GENETIC VARIABILITY OF CAROTENOIDS AND POLYPLOID INDUCTION TOWARDS VITAMIN A BIOFORTIFICATION IN PLANTAIN (MUSA spp.)

By

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ABSTRACT

Vitamin A deficiency (VAD) is one of the most prevalent nutrient deficiencies affecting the health of resource-poor populations in developing countries. Plantains (*Musa* spp. AAB) are a specific group of bananas, which serve as a staple for millions of people in the humid lowlands of West-Central Africa and high provitamin A carotenoid (pVAC) plantain varieties, could have significant impact on VAD in these regions. In this regard, the International Institute of Tropical Agriculture (IITA) based in Nigeria, is developing a breeding pipeline aiming to generate and deploy plantain hybrids combining high pVAC content with other consumer-preferred traits. The overall objective of this study was therefore to assess the variability of fruit pVAC content in banana cultivars and hybrids present in the collection of IITA, Nigeria, and investigate the potential of induced polyploidization as a breeding approach towards plantain biofortification.

A wide collection of 204 genotypes of bananas (AAB-plantains, *M. acuminata* cultivars and bred hybrids) was screened to determine variability in fruit pVAC content using high-performance liquid chromatography (HPLC) and spectrophotometry. Mean total carotenoids (TC) measured by spectrophotometry ranged from 1.28 to 32.03 with a mean of 8.88 µg g^{-1} fresh weight (FW) indicating a high variability of carotenoids in bananas. There was a strong correlation between TC measured by spectrophotometry and that estimated from HPLC, confirming the potential of spectrophotometry as a useful, inexpensive method for rapid screening for pVACs in banana. Predominant carotenoids isolated were α-carotene (38.67%), *trans* β-carotene (22.08%), lutein (22.08%), 13-*cis*-β-carotene (14.45%) and 9-*cis* β-carotene (2.92%), demonstrating that about 78% of the carotenoids in bananas are pVACs. Provitamin A content estimated in terms of β-carotene equivalents (BCE) ranged from 0.24 to 21.06 µg g^{-1} FW with a mean value of 4.42 µg g^{-1} FW across all genotypes. Importantly, 10 plantain cultivars, three *M. acuminata* diploids and four hybrids with relatively high pVAC contents were selected for integration in banana biofortification efforts to tackle VAD.

To assess the effect of ripening on pVACs in plantain fruits, nine cultivars across the three main plantain types (French, False Horn and Horn) were screened at the unripe, ripe and overripe stage. Mean TC measured by spectrophotometry for plantain cultivars at the unripe, ripe and overripe stage was 16.94, 11.98 and 10.11 µg g^{-1} FW while mean BCE was 13.65, 6.95 and 5.05 µg g^{-1} FW, respectively. Notably over 80% of carotenoids in plantain cultivars were pVACs α-carotene and β-carotene across all ripening stages. French plantains had slightly higher BCE contents than False Horn and Horn types but this difference was only significant ($P<0.05$) at the unripe stage. Overall, ripening led to a

decrease in pVACs content from the unripe to the ripe to the overripe stages accompanied by a corresponding increase in lutein content indicating that unripe fruits could yield more provitamin A than ripe and overripe fruits.

To explore the application of induced polyploidization as a breeding strategy for pVAC enhancement in plantains, 10 induced tetraploids derived from six diploid cultivars were evaluated for their agronomic attributes, carotenoid content and fertility. Tetraploids had distinct plant morphology but generally displayed inferior vegetative and yield traits from their corresponding diploids. Similarly, a 50% decrease in pVACs accompanied by a corresponding increase in lutein was recorded in induced tetraploids in comparison to their original diploids. Nevertheless, preliminary fertility assessments indicated over 70% pollen viability for induced tetraploids from four diploid cultivars. These findings demonstrated the use of induced polyploidization to generate useful genetic material that could be incorporated in hybridization programmes aiming to produce high pVAC triploids.

Key words: biofortification, breeding, fruit ripening, high performance liquid chromatography, induced polyploidization, *Musa* spp, plantain, pollen viability, provitamin A carotenoid, spectrophotometry, vitamin A deficiency

DECLARATION

I, Delphine Amah, declare that the thesis that I herewith submit for the Doctoral Degree in Plant Breeding at the University of the Free State, is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

I, Delphine Amah, hereby declare that I am aware that the copyright is vested in the University of the Free State.

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Delphine Amah

October 2018

DEDICATION

I dedicate this thesis work to my father Mutanga Godfrey, my mother Mutanga Pauline (of blessed memory) and to my very special ‘mothers’ Azenwi Christina and Nkeni Anna, for their tireless support and inspiration throughout my entire academic journey.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A4NH	Agriculture for nutrition and health
ANOVA	Analysis of variance
BAP	Benzylaminopurine
BC	β-carotene
BCE	β-carotene equivalents
BCH	Carotenoid β-hydroxylase
BWT	Bunch weight
CCD	Carotenoid cleavage dioxygenase
CHYE	Carotenoid ε-hydroxylase
cm	Centimetre(s)
CRTISO	Carotenoid isomerase
CYP97	Cytochrome P450-type monooxygenase 97
DAH	Days after harvest
DFM	Days to fruit maturity
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DPF	Days to flowering
DRC	Democratic Republic of Congo
DW	Dry weight
DXP	Pyruvate to form 1-deoxy-D-xylulose-5-phosphate
DXR	Deoxyxylulose 5-phosphate reductoisomerase
DXS	Deoxyxylulose 5-phosphate synthase
EAHB	East African highland bananas
EET	Early evaluation trial
FC	Fruit circumference
FHIA	Honduran Foundation for Agricultural Research
FLT	Fruit length
FW	Fresh weight
FWT	Fruit weight
g	Gram(s)
G3P	Glyceraldehyde-3-phosphate
GBS	Genotyping by sequencing
GGPP	Geranyl geranyl pyrophosphate
GM	Genetically modified
GPP	Geranyl pyrophosphate

GS	Genomic selection
GWAS	Genome-wide association study
ha	Hectare(s)
HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
HDS	4-hydroxy-3-methylbut-2-enyl diphosphate synthase
HIV-1	Human immunodeficiency virus type 1
HMBPP	4-hydroxy-3-methyl-2-(*E*)-butenyl-4-diphosphate
HPLC	High performance liquid chromatography
IITA	International Institute of Tropical Agriculture
IPI	Isopentenyl pyrophosphate isomerase
IPP	Isopentenyl pyrophosphate
ITC	International Transit Centre
kg	Kilogram(s)
LCYB	Lycopene β-cyclase
LCYE	Lycopene ε-cyclase
Lut	Lutein
m	Metre(s)
M	Molar(s)
MAS	Marker-assisted selection
MEP	2-C- methyl-D-erythritol 4-phosphate
MET	Multi-locational evaluation trial
MGIS	Musa germplasm information system
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
MS	Murashige and Skoog
MT	Million tonnes
MVA	Mevalonic acid
NAA	1-naphtaleneacetic acid
NCED	9-*cis*-expoxy carotenoid dioxygenase
NF	Number of fingers or fruits
NH	Number of hands or clusters
nm	Nanometer(s)
NSF	Number of suckers at flowering
PDS	Phytoene desaturase
PGT	Pseudostem girth

pH	Potential of hydrogen
PHT	Plant height
PNG	Papua New Guinea
PSY	Phytoene synthase
PTFE	Polytetrafluoroethylene
pVA	Provitamin A
pVAC(s)	Provitamin A carotenoid(s)
PYT	Preliminary yield trial
QTL	Quantitative trait loci
RAE	Retinol activity equivalents
rpm	Revolutions per minute
SAS	Statistical analysis software
SD	Standard deviation
spec	Spectrophometry
SSR	Simple sequence repeat
TC	Total carotenoids
TTC	2,3,5-triphenyltetrazolium chloride
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
VAD	Vitamin A deficiency
v/v	Volume per volume
ZDS	ζ-carotene desaturase
Z-ISO	ζ-carotene isomerase
α-car	α-carotene
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
%	Percent

LIST OF APPENDICES

CHAPTER 1

General introduction

Vitamin A is an essential micronutrient for human growth, vision, immune function, reproduction, cell differentiation and development (WHO/FAO 2004). Inadequate intake of dietary vitamin A results in vitamin A deficiency (VAD), which is a serious health concern. Globally, VAD is estimated to affect about 190 million pre-school children and 19.1 million pregnant women with the highest prevalence in Africa and South-East Asia (WHO 2009). Dietary interventions such as supplementation, food fortification and consumption of vitamin A rich foods have traditionally been employed to combat VAD. However, most low-income populations in developing countries still lack access to these interventions and mainly rely on key staples for their daily nutrient intake (Bai et al. 2011).

To complement existing interventions, agricultural research has focused on increasing the micronutrient content of staples through conventional breeding or biotechnology in a process called biofortification. Biofortification aims to incorporate micronutrient-dense traits in varieties which already have preferred agronomic and consumer desired traits, such as high yield and disease resistance as a cost effective, long-term and sustainable means of ensuring micronutrient delivery (Bouis and Welch 2010; Saltzman et al. 2013). Several crops are being bred conventionally or through genetic modification to increase provitamin A carotenoid (pVAC) content with significant progress recorded for key staples such as rice, sweet potatoes, cassava and maize with conventionally bred vitamin A enriched varieties already being disseminated in some cases (Bouis and Saltzman 2017).

Bananas (*Musa* spp.) rank among the world's top 10 food crops with an annual global production of about 145 million tonnes (FAOSTAT 2017). Over 80% of the world's banana production is mainly by smallholders for home consumption of which about 30% comprises plantains and other starchy cooking banana types (Ortiz and Swennen 2014). Bananas have been identified as an important source of vitamin A and are one of the crops to address VAD in developing countries (Englberger et al. 2003; Davey et al. 2009; Andersson et al. 2017). Plantains (*Musa* spp. AAB) are a distinct subgroup of bananas, particularly important for food security in West and Central Africa, Central America, parts of Asia and the Caribbean islands. Plantain production is constrained by several abiotic and biotic factors, hence breeding efforts have mostly focused on the development and delivery of disease resistant high-yielding hybrids (Tenkouano et al. 2011). While this partially addresses hunger by increasing food supply, it may not address micronutrient deficiencies such as VAD, which is common in regions where the crops are grown. Breeding for consumer acceptable plantain varieties with enhanced fruit pVAC content

offers an added advantage of improving the nutritional status of millions of people who rely on plantain as a staple. Despite this, reports on the development of conventionally bred pVAC enriched plantain/banana hybrids are limited.

HarvestPlus (www.HarvestPlus.org), a global alliance of agriculture and nutrition research institutions, currently supports the development and promotion of biofortified staple crops and has championed conventional breeding efforts towards pVAC biofortification in important staple crops. The International Institute of Tropical Agriculture (IITA; www.iita.org) houses a plantain breeding programme for Africa based in Ibadan and Onne in Nigeria. With support from HarvestPlus, the IITA has incorporated pVAC improvement in the breeding goals and is developing a pipeline towards the generation and deployment of pVAC enriched plantains.

Bananas are known to have high nutritional value; however, reports on adequate quantification of carotenoid content and profiles among popularly consumed banana types is limited. Earlier reports on vitamin A biofortification in banana have focused on exploration and quantification of variability in fruit pVAC content (Englberger et al. 2003; 2006; Amorim et al. 2009; Davey et al. 2009; Fungo and Pillay 2011) and optimization of fruit carotenoid assessment methods (Davey et al. 2006; 2007). While these reports confirm a high variability of carotenoid content in *Musa* spp. indicating possibilities for cross-breeding, representative accessions have not been analysed from all available genome groups. Specifically, data on fruit pVACs content of plantains and other genome groups relevant for plantain breeding is limited. Identification of high pVAC plantains and plantain hybrids in existing germplasm would not only be useful for breeding but also for fast-track delivery of more nutritious plantains.

Plantain production in Africa is mostly for local consumption in various forms (fried, boiled, steamed or roasted) at different ripening stages. Fruit carotenoid composition is complex and is affected quantitatively and qualitatively by several factors including fruit ripening (Rodriguez-Amaya and Kimura 2004). Changes in carotenoid content during ripening in banana have been previously reported (Ngoh-Newilah et al. 2009; Ekesa et al. 2013). However, a more systematic approach to monitoring the changes in carotenoid content and profiles at specific ripening stages for diverse plantain accessions is essential to enable its proper exploitation to alleviate VAD.

Banana breeding programmes have used wild and cultivated *M. acuminata* diploids in hybridizations to generate superior 3x hybrids without paying much attention to provitamin A (pVA). Commonly used breeding strategies involve selecting fertile susceptible 3x

cultivars and crossing to wild-type 2x accessions, which are donors of resistance genes, then selecting 4x and 2x primary hybrids from progenies and crossing 4x and 2x hybrids to produce secondary 3x (Tenkouano et al. 2011; Ortiz and Swennen 2014; Brown et al. 2017). An alternative method of producing 3x banana hybrids involves polyploidization of 2x using anti-mitotic agents to generate induced tetraploids for crossing with 2x (Bakry et al. 2009). Polyploidization of high pVAC diploids offer a useful approach for introgression of high pVAC traits, especially for cultivars with low fertility, but this method has not been fully utilized for plantain breeding. Considering the important role of diploids in these breeding schemes, identification of appropriate high pVAC diploid genotypes as a source of alleles for introduction into elite breeding lines is crucial for vitamin A biofortification in plantains. Similarly, high pVAC diploids would also be useful for genetic studies aiming at elucidating inheritance patterns of pVACs in *Musa* spp. as well as gene identification towards marker development to improve breeding efficiency.

Against this background, this study was carried out with the main objective to assess the variability of fruit pVAC content in banana cultivars and hybrids present in IITA, Nigeria and investigate the potential of polyploidization as a breeding approach towards plantain biofortification.

The specific objectives were:

- To evaluate the variability of fruit carotenoid content and profiles in different types of bananas (plantains, *M. acuminata* cultivars and hybrids) present in the IITA banana germplasm collection.
- To assess the carotenoid profiles in different ripening stages of plantains to understand the effect of ripening on pVAC content.
- To apply *in vitro* polyploidization to selected diploids, assess the effect of doubling on pVAC content and evaluate fertility of induced tetraploids.

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CHAPTER 2

Literature review

2.1 Importance of vitamin A and health implications

2.1.1 Carotenoids and their role as vitamin A precursors

Carotenoids are a group of naturally occurring structurally diverse, lipophilic pigmented compounds. They are synthesized in algae, fungi and higher plants, serving as an important source of vitamin A for animals. About 800 carotenoids have been reported in nature with about 500 fully characterized (Rodriguez-Amaya 2016). Structurally, carotenoids belong to the C40-based isoprenoid polyene compounds with the majority comprising of eight C5 isoprene units. In plants, they are synthesized and located in cellular plastids, particularly in the chloroplasts of photosynthetic tissue and chromoplasts of non-photosynthetic tissue. Carotenoids are essential components of plant photosynthetic systems playing a role in light harvesting and protection from excess photo-oxidation (Britton 2008). In non-photosynthetic tissues such as fruits, vegetables and storage organs, they are present as macro components and impart the characteristic yellow, orange or red colours, while contributing to flavour, aroma and attraction of pollinators (Rao and Rao 2007; Cazzonelli and Pogson 2010; Zhu et al. 2010). Carotenoids and their derivatives are also implicated in plant defence mechanisms and serve as precursors for phyto-hormone apocarotenoids such as abscisic acid and strigolactones (Cazzonelli 2011).

Besides their role in plant growth and development, carotenoids also play an important role in human nutrition and health. This is mainly due to their properties as biological anti-oxidants in the prevention of diseases such as cancers, vascular diseases and eye disorders such as cataract and macular degeneration, and their ability to generate vitamin A (Rao and Rao 2007; Milani et al. 2017). Carotenoids found in human diets are mainly derived from roots, shoots, leaves, tubers, seeds, fruits and flowers of crop plants (Fraser and Bramley 2004**)**. Of the hundreds of carotenoids reported, over 50 have been detected in food and in the human body; the most predominant being α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin (Arscott 2013). Based on chemical structure, carotenoids are classified as (1) carotenes comprising non-oxygenated hydrocarbons such as β-carotene, α-carotene and lycopene; and (2) xanthophylls which are oxygenated derivatives of carotenes such as β-cryptoxanthin, lutein and zeaxanthin (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).

Among the 50 carotenoids that can be cleaved to vitamin A, α-carotene, β-carotene and β-cryptoxanthin are the most commonly found vitamin A precursors in plant-derived human diets, the most abundant being β-carotene (Tanumihardjo et al. 2010; Shete and Quadro 2013). The minimum requirement for vitamin A activity is an unsubstituted β-ionone ring with an 11-carbon polyene chain. β-carotene has two beta ionone rings in its structure, hence can be centrally cleaved by β-carotene-15,15′-dioxygenase to generate two molecules of retinol, while other pVACs can only generate one molecule of retinol (Weber and Grune 2012). Vitamin A activity of pVACs is indicated by retinol activity equivalents (RAE) and as set by the Institute of Medicine, the conversion factor for β-carotene is 12, while that for α-carotene and other carotenoids is 24. This implies that 12 µg β-carotene and 24 µg of other pVACs is equivalent to 1 µg RAE (Tanumihardjo et al. 2010). 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). The importance of carotenoids in plants and animals, particularly their role as vitamin A precursors has generated significant interest worldwide in finding new sources of carotenoids and optimizing its production in existing sources.

2.1.2 Vitamin A and health benefits

Vitamin A is a fat-soluble vitamin, which comprises a group of unsaturated hydrocarbon compounds namely retinol, retinal, and retinoic acid (Dao et al, 2017**)**. These organic compounds are essential micronutrients, which cannot be synthesized by humans, hence must be provided as part of the diet. Dietary sources include preformed retinol (mainly retinyl esters) from animal foods such as dairy products, kidney, oily fish, eggs or liver and pVACs from plant sources such as green-leafy vegetables and deeply coloured yellow and orange fruits/vegetables (WHO/FAO 2004; Tanumihardjo et al. 2016).

Retinol, which is obtained either from conversion of pVACs in the intestinal lining or from hydrolysis of retinyl esters, is stored in the liver and secreted into the blood stream when required. Retinol may be reversibly converted to retinal which can, in turn, be irreversibly oxidized to retinoic acid (Blomhoff and Blomhoff 2006). Retinol, retinal and retinoic acid constitute the active forms of vitamin A, which mediates 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**).** Retinal acts as a chromophore of rhodopsin, a protein which absorbs light in the retinal receptors enabling dim-light vision and ensures normal differentiation and functioning of

the conjunctiva and cornea (Blomhoff and Blomhoff 2006; West and Darnton-Hill 2008). Retinoic acid regulates gene expression representing the pathways through which vitamin A possibly mediates most of it effects on growth and developmental processes such as morphogenesis; organ formation (lung, heart, vascular system, central nervous system, kidney and limb), blood formation; immune function; epithelial tissue formation; and bone formation (Semba 2005; Kam et al. 2012). Therefore, consumption of diets with insufficient preformed vitamin A or pVACs will predispose consumers to abnormalities in related biological processes.

2.1.3 Vitamin A deficiency and associated disorders

Vitamin A deficiency is a health concern predominantly in children and women of reproductive age in developing countries (WHO/FAO 2004). Vitamin A deficiency causes the deterioration of light sensitive rod cells essential for dim-light vision, leading to a condition known as night blindness and in extreme cases can lead to an irreversible form of blindness known as xerophthalmia. Other health consequences include anaemia, stunting in children, weakened immune system and increased susceptibility to infection (WHO/FAO 2004). According to estimates (WHO 2009), 190 million preschool-age children and 19.1 million pregnant women are affected by VAD representing 33.3% of preschool-age children and 15.3% of pregnant women in populations at risk with greatest numbers in Africa and South-East Asia. With about 250 000 to 500 000 children becoming partially or totally blind annually, VAD is the leading cause of childhood visual impairment and blindness in developing countries (Underwood and Arthur 1996; Bailey et al. 2015).

Vitamin A deficiency is also associated with increased severity of infectious diseases such as acute respiratory tract infections, diarrhoea, measles, schistosomiasis, malaria, leprosy, tuberculosis, otitis media, rheumatic fever and human immunodeficiency virus type 1 (HIV-1) (Underwood and Arthur 1996; West and Darnton-Hill 2008). Pregnant women suffering from night blindness are five times more likely to die of infection during or after pregnancy than women without night blindness (Christian et al. 2000). Vitamin A deficiency clusters within countries with endemic areas characterized by poverty, presence of infectious diseases, poor infrastructure, and food insecurity, leading to limited availability and accessibility to vitamin A rich foods (Bailey et al. 2015; Tanumihardjo et al. 2016). Vitamin A deficiency features among the most widespread micronutrient deficiencies with the highest public health burden worldwide (Black et al. 2008; Bailey et al. 2015) hence globally, efforts are being dedicated towards eradicating VAD and its health consequences.

2.1.4 Strategies to alleviate vitamin A deficiency

Strategies to alleviate VAD mostly aim to boost vitamin A status in individuals and populations at risk. Current strategies include supplementation, food fortification and dietary diversification (West and Darnton-Hill 2008; WHO 2009; Tanumihardjo and Furr 2013; Rodriguez-Amaya 2016). The most widely used strategy is supplementation, which employs community-based efforts to supply pharmaceutical formulations containing preformed vitamin A in the form of retinyl esters or β-carotene, or a combination of both, mainly targeted towards children and pregnant women (WHO 2009). This has recorded successes by reducing the risk of maternal infection, anaemia and night blindness in pregnant women (McCauley et al. 2015) and reducing mortality and some diseases in children aged 4 months to 5 years (Imdad et al. 2010; WHO 2011). However, supplementation programmes are expensive and have poor outreach to vulnerable resource-poor populations (Neidecker-Gonzales et al. 2007) due to limited infrastructure, inadequate supplies, budget constraints and sub-optimal health systems for monitoring.

The second strategy; food fortification, entails the incorporation of vitamin A into processed foods and condiments such as milk, margarine, cooking oils, cereal and grain flours, sugars and monosodium glutamate taking advantage of their consumption patterns in target populations. Successes have been recorded in most settings, particularly with sugar fortification in Central and South America (Tanumihardjo and Furr 2013). Food fortification has been successful in developed countries, but less so in developing countries due to challenges with establishing efficient processing and distribution systems limiting accessibility and affordability of commercially processed foods to rural populations (West and Darnton-Hill 2008).

The third strategy, dietary diversification, encompasses increased production and consumption of diverse naturally occurring vitamin A-rich foods to enhance the vitamin A status of vulnerable populations. Through various programmes, populations have been sensitized to grow vitamin rich fruits and vegetables in home and community gardens as well as post harvest handling, to minimize vitamin losses (Britton 2009). Such food-based strategies are considered long-term and sustainable approaches with added advantages of promoting self-sufficiency and food/nutrition security (Rodriguez-Amaya 2016). However, while most people in the developed world have sufficient vitamin A rich diverse diets, populations in developing countries still rely on monotonous diets composed of nutrient poor staples. This has prompted the advancement of new complimentary strategies such as biofortification targeted towards nutrient enhancement of widely consumed staples to increase dietary vitamin intakes.

2.2 Biofortification of food crops for vitamin A enhancement

2.2.1 Biofortification as a strategy to alleviate micronutrient deficiencies

Micronutrient malnutrition or hidden hunger is a condition mainly caused by low dietary intake of essential micronutrients (Kennedy et al. 2003). Plants constitute the main source of nutrients in human diets, but most staples lack essential micronutrients such as vitamins and minerals. Biofortification seeks to enhance the nutrient content of staple food crops by incorporating nutrient-dense traits into preferred crop cultivars through conventional breeding or biotechnology (transgenic) techniques (Nestel et al. 2006). This approach takes advantage of regular consumption of large quantities of key staples to deliver micronutrients to resource-poor, malnourished populations lacking access to diverse diets, food supplements or fortified food products to address micronutrient deficiencies in a sustainable way (Bouis and Welch 2010**;** Saltzman et al. 2013). Biofortification relies on the crop's inherent biosynthetic or physiological capacity to produce or accumulate desired vitamins or minerals (Mayer et al. 2008). When there is sufficient genetic variation in the existing diversity of the target crop, conventional breeding can be carried out, whereby transgressive segregation or heterosis may be exploited. However, in the absence of genetic variability or in a situation where the crop is intractable to breeding, genetic modification offers a suitable alternative, where genes favouring micronutrient accumulation can be introduced from other sources directly into the crop. Biofortification is well established with much progress achieved for some crops as discussed in several reviews (Khush et al. 2012; Saltzman et al. 2013; Birol et al. 2015; Singh et al. 2016; Bouis and Saltzman 2017).

Notably, HarvestPlus has led several initiatives for the development and promotion of biofortified crops. Through these initiatives, biofortification of three globally important micronutrients, vitamin A, iron and zinc, has been achieved in staple food crops, including maize, beans, rice, wheat, pearl millet, potatoes and bananas (Hotz and McClafferty 2007; La Frano et al. 2014; Andersson et al. 2017). This has led to the official release of pVA rich orange-flesh sweet potato, yellow cassava and orange maize as well as iron-rich beans and pearl millet, and zinc-rich rice and wheat in several developing countries (Birol et al. 2015; Bouis and Saltzman 2017).

2.2.2 Carotenoid biosynthesis in plants

A thorough understanding of the regulatory elements and genes involved in the carotenoid pathway is critical to effectively breed plant varieties for carotenoid content. Research using different plant models have contributed to current knowledge on the pathway of

carotenoid biosynthesis in plants, its regulation and enzymes involved, and this has been the subject of several reviews (Cunningham and Gantt 1998; Fraser and Bramley 2004; Giuliano et al. 2008**;** Cazzonelli and Pogson 2010; Hannoufa and Hossain 2012**;** Rosas-Saavedra and Stange 2016). Carotenoids are synthesized in plastids of higher plants while the process is mediated by nuclear-encoded enzymes (DellaPenna and Pogson 2006). Similar to other isoprenoids, carotenoids are built from the 5-carbon compound isopentenyl pyrophosphate (IPP), which originates from two pathways in plants, the cytosolic mevalonic acid (MVA) pathway and the plastid 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodríguez-Concepción 2010).

Most plant carotenoids are synthesized through the MEP pathway (Figure. 2.1) which combines glyceraldehyde-3-phosphate (G3P) and pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DXP) in a reaction catalysed by the enzyme deoxyxylulose 5-phosphate synthase (DXS) (Domonkos et al. 2013; Moise et al. 2014; Rosas-Saavedra and Stange 2016). Subsequently, DXP is converted to MEP in a reductive isomerization reaction mediated by deoxyxylulose 5-phosphate reductoisomerase (DXR). This is followed by a series of reactions leading to the production of IPP and dimethylallyl diphosphate (DMAPP) from 4-hydroxy-3-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP) mediated by the enzyme HMBPP reductase. Geranyl pyrophosphate (GPP) is then formed from the condensation between IPP and its allylic isomer DMAPP and further addition of two units of IPP catalysed by geranyl geranyl pyrophosphate (GGPP) synthase results in the formation of C20 GGPP (Fraser and Bramley 2004). The first committed step towards carotenoid biosynthesis involves the tail to tail condensation of two GGPP molecules, catalysed by phytoene synthase (PSY) to form phytoene. PSY catalyses the first committed step towards carotenoid biosynthesis in plants, hence is considered the most important regulatory enzyme in the pathway (Cazzonelli and Pogson 2010). This enzyme has been extensively studied with the corresponding encoding genes identified and engineered in several crops for carotenoid biosynthesis (Giuliano, 2017). In subsequent steps, the colourless phytoene is converted to red-coloured lycopene through four sequential desaturation reactions catalysed by two desaturases and two isomerases: phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), carotenoid isomerase (CRTISO) and ζ-carotene isomerase (Z-ISO) (Rosas-Saavedra and Stange 2016). These reactions introduce a series of carbon-carbon double bonds constituting the chromophore in carotenoid pigments.

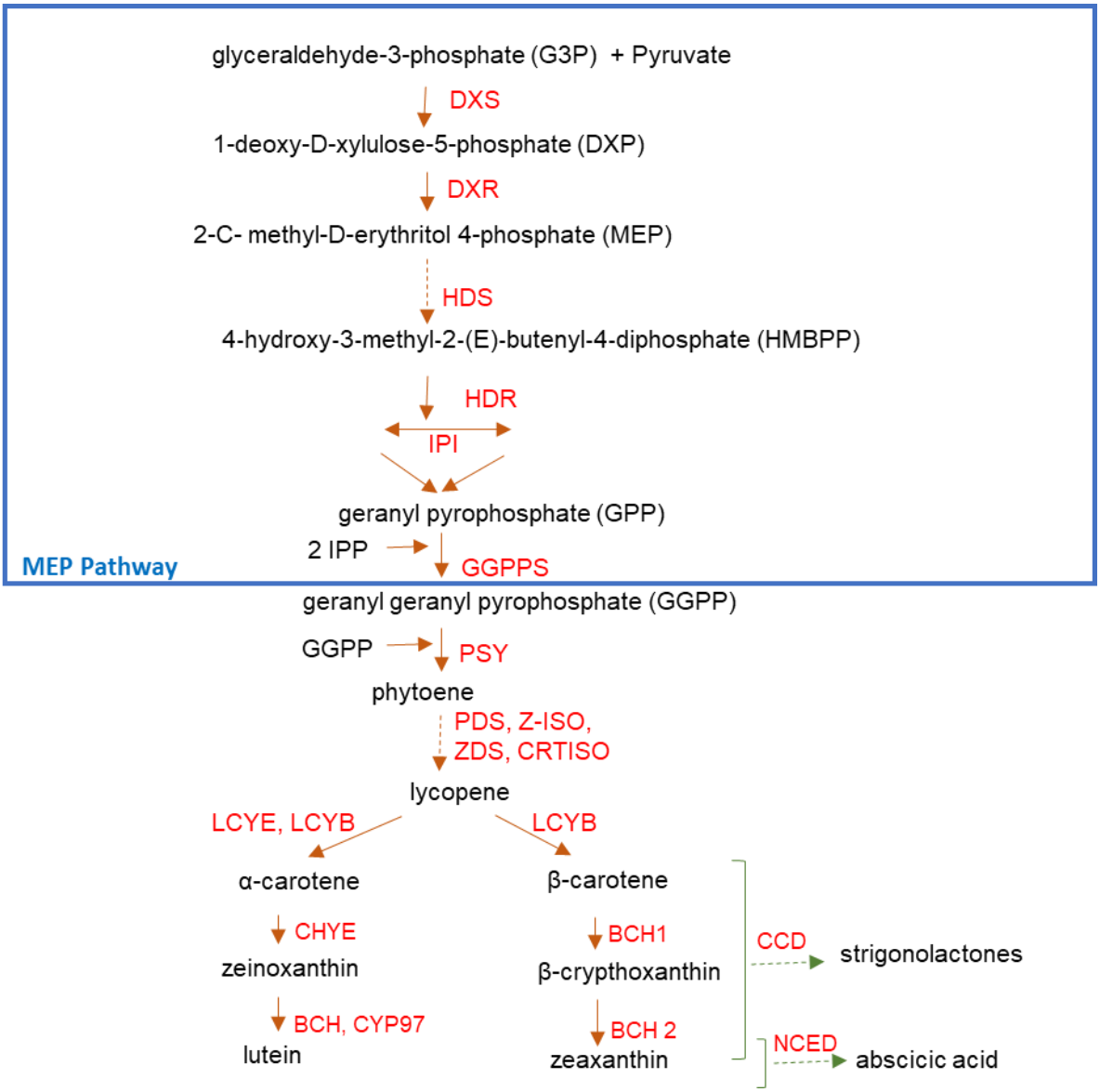

Figure 2.1 Biosynthetic pathway for carotenoids in plants. (Adapted from Cazzonelli and Pogson 2010; Hannoufa and Hossain 2012; Rosas-Saavedra and Stange 2016). Enzymes in red font are defined as: DXS = 1-deoxyxylulose 5-phosphate synthase; DXR = 1-deoxyxylulose 5-phosphate reductoisomerase; HDS = 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR = 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; MEP = 2-C- methyl-D-erythritol 4-phosphate; GGPP = geranyl geranyl pyrophosphate synthase; IPI = isopentenyl pyrophosphate isomerase; PSY = phytoene synthase; PDS = phytoene desaturase; Z-ISO = zeta-carotene isomerase; ZDS = ζ-carotene desaturase; CRTISO = carotenoid isomerase; LCYE = lycopene ε-cyclase; LCYB = lycopene β-cyclase; BCH = carotenoid β-hydroxylase; CHYE = carotenoid ε-hydroxylase; CYP97 = cytochrome P450-type monooxygenase 97; CCD = carotenoid cleavage dioxygenase; NCED = 9-*cis*-expoxy carotenoid dioxygenase.

Lycopene serves as substrate for several downstream reactions, leading to the generation of diverse terminal cyclic carotenes and corresponding xanthophylls. Cyclization of lycopene mediated by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) constitutes the first branching point in the carotenoid biosynthesis pathway, bifurcating into the α-carotene group and the β-carotene group. The enzyme LCYB introduces a β-ionone ring at both ends of lycopene to form β-carotene while two enzymes LCYE and LCYB introduce one ε- and one β-ionone ring at both ends of lycopene to form α-carotene. The final steps in the classic carotenoid biosynthesis pathway involve a series of ring-specific hydroxylation reactions to form xanthophylls, which are basically oxygenated carbon derivatives of carotenoids. Introduction of two subsequent hydroxyl groups to β-carotene results in the formation of β-cryptoxanthin and zeaxanthin, respectively. Similarly, α-carotene is hydroxylated twice to zeinoxanthin and lutein (Cazzonelli and Pogson 2010).

Carotenoid biosynthesis is regulated throughout the life cycle of a plant, resulting in changes in composition based on developmental requirements during germination, photomorphogenesis, photosynthesis, fruit development and response to stimuli (Cazzonelli and Pogson 2010). Carotenoid cleavage plays a role in maintaining physiologically adequate carotenoid levels in plant tissues. In addition to random cleavage by photo-oxidation or peroxidase and lipogenase oxidation, a class of carotenoid cleavage dioxygenase enzymes (CCD) and 9-*cis*-expoxy carotenoid dioxygenase enzymes (NCED) have been associated with carotenoid cleavage (Hannoufa and Hossain 2012; Li and Yuan, 2013). These classes of enzymes catalyse cleavage of carotenoids into apocarotenoids such as strigonolactones, abscisic acid and other compounds (Hannoufa and Hossain 2012).

2.2.3 Breeding for improved carotenoid content in crops

Several crop breeding programmes are engaging in the improvement of carotenoid content and composition of crop plants to enhance their nutritional value. Transgenic and conventional breeding approaches have successfully been employed for improving carotenoid content in crops. As mentioned previously, transgenic approaches involving direct transfer of desirable genes from other sources to elite breeding lines are especially useful for crops where pVAC do not naturally exist at the required levels in available germplasm such as rice, potato and wheat (Guiliano 2017). The classical example for a transgenic pVAC biofortified crop is Golden Rice, resulting from the initial development of a rice line, which expressed a daffodil PSY, leading to phytoene accumulation in rice endosperm (Ye et al. 2000; Paine et al. 2005). Golden Rice paved the way for other transgenic pVAC enriched staple crops such as maize (Aluru et al. 2008; Zhu et al. 2008),

wheat (Wang et al. 2014) potato (Diretto et al. 2006; 2007) and banana (Paul et al. 2017; 2018). Transgenic strategies have potential for pVAC enhancement and are advantageous in that they allow the transfer of specific genes and require less time to generate a crop, which expresses a trait of interest in a stable way. However, they are fraught by regulatory issues and public concerns on the use of genetically modified (GM) crop plants and in addition, most developing countries lack the competence for transgenics. Consequently, to date, none of the developed transgenic high carotenoid crops are approved for release to farmers (De Steur et al. 2017; Lee 2017).

Conventional breeding strategies, on the other hand, attempt to increase the pVAC content of crops using their natural genetic variation, and so far, has been the preferred method for pVAC enhancement in most staple crops. Bouis and Saltzman (2017) elaborated the strategy used by HarvestPlus, which involves exploration of available diversity for pVAC in combination with screening for agronomic or end use characteristics to identify parental stocks for crosses, as well as genetic studies and marker development to increase the speed of breeding. Similarly, existing high pVAC varieties, pipeline varieties or finished germplasm products are identified for fast-track release. Sweet potato, cassava and maize are well known examples of staples, which have been biofortified through conventional breeding with significant increase in vitamin A activity. Ceballos et al. (2013) reported breeding progress in cassava with increased maximum levels of total carotenoid (TC) contents from 10 µg g^{-1} to about 25 µg g^{-1} of fresh root in 5 years through rapid cycling recurrent selection. Pixley et al. (2013) also reported the development of maize lines with up to 20 µg g^{-1} provitamin A activity using marker assisted selection (MAS), whereas existing tropical maize lines had no pVA activity. Biofortified varieties have recently been released and are cultivated in several countries as a food-based approach to combat VAD while biofortified traits are being mainstreamed into crop breeding programmes for scaling-out (Bouis and Saltzman 2017; Lee 2017).

2.2.4 Bioavailability and bioaccessibility of carotenoids

Information on bioavailability and bioaccessibility of carotenoids from foods is important in determining their role in human diets. Bioavailability of carotenoids refers to the proportion of consumed carotenoids that can be absorbed, transported, stored or utilized for normal physiological functions and it is determined using animal models such as mice, rats, chickens, Mongolian gerbils or human volunteers (Rodriguez-Amaya 2016). Bioaccessibility is a component of bioavailability, which refers to the fraction of dietary carotenoids that is liberated from the food matrix during digestion, transferred into mixed

micelles and/or absorbed by enterocytes and delivered in the blood stream and it is measured using *in vitro* models (Tanumihardjo et al. 2010; Giuliano 2017).

Factors affecting bioavailability of carotenoids include food status (cooked or raw), type of carotenoid, food processing method, food matrix in which the carotenoid is incorporated, nutrient status of host, food composition and interaction with other food compounds (Tanumihardjo et al. 2010; Haskell 2012; Rodriguez-Amaya 2016). Among these factors, the food matrix is possibly most critical, because the release of carotenoids from the food matrix determines bioavailability. The food matrix incorporates differences in the composition and storage of carotenoids as well as changes imposed by ripening and food processing (La Frano et al. 2014; Saini et al. 2015). La Frano et al. (2014) pointed out a wide variation of bioavailability and bioaccessibility (2-70%) of β-carotene from different food matrices with higher values obtained in fat rich cooked food matrices. Studies on carotenoid bioavailability specific for bananas are limited. Ekesa et al. (2012) evaluated 2 cultivars, a plantain and an EAHB in Eastern DR Congo and reported a cultivar-dependent response with a relatively high (10-32%) bioaccessibility for β-carotene from boiled bananas and banana-derived dishes. Assessment of micronutrient retention in crops after typical storage, processing, and cooking practices is usually a key component of biofortification efforts and this is done to ensure availability of sufficient levels in foods normally consumed by target populations before biofortified varieties are promoted (De Moura et al. 2015).

2.3 Bananas and their potential for vitamin A biofortification

2.3.1 Importance and nutritional value

Bananas are important food security crops for smallholder farmers worldwide. They are currently cultivated in over 130 countries, on over 5.5 million ha with a global production of about 145 million tons (MT) (FAOSTAT 2017). Over 1000 cultivars exist and the most important banana groups for food security are the dessert bananas (AAA genome), plantains (AAB genome), East African highland bananas (EAHB) (AAA-EA genome) and the bluggoe (ABB) cooking types. Current statistics from FAO (2017) (Figure 2.2) depicts the importance of bananas as well as plantains over seven decades in Africa and parts of Asia where VAD is known to be most prevalent (WHO 2009). The largest average production share of bananas worldwide is from tropical and subtropical regions of Asia, which contribute almost half of the world production (Figure 2.2), while the largest share in plantains and others (65%) from Africa with about two thirds of the worldwide production. For the same period, the share of bananas was negligible while that of plantains and others was non-existent for Europe and Oceania.

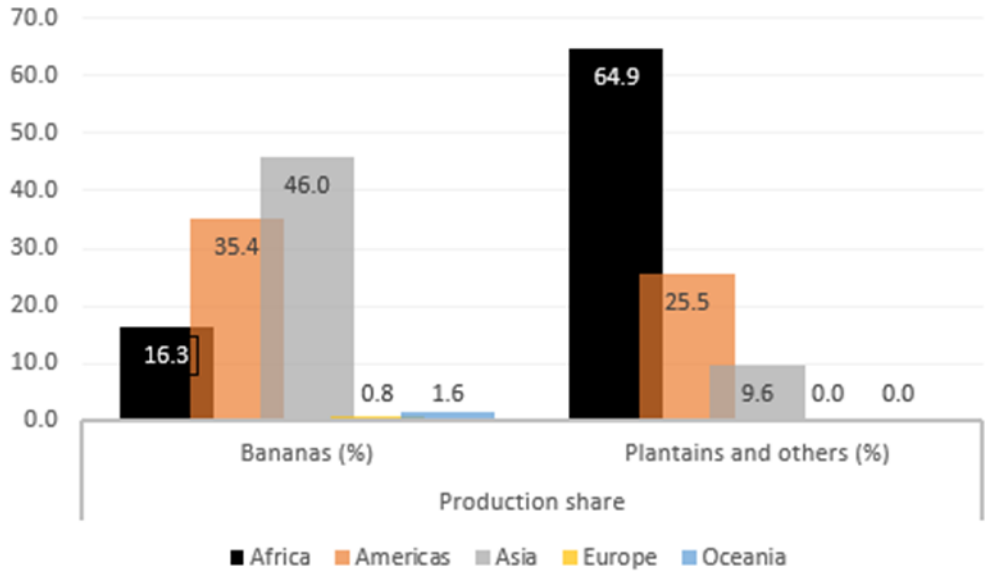

Figure 2.2 Production share of bananas and plantains and others by region, average 1961-2016 (FAOSTAT 2017).

Plantains are cultivated in the humid forest and moist derived Savannas of West and Central Africa, spanning from Guinea Bissau in the west to the Democratic Republic of Congo (DRC) in the south east, with the crop ranking among the top three starchy staples in most of these countries (Norgrove et al. 2014). Their starchy fruits are characterized by a firm orange-yellow pulp and are consumed fried, boiled, steamed or roasted at different stages of ripening. In addition, the fruits are processed into flour, which is further incorporated into other edible products. Plantains are rich in dietary fibre, carbohydrates, potassium, iron, vitamin A, vitamin C and other bioactive compounds such as flavonoids (Robinson and Sauco 2010; Tsamo et al. 2015; Pareek 2016). Being giant herbaceous plants, they are propagated vegetatively through suckers and grow throughout the year, although production varies seasonally. Their perennial nature and ability to grow in diverse environments makes them an attractive all-season crop serving dietary needs of poor populations.

2.3.2 Origin and genetic diversity

Bananas belong to the genus *Musa* within the family Musaceae, of the order Zingiberales (De Langhe et al. 2009). Bananas were 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). Bananas originated from the tropical regions of Asia and Oceania but are now grown in tropical and sub-tropical regions of the world (Ortiz 2015).

Musa spp. is constituted by four genomes; A genome, 2n = 2x = 22 from *M. acuminata*, B genome 2n = 2x = 22 from *M. balbisiana*, S genome 2n = 2x = 22 from *M. schizocarpa* and the T genome 2n = 2x = 20 from the *Australimusa* species. However, most edible bananas are derived from inter and intra specific hybridization of *M. acuminata* and *M. balbisiana* while other cultivars have also originated from *M. schizocarpa* and *Australimusa* species (Davey et al. 2013; Häkkinen 2013; Christelová et al. 2017). Most cultivars are either diploids, triploids or tetraploids with genome configurations AA, BB, AB, AAA, AAB, ABB, AAA, AAAB AABB or ABBB (Carreel 1994); with the most important edible cultivars mentioned previously (dessert bananas, plantains, EAHB and ABB cooking types) predominantly belonging to the triploid configuration. Breeding efforts aimed to tackle various biotic and abiotic stresses have led to the production of many diploid, triploid and tetraploid hybrids also contributing to existing banana diversity (Tenkouano and Swennen 2004; Bakry et al. 2009; Tenkouano et al. 2011; Ortiz and Swennen 2014).

Approximately 120 known plantain cultivars exist comprising selections from existing hybridizations and somatic mutations of a few strains. West and Central Africa harbour the greatest variability, hence are known to be secondary centers of diversity for plantains, as a result of human selection from somatic mutations during cultivation (De Langhe et al. 2005; Ortiz et al. 2015; Adheka et al. 2018). Plantains display the greatest phenotypic diversity among existing triploid banana subgroups with variation occurring for inflorescence type, plant size, fruit orientation, fruit shape, pseudostem colour and fruit colour (Adheka et al. 2018). Based on inflorescence morphology, three main types of plantains have been distinguished, namely: French, False Horn and Horn plantains (De Langhe et al. 2005; Adheka et al. 2018). Despite the huge morphological diversity, there are challenges with elucidating genetic variability using molecular markers, resulting in speculations that genetic diversity originated from epigenetic regulations and not from gametic combinations (Noyer et al. 2005; Hippolyte et al. 2012).

M. acuminata diploids are also phenotypically diverse and are currently differentiated into eight sub-species, *burmanica, siamea, malaccensis, truncata, errans, microcarpa, zebrina* and *banksii* based on deoxyribonucleic acid (DNA) markers (Perrier et al. 2011; Brown et al. 2017). A few cultivars from some of these sub-species have been used as parents in breeding programmes, for example the wild diploid Calcutta 4, (*M. acuminata* ssp *burmanica*) is extensively used as a source of resistance to the Sigatoka complex (Bakry

et al. 2009; Ortiz 2015; Alakonya et al. 2018). A number of these diploids have also been identified as progenitors in the developmental process of edible 3x cultivars and are relevant as parents for evolutionary breeding as described by Tenkouano et al. (2011). Notably, *banksii*-derived AA cultivars originating from Papua New Guinea (PNG) have played a major role in the domestication of AAB plantains of West Africa and the Pacific (Perrier et al. 2011), indicating their potential for plantain breeding.

2.3.3 Variability and carotenoid profiles in bananas

The presence of sufficient variability for a given trait of interest is critical for the success of crop breeding programmes (Acquaah 2012). Several crop plants exhibit a large phenotypic variation in the quantity and types of carotenoids accumulated, and this is more evident in fruits than other plant organs (Lado et al. 2016). Commonly used analysis for quantification of carotenoids, also applicable for banana, are either based on the use of spectrophotometry at 450 nm, or high-performance liquid chromatography (HPLC) following acetone extraction and petroleum ether partition (Rodriguez-Amaya and Kimura 2004). To a large extent, banana biofortification efforts have focused on exploration and quantification of variability in fruit pVAC content (Englberger et al. 2003; 2006; Amorim et al. 2009; Davey et al. 2009; Fungo and Pillay 2011) and optimization of fruit carotenoid assessment methods (Davey et al. 2006; 2007). These studies have revealed high levels of variability in carotenoid content within and across different genomic groups.

In a pioneer study to assess β-carotene in bananas to combat VAD in Micronesia, Engleberger et al. (2003) recorded β-carotene levels ranging from 0.30-27.80 µg g^{-1} from raw and cooked samples from ripe fruits of 12 local banana cultivars. Engelberger et al. (2006) further analysed 12 diverse cultivars (white-yellow-orange flesh) from the Australian field collection for pVACs (*trans* β-carotene, *cis* β-carotene and α-carotene) and recorded the highest value of *trans* β-carotene (14.12 µg g^{-1}) in the yellow/orange fleshed Fe'i banana cultivar Asupina. Their study also pointed out a correspondence between carotenoid content and pulp colour, with orange-yellow fruit pulps having higher carotenoid content than cream fruit pulps. Davey et al. (2007) also studied the variability of pVACs and TC in six widely consumed West and Central African *Musa* varieties grown under standardized field conditions in Cameroon. They found substantial genetic variation of pVACs between cultivars, with orange-fleshed AAB plantains 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. (2009) screened up to 171 cultivars and recorded mean total pVAC values ranging from 0 or undetected to 34.56 µg g^{-1} FW with a mean of 6.97 µg g^{-1} FW, indicating a wide variability in fruit pVAC content.

Amorim et al. (2009) further confirmed variability in carotenoid contents in 42 diverse cultivars screened in Brazil with TC contents in fruit pulps ranging from 1.06 to 19.24 µg $g^{-1}$with a mean of 4.73 µg g^{-1}. Fungo and Pillay (2011) also recorded β-carotene levels ranging from 0.51 to 25.94 µg g^{-1} in 47 diverse *Musa* accessions screened in Uganda.

Borges et al. (2014) evaluated carotenoid profiles of 29 cultivars across different genomic groups in Brazil and identified accessions with pVACs up to 1164 µg g^{-1} dry weight (DW) 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%). Heng et al. (2017) also recorded carotenoid levels ranging from 0.18 to 36.82 µg g^{-1} FW in 38 banana cultivars and hybrids grown in China with the highest values recorded in AAB plantain cultivar Orishele.

From these studies, cultivars with the highest pVACs were widely distributed across different genome groups and the highest levels of pVAC obtained so far in *Musa* spp. were recorded from the Australimusa type Fe'i bananas, specific to the Islands of Micronesia, Eumusa type bananas originating from PNG, and AAB-plantains from Africa. It was also noted that carotenoid profiles of *Musa* fruit pulp consist predominantly of pVACs (mainly α-carotene, *trans* β-carotene and smaller concentrations of *cis*-β carotene isomers) with considerable amounts of non-pVAC lutein (Engleberger et al. 2003; 2006; Davey et al. 2009; Borges et al. 2014) thus confirming banana as a suitable crop for biofortification.

2.3.4 Stability and retention of carotenoids during ripening

Significant changes in nutrient content and composition are often observed during fruit ripening based on the fruit developmental stage, type of fruit tissue and environmental conditions. These changes are also linked to chromoplast development and cause changes in the food matrix, which in turn, affects bioavailability (Lado et al. 2016). Plantain fruits are consumed at varying stages of ripening, necessitating an evaluation of the retention of carotenoids during ripening to ascertain bioavailability. Several studies have investigated the variation of carotenoids during fruit ripening in bananas and reported diverse changes in pVAC contents, which are cultivar dependent. Lokesh et al. (2014) assessed four varieties (two AAA and two AAB type dessert bananas) in India and observed that TC and pVACs remained stable after ripening. Ngoh-Newilah et al. (2009) evaluated 19 cultivars and hybrids (10 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 carotenoids 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 5.6 to 46.8 µg g^{-1} FW in unripe fruit and 16.8 to 106.3 µg g^{-1} FW in ripe fruits, implying an increase in pVAC content from the unripe to the ripe stage in eight banana cultivars evaluated in DRC. In another study on eight banana cultivars in Uganda, Mbabazi (2015) also recorded a consistent increase in β-carotene equivalents (BCE) following ripening in the five EAHB cultivars tested and a decrease in BCE in the two dessert bananas and one plantain cultivar tested.

2.4 Banana breeding strategies and considerations for provitamin A carotenoid improvement

2.4.1 Breeding schemes and constraints

Since most commonly cultivated bananas are of the triploid background, the crop was initially thought to be intractable to breeding. After several attempts of crossing otherwise sterile triploids with fertile pollen from wild diploids, hybrids were successfully generated, indicating possibilities for crossbreeding (Ortiz and Vuylsteke 1996). The main strategy adopted for banana breeding relies on conventional sexual hybridization involving crossing seed-fertile 3x cultivars to 2x wild or improved accessions, selecting 4x and 2x hybrids from intermediate products and crossing selected 4x and 2x hybrids aiming to generate sterile 3x hybrids (Figure 2.3) (Ortiz 2015; Tenkouano et al. 2011; Bakry et al. 2009).

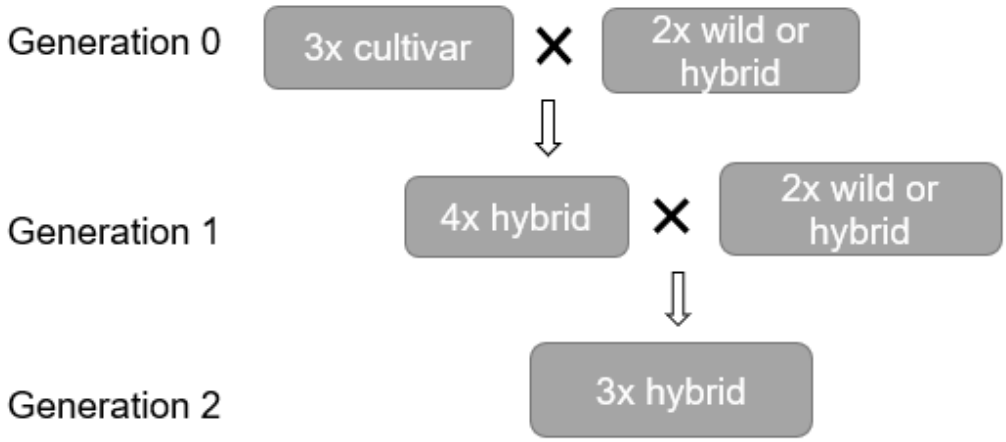

Figure 2.3 Graphical representation of the main banana breeding scheme (adapted from Tenkouano et al. 2011).

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). The use of 2x-2x hybridizations to produce secondary 3x via unilateral sexual polyploidization and 2n pollen production has also been advocated (Ortiz 1997; Oselebe et al. 2006). Considering the importance of diploids in these schemes, efforts are also

devoted to diploid improvement aiming to generate superior diploids combining multiple desirable traits (Ortiz and Vuylsteke 1996; Menon et al. 2011; Brown 2017). Diploid breeding is also important for genetic analysis due to the presence of disomic inheritance in diploids as opposed to the irregular meiotic behaviour observed in higher ploidies. In addition, inclusion of existing diversity present in diploid cultivars and wild relatives in breeding schemes for recurrent selection will broaden the existing narrow genetic base (Ortiz and Swennen 2014).

Regarding the breeding cycle, it may take up to 15 years from crossing to the release of a new variety using existing breeding schemes (Tenkouano et al. 2011). Generally, it takes over a year to obtain seeds from a desired cross and most desired crosses are usually unsuccessful with the few successful crosses having generally low seed set. Seeds obtained from planned crosses rarely germinate and hybrids are often recovered through *in vitro* embryo culture (Bakry 2008). Problems may also arise from poor pollen production and/or germination as well as generation of abnormal seeds lacking embryos and/or endosperms. Banana breeding is therefore complicated by parthenocarpy, low fertility, low seed viability, polyploidy and associated irregular meiotic behaviour, long generation times, diverse genome configurations and the narrow genetic base (Ortiz 2013; 2015; Brown et al. 2017). However, since plantains are highly heterozygous and vegetatively propagated, selected hybrids from crosses have the potential of becoming a suitable cultivar.

Seedlings obtained from crosses are subjected to a series of trials, beginning with the early evaluation trial (EET), usually non-replicated with one to five plants evaluated during two cycles; preliminary yield trials (PYT) where selected clones from EET are evaluated in replicated trials over two cycles possibly with the involvement of farmers and lastly multi-locational evaluation trials (MET) which completes the evaluation process (Tenkouano et al. 2011). According to Ortiz (2013), most growth and yield characteristics of *Musa* display complex inheritance and genetic association patterns and are subject to genotype by environment interaction. Therefore, evaluation under field conditions in different locations and years is essential for a thorough appraisal of performance. Using these strategies, several high yielding disease resistant tetraploid, triploid and diploid plantain hybrids have been produced (Tenkouano and Swennen 2004; Tenkouano et al. 2011).

2.4.2 Polyploidization as a breeding strategy

Polyploidy, which refers to the presence of more than two complete sets of chromosomes per cell nucleus, is a common phenomenon in vegetatively propagated plants. Polyploids can be classified as allo- or auto-polyploids; the former originating from interspecific

hybridization and the latter from doubling of the chromosome number of a diploid species (Dhooghe et al. 2011; Renny-Byfield and Wendel 2014). It is well known that polyploidy has the potential to generate new cultivars with superior agronomic characteristics and other favourable traits, sometimes out-performing their diploid progenitors. Polyploid induction has been advocated as a tool for genetic improvement in several crops to induce new polyploid varieties or to restore fertility of interspecific hybrids (Dhooghe et al. 2011; Sattler et al. 2016).

Mitotic polyploidization, which involves the induction of chromosome doubling in somatic tissues, has been widely used to generate polyploids in vegetatively propagated crops including bananas. This is achieved using antimitotic agents such as colchicine or oryzalin, which inhibits microtubule formation during mitosis thereby preventing chromosome migration during anaphase resulting in the formation of cells with doubled chromosome numbers (Dhooghe et al. 2011). Protocols are well established for *Musa* spp. and autotetraploids have been generated from diploid cultivars (Van Duren et al. 1996; Ganga and Chezhiyan 2002; Bakry et al. 2007). Chromosome doubling in banana is aimed to generate fertile tetraploids from promising diploids for crossing with diploids with the objective of generating sterile triploid hybrids with superior traits. Following doubling, it is important to evaluate induced tetraploids for their agronomic characteristics and fertility to ascertain their capacity to produce promising progenies from crosses. Do Amaral et al. (2015) evaluated autotetraploids derived from the diploid Pisang Lilin and recorded superior agronomic performance, Sigatoka resistance and female fertility, indicating their potential for triploid breeding. Similarly, Goigoux et al. (2013) evaluated two diploid Mlali cultivars Chicame and Paka and their *in vitro* induced tetraploids for use as male parents in Cavendish banana breeding. Pollen fertility assessments using Alexander stain revealed up to 35% viability for both diploid and tetraploid pollen and this viability was later confirmed with test-crosses, indicating their potential to produce triploid hybrids.

2.4.3 Considerations for provitamin A carotenoid improvement

Banana breeding has mainly been aimed at host plant resistance for the devastating fungal disease black Sigatoka, which is prevalent in most producing countries (Tenkouano et al. 2011; Brown et al. 2017; Alakonya et al. 2018). Although there is recent interest in improving the nutritional content of dietary staples, biofortification is currently not routine in the few existing banana breeding programmes worldwide. For the case of pVACs, biofortification breeding is still limited to diversity assessment studies with much progress achieved in documenting variability of pVACs in different sets of germplasm. Concurrently, transgenic techniques are also being explored for pVAC enhancement, specifically for

EAHB which naturally have very low levels of fruit pVAC. Paul et al. (2018) reviewed the progress of the Banana21 project initiated in 2005 aiming to develop EAHB lines with high levels of fruit pVACs for Uganda. Although high pVAC lines were generated for cultivars M9 and Nakitembe, which met the set target of 20 µg g^{-1} BCE DW, the project is still faced with challenges regarding deregulation and release of such GM biofortified varieties. Transgenic techniques appear scientifically appealing, but most research laboratories still lack the competence and there is need to address social, political and environmental hurdles linked with acceptance of GM crops (Lee et al. 2017).

The widely documented natural variability in pVACs in *Musa* germplasm suggests their potential for conventional breeding. Therefore, there is need to explore the utilization of this variability in hybridization programmes using existing and alternative breeding strategies. Furthermore, there is still a considerable diversity of wild and cultivated *Musa* spp. with potential for exploration towards pVAC enhancement. Diploid germplasm will also be useful for genetic studies to elucidate the inheritance of the complex polygenic traits and quantitative trait loci (QTL) analysis in gene discovery programmes. In addition, and for the longer term, the published draft genomic sequence of *Musa* spp. A genome (D'hont et al. 2012; Martin et al. 2016) and B genome (Davey et al. 2013) and the recent progress in the development and use of molecular tools such as genomic selection (GS) (Nyine et al. 2017) will be useful to accelerate pVAC-enriched cultivar development in bananas.

2.5 Conclusions

Vitamin A deficiency is one of the most widespread deficiencies, posing a global health challenge. Biofortified bananas have the potential to address VAD in regions where the crops are grown. To achieve this, research is needed to generate and disseminate high pVAC varieties that are acceptable for consumption. Breeding for high pVAC content in plantains will require a thorough assessment of diverse germplasm relevant for plantain breeding, to identify high pVAC lines for incorporation in existing breeding schemes. In addition, a systematic assessment of pVAC variation during ripening is required to ascertain the ripening stage with the highest retention to ensure bioavailability. Considering the long breeding cycle and other breeding complexities of bananas, it is also necessary to explore alternative breeding strategies such as *in vitro* chromosome doubling for pVAC enhancement. Such studies will generate useful information and relevant genetic stocks to facilitate banana biofortification efforts.

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CHAPTER 3

Carotenoid profiling in *Musa* fruit pulp

3.1 Introduction

Carotenoids are a diverse group of multi-functional lipophilic pigments. They are especially important in plants as a component for photosynthetic systems, but also in human nutrition as biological anti-oxidants and as precursors of vitamin A (Britton 2008). Vitamin A is a fat-soluble vitamin essential for vision, maintenance of epithelial surfaces, immune competence, reproduction and embryonic growth and development (WHO/FAO 2004; Tanumihardjo et al. 2016). Inadequate intakes of vitamin A leads to VAD with health conditions such as xerophthalmia, anaemia and increased susceptibility to and severity of infections (WHO 2009). Vitamin A cannot be synthesized de novo by humans hence can only be obtained from plant sources or as pre-formed vitamin A from animal sources (Blomhoff and Blomhoff 2006). Over 600 carotenoids have been reported with only over 50 detected in food and in humans, the most predominant being α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin (Arscott 2013). Carotenoids with pVA activity such as α-carotene, β-carotene, and β-cryptoxanthin have attracted attention for improvement in crop plants (biofortification) to combat VAD (Bai et al. 2011).

Bananas (*Musa spp*) are an important staple, serving as a source of nutrients and calories for millions of people worldwide, particularly in tropical and sub-tropical regions where it is grown. In 2014, world total production was about 145 MT, of which Africa contributed 28% (FAOSTAT 2017). Over 1000 cultivars exist, mostly derived from intra- or inter- specific hybridization of the wild diploid (2n = 2x = 22 chromosomes) ancestral species *M. acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) (Heslop-Harrison and Schwarzacher 2007). Of these, three main banana groups; dessert bananas (AAA genome), plantains (AAB genome) and EAHB (AAA-EA genome) dominate production. While dessert bananas are of global importance, the latter two banana types are important as starchy staples mainly grown by smallholder farmers (Ortiz and Swennen 2014). Consequently, existing banana breeding programmes have focused on the improvement of these three main important banana groups to address various biotic and abiotic challenges (Ortiz and Swennen 2014; Brown et al. 2017).

Bananas have a considerable diversity in fruit carotenoid content. Englberger et al. (2003) were the first to identify high contents of pVACs in banana, suggesting its value as a good source of vitamin A. Thereafter, other studies emerged with a focus on exploration and quantification of variability in banana fruit pVAC content (Englberger et al. 2006; Amorim

et al. 2009; Davey et al. 2009; Fungo and Pillay 2011) and optimization of fruit carotenoid detection methods (Davey et al. 2006; 2007) towards banana biofortification. The carotenoid profile of bananas includes mainly β-carotene, α-carotene and lutein. However, smaller amounts of β-cryptoxanthin and zeaxanthin have also been reported (Englberger 2006; Borges et al. 2014). About 70% of the carotenoids found in banana are pVAC (Davey et al. 2009; Heng et al. 2017). The non-pVAC lutein is also important in human nutrition for its antioxidant properties and its role as a component of macular pigments in eye health (Britton 2008). Generally yellow-orange flesh banana cultivars are reported to contain higher levels of carotenoids (up to >20 fold) than cream-white flesh cultivars (Englberger et al. 2006; Amorim et al. 2009; Fungo and Pillay 2011).

Efficient screening of large numbers of cultivars is critical for identifying trait variation towards biofortification in crop improvement programmes. Previous studies recorded substantial variability of fruit carotenoid content in *Musa* spp., suggesting possibilities for breeding. However, such studies have been limited to a few genome groups with narrow sets of germplasm and small samples. To our knowledge, few reports exist for carotenoid content in *Musa* spp. where more than 30 genotypes were analysed, despite the vast diversity in *Musa* (Amorim et al. 2009; Davey et al. 2009; Fungo and Pillay 2011; Borges et al. 2014; Heng et al. 2017). Moreover, diverse sampling and analytical strategies employed in some of these studies may have contributed to reported variability. For example, in the most comprehensive study carried out on 171 banana genotypes (Davey et al. 2009), frozen and lyophilized samples were obtained from several locations, and were transported and analysed in a distant lab.

Recently, the IITA banana breeding programme renewed efforts towards vitamin A biofortification and this necessitated a comprehensive assessment of the pVAC content of available genotypes. This study therefore aimed to evaluate the variability of fruit carotenoid content and profiles in different types of bananas (plantains, *M. acuminata* cultivars and hybrids) present in the IITA banana germplasm collection. An understanding of the carotenoid content and profiles will facilitate the identification of high pVAC genotypes for the development of pVA biofortification strategies in banana.

3.2 Materials and methods

3.2.1 Experimental site

Fruits were collected from the banana breeding plots at the IITA Ibadan research station between August 2014 and February 2018. This research station is located at 3° 54' East and 7° 30' North at 240 m above sea level, in the sub humid-derived savanna agro-ecology where the soil type is predominantly ferric luvisols. The rainfall pattern is bimodal, with two distinct rainy seasons, the first from April to July and the second from August to November.

3.2.2 Plant material

Fruits from 204 diverse genotypes available in the IITA Ibadan breeding collection comprising plantains, *M. acuminata* cultivars and hybrids were screened. Plantains (66 genotypes) represented popular farmer preferred varieties within the existing diversity originating from Africa and beyond, as defined by passport data on the Musa germplasm information system (MGIS) database (www.cropdiversity.org) and Swennen (1990). (Appendix 1). Plantains comprised French, False Horn and Horn types based on the bunch morphology as described by Adheka et al. (2018). *M. acuminata* cultivars (80 genotypes) encompassed AA diploids and other cultivars of diverse origins, recently acquired from the International Transit Centre (ITC), Bioversity International in Belgium for evaluation and use for plantain breeding. Cultivars were categorized into PNG cultivars; AA cv. *banksii* cultivars; Indonesia triangle and New Guinea (AA cv. IndonTNg) cultivars; AA cv. mshare cultivars; assorted cultivars and unclassified cultivars, based on passport data on the MGIS database (www.cropdiversity.org) in combination with cytological and molecular groupings (clusters) as described by Christelová et al. (2017) (Appendix 2). Hybrids (59 genotypes) comprised advanced lines/clones selected for high yield and black Sigatoka resistance as well as breeding lines previously developed by the IITA and the Honduran Foundation for Agricultural Research (FHIA) banana breeding programmes. Hybrids comprised diploids, triploids and tetraploids based on ploidy records with pedigree details provided in Appendix 3. All plants were grown under standard field conditions at a spacing of 3 x 2 m and each variety was represented by a minimum of five plants.

3.2.3 Sample processing and preparation

Bunches were harvested at maturity (when ripening was observed at the first hand of the bunch) and stored at ambient temperature in a dark room for ripening. Fruits were taken from the middle of the second hand of each bunch at the fresh ripe stage corresponding to stage 5, when fruit colour had turned yellow with green tips and necks (Dadzie and Orchard 1997).

Sample preparation was carried out under subdued light to minimise light induced degradation of carotenoids. Selected fruits were thoroughly washed, air dried and peeled with sharp stainless-steel knives. The fruit pulp was diagonally cut into halves and each half was further sliced into small pieces and mixed thoroughly. The homogenate was wrapped in aluminium foil and packed in sampling bags from which sub-samples were taken for further analysis. Duplicate samples were prepared for each bunch for all analyses.

3.2.4 Carotenoid extraction and quantification

Total carotenoid estimation was done using ultraviolet-visible (UV-VIS) spectrophotometry while separation and quantification of individual carotenoids was done using HPLC, following the protocol described by Rodriguez-Amaya and Kimura (2004) for cassava, with some modifications.

For each sample, 10 g of fruit pulp homogenate was weighed into a mortar to which about 3 g of Hyflosuperce (celite) was added and the mixture was ground with 50 ml cold acetone. Following thorough maceration, the solution was filtered into a flask by suction through a filter paper in a Büchner funnel. This extraction procedure was repeated 3-4 times until the final residue appeared colourless. The extract was transferred into a 500 ml separating funnel, with a Teflon stopcock, containing 20 ml petroleum ether. Double distilled water was slowly added along the surface of the funnel to remove acetone from the extract without emulsion formation. Following this, the upper organic (petroleum ether) phase was separated from the lower aqueous phase. The lower aqueous phase was discarded, and the washing process was repeated four times to remove any traces of acetone residues. The petroleum ether phase was transferred into a 25 ml volumetric flask through a funnel containing 15 g of anhydrous sodium sulfate to remove residual water and the extract was filled up to 50 ml with petroleum ether. Twenty five ml of this solution was used for spectrophotometry while 25 ml was used for HPLC.

Absorbance of the extract obtained was measured at 450 nm using a Genesys 10 UV-VIS spectrophotometer. Total carotenoid content (TC spec) was calculated as:

$$\text{TC spec } (\mu g\ g^{-1}FW) = \frac{\text{Absorbance at 450nm x volume of extract (ml) x } 10^4}{2\ 592 \text{ x sample weight (g)}}$$

Where:

2 592 = β-carotene absorption coefficient in petroleum ether.

Chromatographic analysis/separation of individual carotenoids was carried out on a Waters Alliance e2695 HPLC separation module (Waters Corporation, Milford, MA) equipped with a polymeric YMC^{TM} C30 5 µm column (4.6 x 250 mm) and a photodiode array detector. The system was operated by Empower software (Waters Corporation, Milford, MA).

Twenty-five ml of the petroleum extract prepared in the previous step was concentrated and dried under nitrogen gas and reconstituted in 1 ml of dichloromethane:methanol (50:50 v/v). The solution was filtered through a 0.22 mm polytetrafluoroethylene (PTFE) syringe filter (Millipore) into 2 ml vials (Waters PTFE/silicone septum) for HPLC. Sample injection volumes were 20 µl and flow rate was set at 1.0 ml min^{-1} at a temperature of 25^{o}C. An isocratic elution was performed for 10 min on extracts with 50:50 v/v methanol: methyl tert-butyl ether. The UV spectra was observed at 200 to 600 nm and carotenoids were detected at 450 nm. Identification of lutein, α-carotene and β-carotene (*cis* and *trans* isomers) were determined using an external standard method based on the calibration curve established from pure standards and verification of absorption spectrum and co-elution with authentic commercial standards (α-carotene, β-carotene and lutein, Sigma-Aldrich, Germany).

Based on individual carotenoid values obtained, TC with pVA activity (pVACs), β-carotene equivalents (BCE) and TC measured from HPLC (TC HPLC) were calculated as follows:

- pVACs (µg g^{-1} FW) = α-car +13-*cis* BC + 9-*cis* BC + *trans* BC
- BCE (µg g^{-1} FW) = 0.5 (α-car +13-*cis* BC + 9-*cis* BC) + *trans* BC
- TC HPLC (µg g^{-1} FW) = Total pVACs + lut

Where FW = fresh weight, α-car = α-carotene, 13-*cis* BC = 13-*cis* β-carotene, 9-*cis* BC = 9-*cis* β-carotene and *trans* BC = *trans* β-carotene, lut = lutein

3.2.5 Data analysis

Three to five bunches were analysed for most genotypes, but only one bunch was available for up to 16 *M. acuminata* cultivars (Appendix 4) and these were not included in the statistical analysis. Results were recorded in µg g^{-1} FW and each value was a mean of duplicate samples. Statistical analysis was carried out using Statistical Analysis Software (SAS) version 9.4 for Windows Copyright © 2002-2015 by SAS Institute Inc., Cary, NC, USA. The PROC GLM statement was used for one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Rest to detect significant differences among means while the PROC CORR statement was used for Pearson correlation analysis.

3.3 Results

3.3.1 Carotenoid content and profiles of banana fruit pulp

3.3.1.1 Carotenoid content and profiles of 189 diverse banana genotypes

The mean and range of carotenoid content determined by HPLC and spectrophotometry for all 189 banana genotypes (771 bunches) analysed is summarised in Table 3.1.

Table 3.1 Mean and range of carotenoids in 189 banana genotypes

Trait	Carotenoid content (µg g^{-1} ± SD)			
	All genotypes (n = 771)	Plantains (n = 270)	*M. acuminata* cultivars (n = 247)	Hybrids (n = 254)
Mean				
Lutein	1.18±1.07	1.24±0.63[a]	1.01±1.4[b]	1.29±1.05[a]
α-carotene	3.50±3.03	5.44±2.29[a]	3.04±3.54[b]	1.87±1.79[c]
13-*cis* β-carotene	1.09±0.90	1.67±0.95[a]	0.90±0.81[b]	0.66±0.55[c]
9-*cis* β-carotene	0.20±0.22	0.28±0.16[a]	0.21±0.31[b]	0.12±0.13[c]
trans β-carotene	2.02±2.09	3.11±1.93[a]	1.87±2.33[b]	1.02±1.35[c]
pVACs	6.82±5.38	10.5±3.92[a]	6.03±6.09[b]	3.67±3.26[c]
BCE	4.42±3.64	6.81±2.80[a]	3.95±4.11[b]	2.34±2.22[c]
TC HPLC	8.00±5.56	11.74±4.01[a]	7.05±6.42[b]	4.96±3.44[c]
TC spec	8.88±5.74	13.41±3.73[a]	7.78±6.11[c]	5.13±3.47[b]
Range				
Lutein	0.13-5.91	0.45-2.33	0.13-5.91	0.18-4.03
α-carotene	0.14-16.51	0.88-8.19	0.26-16.51	0.14-7.84
13-*cis* β-carotene	0.09-4.43	0.46-4.43	0.18-2.79	0.09-1.74
9-*cis* β-carotene	0.02-1.50	0.08-0.67	0.03-1.50	0.02-0.66
trans β-carotene	0.10-11.81	0.23-6.46	0.10-11.81	0.10-5.10
pVACs	0.37-30.30	2.91-14-83	0.89-30.30	0.37-12.68
BCE	0.24-21.06	2.01-10.65	0.57-21.06	0.24-7.78
TC HPLC	1.45-36.21	5.12-15.98	1.56-36.21	1.45-14.47
TC spec	1.28-32.03	3.83-18.71	1.28-32.03	1.76-16.63

SD = standard deviation; n = number of samples; pVACs = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; means with different letters within rows are significantly different at $P < 0.5$.

Generally, banana genotypes were richest in α-carotene (3.50 µg g^{-1} FW), closely followed by *trans* β-carotene (2.02 µg g^{-1} FW), lutein (1.18 µg g^{-1} FW), 13-*cis* β-carotene (1.09 µg g^{-1} FW) and smaller amounts of 9-*cis* β-carotene (0.20 µg g^{-1} FW).

Mean concentrations of individual pVACs (µg g^{-1} FW) recorded for all banana accessions analysed varied substantially from 0.14-16.51 for α-carotene, 0.10-11.81 for *trans* β-carotene, 0.09-4.43 for 13-*cis* β-carotene and 0.02-1.50 for 9-*cis* β-carotene (Table 3.1). This translated to BCE values ranging from 0.24-21.06 µg g^{-1} FW with a mean value of 4.42 µg g^{-1} FW across all genotypes. Lutein concentrations ranged from 0.13-5.91 µg g^{-1} FW. Mean TC (µg g^{-1} FW) calculated from HPLC ranged from 1.45-36.21 with a mean of 8.00, comparable to that measured directly from spectrophotometry, which ranged from 1.28-32.03 with a mean of 8.88. The highest mean TC value was recorded for orange flesh cultivar ITC.0601 Hung Tu and the lowest for cream flesh cultivar ITC.0442 Gu Nin Chio (Figure 3.1)

Figure 3.1 Banana genotypes with high and low carotenoid content. High carotenoid banana ITC.0601 Hung Tu bunch (A) and fruit with longitudinal section (B); low carotenoid banana ITC.0442 Gu Nin Chio bunch (C) and fruit with longitudinal section (D)

Comparatively, the plantain collection had the highest mean concentrations (µg g^{-1} FW) for individual pVACs α-carotene (5.44), *trans* β-carotene (3.11), 13-*cis* β-carotene (1.67) and 9-*cis* β-carotene (0.28) while the hybrid collection had the highest value for lutein (1.29). Consequently, BCE for plantain was over 1.5 times higher than that of cultivars and three times higher than that of hybrids. Figure 3.2 summarises the variation in the proportions of individual carotenoids in all bananas and respective banana groups analysed. Interestingly, over 75% of the isolated carotenoids in most banana genotypes were pVACs α-carotene, and β-carotene (*trans* and *cis* isomers). The plantain collection had the highest proportions of pVACs and the hybrids the lowest.

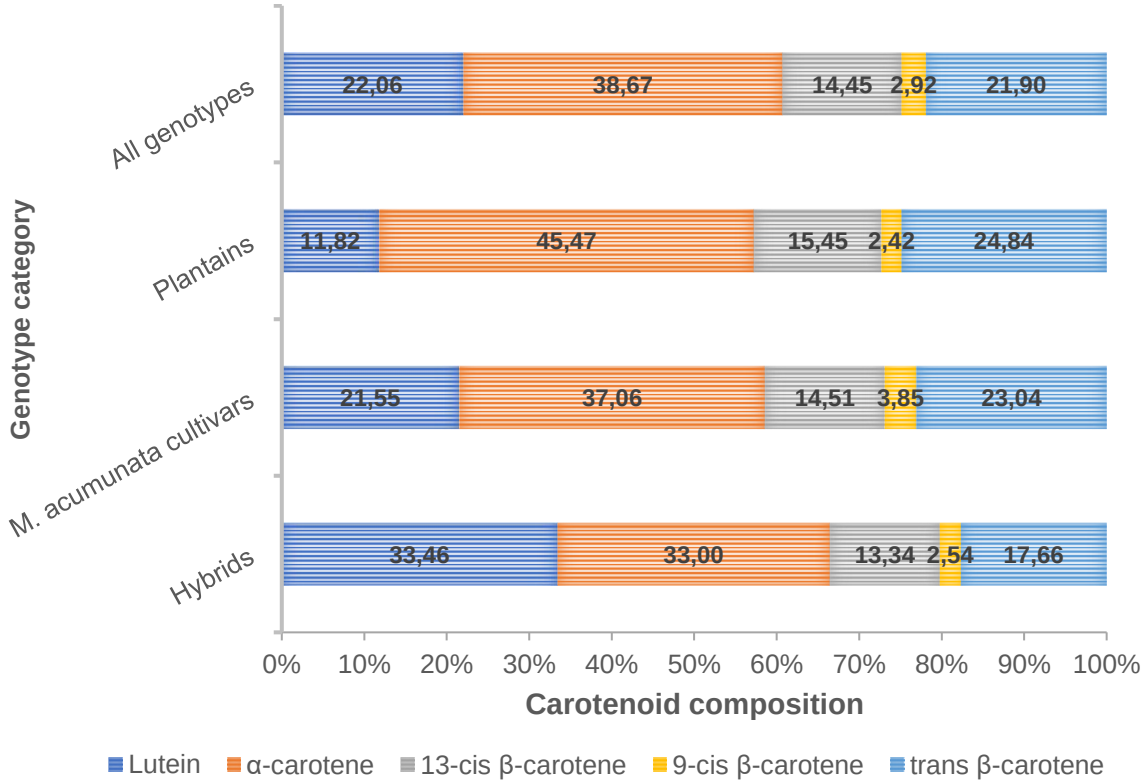
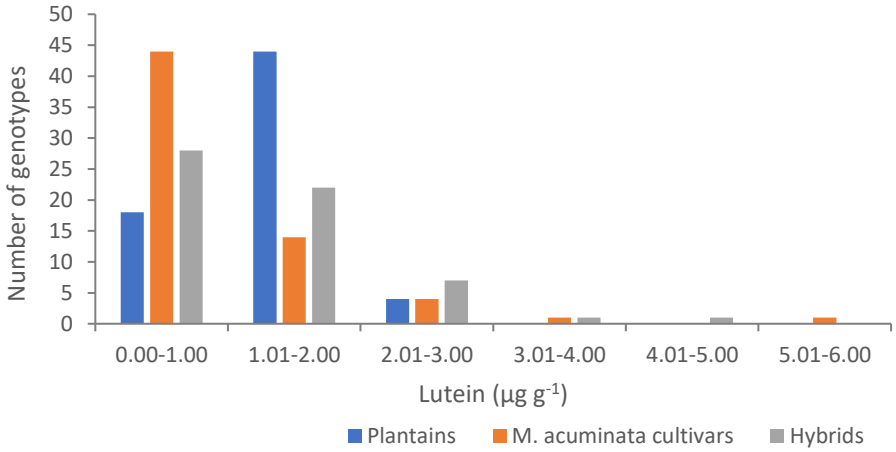


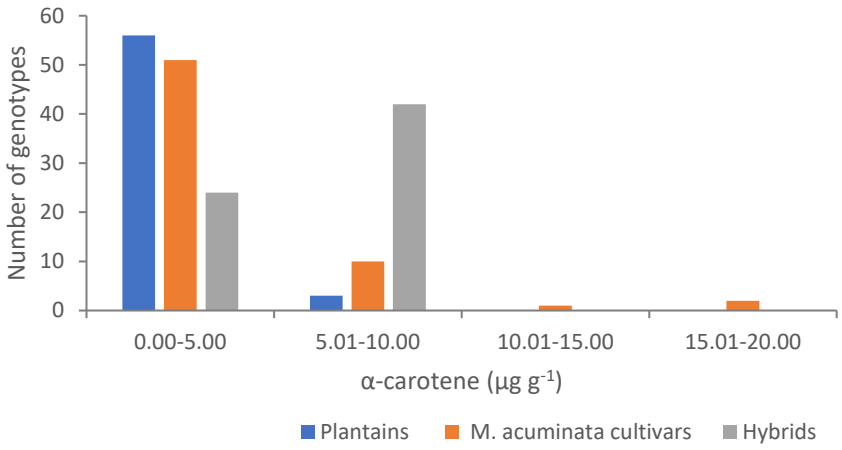
Figure 3.2 Carotenoid composition of 189 banana genotypes.

Frequency distributions for individual carotenoid concentrations and BCE in all banana genotypes are presented in Figure 3.3. All plantain genotypes, 62 *M. acuminata* cultivars and 57 hybrids, had mean lutein concentrations of 0.00-3.00 µg g^{-1} FW while only four genotypes (two cultivars and two hybrids) had concentrations of 3.01-6.00 (Figure 3.3A). The two cultivars richest in lutein were ITC.1552 Ndyali (3.65 µg g^{-1} FW) and ITC.0601 Hung Tu (5.91 µg g^{-1} FW) while the two hybrids were 30804 (3.20 µg g^{-1} FW) and hybrid 30456-1 (4.03 µg g^{-1} FW). The highest lutein concentration in plantain (2.33 µg g^{-1} FW) recorded for ITC.1131 Moto Ebanga, was only 40% of what was recorded for Hung Tu, the accession with the highest value.

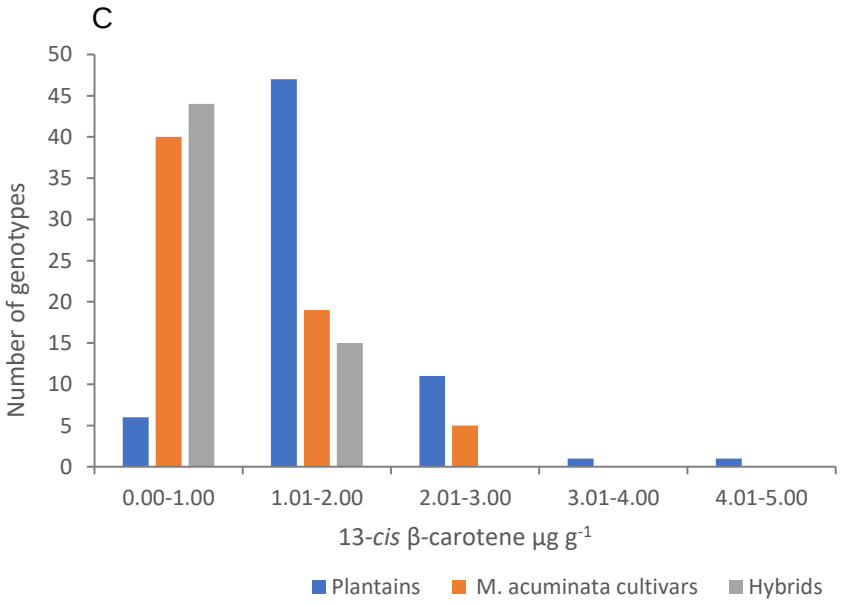
A


Number of genotypes
50
45
40
35
30
25
20
15
10
5
0
0.00-1.00
1.01-2.00
2.01-3.00
3.01-4.00
4.01-5.00
5.01-6.00
Lutein (µg g-1)
Plantains
M. acuminata cultivars
Hybrids

B


Number of genotypes
60
50
40
30
20
10
0
0.00-5.00
5.01-10.00
10.01-15.00
15.01-20.00
α-carotene (µg g-1)
Plantains
M. acuminata cultivars
Hybrids

C


Number of genotypes
50
45
40
35
30
25
20
15
10
5
0
0.00-1.00
1.01-2.00
2.01-3.00
3.01-4.00
4.01-5.00
13-cis β-carotene µg g-1
Plantains
M. acuminata cultivars
Hybrids

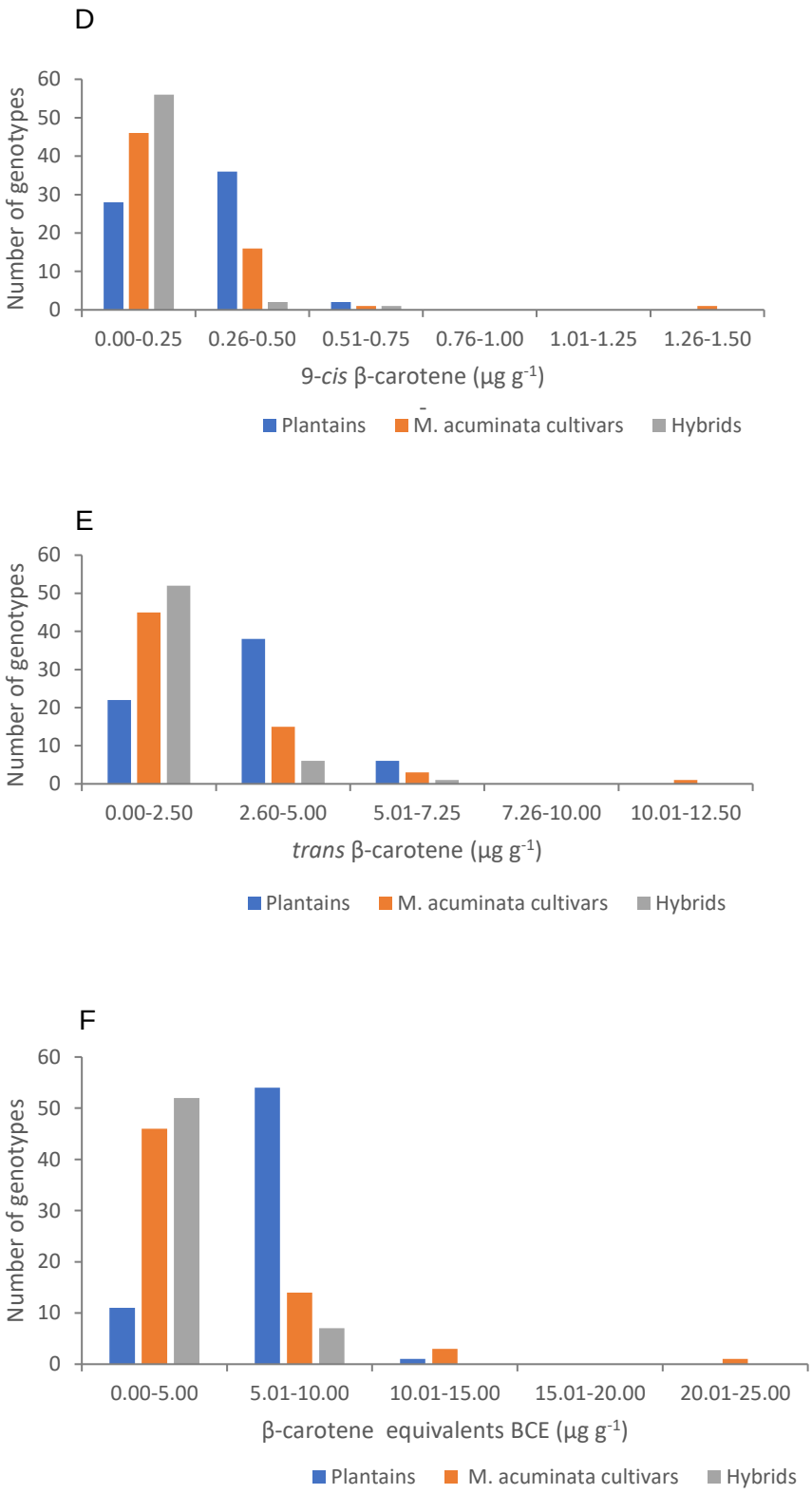


Figure 3.3 **Frequency distributions for carotenoids determined by HPLC in 189 banana genotypes:** lutein (A); α-carotene (B); 13-*cis* β-carotene (C); 9-*cis* β-carotene (D); *trans* β-carotene (E); and BCE (F)**.**

Sixty-one cultivars had α-carotene concentrations from 0.26-9.97 µg g^{-1} FW (Figure 3.3B) while the three cultivars with the highest concentrations (ITC.0809 Maleb, ITC.0894 Tainga and ITC.0601 Hung Tu) had values of 10.74, 15.88 and 16.51 µg g^{-1} FW, respectively. All plantains and hybrids had α-carotene concentrations of 0.00-10.00 ug g^{-1} FW, with the richest plantain accessions ITC.0109 Obino L'Ewai and ITC.0496 Cantebalon having values of 8.14 and 8.19 µg g^{-1} FW, respectively, while the richest hybrid, 33448-2, had a value of 7.84 µg g^{-1} FW.

Fifty-one plantains had mean *trans* β-carotene concentrations of 10.01-20.00 µg g^{-1} FW while only seven plantains had values of 0.00-10.00 µg g^{-1} FW (Figure 3.3E). Contrarily, 50 cultivars and 54 hybrids had β-carotene concentrations of 0.00-10.00 while 14 cultivars and five hybrids had concentrations of 10.01-20.00 µg g^{-1} FW. Sixty cultivars, 65 plantains and all 59 hybrids had BCE of 0.00-10.00 µg g^{-1} FW while four cultivars and one plantain had values of 10.01-25.00 µg g^{-1} FW (Figure 3.3F). With regards to the *cis* isomers of β-carotene, all cultivars and hybrids and most plantains had 13-*cis* β-carotene concentrations ranging from 2.10-3.0 µg g^{-1} with two plantain genotypes, ITC.0022 Mololou (3.26 µg g^{-1} FW) and ITC.0325 Wine Plantain (4.43 µg g^{-1} FW) recording the highest values (Figure 3.3C). All cultivars, plantains and hybrids had 9-*cis* β-carotene values less than 0.75 µg g^{-1} FW while the highest value (1.5 µg g^{-1} FW) was recorded for the cultivar ITC.0809 Maleb (Figure 3.3D).

From the distribution of TC as determined by spectrophotometry (Figure 3.4), 59 plantains had TC levels of 10.01-20.00 µg g^{-1} FW while only six had values of 0.00-10.00 µg g^{-1} FW. Conversely, 50 cultivars and 54 hybrids had TC levels of 0.00-10.00 with 14 cultivars within the 10.00-35.00 µg g^{-1} FW range and five hybrids in the 10.00-20.00 µg g^{-1} FW range. Notably, the cultivar ITC.0601 Hung Tu with the highest BCE, consistently had the highest values for all carotenoids except the *cis* isomers of β-carotene.

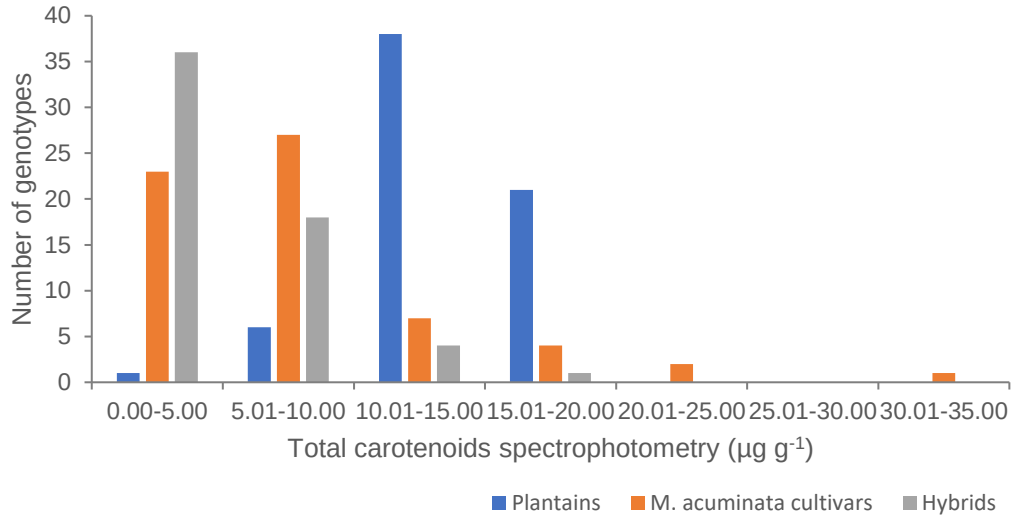


Figure 3.4 Frequency distribution for total carotenoid content determined by spectrophotometry in 189 banana genotypes.

3.3.1.2 Carotenoid content and profiles of fruit pulp of 66 plantain accessions

The mean carotenoid content determined by HPLC and spectrophotometry for 66 diverse plantains analysed is summarized in Table 3.2. The mean and range of carotenoid content determined by HPLC and spectrophotometry for the entire collection of plantains as well as by plantain bunch type is summarized in Table 3.3. The most abundant carotenoid in plantains were α-carotene (5.44 µg g^{-1} FW) and *trans* β-carotene (3.11 µg g^{-1} FW) closely followed by 13-*cis* β-carotene (1.67 µg g^{-1} FW) and lutein (1.24 µg g^{-1} FW) while the least abundant was 9-*cis* β-carotene (0.28 µg g^{-1} FW). This trend was the same for French, False Horn and Horn type plantains with the most abundant carotenoid being α-carotene and the least abundant 9-*cis* β-carotene.

Comparably, mean BCE (µg g^{-1} FW) for French, False Horn and Horn types was 6.89, 6.78 and 6.47, respectively. Although the French type plantains had higher values for all individual carotenoids, there was no significant difference in the means for all three plantain types (Table 3.3). However, the top accessions in terms of BCE content (µg g^{-1} FW) for French, False Horn and Horn types were ITC.0112 BobbyTannap (10.65), ITC.0208 Atali Kiogo (9.74) and ITC.0128 Tshambunu (9.26), respectively (Table 3.2).

In contrast, mean TC obtained from spectrophotometry was 14.05 ,12.86 and 12.41 µg g^{-1} FW for French, False Horn and Horn types respectively, with a significant difference in the means of the French and Horn types (Table 3.3). Top accessions for TC (ug g^{-1} FW) were ITC.0112 BobbyTannap (18.71), ITC.0517 Orishele (16.70), and ITC.0224 75.19S (15.05) (Table 3.2).

Table 3.2 Mean carotenoid content of individual plantain cultivars

Genotype	Type	n	Carotenoid content (µg g^{-1})								
			Lutein	α-caroten	13-*cis* BC	9-*cis* BC	*trans* BC	pVACs	BCE	TC HPLC	TC spec
Dwarf French Plantain	F	3	2.29	2.97	2.03	0.13	2.87	8.00	5.44	10.29	6.96
French Reversion	F	5	1.30	6.04	2.09	0.34	2.75	11.23	6.99	12.53	17.59
ITC.0022 Mulolou	F	4	1.67	4.52	3.26	0.53	0.88	9.19	5.03	10.85	14.07
ITC.0028 Nazika	F	5	1.45	6.46	1.90	0.26	1.86	10.48	6.17	11.92	13.85
ITC.0033 Bungaoisan	F	5	1.00	4.81	1.48	0.23	2.84	9.36	6.10	10.37	13.39
ITC.0054 Diby 2	F	3	1.82	5.64	1.62	0.28	4.50	12.04	8.27	13.86	12.76
ITC.0075 Gabon 4	F	3	2.04	4.30	1.27	0.21	3.88	9.67	6.78	11.71	14.84
ITC.0103 Moungeli	F	4	1.09	3.60	1.06	0.22	0.96	6.38	3.67	7.47	13.36
ITC.0109 Obino L'Ewai	F	4	1.36	8.14	1.86	0.35	3.66	14.01	8.83	15.37	17.31
ITC.0112 BobbyTannap	F	5	0.54	7.18	0.71	0.49	6.46	14.83	10.65	15.38	18.71
ITC.0142 Msisa	F	4	1.01	6.22	1.76	0.31	3.57	11.86	7.72	12.87	15.41
ITC.0203 Niabng	F	4	1.68	5.76	1.96	0.27	3.78	11.77	7.77	13.45	13.69
ITC.0206 Lifongo Liko	F	4	0.67	5.44	1.18	0.67	3.98	11.27	7.62	11.93	11.82
ITC.0215 Mbi Egome 1	F	3	1.50	5.33	1.49	0.27	3.84	10.94	7.39	12.45	15.92
ITC.0219 Apem Pa	F	4	1.14	3.80	1.27	0.18	2.69	7.93	5.31	9.07	11.43
ITC.0231 Apem Onniaba	F	2	0.91	5.35	1.52	0.23	2.22	9.32	5.77	10.23	15.62
ITC.0232 Egjoga	F	5	1.08	6.69	1.97	0.32	3.12	12.10	7.61	13.18	13.55
ITC.0282 Banane Serpent	F	4	1.31	6.19	2.01	0.38	3.80	12.38	8.09	13.69	14.37
ITC.0324 Red Plantain Hembra	F	4	0.91	3.53	0.46	0.13	2.28	6.40	4.34	7.31	5.75
ITC.0325 Wine Plantain	F	4	1.61	5.75	4.43	0.32	3.47	13.97	8.72	15.58	18.40
ITC.0389 Zue Ekon	F	2	1.19	5.23	0.88	0.18	2.79	9.08	5.94	10.27	10.98
ITC.0487 Ntanga 3	F	5	1.72	6.55	1.58	0.14	2.90	11.16	7.03	12.88	17.08
ITC.0496 Cantebalon	F	5	1.45	8.19	2.34	0.40	3.60	14.53	9.06	15.98	16.86
ITC.0499 Obubit Ntanga 2	F	5	0.90	3.35	1.11	0.25	2.82	7.54	5.18	8.43	10.19
ITC.0511 Nselouka	F	5	1.72	6.64	2.23	0.26	2.77	11.9	7.34	13.62	15.53
ITC.0519 Obubit Ntanga GM	F	4	0.95	7.85	1.29	0.26	5.24	14.65	9.95	15.6	15.03
ITC.0635 Dominico Macho	F	3	1.21	5.18	1.98	0.22	1.02	8.41	4.72	9.62	13.73
ITC.0636 Dominico 500	F	2	1.70	6.42	1.14	0.15	2.37	10.08	6.23	11.78	15.50
ITC.0964 Ovang	F	2	1.65	1.61	2.24	0.08	0.23	4.15	2.19	5.80	11.88
ITC.1397 French Reversion Red	F	4	1.25	7.75	1.69	0.34	4.73	14.5	9.62	15.75	17.07
Obubit Ntanga 1	F	5	1.03	4.67	1.47	0.22	1.64	7.99	4.82	9.03	12.38
Purple Plantain	F	5	0.93	5.51	1.76	0.21	1.78	9.26	5.52	10.18	13.20
Red Plantain	F	2	2.21	0.88	0.78	0.13	1.11	2.91	2.01	5.12	3.83

Walungu 8	F	5	1.23	7.24	1.76	0.34	4.29	13.62	8.95	14.85	15.60
ITC.0015 Essang	FH	5	1.01	5.18	1.45	0.35	4.17	11.15	7.66	12.16	12.04
ITC.0017 Gabon 2	FH	4	1.47	3.87	2.58	0.31	2.97	9.73	6.35	11.20	14.20
ITC.0041 Didiedi	FH	4	1.14	6.14	1.54	0.29	2.78	10.74	6.76	11.89	14.13
ITC.0044 Niangafelo	FH	5	1.25	5.73	1.21	0.24	3.91	11.09	7.50	12.34	12.33
ITC.0098 Baka	FH	4	0.94	5.31	1.05	0.24	3.62	10.23	6.93	11.17	11.93
ITC.0111 Agbagba	FH	5	1.22	4.11	2.14	0.29	2.21	8.75	5.48	9.97	12.54
ITC.0208 Atali Kiogo	FH	4	0.80	7.28	1.47	0.38	5.17	14.30	9.74	15.10	14.65
ITC.0209 Bise Egome	FH	5	1.21	3.43	2.57	0.18	1.06	7.24	4.15	8.45	13.41
ITC.0223 Apantu	FH	5	0.98	6.42	1.51	0.37	5.11	13.42	9.27	14.4	15.94
ITC.0229 Abomienu	FH	4	0.96	6.54	1.65	0.36	3.91	12.45	8.18	13.41	16.33
ITC.0233 Mbirinyong	FH	4	0.99	5.34	1.32	0.22	4.07	10.94	7.5	11.94	15.55
ITC.0236 Ngok Egome	FH	4	0.45	2.66	0.86	0.13	1.92	5.56	3.74	6.01	9.08
ITC.0235 Obubit Ukom	FH	5	1.44	4.92	1.56	0.26	2.50	9.25	5.88	10.69	12.83
ITC.0489 Mimi Abue	FH	4	1.60	5.16	1.83	0.28	3.92	11.19	7.55	12.78	11.89
ITC.0515 Okoyo Ukom	FH	3	0.82	6.47	1.76	0.36	5.18	13.77	9.47	14.6	12.34
ITC.0516 Eberedia Ukom	FH	5	1.20	4.51	1.41	0.24	2.22	8.38	5.30	9.57	10.47
ITC.0517 Orishele	FH	5	1.08	7.30	2.06	0.35	2.95	12.64	7.80	13.72	16.70
ITC.0559 Curare Enano	FH	4	1.27	5.00	1.71	0.19	2.33	9.22	5.77	10.49	11.34
ITC.0628 Harton Maqueo	FH	5	0.98	6.85	1.63	0.30	3.07	11.85	7.46	12.84	12.59
ITC.0630 Dominico Harton Rojo	FH	3	1.16	7.38	1.80	0.37	4.80	14.34	9.57	15.50	14.58
ITC.0641 Dominico Rojo	FH	4	1.27	6.09	1.81	0.30	3.72	11.92	7.82	13.18	12.07
ITC.0642 Harton Tigre	FH	5	1.35	6.57	1.83	0.33	3.86	12.59	8.22	13.93	15.72
ITC.0645 Platano Harton	FH	4	0.96	2.37	1.10	0.14	0.65	4.27	2.46	5.23	11.90
ITC.0965 Bo Ahiu-Abue	FH	4	1.16	6.38	1.47	0.30	3.87	12.01	7.94	13.18	15.17
ITC.1129 Big Ebanga	FH	3	1.96	5.28	2.27	0.27	1.38	9.20	5.29	11.16	8.98
ITC.1131 Moto Ebanba	FH	5	2.33	3.31	1.25	0.27	2.87	7.70	5.29	10.03	7.27
MOO9	FH	5	1.21	4.14	1.69	0.27	2.32	8.41	5.36	9.62	10.89
A0 157	FH	5	1.03	3.27	1.59	0.19	1.59	6.64	4.11	7.67	10.62
ITC.0185 3 Hand Planty	H	5	0.92	4.88	1.64	0.24	1.62	8.39	5.00	9.30	11.89
ITC.0121 Ihitism	H	2	1.08	4.98	0.56	0.23	4.14	9.91	7.02	10.99	9.13
ITC.0128 Tshanbunu	H	5	1.03	6.37	1.26	0.32	5.28	13.24	9.26	14.28	14.44
ITC.0224 75.19S	H	3	1.60	5.74	1.42	0.23	4.15	11.54	7.84	13.14	15.05
Standard deviation			0.63	2.29	0.95	0.16	1.93	3.92	2.80	4.01	3.73
Standard error (*N* = 270)			0.04	0.14	0.06	0.01	0.12	0.24	0.17	0.24	0.23

n = number of samples; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACs = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; F = French; FH = False Horn; H = Horn.

Table 3.3 Mean and range of carotenoids in 66 plantain cultivars

Trait	Carotenoid content (µg g^{-1} ± SD)			
	Plantains (n = 270)	French (n = 133)	False Horn (n = 117)	Horn (n = 20)
Mean				
Lutein	1.24±0.63	1.29±0.61	1.20±0.68	1.09±0.38
α-carotene	5.44±2.29	5.64±2.47	5.28±2.17	4.99±1.71
13-*cis* β-carotene	1.67±0.95	1.74±1.04	1.65±0.87	1.39±0.58
9-*cis* β-carotene	0.28±0.16	0.29±0.20	0.28±0.11	0.25±0.10
trans β-carotene	3.11±1.93	3.05±2.00	3.17±1.86	3.16±1.93
Total pVACs	10.50±3.92	10.72±4.24	10.38±3.63	9.79±3.30
BCE	6.81±2.80	6.89±2.99	6.78±2.63	6.47±2.54
TC HPLC	11.74±4.01	12.01±4.32	11.58±3.74	10.88±3.38
TC spec	13.41±3.73	14.05±3.98[a]	12.86±3.41[ab]	12.41±3.04[b]
Range				
Lutein	0.45-2.33	0.54-2.29	0.45-2.33	0.92-1.6
α-carotene	0.88-8.19	0.88-8.19	2.37-7.38	3.27-6.27
13-*cis* β-carotene	0.46-4.43	0.46-4.43	0.86-2.58	0.56-1.14
9-*cis* β-carotene	0.08-0.67	0.08-0.67	0.13-0.38	0.19-0.32
trans β-carotene	0.23-6.46	0.23-6.46	0.65-5.18	1.59-5.28
Total pVACs	2.91-14-83	2.91-14.83	4.27-14.34	6.64-13.24
BCE	2.01-10.65	2.01-10.65	2.46-9.74	4.11-9.26
TC HPLC	5.12-15.98	5.12-15.98	5.23-15.50	7.67-14.28
TC spec	3.83-18.71	3.83-18.71	7.27-16.7	9.13-15.05

SD = standard deviation; n = number of samples; pVACs = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; means with different letters within rows are significantly different at P < 0.5.

3.3.1.3 Carotenoid content and profiles of fruit pulp of 64 diverse *M. acuminata* cultivars

The mean carotenoid content determined by HPLC and spectrophotometry of 64 diverse *M. acuminata* cultivars (247 bunches) analysed is summarised in Table 3.4. The mean and range of carotenoid content determined by HPLC and spectrophotometry for the entire collection of *M. acuminata* cultivars as well as by the different cultivar subgroups/clusters is summarised in Table 3.5. Cultivars are ordered by category: PNG cultivar; AA cv. *banksii;* AA cv. IndonTNg; AA cv. mshare; assorted and unclassified as described in Appendix 2

Table 3.4 Mean carotenoid content of individual *M. acuminata* cultivars

Genotype	Category	N	Carotenoid content (µg g^{-1})								
			Lutein	α-carotene	13-*cis* BC	9-*cis* BC	*trans* BC	pVACs	BCE	TC HPLC	TC Spec
ITC.0078 Wh-o-gu	A	2	0.30	1.71	0.63	0.15	1.40	3.89	2.65	4.19	4.79
ITC.0308 Huwundu Vita	A	2	0.73	2.76	0.52	0.15	3.73	7.15	5.44	7.88	8.87
ITC.0373 Uwati	A	5	1.22	3.10	0.71	0.18	3.05	7.04	5.04	8.26	7.38
ITC.0595 Pagatau	A	5	0.36	1.35	0.50	0.31	0.33	2.48	1.40	2.84	4.41
ITC.0605 Japaraka no.2	A	5	1.08	4.17	0.75	0.08	1.96	6.96	4.46	8.04	8.87
ITC.0777 Pitu	A	4	0.78	2.76	0.96	0.40	2.38	6.49	4.44	7.28	8.64
ITC.0780 Mamakila	A	2	0.72	5.81	2.22	0.34	3.39	11.76	7.58	12.48	12.58
ITC.0792 Niukin	A	2	0.87	1.57	0.40	0.16	2.60	4.73	3.67	5.60	5.17
ITC.0796 Kirkirnan	A	4	0.64	6.44	2.08	0.25	1.99	10.76	6.37	11.39	11.09
ITC.0834 Kungor	A	5	0.60	1.12	0.59	0.09	0.68	2.48	1.58	3.09	5.38
ITC.0838 Bega	A	4	0.26	8.41	2.78	0.27	3.62	15.08	9.35	15.34	21.06
ITC.0868 Porapora	A	5	0.53	2.51	0.88	0.22	1.81	5.43	3.62	5.95	8.16
ITC.0920 Dimaemamosi	A	4	1.24	8.15	1.26	0.39	5.62	15.41	10.52	16.65	15.39
ITC.0932 Gilasalasa	A	4	0.31	8.56	0.93	0.27	4.94	14.70	9.82	15.01	17.96
ITC.0989 Tagomor	A	2	0.49	2.59	1.22	0.11	1.37	5.29	3.33	5.77	9.40
ITC.1011 Kospuke	A	5	0.87	2.48	1.11	0.14	0.75	4.48	2.61	5.35	7.35
ITC.1019 Pongani	A	2	0.63	2.56	1.06	0.10	0.17	3.88	2.02	4.51	7.47
ITC.1214 Terema	A	5	0.91	1.06	0.57	0.11	0.65	2.39	1.52	3.30	5.43
ITC.0266 Sowmuk	B	5	0.54	2.56	0.59	0.12	1.28	4.55	2.92	5.09	7.13
ITC.0269 Niyarma Yik	B	4	0.55	5.14	1.55	0.35	2.71	9.75	6.23	10.29	13.16
ITC.0471 Bebek	B	2	0.13	3.51	1.61	0.20	4.44	9.76	7.10	9.90	11.14
ITC.0600 Waimara	B	2	0.44	8.19	1.17	0.27	3.88	13.52	8.7	13.95	14.89
ITC.0809 Maleb	B	5	0.24	10.74	2.79	1.50	7.11	22.14	14.63	22.38	21.57
ITC.0810 Sihir	B	2	0.28	5.65	1.76	0.18	1.15	8.74	4.94	9.02	13.26
ITC.0849 Sepi	B	2	1.17	0.60	0.25	0.24	0.82	1.91	1.36	3.08	2.84
ITC.0882 Kwosriake	B	2	1.33	1.69	1.52	0.25	0.21	3.67	1.94	5.00	4.72
ITC.0888 Wikago	B	5	1.52	2.78	0.70	0.10	2.04	5.62	3.83	7.14	7.81
ITC.0894 Tainga	B	2	0.26	15.88	1.54	0.34	5.81	23.58	14.69	23.84	16.21
ITC.1187 Tomolo	B	4	0.54	9.97	1.35	0.23	3.75	15.30	9.52	15.83	19.03
ITC.0259 Galeo	C	4	0.78	1.71	0.66	0.08	1.34	3.80	2.57	4.58	6.58
ITC.0294 Pitu	C	3	0.92	2.46	1.04	0.14	0.89	4.53	2.71	5.44	6.64

ITC.0298 Beram	C	2	1.11	0.36	0.34	0.19	0.36	1.24	0.80	2.36	2.70
ITC.0601 Hung Tu	C	4	5.91	16.51	1.72	0.26	11.81	30.30	21.06	36.21	32.03
ITC.0612 Mambee Thu	C	3	0.39	0.86	0.18	0.42	0.98	2.45	1.72	2.83	3.35
ITC.0884 Awondaeke	C	5	0.88	2.56	0.97	0.10	0.98	4.59	2.79	5.47	7.07
ITC.0939 Fu Des	C	5	0.46	5.07	1.51	0.26	4.05	10.90	7.48	11.36	13.82
ITC.1452 Huti (Shumba	D	5	2.45	0.61	0.25	0.27	0.76	1.90	1.33	4.35	3.08
ITC.1454 Makyughu I	D	2	1.84	0.26	0.59	0.08	0.21	1.13	0.67	2.97	1.29
ITC.1456 Huti RB	D	4	2.16	1.25	0.66	0.15	0.36	2.42	1.39	4.59	3.02
ITC.1468 Kahuti	D	5	0.99	0.87	0.39	0.05	0.18	1.49	0.83	2.48	2.50
ITC.1544 Mlelembo	D	4	0.53	0.92	0.40	0.08	0.28	1.68	0.98	2.21	3.34
ITC.1552 Ndyali	D	4	3.65	0.49	0.32	0.17	0.36	1.35	0.86	5.00	2.21
ITC.1559 Huti green bell	D	4	1.02	0.59	0.25	0.09	0.45	1.39	0.92	2.41	3.02
ITC.1561 Makyughu 2-	D	5	0.97	1.29	0.52	0.13	0.33	2.27	1.30	3.23	2.41
ITC.0299 Guyod	E	5	0.54	0.70	0.34	0.03	1.21	2.27	1.74	2.81	3.18
ITC.0317 Umbarim	E	3	2.17	1.99	0.92	0.06	0.62	3.59	2.11	5.75	6.32
ITC.0587 Pisang Rajah	E	2	1.50	4.30	1.00	0.22	2.95	8.48	5.72	9.98	8.25
ITC.0712 AAcv Rose	E	5	0.61	0.64	0.18	0.06	0.64	1.52	1.08	2.13	2.02
ITC.1121 Pisang Lilin	E	4	1.43	0.44	0.25	0.06	0.25	1.00	0.63	2.44	2.87
ITC.1371 Chuoi cau	E	5	1.54	2.41	1.50	0.50	1.01	5.42	3.22	6.95	8.04
ITC.1150 Morong	E	5	2.41	0.77	0.42	0.38	1.69	3.26	2.47	5.66	3.38
ITC.0276 Pisang Madu	F	3	0.35	0.37	0.27	0.05	0.52	1.21	0.86	1.56	3.22
ITC.0279 Bie Yeng	F	5	0.29	2.73	1.07	0.60	3.00	7.41	5.20	7.69	8.30
ITC.0409 Pa Patthalong	F	5	0.60	0.55	0.35	0.06	0.40	1.36	0.88	1.95	2.84
ITC.0413 No. 110	F	5	0.88	0.30	0.21	0.13	0.25	0.89	0.57	1.77	2.80
ITC.0432 Pamoti-On	F	4	0.58	2.56	2.25	0.26	1.33	6.41	3.87	6.99	9.67
ITC.0442 Gu Nin Chio	F	4	0.81	0.91	0.20	0.09	0.10	1.29	0.70	2.10	1.28
ITC.0460 Padri	F	5	0.83	3.46	0.96	0.10	1.15	5.67	3.41	6.50	6.04
ITC.0507 Pisang Madu	F	5	0.53	2.15	0.76	0.12	3.85	6.89	5.37	7.41	9.28
ITC.0532 Khai	F	5	1.21	2.37	1.03	0.17	0.99	4.57	2.78	5.78	7.62
ITC.0663 Khai Nai On	F	2	0.75	1.46	1.03	0.14	2.59	5.22	3.91	5.98	9.47
ITC.0678 Pisang Jeran	F	5	0.43	2.99	1.40	0.16	1.24	5.79	3.52	6.22	8.17
ITC.0695 Pisang	F	4	0.98	1.36	0.80	0.15	1.32	3.64	2.48	4.62	4.05
Morong Princesa	F	5	1.50	1.09	0.66	0.15	1.11	3.02	2.06	4.52	5.65
Standard deviation			1.40	3.54	0.81	0.31	2.33	6.09	4.11	6.42	6.11
Standard error (N = 247)			0.09	0.23	0.05	0.02	0.15	0.39	0.26	0.41	0.39

N= number of samples; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; A = PNG cultivar, B = AA cv. *banksii*, C = AA cv. IndonTNg; D = AA cv. mshare; E = Assorted; F = Unclassified.

Table 3.5 Mean and range of carotenoids in 64 *M. acuminata* cultivars

Trait	Carotenoid content (µg g^{-1} ± SD)						
	Cultivars all (n = 247)	PNG cultivars (n = 67)	AA cv. *banksii* n = 35)	AA cv. IndonTNg (n = 26)	AA cv. mshare (n = 33)	Assorted (n = 29)	Unclassified (n = 57)
Mean							
lutein	1.01±1.40	0.72±0.57^{b}	0.66±0.93^{b}	1.52±3.07^{a}	1.67±1.85^{a}	1.40±1.09^{a}	0.76±0.51^{b}
α-carotene	3.04±3.54	3.73±3.04^{b}	6.05±4.79^{a}	4.68±5.58^{ab}	0.83±0.64^{c}	1.34±1.30^{c}	1.78±1.33^{c}
13-*cis* β-carotene	0.90±0.81	1.04±0.83^{ab}	1.36±0.95^{a}	1.01±0.75^{ab}	0.41±0.36^{d}	0.62±0.72^{dc}	0.84±0.74^{bc}
9-*cis* β-carotene	0.21±0.31	0.21±0.20^{b}	0.40±0.59^{a}	0.20±0.22^{b}	0.13±0.13^{b}	0.20±0.33^{b}	0.17±0.20^{b}
trans β-carotene	1.87±2.33	2.17±2.28^{bc}	3.16±2.55^{ab}	3.23±4.24^{a}	0.38±0.44^{d}	1.09±0.90^{d}	1.36±1.50^{cd}
Total pVACs	6.03±6.09	7.16±5.11^{b}	10.97±7.85^{a}	9.13±9.93^{ab}	1.75±1.07^{c}	3.24±2.42^{c}	4.16±2.70^{c}
BCE	3.95±4.11	4.66±3.48^{b}	7.07±5.10^{a}	6.18±6.18^{ab}	1.07±0.66^{c}	2.17±1.57^{c}	2.76±1.98^{c}
TC HPLC	7.05±6.42	7.88±5.15^{b}	11.63±7.55^{a}	10.65±12.13^{a}	3.43±2.32^{c}	4.65±2.85^{c}	4.92±2.66^{c}
TC spec	7.78±6.11	9.37±5.351^{b}	12.50±6.64^{a}	11.32±9.87^{ab}	2.69±1.02^{d}	4.48±2.66^{cd}	6.00±3.04^{c}
Range							
lutein	0.13-5.91	0.26-1.24	0.13-1.52	0.39-5.91	0.53-3.65	0.54-2.41	0.29-1.50
α-carotene	0.26-16.51	1.06-8.56	0.60-15.88	0.36-16.51	0.26-1.29	0.44-4.30	0.30-3.46
13-*cis* β-carotene	0.18-2.79	0.40-2.78	0.25-2.79	0.18-1.72	0.25-0.66	0.18-1.50	0.20-2.25
9-*cis* β-carotene	0.03-1.50	0.08-0.40	0.10-1.50	0.08-0.42	0.05-0.27	0.03-0.50	0.05-0.60
trans β-carotene	0.10-11.81	0.17-5.62	0.21-7.11	0.36-11.81	0.18-0.76	0.25-2.95	0.10-3.85
Total pVACs	1.56-36.21	2.84-16.65	3.08-23.84	2.36-36.21	2.21-5.00	2.13-9.98	1.56-7.69
BCE	0.89-30.30	2.39-15.41	1.91-23.58	1.24-30.30	1.13-2.42	1.00-8.48	0.89-7.41
TC HPLC	0.57-21.06	1.40-10.52	1.36-14.69	0.80-21.06	0.67-1.39	0.63-5.72	0.57-5.37
TC spec	1.28-32.03	4.41-21.06	2.84-21.57	2.70-32.03	1.29-3.34	2.02-8.25	1.28-9.67

SD = standard deviation; n = number of samples; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; means with different letters within rows are significantly different at P < 0.5.

The most abundant carotenoids for all *M. acuminata* cultivars were α-carotene (3.04 µg g^1 FW) and *trans* β-carotene (1.87 µg g^{-1} FW) closely followed by lutein (1.01 µg g^{-1} FW) and 13-*cis* β-carotene (0.90 µg g^{-1} FW) while the least abundant was 9-*cis* β-carotene (0.21 µg g^{-1} FW). However, carotenoid composition varied between the different *M. acuminata* cultivar categories, with α-carotene being the most abundant carotenoid in PNG cultivars (3.73 µg g^{-1} FW), AA cv. *banksii* (6.05 µg g^{-1} FW), AA cv IndonTNg (4.68 µg g^{-1} FW) and unclassified (1.78 µg g^{-1} FW) category while lutein was most abundant in the AA cv mshare (1.67 µg g^{-1} FW) and assorted (1.40 µg g^{-1} FW) categories (Table 3.5).

The highest mean BCE (µg g^{-1} FW) was recorded for the AA cv. *banksii* (7.07) while the lowest was recorded for the AA cv. mshare (1.07). Similarly AA cv. *banksii* and AA cv. mshare also ranked highest and lowest for TC estimated by spectrophotometry recording mean values of 12.50 and 2.69 µg g^{-1} FW, respectively. These values were not significantly different from the mean BCE and TC for the AA cv IndonTNg category (6.18 and 11.32 µg g^{-1} FW) but were significantly different from the AA cv. mshare category which recorded the lowest values (Table 3.5). Top accessions for each category in terms of BCE (µg g^{-1} FW) were ITC.0894 Tainga (14.69) and ITC.0809 Maleb (14.63) for the AA cv. *banksii*; ITC.0601 Hung Tu (21.06) for the AA cv. IndonTNg and ITC.0920 Dimaemamosi (10.52) for the PNG cultivar category (Table 3.4). The highest TC content from spectrophotometry (µg g-1 FW) was also recorded for ITC.0809 Maleb (21.57), ITC.0601 Hung Tu (32.03) and ITC.0838 Bega (21.06) in their respective categories.

3.3.1.4 Carotenoid content and profiles of fruit pulp of 59 hybrids

The mean carotenoid content determined by HPLC and spectrophotometry for 59 diverse hybrids (254 bunches) analysed is summarised in Table 3.6. The mean and range of carotenoid content determined by HPLC and spectrophotometry for the entire collection of hybrids as well as by the different ploidy categories is summarised in Table 3.7

The most abundant carotenoid in the hybrids was α-carotene (1.87 µg g^{-1} FW), lutein (1.29 µg g^{-1} FW) and *trans* β-carotene (1.02 µg g^{-1} FW) with smaller amounts of 13-*cis* β-carotene (0.66 µg g^{-1} FW) and 9-*cis* β-carotene (0.12 µg g^{-1} FW). However, with the exception of *cis* β-carotene isomers, varying trends in proportions of carotenoids were observed in diploids, triploids and tetraploid hybrids (Table 3.7). Triploids and tetraploids were richest in α-carotene while diploids were richest in lutein.

Table 3.6 Mean carotenoid content of individual hybrids

Genotype	Ploidy	n	Carotenoid content (µg g^{-1})								
			Lutein	α-carotene	13-*cis* BC	9-*cis* BC	*trans* BC	Total pVACs	BCE	TC HPLC	TC spec
1297-3	2x	2	0.18	3.24	1.01	0.14	3.59	7.98	5.79	8.16	7.65
1448-1	2x	3	0.44	1.67	0.28	0.15	1.27	3.38	2.33	3.82	3.78
25291-1 R10P13	2x	5	0.64	0.77	0.40	0.07	0.59	1.83	1.21	2.47	2.60
25291-1 R1P3	2x	5	1.02	0.56	0.19	0.04	0.29	1.08	0.69	2.10	2.49
25291-1 R3P12	2x	5	0.89	0.78	0.31	0.04	0.50	1.62	1.06	2.52	2.91
25291-1 R7P24	2x	3	0.99	0.31	0.17	0.02	0.46	0.97	0.71	1.95	2.26
25291-1A	2x	5	0.89	0.50	0.20	0.07	0.25	1.02	0.64	1.91	2.57
25291-S26	2x	3	0.83	0.91	0.62	0.05	0.26	1.84	1.05	2.68	2.91
25291-S26 R10P34	2x	5	0.43	0.87	0.20	0.17	0.87	2.11	1.49	2.55	3.22
25291-S4 R6P13	2x	4	1.52	0.44	1.02	0.08	0.70	2.24	1.47	3.76	3.44
25291-S62	2x	2	0.58	3.70	0.97	0.15	0.89	5.70	3.30	6.28	9.03
25291-S89	2x	3	1.01	0.37	0.11	0.02	0.23	0.72	0.48	1.74	2.33
25447-S7	2x	4	1.08	1.43	0.52	0.08	0.83	2.86	1.85	3.94	4.33
25447-S7 R11P10	2x	3	1.44	1.47	0.68	0.05	0.75	2.95	1.85	4.39	3.64
25447-S7 R2P8	2x	5	0.92	0.17	0.09	0.04	0.22	0.53	0.38	1.45	1.76
25447-S7 R3P4	2x	3	0.70	2.16	1.05	0.66	5.10	8.97	7.03	9.67	3.17
25447-S7 R4P11	2x	5	1.18	0.14	0.11	0.02	0.10	0.37	0.24	1.56	1.90
25447-S7 R4P30	2x	5	1.67	0.57	0.19	0.03	0.27	1.06	0.67	2.73	1.90
25447-S7 R5P26(4)	2x	5	2.08	0.56	0.26	0.06	0.31	1.18	0.75	3.26	2.55
25447-S7 R8P27	2x	4	0.73	0.95	0.34	0.07	0.69	2.05	1.37	2.78	4.14
2829-62	2x	2	0.68	2.71	0.72	0.13	2.41	5.98	4.20	6.65	7.19
28401	2x	5	1.56	1.33	0.64	0.08	0.33	2.38	1.35	3.94	4.57
29603	2x	5	1.97	0.77	0.29	0.07	0.50	1.62	1.06	3.59	3.27
29650	2x	4	2.88	2.30	0.94	0.10	0.63	3.96	2.29	6.84	3.36
8075-7	2x	4	2.58	0.80	0.36	0.22	0.36	1.73	1.04	4.31	3.40
9128-3	2x	5	1.49	0.57	0.38	0.07	0.80	1.82	1.31	3.31	2.53
9839-3	2x	4	2.18	0.42	0.44	0.11	0.25	1.22	0.73	3.39	2.16
SH 3142	2x	4	0.88	1.44	0.52	0.08	0.73	2.77	1.75	3.65	4.75
SH 3362	2x	5	0.39	0.79	0.70	0.13	1.05	2.67	1.86	3.06	3.67
24408-S22	3x	5	1.51	0.75	0.57	0.10	0.44	1.86	1.15	3.36	2.85
30456-1	3x	4	4.03	1.73	0.82	0.13	0.94	3.63	2.29	7.66	7.02

30456-2	3x	5	2.10	0.28	0.19	0.05	0.18	0.69	0.43	2.80	1.77
33657-2	3x	5	1.78	1.03	0.59	0.08	0.42	2.11	1.26	3.89	3.54
33657-3	3x	5	1.07	0.46	0.28	0.04	0.11	0.89	0.50	1.95	2.39
PITA 21	3x	5	0.79	4.64	1.39	0.19	1.76	7.99	4.88	8.78	10.13
PITA 22	3x	5	0.78	1.28	0.33	0.09	0.58	2.28	1.43	3.06	3.75
PITA 23	3x	5	0.40	4.36	1.17	0.11	1.12	6.76	3.94	7.15	9.86
PITA 24	3x	5	0.35	1.53	0.70	0.11	0.37	2.71	1.54	3.07	3.95
PITA 25	3x	4	0.60	3.41	1.16	0.17	1.01	5.75	3.38	6.35	7.38
PITA 26	3x	4	1.67	4.52	1.18	0.17	1.60	7.47	4.53	9.13	11.77
PITA 27	3x	5	0.62	2.93	0.89	0.15	1.28	5.24	3.26	5.87	7.39
30456-3	4x	5	0.69	2.19	0.83	0.13	0.92	4.07	2.49	4.76	5.40
30804	4x	5	3.20	2.68	0.77	0.10	0.88	4.43	2.65	7.62	4.05
33448-1	4x	5	0.63	1.77	0.88	0.14	0.49	3.28	1.88	3.91	7.51
33448-2	4x	3	1.79	7.84	1.73	0.22	2.89	12.68	7.78	14.47	16.63
612-74	4x	5	2.27	0.64	0.40	0.07	0.23	1.35	0.79	3.62	2.29
BITA 3	4x	5	0.80	0.74	0.28	0.06	0.64	1.72	1.18	2.52	2.98
PITA 1	4x	4	1.79	5.43	1.15	0.26	2.87	9.71	6.29	11.5	13.2
PITA 2	4x	4	1.88	2.71	1.14	0.11	0.89	4.84	2.87	6.72	7.72
PITA 3	4x	2	2.01	5.08	1.38	0.19	3.32	9.98	6.65	11.99	11.15
PITA 4	4x	5	1.00	2.41	0.53	0.11	1.56	4.61	3.09	5.61	6.54
PITA 5	4x	5	1.47	2.82	1.16	0.15	0.91	5.04	2.98	6.51	8.91
PITA 6	4x	4	0.88	4.89	1.74	0.20	3.74	10.57	7.15	11.44	8.46
PITA 7	4x	5	0.60	2.16	1.21	0.15	0.64	4.16	2.40	4.76	3.12
PITA 8	4x	5	1.73	3.33	0.99	0.10	3.70	8.11	5.91	9.85	8.17
PITA 12	4x	5	0.98	2.26	0.70	0.11	0.94	4.01	2.48	4.99	6.86
PITA 14	4x	5	1.68	4.08	0.65	0.32	1.97	7.02	4.49	8.70	9.42
PITA 17	4x	4	1.45	3.65	0.81	0.15	2.10	6.72	4.41	8.17	9.72
PITA 18	4x	4	1.28	3.11	1.21	0.17	2.27	6.76	4.52	8.04	7.93
Standard deviation			1.05	1.79	0.55	0.13	1.35	3.26	2.22	3.44	3.47
Standard error (N=254)			0.07	0.11	0.03	0.01	0.08	0.20	0.14	0.22	0.22

n= number of samples; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; 2x = diploid; 3x = triploid; 4x = tetraploid.

Table 3.7 Mean and range of carotenoids in 59 banana hybrids

Trait	Carotenoid content (µg g^{-1} ± SD)			
	Hybrids (n = 254)	Diploid (n = 117)	Triploid (n = 57)	Tetraploid (n = 80)
Mean				
Lutein	1.29±1.05	1.22±0.90[a]	1.27±1.33[a]	1.42±1.02[a]
α-carotene	1.87±1.79	0.97±0.95[c]	2.19±1.87[b]	2.98±1.99[a]
13-*cis* β-carotene	0.66±0.55	0.43±0.48[c]	0.76±0.52[b]	0.93±0.53[a]
9-*cis* β-carotene	0.12±0.13	0.09±0.15[b]	0.11±0.07[ab]	0.15±0.11[a]
trans β-carotene	1.02±1.35	0.73±1.21[b]	0.80±0.91[b]	1.59±1.61[a]
Total pVACs	3.67±3.26	2.22±2.21[c]	3.86±3.04[b]	5.64±3.65[a]
BCE	2.34±2.22	1.47±1.64[c]	2.33±1.91[b]	3.62±2.55[a]
TC HPLC	4.96±3.4	3.43±2.29[c]	5.13±3.16[b]	7.06±3.90[a]
TC spec	5.13±3.47	3.28±1.8[c]	5.84±3.84[b]	7.33±3.62[a]
Range				
Lutein	0.18-4.03	0.18-2.88	0.35-4.03	0.60-3.20
α-carotene	0.14-7.84	0.14-3.70	0.28-4.64	0.64-7.84
13-*cis* β-carotene	0.09-1.74	0.09-1.05	0.19-1.39	0.28-1.74
9-*cis* β-carotene	0.02-0.66	0.02-0.66	0.04-0.19	0.06-0.32
trans β-carotene	0.10-5.10	0.10-5.10	0.11-1.76	0.23-3.74
Total pVACs	0.37-12.68	0.37-8.97	0.69-7.99	1.35-12.68
BCE	0.24-7.78	0.24-7.03	0.43-4.88	0.79-7.78
TC HPLC	1.45-14.47	1.45-9.67	1.95-9.13	2.52-14.47
TC spec	1.76-16.63	1.76-9.03	1.77-11.77	2.29-16.63

SD = standard deviation; n = number of samples; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; means with different letters within rows are significantly different at $P < 0.5$; Means with different letters within rows are significantly different at $P < 0.5$.

Mean BCE (µg g^{-1} FW) was 2.34 for all hybrids but was significantly different for diploid (1.47), triploid (2.33) and tetraploid (3.62) hybrids. Similarly, mean values for individual carotenoids varied significantly between the different hybrid groups (Table 3.7). The highest mean BCE content was recorded in the hybrids 25447-S7 R3P4 (7.03), PITA 21 (4.88) and 33448-2 (7.78) in the diploid, triploid and tetraploid hybrid categories, respectively (Table 3.6).

Mean TC (µg g^{-1} FW) obtained from spectrophotometry for all hybrids was 5.13 and varied significantly for the different ploidy categories, with tetraploids having the highest mean value (7.33) and diploids having the lowest (3.28) (Table 3.7). The highest mean TC content was recorded in the hybrids 25291-S62 (9.03), PITA 26 (11.77) and 33448-2 (16.63) in the diploid, triploid and tetraploid hybrid categories, respectively. Mean TC

obtained from HPLC and spectrophotometry was two times higher for tetraploids than for diploids and the trend was similar for individual carotenoids except for lutein (Table 3.7).

3.3.2 Correlations between carotenoids

Pearson correlation coefficients computed for all traits (Table 3.8) revealed highly significant (P<0.0001) positive correlations for individual carotenoids (except lutein), pVACs, BCE and TC. Lutein had a weak non-significant negative correlation with 9-*cis* β-carotene and weak positive non-significant correlations with α-carotene,13- *cis* β-carotene and with *trans* β-carotene. The most potent pVAC *trans* β-carotene was strongly positively correlated with α-carotene (r=0.75, P<0.0001) but only moderately correlated with its *cis* isomers 13-*cis* β-carotene (r=0.28, P<0.0001) and 9-*cis* β-carotene (r=0.48, P<0.0001).

Table 3.8 Pearson correlation coefficients among carotenoids and β-carotene equivalents in banana accessions (n=771)

	α-car	13-*cis* BC	9-*cis* BC	*trans* BC	BCE	pVACS	TC HPLC	TC spec
Lutein	0.09	0.09	-0.06	0.03	0.06	0.08	0.26***	0.02
α-car		0.52***	0.44***	0.75***	0.92***	0.96***	0.94***	0.86***
13-*cis* BC			0.37***	0.28***	0.51***	0.58***	0.58***	0.61***
9-*cis* BC				0.48***	0.53***	0.53***	0.51***	0.41***
trans BC					0.93***	0.88***	0.85***	0.70***
BCE						0.99***	0.97***	0.85***
pVACs							0.98***	0.88***
TC HPLC								0.85***

α-car = α-carotene; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; *** $P < 0.0001$.

Notably, TC estimated by spectrophotometry was highly positively correlated with TC calculated from HPLC (r=0.85, P<0.0001), total pVACs (r=0.88, P<0.0001) and vitamin A content (r=85, P<0.0001). TC from spectrophotometry was also highly positively correlated with α-carotene (r=0.86, P<0.0001), *trans* β-carotene (r=0.70, P<0.0001), 13-*cis* β-carotene (r=0.61, P<0.0001) and only moderately correlated with 9-*cis* β-carotene (r=0.41, P<0.0001).

3.4 Discussion

Identification of genetic variability for a target trait is crucial for crop improvement. This study aimed to evaluate the variability of fruit carotenoid content and profiles in different types of bananas (plantains, *M. acuminata* cultivars and hybrids) and identify high pVAC accessions towards pVA biofortification.

Total carotenoid detected by spectrophotometry varied from 1.28 to 32.03 with a mean of 8.88 µg g^{-1} FW across all 189 banana genotypes analysed, indicating a high variability in carotenoids in bananas. The observed variation in TC is comparable to 2.18-32.44 with a mean of 5.24 µg g^{-1} FW observed by Davey et al. (2009) in 171 diverse banana cultivars. Amorim et al. (2009) also detected a wide variability in TC (1.6-19.24 µg g^{-1}) in 42 diverse cultivars in Brazil. These variations will form the basis for future research strategies aimed at increasing the nutritional value of bananas through crossing and selection. Chávez et al. (2005) reported average TC contents of 2.46 µg g^{-1} ranging from 1.02 to 10.40 µg g^{-1} in 1786 genotypes of cassava obtained from diverse geographical locations. High pVAC genotypes were selected and crossed through several cycles to generate high carotenoid cassava genotypes (Ceballos et al. 2017).

Estimating the concentrations as well as relative proportions of pVACs is critical for adequate quantification of vitamin A content in crop plants (Rodriguez-Amaya 2016). While HPLC is considered the gold standard for carotenoid separation, spectrophotometry is often used as a low-cost method for large-scale screening for crops where pVAC profiles are well known and remain consistent among genotypes (Saini et al. 2015; Rodriguez-Amaya 2016). This study found a strong correlation ($r=0.85$, $P<0.0001$) for TC estimated from HPLC with TC measured by spectrophotometry. This is in line with previous observations from Davey et al. (2006; 2009), confirming the potential of spectrophotometry as a useful, inexpensive method for rapid screening for pVACs in banana. Jones et al. (2013) compared both methods in breadfruit cultivars and observed a lack of correspondence between TC obtained from spectrophotometry and that obtained from HPLC, underscoring the limitations of spectrophotometry to estimate pVACs in breadfruit cultivars.

Among the main carotenoids with vitamin A activity in humans, *trans* β-carotene is the most potent, due to its symmetric nature and ability to be cleaved into two molecules of *trans* retinal (Grune et al. 2010). Isomerization of β-carotene into its *cis* isomers results in loss of pVA activity as cleavage rates are known to be lower in *cis* isomers of β-carotene than *trans* isomers (Breshanan et al. 2014). Therefore, HPLC was used to separate the individual carotenoids present in bananas, while further separating the isomeric forms of β-carotene to estimate the pVACs content as previously described (Taleon et al. 2017; Mbabazi 2015). This study indicated that 78% of the carotenoids isolated in banana are pVACs α-carotene and β-carotene (*cis* and *trans*-versions) with β-carotene dominating in most genotypes. Indeed, contents of α-carotene and *trans* β-carotene were strongly positively correlated ($r = 0.75$; $P<0.0001$) (Table 3.8). The other 22% of the carotenoids isolated comprised of lutein, which lacks pVA activity, but has other health benefits, as

previously mentioned. These results agree with previous studies on carotenoids in banana where the predominant carotenoids isolated were α-carotene and β-carotene with smaller amounts of lutein (Englberger et al. 2003; 2006; Davey et al. 2007; 2009; Ekesa et al. 2015; Heng et al. 2017). In contrast, Pixley et al. (2013) reported that only 10-20% of the carotenoids in maize are pVACs (β-cryptoxanthin, β-carotene and α-carotene) with the non pVACs (lutein and zeaxanthin) comprising about 30-50% of the isolated carotenoids. Carvalho et al. (2016) observed that the main pVACs in cassava were β-carotenes with a range of 26.13 to 76.72% recorded in the roots of 23 cassava landraces in Amazonia. Although variable quantities of lutein were detected, no α-carotenes were detected in the observed genotypes.

Among the three groups analysed, the plantain collection had the highest mean total pVACs, corresponding to the highest BCE (Table 3.1). Relative to other banana groups such as the Cavendish and EAHB, higher pVAC contents in plantains have been documented elsewhere, although on a few genotypes not representing the wide plantain diversity (Davey et al. 2009; Ngoh-Newilah et al. 2009; Heng et al. 2017). The current study assessed more plantain genotypes than previously reported and has shown that over 85% of the carotenoids in plantains are pVACs, indicating their suitability for PVA delivery compared to other bananas. While plantains are grouped into French, False Horn and Horn types with the French types based on their bunch morphology (Adheka et al. 2018), Horn types are least preferred because of their smaller bunch size and weight. The results of the current study indicated that there was no significant difference between the mean pVA content in the three different plantain groups, indicating that high pVAC plantains are evenly distributed among the preferred French and False Horn types. This is interesting, because plantains have better yield and quality attributes preferred by consumers in West-Central Africa, hence high pVAC plantains can be further explored for fast-track delivery to complement existing efforts (Ekesa et al. 2017) while breeding is ongoing for further improvement.

Five French plantains (ITC.0112 BobbyTannap, ITC.0519 Obubit Ntanga GM; ITC.1397 French Reversion Red Pseudostem; ITC.0496 Cantebalon) and four False Horn plantains (ITC.0208 Atali Kiogo, ITC.0630 Dominico Harton Rojo, ITC.0515 Okoyo Ukom and ITC.0223 Apantu) and one Horn plantain (ITC.0128 Tshanmbunu) were identified with a high BCE (>9 μg g^{-1} FW) in the plantain collection and their corresponding TCs were >12. μg g^{-1} FW. The French plantain ITC.0112 BobbyTannap had the highest mean TC from spectrophotometry (18.71 μg g^{-1} FW) and the highest BCE (10.65 μg g^{-1} FW) of all plantain accessions screened and this genotype was analysed in this study for the first time. Heng et al. (2017) also recently reported TC values of 36.82, 11.65 and 8.55 μg g^{-1} FW for

mature fruit pulp from Orishele, Dwarf French plantain and Obubit Ntanga GM respectively. In this study, Orishele had high total pVACs content (16.70 µg g^{-1} FW) estimated by spectrophotometry but the corresponding BCE from HPLC data was 7.80 µg g^{-1} FW. Ekesa et al. (2015) also recorded a high BCE for ripe fruits of Apantu (82.38 µg g^{-1} FW), about 10x higher than what was obtained in the current study. Differences in growth environment, sampling, extraction, separation and quantification procedures could account for such huge cultivar differences between studies (Rodriguez-Amaya 2016; Saini et al. 2015).

Utilisation of introduced germplasm is one way to enhance genetic diversity, which forms the backbone of crop breeding programmes. Among all three banana groups, the *M. acuminata* cultivars showed the widest variation for all carotenoids analysed (Table 3.1) and this is expected, because genotypes in this group belong to various subgroups/clusters with diverse geographical origins (Appendix 2). While most are *M. acuminata* (AA) diploid cultivars, the recent classification by Christelová et al. (2017) further groups them into specific 'clusters' based on cytological and molecular characterisation using simple sequence repeat (SSR) markers. According to this classification, the AA cv*. banksii* (AA cv. *banksii* and *banksii* sensu lato clusters) are diploid AA cultivars of the ssp*. banksii* background with origins from New Guinea. The AA cv. IndonTriNG group also originates from New Guinea, but within the triangle shaped by the Eastern Indonesia islands. Mshares are diploid cooking bananas of the Mlali subgroup found in East Africa and neighbouring islands and suspected to be the second donor for the AAA triploid dessert bananas (Perrier et al. 2009; Hippolyte et al. 2012; Christelová et al. 2017). The genepool for diploid banana cultivars has been successfully exploited for banana improvement with respect to disease resistance and agronomic traits (Brown et al. 2017) but not for nutrient quality. Considering the vital role of diploids in banana breeding schemes and their potential for genetic analysis (Brown et al. 2017; Tenkouano et al. 2011), high pVAC diploids will be useful for future investigations towards plantain breeding.

In the *M. acuminata* cultivars, about 22% of carotenoids was lutein while the rest comprised of pVACs with the proportion of β-carotene (*cis* and *trans*) being higher (41%) than that of α-carotene (37%) (Figure 3.2). Comparatively, the AA cv. *banksii* category had a 5-6 fold higher carotenoid and pVA content than the Mshares. The AA cv. IndonTriNG category also had high carotenoid and pVA content, which was not significantly different from that of the AA cv. *banksii* (Table 3.3). Three diploid cultivars ITC.0601 Hung Tu, ITC.0894 Tainga and ITC.0809 Maleb were identified with vitamin A contents higher than that of the highest plantain cultivar. These and other high content AA cv. *banksii* diploids

have potential for plantain breeding as the ssp. *banksii* are suspected to be the contributors of the A genome in plantains (Perrier et al. 2009; Tenkouano et al. 2011). Cultivars originating from PNG have been reported elsewhere to have high carotenoid contents (Fungo and Pillay 2011; Ngoh-Newilah et al. 2008). The only other banana type reported to have higher carotenoid contents than the high pVAC AA cv. *banksii* and AA cv. IndonTriNG cultivars from PNG are the Fe'i bananas (Englberger et al. 2003; 2006) which belong to the Australimusa (now incorporated in the Eumusa) section of the genus *Musa* (Christelová et al. 2017). However, the Fe'i bananas with their characteristic red sap, erect bunches and deep orange fruit pulp are only cultivated in the Pacific islands and are not relevant for plantain breeding.

More hybrid banana genotypes were evaluated in this study for pVAC contents than previously reported, and these hybrids comprised improved diploid and tetraploid parents as well as advanced triploid and tetraploid lines from diverse backgrounds/parentage (Appendix 3). Hybrids had the lowest mean TC estimated by spectrophotometry with a corresponding low mean BCE estimated from HPLC analysis but had the highest mean lutein content (Table 3.1). Up to 33% of the carotenoids in hybrids was lutein (Figure 3.1) while the rest was pVACs. However, while mean lutein contents were highest in the diploid categories, α-carotene was highest in the triploid and tetraploid categories while 9-*cis* β-carotene was lowest in all categories. Except for lutein, all individual carotenoids measured by HPLC and TC measured by both methods increased significantly with ploidy levels (Table 3.7). This may be explained by the predominance of relatively high pVAC plantains Bobby Tannap, Obino L'Ewai and Mbi Egome in the pedigrees of the triploid and tetraploid hybrids analysed (Appendix 3). These three plantain accessions have been used as female (grand) parents in existing plantain breeding schemes owing to their ability to produce seeds from crosses with diploids (Tenkouano et al. 2011).

The tetraploids were selected for high yield and Sigatoka resistance from primary crosses made with plantains and the Sigatoka resistant wild diploid Calcutta 4 (Vuylsteke et al. 1993). Triploids were selected from subsequent crosses made from primary tetraploids and improved diploids (Tenkouano and Swennen 2004). Out of these, two triploid (PITA 21 and PITA 26) and two tetraploid plantain hybrids (PITA 1, and PITA 3) were identified with TC contents >10 and BCE >4 μg g^{-1} FW (Table 3.6). Hybrids generally have low pVAC content because parents were not selected with a focus for pVAC enhancement. The fact that relatively high pVAC PITA hybrids were derived from crosses made with plantains highlights the need to develop strategies which accumulate high pVAC alleles in the diploid background for breeding more nutritious plantains.

Maize and cassava are well known staples where breeding advancements have been made on carotenoid enhancement following the identification of pVAC dense genetic resources (Pixley et al. 2013; Ceballos et al. 2017; Manjeru et al. 2017). Availability of next generation sequencing technologies and the existence of high density genotyping platforms have facilitated the development and incorporation of new breeding tools like MAS to increasing breeding efficiency. QTL mapping has been used to study the DNA regions related to carotenoid accumulation. Identified candidate genes responsible for carotenoid regulation and genome-wide association studies (GWAS) have also been employed in combination with genotyping by sequencing (GBS) to identify trait linked allelic variations valuable for MAS in both crops (Suwarno et al. 2015; Esuma et al. 2016; Rabbi et al. 2017). Until now, banana breeding has been a long and complex process relying on interploidy crosses and phenotypic selection (Brown et al. 2017). MAS has not been effectively deployed for banana improvement but GS based on prediction models is currently being explored with high predictive values for fruit and bunch traits and it is expected that its application will speed up the breeding process and increase genetic gains for useful traits like carotenoids (Nyine et al. 2018). However, it should be noted that genetic gains in carotenoid content should not compromise agronomic performance, productivity and other desired end-use traits to guarantee widespread farmer acceptance of biofortified crops.

3.5 Conclusions

The enrichment of staple crops with essential micronutrients through biofortification is gaining importance as a sustainable means of addressing micronutrient deficiencies. Bananas are an important staple in tropical regions worldwide and their potential as a source of vitamin A was investigated for use in conventional breeding or biofortification programmes. This study has revealed new sources of variability for pVAC in bananas, which are useful for further studies and establishment of biofortification strategies. In this context, high pVAC plantains and hybrids will be useful for further research towards fast-track delivery, while high pVAC AA diploid cultivars will be explored as parents for breeding and genetic analysis of pVAC accumulation in banana, which is currently not known. However, the high variation detected within and among cultivars tested call for further analysis with consistent replications in multiple locations over multiple years to elucidate heritable genetic variation which is relevant for breeding.

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CHAPTER 4

Variation in carotenoid contents during fruit ripening in plantain cultivars

4.1 Introduction

The presence of micronutrients in staple foods is relevant for human nutrition and health. Plantain (*Musa* spp. AAB) is an important staple fruit crop for millions of people worldwide. Africa accounts for about 50% of world plantain production with the main growing region located in West-Central Africa spanning from Guinea to the DRC and Central African Republic. Ghana, Nigeria, Ivory Coast and Cameroon are the top four producing countries with an annual production of over 1.5 MT (FAOSTAT 2017). In these countries, plantain ranks among the top three starchy staples and is principally grown by small scale farmers for home consumption and local trade. Their starchy fruits are consumed when boiled/steamed, fried or roasted at various stages of maturity. A smaller proportion is processed into flour, which is used as a component in other culinary products. Plantains are giant perennial herbs which are generally sterile and develop seedless fruits without pollination. Propagation is typically through suckers or side shoots originating from lateral buds at the base of the main plant.

Cultivated plantains are triploid interspecific hybrids with a complex genome (AAB), derived from two ancestral diploid species *M. acuminata* (AA) and *M. balbisiana* (BB) which originate from South East Asia (Heslop-Harrison and Schwarzacher, 2007). West and Central Africa harbor the greatest variability, hence are known to be secondary centers of diversity for plantains with DRC harboring the largest diversity (De Langhe et al. 2005). Approximately 120 known plantain cultivars exist and cultivar classification based on inflorescence morphology at maturity revealed three main plantain types, namely the French, False Horn and Horn (Adheka et al. 2018) (Figure 4.1). French plantains are characterised by the presence of a complete inflorescence with the presence of a male bud while False Horn plantains are characterised by an incomplete florescence with the male bud degenerating before maturity and Horn plantains have no male inflorescence. According to Adheka et al. (2018) the lack of suitable taxonomic descriptors and the existence of location specific (vernacular) names for the same variety cause complications in distinguishing cultivars.

Figure 4.1 Bunch appearance of the three main plantain types. French: Mbi Egome (A), False Horn: Agbagba (B), Horn: Tsambunu (C).

The fruits are rich in vitamins, minerals and other health related bioactive compounds (Robinson and Sauco 2010; Tsamo et al. 2015) which contribute to its quality and nutritional value. Their orange-yellow fruit pulp is a source of carotenoids, which are important for human nutrition (Davey et al. 2007; Ngoh-Newilah et al. 2008; 2009; Heng et al. 2017). Carotenoids are isoprenoid compounds, which play essential roles in plants, serving as light harvesting pigments in photosynthesis and as precursors of plant volatiles and hormones. These compounds are implicated in human nutrition and health, serving as antioxidants and as precursors for vitamin A (Fraser and Bramley 2004; Cazzonelli and Pogson 2010). Carotenoids with pVA activity are normally found in plant tissues and the most abundant pVACs include α-carotene, β-carotene and β-cryptoxanthin (Saini et al. 2015). These compounds can produce one or two retinal molecules, which are the structural basis for vitamin A molecules upon cleavage. Other carotenoids, like the xanthophyll lutein, is also important for health as antioxidants and as components of macular pigment implicated in eye health (Britton 2008). The growing importance of plant-based carotenoids (carotenes and xanthophylls) has inspired efforts aimed at assessing their contents in staple food crops (Saini et al. 2015). Micronutrient concentrations in raw foods, as well as bioavailability in the food matrix and degradation or breakdown, has a consequence on the final micronutrient intake from consumed foods.

Plantain fruits are normally harvested when mature and either processed immediately for consumption or stored (for a few days to several weeks), before processing at various stages of ripeness. Changes in carotenoid content and composition observed during ripening in fruits are influenced by type of tissue, developmental stage and environmental conditions and such changes are highly variable among cultivars (Arscott 2013; Lado et al. 2016). Considering that vitamin A intake from consumed foods depends on the concentrations in raw foods, it is important to determine the appropriate maturity/ripeness

stage of fruits with the highest amount of pVACs to obtain optimum health benefits. Carotenoid composition and profiles during ripening or storage have been investigated in various plant species, with different patterns/tendencies observed among species and even among cultivars of the same species (Lado et al. 2016). Specifically, studies on *Musa* spp., a fleshy fruit with a relatively simple carotenoid profile comprising of mainly α-carotene, β-carotene and lutein, have recorded variable cultivar-dependent quantitative and qualitative changes during ripening, with carotenoids either increasing, decreasing or remaining the same (Ngoh-Newilah et al. 2009; Ekesa et al. 2013; 2015; Lokesh et al. 2014; Mbabazi 2015). However, reports specific to plantains are limited to a few cultivars obtained from different locations and analysed in the same laboratory, thereby not being representative of the vast plantain diversity in Africa.

Thus, the aim of this study was to systematically evaluate the carotenoid composition and profiles of representative accessions within the three types of plantains at three stages of ripeness (unripe, ripe and overripe). The focus was to identify the best stage of post-harvest ripening for maximising pVAC bioavailability in plantains.

4.2 Materials and methods

4.2.1 Cultivars and sampling site

A total of nine plantain cultivars were used in this study, comprising of three cultivars representative of each of the three plantain types (Table 4.1). Plantain bunches were obtained from the banana breeding plots at the IITA Ibadan research station between November 2016 and February 2018. This research station is located at 3° 54' East and 7° 30 ' North at 240 m above sea level, in the sub humid-derived savanna agro-ecology where the soil type is predominantly ferric luvisols. The rainfall pattern is bimodal, with two distinct rainy seasons, the first from April to July and the second from August to November. All plants were grown under standard field conditions at a spacing of 3 x 2 m and each cultivar was represented by a minimum of five plants.

4.2.2 Sampling and carotenoid quantification

The unripe, ripe and overripe stages were determined by the colour of fruit peels, corresponding to ripening stages 1, 5 and 8 previously described by Dadzie and Orchard (1997). Unripe fruits had green fruit peels while ripe fruits had completely yellow fruit peel with green tips (Figure 4.2). Overripe fruits had yellow peels with big brown/black patches due to senescence of fruit peel tissue. These three stages represent the stages at which plantains are normally processed for consumption.

Table 4.1 Cultivar characteristics of nine plantain cultivars used for carotenoid evaluation during ripening

Cultivar name	Plantain type	Origin	BWT	DAH R	DAH OR
ITC.0112 Bobby Tannap (BT)	French	Cameroon	14.0±4.4	4.4±2.3	7.6±2.9
ITC.0215 Mbi Egome (ME)	French	Nigeria	14.0±1.2	3.4±0.9	6.4±0.6
ITC.0109 Obino L'Ewai (OL)	French	Nigeria	14.0±1.0	3.8±1.8	6.6±1.1
ITC.0111 Agbagba (Ag)	False Horn	Nigeria	9.0±3.4	6.4±3.0	6.9±1.8
ITC.1129 Big Ebanga (BE)	False Horn	Cameroon	11.8±1.5	3.2±2.0	10.6±3.0
ITC.0517 Orishele (Or)	False Horn	Nigeria	9.0±1.6	4.4±0.9	6.6±0.9
ITC.0185 Three Hand Planty (THP)	Horn	Cameroon	7.4±2.3	4.6±3.7	10.2±2.0
ITC.0224 7519S	Horn	Nigeria	8.0±1.9	6.0±4.0	9.4±4.3
ITC.0128 Tshambunu (Ts)	Horn	Burundi	10.0±1.4	2.8±0.8	7.0±0.7

BWT = bunch weight; DAH R = days after harvest for ripe fruit; DAH OR = days after harvest for overripe fruits; values are expressed as means ± standard deviation.

Figure 4.2 Unripe (A), ripe (B) and overripe (C) fruits of the plantain cultivar Big Ebanga.

For all cultivars, bunches were harvested at maturity when ripening was observed at the first hand of the bunch. Two fruits at the unripe stage were sampled at harvest. Bunches were then kept in a dark room at ambient temperature for ripening, after which two fruits were sampled at the ripe and overripe stages, respectively. Except for Horn plantains with a single hand, all fruits sampled were obtained from the second hand of the bunch. Sample preparation, and carotenoid quantification through HPLC and spectrophotometry were carried out as described in Chapter 3 section 3.2.4.

4.2.3 Data analysis

Results were recorded in µg g^{-1} FW and each value was a mean of duplicate samples from the two fruits analysed. Statistical analysis was carried out using SAS version 9.4 for Windows Copyright © 2002-2015 by SAS Institute Inc., Cary, NC, USA. The PROC GLM statement was used for one-way ANOVA followed by Duncan's Multiple Range Rest to detect significant differences among means while the PROC CORR statement was used for Pearson correlation analysis.

4.3 Results

4.3.1 Carotenoid content and profiles of plantain cultivars at the unripe, ripe and overripe stage

The main carotenoids detected by HPLC in all plantain cultivars at all ripening stages were α-carotene and *trans* β-carotene with smaller amounts of lutein, 13-*cis*-β-carotene and 9-*cis* β-carotene (Table 4.2). Total carotenoids recorded from spectrophotometry (TC spec) for plantain cultivars at the unripe stage ranged from 11.06-23.18 with a mean of 16.94 µg g^{-1} FW. Generally, the carotenoid content of green fruits was dominated by α-carotene with a mean of 9.39 µg g^{-1} FW representing 47.59% of the TC content. This was closely followed by *trans* β-carotene with a mean of 8.05 µg g^{-1} FW representing 40.80%, with smaller quantities of 13-*cis* β-carotene with a mean of 1.49 µg g^{-1} FW representing 7.55%, lutein with a mean of 0.47 µg g^{-1} FW representing 2.38% and minor amounts of 9-*cis* β-carotene 0.33 µg g^{-1} FW representing 1.67% of the TC (Figure 4.3; Table 4.2). Beta carotene equivalents for unripe plantains ranged from 8.64-19.62 with a mean of 13.65 µg g^{-1} FW. High TC spec accessions were Obino l'Ewai (23.18 µg g^{-1}), Mbi Egome (22.05 µg g^{-1}) and Bobby Tannap (20.44 µg g^{-1}) while Tsambunu (11.06 µg g^{-1}) had the lowest content. The highest BCE values (µg g^{-1} FW) were recorded for Obino L'Ewai (19.62), Bobby Tannap (18.19) and Orishele (16.8) while the lowest values were recorded for 75 19s (8.64) and Three Hand Planty (9.38) (Table 4.2).

Ripe plantains had lower TC spec contents ranging from 8.84-15.06 with a mean of 11.98 µg g^{-1} FW. Carotenoid content was still dominated by α-carotene (46.17%) followed by *trans* β-carotene (24.48%) with equal proportions of lutein and 13-*cis* β-carotene (3.64% each) and minor amounts of 9-*cis* β-carotene (2.12%) corresponding to mean values of 5.79, 3.07, 1.71, 1.71 and 0.26 µg g^{-1} FW respectively (Figure 4.3; Table 4.2). BCE content in ripe fruits ranged from 4.66-10.22 with a mean of 6.95 µg g^{-1} FW. TC spec values were highest for Obino L'Ewai (15.06 µg g^{-1}) Mbi Egome (15.00 µg g^{-1}) and Orishele (13.42 µg g^{-1}) and lowest for Tsambunu (8.84 µg g^{-1}) (Table 4.2).

Table 4.2 Carotenoid content and profiles in the fruit pulp of nine plantain cultivars

Cultivar	B type	R stage	Carotenoid content (µg g^{-1} FW)								
			Lutein	α-carotene	13-*cis* BC	9-*cis* BC	*trans* BC	pVACs	BCE	TC HPLC	TC Spec
	F	Ur	0.30±0.12^{b}	13.21±6.26^{a}	1.84±1.79^{a}	0.44±0.30^{a}	10.44±3.42^{a}	25.93±11.19^{a}	18.19±7.18^{a}	26.23±11.27^{a}	20.44±2.13^{a}
BT		R	2.21±1.45^{a}	7.22±3.14^{b}	1.23±0.47^{a}	0.32±0.18^{a}	5.02±2.50^{b}	13.79±6.07^{b}	9.41±4.25^{b}	16.00±7.34^{ab}	12.16±2.14^{b}
		Or	2.56±1.34^{a}	4.53±2.26^{b}	1.05±0.61^{a}	0.18±0.07^{a}	2.61±1.38^{b}	8.38±4.14^{b}	5.50±2.73^{b}	10.94±5.10^{b}	8.15±3.70^{c}
	F	Ur	0.19±0.09^{b}	9.34±2.16^{a}	1.33±1.07^{a}	0.30±0.20^{a}	8.19±1.70^{a}	19.16±2.97^{a}	13.68±1.83^{a}	19.36±2.94^{a}	22.05±1.70^{a}
ME		R	2.33±1.15^{a}	6.62±3.00^{a}	1.35±0.76^{a}	0.25±0.18^{a}	2.64±2.18^{b}	10.87±4.86^{b}	6.75±3.41^{b}	13.20±5.84^{a}	15.00±1.81^{b}
		Or	1.74±1.16^{a}	7.27±3.79^{a}	1.57±0.86^{a}	0.24±0.16^{a}	4.36±2.82^{b}	13.44±7.02^{ab}	8.90±4.86^{ab}	15.18±7.50^{a}	11.86±2.97^{c}
	F	Ur	0.35±0.21^{b}	13.63±4.81^{a}	1.27±0.71^{a}	0.44±0.28^{a}	11.96±6.60^{a}	27.29±11.71^{a}	19.62±9.09^{a}	27.64±11.8a	23.18±1.49^{a}
OL		R	2.14±1.03^{a}	7.87±4.15^{ab}	0.99±0.57^{a}	0.24±0.16^{a}	3.25±4.12^{b}	12.34±8.71^{b}	7.79±6.37^{b}	14.48±9.70^{ab}	15.06±2.77^{b}
		Or	2.03±1.27^{a}	6.06±3.60^{b}	1.34±0.58^{a}	0.29±0.15^{a}	3.28±3.41^{b}	10.96±7.46^{b}	7.12±5.41^{b}	12.99±8.56^{b}	13.47±3.73^{b}
	FH	Ur	0.27±0.21^{a}	6.66±6.88^{a}	0.39±0.17^{b}	0.25±0.15^{a}	7.02±3.82^{a}	14.32±10.28^{a}	10.67±6.95^{a}	14.59±10.48^{a}	15.24±3.79^{a}
Ag		R	0.43±0.74^{a}	3.48±2.03^{a}	1.37±0.38^{a}	0.30±0.29^{a}	2.08±2.01^{b}	7.23±4.26^{a}	4.66±3.11^{ab}	7.66±4.98^{a}	10.41±2.81^{ab}
		Or	0.43±0.66^{a}	2.93±2.14^{a}	0.74±0.39^{b}	0.15±0.06^{a}	1.17±0.44^{b}	5.00±2.41^{a}	3.09±1.32^{b}	5.42±2.90^{a}	9.14±3.69^{b}
	FH	Ur	1.08±1.86^{a}	8.92±5.00^{a}	1.02±1.11^{a}	0.21±0.20^{a}	8.24±3.86^{a}	18.39±10.05^{a}	13.31±6.95^{a}	19.46±10.35^{a}	17.18±3.96^{a}
BE		R	1.40±0.49^{a}	5.71±0.86^{ab}	1.13±0.85^{a}	0.16±0.07^{a}	2.57±1.10^{b}	9.57±2.07^{b}	6.07±1.48^{b}	10.97±2.24^{ab}	12.45±1.11^{b}
		Or	1.38±0.54^{a}	3.95±1.45^{b}	0.77±0.47^{a}	0.13±0.04^{a}	1.81±1.87^{b}	6.66±2.89^{b}	4.23±2.32^{b}	8.04±2.77^{b}	10.60±2.36^{b}
	FH	Ur	0.85±0.62^{a}	13.16±5.70^{a}	1.92±1.36^{a}	0.52±0.29^{a}	9.00±4.90^{a}	24.60±10.81^{a}	16.80±7.61^{a}	25.45±10.27^{a}	18.38±3.50^{a}
Or		R	1.72±1.25^{a}	7.84±2.76^{ab}	1.93±1.28^{a}	0.34±0.13^{a}	5.16±1.45^{a}	15.27±3.45^{ab}	10.22±1.64^{ab}	16.99±4.52^{ab}	13.42±1.82^{b}
		Or	0.99±1.11^{a}	5.66±1.03^{b}	1.20±0.75^{a}	0.29±0.07^{a}	4.14±3.14^{a}	11.30±3.59^{b}	7.72±3.32^{b}	12.29±3.57^{b}	12.56±1.45^{b}
	FH	Ur	0.16±0.06^{b}	4.80±4.06^{a}	2.48±2.16^{a}	0.26±0.09^{a}	5.61±1.89^{a}	13.15±4.26^{a}	9.38±3.06^{a}	13.32±4.30^{a}	11.12±3.41^{a}
THP		R	1.38±0.39^{a}	4.58±4.67^{a}	3.55±1.81^{a}	0.26±0.13^{a}	2.05±2.58^{b}	10.44±7.52^{ab}	6.25±5.01^{ab}	11.82±7.78^{a}	10.74±4.05^{a}
		Or	1.84±0.60^{a}	1.74±0.41^{a}	1.44±1.46^{a}	0.15±0.08^{a}	0.64±0.41^{b}	3.96±1.96^{b}	2.30±1.10^{b}	5.80±2.19^{a}	7.83±1.47^{a}
	FH	Ur	0.76±0.83^{a}	6.12±4.88^{a}	1.68±1.60^{a}	0.25±0.08^{a}	4.61±1.37^{a}	12.67±4.68^{a}	8.64±2.69^{a}	13.43±4.82^{a}	14.10±6.51^{a}
75		R	1.52±0.56^{a}	3.79±2.63^{a}	1.60±1.12^{a}	0.21±0.11^{a}	3.47±4.96^{a}	9.06±7.38^{a}	6.26±6.16^{a}	10.58±7.21^{a}	10.07±4.79^{a}
		Or	1.03±0.75^{a}	2.41±2.54^{a}	1.16±0.95^{a}	0.17±0.11^{a}	1.63±2.18^{a}	5.37±5.11^{a}	3.50±3.64^{a}	6.40±4.67^{a}	9.35±3.68^{a}

Ts	FH	Ur	0.31±0.04 [b]	9.41±3.46 [a]	1.55±0.57 [a]	0.30±0.06 [a]	7.60±4.85 [a]	18.86±7.98 [a]	13.23±6.40 [a]	19.17±7.99 [a]	11.06±1.81 [a]
		R	2.27±0.69 [a]	5.38±3.17[b]	2.26±1.12 [a]	0.27±0.17 [a]	1.79±1.48 [b]	9.71±5.84[b]	5.75±3.64 [b]	11.97±6.48 [ab]	8.84±2.25 [a]
		Or	2.73±0.82 [a]	3.66±1.88 [b]	1.64±0.63 [a]	0.14±0.09 [a]	1.07±1.45[b]	6.51±3.65 [b]	3.79±2.50 [b]	9.24±4.01 [b]	8.83±1.72 [a]
All cultivars		Ur	0.47±0.72[b]	9.39±5.46[a]	1.49±1.31[a] [b]	0.33±0.21[a]	8.05±4.15[a]	19.26±9.48[a]	13.65±6.69[a]	19.72±9.52[a]	16.94±5.32[a]
		R	1.71±1.02[a]	5.79±3.23[b]	1.71±1.20[a]	0.26±0.16[ab]	3.07±2.77[b]	10.82±5.87[b]	6.95±4.21[b]	12.53±6.54[b]	11.98±3.33[b]
		OR	1.70±1.11[a]	4.21±2.80[b]	1.23±0.79[b]	0.19±0.11[b]	2.24±2.29[b]	7.86±5.26[c]	5.05±3.72[b]	9.56±5.68[c]	10.11±3.27[c]

BT = ITC.0112 Bobby Tannap; ME = ITC.0215 Mbi Egome; OL = ITC.0109 Obino L'Ewai; Ag = ITC.0111 Agbagba; BE = ITC.1129 Big Ebanga; OR = ITC.0517 Orishele; THP = ITC.0185 Three Hand Planty 7 = ITC.0224 75 19S; Ts = ITC.0128 Tshambunu; F = French; FH = False Horn; H = Horn; Ur = unripe; R = ripe; Or = overripe; B type = Bunch type; R stage = Ripening stage; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; Values represent means ± standard deviation (n=5, except for Orishele and Agbaba where n= 3 or 4); means with the same letter within the column for each cultivar are not significantly different from each other at p<0.05.

Table 4.3. Carotenoid content (µg g^{-1} FW) and profiles of French, False Horn and Horn plantain cultivars at different ripening stages

Traits	French plantains			False Horn plantains			Horn plantains		
	Ur (n=15)	R (n=15)	Or (n=15)	Ur (n=14)	R (n=14)	Or (n=12)	Ur (n=15)	R (n=15)	Or (n=15)
Lutein	0.28±0.15[d]	2.23±1.13[a]	2.11±1.22[a]	0.72±1.14[cd]	1.15±0.96[bc]	0.97±0.80[cd]	0.41±0.52[d]	1.72±0.66[ab]	1.87±0.99[a]
α-carotene	12.06±4.81[a]	7.24±3.25[bc]	5.96±3.26[cd]	9.32±6.09[ab]	5.52±2.55[cde]	4.04±1.84[de]	6.78±4.36[bcd]	4.58±3.39[cde]	2.60±1.89[e]
13-*cis* BC	1.48±1.21[bc]	1.19±0.59[bc]	1.32±0.68[bc]	1.05±1.10[bc]	1.44±0.87[bc]	0.87±0.51[c]	1.91±1.53[ab]	2.47±1.54[a]	1.41±1.01[bc]
9-*cis* BC	0.39±0.25[a]	0.27±0.17[abc]	0.24±0.13[bc]	0.32±0.24[ab]	0.26±0.20[abc]	0.18±0.09[bc]	0.27±0.08[abc]	0.25±0.13[bc]	0.15±0.09[c]
Trans BC	10.19±4.38[a]	3.64±3.01[c]	3.41±2.59[c]	8.02±3.91[ab]	3.14±1.98[c]	2.18±2.14[c]	5.94±3.15[b]	2.44±3.18[c]	1.11±1.48[c]
PVACs	24.13±9.54[a]	12.33±6.36[cd]	10.93±6.28[cd]	18.71±10.43[b]	10.36±4.59[cde]	7.26±3.67[de]	14.9±6.17[bc]	9.74±6.47[cde]	5.28±3.68[e]
BCE	17.16±6.8[a]	7.98±4.62[cd]	7.17±4.39[cd]	13.37±7.04[b]	6.75±3.14[cde]	4.72±2.82[de]	10.42±4.56[bc]	6.09±4.68[de]	3.20±2.52[e]
TC HPLC	24.41±9.62[a]	14.56±7.31[bc]	13.04±6.90[cd]	19.43±10.54[ab]	11.51±5.38[cde]	8.23±3.84[de]	15.30±6.18[bc]	11.46±6.68[cde]	7.15±3.82[e]
TC spec	21.89±2.03[a]	14.07±2.53[c]	11.16±3.96d[e]	16.83±3.72[b]	12.00±2.29[cd]	10.6±2.82[de]	12.09±4.3[cd]	9.88±3.66[de]	8.67±2.40[e]

Ur, unripe; R, ripe; Or, overripe. 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; Means with the same letter within the same row are not significantly different at p<0.05.

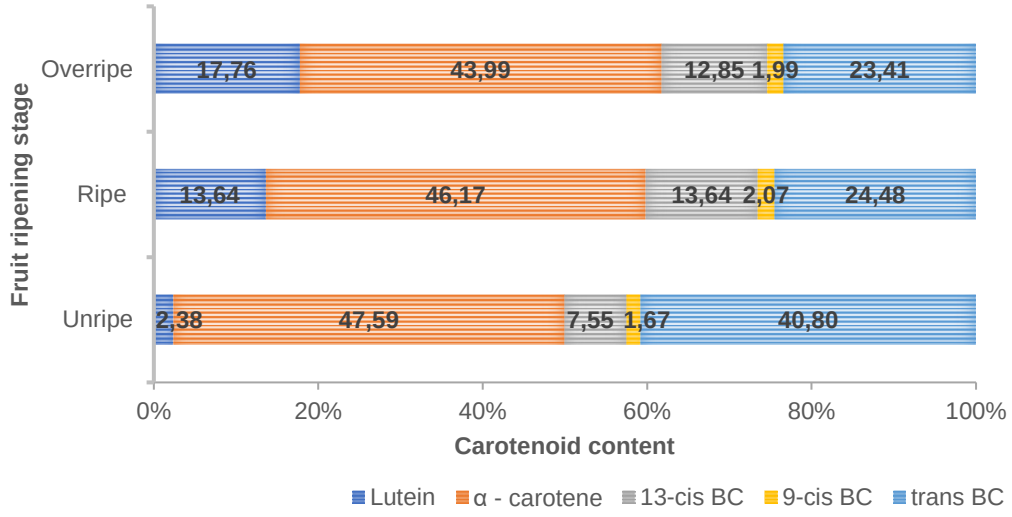

Figure 4.3 Relative carotenoid composition in plantain cultivars at different ripening stages. Data presented is the mean of at least three bunches sampled from all nine cultivars (unripe, n=44; ripe n=44; and overripe, n=42).

BCE values (µg g^{-1} FW) were highest for Orishele (10.22), Bobby Tannap (9.41) and Obino L'Ewai (7.79) and lowest for Agbagba (4.66) and Tsambunu (5.75) (Table 4.2).

For overripe plantain fruits, TC spec values ranged from 7.83-13.47 with a mean of 10.11 µg g^{-1} FW (Table 4.2). The main carotenoid in overripe fruits was α-carotene (43.99%) followed by *trans* β-carotene (23.41%), lutein (17.66%), 13-*cis* β-carotene (12.85%) and 9-*cis*-β-carotene (1.99%) corresponding to mean values of 4.21, 2.24, 1.70, 1.23 and 0.19 µg g^{-1} FW respectively (Figure 4.3; Table 4.2). BCE content in overripe fruits ranged from 2.30 to 8.90 with a mean of 5.05 µg g^{-1} FW. TC spec values in overripe fruits were highest for Mbi Egome (11.86 µg g^{-1}), Obino L'Ewai (13.47 µg g^{-1}), Orishele (12.56 µg g^{-1}) and lowest for HP (7.83 µg g^{-1}) and Tsambunu (8.83 µg g^{-1}). Similarly, BCE values (µg g^{-1} FW) were highest for Mbi Egome (8.9), Orishele (7.72) and Obino L'Ewai (7.12) and lowest for Three Hand Planty (2.3) and Agbagba (3.09) (Table 4.2).

Among the three plantain types, the French plantains had significantly higher ($p>0.05$) TC contents than the False Horn and Horn types at all ripening stages (Table 4.3). Similarly, the pVACs and BCE were significantly higher for the French than for False Horn and Horn, and plantains at all three ripening stages. This trend was the same for *trans* β-carotene and α-carotene but different for lutein. The French plantains had the highest contents only at the ripe and overripe stages while the False Horn plantains had the highest lutein content at the green stage (Table 4.3).

4.3.2 The influence of ripening state on carotenoid contents and profiles of plantain cultivars

Figure 4.4 summarises the changes in all carotenoids at different ripening stages. Overall, pVACs content decreased from the unripe to the ripe stages, with the most drastic pVAC β-carotene recording up to 62% and 72% loss at the ripe and overripe stage respectively. Similarly, there was a 38% and 55% decrease in α-carotene content at the ripe and overripe stages. Levels of 9-*cis*-β-carotene decreased by 21% and 42% at ripe and overripe stage but 13-*cis* β-carotene levels increased by 15% at the ripe stage and decreased by 17% at the overripe stage.

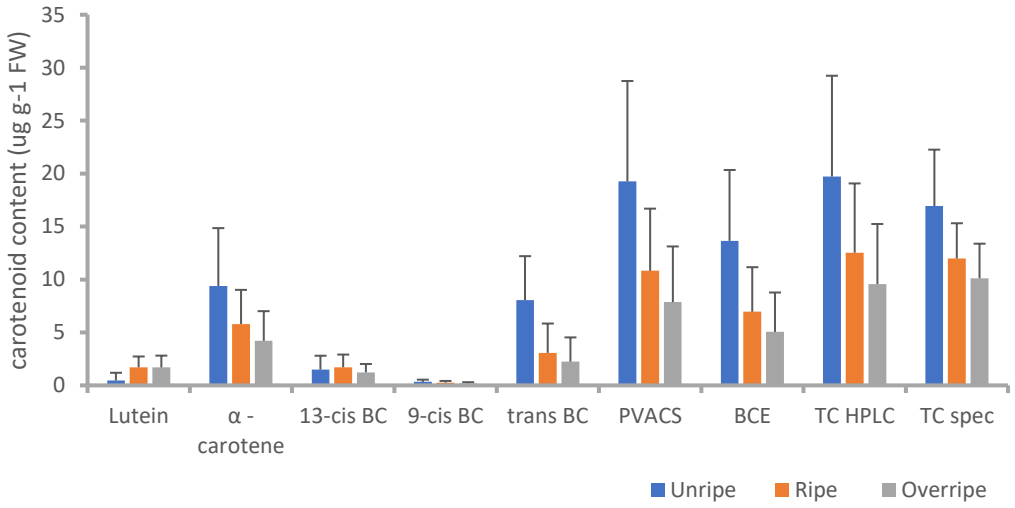

Figure 4.4 Carotenoid composition of fruit pulp of nine plantain cultivars across three ripening stages. 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry.

This decreasing trend was also reflected in pVA content with a 41% and 37% loss in BCE at the ripe and overripe stages. Changes in BCE contents from the unripe to the ripe stage were statistically significant (P>0.05) for all plantain types (Table 4.2). Conversely, contents of the non-pVAC lutein increased (up to four-fold) from the unripe to the ripe and overripe stages. Spectrophotometry results also indicated that TC contents decreased by 29% and 40% at the ripe and overripe stages. All three plantain types recorded a decrease in pVACs at the ripe (35-49%) and overripe (55-65%) stages. The trend was similar for TC with the French plantains recording relatively higher losses than the False Horn and Horn types (Table 4.3).

Changes in the percent composition and profiles of individual carotenoids with ripening for each of the nine plantain cultivars sampled is indicated in Figure 4.5. All cultivars showed

an increase in accumulation of lutein during ripening except ME and OR which later recorded reduced content as ripening progressed from the ripe to the overripe stages.

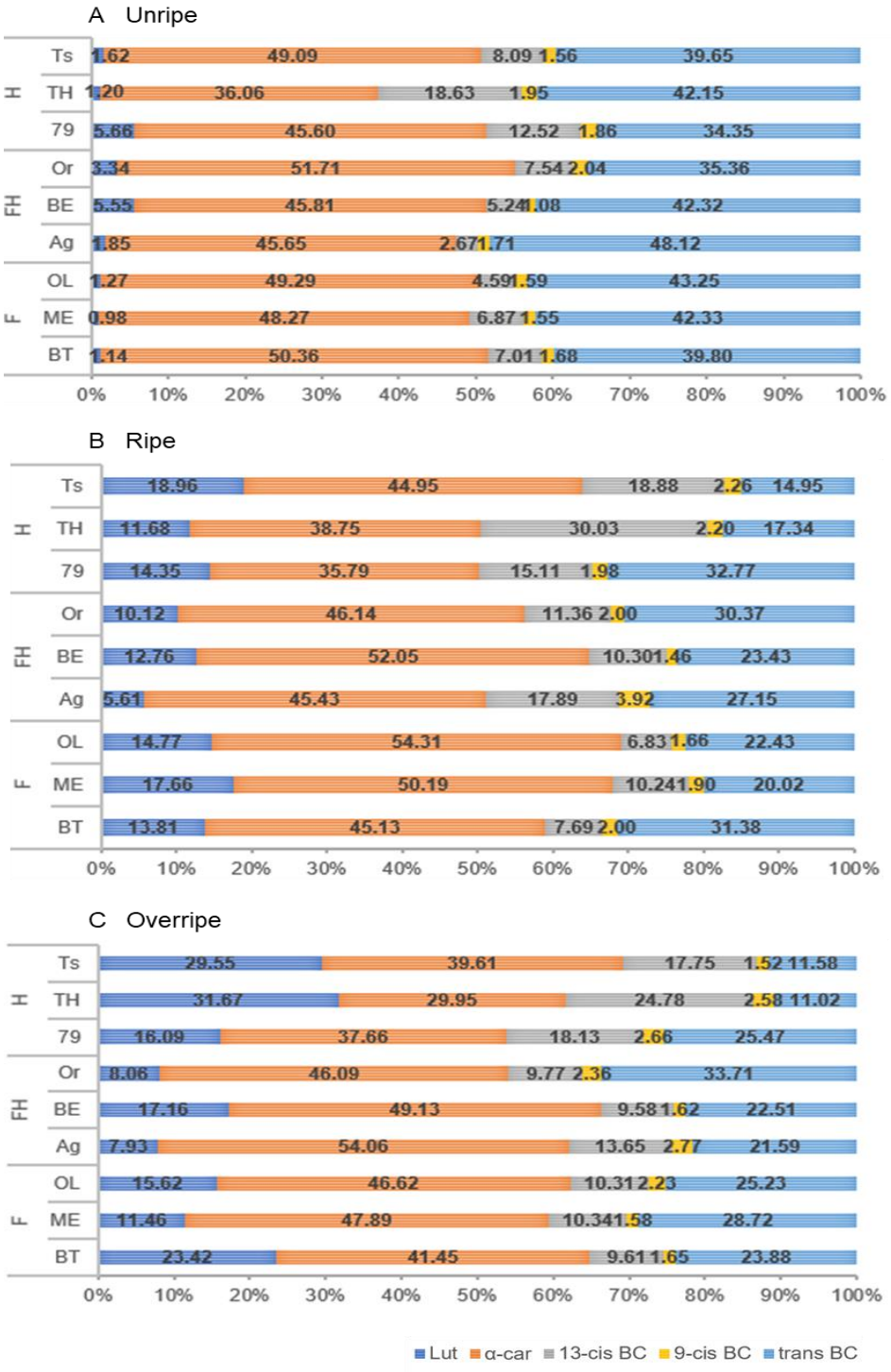

Figure 4.5 Percentage composition of individual carotenoids in the fruits of nine plantain cultivars across three ripening stages; **Unripe (A), Ripe (B) and Overripe (C).** F = French; FH = False Horn; H = Horn; BT = ITC.0112 Bobby Tannap; ME = ITC.0215 Mbi Egome; OL = ITC.0109 Obino L'Ewai; Ag = ITC.0111 Agbagba; BE = ITC.1129 Big Ebanga; OR = ITC.0517 Orishele; THP = ITC.0185 Three Hand Planty 75 = ITC.0224 75 19S; Ts = ITC.0128 Tshambunu.

Changes in lutein levels from the unripe to the ripe stage were significant (P>0.05) for all cultivars except Agbagba, Big Ebanga, Orishele and 7519S but changes from the ripe to the overripe stages were not significant for all cultivars. With the exception of Mbi Egome, all cultivars showed a decrease in the main pVACs (*trans* β-carotene and α-carotene) during ripening from the unripe to the ripe, to the overripe stage with a consequent decrease in total pVACs. This was reflected in the reduction in BCE from the unripe to the ripe and overripe stages and changes were statistically significant (P > 0.05) for all cultivars except 7519S (Table 4.2).

4.3.3 Correlations

Pearson correlation coefficients were estimated for carotenoid traits for unripe, ripe and overripe plantains (Table 4.4).

Table 4.4. Pearson correlation coefficients among carotenoids in unripe (n=44), ripe (n=44) and overripe (n=42) fruits of nine plantain cultivars

	α-car	13-*cis* BC	*trans* BC	9-*cis* BC	pVACS	TC HPLC	BCE	TC spec
Unripe								
Lut	0.07	-0.12	-0.03	-0.05	0.02	0.09	0.00	0.15
α-car		0.13	0.78***	0.74***	0.95***	0.95***	0.92***	0.58***
13-*cis* BC			0.08	0.45**	0.26	0.25	0.21	-0.08
trans BC				0.48**	0.91***	0.91***	0.96***	0.49***
9-*cis* BC					0.72***	0.71***	0.66***	0.37*
pVACS						1.00***	0.99***	0.54***
TC HPLC							0.99***	0.55***
BCE								0.54***
Ripe								
Lut	0.68***	0.14	0.41**	0.59***	0.61***	0.71***	0.56***	0.29
α-car		0.11	0.70***	0.66***	0.92***	0.93***	0.88***	0.67***
13-*cis* BC			0.01	0.31*	0.27	0.27	0.19	-0.08
trans BC				0.66***	0.88***	0.85***	0.94***	0.57***
9-*cis* BC					0.77***	0.78***	0.76***	0.38*
pVACS						0.99***	0.99***	0.63***
TC HPLC							0.97***	0.61***
BCE								0.63***
Overripe								
Lut	0.33*	0.41**	0.10	0.26	0.29	0.46**	0.23	0.04
α-car		0.33*	0.85***	0.74***	0.97***	0.96***	0.95***	0.71***
13-*cis* BC			0.19	0.51***	0.42**	0.47**	0.35*	0.38*
trans BC				0.75***	0.93***	0.88***	0.97***	0.51***
9-*cis* BC					0.82***	0.81***	0.81***	0.61***
pVACS						0.98***	0.99***	0.67***
TC HPLC							0.97***	0.63***
BCE								0.63***

α-car = α-carotene; 13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACS = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

Highly significant positive correlations were observed between α-carotene and β-carotene at all three ripening stages (r= 0.78; r= 0.70 and r=0.85 for unripe, ripe and overripe fruits, respectively). Significant positive correlations were also observed for TC calculated from HPLC and TC spec at the green (r=0.55), ripe (r=0.61) and overripe stage (r=0.63), indicating a correspondence between the carotenoid quantification methods. Similarly, a positive correlation was noted for pVACs and TC spec at the unripe (r=0.54), ripe (r=0.63) and overripe stage (r=0.67). There were no significant correlations for lutein at the unripe stage but there was a significant correlation with α-carotene, which was strong (r=0.68) at the ripe stage and moderate (r=0.33) at the overripe stage. At all three stages, α-carotene and *trans* β-carotene were positively correlated with TC spec (unripe, r= 0.58 and r= 0.49; ripe, r=0.67 and r=0.57 and overripe, r=0.71 and r= 0.51, respectively) (Table 4.4).

4.4 Discussion

Fruit carotenoid content is quantitatively and qualitatively affected by several factors, including stage of ripeness. Therefore, characterisation of carotenoid composition in edible portions of fruit cultivars and their variations during ripening is valuable for biofortification/dietary diversification programmes. Over 120 plantain cultivars exist and fruits are typically harvested at maturity and processed for consumption at different stages of ripeness. Considering the vast plantain diversity, nine representative cultivars were selected across the three main plantain types: French, False Horn and Horn. The aim of this investigation was to assess their carotenoid composition at three stages of ripeness: unripe, ripe and overripe, using two analytical techniques, HPLC and spectrophotometry.

Recently, the carotenoid profiles and contents have been characterised for various types of bananas and it was reported that the major carotenoids isolated were α-carotene, β-carotenes and small amounts of lutein (Englberger et al. 2006; Davey et al. 2009; Ngoh-Newilah et al. 2009; Heng et al. 2017). This has consequences for human nutrition and health, as α-carotene and β-carotene are carotenes which contribute to vitamin A activity, while lutein is a xanthophyll that is an antioxidant and a component in macular pigments implicated in eye health. The HPLC results indicated that over 80% of the carotenoids detected in plantains at all ripening stages were pVACs; α-carotene, *cis*- and *trans*- as well as β-carotene (>80%) while less than 20% comprised the non pVAC lutein. A higher proportion of α-carotene (60%) was also recorded by Heng et al. (2017) in three plantain cultivars sampled in China. Contrarily, Ekesa et al. (2013) recorded higher proportions of β-carotene (about two times higher) than α-carotene in two French plantain cultivars sampled in Eastern DRC.

Total and individual carotenoid contents varied within and between all nine tested cultivars. The French plantains had significantly higher carotenoid contents than the False Horn and Horn types. Indeed, TC measured by HPLC and spectrophotometry indicated a two-fold variation across all cultivars tested at all ripening stages. These findings confirm earlier reports on the variability in individual and TC contents within genomic groups in bananas (Englberger et al. 2006; Davey et al. 2009). While the synthesis and accumulation of carotenoids are genetically determined, it is also affected by season, geographical location, climate, growth conditions and stage of maturity (Rodriguez-Amaya 2004; Arscott 2013). Differences in analytical techniques, coupled with complications of simultaneous extraction of carotenes and xanthophylls also constitute a source of variation, further complicating comparisons among studies (Amorim-Carrilho et al. 2014; Mercadante et al. 2017). Lado et al. (2016) reviewed the carotenoid composition in the pulp of fleshy fruits, noting the variable carotenoid contents and profiles within and across species, indicative of variable mechanisms in carotenoid regulation and accumulation. While some fruits like strawberries, recorded low contents of <1 µg g^{-1} FW with simple profiles. Others, like red peppers recorded up to about 900 µg g^{-1} with complex carotenoid profiles. In this study, mean TC for plantains sampled at all ripening stages ranged from 10.11 (in overripe fruits) to 16.94 µg g^{-1} FW (in ripe fruits), qualifying plantains as high carotenoid fruits (5-20 µg g^{-1} FW) based on the carotenoid classification criteria reviewed by Lado et al. (2016).

Orange-yellow coloured fruits and vegetables have often been associated with high contents of carotenes (such as α-carotene, β-carotene and β-cryptoxanthin) and smaller quantities of xanthophylls (such as lutein) (Saini et al. 2015; Britton 2008). In yellow papaya, which accumulate up to 33.64 µg g^{-1} FW TC, the predominant pVACs β-cryptoxanthin and β-carotene make up 60% and 7% of this total, respectively (Schweiggert et al. 2011). In a recent comparative study, Chan-León et al. (2017) reported relatively higher contents of β-carotene in orange flesh papaya than yellow and red flesh types. Similarly, in yellow-orange landrace pumpkins (*Cucurbita* spp*.)* accumulating up to 405 µg g^{-1} pVACs, β-carotene (62%) and α-carotene (17%) were dominating (De Carvalho et al. 2012). The results from the current study indicate that plantains with their characteristic yellow-orange fruit pulp, have a simple carotenoid profile comprising high proportions of α-carotene and β-carotene, which contribute to pVA content and smaller amounts of lutein, confirming their potential as a dietary source of vitamin A. A recent study revealed that plantain has by far the highest pVACs β-carotene (>6.25 ug g^{-1} FW) and α-carotene (>3.75 µg g^{-1} FW) than other main starchy staples (cassava, yam, cocoyam and maize) sampled in Ghana (Wald et al. 2018).

This study also recorded a significant positive correlation of the carotenoids, α-carotene, β-carotene and 9-*cis* β-carotene (Table 4.4). This could be an indication that high levels of one carotenoid can lead to high levels of the other. Lutein levels were not significantly correlated with other carotenoids at the green stage, possibly because of its low content relative to other carotenoids. The highly significant positive correlation for TC measured by spectrophotometry and TC measured by HPLC at all ripening stages indicate an agreement of both carotenoid assessment methods. While HPLC remains the method of choice for carotenoid separation to adequately determine their nutritional value (Rodriguez-Amaya 2016), spectrophotometry, which is relatively easier and more rapid, is feasible for rough estimations of carotenoid contents in plantains as previously suggested by Davey et al. (2006; 2009).

Several studies have investigated the accumulation of carotenoids in fruit pulp during ripening in bananas and reported variable tendencies (Lokesh et al. 2014; Ekesa et al. 2015; Mbabazi 2015). The present study, which focused on representative cultivars of the AAB plantain sub group, generally depicted a decrease in pVACs and TC contents from the unripe to the ripe and overripe stages for all cultivars sampled. As depicted in Table 4.2, this decreasing trend was significant for most cultivars between the green and the ripe and overripe stages, but was mostly not significant between the ripe and the overripe stage. Conversely, an increasing trend in lutein contents was observed with ripening for most accessions. These results are in contrast with the results of Ekesa et al. (2015) who reported an increase in mean total pVACs content in eight banana cultivars (including two plantains) evaluated in DRC, which was, in some cases, associated with an increase in lutein. Ngoh-Newilah et al. (2009) also recorded a decreasing trend in pVACs with ripening for some plantain cultivars evaluated at three ripening stages in Cameroon. In another study with eight banana cultivars in Uganda, Mbabazi (2015) also recorded a decrease in BCE following ripening in the single plantain and two dessert banana cultivars tested, contrary to consistent increase in BCE in five EAHB cultivars tested. When Lokesh et al. (2014) assessed four varieties (two AAA and two AAB type dessert bananas) in India it was observed that TC and pVACs remained stable after ripening.

Fruit ripening is characterised by biochemical and structural changes, including the gradual transformation of chloroplasts into chromoplasts, which are plastids specialised in carotenoid storage (Li and Yuan 2013; Sun et al. 2017). The climacteric fleshy fruit, tomato is characterised by increased carotenes from the mature green stage to the fully ripe red stage, associated with the conversion of chloroplast into chromoplast. Beta-carotene and xanthophylls are predominant in the mature green stage while lycopene is predominant in the fully ripe stage (D'Andrea et al. 2018). Isolation of transcriptional regulatory genes

associated with carotenoid biosynthesis and their implication in carotenoid accumulation during ripening have been investigated in several crop species (Lado et al. 2016). Buah et al. (2016) investigated carotenogenesis during fruit development for a low carotenoid accumulating cultivar AAA Cavendish and a high accumulating F'ei cultivar Asupina. They observed an increase in TC from the mature green to the fully ripe stage with a predominance of β-carotene in Asupina and lutein in Cavendish and noted the conversion of chloroplasts into chromoplasts in Asupina but not in Cavendish. However, although they observed changes in isoprenoid pathway gene expression patterns at different ripening stages and between the two cultivars, these did not correlate with observed carotenoid accumulation patterns. Increased levels of carotenoids with ripening has also been reported in mango (Vásquez-Caicedo et al. 2005) and papaya cultivars (Schweiggert et al 2011). In contrast, Zhu et al. (2015) noted that the predominant carotenoids in strawberry were lutein and β-carotene, with variable levels across genotypes and contents dramatically decreased with ripening and changes were consistent with decreased expression of major isoprenoid pathway genes.

A decrease in carotenoid contents during post-harvest ripening may also be associated with degradation or rapid turnover of metabolites from the carotenoid pathway. Carotenoid molecules in intact fruits are less susceptible to degradation because they are protected by natural processes within the living tissues in the growing plant. While the underlying mechanisms of carotenoid accumulation during ripening in plantains is still unclear, it is interesting to note that mature green fruits of plantains would yield more pVA than ripe and overripe fruits, which is relevant to its use as a dietary source of vitamin A. However, the effect of processing (retention studies) should be taken into consideration in the nutritional evaluation of plantains.

4.5 Conclusions

Carotenoid content and variation during ripening has been extensively studied in fruits due to their nutritional and health promoting properties. The present study reports for the first time, the carotenoid composition of fruit pulp of plantain cultivars representing the three main bunch types (French, False Horn and Horn) at three ripening stages at which they are commonly processed for consumption. Total carotenoids decreased in all cultivars, across all three bunch types during ripening, reflecting a mean decrease from 16.94 to 10.11 µg g^{-1} FW. Carotenoid profiles revealed high proportions of pVACs β-carotene (*trans* and *cis* isomers), α-carotene and smaller proportions of the non-pVAC lutein at all ripening stages. Provitamin A carotenoid contents were highest at the unripe stage but were reduced significantly at the ripe and overripe stages, whereas lutein concentrations

were lowest at the unripe stage and increased significantly with ripening. Plantain cultivars recorded mean BCEs of 5.05-13.65 µg g^{-1} FW, with the French recording higher values than the False Horn and Horn plantain types, indicating their potential as a dietary source for vitamin A. However, further studies will be required to determine pVACs stability/retention following the most prevalent methods of processing (frying, boiling and roasting) and to further investigate their bioavailability when consumed.

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CHAPTER 5

In vitro polyploidization: a breeding strategy towards banana biofortification

5.1 Introduction

Micronutrient deficiency, also known as 'hidden hunger', is a major public health concern, caused by inadequate dietary intake of essential nutrients or minerals. One in three people worldwide are malnourished, with two billion people lacking key micronutrients like iron and vitamin A (Global Nutrition Report 2017). Traditional strategies such as mineral supplementation and food fortification have failed to eradicate micronutrient deficiencies due to lack of economic, social and cultural mechanisms for efficient implementation. Recently, crop improvement efforts have been focusing on producing nutrient-rich high yielding crops through biofortification, as a sustainable solution to address micronutrient malnutrition (Bouis and Welch 2010). Unfortunately, major food crops have insufficient amounts of micronutrients required for healthy human growth. Biofortification involves the use of conventional breeding or transgenic approaches to enhance the nutritional value of crops. Conventional breeding which exploits the natural genetic variability has been the preferred method for pVA enhancement of potato, maize and cassava, with biofortified varieties already available in farmers' fields (Bouis and Saltzman 2017; Garg et al. 2018).

Among the fruit crops, plantains (*Musa* spp., subgroup AAB) are an important staple for millions of people in tropical regions worldwide, particularly for Africa, which accounts for over half of the world's production (FAOSTAT 2017). Besides being a principal source of calories, plantains are a good source of micronutrients such as vitamin A, which can be enhanced by biofortification. *Musa* spp. exhibit considerable genetic variability for pVACs, with some cultivars accumulating up to 35 µg g^{-1} FW of fruit pulp (Davey et al. 2009), that can be useful for breeding vitamin A biofortified plantains. Conventional banana breeding strategies rely on sexual hybridization across ploidy categories and phenotypic selection from viable offspring. This involves crosses between 3x cultivars and 2x wild or improved accessions, selecting 4x and 2x hybrids from intermediate products and crossing selected 4x and 2x hybrids in order to generate sterile 3x plantain hybrids (Bakry et al. 2009; Tenkouano et al. 2011; Ortiz 2015). However, this process takes a long time and is further complicated by parthenocarpy, polyploidy and associated irregular meiotic behaviour, low fertility, low seed viability, long generation times, diverse genome configurations and the narrow genetic base (Ortiz 2013; 2015; Brown et al. 2017).

Polyploid induction has been advocated as a tool for genetic improvement in several crops with the aim of creating new polyploid varieties or increasing fertility of interspecific hybrids

(Dhooghe et al. 2011; Sattler et al. 2016). Polyploidy has the potential to generate new cultivars with variable agronomic characteristics and other favourable traits sometimes out-performing their diploid progenitors. An alternative breeding approach involving polyploid induction has been proposed for bananas. This approach aims to create triploids directly from ancestral diploid cultivars or improved diploid hybrids through reconstitutive breeding (Bakry et al. 2009; Tenkouano et al. 2011). Polyploid induction in *Musa* spp. is achieved through treatment of highly proliferating totipotent explants with anti-mitotic compounds (colchicine or oryzalin), which interfere with microtubule formation, followed by plantlet regeneration, isolation and propagation of identified polyploids (Van Duren et al. 1996; Ganga and Chezhiyan 2002; Bakry et al. 2007; Goigoux et al. 2013; Salles Pio et al. 2014; Do Amaral et al. 2015). Although there are several reports on induction of polyploidization in banana to generate tetraploid breeding lines for use in triploid breeding, information on field evaluation of induced tetraploids are scarce. Once induced tetraploids are created, there is need for field evaluation to ascertain their suitability for use in breeding. Moreover, thus far, the doubled diploid strategy has not been applied for plantain breeding.

Plantains are predominantly triploid cultivars with limited fertility and narrow genetic variability, which necessitates the use of diploid bananas for genetic improvement. Diploid cultivars with AA genome configuration, have been implicated in the evolutionary process of present cultivated varieties and constitute a wide diversity of *Musa* spp. that has been useful for banana improvement programmes (Bakry et al. 2009; Brown et al. 2017). Specifically, the AA cultivars of the ssp. *banksii* cluster from PNG are thought to have contributed to the A genome in plantain cultivars (Perrier et al. 2009), hence are of relevance to plantain breeding. AA diploid cultivars with origins from PNG have also been reported to have high pVAC content with potential for vitamin A biofortification (Fungo and Pillay 2011). Against this background, the aim of this study was to determine the effect of induced polyploidization on agronomic attributes and pVAC content and to evaluate the fertility of induced tetraploids as a strategy towards banana biofortification.

5.2 Materials and methods

5.2.1 Genotypes

Six diploid AA genotypes were used in this study, selected based on availability (Table 5.1).

Table 5.1 Diploid banana genotypes used to study the effect of induced polyploidization on agronomic characteristics and provitamin A carotenoids

Genotype	Status	Origin*	Cluster**	Induced 4x ***
ITC.0266 Sowmuk	cultivar	Unknown	AA cv. *banksii* sensu lato	2
ITC.0298 Beram	cultivar	Indonesia	AA cv. IndonTriNG	2
ITC.0259 Galeo	cultivar	Unknown	AA cv. IndonTriNG	2
ITC.0507 Pisang Madu	cultivar	Unknown	n/a	2
ITC.0712 AAcv Rose	cultivar	Indonesia	*M. acuminata* ssp. *Malaccensis*	1
25447-S7	hybrid****	Nigeria	n/a	2

* Country of origin and classification as stated in MGIS database (www.cropdiversity.org); ** Specific cluster as described in Christelová et al. (2017); *** Number of isolated tetraploid lines used for further evaluation; **** Pedigree 2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin).

5.2.2 *In vitro* chromosome doubling

Genotypes were available as proliferating cultures introduced from the ITC, Belgium or from the IITA *in vitro Musa* germplasm collection. *In vitro* polyploidization was carried out with oryzalin [3,5-dinitro-N4, N4-dipropylsulfanilamide] as described by Bakry et al. (2007). Briefly, proliferating shoot clusters were established from *in vitro* shoot tips on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 4 mg l^{-1} 6-benzylaminopurine (BAP), 30 g l^{-1} sucrose and 2.0 g l^{-1} Gelrite with pH adjusted to 5.8 ± 0.1. Selected bud clusters were subjected to oryzalin treatment (45 µM; 15.6 mg l^{-1}) for 48 hours on a gyratory shaker at 90 rpm in liquid MS medium, after which they were rinsed in sterile distilled water for 24 hours at 90 rpm. Clusters were transferred to semi-solid MS medium with 1 mg l^{-1} BAP and surviving plants were cultured at 30 day intervals four times to allow chimera dissociation and plantlet development. Young leaf tissue was obtained from selected fully developed plantlets for ploidy analysis. Ploidy level of each accession was estimated by flow cytometry according to Dolezel et al. (2007), using a reference diploid, Calcutta 4 (a male fertile pollen parent used in banana breeding programmes) as external standard.

From each genotype one or two tetraploids were isolated from distinct doubling events for further evaluation (Table 5.1). Confirmed tetraploids and diploid progenitors were established on proliferation medium to generate clones and plantlets were rooted on MS medium devoid of BAP and supplemented with 1 mg l^{-1} 1-naphtaleneacetic acid (NAA), for 40 days, after which they were simultaneously acclimatized in a screenhouse for field establishment.

5.2.3 Site characteristics and experimental design

Experimental plants were established at the IITA Ibadan research station located at 3° 54' East and 7° 30' North at 240 m above sea level, in the sub humid-derived savanna agro-ecology where the soil type is predominantly a ferric luvisol. The rainfall pattern is bimodal, with two distinct rainy seasons, the first from April to July, and the second from August to November. Fully acclimatized plants of all six diploid progenitors and 10 induced tetraploids (Table 5.1) were planted in a completely randomized design with nine replications. All plants were grown under standard field conditions at a spacing of 3 m x 2 m at a density of 1666 plants ha $^{-1}$. Plants were evaluated between the months of April 2017 and May 2018.

5.2.4 Data collection

Agronomic, pVAC, male and female fertility traits were assessed and compared between diploid progenitors and induced tetraploid lines.

5.2.4.1 Agronomic assessment

Agronomic characteristics (vegetative and yield-related traits) were evaluated at flowering and at harvest. Plant height (PHT), pseudostem girth at 100 cm from soil surface (PGT) and number of suckers (NSF) were recorded at flowering. Bunch weight (BWT), number of hands or clusters (NH), total number of fingers or fruits (NF), fruit weight (FWT), fruit length (FLT) and fruit circumference (FC) were recorded at harvest when fruits were completely filled or when a fruit showed signs of yellowing. Fruit measurements were taken from the middle fruit of the third hand. Flowering date was recorded upon emergence of the flag leaf and harvest date was recorded when ripening was observed on the first hand of the bunch or when fruits were rounded with black tips. Days to flowering (DPF) was recorded as the number of days between planting and flowering. Days to fruit maturity (DFM) were recorded as number of days between flowering and harvesting.

5.2.4.2 Carotenoid quantification

For each genotype, three plants were selected for pVAC assessment using HPLC. Harvested bunches were stored at ambient temperature in a dark room for ripening. Fruits were taken from the middle of the second hand of each bunch at the fresh ripe stage corresponding to stage 5 when fruit colour had turned yellow with green tips and necks (Dadzie and Orchard 1997). Sample preparation and carotenoid quantification through HPLC and spectrophotometry were carried out as described in Chapter 3 section 3.2.4

5.2.4.3 Fertility assessment

Of each genotype, three plants were assessed for pollen viability using 1% 2,3,5-triphenyltetrazolium chloride (TTC) stain diluted in Tris buffer (hydrochloric acid 0.15 M, pH 7.8) as described by Soares et al. (2015). Male flowers were collected from each genotype at anthesis between 7.30-10.30 am and immediately conveyed to the laboratory for analysis. Pollen grains were collected from two anthers of the same flower and manually spread on a glass slide, after which a drop of TTC stain was added. The preparation was covered with a cover slip and allowed to stand for two hours to allow uptake of the stain. Two slides were prepared of each genotype (four flowers from each individual plant) and preparations were observed under bright field illumination using a light microscope (Olympus BX51). Pollen grains stained with TTC appears light pink or red when viable and remains transparent when non-viable. Viable and non-viable pollen grains were recorded from observation of three selected microscopic fields of each slide. The percentage of stained pollen grain was calculated from the pollen counts and expressed as percentage pollen viability.

At flowering, all plants were bagged and pollinated with Calcutta 4 (the standard male fertile pollen parent). Seed set was assessed in fully mature plants at harvest as an indicator of female fertility.

5.2.5 Data analysis

Statistical analysis was carried out using SAS version 9.4 for Windows Copyright © 2002-2015 by SAS Institute Inc., Cary, NC, USA. The PROC GLM statement was used for one-way ANOVA followed by Student-Newman-Keuls Test and Duncan's Multiple Range Rest to detect significant differences among means as applicable.

5.3 Results

5.3.1 Agronomic characteristics

Up to nine plants of each genotype were planted, but results are only presented for plants harvested at full maturity, as some plants were lost due to wind damage before maturity. Significant phenotypic differences were observed between diploid plants and their induced tetraploids for vegetative and yield traits. Tetraploid plants had longer curved leaves, which were drooping, while diploid plants had shorter leaves which were more erect (Figure 5.1). Data for vegetative characteristics are presented in Table 5.2. Generally, mean number of days to flowering (384.04) was significantly longer for induced tetraploids plants than for diploids (299.71) but the maturity time was significantly shorter for induced tetraploids

(81.21) than for original diploids (101.55). Plant height and plant girth did not differ significantly for both groups but the number of suckers at flowering was higher for diploids (7.34) than for induced tetraploids (3.87).

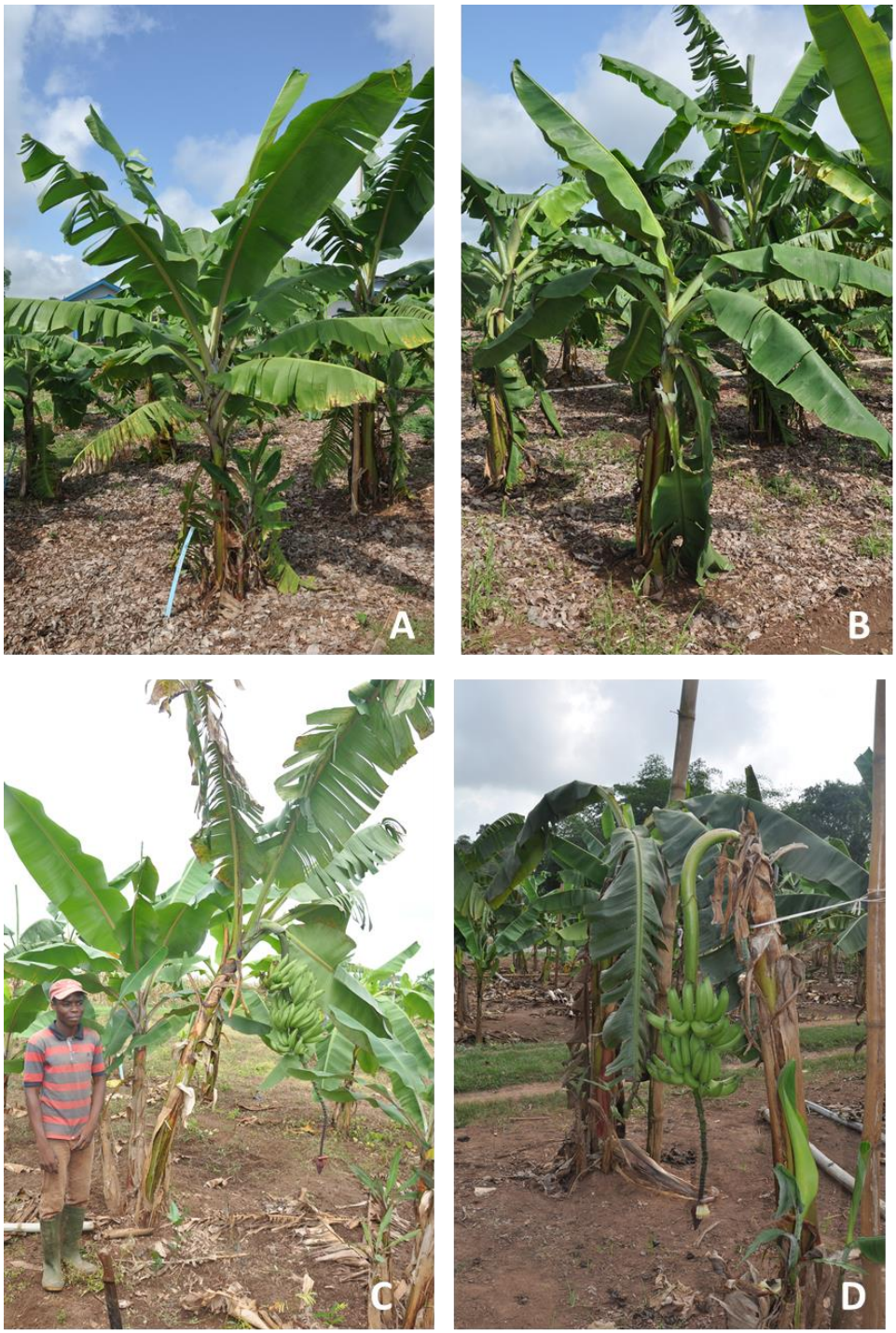

Figure 5.1 Leaf characteristics of diploid and induced tetraploid banana

Pre-flowered plants of diploid (A) and induced tetraploid (B) and mature plants for diploid (C) and induced tetraploid (D) of the cultivar Galeo.

Table 5.2 Vegetative characteristics of diploid and induced tetraploid banana genotypes

Genotype	Ploidy	n	Vegetative characteristics (mean ± standard deviation)				
			DPF	PHT (cm)	PGT (cm)	NSF	DFM
Sowmuk	2x	6	272.00±41.53^{ef}	231.33±19.61^{bcd}	36.83±4.31^{de}	5.50±1.87^{bcde}	53.50±2.66^{g}
Sowmuk 1	4x	5	460.40±60.87^{ba}	270.00±9.35^{ab}	39.60±4.88^{cde}	4.00±1.22^{bcde}	48.60±2.07^{g}
Sowmuk 2	4x	5	481.80±39.77^{a}	238.00±8.37^{bcd}	37.40±2.19^{de}	3.20±1.92^{de}	51.80±6.53^{g}
Beram	2x	6	343.33±25.07^{cde}	296.67±41.19^{a}	49.17±9.24^{ab}	13.50±6.35^{a}	87.17±28.48^{cdef}
Beram 3A	4x	3	395.33±76.79^{bcd}	271.67±55.08^{ab}	44.33±13.05^{abcd}	9.00±4.36^{bc}	65.33±6.66^{fg}
Galeo	2x	8	309.13±32.38^{de}	226.25±8.35^{bcd}	34.50±5.58^{def}	8.00±2.33^{bcd}	101.00±2.98^{cd}
Galeo 1	4x	4	395.75±73.22^{bcd}	220.00±20.41^{cd}	40.75±2.87^{bcde}	4.25±1.26^{bcde}	74.75±23.95^{ef}
Galeo 2	4x	3	394.67±19.09^{bcd}	223.33±17.56^{cd}	39.67±5.86^{cde}	5.33±2.52^{bcde}	77.67±8.74d^{ef}
P Madu	2x	8	351.13±49.92^{cde}	226.88±19.81^{bcd}	36.88±3.23^{de}	4.75±1.04^{bcde}	100.75±8.24^{cd}
P Madu 1	4x	4	353.25±10.72^{cde}	283.75±14.93^{a}	50.00±2.58^{a}	3.75±0.50^{cde}	79.50±15.72^{def}
P Madu 2	4x	7	415.86±50.94^{abc}	261.43±22.86^{abc}	48.14±1.95^{abc}	3.00±1.53^{de}	89.14±3.63^{cde}
AA cv. Rose	2x	5	217.00±42.77^{f}	170.00±7.91^{e}	26.20±2.86^{fg}	9.20±4.38^{b}	132.20±7.40^{b}
AA cv. Rose 1	4x	3	319.33±20.50^{de}	173.33±17.56^{e}	25.33±4.51^{g}	6.67±0.58^{bcde}	97.00±6.56^{cde}
25447-S7	2x	5	266.00±58.61^{ef}	221.00±42.92^{cd}	30.60±5.37^{efg}	3.40±1.52^{de}	148.00±16.19^{a}
25447-S7 1	4x	5	278.20±27.19^{ef}	205.00±20.31^{de}	37.60±4.51^{de}	2.80±1.48^{de}	105.40±10.48^{c}
25447-S7 2	4x	8	339.13±39.70^{cde}	175.63±19.35^{e}	30.63±3.25^{efg}	2.00±0.76^{e}	103.38±9.91^{c}
All 2x		38	299.71±60.49^{B}	230.21±42.40^{A}	36.08±8.47^{A}	7.34±4.50^{A}	101.55±31.40^{A}
All 4x		47	384.04±74.35^{A}	230.21±43.29^{A}	39.28±8.21^{A}	3.87±2.36^{B}	81.21±22.28^{B}
All genotypes		85	346.34±80.11	230.21±42.64	37.85±8.43	5.42±3.87	90.31±28.46

n = number of plants sampled; DPF = days to flowering, PHT = plant height; PGT = Plant girth; NSF = number of suckers at flowering; DFM = days to fruit maturity; means followed by the same case letter within a column are not significantly different at p<0.05.

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In terms of yield related traits (Figure 5.2; Table 5.3), induced tetraploids generally had significantly lower mean bunch weights (6.01 kg) than diploids (7.76 kg) but variable changes were observed for individual genotypes. Notably, tetraploids from the hybrid 25447-S7 (25447-S7 1 and 2) and Pisang Madu (P Madu 1) had slightly higher bunch weights than their diploid counterparts while Sowmuk recorded the highest reduction in bunch weight (>50%) following polyploidization, but these changes in bunch weights were not significant (Figure 5.2). Similarly, the number of hands per bunch was higher (7.39) for diploids than for induced tetraploids (6.00), but this difference was only significant for Beram and AA cv. Rose (Table 5.3). Mean number of fruits was significantly higher for diploids (107.63) than tetraploids (82.43) but mean fruit weight and circumference were variable among doubled and non-doubled genotypes.

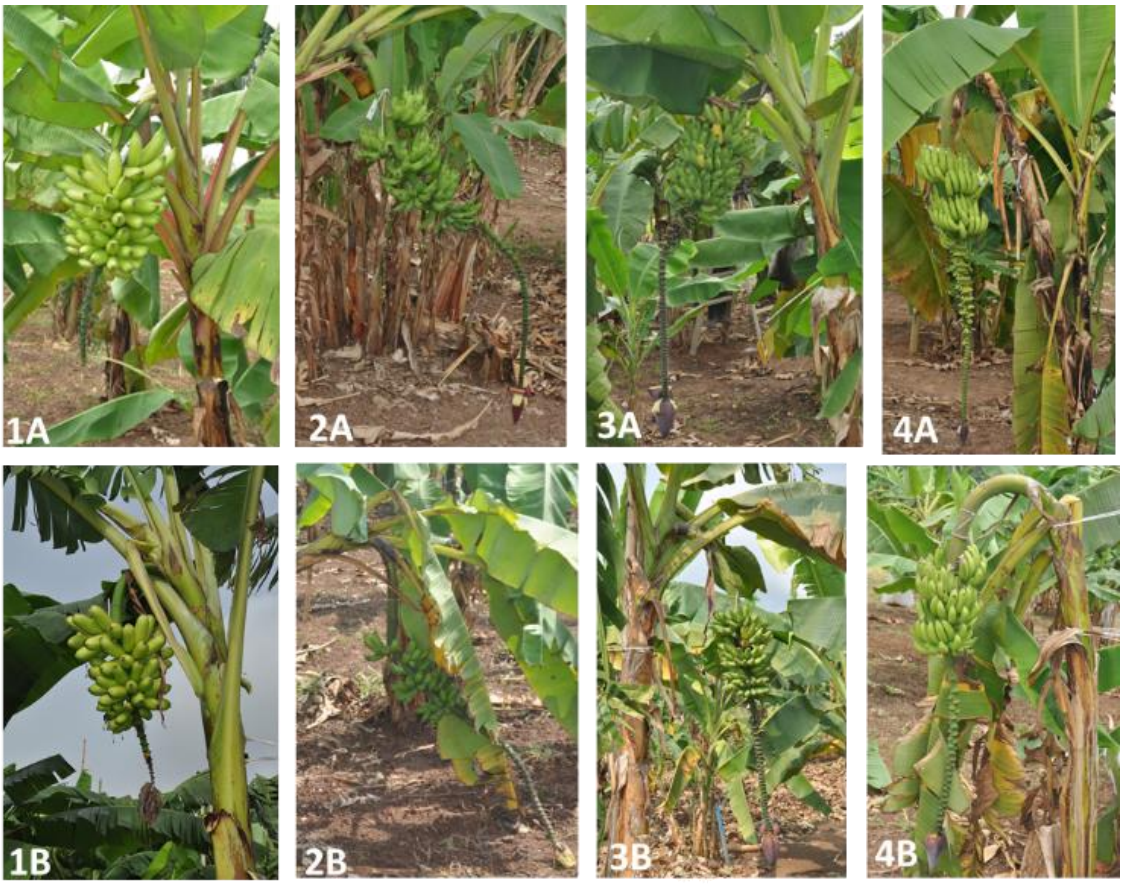

Figure 5.2 Bunch characteristics of diploid and induced tetraploid bananas

2x Sowmuk (1A); 2x Sowmuk (1B); 2x AA cv. Rose (2A); 4x AA cv. Rose(2B) 2x P Madu (3A); 4x P Madu (3B); 2x 25447-S7 (4A); 4x 25447-S7 (4A).

Table 5.3 Yield attributes of diploid and induced tetraploid banana genotypes

Genotype	Ploidy	n	Yield - related traits (mean ± standard deviation)					
			BWT (kg)	NH	NF	FLT (cm)	FC (cm)	FWT (cm)
Sowmuk	2x	6	7.25±2.72^{abc}	7.33±1.37^{abcd}	107.83±42.37^{abcd}	14.67±1.21^{d}	11.50±1.05^{abc}	55.33±19.36^{cd}
Sowmuk 1	4x	5	4.20±2.17^{bc}	5.40±0.55^{def}	67.20±7.60^{de}	12.60±1.34^{d}	10.80±1.64^{abc}	58.80±22.99^{cd}
Sowmuk 2	4x	5	2.80±1.48^{bc}	6.60±0.55^{bcde}	63.80±10.64^{de}	11.40±0.89^{d}	10.60±1.14^{abc}	42.80±8.35^{cd}
Beram	2x	6	7.50±2.95^{abc}	7.50±1.22^{abc}	107.17±20.64^{abcd}	17.17±1.72^{c}	11.33±0.52^{abc}	71.50±14.18^{cd}
Beram 1	4x	3	3.50±0.50^{bc}	4.00±1.00^{f}	41.67±23.67^{e}	13.00±0.00^{d}	12.00±1.73^{ab}	61.33±6.35^{cd}
Galeo	2x	8	12.56±2.44^{a}	6.38±0.74^{cde}	92.00±8.28^{bcd}	22.50±1.31^{a}	11.00±0.53^{abc}	115.63±39.42^{b}
Galeo 1	4x	4	8.00±4.32^{abc}	4.75±0.50^{ef}	59.50±4.80^{de}	21.00±1.83^{ab}	13.00±0.82^{a}	168.75±18.84^{a}
Galeo 2	4x	3	12.33±8.50^{a}	5.33±0.58^{def}	72.67±5.77^{cde}	18.00±3.46^{c}	10.33±2.08^{bcd}	87.00±35.93^{bc}
P Madu	2x	8	7.75±1.83^{abc}	8.50±1.07^{a}	138.25±42.24^{a}	13.75±1.39^{d}	9.00±1.31^{cd}	38.00±11.14^{d}
P Madu 1	4x	4	8.75±2.63^{ab}	7.25±0.50^{abcd}	127.25±8.88^{ab}	13.00±1.41^{d}	9.75±0.50^{bcd}	52.75±5.91^{cd}
P Madu 2	4x	7	6.71±1.11^{bc}	6.86±1.07^{abcd}	116.86±18.73^{ab}	12.29±1.60^{d}	9.29±1.25^{bcd}	64.43±40.8^{cd}
AA cv. Rose	2x	5	3.00±0.71^{bc}	8.40±0.55^{ab}	105.40±2.07^{abcd}	11.60±0.89^{d}	7.80±0.45^{d}	26.40±4.39^{d}
AA cv. Rose 1	4x	3	2.33±0.58^{c}	6.33±1.53^{cde}	58.33±16.17^{de}	11.00±1.00^{d}	9.00±1.00^{cd}	32.00±7.00^{d}
25447-S7	2x	5	5.80±2.17^{bc}	6.20±1.10^{cde}	86.20±30.59^{bcde}	19.40±5.03^{bc}	9.40±3.21^{bcd}	31.60±20.28^{d}
25447-S7 1	4x	5	6.20±2.28^{bc}	6.40±0.89^{cde}	106.40±19.03^{abcd}	13.60±0.55^{d}	8.80±0.45^{cd}	48.00±11.47^{cd}
25447-S7 2	4x	8	6.00±3.42^{bc}	5.88±0.99^{cde}	75.50±15.93^{cde}	12.75±1.04^{d}	9.00±1.07^{cd}	38.38±14.73^{d}
All 2x		38	7.76±3.62^{A}	7.39±1.33^{A}	107.63±32.72^{A}	16.74±4.30^{A}	10.08±1.87^{A}	60.00±38.75^{A}
All 4x		47	6.01±3.78^{B}	6.00±1.18^{B}	82.43±29.35^{B}	13.57±3.03^{B}	10.06±1.67^{A}	62.40±40.71^{A}
All genotypes		85	6.79±3.79	6.62±1.42	93.69±33.20	14.99±3.96	10.07±1.75	61.33±39.63

n = number of plants sampled, BWT = bunch weight; NH = number of hands; NF = number of fruits; FLT = fruit length; FC = fruit circumference; FWT = fruit weight; means followed by the same case letter within a column are not significantly different at $p<0.05$.

5.3.2 Carotenoid traits

Data of carotenoid content of fresh ripe fruit pulp of diploids and induced tetraploids are presented in Table 5.4. The predominant carotenoids isolated were pVACs α-carotene, β-carotene (*cis* and *trans* versions) and lutein. Total carotenoids determined from HPLC (µg g^{-1} FW) was highest for diploid Sowmuk (8.58) and Galeo (6.80) and lowest for AA cv. Rose (1.50) and 25447-S7 (1.66). Figure 5.3 depicts the pulp colour variation of high and low carotenoid genotypes. Generally lutein content was higher in tetraploids (1.09 µg g^{-1}FW) than in diploids (0.54 µg g^{-1}FW) while all other carotenoids were higher in diploids than in tetraploids except for 25447-S7. Individual genotypes showed increase in lutein content after doubling, but this was only significant for the tetraploid Sowmuk and Galeo, which had the highest carotenoid content at the diploid and tetraploid level. In contrast, induced tetraploids for the genotypes Sowmuk, Beram, Galeo and P Madu had significantly lower pVACs α-carotene and *trans* β-carotene with consequently lower BCE and TC content (Table 5.4). Cis carotenes 13-*cis* β-carotene and 9-*cis* β-carotene decreased with doubling in all genotypes, but the decrease was not significant.

Figure 5.3 Fruit pulp colour of banana diploids and induced tetraploids with high and low carotenoid content. High content: 2x Sowmuk (1A); 4x Sowmuk (1B); 2x Galeo (2A); 4x Galeo (2B). Low content: 2x 25447-S7 (3A) and 4x 25447-S7 (3B).

Table 5.4 Carotenoid content of diploid and induced tetraploid banana genotypes

Genotype	Ploidy	Carotenoid content (Mean µg g^{-1} ± standard deviation)							
		Lutein	α-carotene	13-*cis*-β-carotene	9-*cis* β-carotene	*trans*-β-carotene	BCE	pVACs	TC HPLC
Sowmuk	2x	0.36±0.38^{cde}	3.76±0.54^{a}	0.64±0.22^{abc}	0.17±0.04^{ab}	3.65±1.09^{a}	5.94±1.16^{a}	8.22±1.32^{a}	8.58±1.54^{a}
Sowmuk 1	4x	0.88±0.58b^{cde}	0.99±0.23^{cde}	0.40±0.25^{cde}	0.07±0.03^{b}	0.74±0.37^{efg}	1.46±0.24^{efg}	2.19±0.28^{cd}	3.07±0.37^{de}
Sowmuk 2	4x	1.45±1.67^{b}	1.08±0.98^{cd}	0.47±0.39^{bcd}	0.05±0.02^{b}	1.37±0.49^{cde}	2.17±0.18^{cde}	2.96±0.86^{c}	4.41±2.52^{cd}
Beram	2x	1.41±0.13^{bc}	0.93±0.38^{cdef}	0.21±0.1^{def}	0.42±0.61^{ab}	1.77±0.55^{bcd}	2.54±0.66^{cd}	3.32±0.83^{c}	4.74±0.76^{c}
Beram 1	4x	0.54±0.68^{bcde}	0.08±0.04^{f}	0.01±0.00^{f}	0.78±1.08^{a}	0.13±0.16^{g}	0.56±0.69^{gh}	0.99±1.21d^{e}	1.53±0.53^{ef}
Galeo	2x	0.34±0.32^{de}	3.07±0.63^{ab}	0.74±0.32^{ab}	0.08±0.02^{b}	2.57±0.16^{b}	4.52±0.33^{b}	6.47±0.52^{b}	6.80±0.73^{b}
Galeo 1	4x	1.42±0.89^{bc}	0.99±0.57^{cde}	0.26±0.14^{def}	0.09±0.04^{b}	2.06±0.75^{bc}	2.73±1.04^{c}	3.40±1.34^{c}	4.83±0.89^{c}
Galeo 2	4x	2.87±1.35^{a}	1.14±0.21^{c}	0.20±0.07^{def}	0.07±0.05^{b}	1.56±0.88^{cde}	2.26±0.98^{cd}	2.97±1.07^{c}	5.84±0.28^{bc}
P Madu	2x	0.09±0.02^{e}	2.41±0.83^{b}	0.92±0.31^{a}	0.41±0.56^{ab}	1.97±0.74^{bc}	3.83±0.41^{b}	5.70±1.10^{b}	5.79±1.08^{bc}
P Madu 1	4x	0.27±0.09^{e}	0.35±0.08^{cdef}	0.19±0.03^{def}	0.47±0.56^{ab}	1.29±0.05^{cdef}	1.79±0.36^{def}	2.29±0.69^{cd}	2.57±0.77^{ef}
P Madu 2	4x	0.89±0.13^{bcde}	0.58±0.18^{bcde}	0.24±0.20^{def}	0.05±0.03^{b}	0.37±0.09^{fg}	0.80±0.18^{fgh}	1.23±0.34^{de}	2.11±0.22^{ef}
AA cv. Rose	2x	0.28±0.17^{e}	0.79±0.38^{cdef}	0.04±0.02^{ef}	0.25±0.06^{b}	0.15±0.05^{g}	0.68±0.11^{gh}	1.22±0.26^{de}	1.50±0.15^{ef}
AA cv. Rose 1	4x	0.71±0.06^{bcde}	0.23±0.03^{def}	0.08±0.01^{ef}	0.01±0.00^{ab}	0.28±0.04^{g}	0.43±0.05^{gh}	0.59±0.06^{e}	1.30±0.10^{f}
25447-S7	2x	0.74±0.14^{bcde}	0.43±0.03^{cdef}	0.21±0.10^{def}	0.01±0.00^{b}	0.28±0.04^{g}	0.60±0.03^{gh}	0.93±0.06^{de}	1.66±0.19^{ef}
25447-S7 1	4x	0.98±0.11^{bcde}	0.16±0.00^{ef}	0.08±0.04^{ef}	0.01±0.00^{b}	0.06±0.01^{g}	0.18±0.02h	0.30±0.04^{e}	1.28±0.11^{f}
25447-S7 2	4x	1.40±0.18^{bcd}	0.35±0.11^{cdef}	0.12±0.05^{def}	0.02±0.02^{b}	0.86±0.64^{defg}	1.10±0.72^{efgh}	1.35±0.79^{de}	2.76±0.84^{ef}
All 2x (n=18)		0.54±0.49^{B}	1.90±1.36^{A}	0.46±0.38^{A}	0.22±0.33^{A}	1.73±1.36^{A}	3.02±2.07^{A}	4.31±2.87^{A}	4.84±2.76^{A}
All 4x (n=27)		1.09±0.83^{A}	0.58±0.47^{B}	0.2±0.18^{B}	0.14±0.35^{A}	0.85±0.75^{B}	1.31±0.95^{B}	1.77±1.21^{B}	2.86±1.59^{B}
mean		0.87±0.76	1.11±1.13	0.3±0.30	0.17±0.34	1.20±1.11	2.00±1.70	2.79±2.37	3.66±2.32

BCE = β-carotene equivalents; pVACs = total carotenoids with vitamin A activity; TC HPLC = total carotenoids determined by HPLC; means were calculated from three plants sampled for each genotype; means followed by the same case letter within a column are not significantly different at $p<0.05$

5.3.3 Fertility attributes

Pollen viability via staining with TTC was used as an estimate of male fertility and the results obtained for four diploid genotypes and six induced tetraploids studied are illustrated in Figures 5.4 and 5.5. Calcutta 4 was added as a known fertile diploid control for comparison.

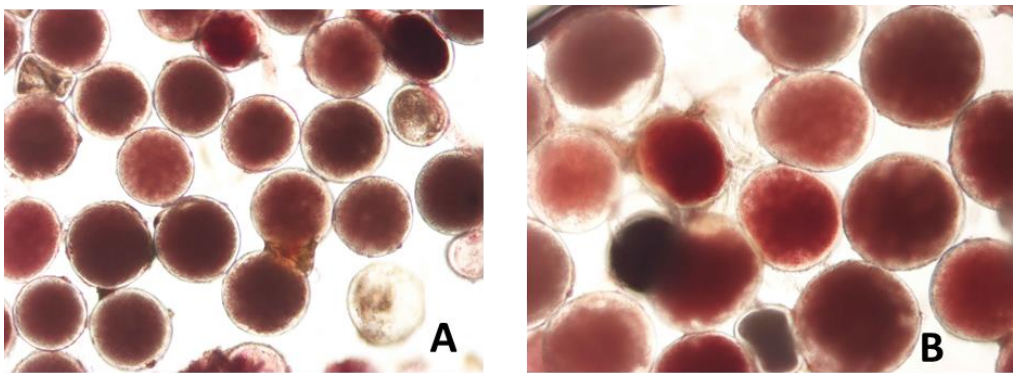

Figure 5.4 TTC stained pollen from diploid (A) and tetraploid (B) banana plants of the cultivar Galeo, showing high viability. Magnification 200x.

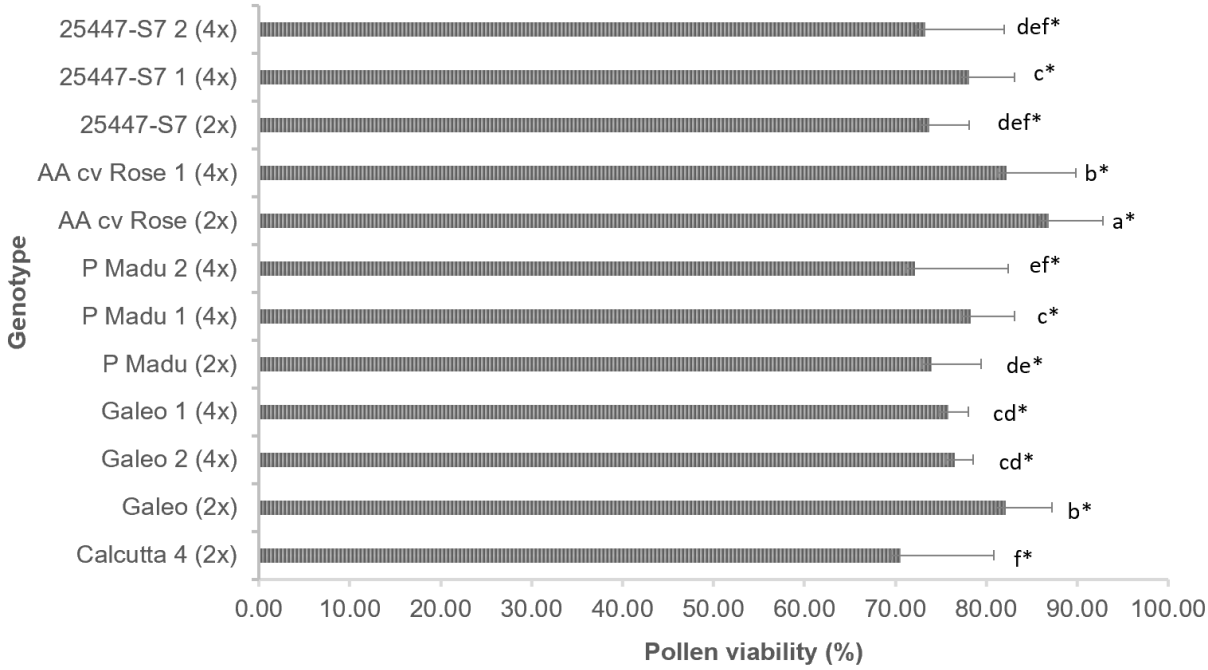

Figure 5.5 Difference in pollen viability of diploid and induced tetraploid bananas. Values represent mean ± standard deviation. Different lowercase letters between rows indicate a significant difference at $p < 0.05$.

Genotypes ITC.0266 Sowmuk and ITC.0298 Beram and their corresponding induced tetraploids were not included in the viability assessment because they recorded very low pollen counts (<10 per field) and degenerated male buds (Figure 5.2:1A and 1B).

Seed set was recorded following pollination of genotypes with pollen from Calcutta 4 as an estimate of female fertility (Table 5.5). All diploid genotypes set seeds when pollinated with Calcutta 4, with average number of seeds per bunch ranging from 1 to 39.83 for 25447-S7 and Galeo respectively. Interestingly, ITC.0266 Sowmuk and ITC.0298 Beram which had very low pollen counts (<10 per field) and were not used for viability assessment were also seed fertile. However, regarding the induced tetraploids, seeds were only obtained for tetraploid versions of two genotypes Pisang Madu and 24447-S7 with some clones recording more seeds per bunch than their diploid counterparts.

Table 5.5 Seed set of diploid and induced tetraploid banana genotypes pollinated with Calcutta 4

Genotype	Ploidy	Number of crosses harvested	Number of crosses with >1 seed	Total number of seeds	Number of seeds per bunch
Sowmuk	2x	7	2	14	7.00
Sowmuk 1	4x	3	0	0	0.00
Sowmuk 2	4x	4	0	0	0.00
Beram	2x	6	1	2	2.00
Beram 1	4x	4	0	0	0.00
Galeo	2x	9	6	239	39.83
Galeo 1	4x	3	0	0	0.00
Galeo 2	4x	2	0	0	0.00
P Madu	2x	4	2	4	2.00
P Madu 1	4x	4	2	27	13.50
P Madu 2	4x	7	3	8	2.67
AA cv. Rose	2x	8	2	42	21.00
AA cv. Rose 1	4x	7	3	0	0.00
25447-S7	2x	8	1	1	1.00
25447-S7 1	4x	5	4	40	10.00
25447-S7 2	4x	8	2	6	3.00
Total 2x		42	14	302	21.57
Total 4x		47	14	81	5.79

5.4 Discussion

Induced polyploidization is a useful breeding tool for creating novel genetic variation for cultivar improvement and could open possibilities to utilize existing pVAC diversity within the diploid genepool for biofortification in *Musa* spp. Polyploidy is believed to impart

phenotypic changes such as increased cell nuclei and size, leading to increased plant organs (gigas effect) as a consequence of gene duplications (Hegarty et al. 2013; Sattler et al. 2016). Other resulting physiological or phenological characteristics may include larger leaves, greater vigour and biomass, increased resistance to biotic and abiotic stress, higher content of chemical substances, altered flowering time, increased fertility and better post-harvest quality (Dhooghe et al. 2011). Although there are several reports in the induction of polyploidy in bananas, most of these reports focus on *in vitro* polyploidization protocols without a comprehensive report on evaluation of induced tetraploids in the field. Thus, in this study, tetraploids obtained from six diploid genotypes were characterized for their agronomic attributes, pVAC contents and fertility, in comparison with their diploid progenitors.

From field observations, induced tetraploids showed distinct morphological characteristics when compared to the diploid progenitors. Induced tetraploids were characterized by long curved leaves with weak petioles resulting in a drooping leaf habit or fallen/broken leaves, as previously observed in banana (Van Duren et al. 1996; Bakry et al. 2007; Do Amaral et al. 2015). Increased leaf size following polyploidization as a result of cell division and expansion has also been reported in several other plant species, with wide applications in the improvement of leaf size and flowers of ornamental plants (Huang et al. 2015; Tamayo-Ordóñez et al. 2016).

With regards to phenology (Table 5.2), longer days to flowering (28%) were observed in induced tetraploids than original diploids, but once plants had flowered, tetraploids matured faster (25%) than diploids. Plant height and girth at flowering was not significantly affected by doubling but 47% less suckering was observed in induced tetraploids than in diploid plants. This was in contrast to observations by Do Amaral et al. (2015), who reported that plant height and pseudostem diameter increased while number of suckers decreased with polyploidization in Pisang Lilin. Variable changes in yield attributes following polyploidization, were also recorded. Although tetraploids had larger fruits, this did not necessarily result in increased bunch weight because of the associated decrease in number of hands, number of fruits and fruit length. Do Amaral et al. (2015) also observed fewer fruits in the tetraploid Pisang Lilin in the first cycle of evaluation, which later increased in the second cycle.

Polyploidization is known to have played a key role in plant evolution and speciation, leading to better adaptation. While it is expected that induced polyploidization would result in superior traits, this varies among crop species. For example, more vigorous plants with larger flowers were observed in the tetraploid Snowdrop windflower (Zahumenická et al.

2018) while a decreased growth rate or plant vigour was observed in tetraploid apples (Xue et al. 2017). Generally, polyploidy is induced *in vitro* via the use of toxic anti-tubulin compounds such as oryzalin, which prevent microtubule formation and inhibits mitosis during metaphase, affecting plant growth and vigour. Podwyszyńska et al. (2015) reported that induced tetraploids of daylily exhibited poorer growth than original diploids in the first year but became more vigorous in the second year, possibly attributable to residual toxic effects of anti-mitotic agents. In the current study, only a small number of plants were analysed for one cycle, hence evaluation of larger numbers of plants are necessary.

Carotenoids are widely distributed in nature and specifically pVACs present in edible plant parts are important for human health. The effect of polyploidy on compositional traits have been studied in some plant species, indicating increased or decreased concentrations (Trojak-Goluch and Skomra 2013; Zahumenická et al. 2018). However, studies specific to carotenoids are limited. Carotenoids often present in banana fruit pulp are mainly the pVACs α-carotene and β-carotene (*cis* and *trans* versions) with smaller quantities of lutein (Davey et al. 2009). Using HPLC, the carotenoid content of banana fruit pulp of tetraploids and diploid plants were assessed to determine the effect of doubling. Polyploidization led to a significant decrease in pVACs in induced tetraploids when compared to diploid progenitors, which was associated with a corresponding increase in contents of the non-pVAC lutein. Trojak-Goluch and Skomra (2013) also reported significantly lower contents of essential oils in tetraploid hops in comparison to their diploid counterparts. On the other hand, Sanwal et al. (2010) reported higher gingerol content and antioxidant activity in tetraploid clones of ginger than that of the original diploids. The expectation is that whole genome duplication may lead to increased gene expression from increased gene dosage, resulting in enhanced contents of compositional traits. However, this is not always the case as polyploidization may also lead to genome alterations affecting the expression of key genes implicated in hormonal regulation of plant developmental pathways (Sanglard et al. 2017; Xue et al. 2017). Polyploidization may also result in a changed post-transcriptional regulation and translational modification changes of proteins, which play a role in plant biological processes (Wang et al. 2017).

The ability to generate progeny depends on gamete fertility, which is known to vary among genotypes. Understanding the pollen viability as an indicator of male fertility and seed set as an indicator of female fertility is critical to ascertain the potential for use of induced tetraploids in hybridization. The absence of pollen in diploid and tetraploid Sowmuk and Beram (not included in pollen viability studies) is linked to the rapid degeneration of the male bud before maturity. While some diploids may be infertile, fertility may be restored at the tetraploid level. Bakry et al. (2009) reported male and female fertility in tetraploids

induced from interspecific AB clones, which were otherwise sterile at the diploid level. In this study, it was observed that diploid and tetraploid versions of genotypes tested had high pollen viability (>70%), which was indeed higher than that of the male fertile diploid Calcutta 4. Notwithstanding, the tetraploid Galeo and AA cv. Rose recorded lower pollen viability than their original diploids. However, for P Madu and 25447-S7, pollen viability of tetraploid versions were comparable to, or higher than that of their corresponding diploids. The pollen viability values for induced tetraploids were higher than the 31 to 62.6% viability observed by Soares et al. (2016) on 12 banana tetraploid hybrids assessed for pollen viability using TTC stain, which is a fast and reliable method of accessing pollen viability. Goigoux et al. (2013) also detected viable pollen in doubled diploid Mlali clones Chicame 4x and Paka 4x, which was not significantly different from the diploids, indicating their potential as male parents in banana breeding.

All diploid genotypes set seeds when pollinated with Calcutta 4 as male parent. The fact that Sowmuk and Beram, which had no pollen, also set seeds, indicates that both diploids could be used as female parents in crosses for further improvement. The top two accessions with high pollen viability (Galeo 2x and AAcv Rose 2x) also had the highest number of seeds per bunch when pollinated with Calcutta 4. This study has shown that seed fertility in induced tetraploids is variable. Cultivars P Madu and 24447-S7 were seed fertile at the diploid and tetraploid level while all other cultivars were only seed fertile at the diploid level. More seeds per bunch in some clones of P Madu and 25447-S7 were also observed compared to their original diploid. Do Amaral et al. (2015) also observed that the tetraploid Pisang Lilin pollinated with AA cultivars set seeds, while the original diploids were seed sterile. However, it would be precarious to conclude that induced tetraploids were seed sterile where only four or less pollinated bunches were harvested. Further evaluations of larger numbers of crosses are needed for confirmation.

The prevailing ploidy levels in banana include diploids, triploids and tetraploids, but triploids are believed to be the optimum ploidy level, since they exhibit better agronomic characteristics and produce non-functional gametes, which ensure seedlessness. In the banana breeding scheme, tetraploids are synthesized from 3x-2x crosses, and are further crossed with male diploid plants to generate triploids. Therefore, the goal for induced polyploidization in banana breeding is to directly synthesize tetraploids which can be crossed with elite diploids to generate triploids, while keeping the maximum genetic constitution of the diploid at the tetraploid level. Bakry et al. (2009) crossed induced tetraploid clones from Guyod, Galeo, IDN110 and Tjau Lagada with Calcutta 4 and obtained 347 hybrids, which were predominantly (98%) triploids. Oselebe et al. (2010) also pointed out the predominance of triploids in progenies from 4x - 2x crosses. Although

induced tetraploids generally showed inferior agronomic and pVAC traits in comparison to original diploids, it is expected that these tetraploids will be useful for the generation of triploids with superior traits when crossed with elite diploids. However, it will be necessary to further evaluate hybrids from crosses with induced tetraploids to ascertain that traits of interests are ultimately expressed in the triploid progeny. Moreover, once tetraploid lines are produced, other desirable traits such as disease and pest resistance may be introgressed by incorporating different diploid cultivars and hybrids in the breeding scheme.

5.5 Conclusions

Biofortification through conventional breeding is becoming popular as a trusted approach to tackle micronutrient deficiency and breeding programmes are utilizing available diversity to enhance micronutrient levels in staple crops. Although considerable diversity exists for pVACs in *Musa* genetic resources, genetic enhancement for pVAC content is still limited. The use of *in vitro* polyploidization was explored as a breeding tool for banana biofortification through conventional breeding. Tetraploids generated from *in vitro* polyploidization of diploid banana cultivars were characterized for their agronomic traits, pVACs content and fertility. Induced tetraploid plants showed distinct morphological characteristics but generally had inferior agronomic characteristics and pVACs when compared to the original diploids. Preliminary fertility assessments indicated that induced tetraploids were pollen fertile and seed fertile, hence could be used for the synthesis of triploid hybrids. It was concluded that polyploidization has potential for inducing variation in bananas and would be useful in the context of biofortification to utilize valuable traits in high pVAC diploids, which will otherwise remain inaccessible for breeding.

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CHAPTER 6

General conclusions and recommendations

Micronutrient deficiencies, such as VAD, are serious public health issues in most developing countries. Strategies like dietary diversification, food fortification and nutrient supplementation have been utilized to combat micronutrient deficiencies. Although these strategies are quite affordable and accessible in developed countries, the majority of resource-poor populations in developing countries particularly in sub-Saharan Africa still rely predominantly on starchy staples (such as rice, maize, cassava, potatoes and banana) with limited amounts of basic micronutrients. Biofortification, the process by which the nutritional quality of food crops is improved through agronomic practices, conventional plant breeding, or modern biotechnology, offers an affordable long-term strategy to provide essential nutrients to resource-poor populations. Consequently, crop breeding programmes have recently engaged in biofortification, tapping into natural variability of high pVAC in crop varieties to tackle VAD in tropical countries. Conventional breeding of maize, cassava and sweet potato has led to enhanced levels of pVACs and biofortified varieties have been distributed to several countries.

The IITA plantain breeding programme aims at developing improved plantain cultivars with good yield, disease and pest resistance and good fruit quality for processing and consumption as desired by consumers. Recently, pVAC enhancement was added to the breeding goals aiming towards the deployment of high pVAC varieties to tackle VAD. Although conventional breeding is possible in banana, it is often limited by ploidy level variations, sterility, parthenocarpy, long crop cycles and poor seed germination. Thus, the broad goal of this study was to assess the variability of fruit pVAC content in banana cultivars and hybrids present in the breeding programme and investigate the potential of induced polyploidization as a breeding approach towards plantain biofortification. The specific objectives contributing to this goal were to 1) evaluate the variability of fruit carotenoid content and profiles in different types of bananas (plantains, *M. acuminata* cultivars and hybrids) present in the IITA banana germplasm collection 2) assess the carotenoid composition and profiles of plantains at different stages of ripeness to understand the effect of ripening on pVAC content and 3) determine the effect of induced polyploidization on agronomic attributes and pVAC content and to evaluate the fertility of induced tetraploids.

HPLC and spectrophotometry were used to screen a wide collection of banana cultivars and hybrids to determine variability in fruit pVAC content and identify genotypes with potential for biofortification. HPLC is most appropriate for the separation and quantification

of pVACs to determine vitamin A activity but spectrophotometry is also a useful low-cost method for large scale screening, especially for crops where the carotenoid profiles are well known. A strong correlation observed between these methods in this study confirms the potential for spectrophotometry as a rapid method for screening large sets of banana germplasm especially in a breeding programme. Moreover, results from this study also confirms that banana has a relatively simple carotenoid profile consisting mainly of pVACs α-carotene and β-carotene and the non-pVAC lutein. The use of spectrophotometry as opposed to HPLC will be advantageous as a time-saving and cost-efficient option for initial selection in large population screening trials.

Critical sets of germplasm required for plantain breeding include plantain cultivars (local cultivars requiring improvement), *M. acuminata* cultivars (with desirable traits for introgression) and bred-hybrids (parents and advanced lines that already have a combination of desirable traits). This study recorded a wide variation in pVAC content (>30 fold) within the IITA collection containing all three sets of germplasm. The IITA collection represents only a fraction of the existing banana diversity, but constitutes the highest number of genotypes evaluated for pVACs from a single location for plantain cultivars, *M. acuminata* cultivars or bred hybrids. High pVAC *M. acuminata* cultivars were identified with BCE values higher than that of the plantain cultivar with the highest content (10.65 µg g^{-1} FW), which will primarily be explored for use as parents in hybridization schemes aiming towards pVAC enhancement, since diploids are essential for banana breeding. Such high pVAC diploids will also be used to generate populations aiming to study the genetics of pVAC accumulation in banana fruit pulp, which is currently not known. On the other hand, high pVAC plantains and hybrids with consumer preferred traits will be explored for direct dissemination to rural communities. A first step towards this would be to evaluate high pVAC genotypes in replicated trials in several locations to elucidate stable and heritable genetic variance for pVACs in combination with other preferable traits, and to assess pollen and seed fertility towards hybridization and selection.

Plantains are a specific banana sub group with a characteristic orange-yellow starchy pulp, which are mainly consumed after cooking. Plantains are a major staple in West and central Africa, particularly in Ghana, Nigeria, Ivory Coast, Cameroon and Congo. They are commonly processed for direct consumption or as components of other food preparations through several methods including roasting, boiling, frying and drying at various stages of ripeness. Nine plantain cultivars across the three main plantain types (French, False Horn and Horn) were screened for pVACs at the unripe, ripe and overripe stage. This study indicated that 1) over 80% of carotenoids in cultivars across all plantain types and all ripening stages are pVACs 2) French plantains had slightly higher BCE contents than

False Horn and Horn types, but this difference was only significant at the unripe stage and 3) pVACs and TC decreased significantly from the unripe to the ripe and overripe stages for all cultivars sampled. In summary, this indicates that plantains are a good target for biofortification and that green fruits could yield more pVA than ripe and overripe fruits. Since plantains are consumed after cooking, which sometimes involves the addition of high pVA ingredients such as palm oil, there is a need to access the retention of pVA in plantain-based food preparations to effectively ascertain their potential for biofortification. It is also possible that enzymatic or oxidative degradation may be responsible for the decrease in carotenoid contents with ripening. Therefore, more research is warranted to understand the mechanism of carotenoid accumulation and degradation during fruit development in plantains and to ascertain the possible influence of genotypes or plantain types.

Induced polyploidization was also explored as a breeding approach for pVAC improvement in bananas. An important output of this study is the 10 induced tetraploids derived from six diploid cultivars, which will serve as new sources of genetic variation. Induced tetraploids developed like normal plants in the field and had distinct morphological characteristics, but unfortunately displayed inferior agronomic and pVAC traits compared to the original diploid cultivars, limiting their possible use as cultivars. However, preliminary fertility assessments indicated that induced tetraploids are seed fertile and pollen fertile, hence could be used in crosses with elite diploids to generate triploid hybrids. In this regard, it will be paramount to evaluate progeny from cross combinations with induced tetraploids to confirm that tetraploids can indeed transmit the phenotypes of the original diploids to their progeny or serve as high combiners for pVAC and other desirable agronomic and disease/pest resistance traits. Consequently, it will be interesting to subject other high pVAC diploids to polyploidization to generate new genetic stocks for breeding pVAC enhanced triploid varieties.

This study is the first comprehensive investigation on prospects of pVAC biofortification in plantains through conventional breeding. The findings underpin the integration of pVAC improvement in existing breeding efforts targeting the development of plantain hybrids which combine disease and pest resistance with other farmer preferred traits. A combination of high pVACs with disease/pest resistance and other farmer preferred agronomic traits is imperative for plantain biofortification to have nutritional impact and effectively address micronutrient deficiency. Induced polyploidization in combination with other emerging molecular tools such as GWAS and GS, which facilitate genetic studies and marker development, will speed up banana breeding. In a broader context, outputs of

this research is also relevant for pVAC biofortification efforts targeting other types of bananas worldwide.

APPENDICES

Appendix 1 Origin and classification of plantain cultivars

Cultivar name	Bunch type	Origin [a]
Dwarf French Plantain	French	Honduras
French Reversion	French	Nigeria
ITC.0022 Mulolou	French	Gabon
ITC.0028 Nazika	French	Congo
ITC.0033 Bungaoisan	French	Philippines
ITC.0054 Diby 2	French	Ivory Coast
ITC.0075 Gabon 4	French	Gabon
ITC.0103 Moungeli	French	Gabon
ITC.0109 Obino L'Ewai	French	Nigeria
ITC.0112 BobbyTannap	French	Cameroon
ITC.0142 Msisa	French	Burundi
ITC.0203 Niabang	French	Cameroon
ITC.0206 Lifongo Liko	French	Cameroon
ITC.0215 Mbi Egome 1	French	Nigeria
ITC.0219 Apem Pa	French	Ghana
ITC.0231 Apem Onniaba	French	Ghana
ITC.0232 Egjoga	French	Nigeria
ITC.0282 Banane Serpent	French	Guadeloupe
ITC.0324 Red Plantain Hembra	French	Honduras
ITC.0325 Wine Plantain	French	Honduras
ITC.0389 Zue Ekon	French	Cameroon
ITC.0487 Ntanga 3	French	Nigeria
ITC.0496 Cantebalon	French	Cameroon
ITC.0499 Obubit Ntanga 2	French	Nigeria
ITC.0511 Nselouka	French	Ivory Coast
ITC.0519 Obubit Ntanga GM	French	Nigeria
ITC.0635 Dominico Macho	French	Colombia
ITC.0636 Dominico 500	French	Colombia
ITC.0964 Ovang	French	Cameroon
ITC.1397 French Reversion Red Pseudostem	French	Costa Rica
Obubit Ntanga 1	French	Nigeria
Purple Plantain	French	n/a
Red Plantain	French	Nigeria
Walungu 8	French	Burundi
ITC.0015 Essang	False Horn	Gabon
ITC.0017 Gabon 2	False Horn	Gabon
ITC.0041 Didiedi	False Horn	Ivory Coast
ITC.0044 Niangafelo	False Horn	Ivory Coast
ITC.0098 Baka	False Horn	Gabon
ITC.0111 Agbagba	False Horn	Nigeria
ITC.0208 Atali Kiogo	False Horn	Nigeria
ITC.0209 Bise Egome	False Horn	Nigeria
ITC.0223 Apantu	False Horn	Ghana
ITC.0229 Abomienu	False Horn	Ghana
ITC.0233 Mbirinyong	False Horn	Nigeria
ITC.0236 Ngok Egome	False Horn	Nigeria
ITC.0235 Obubit Ukom	False Horn	Nigeria
ITC.0489 Mimi Abue	False Horn	Nigeria
ITC.0515 Okoyo Ukom	False Horn	Nigeria

ITC.0516 Eberedia Ukom	False Horn	Nigeria
ITC.0517 Orishele	False Horn	Nigeria
ITC.0559 Curare Enano	False Horn	Costa Rica
ITC.0628 Harton Maqueo	False Horn	Colombia
ITC.0630 Dominico Harton Rojo	False Horn	Colombia
ITC.0641 Dominico Rojo	False Horn	Colombia
ITC.0642 Harton Tigre	False Horn	Colombia
ITC.0645 Platano Harton	False Horn	Colombia
ITC.0965 Bo Ahiu-Abue	False Horn	Nigeria
ITC.1129 Big Ebanga	False Horn	Cameroon
ITC.1131 Moto Ebanga	False Horn	Cameroon
MOO9	False Horn	n/a
A0 157	False Horn	n/a
ITC.0185 3 Hand Planty	Horn	Cameroon
ITC.0121 Ihitism	Horn	Nigeria
ITC.0128 Tshambunu	Horn	Burundi
ITC.0224 75.19S	Horn	Nigeria

[a] origin as stated on MGIS database (www.cropdiversity.org) and (Swennen, 1990).

Appendix 2 Origin and classification of *M. acuminata* cultivars

Accession name	Origin [a]	ssp., group, subgroup [a]	Cluster [b]	Ploidy	Category [c]
ITC.0308 Huwundu Vita	PNG	AA Subgr. Pisang jari buaya		2x	PNG cultivars
ITC.0373 Uwati	PNG	AA	AA cv. ISEA2	2x	PNG cultivars
ITC.0591 Kasaska*	PNG	AA		2x	PNG cultivars
ITC.0605 Japaraka no.2	PNG	AA		2x	PNG cultivars
ITC.0777 Pitu	PNG	AA		2x	PNG cultivars
ITC.0779 Tangamor*	PNG	AA		2x	PNG cultivars
ITC.0780 Mamakila	PNG	AAA		2x	PNG cultivars
ITC.0792 Niukin	PNG	AA		2x	PNG cultivars
ITC.0794 Lalalur*	PNG	AA		2x	PNG cultivars
ITC.0796 Kirkirnan	PNG	AA		2x	PNG cultivars
ITC.0834 Kungor	PNG	AA		2x	PNG cultivars
ITC.0838 Bega	PNG	AA		2x	PNG cultivars
ITC.0868 Porapora	PNG	AA		4x,2x/4x	PNG cultivars
ITC.0932 Gilasalasa	PNG	AA		2x	PNG cultivars
ITC.0989 Tagomor	PNG	AA		2x	PNG cultivars
ITC.1011 Kospuke	PNG	AA		2x	PNG cultivars
ITC.1019 Pongani	PNG	AA		2x	PNG cultivars
ITC.1210 Marakudu*	PNG	AA		2x	PNG cultivars
ITC.1214 Terema	PNG	AA		2x	PNG cultivars
ITC.0078 Wh-o-gu	PNG	AAA		3x	PNG cultivars
ITC.0595 Pagatau	PNG	AAA		3x	PNG cultivars
ITC.0920 Dimaemamosi	PNG	AA		3x	PNG cultivars
ITC.1446 Makyughu II*	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1452 Huti (Shumba Nyeelu)	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1454 Makyughu I	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1455 Mshale Mlelembo*	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1456 Huti RB	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1466 Nshonowa*	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1468 Kahuti	Tanzania	AA Mshare		2x	AA cv. mshare
ITC.1544 Mlelembo	Tanzania	AA Mshare		3x	AA cv. mshare

ITC.1552 Ndyali	Tanzania	AA Mshare	AA cv. African	2x	AA cv. mshare
ITC.1559 Huti green bell	Tanzania	AA Mshare	AA cv. African	2x	AA cv. mshare
ITC.1561 Makyughu 2-Mshare	Tanzania	AA Mshare	AA cv. African	2x	AA cv. mshare
ITC.0254 Madang*	Unknown	acuminata ssp. *banksii*		2x	AA cv. *banksii*
ITC.0266 Sowmuk	Unknown	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0269 Niyarma Yik	Unknown	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0471 Bebek	Indonesia	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0600 Waimara	PNG	AA	AA cv. *banksii*	2x	AA cv. *banksii*
ITC.0603 Somani*	PNG	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0809 Maleb	PNG	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0810 Sihir	PNG	AA	AA cv. *banksii*	2x	AA cv. *banksii*
ITC.0849 Sepi	PNG	AA	AA cv. *banksii* sensu	2x/4x	AA cv. *banksii*
ITC.0882 Kwosriake	PNG	AA	AA cv. *banksi*i sensu	2x	AA cv. *banksii*
ITC.0888 Wikago	PNG	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0892 Pai ka*	PNG	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0894 Tainga	PNG	AA	AA cv. *banksii*	2x	AA cv. *banksii*
ITC.1187 Tomolo	PNG	AA	AA cv. *banksii* sensu	2x	AA cv. *banksii*
ITC.0259 Galeo	Unknown	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0294 Pitu	Unknown	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0298 Beram	Indonesia	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0601 Hung Tu	PNG	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0612 Mambee Thu	Unknown	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0869 Mala*	PNG	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0884 Awondaeke	PNG	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0939 Fu Des	PNG	AA	AA cv. IndonTriNG	2x	AA cv.
ITC.0310 Morong Princesa*	Philippines	AA Subgr. Pisang jari buaya	AA cv. P. Jari Buaya	2x	Assorted
ITC.0312 Pisang Jari Buaya*	Malaysia	AA Subgr. Pisang jari buaya	AA cv. P. Jari Buaya	2x	Assorted
ITC.1121 Pisang Lilin	Unknown	AA	AA cv. ISEA 1	2x	Assorted
ITC.1150 Morong Princesa	Unknown	AA	AA cv. ISEA 2	2x	Assorted
ITC.0299 Guyod	Philippines	AA	AA cv. Indon TriPh	2x	Assorted
ITC.0587 Pisang Rajah (South	Unknown	AAB Subgr. Pisang Rajah	40. AAB P. Raja(h)	3x	Assorted
ITC.0712 AAcv Rose	Indonesia	AA	M. acuminata ssp.	2x	Assorted
ITC.0317 Umbarim	Unknown	AA Subgr. Pisang jari buaya		2x	Assorted

ITC.1371 Chuoi cau man	Vietnam	AA sucrier	2x	Assorted
ITC.0306 Medja*	Indonesia	AAA	3x	Unclassified
ITC.0460 Padri	Indonesia	AA	4x	Unclassified
ITC.0678 Pisang Jeran	Indonesia	AA	2x	Unclassified
ITC.0695 Pisang Perecet	Indonesia	AA	2x	Unclassified
ITC.0409 Pa Patthalong	Thailand	AA	2x	Unclassified
ITC.0413 No. 110	Thailand	AA	2x	Unclassified
ITC.0663 Khai Nai On	Thailand	AA	2x	Unclassified
ITC.0432 Pamoti-On	Philippines	AA	2x	Unclassified
ITC.0436 Tin-Yo I-King*	Malaysia	AA	2x	Unclassified
ITC.0442 Gu Nin Chio	Malaysia	AA	2x	Unclassified
ITC.0276 Pisang Madu	Unknown	AA	2x	Unclassified
ITC.0279 Bie Yeng	Unknown	AA	2x	Unclassified
ITC.0507 Pisang Madu	Unknown	AA	2x	Unclassified
ITC.0532 Khai (Kampengpeth)	Unknown	AA	2x	Unclassified
ITC.1252 Datil*	Unknown	AA	2x	Unclassified
Morong Princesa	Unknown	AA	2x	Unclassified

PNG = Papua New Guinea, [a] country of origin and classification (group and subgroup) as stated on MGIS database (www.cropdiversity.org), [b] specific molecular grouping (cluster) as described in Christelová et al. 2017, [c] PNG cultivars originated from Papua New Guinea; AA cv. Mshare are cultivars from the 'Mlali' subgroup which originated from East Africa, AA cv. *banksii* are cultivars of the ssp. *banksii* clusters; AA cv. IndonTriNG are cultivars of the Indonesian Triangle and new Guinea cluster; Assorted represents cultivars with diverse subgroups or clusters; unclassified represents cultivars with undefined subgroup and cluster, *cultivars with only one replicate hence not included in analysis but results are presented in Appendix 4.

Appendix 3 Pedigree of hybrids

Genotype	Ploidy	Pedigree
1297-3	2x	French reversion x Calcutta 4
1448-1	2x	0bino l'Ewai x Calcutta 4
2829-62	2x	Bobby Tannap x Calcutta 4
28401	2x	11669-1 (0bino l'Ewai x Calcutta 4) x 9839-3 (Calcutta 4 x Padri)
29603	2x	612-74 (Bluggoe x Calcutta 4) x 1297-3 (French reversion x Calcutta 4)
29650	2x	9839-2 (Calcutta 4 x Padri) x 8075-7 (SH 3362 x Calcutta 4)
25291-1 R10P13	2x	n/a
25291-1 R1P3	2x	n/a
25291-1 R3P12	2x	n/a
25291-1 R7P24	2x	n/a
25291-1A	2x	2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
25291-S26	2x	2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
25291-S26 R10P34	2x	n/a
25291-S4 R6P13	2x	n/a
25291-S62	2x	2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
25291-S89	2x	2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
25447-S7	2x	2829-62 (Bobby Tannap x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
25447-S7 R2P8	2x	n/a
25447-S7 R3P4	2x	n/a
25447-S7 R4P11	2x	n/a
25447-S7 R4P30	2x	n/a
25447-S7 R5P26(4)	2x	n/a
25447-S7 R8P27	2x	n/a
25447-S7 R11P10	2x	n/a
8075-7	2x	SH 3362 x Calcutta 4
9128-3	2x	Tjau lagada x Pisang lilin
9839-3	2x	Calcutta 4 x Padri
SH 3142	2x	Intermating Pisang Jari Buaya

SH 3362	2x	SH3362 (SH3217 X SH3142), SH3217(SH2095 X SH2766), SH2095 [(Sinwobogi X Tjau lagada) X (wild malaccensis X Guyod)], SH2766 [Tjau lagada X (wild malaccensis X Guyod)], SH3142 (Intermating Pisang Jari Buaya)
24408-S22	3x	612-74 (Bluggoe x Calcutta 4) x Calcutta 4
30456-1	3x	612-74 (Bluggoe x Calcutta 4) x 8075-7 (SH 3362 x Calcutta 4)
30456-2	3x	612-74 (Bluggoe x Calcutta 4) x 8075-7 (SH 3362 x Calcutta 4)
33657-2	3x	612-74 (Bluggoe x Calcutta 4) x 1297-3 (French reversion x Calcutta 4)
33657-3	3x	612-74 (Bluggoe x Calcutta 4) x 1297-3 (French reversion x Calcutta 4)
PITA 21	3x	1658-4 (0bino l'Ewai x Pisang lilin) x 2829-62 (Bobby Tannap x Calcutta 4)
PITA 22	3x	2796-5 (Bobby Tannap x Pisang lilin) x 4400-8 (Bobby Tannap x Calcutta 4)
PITA 23	3x	4698-1 (0bino l'Ewai x Calcutta 4) x 5105-1 (Pisang lilin x Calcutta 4)
PITA 24	3x	7152-2 (Mbi Egome x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
PITA 25	3x	4698-1 (0bino l'Ewai x Calcutta 4) x D. F. Plantain
PITA 26	3x	548-9 (0bino l'Ewai x Calcutta 4) x 1297-3 (French reversion x Calcutta 4)
PITA 27	3x	6930-1 (0bino l'Ewai x Calcutta 4) x 2829-62 (Bobby Tannap x Calcutta 4)
30456-3	4x	612-74 (Bluggoe x Calcutta 4) x 8075-7 (SH 3362 x Calcutta 4)
30804	4x	6930-1 (0bino l'Ewai x Calcutta 4) x 4400-8 (Bobby Tannap x Calcutta 4)
33448-1	4x	612-74 (Bluggoe x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
33448-2	4x	612-74 (Bluggoe x Calcutta 4) x 9128-3 (Tjau lagada x Pisang lilin)
612-74	4x	Bluggoe x Calcutta 4
BITA 3	4x	Laknau x Tjau lagada
PITA 1	4x	0bino l'Ewai x Calcutta 4
PITA 2	4x	0bino l'Ewai x Calcutta 4
PITA 3	4x	0bino l'Ewai x Calcutta 4
PITA 4	4x	Bobby Tannap x Calcutta 4
PITA 5	4x	Bobby Tannap x Pisang lilin
PITA 6	4x	0bino l'Ewai x Calcutta 4
PITA 7	4x	0bino l'Ewai x Pisang lilin
PITA 8	4x	0bino l'Ewai x Calcutta 4
PITA 12	4x	0bino l'Ewai x Calcutta 4
PITA 14	4x	Mbi Egome x Calcutta 4
PITA 17	4x	Bobby Tannap x Calcutta 4
PITA 18	4x	0bino l'Ewai x Calcutta 4

Appendix 4 Carotenoid content of *M. acuminata* cultivars with a single replicate

Genotype	Category	Carotenoid content (µg g^{-1} FW)								
		Lutein	α-carotene	13-*cis* BC	9-*cis* BC	*Trans* BC	pVACs	BCE	TC HPLC	TC spec
ITC.0591 Kasaska	PNG cultivar	0.05	7.12	0.79	0.03	5.68	13.62	9.65	13.67	17.43
ITC.0779 Tangamor	PNG cultivar	0.39	6.14	0.98	0.76	2.42	10.29	6.35	10.68	8.56
ITC.0794 Lalalur	PNG cultivar	0.46	13.07	1.62	0.38	6.55	21.62	14.08	22.08	12.70
ITC.1210 Marakudu	PNG cultivar	0.39	7.01	1.01	0.20	3.78	12.01	7.90	12.40	12.30
ITC.1446 Makyughu II	AA cv. Mshare	1.31	0.83	0.13	0.03	0.29	1.27	0.78	2.58	2.68
ITC.1455 Mshale Mlelembo	AA cv. Mshare	1.23	0.07	0.07	0.03	0.04	0.21	0.13	1.44	1.51
ITC.1466 Nshonowa	AA cv. Mshare	2.82	0.26	0.10	0.03	0.02	0.41	0.21	3.22	2.70
ITC.0254 Madang	AA cv. *banksii*	0.90	4.86	0.59	0.14	2.97	8.57	5.77	9.47	9.99
ITC.0603 Somani	AA cv. *banksii*	0.37	9.09	1.28	1.59	5.86	17.82	11.84	18.19	19.93
ITC.0892 Pai ka	AA cv. *banksii*	0.47	0.20	0.13	0.11	1.09	1.53	1.31	2.00	1.74
ITC.0310 Morong Princesa	Assorted	1.30	1.06	0.58	0.17	2.11	3.92	3.02	5.23	3.21
ITC.0312 Pisang Jari Buaya	Assorted	0.18	1.71	0.70	0.14	0.64	3.19	1.92	3.37	4.52
ITC.0869 Mala	Unclassified	0.05	2.99	0.43	0.11	1.42	4.96	3.19	5.01	2.36
ITC.0436 Tin-Yo I-King	Unclassified	0.28	1.67	0.57	1.04	1.65	4.93	3.29	5.21	5.45
ITC.0306 Medja	Unclassified	0.97	1.14	0.64	0.05	0.52	2.34	1.43	3.31	3.63
ITC.1252 Datil	Unclassified	0.45	1.75	0.46	0.11	2.32	4.64	3.48	5.10	7.33

13-*cis* BC = 13-*cis* β-carotene; 9-*cis* BC = 9-*cis* β-carotene; *trans* BC = *trans* β-carotene; pVACs = total carotenoids with vitamin A activity; BCE = β-carotene equivalents; TC HPLC = total carotenoids determined by HPLC; TC spec = total carotenoids determined by spectrophotometry.