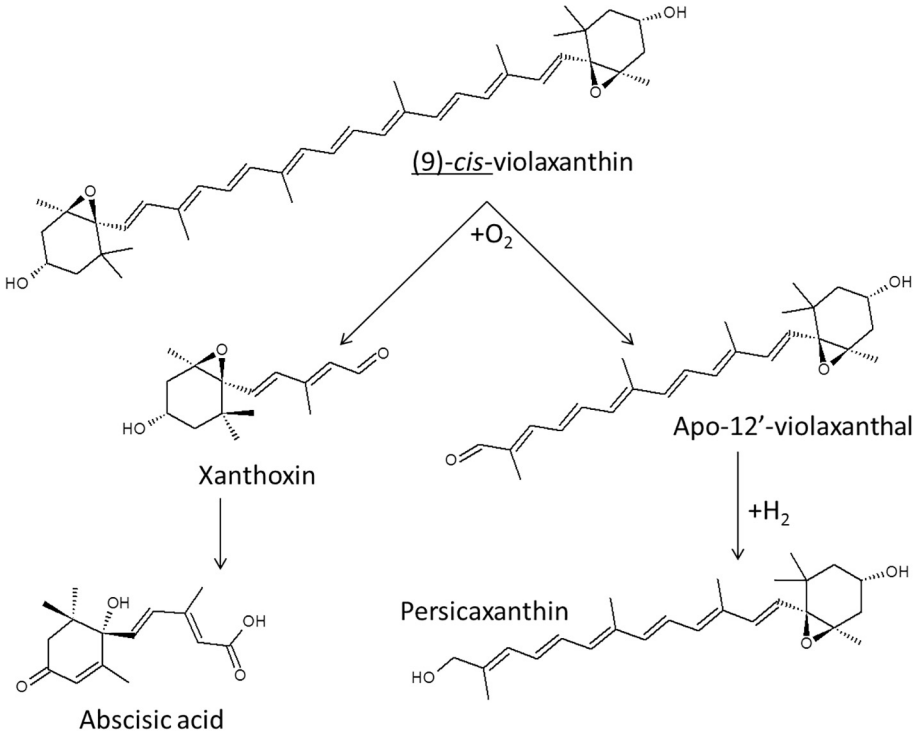

Fig. 4. Putative route to the C_{25}-epoxy-apocarotenoid persicaxanthin proceeds *via* oxidative cleavage of violaxanthin (likely the 9-*cis* isomer) to an apo-12′-violaxanthal intermediate and subsequent hydrogenation. Xanthoxin, a product from the oxidative cleavage, is an intermediate in the biosynthesis of the plant hormone abscisic acid (ABA) which is speculated to play a regulatory role in yam tuber dormancy.

accessions vary in their susceptibility to oxidative degradation and photo-isomerisation. Future study investigating these alterations over the storage time of yam and also following processing would be required to allow in-depth analysis of nutritional values.

3.3. Implication for biofortification and Dioscorea improvement programmes

Breeding strategies for biofortification of *Dioscorea* are currently hindered, due to a lack of genetic resources coupled with inaccurate or unclear historical work regarding nutritional contents, which has led to a limited understanding of biochemical composition and diversity of the global germplasm collection. Whilst broad metabolomics profiling has been conducted on elite breeding lines (Price et al., 2017), this did not encompass carotenoids, yet most biofortification efforts have focused on enhancing provitamin A activity through increasing the abundance of β-carotene in crops. Since yams are prevalently grown in regions with the highest Vitamin A deficiency, carotenoid screening of breeding lines of this multi-species crop collection provides a basis for improving provitamin A content. Typically the β-carotene contents of all 46 accessions used in the study were relatively low (96.3–326 μg/100 g DW, excluding TDd 3109 where β-carotene was absent) compared to many plant-derived foods (Giuliano, 2017). Alternatively, if the β-carotene epoxides are included, then provitamin A content of some accessions of *D. dumetorum* rivals that of mutant yellow cassava and the transgenic golden potato. As such, it would seem imperative that the stability and formation of these epoxides is investigated, alongside establishing their true provitamin A activity in humans.

4. Conclusion

The carotenoid composition and β-carotene contents of the major edible yam species has been determined, clarified and directly compared. Although the sample size was small, results from the current study aid identification of accessions within the current yam breeding program that can be used as a basis for provitamin A biofortification of the crop. Furthermore, the diversity of carotenoid profiles residing within current collections has been highlighted and provides large scope for future studies, e.g., profiling carotenoids throughout tuber growth and storage periods has potential to elucidate the regulatory mechanisms of carotenoid accumulation and stability in yam tubers. Since carotenoids and their derivatives play a vital role in plant growth and development, detailed understanding is needed in order to breed towards, for example, potentially reduced dormancy periods or tolerance to postharvest physiological deterioration.

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Author contributions

Experiments were developed by P.D.F. and E.J.P. All experimental work was performed by E.J.P. Planting materials were provided by R.B. and A.L.P. E.J.P., P.D.F. and R.B. contributed to the interpretation of the results and preparation of the manuscript.

Declaration of interest

All authors declare no conflicts of interest exist.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.03.066.

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