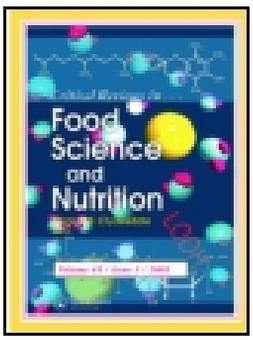

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REVIEW

Recent advances in banana (*musa* spp.) biofortification to alleviate vitamin A deficiency

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ABSTRACT

Vitamin A deficiency (VAD) is one of the most prevalent micronutrient deficiencies that disproportionately affects low income populations in developing countries. Traditional breeding and modern biotechnology have significant potential to enhance micronutrient bioavailability in crops through biofortification. Bananas (*Musa* spp.) are economically important fruit crops grown throughout tropical and sub-tropical regions of the world where VAD is most prevalent. Some banana genotypes are rich in provitamin A carotenoids (pVACs), providing an opportunity to use bananas as a readily available vehicle for provitamin A delivery. This review summarizes the progress made in carotenoid research in bananas relative to banana diversity and the use of conventional breeding and transgenic approaches aimed at banana biofortification to address vitamin A deficiency. Existing reports on sampling strategies, pVAC retention and bioavailability are also evaluated as essential components for a successful banana biofortification effort. The wide variability of pVACs reported in banana cultivars coupled with recent advances in unraveling the diversity and genetic improvement of this globally important but often-neglected staple fruit crop underscores their importance in biofortification schemes.

KEYWORDS

Banana; biofortification; micronutrient deficiency; provitamin A

Introduction

Vitamin A deficiency (VAD) is one of the most prevalent micronutrient deficiencies worldwide, disproportionately affecting low income and vulnerable populations in developing countries. It is the leading cause of preventable blindness in children and has been associated with increased risk of disease and death from severe infections such as malaria, diarrhea and measles (Black et al., 2008; Hamer and Keusch, 2015). VAD affects 40-60% of African children and 10-20% pregnant women in low income countries (WHO, 2009; Bailey et al., 2015). Retinol, retinal and retinoic acid constitute the physiologically active forms of vitamin A which underpins its role in major biological processes responsible for vision, maintenance of epithelial surfaces, immune competence, reproduction and normal embryogenesis (WHO/FAO, 2004; Tanumihardjo et al., 2016). Deficiency of this essential vitamin results in impaired vision, anemia and weakened immune responses, leading to increased morbidity and mortality. Other health consequences include impaired cell development, growth and tissue function during childhood development, pregnancy and lactation (WHO, 2009; Wiseman et al., 2017).

Causes of VAD can include insufficient dietary intake of vitamin A rich foods, and impaired absorption or significant loss of vitamin A due to illness (WHO/FAO, 2004). Vitamin A exists either as preformed retinoids in animal tissues or as provitamin A carotenoids (pVACs) in plant tissues. Vitamin A malnutrition clusters within geographical regions and endemic areas are characterized by poverty, presence of infectious diseases, poor infrastructure and food insecurity, leading to restricted availability and accessibility to foods rich in vitamin A (Bailey et al., 2015; Tanumihardjo et al., 2016).

Strategies such as food fortification, supplementation or dietary diversification have been proposed to reduce the prevalence of VAD worldwide (WHO, 2009; Tanumihardjo and Furr, 2013). Despite these strategies, the prevalence of VAD has remained unchanged in sub-Saharan Africa and South Asia between 1991 and 2013 (Hamer and Keusch, 2015) due to budget constraints, lack of efficient processing, distribution and health monitoring systems, and other reasons. Such constraints led to the emergence of new complimentary strategies such as biofortification targeted towards nutrient enhancement of major staple crops to increase dietary vitamin intake. Biofortification exploits the regular consumption of large quantities of major food staples as a means to deliver micronutrients to malnourished populations lacking access to diverse diets, food supplements or

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fortified food products (Bouis et al., 2011; Saltzman et al., 2013). This approach has shown great potential to efficiently address deficiencies of vitamin A and other micronutrients in developing countries where millions of people are now growing biofortified crops (Bouis and Saltzman, 2017). Biofortification can be achieved through conventional crop breeding, genetic modification (transgenic) or agronomic (fertilization) strategies.

Importance, diversity and nutritional value of bananas

Bananas (*Musa* spp.) are economically important fruit crops grown in tropical and sub-tropical regions of the world. Bananas are currently cultivated in over 130 countries, on over 5.5 million hectares with a global production of about 145 million tons (FAOSTAT, 2017) and serves as a principle source of carbohydrates for millions of people worldwide. Bananas are vegetatively propagated through suckers and are grown throughout the year, which makes them an attractive all-season crop.

Bananas belong to the family *Musaceae* together with two other genera *Musella* and *Ensete*, of the order Zingiberales (De Langhe et al., 2009). The genus *Musa* was previously classified into four sections: Eumusa (x = 11), Rhodochlamys (x = 11), Australimusa (x = 10) and Callimusa (x = 9, 10), and later a fifth section Ingentimusa (Häkkinen and Wallace, 2011). Recently this was revised to two sections; a new section Musa, combining the section Eumusa and Rhodochlamys and the section Callimusa now also including the section Australimusa and Ingentimusa (Häkkinen 2013; Christelová et al., 2017).

Most edible bananas belong to the section Eumusa although Fe'i type edible bananas belong to the section Australimusa. On the basis of ploidy and genome configuration, domesticated bananas are classified as diploid ($2n = 2x = 22$; AA and AB), triploid ($2n = 3x = 33$; AAA, AAB, and ABB), or tetraploid ($2n = 4x = 44$; AAAA, AAAB, and AABB) (Heslop-Harrison and Schwarzacher, 2007) with most cultivated varieties in the triploid category. Bananas are derived from intra or inter-specific hybridization of two wild diploid ancestral species, *M. acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) originating from South-East Asia, although *M. schizocarpa* (S genome) and *M. textilis* (T genome) is also reported to have contributed to the origin of some cultivars (Heslop-Harrison and Schwarzacher, 2007; Häkkinen, 2013; Christelova et al., 2017). The most important edible triploids are dessert bananas (AAA genome), plantains (AAB genome), East African highland bananas (EAHB) (AAA-EA genome) and the Bluggoe and Pisang awak cooking types (ABB genome). The relative importance of these banana subgroups vary by sub-region, and cultivars differ mainly by the amounts of starch and sugars produced in their fruits. While dessert cultivars are globally the most popular and most valued in the industrialized nations, all other cooking types are more relevant for subsistence in developing countries, particularly in Africa and South-East Asia. Many diploid, triploid and tetraploid hybrids have been produced from various breeding programs and contribute to existing banana diversity (Tenkouano and Swennen, 2004; Bakry et al., 2009; Tenkouano et al., 2011; Ortiz and Swennen, 2014).

Dessert bananas are consumed uncooked when ripe, while starchy cooking types are consumed when cooked at varying stages of maturity. In addition, bananas of both types can be processed into juice, puree or flour for use in other edible products. Bananas have a relatively high nutritional value, being rich in dietary fiber, carbohydrates, vitamins and minerals (Robinson and Saúco, 2010; Borges et al., 2014; Pareek, 2016). Vitamins A and C in banana fruit pulp are cultivar dependent (Wall et al., 2006; Amorim et al., 2009, Davey et al., 2009a). In addition, bananas contain other bioactive substances with useful antioxidant properties such as phenolics, carotenoids, flavonoids and biogenic amines (Tsamo et al., 2015; Singh et al., 2016). Banana pulp color can be white, cream, ivory, yellow, or orange; cultivars with yellow-orange pulp color have been associated with high provitamin A content (Englberger et al., 2006a; Fungo and Pillay, 2011). As bananas are key staples in Africa and South-East Asia where prevalence of vitamin A malnutrition is high (WHO, 2009), biofortification efforts have recently been pursued targeting bananas to enhance the nutritional status of vulnerable populations in these regions.

Variability of carotenoids in *musa* spp

Carotenoids are naturally occurring and structurally diverse plant pigments that are important components in both plant development and human health. These pigments are essential for plant photosynthesis, playing a role in light harvesting and protection from excess photo-oxidation (Britton, 2008). They are present as macro-components in fruits, vegetables and storage organs where they impart the characteristic yellow, orange or red colors, contribute to flavor and aroma, and aid in the attraction of pollinators (Rao and Rao, 2007; Cazzonelli and Pogson, 2010; Zhu et al., 2010). Carotenoids are synthesized in algae, fungi and higher plants and serve as the sole source for humans and animals. About 800 carotenoids have been reported in nature and over 50 have been detected in food and in the human body (Arscott, 2013; Rodriguez-Amaya, 2016). A group of carotenoids (α-carotene, β-carotene and β-cryptoxanthin) serve as precursors of vitamin A in humans, hence are classified as pVACs (Weber and Grune, 2012; Shete and Quadro, 2013). Beta carotene contains two replaceable β-ionone rings compared to one ring in α-carotene and β-cryptoxanthin. The extra ring contributes approximately twice as much activity hence β-carotene is the most potent pVAC (Shete and Quadro, 2013). Vitamin A activity of carotenoids is indicated by retinol activity equivalents (RAE) and as described by the Institute of Medicine, the conversion factor for β-carotene is 12, while that for α-carotene and other carotenoids is 24 (Tanumihardjo et al., 2010). Thus, 1 µg RAE is equal to 12 µg β-carotene or 24 µg of the other provitamin A carotenoids α-carotene and β-cryptoxanthin.

Provitamin A carotenoids are an important source of vitamin A, supplying up to 35% and 80% of vitamin A intake in western societies and developing countries respectively (Rodriguez-Amaya, 2016). Other predominant carotenoids include xanthophyls like lutein and zeaxanthin, which are associated with eye health. Both carotenoids are macular pigments and play a role in reducing age-related macular degeneration (Vishwanathan and Johnson, 2013). The nutritional value and health-related benefits of carotenoids have led to considerable effort in identification, quantification and improvement of content in staple crops (Bai et al., 2011).

Early reports on carotenoids in *Musa* spp. established the carotenoid content of Cavendish banana leaves, peel and pulp, and changes in carotenoids relative to fruit maturation (Thomas and Janave, 1992; Subagio and Morita, 1997). These studies were followed by exploratory screening for pVACs (and sometimes non-pVACs) for micronutrient enhancement. Carotenoid variability in bananas is perhaps the most researched area in carotenoid biofortification, and these studies are summarized in Table 1. Englberger et al. (2003a; 2003b; 2006a) were the first to assess carotenoids in raw and cooked samples of Micronesian bananas with a biofortification objective. These studies showed that bananas were consistently higher in β-carotene than other carotenoids, with as much as 8508 µg 100 g^{-1} recorded in Fe'i bananas having a characteristic deep yellow-orange pulp. Other carotenoids reported were α-carotene and lutein, zeaxanthin and β-cryptoxanthin (Table 1).

Englberger et al. (2006b) further analyzed banana cultivars (white-yellow-orange flesh) from the Australian field collection for pVACs and recorded the highest value of trans β-carotene (1 412 µg 100 g^{-1}) in the yellow/orange fleshed Fe'i banana cultivar 'Asupina'. This study also pointed out a correspondence between carotenoid content and pulp color, with orange-yellow fruit pulps having higher carotenoid content than cream fruit pulps (Figure 1). A similar study on Fe'i banana cultivars in the Solomon Islands (Table 1) further confirmed their high pVAC contents (Englberger et al., 2010). However, while the Fe'i bananas had the highest recorded β-carotene levels in these studies and were recommended, it is unclear if they are widely cultivated in the Pacific Islands and have an impact on VAD. Nevertheless, owing to their high pVAC concentrations, Fe'i bananas, notably 'Asupina', with high trans β-carotene levels of up to 1 412 µg 100 g^{-1}, were identified as a source of genes for carotenoid biofortification (Englberger et al., 2006; Paul et al., 2017).

In contrast to Englberger et al. (2010) who found β-carotene highest in banana, Wall (2006) reported that lutein was highest and β-crypotxanthin lowest in bananas (Table 1). Wall sampled 'Dwarf Brazilian' (Prata) and 'Williams' (Cavendish) bananas from different locations in Hawaii.

Arora et al. (2008) investigated carotenoid contents in fruit pulp of 11 Indian banana cultivars and reported β-carotene contents of 27 - 117.2 µg 100 g^{-1} (Table 1) with highest content in the fruit pulp of 'Red banana'. Davey et al. (2007) also studied the variability of pVACs and total carotenoids in six widely consumed West and Central African *Musa* cultivars grown under standardized field conditions in Cameroon (Table 1). They found substantial variation of pVACs between cultivars, with orange-fleshed AAB plantain cultivars recording higher pVAC contents than AAA dessert bananas. In a more comprehensive study to understand the variability of pVACs (α- and β-carotene) and lutein, Davey et al. (2009a) screened fruit pulps of 171 cultivars from all the major *Musa* genome groups obtained from five different countries (Table 1). Mean total pVAC values recorded ranged from 0 or undetected to 211.2 nmol g^{-1} (11 337 µg 100 g^{-1}) dry weight with a mean of 31 nmol g^{-1} (1 636 µg 100 g^{-1}) dry weight, indicating a wide variability in fruit pVAC content. Amorim et al. (2009) further confirmed variability in carotenoid contents in *Musa* spp. in 42 diverse cultivars grown in standard field conditions in Brazil (Table 1), with total carotenoid in fruit pulps ranging from 1.06 to 19.24 µg g^{-1} with a mean of 4.73 µg g^{-1}. Similarly, Fungo and Pillay (2011) screened 47 diverse *Musa* accessions grown under standard field conditions in Uganda (Table 1) where β-carotene concentrations in fruit pulp ranged from 50.6 to 2594.0 µg 100 g^{-1} with the highest contents recorded in genotypes from Papua New Guinea.

Borges et al. (2014) evaluated carotenoid profiles of 29 cultivars across different genomic groups in Brazil and confirmed significant variability in carotenoid contents among genotypes studied (Table 1). They identified accessions with appreciable amounts of pVACs (up to 1 164 µg g^{-1} dry weight) and demonstrated that most of the pVAC in fruit pulp is composed of trans α-carotene (44.9%) and trans β-carotene (42.4%) and only a small amount of cis β-carotene (12%). Recently, Heng et al. (2017) recorded total carotenoid levels ranging from 0.18–36.82 µg fresh weight in 38 banana cultivars and hybrids grown in China (Table 1), with the highest values recorded in AAB plantain cultivar 'Orishele'.

In summary, most of the above-mentioned studies indicate a wide variability of pVACs in bananas suggesting the need to further assess the vast diversity of *Musa* germplasm. They also indicate a positive correlation of pVAC content with fruit pulp color, with the white AAA Cavendish/EAHB types having a lower content while the yellow-orange plantains (AAB), Papua New Guinea diploids (Eumusa) and Micronesian bananas (Australimusa) have relatively high carotenoid contents. Another interesting finding is that carotenoids in bananas comprise predominantly of the pVACs α- and β-carotene (*cis* and *trans* isomers) and the non-pVAC lutein, with significant health benefits, although the relative proportions vary among studies (Table 1). While, these studies form a strong basis for the use of banana as a vehicle for provitamin A delivery, it is challenging to assess the qualitative and quantitative differences in pVAC content in cultivars studied. This is because some of the reported variability may be linked to challenges with sampling and quantification as discussed in a later section. Moreover, considering that these studies are single environment studies, there is need for further studies to assess the effect of

Table 1. Overview of studies on variability of carotenoids in banana fruit pulp.

Number of genotypes and collection site	Fruit stage	Sample type	Extraction solvent	Column and mobile phase	Type of carotenoid	Content	Vitamin A content[1]	Units	Reference
12 banana cultivars from farmers in Micronesia	Ripe	Raw and cooked, frozen samples analyzed overseas	Acetone, saponification in ethanol, tert-butyl ethanol and 50% v/v KOH (85:14:1 v/v/v)	Reverse phase (RP)-HPLC with C18-column (Suplex pKb-100, Supleco); mobile phase: methanol/acetonitrile for carotenes. Normal-phase HPLC with LiChrosorb-Si60-column (Merck); mobile phase: hexane / acetone (81:19 v:v) (xanthophylls)	β-carotene α-carotene Lutein zeaxanthin Total carotenoids	30-2780 10-950 10-260 ud-40 60-5730		µg 100 g^{-1}	Englberger et al. (2003b)
13 banana cultivars from farmers in Micronesia	Ripe	Raw and cooked, frozen samples analyzed overseas	Acetone with magnesium carbonate and sodium sulfate	HPLC C18 300mm x 3.9mm stainless-steel column (Novapak) with C18 guard column; mobile phase: methanol / tetrahydrofuran (90:10 v/v)	β-carotene α-carotene Total carotenoids	56-6360 42-1472 370-4320	BCE: <8-939 RAE: 1-78	µg 100 g^{-1}	Englberger et al. (2003a)
16 banana cultivars from local markets or growers in Micronesia	Ripe	Raw and cooked	–	–	β-carotene α-carotene Lutein Zeaxanthin β-cryptoxanthin	ud -8508 ud-3408 40-1293 ud-137 ud-30	BCE: ud-8508	µg 100 g^{-1}	Englberger
12 banana cultivars from field collection and local growers in Australia	Ripe	Raw, frozen samples analyzed overseas	Ethanol/hexane (3:4 v/v) with sodium carbonate and celite	Vydac 201TP54 RP column fitted in a Shimadzu Model 10 HPLC system; mobile phase: acetonitrile / methanol / dichloromethane (75:20:5 v/v/v), containing 0.1% Butylated hydroxytoluene (BHT) and 0.05% triethylamine (TEA)	*cis* β-carotene *trans* β-carotene α-carotene Lutein Total carotenoids	6 to 85 50-1412 61-1055 7-146 130-9400	BCE: 119-1554 RAE: 10-130	µg 100 g^{-1}	Englberger et al. (2006a)
10 banana cultivars from markets and growers in Makira, Solomon Island	Ripe	Frozen samples analyzed overseas	Acetone with magnesium carobonate and sodium sulfate	C18 300 x 3.9 mm column (Waters Novapak) with Waters, Sydney, Australia C18 guard column; mobile phase: methanol / tetrahydrofuran (THF) (90:10 v/v)	β-carotene α-carotene Other carotenoids Total carotenoids	35-5945 <2-2358 <2-1097 130-9400	BCE: 98-5227 RAE: 8-435	µg 100 g^{-1}	Englberger et al. (2010)
2 banana cultivars from plantations in several locations in Hawaii	Unripe	Raw	THF with Magnesium carobonate sodium sulfate, and 0.01% BHT	ODS Hypersil C-18 100 x2.1 mm, 5µm narrow-bore column (Agilent Technologies); mobile phase: acetonitrile/THF/water (85:12.5:2.5 v/v/v)	β-carotene α-carotene Lutein	42.8-131.4 60.0-155.6 86.2-192.2	RAE: 6-17	µg 100 g^{-1}	Wall (2006)
7 Central and West African Musa cultivars from germplasm collection in Cameroon	Ripe	Fresh fruit transported, lyophillized and analyzed in overseas lab,	THF/methanol (1:1 v/v) with 0.25% BHT	LiChrosphere, C18, 150 x 4.6mm 3 µm RP-HPLC column (Alltech, Eke, Belgium), or aWaters, ODS-2 150mm ×4.6 mm 3 µm RP-HPLC column (Millipore, Brussels, Belgium); Mobile phase: (A) acetonitrile with 0.05% TEA and 0.1% BHT, (B) methanol/ethyl acetate (1:1, v/v) with 0.05% TEA and 0.1% BHT)	*cis* β-carotene *trans* β-carotene α-carotene Total carotenoids (S)	0-5.4 1.05-37.6 1.67-35.2 15.4-84.7		nmol g^{-1} d.w.	Davey et al. (2007)
11 main banana cultivars from various sources in India	Ripe	Raw	Acetone with 0.1% BHT	HPLC only for 4 selected high and low content accessions; 10 x 1 cm alumina column with 1 cm layer of sodium sulfate, mobile phase: acetone/hexane (2:1, v/v)	Total carotenoids (S) β-carotene	2.5-4 27.99-117.2		µg g^{-1} d.w. µg 100 g^{-1}	Arora et al. (2008)
		Raw	Acetone	/		1.06-19.24	/	µg g^{-1}	

(*continued*)

Table 1. Continued.

Number of genotypes and collection site	Fruit stage	Sample type	Extraction solvent	Column and mobile phase	Type of carotenoid	Content	Vitamin A content[1]	Units	Reference
42 diverse cultivars from field collection in Brazil					Total carotenoids (S)				Amorim et al. (2009)
171 diverse cultivars, from 5 different countries (Cameroon, Uganda, USA, Phillipines, Cambodia, Belgium)	Immature green fruits	Raw frozen and lyophillized samples transported and analyzed in overseas lab	THF/methanol (1:1, v/v) with 0.1% BHT	C18 RP-HPLC using a 150 x4.6 mm, Waters ODS-2 3 μm (Millipore, Brussels, Belgium); mobile phase (A) acetonitrile containing 0.05% TEA and 0.1% BHT, (B) methanol/ethyl acetate, (1:1, v/v) containing 0.05% TEA and 0.1% BHT OR 150 x 4.6 mm, 3 μm, C30 RP-HPLC column (YMC Europe, Gmbh, Germany); methyl tert-butyl ether (MTBE) in methanol, containing 0.1% TEA and 0.25% BHT	*cis* β-carotene *trans* β-carotene α-carotene Lutein Total carotenoids (S)*	0-10.7 0-130.2 0-98.8 0-13.1 1.5-198.1	BCE: 0-171.5	nmol g^{-1} d.w.	Davey et al. (2009)
47 diverse banana genotypes from IITA collection in Uganda	Mature	Raw, frozen	Chloroform with 0.25% BHT	5 u C-18 steel column (Waters Associates, Milford, MA); mobile phase: methanol/acetonitrile/water (490:40:20, v/v/v)	β-carotene	50.6-2594.0	RE: 14.86-403.59	μg 100 g^{-1}	Fungo and Pillay (2011)
29 banana genotypes from different genomic groups	Ripe (fully yellow)	Oven dried fruit pulp flour	Hexane/acetone solution (1:1, v/v) with 0.1% BTH	C18 reversed-phase 250 x 4.6 mm 5μm column (Vydac 201TP54) coupled to a 30 x 4.6mm 5μm C18 pre-column (Vydac 201TP54); mobile phase: methanol/acetonitrile (90:10, v/v)	*cis* β-carotene *trans* β-carotene α-carotene Lutein Zeaxanthin Total carotenoids	0-224 0/trace -525 0/trace -415 0-4.61 ud-3.72 4.04-1167	RAE: 0-70.4	μg g^{-1} d.w.	Borges et al. (2014)
34 genotypes, China	Fully developed	Raw	Hexane/acetone/ethanol (2.1.1, v/v/v), Saponification with MTBE, 10% KOH and methanol	Agilent 1260 infinity system C30 250 x 4.6 mm, 5 μm HPLC column; mobile phase: (A) methanol with 0.05% triethylamine (TEA) and 0.1% BHT, (B) acetonitrile with 0.05% TEA and 0.1% BHT, (C) MTBE with 0.1% BHT	β-carotene α-carotene Lutein Total carotenoids	0.02-8.53 0.03-27.27 0.03-1.48 0.18-36.8	RAE: <0.01-1.85	μg g^{-1} f.w.	Heng et al. (2017)

HPLC = High performance liquid chromatography; TC = total carotenoids; ud = undetermined; f.w. = fresh weight; d.w. = dry weight; BCE = β-carotene equivalents; RAE = retinol activity equivalents; RE = retinol equivalents; (S) = Total carotenoids measured using spectrophotometry.*TC values also provided as 0-7.7 ug g^{-1} d.w. and 0-576.6 ug 100 g^{-1} f.w.

Figure 1. Appearance of mature (green) fruit pulp of banana cultivars with contrasting pVAC content. A: low (<2 ug g^{-1} f.w.) pVAC M'chare cultivar ITC1544 Mlelembo. B: high (<12 ug g^{-1} f.w.) pVAC plantain cultivar ITC0519 Obubit Ntanga green mutant.

locations and years to accurately quantify genetic and environmental components of observed variations.

Stability and retention of pVACS during banana fruit ripening and processing

Banana fruits are normally harvested when mature, then stored (for a few days to several weeks) and consumed raw or after processing, at various stages of ripeness. According to Dadzie and Orchard (1997) banana fruits are mature when fingers of the first hand on the bunch show signs of ripening or when the fruit tips turn black. In order to impact human nutritional status, the amount and availability of carotenoids in the raw mature banana fruit at harvest must be determined. Carotenoids are highly saturated molecules and are prone to degradation from reaction with light, atmospheric oxygen, heat, free radicals, singlet oxygen, acid and metals (De Moura et al., 2015; Rodriguez-Amaya, 2016). Carotenoid molecules in intact fruits are less susceptible to degradation because they are protected by natural processes within the living tissues. However, any form of tissue disruption from storage or processing following harvest will inadvertently lead to changes in carotenoid contents.

Changes in carotenoid content and composition observed during ripening are influenced by type of fruit tissue, fruit developmental stage and environmental conditions. Such changes are highly variable among cultivars, and fruit peel is often richer in carotenoids than fruit pulp (Lado et al., 2016). Several studies have investigated the variation of carotenoids during fruit ripening in bananas and reported significant changes in pVAC contents which are cultivar dependent. Lokesh et al. (2014) assessed four cultivars (two AAA and two AAB type dessert bananas) in India and observed that total carotenoids (TC) and pVACs remained stable after ripening. Ngoh-Newilah et al. (2009) evaluated 19 cultivars and hybrids (ten plantains, three cooking bananas, three dessert bananas and three hybrids) in Cameroon at three ripening stages and noted a significant increase or decrease in carotenoid contents. Similarly, Ekesa et al. (2013) documented variable trends in changes in total and individual cultivars at four ripening stages in popular cultivars (two cooking bananas and one plantain) in Uganda. Ekesa et al. (2015) also recorded mean total pVACs ranging from 560 to 4 680 mg 100 g^{-1} fresh weight in unripe fruit and 1 680 to 10 630 mg 100 g^{-1} fresh weight in ripe fruits implying an increase in pVAC contents from the unripe to the ripe stage in eight banana cultivars evaluated in DR Congo. In another study with eight banana cultivars in Uganda, Mbabazi (2015) also recorded a consistent increase in β-carotene equivalent following ripening in the five EAHB cultivars tested and a decrease in β-carotene equivalent in the two dessert bananas and one plantain cultivar tested.

Few reports exist on retention of micronutrient content in banana following processing. These mostly highlight variable effects attributable to cooking of bananas. Earlier work of Englberger et al. (2003a) recorded no consistent effect of cooking (boiling or baking) on pVAC content in Micronesian bananas. Cooking bananas help to release carotenoids from plastids and can concentrate amounts through water loss. Type of cooking can also affect carotenoid content. Englberger et al. (2003b) found that steaming or boiling Micronesian bananas increased carotenoid content by about 30%. Ekesa et al. (2013) recorded a 90-96% retention and even a 46–45% increase in pVAC contents following steaming of two Ugandan cooking banana cultivars. A similar trend was observed by Mbabazi (2015), documenting retention levels of 55.4 to 93.9% following boiling and 57.3 to 76.9% following steaming of EAHB and plantain cultivars in Uganda. A high loss (58%) in total pVACs was recorded from plantains fried in refined vegetable oil in Uganda and 95% retention of pVACs was observed in popular bananas fried in palm oil in Eastern DR Congo (Ekesa et al., 2013a; 2013b). However, these results should be interpreted with caution as inclusion of additional ingredients (such as banana leaves, banana peels and oil) during processing may contribute to carotenoid contents independently, with a consequence on retention rates. De Moura et al. (2015) reported a strong genotype effect on pVAC retention within crops.

As some carotenoids are also prone to loss with cooking, retention rates following commonly used methods of processing should be assessed for representative cultivars of popular banana subgroups.

Sampling and analysis to support provitamin A enhancement and comparison

Efficient carotenoid analysis methods and high throughput screening techniques that ensure sensitivity, specificity and precision, are required for any biofortification breeding effort. Over the years, attention has been paid to optimization of carotenoid extraction and quantification protocols to ensure maximum recovery from samples. However, this remains challenging because of the presence of diverse carotenoids with varying levels of polarity and concentrations, their susceptibility to degradation from exposure to light, heat, acids and long extraction times (Rodriguez-Amaya and Kimura, 2004; Rodriguez-Amaya, 2016). Carotenoid analysis has mostly been done through colorimetric, spectrophotometric or chromatographic methods and several reviews have been published on this subject (Rivera and Canela-Garayoa, 2012; Amorim-Carrilho et al., 2014; Saini et al., 2015; Rodriguez-Amaya, 2016). Colorimetric and spectrophotometric methods are used for qualitative analysis to obtain a rapid overview of carotenoids present in a sample, while chromatographic methods are preferred for more precise qualitative and quantitative analysis for the separation and quantification of individual carotenoids in samples.

Due to the diverse properties and functions of carotenoids, it is important to determine the carotenoid profile and quantify the different types of carotenoids in banana samples to ascertain their nutritional value for biofortification. Chromatographic methods [high performance liquid chromatography (HPLC) and reverse phase-HPLC] have found wide application for detailed quantitative analysis in bananas (Englberger et al., 2006b; 2010; Davey et al., 2009a), while colorimetric and spectrophotometric methods have been applied for the rapid assessment of carotenoids (Amorim et al., 2009), sometimes prior to quantitative analysis (Borges et al., 2014). Several studies on bananas confirmed that pVAC contents show a positive correlation with fruit pulp color (Englberger et al., 2006b; Ngoh-Newilah et al., 2008; Amorim et al., 2009; Aquino et al., 2016). While color assessment and spectrophotometry may be inappropriate for precise calculation of pVACs, they are useful for initial screening of large sets of germplasm for breeding purposes.

Efforts have been made towards establishing reliable protocols for sampling and analysis of bananas for pVACs (Davey et al., 2006; 2007). Due to the varying developmental stages of banana fruits across the bunch, following sequential emergence of flowers along the floral axis, standardized sampling methods were sought to ensure representativeness, while accommodating within bunch variation. Similarly, issues with long extraction times were addressed to optimize protocols for large scale screening. Davey et al. (2006) developed a rapid reproducible protocol which incorporated the use of lyophilization and extensive tissue disruption to increase pVAC recovery and avoidance of saponification/concentration steps often linked with significant losses of pVACs. Considering the limitations of carotenoid quantification using visible range spectrophotometry (long processing times) and HPLC (expensive), Davey et al. (2009a; 2009b) further developed a protocol using visible and near-infrared reflectance spectroscopy (Vis/NIRS) for high throughput analysis, which proved more cost and time effective.

Quantification of PVAC has been challenging for bananas as depicted by the inconsistencies in sampling and analysis strategies depicted in Table 1. Such inconsistencies may have contributed to variable results obtained within and between genotypes. For example, some reports recorded the sampling of fruits in diverse locations and transportation to distant laboratories in frozen or freeze-dried state which may contribute to carotenoid degradation. From these studies, it is also unclear if fruits from different cultivars were collected at the same stage of maturity for analysis, as this is crucial for comparisons among cultivars, since carotenoid composition is influenced by stage of maturity. Other complications arise from the differences in solvents used for extraction and analysis as well as the HPLC columns used for separation (Table 1) which has an effect on the yields of individual carotenoids among studies. Non-polar solvents like hexane are most appropriate for carotenes or esterified carotenoids while polar solvents are more appropriate for xanthophylls, while a combination of acetone/ethanol/hexane is frequently applied for the simultaneous extraction of polar and non-polar carotenoids (Amorim-Carrilho et al., 2014; Saini and Keum, 2018). Likewise, the inclusion of a saponification (alkaline hybrolysis) step aimed at removing interfering compounds (like chlorophyls and lipids) and breaking down esterified carotenoids may be beneficial for the recovery of esterified carotenoids but detrimental to un-esterified carotenoids like β-carotene which could be destroyed in the process. Neverthelesss, Davey et al. (2006) found no detectible effect of saponification on the fruit pulp carotenoid profiles of *Musa* genotypes.

Further, some studies estimated pVAC from total carotenoids without separating and quantifying individual carotenoids with pVAC activity, while in some cases only β-carotene (sometimes with α-carotene) is measured. Considering that specific carotenoids contribute to provitamin A activity, individual carotenoids have different levels of pVAC activity, and such assessments may be misleading, especially for crops where carotenoid profiles are not consistent among studies. Where possible, it is preferable to identify and quantify the individual pVACs present in food samples to efficiently ascertain vitamin A content. Fruit carotenoid composition is quantitatively and qualitatively affected by cultivar/variety, stage of maturity, geographical origin, seasonal variation, production conditions, post-harvest handling and storage (Rodriguez-Amaya and Kimura, 2004). Therefore there is a need for more systematic sampling and quantification protocols that address these factors to distinguish natural variation from possible analytical

errors and to properly rank cultivars for their provitamin A content.

Strategies to enhance provitamin A carotenoid content in bananas

Productivity, diseases resistance and yield have been priorities of banana breeding, with an emphasis on simply inherited agronomic traits. Nutritional quality aspects has been secondary due to limited resources, limited knowledge on the genetics of these traits, and the overwhelming dire impacts associated with major diseases of banana. The recent advances in banana genomics and increased awareness of the nutritional importance of biofortified bananas has renewed interest in this area. Breeding for carotenoid enhancement to achieve target levels has recorded significant progress in many crops through conventional breeding and transgenic techniques (Alós et al., 2016; Giuliano, 2017). While conventional breeding exploits inherent natural genetic variation of existing populations for improvement, transgenic techniques employ the transfer of specific genes of interest, sometimes across taxonomic groups. The use of these techniques for biofortification has been previously reviewed (Bai et al., 2011; Alós et al., 2016; De Steur et al. 2017; Giuliano, 2017; Lee 2017) with varying levels of progress. In terms of release of pVAC biofortified crops to farmers, conventional breeding has achieved much more progress with the release of several conventionally bred high carotenoid crops such as cassava, maize and sweet potato (Bouis and Saltzman, 2017). Transgenic high carotenoid crops on the other hand are yet to be approved for release (De Steur et al., 2017; Lee, 2017). Both techniques have potential for pVAC enhancement in banana and are currently being explored. HarvestPlus (www.harvestplus.org), part of the Consultative Group for International Agricultural Research (CGIAR) Research Program on Agriculture for Nutrition and Health (A4NH), currently supports the development and promotion of biofortified staple crops and has so far championed conventional breeding efforts towards provitamin A biofortification in staple crops, including banana. To date, HarvestPlus partners have released more than 140 biofortified varieties of 10 crops in 26 countries (Andersson et al., 2017). Similarly, through the Banana 21 project (www.banana21.org), the Queensland University of Technology (QUT), Australia and the National Agricultural Research Organization (NARO) are leading efforts towards transgenic pVAC enriched cooking banana biofortification in Uganda, with significant progress (Paul et al., 2018).

Conventional breeding

Substantial variability in pVAC contents have been widely documented across different banana subgroups, suggesting their potential for breeding. However, unlike other major staples, conventional banana breeding is a long and complex process fraught with many challenges. Banana breeding is complicated by parthenocarpy, low fertility, low seed viability, polyploidy and associated irregular meiotic behavior, long generation times, diverse genome configurations and a narrow genetic base (Ortiz, 2013; 2015; Brown et al., 2017). The entire breeding cycle from crossing to release of a new variety may take up to 15 years (Tenkouano et al., 2011). Despite the long and complex breeding cycle, banana breeding programs have successfully developed hybrids with diverse agronomic and disease resistance traits, some of which have been widely disseminated (Ortiz and Swennen, 2014; Brown et al., 2017).

The main strategy adopted for conventional banana breeding involves crossing seed-fertile 3x cultivars to 2x accessions that are donors of the genes of interest, selecting 4x and 2x hybrids from intermediate products and crossing these selected hybrids to generate sterile 3x hybrids (Tenkouano et al., 2011). Alternative schemes involving polyploidization of diploid hybrids or cultivars to obtain tetraploids for crossing with diploid lines to generate triploids, are also pursued (Bakry et al., 2009; Do Amaral et al., 2015). The use of 2x-2x hybridizations to produce secondary 3x via unilateral sexual polyploidization and 2n pollen production has also been advocated (Oselebe et al., 2006). Efforts are also devoted to diploid improvement due to their importance in breeding strategies, but also to explore their potential for use in genetic analysis (Ortiz and Vuylsteke, 1996; Menon et al., 2011).

Owing to the high heritability for the trait in most crops, substantial progress has been made in improvement of β-carotene in maize, sweet potato and cassava using conventional breeding achieving target levels (Ceballos et al., 2013; Saltzman et al., 2013; Bouis and Saltzman, 2017; Low et al., 2017). To date, banana pVAC biofortification has centered around cultivar characterization for carotenoid amount and diversity for potential parental selection. Aguilar Morán (2014) documented the work of the Honduran Agricultural Research Foundation (FHIA) breeding program on the development of biofortified plantains in 2009-2011, which resulted in the selection of two sigatoka-resistant high pVAC hybrids SH-4008 and SH 4037, with pVAC values of 122.86 and 129.36 nmol g^{-1} dry weight, respectively. The IITA breeding program also recently incorporated high pVAC diploids from Papua New Guinea in the plantain breeding strategy aiming for pVAC improvement.

The limited progress in conventional breeding for pVAC enhancement in banana is no doubt linked to the previously mentioned complexities in banana breeding. However, the recent release of draft genomic sequences in *Musa* spp. (D'Hont et al., 2012; Davey et al., 2013; Martin et al., 2016) provides a useful resource for functional genomics and identification of candidate genes relevant for the improvement of desired agronomic, resistance and quality traits. This has opened new opportunities and spurred interest in the development and use of molecular techniques to speed up banana breeding. Following advancements with genotyping and sequencing platforms, marker-assisted selection, linkage/association mapping and genomic selection are now being explored (Brown et al., 2017). Progress recorded so far with these techniques regarding other traits (Biswas and Yi, 2016;

Nyine et al., 2018) suggest their potential for provitamin A enhancement in combination with other breeding strategies.

Transgenic approach

Provitamin A biofortification through metabolic engineering in crop plants, has mostly been targeted towards a controlled increase of β-carotene, which is the most efficient pVAC. Giuliano (2017) elaborated the main classes of strategies that have been exploited for transgenic pVAC accumulation. These strategies involve insertion of genes encoding the rate limiting steps in the carotenoid biosynthetic pathway (push strategy); silencing the biosynthetic step immediately downstream of the carotenoid of interest (block strategies) and the regulation of the size of carotenoid storage structures and stable storage of carotenoids (sink strategies). Extensive knowledge of the metabolic pathways and the genes involved in carotenoid biosynthesis is essential for any carotenoid metabolic engineering.

Progress on the elucidation of the carotenoid biosynthesis pathway in crop plants has been extensively reviewed (Cazzonelli and Pogson, 2010; Hannoufa and Hossain, 2012; Giuliano, 2014; Rosas-Saavedra and Stange, 2016). Phytoene synthase (PSY) is known to be an important rate-limiting enzyme for carotenoid biosynthesis. Pioneering work on transgenic β-carotene enhancement involved insertion of genes which encode this rate-limiting step (the push strategy) and led to the development of golden rice (Ye et al., 2000). Following the genetic success with golden rice, this strategy has gained application in several other crops including wheat, maize, potato and banana (Kaur et al., 2016; Lee, 2017). Mlalazi (2010) investigated the role of PSY in carotenoid synthesis in the high carotenoid content Fe'i banana (Asupina) and low carotenoid content banana (Cavendish), based on the hypothesis that differences in carotenoid contents could be due to enzyme activity or factors regulating PSY gene expression. Their study recorded similarities between mRNA accumulation and promoter activity of PSY genes studied between the high and low carotenoid cultivars, with some differences in splicing suggesting the implication of other genes or regulatory mechanisms. Mlalazi et al. (2012) further characterized PSY genes in banana and proposed two paralog *PSY 1* and *PSY 2* genes similar to those in other monocots. They also demonstrated that both genes contain functional enzymes, with Asupina PSYs having twice as much activity as Cavendish PSYs, suggesting the use of Asupina PSYs (cisgenic or intragenic) for β-carotene improvement in banana. Paul et al. (2017) recently documented the first report on genetic modification of Cavendish banana using the Asupina-derived banana phytoene synthase gene (*MtPsy2a*), leading to enhanced pVAC levels in field grown transgenic plants in Australia. Transgenic lines with fruit pVAC levels up to 55.0 μg g^{-1} (a 20–50 fold increase and more than double the target level of 20 μg g^{-1}) serves as a proof of concept for the genetic modification of EAHB for pVAC enhancement. To further elucidate the regulation of carotenogenesis in banana, Buah et al. (2016) studied the key differences in mechanisms that regulate carotenoid accumulation, isoprenoid gene expression and carotenoid degradation in Asupina and Cavendish. They reported a higher expression of carotenoid cleavage dioxygenase 4 (CCD4) in Cavendish, possibly associated with a decrease in β-carotene, and the conversion of amyloplasts to chromoplasts in Asupina, possibly associated with increased β-carotene. CCD4 genes are known to be responsible for cleavage of β-carotenes to form apocarotenoids while chromoplasts are critical for β-carotene storage under the Orange gene (*Or*) control (Li and Yuan, 2013). Using comparative proteomic analysis, Amoako-Andoh (2016) also identified proteins for enzymes involved in carotenoid biosynthesis, sequestration, storage and degradation, but failed to identify proteins for the enzymes in major rate-limiting steps in the pathway. More insight is needed regarding the structural and regulatory genes involved in the carotenoid biosynthetic pathway as a prerequisite for genetic engineering to enhance pVAC in banana.

While developing genetically modified (GM) crops is daunting but scientifically possible, their release and utilization by target populations are complicated by political and ethical concerns (Lee, 2017). Preliminary sensory tests for transgenic PVA biofortified bananas in Uganda indicated a preference for their texture and appearance (but not the taste), indicating consumer acceptability, but release is still pending due to the tedious regulatory framework and skepticism of consumers. Nevertheless, Paul et al. (2018) postulates that 'Golden bananas' may be released by 2021 for adoption to ultimately alleviate VAD, with the use of efficient stewardship and communication strategies to demystify the use of GM crops.

Bioavailability and bioaccessibility

Bioavailability of carotenoids is the portion of ingested carotenoids that can be absorbed, transported, stored or utilized for normal physiological functions, determined from analyses done with *in vivo* or *in vitro* methodologies (La Frano et al., 2014; Rodriguez-Amaya, 2016). Bioaccessibility refers to the fraction of dietary carotenoids that is released from the food matrix and transferred into mixed micelles during digestion, a process necessary for carotenoid absorption by intestinal mucosa (La Frano et al., 2014; Kopec and Failla, 2018). Information on bioavailability and bioaccessibility of carotenoids from food is important in determining the role of carotenoids in human diets.

Several food and host related factors affect the bioavailability of carotenoids. These factors include food status (cooked or raw), type of carotenoid, food processing method, food matrix in which the carotenoid is incorporated, nutrient status of the host, food composition and interaction with other food compounds (Tanumihardjo et al., 2010; Rodriguez-Amaya, 2016). As carotenoids are fat-soluble, bioavailability appears to be associated with food matrix and other components present in the diet. In fruits, carotenoids primarily occur in the chromoplasts where they are located mainly in the plastoglobili and can be deposited as crystals. Bioaccessibility through carotenoid absorption is

influenced by cell walls, the carotenoid protein complex and fibers which trap carotenoids, as well as the type and amount of fat in the diet (Díaz-Gómez et al., 2017). In animals, fats promote the excretion of bile salts, which enhance micelle formation and carotenoid solubilization. Bioaccessibility and bioavailability of pVACs in biofortified food has been extensively reviewed (La Frano et al., 2014; Giuliano, 2017; Kopec and Failla, 2018). These reviews indicate that pVACs from biofortified crops are highly bioaccessible and bioavailable and have the potential to enhance vitamin A status.

While processing, storage and cooking are the main factors affecting bioavailability only few of these reports are specific to pVACs in banana. Ekesa et al. (2012) used an *in vitro* digestion model to estimate bioaccessibility from boiled bananas and banana-derived dishes in Eastern DR Congo. Two commonly used cultivars, a plantain and an EAHB, were evaluated. The study revealed a cultivar-dependent response recording a relatively high (10-32%) bioaccessibility for β-carotene, which was modified when high pVAC ingredients were added. These values were higher in banana than the 0.6-3% recorded for potato (Failla et al., 2009). Thakkar et al. (2009) and Failla et al. (2012) recorded similar high bioaccessibility values of 25-30% and 27-45% for pVACs in processed cassava roots.In an animal study using Mongolian gerbils fed with processed banana flour, a bioconversion of 28.1 was recorded, which was 17 times higher than other fruits tested. This high bioefficiency demonstrates that banana is less effective in maintaining liver reserves (Arscott et al., 2010). These results inspired further investigations on elucidating matrix effects and was especially relevant as bananas contain significant amounts of resistant starch, which is thought to limit bioavailability. Bresnahan et al. (2012) found that cooking (but not ripening), improved the retinol bioefficacy in bananas, and demonstrated that bananas prevented retinol depletion in Mongolian gerbils. These results were in contrast to the previous hypothesis that ripening increases bioavailability by breaking down insoluble starches to soluble sugars and were further supported by the higher conversion factor in ripe compared to green banana. Appropriate processing and cooking methods that maximize the bioavailability of pVACs are essential for realizing the full potential for biofortification.

Conclusions

The importance of vitamin A and its health benefits are well known, and several food fortification and supplementation programs exist to boost vitamin A supply worldwide. Despite this, VAD is still a widespread health challenge, especially in developing countries with limited access to fortified foods. Biofortification provides a feasible means of addressing micronutrient deficiency by increasing the bioavailable concentrations of micronutrients in crop plants, using conventional breeding or genetic engineering approaches. Bananas are important staple crops for home consumption by populations in developing countries. From the studies cited in previous sections, it is evident that bananas have substantial levels of pVACs, underscoring the crop's potential as a vehicle for vitamin A delivery.

The pVAC contents vary widely between cultivars, with lowest values in Cavendish and highest values in the AAB plantains and diploid cultivars from Papua New Guinea. The variability of pVACs in diverse *Musa* germplasm has also incited the exploration of biofortification breeding. Notwithstanding, there is need for further assessment of representative accessions of the main banana sub-groups relevant for consumption or for breeding in specific regions, since preference for banana cultivars tend to be location/region-specific. Moreover, there is need for in-depth studies to adequately quantify genetic variation and to further investigate the genetics of inheritance of pVACs in Musa spp towards conventional breeding. Transgenic techniques are also being investigated, with promising results, but will require further insights on the technical and economic feasibility for proper exploitation. The rapid advancement of transcriptomics and metabolomics offer opportunities for research to further elucidate the regulatory mechanisms of carotenoid biosynthesis and suggest ways to modify metabolism to increase pVAC content or bioavailability.

While these methodologies are being developed as a long-term strategy, the 'fast-tracking' approach remains useful as a short-term strategy. This strategy involves the identification and dissemination of adapted varieties with significant pVAC contents to vitamin A endemic regions (Davey et al., 2009b; Bouis and Saltzman, 2017), and has been adopted for the dissemination of high pVAC cooking and dessert bananas in Burundi, Rwanda and DR Congo (Andersson et al., 2017; Ekesa et al., 2017). Such 'fast-track' cultivars will need to possess other desirable quality related traits that meet the needs of consumers in target regions for adoption.

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