ABSTRACT

MUNOZ CASTILLO, BRYAN. Characterization of Edible Banana Germplasm for Nutritional Improvement and Resistance to Black Sigatoka Disease. (Under the direction of Dr. Massimo Iorizzo).

Bananas (Musa spp.) is the second most consumed fruit globally and a staple crop in tropical and sub-tropical regions. Given its high consumption, extensive efforts are ongoing to improve provitamin A carotenoid and bioactives contents and resistance to disease through breeding and other strategies. By achieving these goals, will make banana production more sustainable, and in the long term, it will contribute to improving consumer health.

To contribute towards these efforts, a collection of 27 edible banana accessions were evaluated in this study for carotenoid content, bioaccessibility, starch and sugar content, total phenolic content, and resistance to Black Sigatoka. The results of these analyses were used to: a) understand the relationships between carotenoid content and bioaccessibility and how these characteristics are affected by ripening treatments (natural vs. artificial) and the ripening stages; b) identify banana accessions with high phenolic content in the pulp and evaluate the effect that the post-harvest ripening has on phenolic content and sugar accumulation; c) understand how stable is Black Sigatoka resistance within a group of 18 edible banana accessions; and d) to characterize the defense response triggered by the *P. fijensis* effector protein PfAVR4 on the expression of five genes involved in the plant-pathogen interaction.

Carotenoid, sugar, total phenolic content, and Black Sigatoka resistance were found to be genotype dependent. Compared with Cavendish, the most commonly grown banana cultivar, several accessions had higher carotenoid, sugar, and phenolic content. In most accessions, carotenoid and sugar content increased from unripe to ripe fruit, while total phenolic content stayed stable during ripening. Bananas ripened with exogenous ethylene had a significantly

higher carotenoid and sugar content and carotenoid bioaccessibility, but a lower total phenolic content. Relative bioaccessibility was not significantly correlated with carotenoid content, but it had a low positive correlation with resistant starch. Six accessions had phenolic content in the pulp almost as high as in the peel and up to three-fold higher than what has been previously reported in banana.

Response to Black Sigatoka (*P. fijensis*) infection was evaluated under three conditions: 1) field; 2) in vitro using leaf discs; and 3) in controlled conditions using whole plants. These experiments confirmed that Calcutta IV, FHIA 25, and Ducasse are highly resistant, while Cavendish is highly susceptible. Bri Bri, an uncharacterized accession collected in Costa Rica, was identified as a new source of moderate resistance to Black Sigatoka, and that also had high carotenoid content and bioaccessibility, and sugar content. Resistant plants but not susceptible plants inoculated with the PfAVR4 effector - expressed a hypersensitive response (HR), compatible with a gene-for-gene resistance mechanism. The expression level of three resistance genes, named PR4, PR10, and R4, was consistently upregulated at 6-36 hours post-inoculation (HAI) with the PfAVR4 effector or conidia. Interestingly, in resistant plants inoculated with the - PfAVR4 effector, a very high expression of PR4 was detected 6 HAI, which coincided with the initial appearance of HR symptoms, including small necrotic lesions and upregulation of the peroxidase enzyme (POX). These observations suggest that PR4, which was previously identified to be upregulated in resistant plants inoculated with *P. fijensis*, is a strong candidate for being the gene that recognizes the PfAVR4 effector, which triggers the HR response and ultimately confers resistance to Black Sigatoka.

Overall the results of this study can inform banana breeding and production programs about material and strategy to improve nutrient delivery, bioactive content, and Black Sigatoka

resistance. In addition, it establishes the foundation to further understand the genetic and molecular mechanisms controlling these traits, which represent a critical step to developing an effective and sustainable crop improvement program.

Characterization of Edible Banana Germplasm for Nutritional Improvement and Resistance to Black Sigatoka Disease

by
Bryan Muñoz Castillo

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APPROVED BY:

_____	_____
Dr. Massimo Iorizzo Committee Chair	Dr. Colin Kay
_____	_____
Dr. Jean Beagle Ristaino	Dr. Mario Feruzzi
_____	_____
Dr. Mary Ann Lila	D. Penelope Perkins -Veazie

DEDICATION

You know how it is. You pick up a book, flip to the dedication, and find that, once again, the author has dedicated a book to someone else and not you.

Not this time.

Because we/

haven't yet met/

have only a glancing acquaintance/

are just crazy about each other/

haven't seen each other in much too long/

are in some way related/

will never meet, but will,

I trust, despite that, always think fondly of each other...

This one's for you.

With you know what, and you probably know why.

Neil Gaiman

BIOGRAPHY

Bryan Munoz was born in San Jose, Costa Rica. In 1994 at age 13, while reading the newspaper, he found a fascinating article about the Human Genome and how it was going to change the history of humankind. At that moment, he decided that he wanted to pursue a career in science.

He earned a scholarship for EARTH University in CR, where he graduated Magna Cum Laude with a degree in Agriculture in 2000. In 2004 he moved to Lincoln, Nebraska, to pursue a Master's degree in plant breeding and genetics at the University of Nebraska in Lincoln.

In 2008, he moved back to Costa Rica, where he started a career in Biodiplomacy at the prestigious Inter-American Institute for Cooperation on Agriculture (OAS). During his tenure, he learned the impact that science can have on policy and decision making, so he decided to go back to school in 2012 to pursue a Law degree at ULACIT University.

In 2017, while working as a faculty member at TEC University in Costa Rica, he started a project on Black Sigatoka detection in banana in conjunction with DOLE Costa Rica. That is how he learned about the opportunity of earning a Ph.D. degree at Dr. Iorizzo's Lab from NCSU, which brings us to now.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Introduction

1.1 Economic importance

Bananas constitute an essential source of income, food security, and nutrition for rural areas in many developing countries. In some countries, banana production represents one of the most profitable crops and primary drivers for economic growth. For example, in tropical countries, such as Costa Rica, banana exports constitute about 25% of the total agricultural exports' economic value, worth about 1.2 billion dollars in 2019 (PEN, 2019).

FAO's production index indicates that banana is a staple food in tropical countries. According to FAOstat, banana production reached 22.2 million metric tons in 2020, making it one of the world's most important food crops. This number may be underestimated as most bananas are produced and traded in local and regional production areas. It is estimated that in Africa and Asia, 70-80% of the production derives from small farmers using local varieties adapted to their region for over 1000 years (MUSANET, 2016, FAO 2021).

India, China, The Philippines, and Brazil are the largest banana producers (around 40 million tons per year) and consumers; their production is primarily sold in the domestic market. On the other hand, Ecuador and Costa Rica are the largest exporters of bananas, selling over 15 million tons per year during the past three years. The United States and the European Union are the two largest importers of banana (50% share of the market) (FAO,2021).

Preliminary estimates indicate that global exports of bananas (excluding plantain) have grown 15% in the past five years, despite an all-time low in production due to adverse weather and the spread of the *Fusarium* Wilt (FAO, 2016). This increase resulted from banana supply growth from Ecuador, Costa Rica, and Colombia, three of the five leading exporters. The successful containment of the *Fusarium* TR4 outbreak and the implementation of mitigation

strategies for COVID-19 in their plantations at the early stages of the pandemic helped minimize disruptions to their ability to supply bananas. Following this trend, exports from Latin America and the Caribbean grew by an estimated 6.6% in 2020. Ecuador, the largest exporter of bananas, registered an estimated 5.6% percent growth in shipments reaching 6.9 million tons due to a rise of 22% in exports to the United States (FAO, 2021).

In contrast, the global net imports of bananas have only grown 0.2% since 2019. In the United States, the first net importer of bananas, the market remained unchanged at 4.1 million tons, while in the European Union, imports grew about 4.8 %. The difference between these two regions is that in Europe, there is an increasing demand for specialty bananas, where varieties that are considered more nutritious or environmentally friendly are gaining popularity. At the same time, in the United States, the increment of import prices with the rising unemployment rates in 2020 hampered the domestic demand for fresh fruits and produce (Mead *et al.*, 2020; Voora *et al.*, 2020, FAO, 2021).

When talking about banana production and banana trade, it is essential to note the substantial differences between production for the domestic market and trade production. While exporting countries tend to have plantation systems that require significant investments, countries that produce primarily for the domestic market have a more substantial share of family farming systems, growing local landraces with traditional farming techniques. Based on this distinction, about 30% of the world's trade production is controlled by major corporations (Chiquita, Del Monte, and Dole), and within that structure, around 30% of major corporation production is outsourced to small farmers (Vezina, 2016; Dodo, 2014). Another significant difference is that the export market is dominated by a single cultivar, Cavendish, and very limited efforts have been made to diversify this market.

Historically, banana cultivation for the trade market has had a tremendous socioeconomic impact in developing countries. Since the introduction of this crop for trade by the late 1800s, companies involved in the banana business contributed to building infrastructure and services that benefited not only the plantations but also the population living in the production regions (Elías-Caro & Vidal Ortega, 2013). At the same time, this crop has always been associated with unfair working conditions and exploitation of the so-called Banana Republics, where corporations owned the land, the production, the distribution network, and the infrastructure. However, since the mid-nineties, this trend has been changing due to several lawsuits of worker unions, economic distress, and mainly a switch in the political views of buyers within the US and EU (Douglas & Reynolds, 2000; Dodo, 2014).

Large banana corporations (e.g., Dole, Chiquita) started switching from farming to specializing in marketing and distribution while outsourcing production to smaller farmers. However, most of these companies still exert tremendous power over markets and distribution chains. And at the same time, new smaller and domestic fresh produce companies entered the banana export/distribution markets, creating more opportunities for small and medium farmers to access the import market (Dodo, 2014). This new scenario is creating more competition in the export market, and it might open more opportunities for diversifying banana cultivars offered in this large trade market.

1.2 Banana importance as a source of nutrients and bioactives

Most bananas are produced by small-scale farmers and used for local consumption as a food security crop. In multiple countries and regions of the world such as Africa, Asia Pacific, India, and Latin America, banana is consumed up to three times a day, making it a staple food

for millions of people and providing 25% of the carbohydrates required in their diet (FAO, 2018). Banana is rich in nutrients and carbohydrates, but its nutrient profile and composition complexity are still a vast field to explore (MUSANET, 2016).

East Africa is the largest banana-producing and consuming region in Africa. Uganda is the world's second-leading banana producer, and the daily consumption of bananas in this country exceeds 1.6 kilograms per person, the highest in the world. At the same time, the Philippines and Papua New Guinea are the leading consumer countries for the Asia-Pacific region, and Colombia and Peru have the highest per capita consumption of plantain in Latin America (Van Asten & Staver, 2012).

Bananas have several uses other than food in some of these regions. The fruit and the plant are recognized as natural remedies and supplements for processed food and beverages. They have been used as a flavoring agent in cooking, for garment and furniture construction, and as a token in ceremonial practices (Paul *et al.*, 2017). At the same time, banana peels constitute 40% of the total weight of the fruit and have often been considered a by-product with no useful application or waste. Recent studies suggested that banana peel can be used as an ingredient for food enrichment and the extraction and isolation of health-beneficial bioactives. It also contains different types of starch and cellulose that can be used in various industries. Even as food, banana peels are gaining popularity as a meat replacement within the vegan community in the United States and Europe (Padam *et al.*, 2014; Heil 2021).

Banana is ubiquitous, popular, a fruit easy to digest by all age groups; it is a rich source of nutrients such as carbohydrates, carotenoids, vitamin C, and other bioactives, including polyphenols, active amines. Combined with the fact that banana is one of the most consumed fruits globally, especially in developing countries where a significant fraction of their population

suffers from malnutrition problems, makes this crop an ideal target for biofortification programs (Khoozani & Bekhit, 2019). Nowadays, banana's most studied nutrients and bioactives are carotenoids, polyphenols, and active amines due to their importance in nutrition (Van Asten & Staver, 2012).

Vitamin A is an essential component of a healthy diet. Indeed, Vitamin A deficiency can cause blindness, chronic infections, and death, and it affects about 20 million pregnant women, 140 million children in 118 countries (Borges & Maraschin, 2016). This vitamin is metabolized from fat-soluble pigments called carotenoids. Yet, not all the carotenoids are precursors of vitamin A (pVAC), and the most important carotenoids for the human diet are α-carotene, β-carotene, and β-cryptoxanthin (Davey *et al.*, 2009; Combs, G. *et al.*, 2017). Several studies have shown that bananas have consistently higher amounts pVAC content than other carotenoids. Fe'I banana, for example, is a variety that grows in the pacific islands; it has a characteristic deep yellow-orange pulp that contains the highest amount of β-carotene ever recorded in banana (8,508 µg/100g of fresh weight). Other carotenoids reported in banana pulp and peel α-carotene, lutein, zeaxanthin, and β-cryptoxanthin (Amah *et al.*, 2019; Lopes *et al.*, 2020).

Among other healthy metabolites present in banana fruit are phenolics. Polyphenols or total phenolics are a complex class of chemical compounds generally present as glycosides, esters, hydroxylated forms, and polymers in plants. Once consumed, they can be extensively modified by the digestive system and the liver, generating a diversity of bioactive metabolites that act as antioxidants and are related to numerous health benefits as an anti-inflammatory, anti-carcinogenic, and antimutagenic agents (Santangelo *et al.*, 2016). Banana pulp and peel can potentially be exploited in the food and pharmaceutical industries because of their high content of catechin and rutin. Several studies have reported catechin, epicatechin, and gallocatechin as

major compounds in the Musa family, while gallic acid, protocatechuic acid, 7-O-neohesperoside naringenin, and hydroxycinnamic acids have also been identified in lesser quantities in banana pulp (Borges *et al.*, 2019; Lopes *et al.*, 2020).

Biologically active amines are a group of chemicals whose function in fruit and vegetables has been related to shelf life and the overall quality of the product (Islam *et al.* 2016). These chemicals also function as psychoactive or vasoactive metabolites in humans and are involved as hormonal regulators on glycogen metabolism. Banana pulp, and especially peel, are rich sources of active amines such as epinephrine, norepinephrine, serotonin, and dopamine, being the last two of particular interest for the pharmaceutical industry as they act as mood modulators. Insufficient amounts of dopamine and serotonin can lead to depression, anxiety, and loss of motor control (Ghag & Ganapathi, 2018; Pereira & Maraschin, 2015).

Overall, given the large consumption of banana globally and its diverse content of nutrients and bioactive, banana can serve as a vehicle to boost the human diet with their healthy nutrients and bioactives.

1.3 Banana production challenges

The growth and intensification of agricultural commodities (e.g., bananas, pineapples, coffee) in large plantations happened worldwide during the 1980s and early 1990s. For instance, in Costa Rica, the acreage dedicated to bananas grew 20 fold during the 1980s. This growth was only possible under a mono-crop system, which usually requires a high density of plants under large amounts of agrochemicals inputs to sustain production and keep diseases at a manageable level (Arias *et al.*, 2004).

Another factor that contributed to expanding banana production was the use of a single cultivar (or somaclonal variants) over-time, which facilitated the development of standardized agronomical management practices and maximized profits. On the other end, due to the permanent cultivation of the same cultivar in the same area over the years, the agroecosystems within a commercial banana plantation have experienced a long-term evolutionary selection pressure that has left the crop susceptible to an increasing amount of pests and foliar diseases. At the same time, the number of pesticides needed to limit production losses has increased the diversity/number of pathogens that became resistant to those pesticides (Llaguno *et al.*, 2014). For these reasons, managing banana diseases represents the most important production challenge in banana cultivation. Disease management strategies need to guarantee production needs at a sustainable cost, but at the same time, they have a direct effect on the environment where large banana plantations are grown.

Banana production is affected by numerous pests and diseases, including bacterial infections such as Moko/Bugtok (*R.solanacearum*) and Xanthomonas wilt (*X.campestris*), pathogenic nematodes (*R.similis),* or fungal diseases such as *Fusarium* wilt of banana, also known as Panama disease (*F.oxysporum*) and Black Sigatoka (*P.fijensis*) (Pegg *et al.*, 2019; Blomme *et al.*, 2017; Soares *et al.* 2021).

Black Sigatoka has been identified as a significant constraint to the global production of bananas and plantain (Yonow *et al.*, 2014). The disease induces severe defoliation and reduces photosynthesis, biomass accumulation, and yield across most tropical and sub-tropical environments where bananas and plantains are cultivated. This disease represents one of the most severe biological threats to banana production for food security and export. In South America, where it is the most prevalent disease in banana production, small producers are most affected as

they do not have the technology or financial resources to sustain efficient disease control. Also, controlling Black Sigatoka disease by fungicide applications is possible, but it negatively impacts the environment.

One of the most controversial issues with banana cultivation is the environmental and health problems caused by the excessive use of pesticides and the transformation of ecosystems. In Costa Rica, pesticides are applied continuously throughout the growing season. Banana plantations are aerially sprayed with fungicides with up to 40-60 applications a year in order to control Black Sigatoka. At the same time, nematicides are applied manually up to two to four times a year, and herbicides eight to twelve times a year. Altogether, it sums up to 50 kg of active ingredient per hectare per year. This amount of active ingredient percolates the soil reaching the aquifers, leaving behind a high amount of heavy metals in water reservoirs and contaminating the drinking water and the soils (Corbana, 2011).

Public health hazards from using agricultural pesticides in banana have received increasing attention from the general public. During the ’80s, in Costa Rica, approximately 1,500 workers suffered permanent sterilization due to the continued exposure to a toxic nematicide called DBCP (1,2-dibromo-3-chloropropane) (Thrupp, 1991). At the same time, Mancozeb (manganese–zinc complex of ethylene-bis-dithiocarbamate), a common fungicide used to control Black Sigatoka, has become one of the most significant contaminants in banana plantations because of its toxicity. Studies have linked the accumulation of Mn and ethylene thiourea (byproducts of mancozeb degradation) with alteration of the thyroid function in women and poorer cognitive abilities in babies, both being a problem with the population that live in or near a banana plantation (Mora, 2018). Intensive agrochemical use pollutes water supplies,

contaminates soils, and can directly or indirectly affect workers' health and communities in general.

Given these challenges, developing sustainable (economically and environmentally) strategies to manage disease in banana production is crucial. Identifying and breeding new sources of resistance to control these diseases is a critical step to build toward these strategies (Cordoba & Jansen, 2014).

1.4 Banana biodiversity

Banana plants are monocots that belong to the order of *Zingiberales*, *Musaceae* family, distinguished by being large herbaceous plants with a rhizome, and a pseudostem, lacking an outer stem except when they are flowering. In that sense, banana plants are vaguely related to Poales, such as rice and sugarcane, but more closely related to ginger and ornamental plants like Heliconias and Bird of paradise. (Martinez *et al.*, 2012; Wang *et al.*, 2019).

The *Musaceae* family includes two genera: *Musa* and *Encete*, but it is open for debate if there is a third genus called *Musella*. Members of both genera are classified into four sub-groups: Eumusa, Rhodochlamys, Australimusa, and Callimusa. The first two groups have 11 chromosomes, and the second group with ten chromosomes (most of the edible bananas fall into the *Eumusa* group) (Wang *et al.*, 2019).

Additionally, members in *Eumusa* can be divided into four genetic backgrounds: *Musa acuminata* (the A genome), *Musa balbisina* (the B genome), *Musa schizocarpa* (the S genome), and *Australimusa* (the T genome). Edible banana first originated from the domestication of *M. acuminata* that lately crossed with *M. balbisiana*, resulting in modern cultivars with one or more copies of the A and B genome or a mix between the two (Wang *et al.*, 2019).

The *M. acuminata* genome displays unique intra-specific variation, while the *M. balbisiana* genome shows more enrichment in genes related to photosynthesis and secondary metabolism, supporting the idea that varieties containing the B genome display more vigor and resistance to biotic and abiotic stress (Chase & Laliberte, 2016; Wang *et al.*, 2019).

Currently banana groups are classified according to their genomes: diploids edible/inedible-wild varieties (AA, AB), triploids (AAA, AAB and ABB), and tetraploids (AAAA, AAAB). Some other edible species such as Fe'i, basjoo, or itinerans, belong to the S genome and cannot be classified within those groups (Chase & Laliberte, 2016).

According to MUSANET (2016), there are around 9,051 accessions of bananas curated by 56 institutes worldwide. Approximately 1,000 edible cultivars have been identified; nevertheless, most of the same varieties are grown in different places under different local names, and the lack of a standardized cultivar nomenclature is slowing banana research. Given all this biodiversity, 87% of banana production is grown at a small scale, using local varieties. But, only a few varieties are produced at a large scale for international trade, being Cavendish the most popular.

Millions of people worldwide depend on bananas as a source of food, income, and as part of their cultural heritage. Nonetheless, despite the growth of crop production and consumption, the continuous use of only a few cloned varieties has limited the use of banana biodiversity for crop improvement. Several banana accessions with improved nutritional characteristics (Amah *et al.*, 2019), drought tolerance (Vanhove *et al.*, 2012), and disease resistance (Zorilla-Fontanessi *et al.*, 2020), just to name a few, have been already identified, which demonstrates the potential of using banana biodiversity to face production challenges while improving its nutritional/health

potential. In the face of climate change, worldwide malnourishment, and growing diseases, and pest pressure, protecting and exploring banana biodiversity is imperative (Rai *et al.*, 2018).

1.5 Challenges and opportunities for banana breeding.

Given the environmental problems surrounding banana production, breeding has mainly been focused on improving resistance for diseases such as Black Sigatoka and, more recently, *Fusarium* TR4 (Alakonya *et al.* 2018). Also, since banana is a staple crop in many regions of the world suffering malnutrition, there has been an increasing interest in focusing some breeding efforts on nutrient biofortification. For example, for Vitamin A, very promising advances have been made towards identifying varieties rich in Provitamin A (pVAC) (Amah, 2018). Productivity, diseases resistance, yield, and high carotenoids are traits that have been prioritized in banana breeding, and accessions harboring these traits have been identified. However, using traditional breeding to introduce these traits into new banana cultivars is somewhat challenging (Amah *et al.*, 2019).

A breeding cycle in banana can take up to 20 years from crossing to the release of a new variety using traditional breeding schemes (CGIAR 2021). Seeds obtained from planned crosses rarely germinate, and hybrids are often recovered through in vitro embryo culture (Bakry, 2008). In summary, traditional breeding in banana is limited by parthenocarpy, low fertility, low seed viability, polyploidy and associated irregular meiotic behavior, long generation times, diverse genome configurations, and the narrow genetic base (Ortiz, 2015; Brown *et al.*, 2017).

On the other hand, the genetic engineering approach for biofortification is a viable technique in crops with slight genetic variability for nutritional characteristics (cassava, rice). For crops that are mass-produced and can easily reach people through markets (cereals, oilseeds,

banana), and for crops where traditional breeding gets extremely hard, notwithstanding their genetic variability (banana) (Garg *et al.,* 2018).

Some of the most noticeable benefits of the genetic engineering approach (including cisgenics, transgenics, gene editing) are: there are no taxonomic constraints and can use the entire genetic pool to reach their goal. It can use multiple genes that not only focus on the biofortification but also on bioavailability and agronomic traits such as water use or pest's resistance, and overall it can shorten the amount of time of development of a new variety as it focuses directly on the trait of interest without modifying other characteristics (Garg *et al.,* 2018). Nonetheless, most countries where crop biofortification is needed also have limited technological capacity to develop genetically engineered varieties. And even when those varieties can be created in other countries, there is a disconnection between the technology holder and the final user, which might result in mistrust of the product by the general public, and can make a biofortification project fail (Stone & Glover, 2017).

Transgenic approaches for pVAC biofortification in bananas have been explored. For example, Paul *et al.* (2018) reviewed the progress of the Banana21 project that started in 2005, aiming to develop banana lines high in pVACs for Uganda. Although some progress was made, the project faced the challenge of regulating and releasing the new genetically modified (GM) and biofortified variety. Genetic engineering techniques seem to offer a way for biofortification. However, most research laboratories still lack the skills to address the social, political, and environmental hurdles linked with the acceptance of GM crops (Lee, 2017; Amah, 2018).

Limited regulatory capacity and regulatory issues between countries and regions may become an issue when authorizing the release of a new variety. Whether for consumption or cultivation, some of the countries in need of bio-fortified crops have policies in place that

hamper the adoption of GM varieties. Thus, a hybrid approach between genetic engineering and the adoption of enriched varieties for local consumption would probably be best to deliver bio-fortified bananas to producers (IICA, 2008). Also, it is important to note that breeding efforts for biofortified banana are still limited to the assessment of pVAC content with narrow integration of research in food/human nutrition science.

Bananas have a considerable amount of health-related bioactive compounds that can lead to a more nutritious diet. It is cultivated by small farmers and large corporations alike, and it is consumed worldwide, becoming a reliable crop and a stable food source. It has vast biodiversity, and researchers are just starting to unveil the genetic diversity in local landraces. At the same time, because it is produced as a mono-crop, its production is threatened by diseases such as Black Sigatoka and *Fusarium* TR4.

The objectives of the studies presented here are to contribute to advancing breeding strategies for nutrition improvement, biofortification programs, and resistance to common diseases in the crop. A collection of 27 very diverse edible banana accessions were evaluated for carotenoid content, bioaccessibility, starch and sugar content, total phenolic content, and resistance to Black Sigatoka. The results of these studies were used to: a) understand the relationships between carotenoid content and bioaccessibility and how these characteristics are affected by ripening treatments (natural vs. artificial) and the ripening stages; b) identify banana accessions with high phenolic content in the pulp and evaluate the effect that the post-harvest ripening has on phenolic content and sugar accumulation; c) understand how stable is Black Sigatoka resistance within a group of 18 edible banana accessions; and d) to characterize the defense response triggered by the *P. fijensis* effector protein PfAVR4 on the expression of five genes involved in the plant-pathogen interaction.

These work outcomes will help to better understand how integration of food/human nutrition studies and post-harvest maturation strategies can successfully advance banana biofortification programs. Additionally, evaluation is made of genetic engineering or traditional breeding strategies for developing new banana cultivars resistant to Black Sigatoka, one of the most economically significant diseases in banana production.

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Chapter 2. Carotenoid content and bioaccessibility in a diverse collection of banana accessions ripened in different conditions

2.1 Introduction

Vitamin A is essential for human health (Gilbert, 2013) since it supports systemic functions such as vision, immunity response, and growth. For tissue maintenance, Vitamin A seems to signal the replacement of normal mucus-secreting cells with cells that produce keratin, such as the conjunctiva and cornea of the eye, the trachea, the skin, etc. For immunity, individuals with a micronutrient deficiency are typically more susceptible to infection than individuals with adequate vitamin A levels. This vitamin has also been observed to have antioxidant properties and has been associated with a reduced risk of cardiovascular disease, diabetes, and several cancer forms (Amah *et al.*, 2019; Murillo & Fernandez, 2016).

Vitamin A deficiency, or VDA, is the leading cause of blindness in children around the world. VDA may also cause death or fatal disease from severe infections and has been linked to HIV transmission from mother to baby. VDA has become a public health problem in more than half of all countries, especially in Africa and South-East Asia (FAO, 2018).

Vitamin A is the generic descriptor for chemical compounds with the qualitative biological activity of retinol, and they exist in natural products in various forms. One of the most common forms of these compounds is pre-formed retinoids stored in animal tissues. While in milk, meat, and eggs, vitamin A exists in several forms, mainly as long-chain fatty acid esters of retinol, the predominant one being retinyl palmitate (Combs, G. *et al.*, 2017).

Carotenoids are fat-soluble pigments synthesized by plants and microorganisms and can be found in green, orange, and yellow tissues. There are approximately 700 different types of carotenoids in nature; however, it is estimated that only 40 carotenoids can be metabolized in

humans, and only six of them can be detected in blood plasma (α-carotene, β-carotene, lutein, lycopene, β-cryptoxanthin, and zeaxanthin). Yet, not all of them are precursors of vitamin A (Fernandez-Garcia *et al.*, 2012). Only about 60 carotenoids can be cleaved by animals to yield at least one retinol molecule, the active form of vitamin A. These pigments are called pro-vitamin A carotenoids or pVACs, and the most important for the human diet are α-carotene, β-carotene, and β-cryptoxanthin. (Davey *et al.*, 2009; Combs, G. *et al.*, 2017).

To reduce VDA in developing countries, breeders, farmers, and plant and food scientists have been focusing their efforts on vitamin A biofortification in staple crops. Given that banana is produced worldwide and is already a staple crop in many countries, it makes it an ideal target for nutrient biofortification (Garg *et al.*, 2018).

Cultivated in over 160 countries, bananas (*Musa Spp.*) have their origin in South East Asia, and their adaptability and versatility contributed to their spread worldwide. Many cultures from Latin America, the Caribbean, South East Asia, Polynesia, and Africa have adapted banana fruit to their needs, making it a staple in their diets. Although there are over a thousand accessions of bananas and 87% of the banana production is for local food consumption, only a few varieties are available for exports, with Cavendish being the most popular (De Lagne *et al.*, 2009; Crouch & Crouch, 1996; MusaNet, 2016; Dale *et al.*, 2017).

Edible banana germplasm can be classified by consumption or use into: dessert bananas, cooking bananas, and plantains. The first group distinguishes itself from the other two because they are likely to be sweeter and eaten fresh while cooking bananas, and plantains have higher starch content and are consumed cooked. This classification is not always obvious, especially for regionally adapted landraces where the varieties follow the traditional use in the location where they are grown (Vezina, 2013) (Figure 1-A).

Banana is a climacteric fruit as ripening involves a rapid conversion of starch into sugar and a sudden increase in ethylene production. To avoid spoilage and increase marketability, commercial bananas are harvested unripe. They remain as such until they reach their distribution center, where they are treated with exogenous ethylene to promote ripening (Kaur *et al.*, 2017). Several studies covered the effect of this artificial ripening system on sugars, starch, soluble solids, chlorophylls, and some market quality traits (texture, flavor, color), but little is known about the effect of such treatment on pVACs in comparison to the fruit ripening on the plant (Ekesa *et al.*, 2013; Hailu *et al.*, 2013; Akter *et al.*, 2013; Sadat Hossein *et al.*, 2018).

Carotenoids in bananas are probably one of the most studied groups of phytochemicals to use for biofortification. The most abundant carotenoids in bananas are α-carotene, β–carotene, lutein, β-cryptoxanthin, and zeaxanthin. Empirical evidence indicates that genotypes with darker orange color have higher carotenoid content, especially β-carotene. However, the carotenoid profile varies across banana germplasm and variety (Amah *et al.*, 2019). Previous studies have pointed out that the differences in carotenoid accumulation in banana varieties can be caused by differential expression of genes in the carotenoid pathways and the accumulation of this pigment in specialized structures called chromoplasts (Mlalazi, 2012; Buah *et al.*, 2016).

Fe'I bananas, originally from Polynesia (Australimusa and Callimusa species), have the highest carotenoid content reported in the literature, varying from 6360 µg/100g to 14,212 µg/100g F.W. Davey *et al.* (2009) studied carotenoid content across 170 accessions from the Musa genus and observed values ranging from 0 to 11,337 µg/100g of dried weight and indicated that plantains have a higher carotenoid content. Other studies have also noted that plantains have higher carotenoid content than dessert bananas (Amah *et al.*, 2019; Amah, 2018; Englberger, 2003).

Even though carotenoid content plays an essential role in pVAC biofortification, other factors should be considered when breeding for biofortification. For instance, it is critical to understand what fraction of the carotenoids accumulated in the fruit are bioaccessible and bioavailable after ingestion. Bioavailability refers to the proportion of consumed carotenoids that the body can absorb, transport, store, or utilize. Bioaccessibility refers only to the fraction of carotenoids released from the food matrix to the digestive system (Dıaz-Gomez *et al.*, 2017; Amah, 2018).

Factors such as the type of carotenoid, the fruit's fiber content, and the amount of fat ingested in the diet affect bioaccessibility. Efficient absorption of retinol is also dependent on the health of the intestinal lining, and it is compromised in people with inflammatory bowel disease, protein-calorie malnutrition, zinc deficiency, and alcohol abuse (Fairweather-Tait, S., 2003)

For scientists, nutritionists, and breeders, considerations about crop intake, bioavailability, bioaccessibility, nutrient requirement, and the retinol equivalency of pVACs, are essential to establish goals for vitamin A biofortification programs (Amah, 2019). Studies on bioaccessibility in bananas are minimal and primarily focused on the effect of the cooking process on pVACs bioaccessibility. Key findings highlighted that β-carotene bioaccessibility ranges from 10% to 32% and that ingredients such as palm oil and amaranth have a significant effect on increasing pVAC bioaccessibility (Schweiggert *et al.*, 2012, Ekesa *et al.*, 2012).

Despite the ongoing efforts on carotenoid biofortification in bananas, little is known about the variation of pVAC bioaccessibility in banana germplasm and the effect of the ripening conditions (Amah *et al.*, 2019). This study aimed to characterize 27 accessions of edible bananas to evaluate the impact of the ripening treatments (natural vs. artificial) and the ripening stages on carotenoid content and bioaccessibility.

2.2 Materials and methods

2.2.1 Plant material and sample collection

A set of 27 banana accessions were collected from the Dole Food Company's banana collection in Rio Frio, Costa Rica (10 ° 18'29.9" North and 83 ° 52'59, 6"). The 27 accessions were all edible banana types and represented the three major banana market types, including the dessert banana, cooking banana, and plantain banana. Besides the market category, accessions were also selected to represent different fruit colors (yellow and orange), flavor/taste, ploidy level (2x, 3x, 4x), and other plant characteristics (e.g., resistance to diseases).

A modified ripening scale from the one developed by Soltani (2011) was used to decide the appropriate stages for sampling (Figure 1-B) in order to accommodate the different colors and textures of all accessions included in this study. The Soltani scale ranging from 1-7 stages of ripening was simplified into three stages where A= unripe; B= controlled conditions, semi-ripe (break in color and texture), and C= controlled conditions, fully ripe (C_{cc}) and fully ripe naturally on the plant (C_{nc}). Fruits were collected from three biological replicates for each accession, totaling 324 samples.

For ripening in controlled conditions, unripe green banana fruits were harvested and stored in a ripening chamber at 14.5 ℃. To initiate the process, the temperature was raised to 17 ℃ for 24 h. Then ethylene was applied by employing a catalytic generator with 1 L of technical grade ethylene at 95% for 24 h. Following the ethylene treatment, the room was ventilated for 20-30 minutes, and the fruit was held at 17 ℃ for two days, at 15.5 ℃ for one day, and finally at 14.5 ℃ for one day or two days depending on the ripening stage. During this treatment, semi-ripe bananas (B) were sampled by visually inspecting the ripening stage. Fully ripe bananas were

collected at the end of the ripening treatment. The banana fruits were evaluated visually and collected from the plants at the fully ripened stage (Ccn) for natural ripening.

2.2.2 Sample preparation

Banana fruits were peeled for each biological replicate, and the pulp was sliced and immediately frozen in liquid nitrogen to avoid oxidation. Frozen samples were stored at -80 ℃ for approximately 12 hrs and then lyophilized for five days. To avoid phytochemical oxidation and degradation, the lyophilizer was light sealed, and each sample was placed into Mylar bags. Dried samples were stored in light-protected falcon tubes at -80 ℃ until processed.

2.2.3 Carotenoid bioaccessibility

Ten banana accessions with a total carotenoid content >1 mg/100g of dry weight in the fully ripened fruits (natural and controlled conditions), plus Cavendish (Landrace Ecuadorian Dwarf – Super Green), were selected to evaluate carotenoid bioaccessibility. This analysis was performed using a three-phase, high-throughput digestion method adapted from Hayes *et al.* (2020) that uses a Tecan Freedom EVO liquid handling robot (Tecan Group Ltd., Männedorf, Switzerland) to automate all of the liquid handling steps during the protocol.

Briefly, 150 mg of lyophilized banana fruits were dispersed in 5 mL of boiling water, and 5% (w/ w) canola oil was added to facilitate micellization of carotenoids and the digestion process.

The digestion consisted of three phases: a) the oral phase, where the banana solution is incubated for 10 min at 37 °C, 120 rpm with α-amylase (10 units/mg); b) the gastric phase where the oral phase solution was incubated 60 min at 37 °C, 120 rpm with of 0.6 mL of pepsin (final

concentration of 0.4 g/L) and adjusted to pH 2.5 ± 0.1; c) the intestinal phase, where the gastric phase solution was incubated for 120 min at 37 °C, 120 rpm with 0.6 mL of pancreatin-lipase solution (final concentration of 0.8 g/L for both pancreatin and lipase) and 0.9 mL of bile solution (final concentration of 1.8 g/L) and adjusted to pH 7.0 ± 0.1.

After digestion, an aliquot of the final digesta was transferred and stored at −80 °C, and a second aliquot of digesta was centrifuged (BeckmanCoulter, Allegra X-30R Centrifuge, Indianapolis, IN, USA) at 4,255g for 60 min. The supernatant was collected and filtered through a 0.2 μm cellulose acetate filter to isolate the fraction containing the micellarized carotenoids (aqueous fraction).

2.2.4 Extraction of carotenoids from raw banana materials and digestive fractions

All samples were processed under yellow lights to minimize the potential for photodegradation of carotenoids. An aliquot of 50 mg of lyophilized banana powder was dispersed in 1 ml of chilled methanol and slurried, and 100 μL of β-apo-8'-carotenal was spiked into the slurry as an internal standard. Samples were sonicated for 5 minutes, then placed on ice for 10 minutes, before extraction with 5 ml of acetone: petroleum ether (0.1%BHT) (1:3). A total of 3 sequential extracts were combined and dried under nitrogen gas, re-solubilized in 300 μL of ethyl acetate:methanol (1:4), filtered using a 0.45 μm cellulose acetate filter in preparation for analysis by liquid chromatography (LC) with photodiode array detection (LC-PDA). The same carotenoid method was used for the extraction from 1 mL of aqueous and digesta fractions obtained from in vitro digestion. Extraction recovery of aqueous and digesta was 97% as determined by using trans-β-apo-8'-carotenal spiked into samples.

2.2.5 Carotenoid analysis

Carotenoid analysis was accomplished by LC-PDA using a Waters Alliance 2695 Liquid Chromatograph system (Waters, Milford, MA, USA) equipped with a model 2998 Photo Diode Array Detector (Milford, MA, USA). Carotenoids separation was performed using a YMC C30 column (3 μm 150 mm x 2 mm) thermostated at 35°C and a gradient elution method, 17-minutes run consisting of: 1) 95% solvent A (methanol: ammonium acetate, adjusted to pH 4.6, 98:2) and 5% solvent B (ethyl acetate) for three minutes; 2) 85% solvent A and 15% solvent B for five minutes; 3) 20% solvent A and 80% solvent B for one minute; 4) 100% solvent B for four minutes, and 5) 95% solvent A and 5% solvent B. The flow rate was 0.37 mL/min, with an injection volume of 10 μl. External standards of reference materials were run daily, which ensured inter-day consistency of the LC measurements. All compounds of interest, including the main banana carotenoids lutein, β-carotene, α-carotene, 13-cis-α-carotene, and 13-cis-β-carotene, were quantified at 450 nm. Quantification was achieved using calibration curves developed in the concentration range of 0.01−7.5 293 μM for all carotenoids.

2.2.6 Starch analyses

Megazyme's K-RSTAR 05/19 kit was used for measuring starch. Approximately 100 mg of lyophilized banana powder was dispersed in a 4 mL solution of sodium maleate (100 mM) with pancreatic α-amylase (10 mg/ mL) and amyloglucosidase (AMG) (3U/mL) and incubated for 16 h at 37 ℃ with continuous lateral shaking. The reaction was stopped by adding an equal volume of 100% ethanol, and the resistant starch was recovered by centrifugation at 1,800g for 10 minutes. The pellet was washed twice by adding 8 mL of 50% ethanol. All the supernatants

from this step were kept and diluted to 100 mL with sodium Maleate buffer (100mM) to measure soluble starch.

The pellet was dissolved in 2 mL of KOH solution (2M) by continuously stirring in a cold room at 3 ℃ for 20 min. Then, it was neutralized with 8 mL of NaOAc buffer (1.2 M) at room temperature. The resistant starch was quantitatively hydrolyzed to D-glucose with 0.1 mL of AMG (1:3,300 U/mL) at 50 ℃ for 30 min. Both resistant and soluble starch were measured as D-glucose by the reaction of glucose oxidase/peroxidase reagent (GOPOD) in a microplate reader at 510 nM (MaClearly *et al.*, 2019)

2.2.7 Data analyses

The data used for statistical analysis included three biological replicates for each accession, ripening stage, and ripening treatment. The results are presented as the mean ± standard error.

Total carotenoids were calculated as the sum of individual lutein, β-carotene, α-carotene, 13-cis-α-carotene, and 13-cis-β-carotene as determined by LC analysis. Relative bioaccessibility was calculated as the ratio of a particular carotenoid or total carotenoids from the aqueous phase to their concentration in the crude digesta. Absolute bioaccessibility was calculated as the relative bioaccessibility expressed as a percentage multiplied by total carotenoid content and expressed as mg per 100 g. All carotenoid concentration data were calculated per 100 g of banana (dry weight, D.W.)

The recommended dietary allowances for Vitamin A were estimated as Retinol Equivalence (RAE). One mg of RAE is equivalent to 12 mg of β-carotene and 24 mg of all other dietary pVACs (α-carotene and β-cryptoxanthin) (FNB, 2001).

All statistical analyses were performed using the software R Studio (R Core Team, 2014; RStudio Team (2020), and results were plotted using the package ggplot2 (Wickham, 2009). Means were compared using a one-way analysis of variance (ANOVA) to determine the significant difference ($\alpha < 0.05$) among accessions and ripening stages. In contrast, a paired t-test was used to determine any significant difference between ripening methods ($\alpha < 0.05$).

2.3 Results

2.3.1 Carotenoid content

Lutein, β-carotene, α-carotene, 13-cis-α-carotene, and 13-cis-β-carotene were found to be the main carotenoids in all banana accessions. These individual carotenoid species were measured in 27 banana accessions, three ripening stages (A, B, C), and for two ripening methods, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc) (Figure 1.1-B).

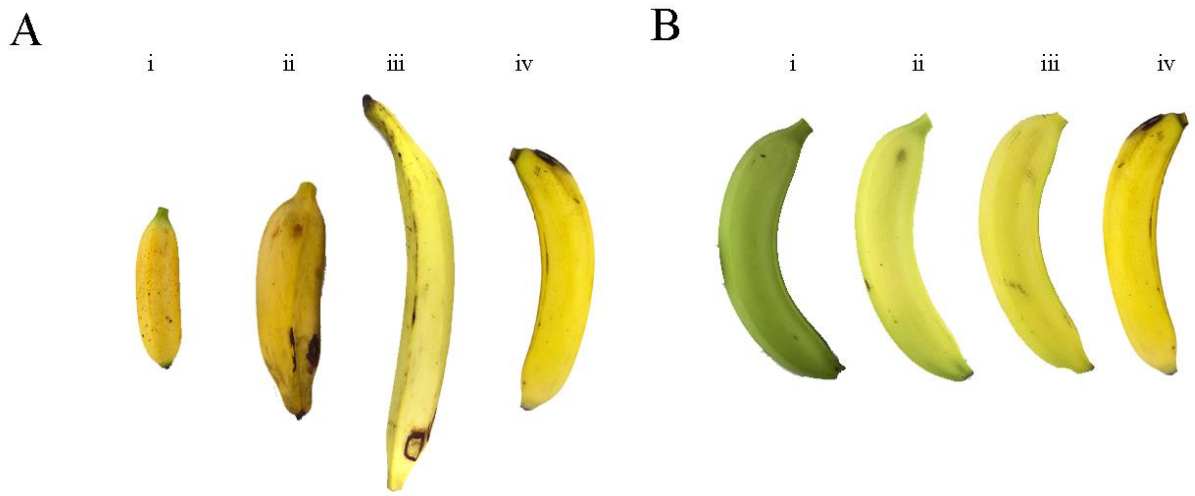

Figure 2.1. Banana market categories and ripening stages. A) Banana subgroups: (i) Dessert banana, Bri Bri, (ii) Cooking banana, Bluggoe, (iii) Plantains, Tindok, (iv) Cavendish Banana; B) Standard banana ripening scale of Cavendish banana: (i) unripe = sample A, (ii) semi-ripe = sample B, (iii) fully ripe in controlled conditions = sample Ccc; (iv) fully ripe naturally on the plant = sample Cnc.

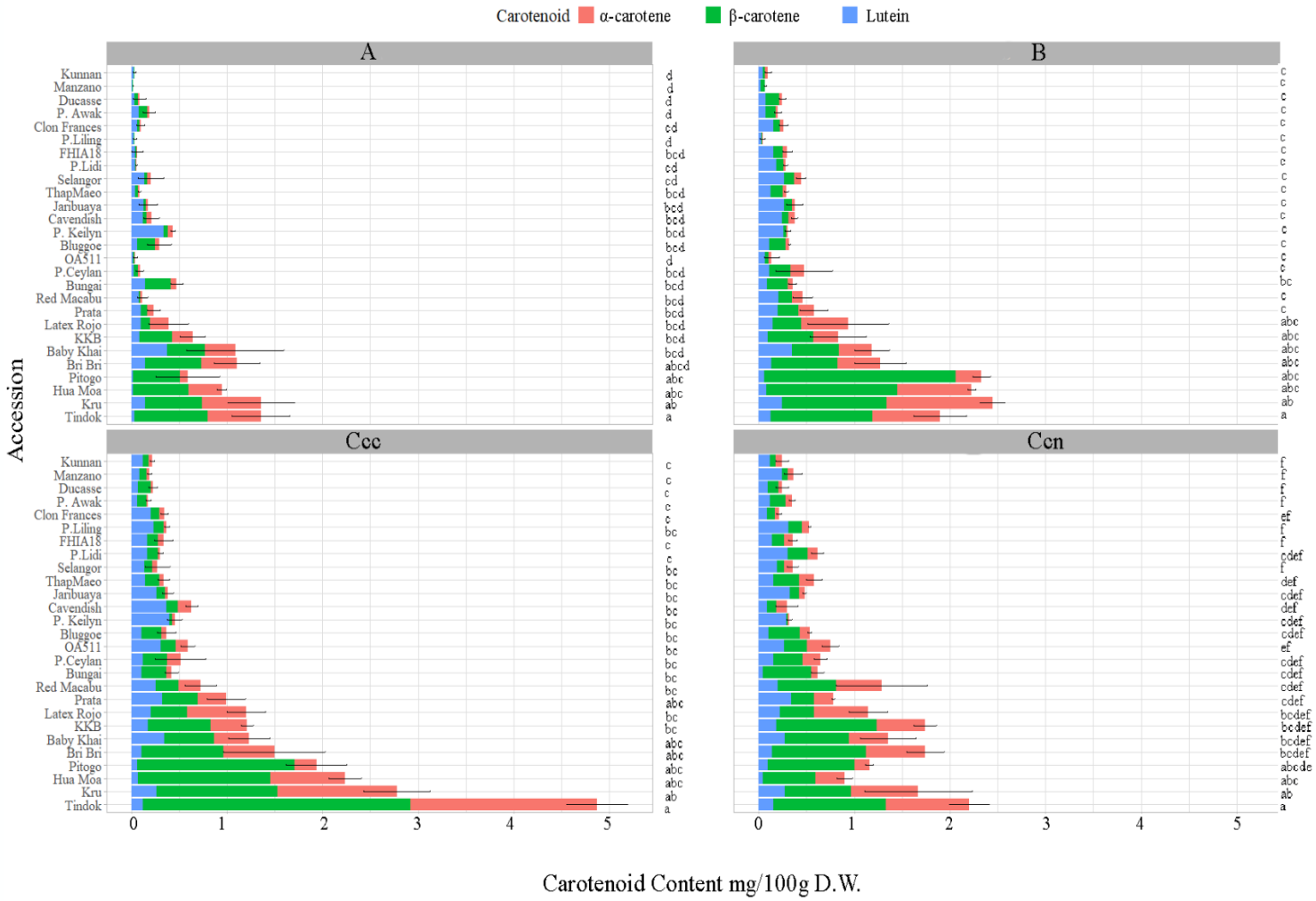

Figure 2.2. Carotenoid content and profile in 27 banana accessions, at three ripening stages (A, B, C) and two ripening methods at stage C, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). Letters on the right side of each graph represent Tukey's HSD grouping results

Total carotenoid content (TCC) showed no clear pattern with ripeness and appeared to be dependent more on the accession response to the ripeness stage. In unripe banana (stage A) ranged from 0.02±0.002 mg/100 g in Manzano to 1.36±0.030 mg/100 g in Tindok and 1.36±0.035 mg/100 g in Kru. In comparison, among semi-ripe banana samples (stage B), TCC ranged from 0.41±0.025 mg/100 g in Pisang Liling to 1.36±0.030 mg/100 g in Tindok (Figure 1.2). For fully ripe banana samples, at stage Ccc, TCC varied from 0.178±0.028 mg/100 g in Pisang Awak to 4.872±0.032 mg/100 g in Tindok. For the banana samples ripened on the plant (stage Ccn), TCC ranged from 0.212±0.026 mg/100 g in Clon Frances to 2.202±0.21 mg/100 g in Tindok (Figure 1.2). The accessions with the highest TCC were Tindok, Kru, Hua Moa, Pitogo, Bri Bri, and Baby Khai (Ccc samples), which had from 7 to 4-fold higher carotenoid content than in Cavendish, respectively (Figure 1.2).

Significant variation in TCC based on ANOVA analysis was found among the 27 banana accessions ($p<0.05$). The average α-carotene content ranged from 0.017±0.006 mg/100 g in Pisang Awak (Ccc) to 1.95±0.04 mg/100 g in Tindok (Ccc); β-carotene ranged from 0.009±0.005 mg/100 g in Pisang Liling (Ccc) to 2.00±0.06 mg/100 g in Tindok (Ccc). Lutein ranged from 0.06±0.03 mg/100 g in Pisang Awak (Ccc), to 0.40 ±0.02 mg/100 g in Pisang Keilyn (Ccc). In Cavendish, the average content for α-carotene was 0.14±0.04 mg/100 g (Ccc), 0.07±0.002 mg/100 g (Ccc) for β-carotene, and 0.37±0.03 mg/100 g (Ccc) for lutein. The average lutein and α-carotenoid content for all accessions were not significantly different between ripening methods and stages. Similar results were obtained for the average β-carotenoid content between stages A, B, and Ccc. In contrast, the average β-carotene content was significantly higher in the samples fully ripe under controlled conditions (Ccc) (0.45 ±0.01

mg/100 g) than the samples fully ripe on the plant (Ccn) (0.37 ±0.06 mg/100 g) (paired t-test $p<0.05$).

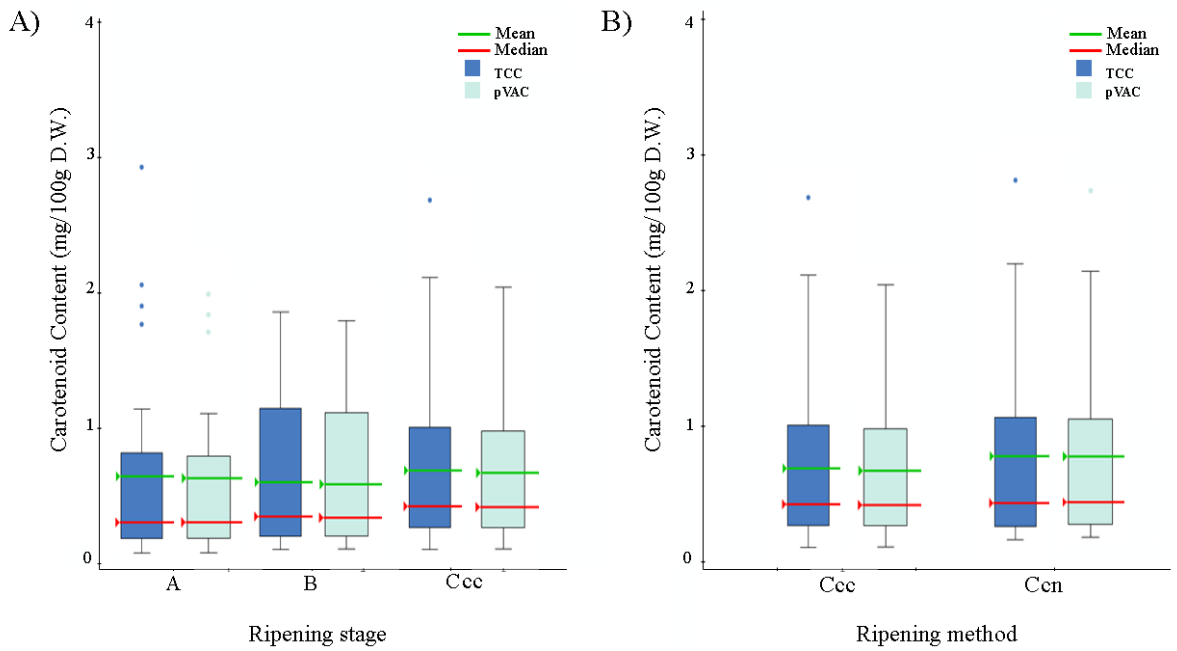

Figure 2.3. Average total carotenoid content (TCC) and pro vitamin A (pVAC) content (mg/100g D.W.) in 27 banana accessions. (A) ripening stage (A, B, C); and (B) ripening method, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc).

Across all conditions, the carotenoid profile was significantly different among the 27 accessions (ANOVA $p<0.005$). The average carotenoid profile was 38% lutein, 22% α-carotene, and 40% β-carotene, which indicates that about 66% of carotenoids are represented by pVAC. Accessions with a high content of β-carotene had lower lutein content and vice-versa. However, no significant correlation between TCC and profile was found.

Based on the TCC and pVAC aggregate average, carotenoid content and composition were accession dependent, and no significant differences were found among the ripening method or stages (Figure 1.3 A-B).

Variation in TCC across maturation stages was also assessed by accessions. In most of the accessions (N=25), TCC from unripe (stage A) to fully ripe fruits (stage Ccc) increased (Figure 1.4 A). Tindok, the accession with the highest TCC in all conditions, showed an almost four-fold (3.52 mg/100 g) increase in TCC from stage A to Ccc. In accessions such as Bungai, Pisan Awak, Pisang Keilyn, Selangor, and Bluggoe, TCC was very stable during the maturation process (Figure 4A). In contrast, P. Keylin was the only accession that showed a decrease of 0.123 mg/100 g from stage A to stage Ccn, while the remaining 26 accessions showed an increase in TCC (Figure 1.4 B).

When comparing naturally ripened vs. those ripened under controlled conditions, TCC was lower in controlled conditions for 18 accessions and higher for nine accessions (Figure 1.5). Among accessions with a lower TCC in the Ccc stage, the reduction ranged from 5% in FHIA18 to 50% in Pisan lidi. Among accessions with higher TCC in the Ccc stage, the increment ranged from 4% in Latex Rojo to 55 % in Tindok with 2.67 mg/100 g TCC (Figure 1.5).

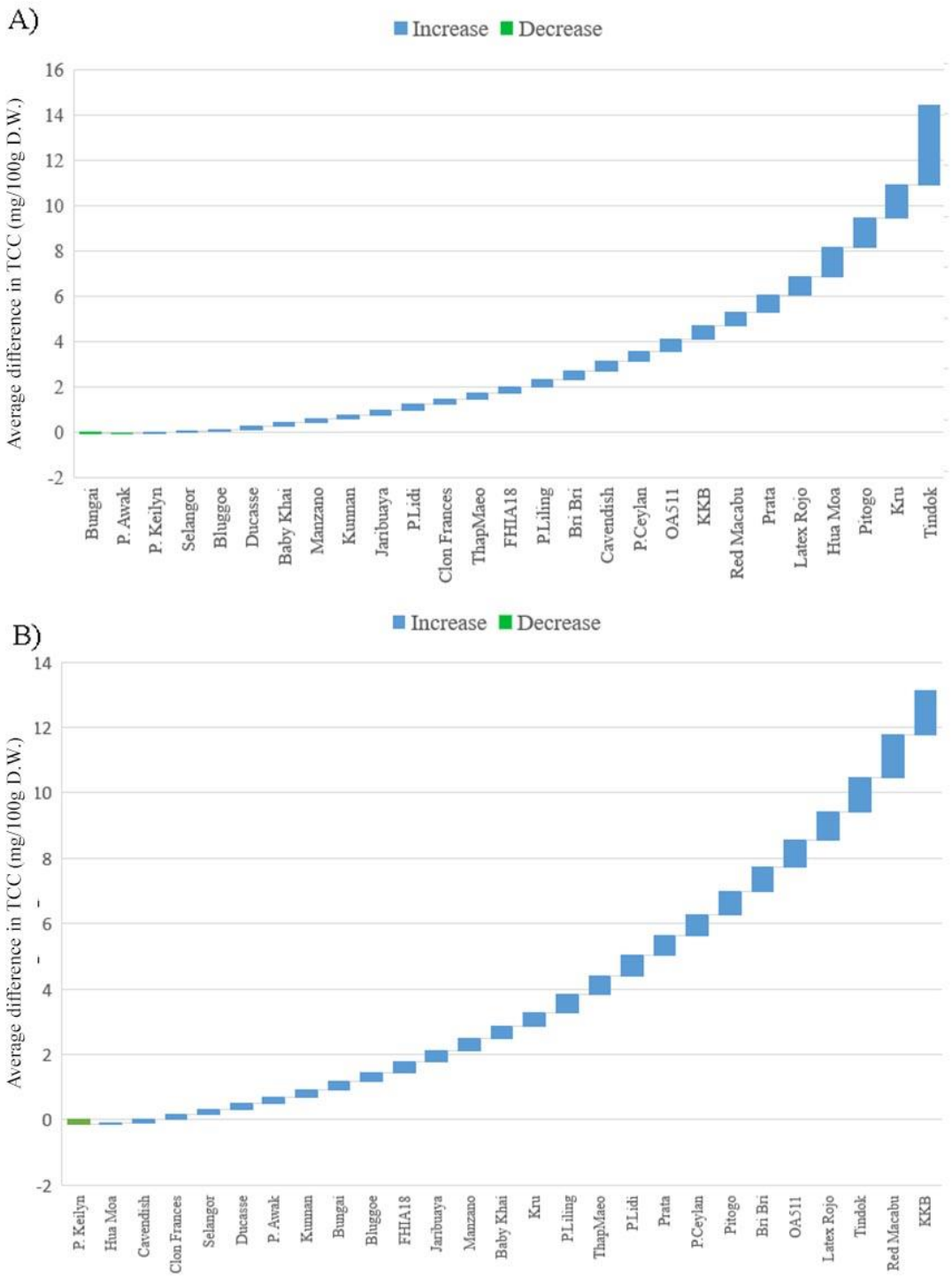

Figure 2.4. A) Average difference in carotenoid content (mg/100g D.W.) from stage A (unripe) to stage Ccc (Fully ripe in controlled conditions). B) Average difference in carotenoid content (mg/100g D.W.) from stage A (unripe) to stage Ccn (Fully ripe on the plant).

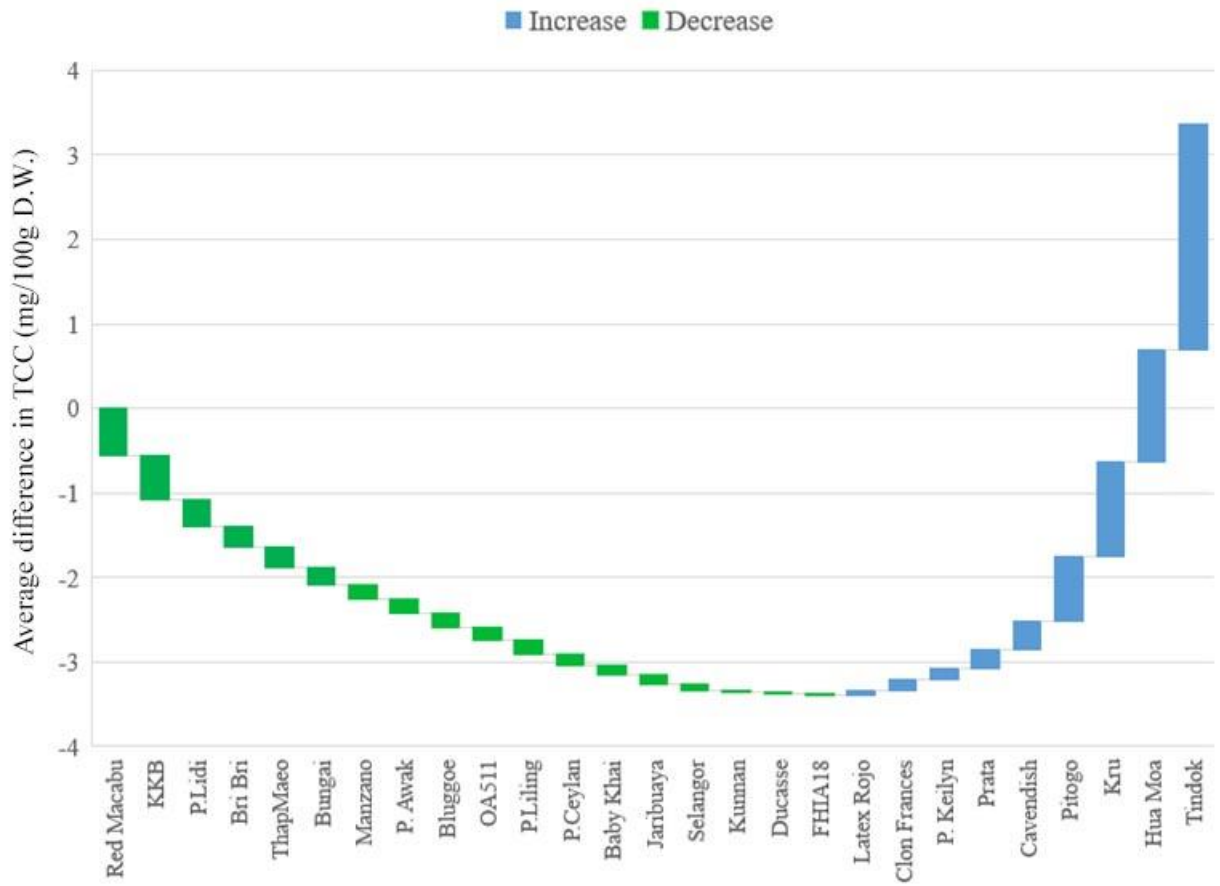

Figure 2.5. The average difference in carotenoid content (mg/100g D.W.) between bananas fully ripe naturally, on the plant (Ccn) vs. fully ripe in controlled conditions (Ccc). For each sample, this value was estimated using the following formula: avg. TCC at Ccn – TCC at Ccc.

The 27 accessions were further divided by market type (cooking, dessert, and plantains), and effects of natural or controlled ripening over carotenoid accumulation were examined. For fully ripe bananas (Ccc and Ccn), cooking bananas had the lowest average amount of TCC (0.45 ±0.07 mg/100 g at stage Ccn), followed by dessert bananas (0.75 ±0.09 mg/100 g at stage Ccn) and plantains, which had the highest average TCC (2.44 ±0.40 mg/100 g at stage Ccc) (Figure 1.6). Overall, plantains at stage C (Ccc and Ccn) samples had a significantly higher TCC (ANOVA $p<0.05$) than the rest of the accessions.

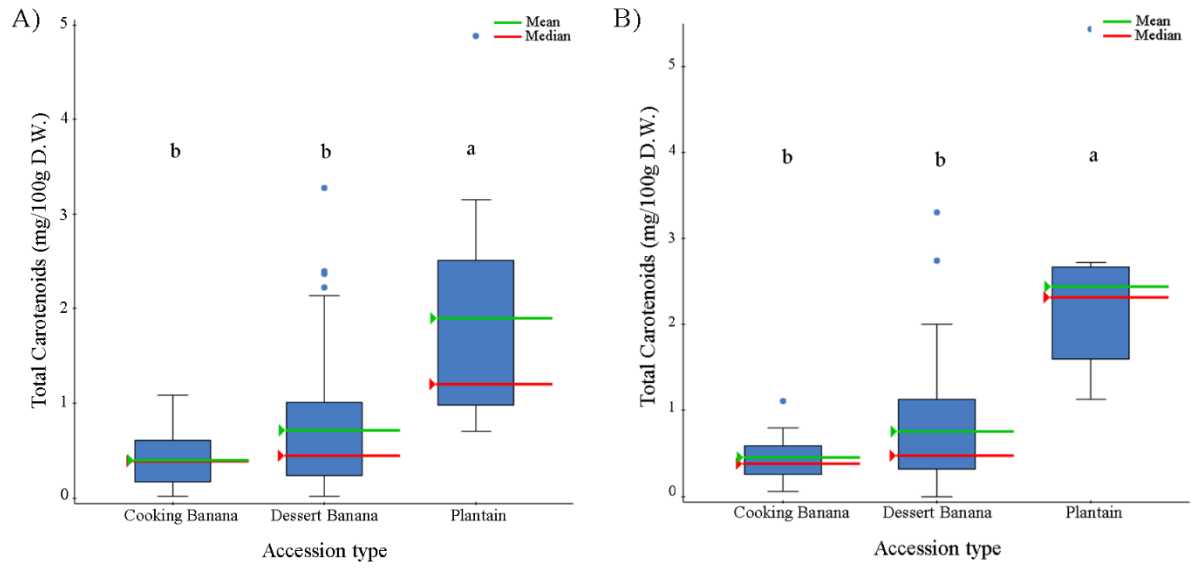

Figure 2.6. Average total carotenoid content (mg/100g D.W.) for 27 banana accessions classified by market type. (A) fully ripe in controlled conditions (Ccc), and (B) fully ripe naturally on the plant (Cnc). Letters on top of each bar represent Tukey's HSD grouping.

2.3.2 Carotenoid bioaccessibility

2.3.2.1 Relative bioaccessibility

Relative bioaccessibility of TCC varied among accessions, from 7.8%± 1.25 in Latex Rojo at stage Ccn to 34.68%± 0.45 in Bri Bri at stage Ccc. For pVAC, the value ranged from 8.85± 1.87 in Latex Rojo at stage Ccn to 34.67%± 0.55 in Bri Bri at stage Ccc (Table C.1).

Lutein had the highest average relative bioaccessibility (23.50%± 1.55), followed by α-carotene (18.60%± 1.63) and β-carotene (16.40%± 1.48). Analysis of variance showed significant differences between accessions and a significant interaction between accessions and ripening stages ($p<0.05$).

The ripening method has a significant effect on relative bioaccessibility (paired T-test $p<0.05$, Figure 1.7 A). Banana samples at stage Ccn had a lower average relative bioaccessibility (18%± 1.80) than the corresponding samples ripened with exogenous ethylene (stage Ccc, 21 % ± 1.32).

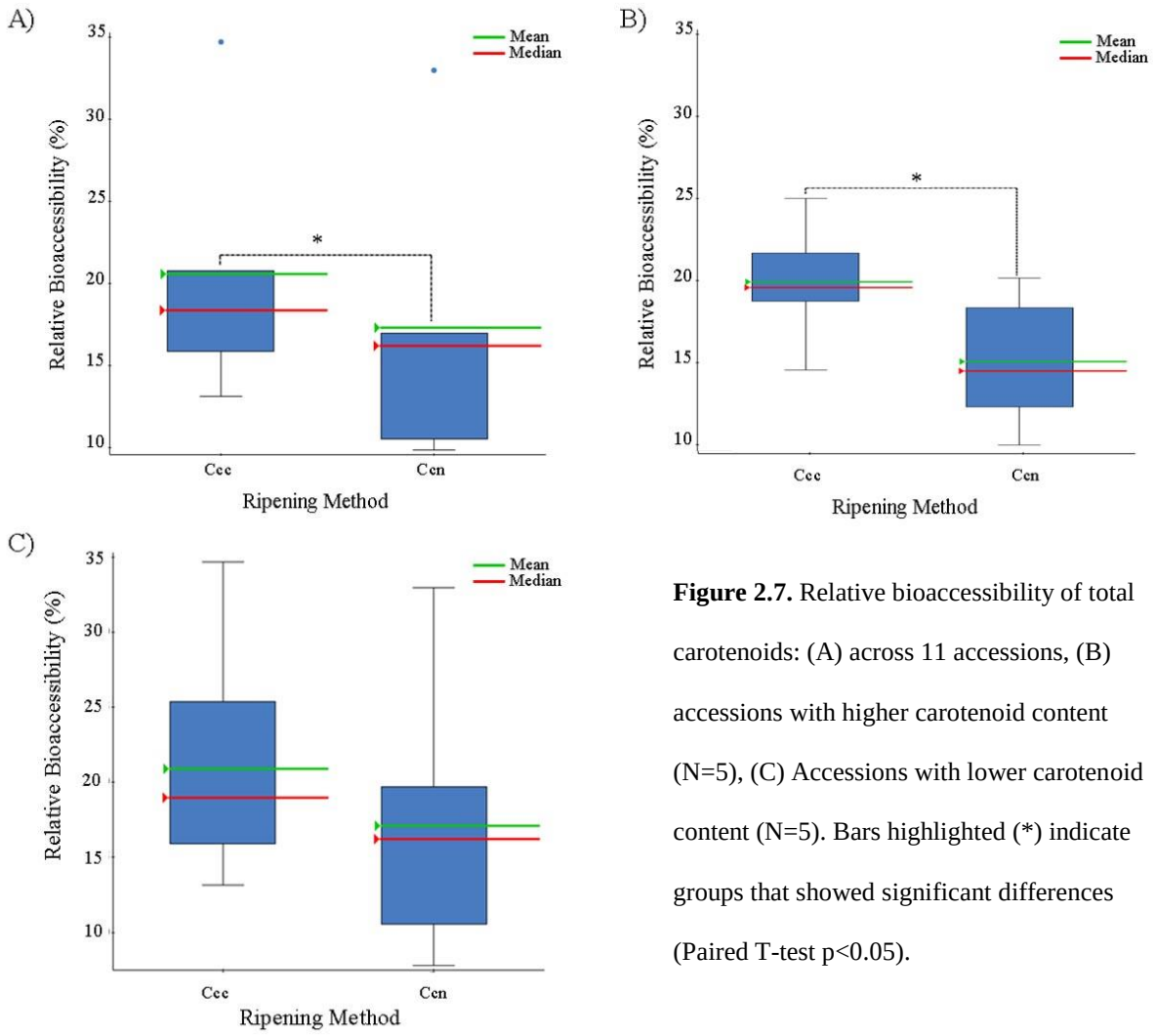

Figure 2.7. Relative bioaccessibility of total carotenoids: (A) across 11 accessions, (B) accessions with higher carotenoid content (N=5), (C) Accessions with lower carotenoid content (N=5). Bars highlighted (*) indicate groups that showed significant differences (Paired T-test $p<0.05$).

When accessions were divided by carotenoid content, banana accessions with the highest content (Tindok, Pitogo, Hua moa, Bri Bri, and Kru) at stage Ccc had a significantly higher relative bioaccessibility than the corresponding samples at stage Ccn (paired T-test $p<0.05$,

Figure 7-B). In contrast, for the accessions with the lowest TCC (KBB, Prata, Latex Rojo, Baby Khay, and Red Macabu), ripening conditions did not significantly affect relative bioaccessibility (paired T-test p=0.14, Figure 1.7 C).

2.3.2.2 Bioaccessible content

Red Macabu had the lowest total carotenoid bioaccessible content (Ccc=0.097 ±0.01mg/100 g), and Bri Bri had the highest total carotenoid bioaccessible content (Ccc= 0.63 ±0.13mg/100 g) (Figure 1.8). The total carotenoid bioaccessible content of Cavendish was 0.19 ±0.03mg/100g for Ccc and 0.10 ±0.01mg/100 g for Ccn (Figure 1.8 A). Bioaccessible content measured as pVAC ranged from 0.06 ±0.01mg/100 g for Red Macabu to 0.58 ±0.11mg/100 g for Tindok at stage Ccc. The pVAC bioaccessible content of Cavendish was 0.07 ±0.03mg/100g and 0.06 ±0.01mg/100 g for Ccc and Ccn, respectively (Figure 1.8 B).

Total bioaccessible carotenoid content differed among accessions ($p<0.05$), and the ripening treatment and the accession interaction was significant ($p<0.005$). β-carotene ($P<0.05$) also differed, ranging from 0.03 ±0.0005mg/100 g in Cavendish (stage Ccc) to 0.34 ±0.36 mg/100 g in Bri Bri (stage Ccc) (Figure 1.8 A).

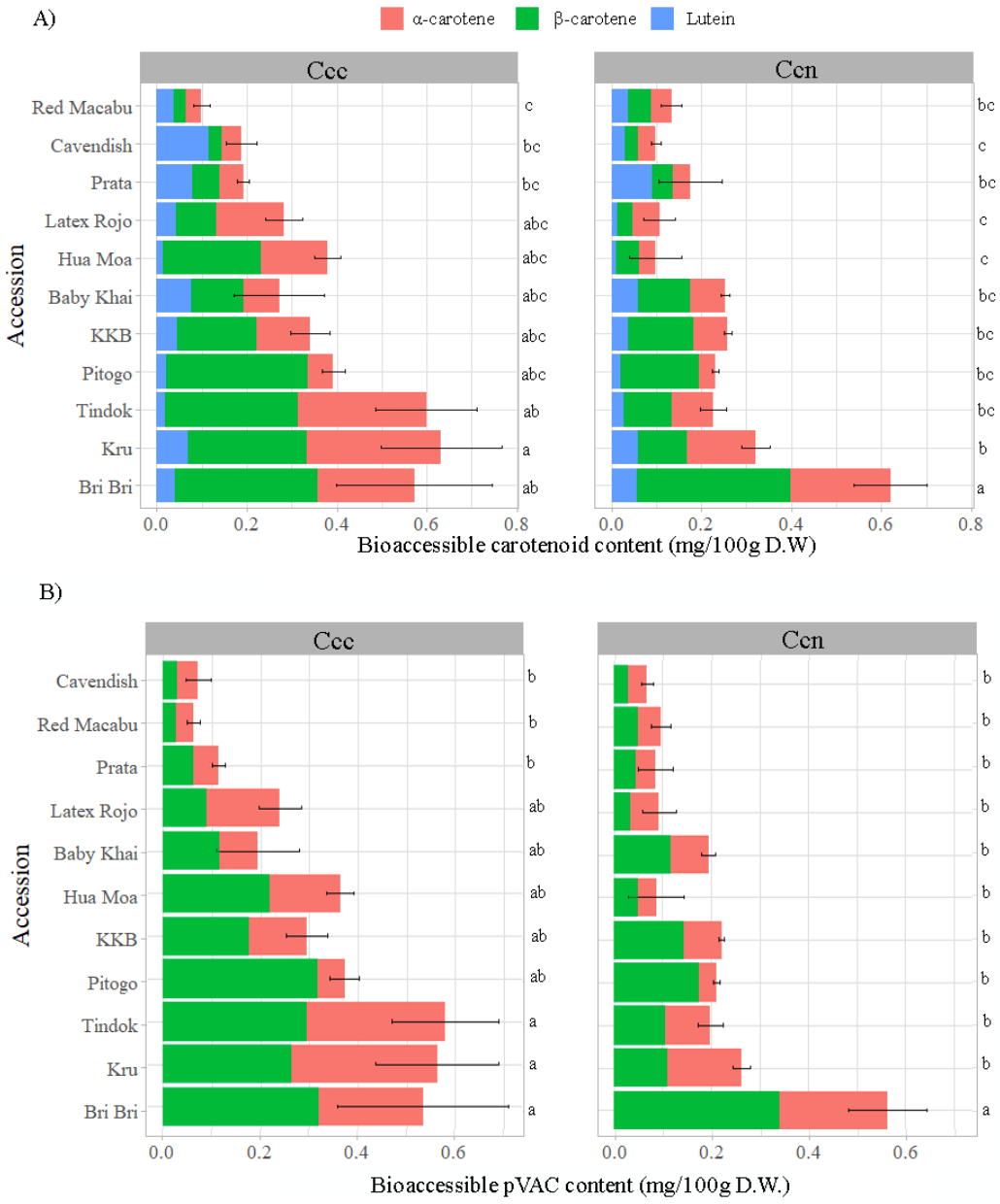

Figure 2.8. Bioaccessible content for 11 banana accessions evaluated at fully ripe stage under two ripening conditions, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). A) Bioaccessible TCC, B) Bioaccessible pVAC. Letters on the right side of each graph represent Tukey's HSD grouping.

The bioaccessible content expressed in retinol equivalents (RAE) ranged from 0.007±0.001 mg/100 g in Red Macabu (stage Ccn) to 0.041 ±0.005 mg/100 g in Bri Bri (stage Ccn). For Cavendish it varied from 0.006 ±0.001mg/100 g (stage Ccc) to 0.007 ±0.005mg/100g (stage Ccn) (Table C.2). The ripening method significantly affected the carotenoid bioaccessible content. With an average of 0.29 ±0.04 mg/100 g accessions ripened with exogenous ethylene (stage Ccc) had a significantly (paired T-test $p<0.05$) higher carotenoid bioaccessible content than accessions ripened on the plant (stage Ccn, avg. 0.24 ±0.03 mg/100 g) (Table C.1; Figure 1.8).

When individual carotenoids were compared across ripening conditions (stages Ccc and Ccn), accessions showed significant differences in bioaccessible content for α-carotene and β-carotene (paired T-test $p<0.05$), but not for lutein (Appendix C; Figure 1.8).

As observed for the relative bioaccessibility data, there were differences in bioaccessible content when accessions with the highest and the lowest total carotenoid content were compared by the ripening method (stages Ccc and Ccn). Indeed, considering the five accessions with the highest carotenoid content (Tindok, Pitogo, Hua moa, Bri Bri, and Kru), the Ccn samples had a significantly lower carotenoid bioaccessible content than the Ccc samples (paired T-test $p<0.05$). For the accessions with the lowest carotenoid content (KBB, Prata, Latex Rojo, Baby Khay, and Red Macabu), the carotenoid bioaccessible content of the samples ripen in the two conditions (Ccc vs. Ccn) were not significantly different (paired T-test $p=0.23$) (Appendix C; Figure 1.8).

2.3.3 Starch content

Total starch, resistant starch, and soluble starch content were evaluated to assess their effect on carotenoid bioaccessibility.

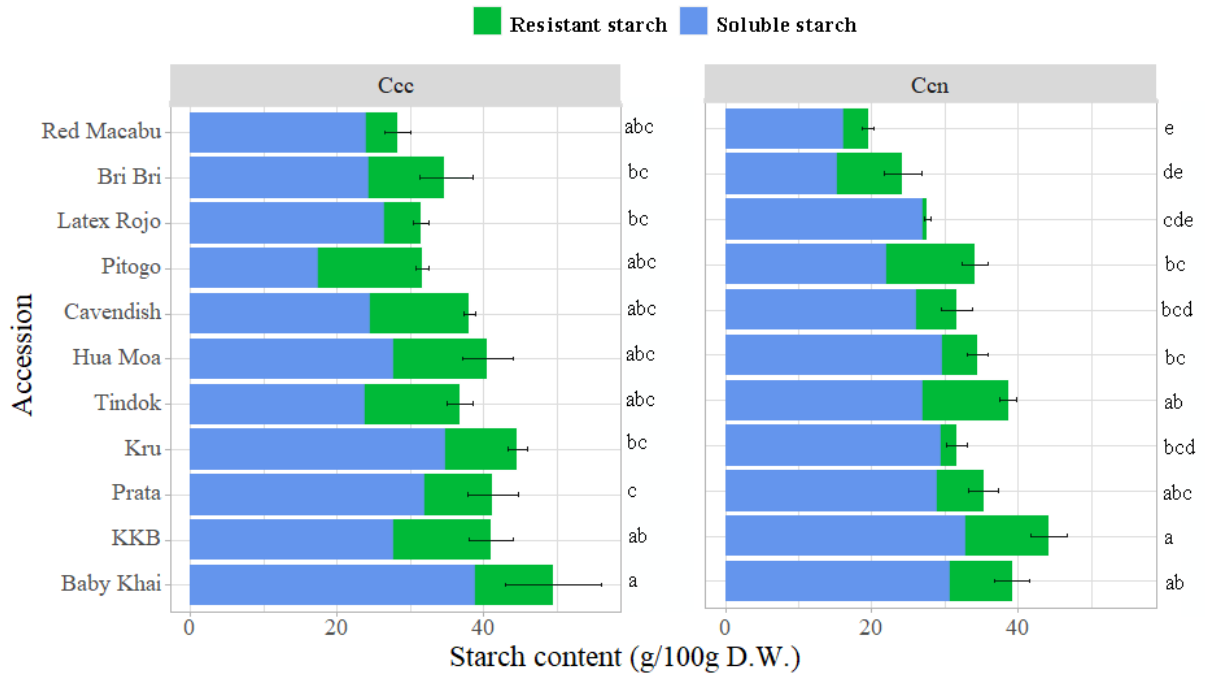

Figure 2.9. Total starch content for 11 banana accessions evaluated at fully ripe stage under two ripening conditions, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). Letters on the right side of each graph represent Tukey's HSD grouping.

Red Macabu had the lowest total starch content (Ccc=28.24 ± 1.8 g/100 g; Ccn= 19.53±0.81 g/100 g), and Baby Khai had the highest starch content (Ccc=49.51 ± 6.54 g/100 g; Ccn= 39.18±2.34 g/100 g). In Cavendish, the total starch content was 38.11 ± 0.78 g/100 g at stage Ccc and 31.63±2.16 g/100 g at stage CCn (Figure 1.9).

On average, resistant starch represented about 25% of the starch content in all banana samples evaluated here. Its value ranged from 0.49±0.14 g/100 g (2% of total starch) at stage Ccn in Latex Rojo to 14.09±1.31 g/100 g (44% of total starch) at stage Ccc in Pitogo. The resistant starch content in Cavendish was 13.56±0.36 g/100 g at stage Ccc and 5.49±0.79 g/100 g at stage Ccn (25% of total starch) (Table C.3; Table C.3).

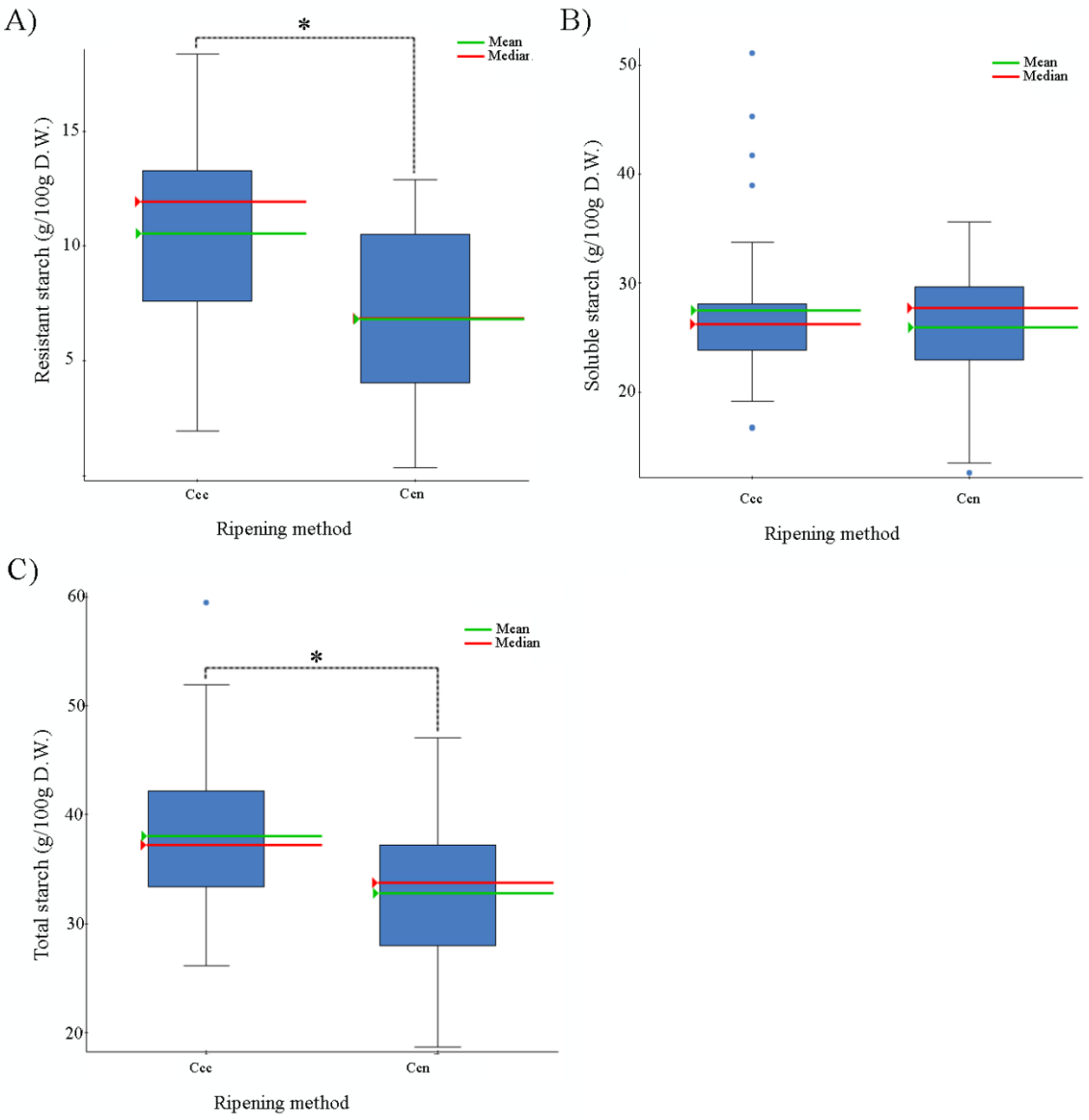

Figure 2.10. Starch content for 11 banana accessions evaluated at fully ripe stage under two ripening conditions, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). (A) Resistant starch, (B) Soluble starch, (C) Total starch. Bars highlighted (*) indicate groups that showed significant differences (Paired T-test $p<0.05$).

When analyzed by starch type (total, soluble, and resistant starch), accessions showed significant differences across ripening conditions (stages Ccc and Ccn) for total and resistant starch (ANOVA, $p<0.05$), with an average decrease of 3.73 g/100 g of resistant starch, and 4.96 g/100 g of total starch in natural ripening (Ccn). No significant differences were observed for soluble starch (Figure 1.10).

Resistant starch content had a significant positive correlation with total bioaccessible carotenoid content (R^2 0.20, $p<0.05$) and bioaccessible β-carotene content (R^2 0.25, $p<0.05$). Even though both correlation coefficients of determination are very close to each other and have a high statistical significance level, they are weak. No other significant correlations were found between starch content and relative bioaccessibility.

2.4 Discussion

2.4.1 Carotenoid content and profile at different ripening stages

While the synthesis and accumulation of carotenoids are under genetic control, these processes are also affected by season, geographical location, climate, growth and ripening conditions, and maturity stage (Rodriguez-Amaya 2004; Arscott 2013). Differences in analytical techniques, experimental design, and growing environments must be considered when comparing carotenoid content across multiple studies (Amorim-Carrilho *et al.*, 2014; Mercadante *et al.*, 2017).

In this study, total carotenoid content (TCC) varied greatly with ripeness and accession, from 0.02±0.002 mg/100 g in Manzano at the unripe stage to 4.87±0.032 mg/100 g D.W. in ripe Tindok banana. TCC was accession dependent, and multiple accessions were found to exceed the TCC of Cavendish (0.397±0.082 mg/100 g to 0.422±0.043 mg/100 g), which is a standard banana used for export. The levels of TCC are within the overall range of values reported for green or ripened bananas in previous studies by Davey *et al.* (2007) and Amah (2018) TCC was found to be genotype-dependent (Amorim *et al.*, 2009).

At fully ripe stage and all ripening conditions (Ccc and Ccn), plantain bananas had the highest pVAC content with 2.44 ±0.40 mg/100 g at stage Ccc compared to dessert bananas with a pVAC content of 0.75 ±0.09 mg/100 g at stage Ccn. These results are consistent with previous studies indicating that plantains have a higher TCC and pVAC (Amah 2018; Davey *et al.* 2009; Ngoh-Newilah *et al.* 2009; Heng *et al.* 2017) than other banana types (dessert and cooking banana). In a recent study, plantain had the highest pVACs content with 1.38 mg/100 g of β-carotene and 0.82 mg/100 g of α-carotene, which was higher in pVAC content than other starchy staples crops such as cassava, yam, cocoyam, and maize (Wald *et al.* 2018). Although this trend

has been described for only a few genotypes, pVAC is of particular interest, as plantains have a high potential of becoming suitable cultivars in pVAC biofortification programs (Amah, 2018).

On average, 66% of the carotenoids isolated in the banana samples evaluated in this study were pVACs (α-carotene and β-carotene), with β-carotene being the most abundant carotenoid in most accessions, contributing up to 40% TCC. We also noted that α-carotene and β-carotene content were strongly correlated ($r^2 = 0.70$; $P<0.05$). The remaining 22% of the carotenoids detected were lutein, which lacks pVAC activity but has other health benefits (Borges *et al.*, 2019). These results are consistent with previous research on carotenoids in bananas, where most samples had a higher content of α-carotene and β-carotene with a smaller proportion of lutein. For instance, Amah (2018) found that the average content across 189 accessions was 78% pVACs, with a high positive correlation between α-carotene and β-carotene ($r^2=0.75$, $p<0.001$). Our study also identified a significant positive correlation between α-carotene and β-carotene and a negative correlation with lutein. These observations and results are consistent with the knowledge that lutein is synthesized by a parallel branch of the carotenoid pathway, and the enzymes used to synthesize lutein or α-carotene compete for the same substrates (Englberger *et al.* 2003; Davey *et al.* 2007; 2009; Rodrigo, 2007, Ekesa *et al.* 2015; Heng *et al.* 2017).

Fruit maturation, or ripening, is defined by four significant physiological changes: color (due to the loss of chlorophyll and the gain of carotenoids and anthocyanins); texture (softening of the cell wall); flavor (an increase in sugars and/or accompanied by decreased organic acids); and smell (production of volatile compounds) (Osorio *et al.*, 2013). These physiological changes can often directly or indirectly affect carotenoids' content and profile (Kaur *et al.*, 2017).

In our study, in most of the accessions (N=25), we observed an increase of TCC from unripe (stage A) to fully ripe fruits (stage Ccc), but in some (N=2), TCC decreased. In Tindok,

the accession with the highest TCC in all conditions, TCC increased almost four-fold (3.52 mg/100 g) from stage A to Ccc, while in other five accessions of dessert banana (Bungai, Pisan Awak, Pisang Keilyn, Selangor, and Bluggoe) TCC was very stable during ripening, as reported by Lokesh *et al.* (2014).

Several studies have described a decrease in pVACs and TCC contents from the unripe to the ripe and overripe stages. Amah (2018) found that mean TCC in 9 plantain cultivars sampled at three ripening stages (unripe, ripe, and overripe) ranged from 2.24 mg/100g (in overripe fruits) to 3.75 mg/100g (in ripe fruits). Ekesa *et al.*(2015) reported an increase in mean total pVACs content in eight banana cultivars (including two plantains). Ngoh-Newilah *et al.* (2009) found a decreasing trend in pVACs with ripening for some plantain cultivars evaluated at three ripening stages, while Mbabazi (2015) found a decrease in β-carotene content following ripening in a single plantain and two dessert banana cultivars tested, diverging from a consistent increase in β-carotene in five plantains. In contrast, Lokesh *et al.* (2014) assessed four banana varieties and reported that TCC and pVAC remained stable after ripening. However, the ripening method and ripening stages used for TCC analysis were not always specified in some of these studies, making a comparison across data more challenging (Amorim-Carrilho *et al.*, 2014; Mercadante *et al.*, 2017).

In comparison, our study did not identify a single pattern in TCC accumulation, while in some accessions, TCC increased from ripening stages A to C; in some others, it decreased or was kept stable. Overall, our results suggest that the pattern of TCC across maturation stages varies according to the accessions, which is important when considering different banana varieties for biofortification programs (Saini *et al.*, 2017).

2.4.2 Carotenoid content under different ripening methods

When comparing ripening methods (Ccc and Ccn samples), 18 accessions had lower TCC in the Ccn than Ccc samples. In comparison, in nine accessions, TCC was higher in the Ccn samples than in the Ccc samples, suggesting that treating bananas with exogenous ethylene directly affects TCC accumulation. This factor should be considered in future studies when measuring carotenoids in bananas for breeding programs, as quantification of PVAC is often challenging due to the inconsistencies in sampling and analysis strategies. Such discrepancies may contribute to variable results found in different studies (Amah *et al.*, 2019).

The effect of ripening with exogenous ethylene on carotenoid accumulation is yet to be understood in bananas but has been studied in other crops. Previous studies in tomatoes and citrus have found that post-harvest-ripened fruits accumulated more carotenoids than those ripened on the plant (Giovanelli *et al.*, 1999). In citrus, it was observed that ethylene upregulates the expression of carotenoid biosynthetic genes (phytoene synthase, ζ-carotene desaturase, and β-carotene hydroxylase). This study concluded that exogenous ethylene reproduces and accelerates the physiological and molecular changes in the carotenoid biosynthesis naturally occurring during maturation (Rodrigo & Zacarias, 2007).

Becatti *et al.* (2009) reported that TCC and carotenoid profiles in tomatoes were controlled by ethylene (endogenous and exogenous) and UV-B radiation. The latter influenced carotenoid metabolism either in an ethylene-dependent or -independent way, acting antagonistically.

Rodrigo (2007) observed an ethylene-dependent up-regulation of carotenoid biosynthetic genes involved in phytoene formation and desaturation of lycopene β-cyclase during citrus degreening with exogenous ethylene. In contrast, the expression of lycopene ε-cyclase (involved

in lutein synthesis) was down-regulated by ethylene, redirecting the accumulation flux towards β-carotenoids. This suggested that the application of exogenous ethylene has a direct effect on TCC accumulation in citrus. At the same time, Rodrigo & Zacarias (2007) also observed that citrus protected against UVB radiation on the field showed a higher β-carotene accumulation, suggesting a particular sensitivity of lycopene β-cyclase to UV-B radiation.

Previous studies have indicated that the application of exogenous ethylene directly affects metabolite accumulation in bananas, especially on sugars, vitamin C, organic acids, volatile compounds, and minerals. However, the underlying mechanisms of how this happens are not well understood (Hakim *et al.*, 2012; Sonmezdag *et al.*, 2014; Maduwanthi & Marapana, 2019; Maduwanthi & Marapana, 2021).

Hakim (2012) studied the effects of ethylene treatments on the carotenoid accumulation in bananas, tomato, and pineapple; the study concludes that there is a decrease in TCC accumulation in all fruits treated with exogenous ethylene. At the same time, Maduwanthi (2021) studied the effect of exogenous ethylene on harvested bananas; this study also finds a decrease in TCC in treated bananas. Both studies do not report the specific variety.

Our study has found that out of 27 accessions, nine accessions had higher TCC in natural ripening conditions (Ccn), as reported by Hakim *et al.* (2012) and Maduwanthi & Marapana (2021). In contrast, 18 accessions had higher TCC when treated with exogenous ethylene (Ccc), as reported in tomato and citrus when compared with untreated fruit ripened in the plant (Rodrigo & Zacarias, 2007; Becatti *et al.* 2009).

Overall the results of our study, considering a significant number of banana accessions, indicated that while ethylene treatment has a significant effect on TCC accumulation, this effect is genotype dependant, as has been previously suggested by the influence of ethylene in the

carotenoid pathway in apricots (Marty *et al.* 2005), tomatoes (Su *et al.* 2015), and citrus (Rodrigo & Zacarias, 2007).

2.4.3 Bioaccessibility

The potential for carotenoids from plant-based foods to have their effect on the human body is contingent on its release from the matrix into the digestive tract. This characteristic is commonly referred to as bioaccessibility, and it is easily affected by morphological traits such as the fiber content, the method of cooking, or even other ingredients used during cooking (Tydeman *et al.*, 2010). Bioaccessibility is related to the concept of bioavailability, which is defined as the amount of bioaccessible nutrient (or bioactive) that reaches the tissues and participates in metabolic processes. Both concepts are highly correlated and crucial in determining the vitamin A activity of a particular food product and setting breeding objectives for biofortification (Dima *et al.*, 2020).

Our study measured the bioaccessibility of 11 banana accessions at fully ripened stage and two maturation methods (Ccc and Ccn), in which its carotenoid content was higher than 1 mg/100 g D.W. Relative bioaccessibility of TCC varied among accessions, from 7.82%± 1.25 in Latex Rojo at stage Ccn to 34.68%± 0.45 in Bri Bri at stage Ccc, lutein showed the highest average relative bioaccessibility (23.50%± 1.55), followed by α-carotene (18.60%± 1.63) and β-carotene (16.40%± 1.48). The bioaccessible content ranged from 0.097 ±0.01mg/100 g in Red Macabu (stage Ccc) to 0.63 ±0.13mg/100 g in Bri Bri (stage Ccc). Interestingly, no correlation between TCC and relative bioaccessibility was found, suggesting that this trait in bananas is accession-dependent and unrelated to the carotenoid content or profile.

Bioaccessibility and bioavailable content of pVACs in biofortified food has been extensively reviewed (La Frano *et al.*, 2014; Giuliano, 2017; Kopec and Failla, 2018). However, little is known about carotenoids' bioaccessibility in bananas or the effect that the ripening methods have on them. Our study is a pioneer in the field because it includes different banana accessions and measures the effect of two ripening methods on bioaccessibility.

Ekesa (2012) studied the bioaccessibility of two plantain cultivars in Beni territory Eastern DR Congo cooked in 6 different ways. The study found that the bioaccessibility of α-carotene and β-carotene and ranged from 10% to 32%, dependent on the food recipes and the cultivar. Hempel *et al.* (2017) found that lutein tends to have a higher bioaccessibility than other carotenoids because of its deposition in tubular ultrastructures that are easily liberated from the matrix and digested.

In our study, the ripening method was found to have a significant effect on relative bioaccessibility and bioaccessible content. Bananas ripened on the plant (stage Ccn) had a lower average relative bioaccessibility and bioaccessible TCC (18%± 1.80 and 0.24 ±0.03 mg/100 g) than the corresponding samples ripened with exogenous ethylene (stage Ccc, 21 %± 1.32 and of 0.29 ±0.04 mg/100 g). Bresnahan *et al.* (2012) found that cooking but not ripening affects pro-vitamin A carotenoid bioavailability from bananas feed to Mongolian gerbils. Nonetheless, this study only compares ripened bananas versus unripened bananas.

According to FDA (2021) and their guidelines for a healthy diet, an average adult must consume at least 0.9 mg RAE of Vitamin A per day (Daily Value, DV). In their calculations, one portion of banana (based on Cavendish banana with an average weight of 126g) contributes 2% of the DV for this vitamin. Nonetheless, it is not specified if such an amount is calculated using bioaccessible content.

Based on bioaccessible TCC, our study found that one portion of Cavendish banana has 1% of DV at stage C, regardless of the ripening method, while the same size portion of Tindok has 17% DV at stage Ccc and 8% DV at stage Ccn. At the same time, one whole portion of Tindok (326g) could provide up to 54% of Vitamin A daily value, which could make a difference in countries where banana is a staple fruit, and vegetables and fruits with high pVAC content are hard to find or expensive to buy. For example, in Costa Rica, one portion of Tindok is valued at USD 0.30, and the same portion of sweet potato is valued at USD 1 (CNP, 2021).

For Tindok, Pitogo, Kru, Hua Moa, which have the highest TCC at stage Ccc, the Percent Daily Value (%DV) for a standard portion was lower in the samples ripened in the tree (Ccn) when compared to those ripened with exogenous ethylene (Ccc). For the remaining accessions, the %DV for a standard portion was the same or slightly different (>1% difference) for both maturation methods. The data suggest that this trait is also accession dependant (Appendix C).

Relative bioaccessibility has been inversely correlated with the fiber content of food. High pectin concentration and other dietary fibers such as cellulose, guar, and alginate significantly reduce carotenoid micellization by increasing the viscosity of the intestinal content (Schweiggert *et al.*, 2012). For this reason, our study measured the amount of resistant starch and soluble starch content and their effect on carotenoid bioaccessibility.

Our result found that Red Macabu had the lowest total starch content (Ccc=28.24 ± 1.8 g/100 g; Ccn= 19.53±0.81 g/100 g), while Baby Khai had the highest starch content (Ccc=49.51 ± 6.54 g/100 g; Ccn= 39.18±2.34 g/100 g). On average, resistant starch represented about 25% of the starch content in all banana accessions. While resistant starch showed significant differences according to maturation stage (Ccc and Ccn), soluble starch did not; both types of starch are accession dependant.

Studies on starch content and composition in bananas have found that the starch content on average is approximately 65% of the total dry weight, while resistant starch may vary from 37.2 % to 79.2%, the starch content and composition is accession dependant (Vatanasuchart *et al.*, 2012; Faissant *et al.*, 1995, A.Chávez-Salazar *et al.*, 2019)

In contrast with the original hypothesis, where bioaccessibility is theorized to be inversely correlated with resistant starch, our study found that resistant starch content had a significant positive correlation with total bioaccessible carotenoid content (R^2 0.20, $p<0.05$). and bioaccessible β-carotene content (R^2 0.25, $p<0.05$). However, such correlation is relatively low.

A study by Vatanasuchart *et al.* (2019) on eleven banana cultivars grown in Thailand showed that the R.S. content observed in the common cultivars ranges between 52.2- 61.4%, and values for indigenous cultivars are between 50.7-68.1%, most of them being resistant starch type II which has less impact on carotenoids bioaccessibility in comparison to soluble fiber. The composition and type of resistant starch in our sample might explain our results.

Our study is first in comparing the carotenoid content and bioaccessibility at multiple ripeness stages and ripening methods in a relatively large set of banana accessions. Our data shows that carotenoid content and carotenoid bioaccessibility vary across accessions. Moreover, the application of exogenous ethylene during ripening affects both of these traits and the Percent Daily Value for Vitamin A, which is essential knowledge for a successful banana biofortification effort.

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Chapter 3. Total phenolic and sugar content changes with ripening in a diverse collection of banana accessions ripened in different conditions

3.1 Introduction

Nutrients are those chemicals found in food that have an effect on the organism or have a particular function at specific tissues or cells, and they are essential for metabolism maintenance. Bioactives also have an effect on the organism but are non-essential for life; neither is there a required nor recommended intake amount. However, they have a positive impact on maintaining body functions and preventing disease. Unlike other micronutrients, phenolics in human nutrition are considered bioactive, as they are not essential for short-term wellbeing. New research shows that moderate consumption of these phytochemicals has a favorable role in minimizing the incidence of common diseases (Del Rio *et al.*, 2013).

Phenolics are a large group of secondary metabolites in plants composed of at least 8,000 chemicals, derived primarily from the pentose phosphate, shikimate, and phenylpropanoid pathways. They are responsible for color, taste, and smell in foods, while in most plants, their principal function is related to defense mechanisms to overcome biotic and abiotic stresses. Phenolics are divided into four major chemical groups: phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (anthocyanins and anthoxanthins), tannins (derived tannins and hydrolyzable tannins), and stilbenes (Ozcan *et al.*, 2014).

Diverse phytochemicals within the phenolic family have been linked to numerous health benefits. Initially, it was thought that its role in human health was related to antioxidant activity because of its radical-scavenging nature. New evidence shows that minimal amounts of some of these chemicals may exert action on the intracellular signaling cascades responsible for growth, proliferation, and apoptosis (Ozcan *et al.*, 2014).

Some phenolic acids, tannins, and flavonoids have anti-carcinogenic and antimutagenic effects by protecting DNA from oxidation. Flavonoids and catechins are considered therapeutic for neurodegenerative diseases and brain aging processes. Resveratrol is known for its DNA protectant properties and has been shown to have beneficial effects on cognitive functions. Also, tannins have been reported to accelerate blood clogging, reduce blood pressure and modulate immune responses (Ozcan *et al.*, 2014).

Bananas are not generally considered to be especially rich sources of phenolics as compared to blueberries, black grapes, cherries, citrus, or even banana peels (Kumpulainen & Salonen, 1999). On the other hand, banana peel is very rich in phenolics, and it contains from 1.5 to 3 times the amount of phenolics than the pulp (Bennet *et al.*, 2010; Vu *et al.*, 2018; Qamar& Shaik 2018). Several studies have shown that total free phenolics in banana pulp range from 11.8 to 90.4 mg of gallic acid equivalents (GAE) in 100 g of fresh weight, and different banana accessions may be as high in phenolic content in the pulp as they are in the peel (Bennet *et al.*, 2010).

Multiple studies in bananas have evaluated total phenolic content, type of phenolics, and tissue-specific accumulation. Banana peel tends to have higher amounts of phenolics content than the pulp, ranging from 0.01 to 5.5 g gallic acid equivalents (GAE)/100 g, while the amount found in pulp goes from 0.02 to 0.91 g GAE/100g (Bennet *et al.*, 2010; Vu *et al.*, 2018; Qamar& Shaik 2018).

More than 40 phenolic compounds have been identified in bananas. Some of the most common phenolics are hydroxycinnamic acids in pulp (e.g., ferulic acid-hexoside) and flavonol glycosides (e.g., rutin) in the peel. Dopamine and serotonin have always been known for their place in metabolic processes involved with the brain, such as neurotransmission, and they are

gaining interest in banana research as they are also found endogenously in the pulp and peel. Cavendish banana has been found to have a dopamine amount ranging from 80-560 mg/100g in peel and 2.5-10 mg/100 in the pulp. (Borges *et al.*, 2019; Signh *et al.*, 2016; Kanazawa & Sakakibara, 2000). Some of the other phenolics found in bananas have been linked to preventing cardiovascular diseases, cancer, diabetes, and obesity; even the phenolics in the peel can be used as functional ingredients in food, given their activity as antibacterial, antiviral, anti-inflammatory, and antiallergenic (Sidhu *et al.*, 2018; Vu *et al.*, 2018). Most of these studies focused on Cavendish and very few other accessions, and the amount of information on phenolic content across multiple banana varieties is minimal compared to other nutrients like carotenoids (Singh B. *et al.*, 2016). Also, it is unknown if there are banana accessions with high phenolics in the pulp, which could potentially increase the uptake of these bioactives in our diet (Morais *et al.*, 2015).

Compared to phenolics, carbohydrates are the most abundant nutritional compound in bananas, including soluble and insoluble starch and soluble sugars. It is considered one of the most critical characteristics in quality control for the fruit (Niemman *et al.*, 2012). Given the importance of sugar in bananas as a quality trait, many studies address the topic; however, few studies have focused on the kinetic accumulation of carbohydrates during ripening or the effect of postharvest treatments on them.

Ripening in bananas is a genetically programmed and highly organized process of organ transformation from unripe to the ripe stage. Commercial marketing of bananas usually requires harvesting green fruit to ensure a uniform and controlled ripening process and delivery of bananas optimal in color, smell, and sweetness to market (Tarkosova & Copikova. 2000). Wills *et al.* (1984) reported an ample profile of many of the compositional changes that occur during

ripening in Cavendish bananas. The study found that the most marked changes in this process were in the carbohydrates due to starch hydrolysis during ripening.

This study aims to characterize the total phenolic accumulation in 27 banana accessions to evaluate differences in total phenolic content in the pulp relative to peel, sugar content, and the effect of the post-harvest ripening on total phenolics and sugar content. And this study aims to identify banana accessions with high phenolic content in the pulp, which can be used as starting material in future biofortification programs.

3.2 Materials and methods

3.2.1 Plant material and sample collection

A set of 27 banana accessions was collected from the Dole Food Company's banana collection in Rio Frio, Costa Rica (10 ° 18'29.9" North and 83 ° 52'59,6") as described in Chapter 2, materials and methods section.

3.2.2 Sample preparation

Three hundred twenty-four banana fruits from 27 accessions were peeled; both the pulp and the peel were sliced and immediately frozen in liquid nitrogen to avoid oxidation. Frozen samples were stored at -80 ℃ for approximately 12 hrs and then lyophilized for five days. To prevent oxidation and degradation of the phytochemicals, the lyophilizer was light sealed, and each sample was placed into Mylar bags. Dried samples were stored in light-protected falcon tubes at -80 ℃ until processed.

3.2.3 Extraction of total phenolics (TPC) from lyophilized banana pulp and peel

For banana pulp, 130-150 mg (not exceeding 200 mg) of freeze-dried powder was added to 3 ml of 70% methanol (270 mM HCL), vortexed for 1 min, and centrifuged at 4,800 rpm 4 °C for 30 min. Samples were filtered through 0.20 μm PTFE filters, and two aliquots of 1 ml were stored at -80 ºC. Aliquots were thawed on ice in darkness and diluted 5-fold by adding 200 μl of extract to 800 μl DI water before analysis.

Total phenolics were extracted from banana peel by adding 80 mg of freeze-dried powder to 10 ml of acidified methanol (70% methanol and 270 mM HCl), vortexed for 1 min, and centrifuged at 4,800 rpm 4 °C for 30 min. Samples were filtered through 0.20 μm PTFE filters,

and two aliquots of 1 ml were stored at -80 ºC. Before analysis, the aliquots were thawed on ice in darkness and diluted 100-fold by adding 100 µl of extract to 900 µl DI water twice.

3.2.4 Total Phenolics analysis

Total phenolic measurement was performed in 96 well plates. 25 µl of the extract was transferred to the 96 well plate for each sample, and 75 µl of water was added. 25 µl Folin-Ciocalteu (F.C.) reagent (1:1) was added to each well and incubated for 6 min in the dark at room temperature. After incubation, 100 µl of 7.5% sodium carbonate solution was added to each well, and samples were incubated in the dark at room temperature for 90 min for color development before plate reading.

Simultaneously, a standard stock solution of gallic acid was prepared by dissolving 500 mg of the acid (97.5-102.5% titration) in 10 ml of 100% ethanol and filled to a 100 mL volumetric flask with DI water. A series of aliquots, of 50 µl, 100 µl, 200 µl, 350 µl, 500 µl, 750 µl, and 1,000 µl gallic acid stock standards were diluted to a total of 10.0 ml with DI water and used to prepare the standards for calibration. The standards were processed in the same fashion as the samples for analysis.

Absorbance was measured for each plate at wavelengths 765 nm and 970 nm. Total phenolics results were expressed as mg gallic acid equivalent/100 g fresh flesh or peel.

3.2.5 Extraction of sugars from lyophilized banana pulp

Approximately 0.01 g freeze-dried banana powder was dispersed in 350 µl of 100% ethanol and 150 µl 10 mM of mannitol (in water solution as internal standard). The samples were mixed and vortexed for 1 min and incubated in a water bath at 80 °C for 30 min.

The samples were cooled down for 5 min on ice and diluted with 1 ml of 80% ethanol, then centrifuged at 14,000 rpm and 4 °C for 20 min (centrifuge 5417 R, Eppendorf). One ml of supernatant was dried under a vacuum for 60 min (Savant, DNA120, SpeedVac Concentrator, Thermo: Heater, Manual, High). The samples were diluted with 1.0 ml Milli-Q filtered H2O and filtered into HPLC vials with a 0.2 µm filter (17 mm nylon syringe filter, F2513-2, Thermo Scientific).

3.2.6 Sugar analysis

Sugar analysis was performed by liquid chromatography in an Elite LaChrom system (Hitachi) coupled with a 1260 RID detector (L-2490), column oven (L-2350), autosampler (L-2220) and pump (L-2130)

Sugar separation was accomplished using a Rezex RCM-Monosaccharide Ca+2 (8%), 00H-0130-KO, Phenomenex column, coupled with securitygard cartridges Carbo-Ca 4X 3.0 mm ID, AJ0-4493. Mobile phase of 100 water was run isocratically at 0.6 mL/min for 15 min; injection volume 20µL; Autosampler compartment temperature was 4ºC; the column oven 50ºC and the RID detector were set at 45ºC. The mobile phase was prepared fresh, and the RID was flushed with the mobile phase for at least 20 min, followed by a 2-hr warm-up before experiments. The recovery rate for the internal standard was about 98.29%.

3.2.7 Near-Infrared determination of sugar

Spectra were collected using a Perten DA 7250 near-infrared (NIR) spectrophotometer (Springfield, IL, USA). To develop a calibration curve, lyophilized banana powder samples from 12 accessions at three ripening stages (Stage A, B, and Ccc) and two ripening methods (Ccc and Ccn) (1g-3g) were placed into an aluminum cup and scanned in triplicate. In between readings, the powder was redistributed in the cup.

All samples were scanned in the reflectance mode using a wavelength range from 950-1,650 nm at approximately +/- 2 nm spacing depending on the wavelength with an optical resolution of approximately 10 mm and interpolated back to an even data spacing of every 5 nm at room temperature (~20 °C). The spectral resolution was about 10 nm, with data spacing at approximately 5 nm. Background corrections were made before each sample was scanned, using a reference scan of a white ceramic piece for 3 seconds every time the scanner was started.

The average of three different position spectra for each sample was used as input. Calibration models for every individual sugar were derived using the unscrambler package (CAMO Software AS, Norway)(www.camo.com/unscrambler) by using Honigs regression (H.R.) to calculate the correlation between spectra data and HPLC data of reference.

The raw spectra were preprocessed using detrending which fit the data with a second degree polynomial of x0, x1, and x2, and then subtracting that fit from the spectrum. The pretreated spectrum correlates better and is more repeatable to chemical analysis than the raw spectrum. Data were treated using Standard Normal Variate (SNV), in which the total area of the detrended polynomial is divided to make it equal to 1. SNV treatment helps remove light path length differences caused by the heterogeneous particle sizes of the material.

The statistical methods used for the calibration (cross-validation) and validation (external-validation) datasets included the coefficient of determination (R^2), the root mean square error (RMSE), the ratio of prediction to deviation (RPD), and the ratio between the standard deviation (S.D.) of the HPLC analyzed data and prediction data by NIR model (RMSE), in order to estimate the prediction ability of the model.

The R^2 and residual predictive deviation (RPD) were calculated between the measured sugar content using HPLC and predicted values from the validation dataset to determine which pretreatment gave greater values. An R^2 value greater than 0.85 and an RPD value greater than 2.5 were used as the cut-off value to evaluate the calibration curves' performance.

3.2.8 Data analyses

The statistical analysis data included three biological replicates for each accession, ripening stage, and ripening treatment. The results are presented as the mean ± standard error.

All statistical analyses were performed using the software R Studio (R Core Team, 2014; RStudio Team (2020), and figures were produced using the package ggplot2 (Wickham, 2009). Means were compared using a one-way analysis of variance (ANOVA) to determine the significant difference ($\alpha < 0.05$) among accessions and ripening stages. In contrast, a paired T-test was used to determine any significant difference between ripening methods ($\alpha < 0.05$).

3.3 Results

3.3.1 Total phenolics

Total phenolic content in pulp (TPCP) in unripe banana (stage A) ranged from 0.04±0.01 g GAE/100 g in Tindok to 2.61±0.57 g GAE/100 g in Kru (Figure 2.1). Compared to semi-ripe banana samples (stage B), TCCP ranged from 0.09±0.01 g GAE /100 g in Kru to 2.99±0.16 g GAE /100 g in Pisang Lidi. For fully ripe banana samples at stage Ccc, TPCP varied from 0.09±0.01 g GAE mg/100 g in Hua Moa to 3.26±0.21 g GAE/100 g in Pisang Lidi. For the banana samples ripen on the plant (stage Ccn), TPCP ranged from 0.02±0.001 g GAE g/100 g in Ducasse to 1.13±0.77 g GAE/100 g in Bungai (Figure 2.1; ANNEX B).

The content of phenolics in the pulp of Cavendish samples was 0.25±0.09, 0.46±0.03, and 0.46±0.04 g GAE/100 g for stages A, B, and fully ripe in controlled conditions (Ccc), and 0.19±0.001 g GAE/100 g for ripened on the plant (Ccn) (Figure 2.1).

Overall, the total phenolic content in the peel (TPCLP) was higher than that in the pulp. TPCPL for bananas in stage A (unripe) ranged from 0.17±0.01 g GAE/100 g in Hua Moa to 4.51±0.11 g GAE/100 g in Prata (Figure 2.1). Partly ripe (stage B) samples ranged from 2.17±0.16 in Hua Moa to 4.78±0.75 g GAE mg/100 g in Kunnan. For fully ripe banana samples at stage Ccc, TPCPL varied from 1.96±0.17 g GAE mg/100 g in Hua Moa to 5.18±0.25 g GAE/100 g in Prata. For bananas ripened on the plant (stage Ccn), TPCPL ranged from 1.26±0.09 in KKB to 4.84±0.42 g GAE/100 g in Prata (Figure 2.1).

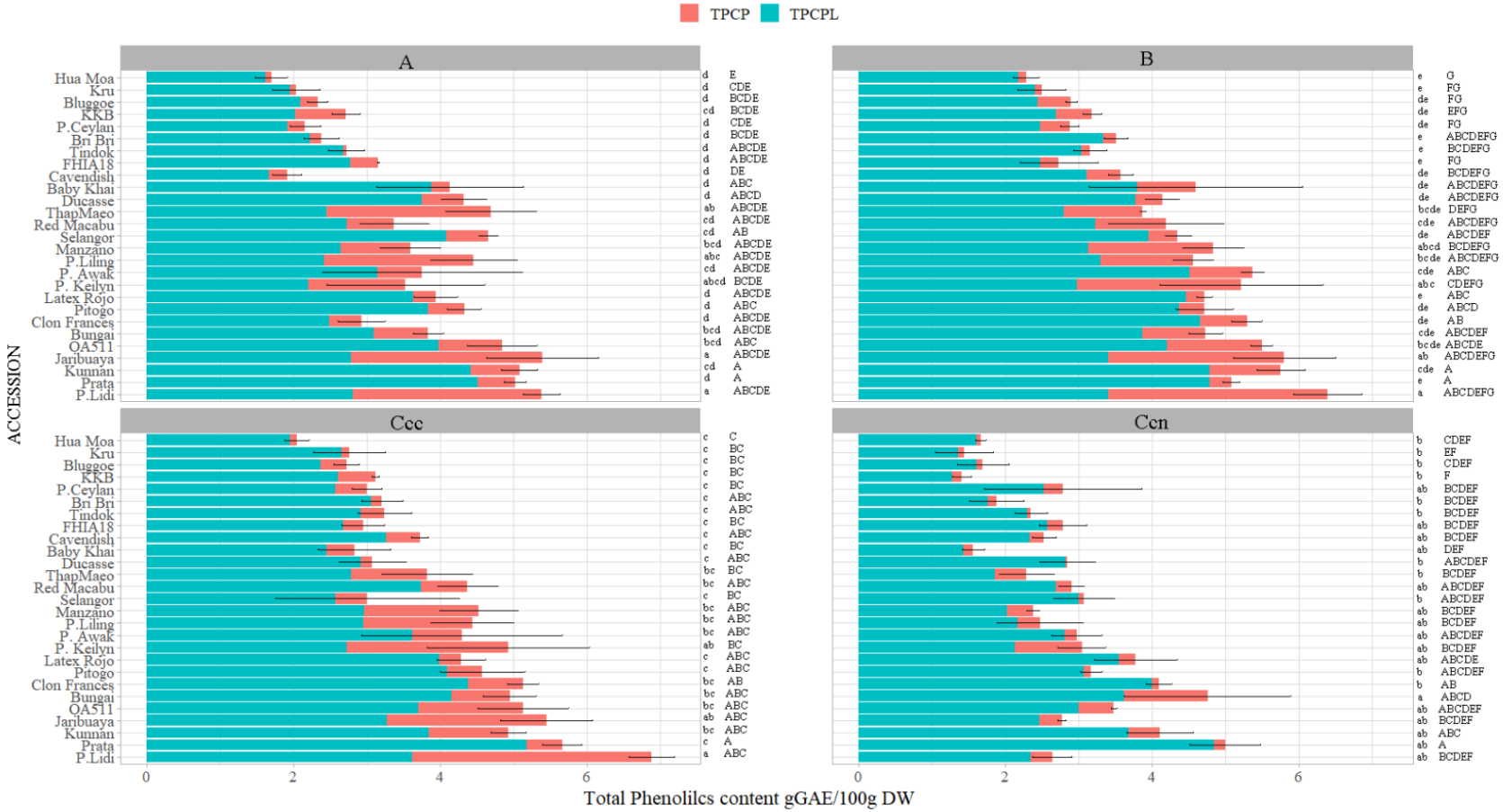

Figure 3.1. Total phenolics content in pulp (TPCP) and peel (TPCPL) in 27 banana accessions expressed in gallic acid equivalents (GAE)/100 g D.W, at three ripening stages (A, B, C) and two ripening methods at stage C, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). Letters on the right side of each graph represent Tukey's HSD grouping, the lower case represents the grouping in the pulp, and the upper case represents the grouping in the peel.

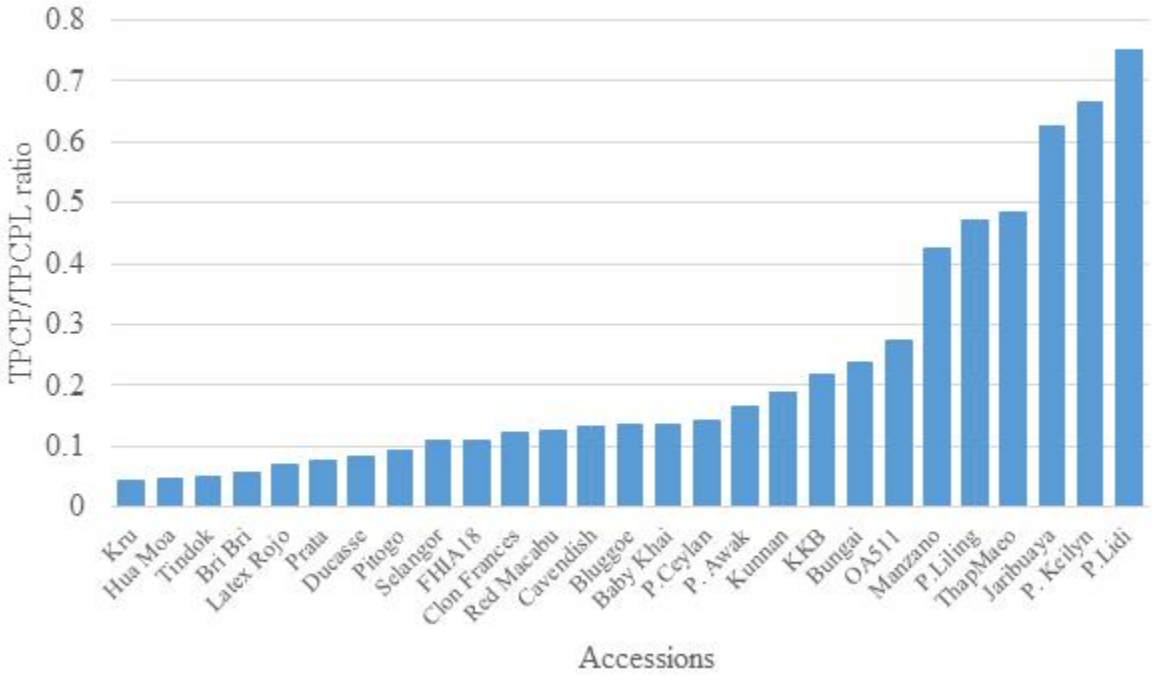

Figure 3.2. The ratio of total phenolic content in pulp (TPCP) and peel (TPCPL) in 27 banana accessions. Values were calculated for each accession using mean TPCP/TPCPL averaged across at all ripening stages.

TPCPL in Cavendish was 1.67±0.26 g GAE/100 g for stage A, 3.11±0.15 g GAE/100 g for stage B, 3.26±0.08 g GAE/100 g for the fully ripe stage in controlled conditions (Ccc), and 2.33±0.17 g GAE/100 g for the fully ripe stage on the plant (Ccn). There were significant differences in TPCPL in all 27 banana accessions (ANOVA $p<0.05$) (Figure 2.1), and several accessions had a significantly higher TPC than Cavendish banana in both peel and pulp.

The ratio of TPCPP to TCPL showed that the accessions Pisang Lidi (0.74), Pisang Keilyn (0.67), and Jaribuaja (0.62) have the highest proportional content of phenolics between pulp and peel. The accessions Thap Maeo, Pisang Liling, and Manzano have a ratio between 0.40 and 0.50. The remaining accessions have a value lower than 0.30 (Figure 2.2).

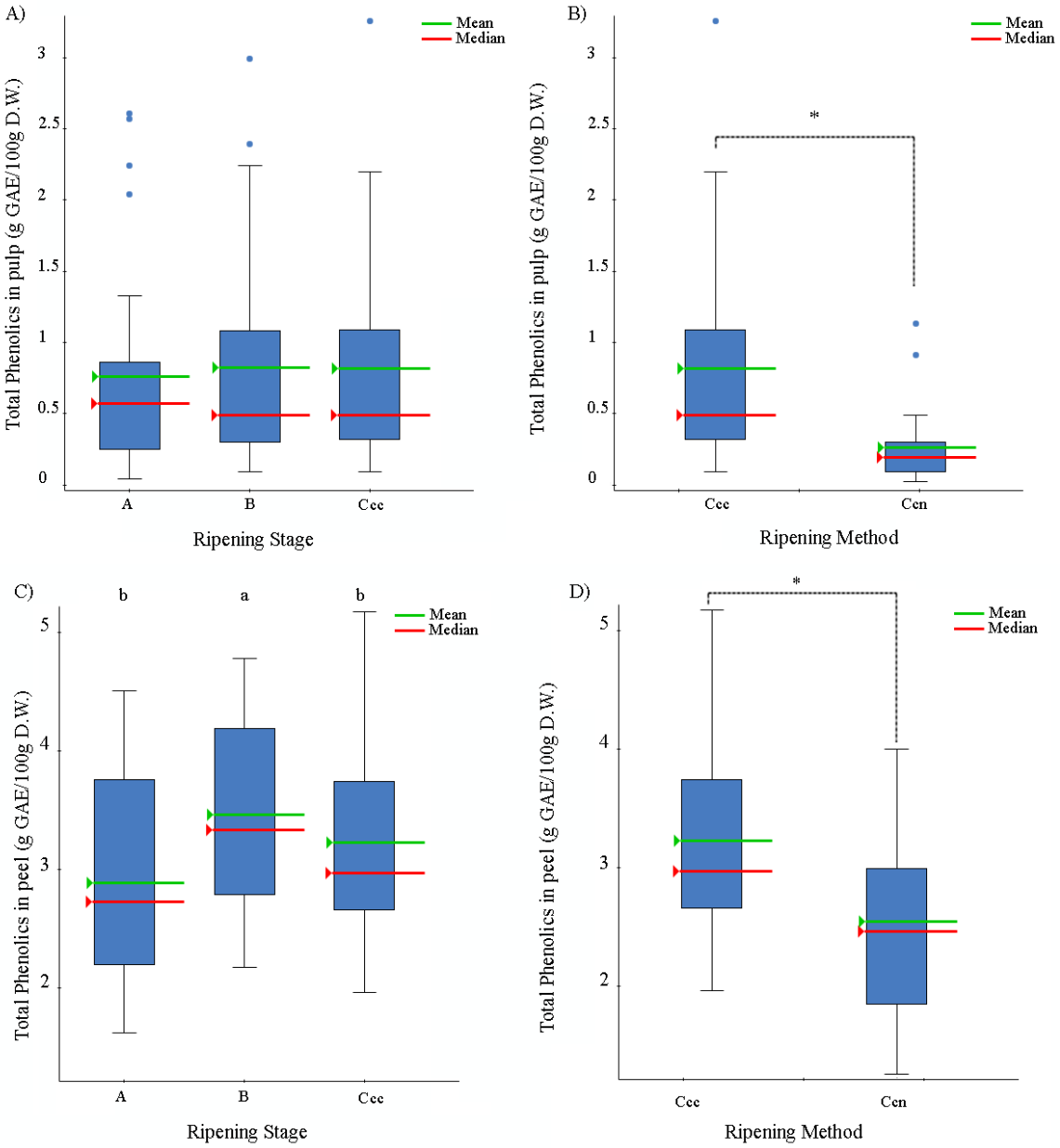

Figure 3.3. Average TPC (g GAE /100g D.W.) in pulp and peel in 27 banana accessions. (A) TPCP by ripening stage (A, B, C); and (B) TPCP by ripening method, ripened fully in controlled conditions (Ccc) and ripened fully on the plant (Cnc). (C) TPCPL by ripening stage (A, B, C); and (D) TPCPL by ripening method, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). (*) indicate groups that were significantly different (Paired T-test $p<0.05$). Letters above each bar represent Tukey's HSD grouping.

TPCP for the 27 accessions varied according to the ripening stage and ripening treatment. The aggregate average was 0.76±0.14, 0.82±0.14 g GAE/100 g, for stage Ccc was 0.82±0.15 g GAE/100 g, and for stage, Ccn was 0.26±0.05 g GAE/100 g (Appendix D). ANOVA analysis found no significant differences in all maturation stages (A, B, Ccc). However, there was a significant difference (paired T-test $p<0.05$) between maturation methods (Ccc, Ccn), where there was a 3-fold reduction in the aggregate average from stage Ccc to stage Ccn (Figure 2.3).

The aggregate average of TPCPL for unripe bananas (stage A) was 2.88±0.16 g GAE/100 g. For stage B, or semi-ripe banana was 3.72±0.15 g GAE/100 g. For stage Ccc, or banana ripened in controlled conditions was 3.23±0.14 g GAE/100 g, and for stage Ccn, or banana ripened on the plant was 2.54±0.17 g GAE/100 g (Figure 2.3).

There were significant differences among ripening stages (ANOVA $p<0.05$) and ripening methods (paired T-test $p<0.05$). Accessions reached their peak of TPCPL accumulation at stage B compared to stages A and Ccc, while there was a 10% reduction of TPCPL from ripening in controlled conditions (Ccc) to ripen on the plant (Ccn). No significant correlation was found between phenolic content in the pulp (TPCP) and the peel (TPCPL) ($R^2<0.03$).

3.3.2 Sugar content

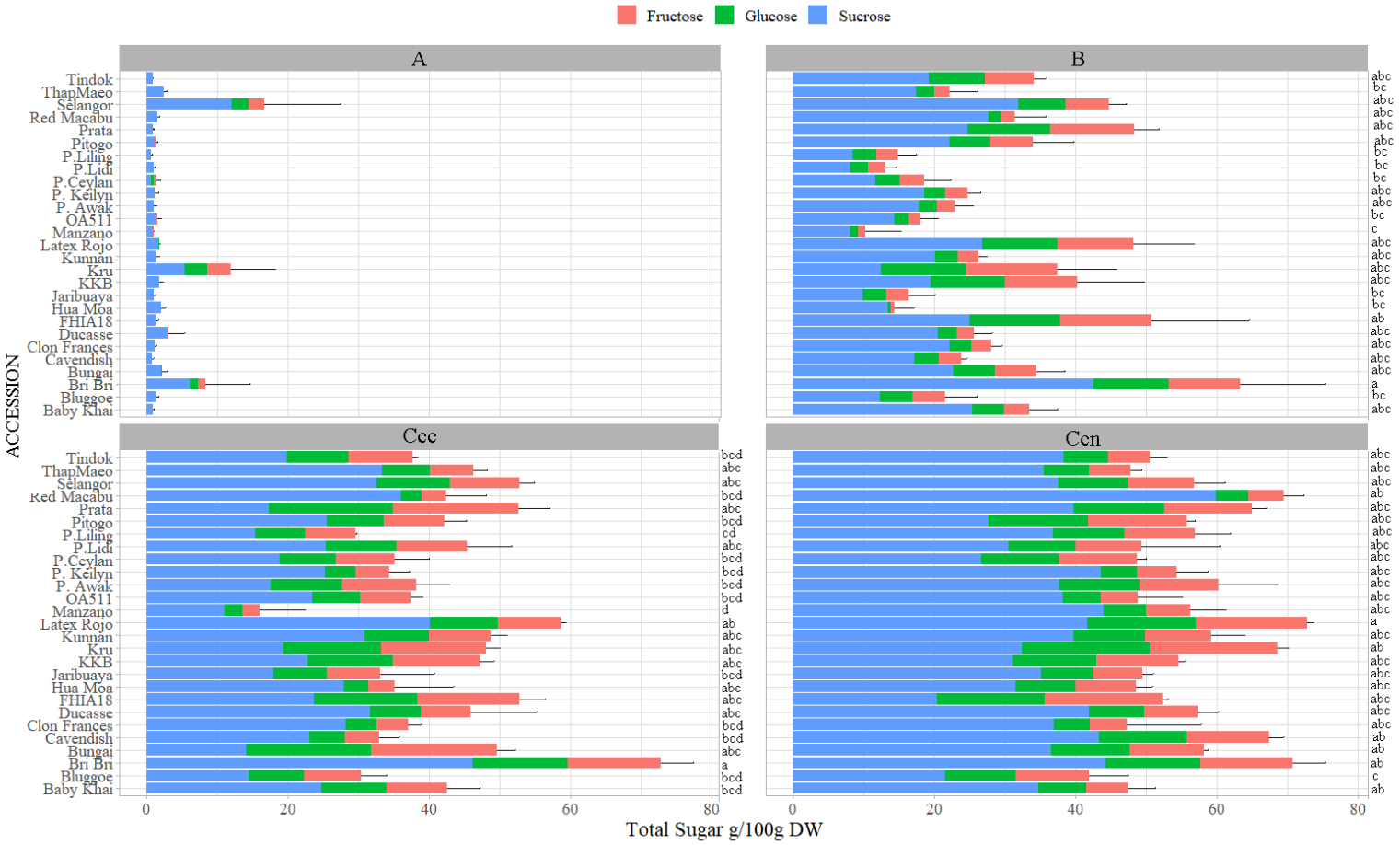

Figure 3.4. Total sugar content (TSC) and profile in 27 banana accessions, in the pulp samples at three ripening stages (A, B, C) and two ripening methods at stage C, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). Letters on the right side of each graph represent Tukey's HSD grouping.

Total sugar content (TSC) and individual sugars (glucose, fructose, and sucrose) were measured in the pulp samples to evaluate the variation of sugars in the 27 accessions and the effect of reducing sugars on total phenolic quantification in pulp (TPCP). Indeed, the F.C. assay used to measure TCC could be affected by sugars (Huang *et al.*, 2005). TSC was calculated by assessing the glucose, fructose, and sucrose content in 27 banana accessions, three ripening stages (A, B, C), and two ripening methods, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). A NIR methodology was also developed for measuring total and individual sugars (ANNEX D).

TSC ranged from 0.02±0.027 g/100 g in Pisang Keilyn to 3.72±0.81 g/100 g in Bungai. In semi-ripe banana samples (stage B), TSC ranged from 5.2±5 g/100 g in Manzano to 57.59±6.41 g/100 g in Bri Bri (Appendix D). For fully ripe banana samples, at stage Ccc, TSC varied from 10.11±6.41 g/100 g in Manzano to 78.71±4.58 g/100 g in Bri Bri, and for fully ripe on the plant (stage Ccn), TSC ranged from 26.05±10.58 g/100 g in Clon Frances to 76.14±4.7 g/100 g in Bri Bri. The accessions with the highest TSC were Bri Bri (stages B, Ccc, and Ccn), Latex Rojo, Cavendish, Pisang Awak, and Kru (all at stage Ccn) (Figure 2.4).

Significant variation in TSC based on ANOVA analysis was found among the 27 banana accessions ($p<0.05$). The average fructose content varied from 0 in several accessions at stage A to 18.15±1.94 g/100 in Kru at stage Ccn (Appendix D). Similarly, the average glucose content ranged from 0 in several accessions to 18.05±1.7 g/100 in Kru at stage Ccn. With the highest average content across all accessions and maturation stages, sucrose went from 0.67±0.53 g/100 in Pisang Ceylan at stage A to 59.83±1.5 g/100 in Red Macabu at stage Ccn. The average sucrose content for Cavendish was 21.10±0.9 g/100, 5.19±0.34 g/100 for glucose, and 4.96±0.27 g/100 for fructose.

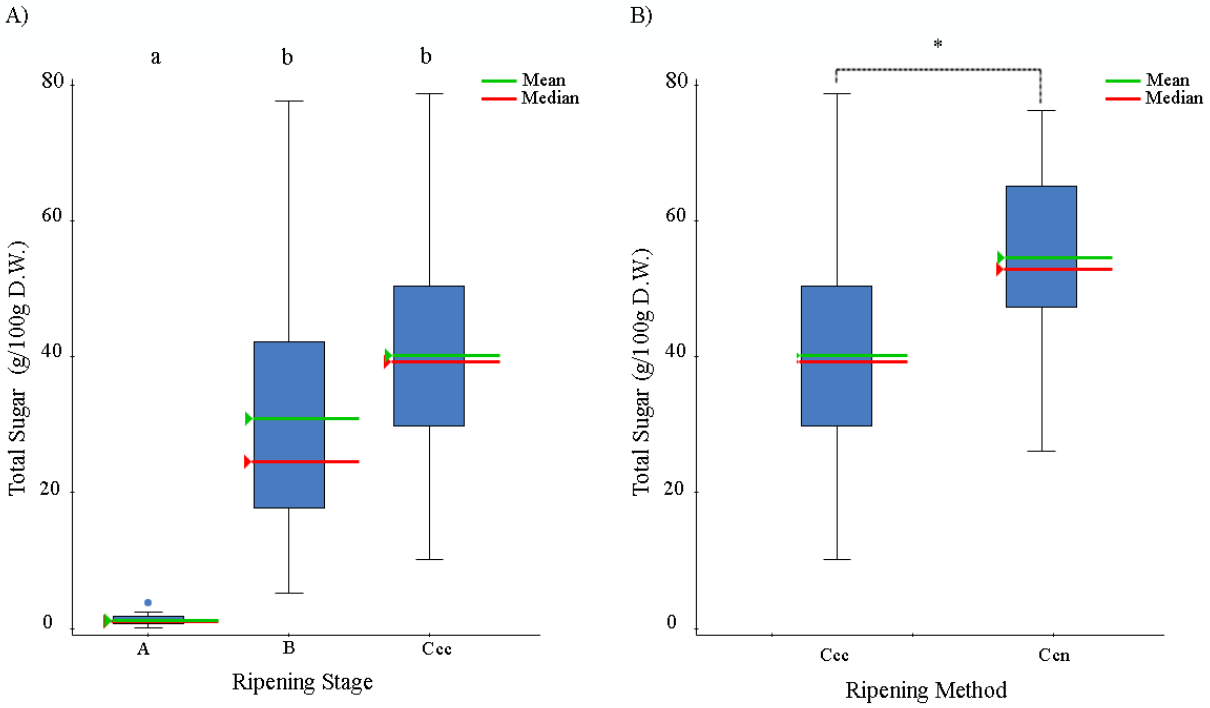

Figure 3.5. Average TSC (g /100g D.W.) in 27 banana accessions. (A) Ripening stage (A, B, C); and (B) Ripening method, fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc). Letters on top of each bar represent Tukey's HSD grouping. Bars highlighted (*) indicate groups that showed significant differences (Paired T-test $p<0.05$).

There was significant variation among the average glucose, fructose and glucose content in all accessions. However, there was also a significant interaction between the type of sugar and the maturation stage (ANOVA $p<0.05$).

The aggregate average TSC for unripe banana (stage A) was 1.23±0.14 g/100, for semi-ripe banana (stage B) was 30.90±3.65 g/100 (Figure 2.4). For fully ripe bananas, the aggregate average was 40.14±2.90 g/100 for stage Ccc and 54.48±2.18 g/100 for stage Ccn. There were significant differences among ripening stages (ANOVA $p<0.05$) and ripening methods (paired T-test $p<0.05$), and such differences are independent of the accessions. TSC increased 32-fold from

stage A to Stage Ccc, and TSC is 1.36 fold higher when comparing accessions ripened by natural maturation (Ccn) to those ripened in controlled conditions (Ccc) (Figure 2.5).

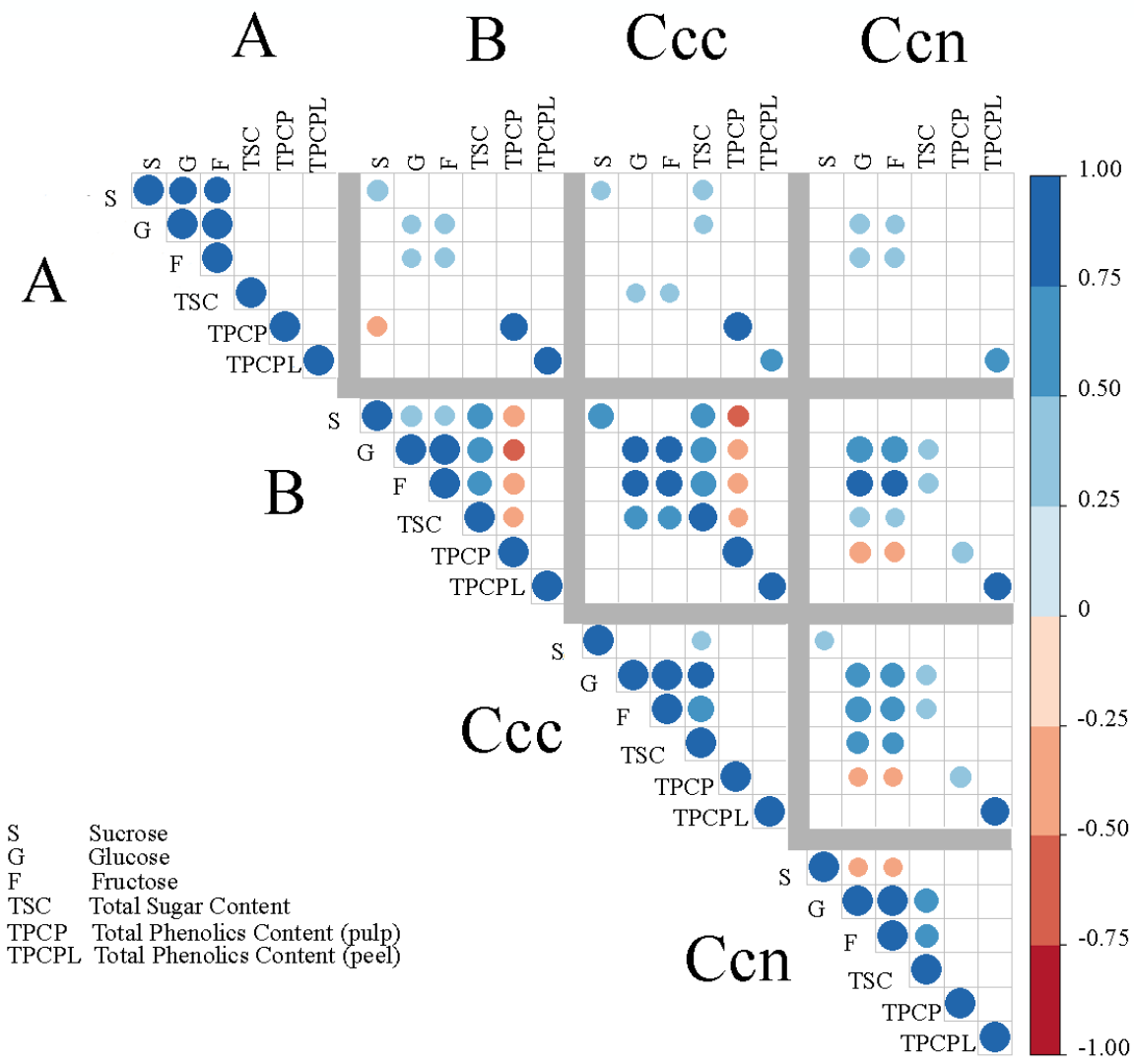

Figure 3.6. Correlation matrix among sugar (Glucose, fructose, sucrose, and TSC) and total phenolics (TPCP and TPCPL) in 27 banana accessions at three ripening stages (A, B, Ccc); and two ripening methods (fully ripe in controlled conditions (Ccc) and fully ripe naturally on the plant (Cnc)). Only those coefficients of determination statistically significant ($p<0.05$) are shown.

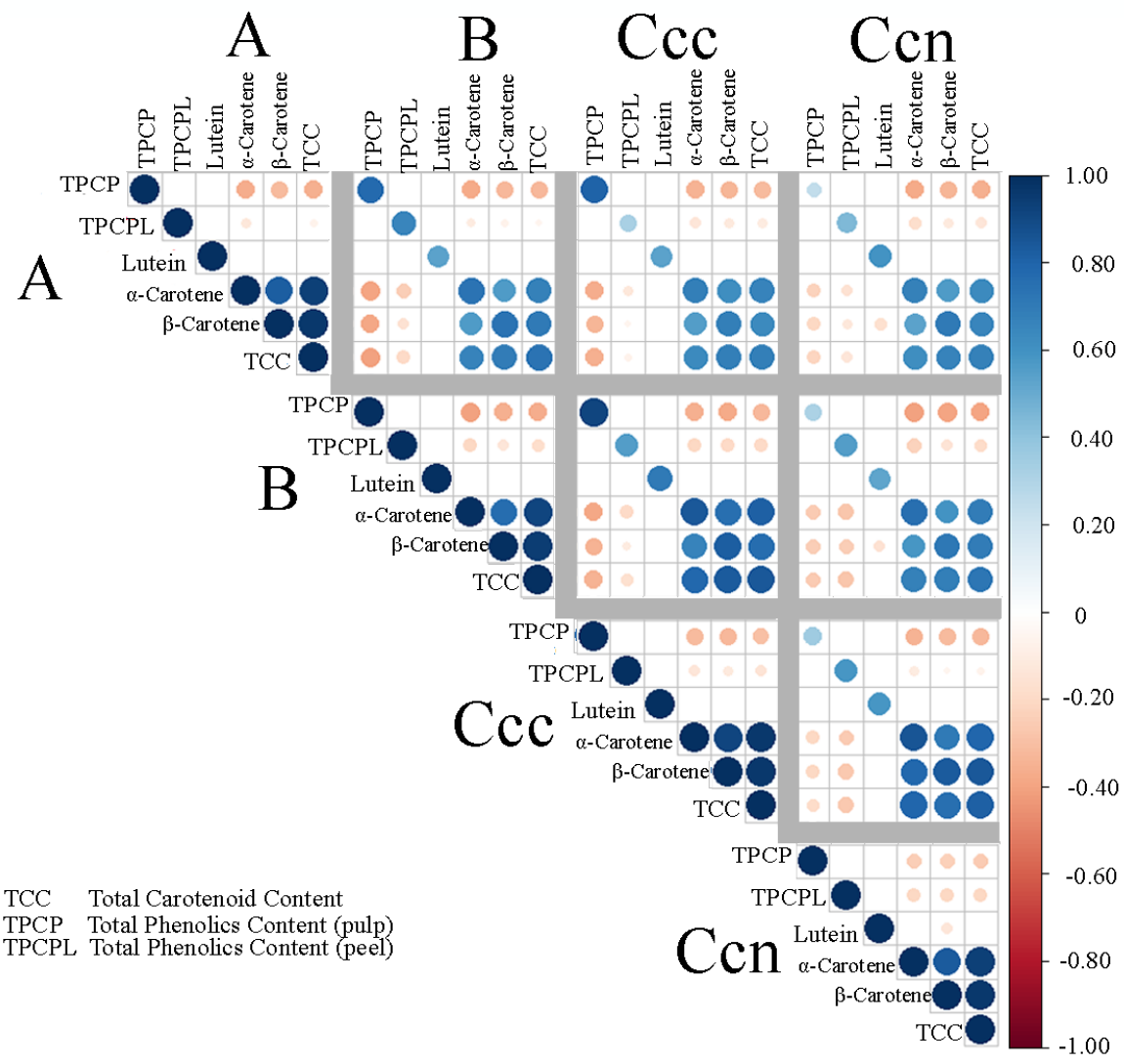

Figure 3.7. Correlation matrix among carotenoids (lutein, α-carotene, β-carotene, and TCC) and total phenolics (TPCP and TPCPL) in 27 banana accessions at three ripening stages (A, B, Ccc); and two ripening methods (fully ripe in controlled conditions -Ccc and fully ripe naturally on the plant -Cnc). Only those coefficients of determination statistically significant ($p<0.05$) are shown.

Correlation analysis showed a strong significant correlation for all accessions across ripening stages (A, B, and Ccc)(Figure 2.6). The correlation between TPCP between stages A and B was R^2 0.71, and for stages B and Ccc were R^2 0.95. For TPCPL between stages A and B,

the coefficient was R^2 0.65, and between stages B and Cc was R^2 0.64. In contrast, when comparing ripening methods Ccc and Ccn, only phenolics in the peel showed a significant and robust correlation (R^2 0.74). No significant correlations were found between TPCP and TPCPL (Figure 2.6).

Comparison of sugar and phenolic content across ripening stages (A, B, and Ccc) indicated that a significant but weak correlation ($p<0.05$) exists between TPCP and the individual sugars (glucose, fructose, and sucrose) for stages B and Ccc (Figure 2.6). The most significant coefficient of determination was found between sucrose at stage B with TPCP at stage Ccc (R^2 - 0.62) (Figure 2.5). No significant correlation was observed between sugars and phenolics content within each set of samples at the same ripening stage and methods (e.g., Ccc vs. Ccc), indicating that sugars content had no effect on the TPCP and TPCPL measurement.

To integrate data from chapter 1 and chapter 2, all the data were correlated to understand the possible interaction between carotenoid content, carotenoid bioaccessibility, total phenolic content, and sugar content. When analyzing carotenoid content and phenolic content across ripening stages (A, B, and Ccc) and ripening methods (Ccc, Ccn), significant but weak correlations ($p<0.05$) were found between TPCP and individual carotenoids content (α-carotene, β-carotene). The most robust coefficient of determination (R^2) was found between TPCP and β-carotene at stage B (R^2 0.16) and the weakest between TPCP and β-carotene at stage Ccn (R^2 0.05) (Figure 6). No significant correlations were found among TPCP and TPCL and relative and absolute bioaccessibility of carotenoid content ($R^2<0.05$) (Figure 2.7).

3.4 Discussion

3.4.1 Total phenolic content variation in banana pulp (TPCP) and peel (TPCPL)

Many available quantification methods for total phenolic content in food are based on the reaction of the phenolic compounds with a colorimetric reagent, allowing measurement in the visible light spectrum. One of these methods is the Folin–Ciocalteu (F.C.) reaction, a standardized technique for measuring phenolics in food. This methodology relies on the transfer of electrons in an alkaline medium from phenolic compounds to other acid complexes to form color complexes that can be measured via a spectrophotometer (Waterhouse, A., 2002).

The F.C. assay has the limitation that other oxidative substances in the sample can interfere with the reaction by inhibiting, enhancing, or interfering with it. Inhibition could occur due to oxidants competing with the F–C reagent or air oxidation. Interference may arise from other substances in the sample, such as phenols, aromatic amines, high sugar levels, or ascorbic acid. Additional literature (Dai & Mumper, 2010; Nardini & Garaguso, 2018) has discussed that high levels of ascorbic acid, sugars, or sulfites might have an additive effect on the results; therefore, they need to be considered when designing the essay. Nonetheless, the F.C. assay is reproducible and straightforward and should be considered as a base approximation for the total phenolic content (Huang *et al.*, 2005).

At the same time, the extraction method for phenolics might be significantly affected by sample preparations and solvents used for extraction. Several authors have found that chloroform tends to be a better solvent for phenolics extraction from dried peels and pulps, while water and methanol have been successfully used in fresh pulps and peels (Nicoli *et al.*, 1999; Orhan *et al.*, 2007; Policegoudra *et al.*, 2007).

Sultana *et al.* (2008) showed that the extract yield from banana peels with ethanol was around was 24.6%, while Gonzalez-Montelongo *et al.* (2010) obtained a yield of 54% with a mixture of acetone and water. These results showed that it is essential to consider the methodology and the extraction solvent when comparing results with other authors.

Various phenolics in banana have been identified (gallic acid, catechin, epicatechin, tannins, and anthocyanins) in the pulp, the peel, and even the plant's rhizome (Sidu & Zafar, 2018). Most studies of phenolics in banana focus their efforts on the peel rather than the pulp, as the peel has been regarded as higher in phenolic content and can be used as a functional ingredient in food or food supplements (Vu *et al.*, 2016). Nonetheless, accession with high phenolic content in the pulp is yet to be identified (Sidu & Zafar, 2018).

In our study, the accessions showed a total phenolic content in pulp (TPCP) that ranged from 0.04±0.01 g GAE/100 g in Tindok at stage A (unripe) to 3.26±0.21 g GAE/100 g in Pisang Lidi at Stage Ccc (ripe with exogenous ethylene). In the peel, the phenolic content (TPCPL) ranged from 0.17±0.01 g GAE/100 g in Hua Moa at stage A to 5.18±0.25 g GAE/100 g in Prata at stage Ccc. On average, banana peels show a phenolic content fivefold higher than in the pulp. The accessions with the highest phenolics content in pulp were Pisan Lidi (stage Ccc), Jaribuaya (stage A), Pisang Keilyn (stage B), Thap Maeo (stage A), and Pisang Lilling (stage A). At the same time, Prata showed the highest phenolic content in peel at all stages.

Most studies agree that TPC ranges widely according to the banana variety, and all of them agree that peel tends to have a higher TPC than pulp. Fatemeh *et al.*(2012) reported a TPC range in pulp from 0.075 to 0.68 g GAE/100 g. Sun *et al.* (2012) reported an average of 0.056 g GAE/100 g, while Choo (2010) reported Awak as a high phenolic content banana pulp with 0.090 g GAE/100 g. Anal *et al.* (2014) found that TPC in banana peel ranged from 0.18 to 0.35 g

GAE/100 g, Sultana *et al.* (2008) found an average of 1.1 g GAE/100g (ethanol extraction), and Gonzalez-Montelongo (2010) 1.4 g GAE/100 g when extracted with methanol.

In the same fashion, in our study, TPC in pulp varied according to the ripening stage or ripening method for each accession. ANOVA analysis showed no significant differences among maturation stages (A, B, and Ccc), but significant differences were found among maturation methods (Ccc and Ccn). According to the aggregate average, stages B and Ccc showed the highest phenolic content in pulp with 0.82±0.14 g GAE/100 g, while stages A and Ccn showed the lowest TPC with 0.76±0.14 g GAE/100 g 0.26±0.05 g GAE/100 respectively. The general trend showed an increase of TPC from stages A to B and decreased from stages B to Ccc. In comparison, TPC in peel revealed significant differences among ripening stages (ANOVA $p<0.05$) and ripening methods (paired T-test $p<0.05$). In most accessions, the peak for TPC accumulation was at stage B (3.72±0.15 g GAE/100 g, aggregate average).

Similar studies have reported that fruit maturity significantly affects the phenolic content in the peel as in the pulp. TPC was found to decrease in pulp with the ripening process, while ripe banana peel has 52% to 15% less phenolic content than in the green peel (Fatemeh *et al.*, 2012, Sundaram *et al.*, 2011).

Little is known about the effects of exogenous ethylene during maturation over TPC accumulation. Our study has found a significant decrease of phenolics in pulp (3-fold) and peel (10%) from bananas ripened with exogenous ethylene compared to those ripened on the plant. Earlier studies have shown that cultivation conditions might influence the levels of bioactive compounds within bananas up to a 38.2% difference (Faller & Fialho 2010).

Heredia *et al.* (2009) studied the effect of exogenous ethylene on different wounded fruit and vegetables (lettuce, cilantro, cabbage, green beans, apples, plums, peaches, table grapes,

strawberries, bell peppers, asparagus, celery, carrots, radishes, potatoes, and jicama). They found an increase in TPC accumulation within the treated samples. They hypothesized that a common signal between ethylene as a stress response and differences in phenolic synthesis and degradation kinetics might change the accumulation of these bioactives.

In contrast, Maduwanthi and Marapana (2021) found that inducing ripening with exogenous ethylene in bananas lowered the content of phenolics, flavonoids, and antioxidant activity considerably compared to the non-treated group. It is worth mentioning that this study did not compare banana ripened on the tree, but rather green banana ripened without exogenous ethylene, and it also only considers one variety of banana (Ambul banana, *Musa acuminata*, AAB).

Considering the potential confounding effect introduced by sugar in the F-C method, our study measured the amount of glucose, sucrose, and fructose in every pulp sample. We found that there was no correlation among total sugar content (TSC), individual sugars (glucose, fructose, and sucrose), and TPC across ripening stages or methods ($R^2<0.10$, $p<0.05$), suggesting that there is no influence of any of these sugars in our TPC report.

On the other hand, significant but weak negative correlations ($p<0.05$) were found between TPCP and individual carotenoids (α-carotene, β-carotene). Similar findings to our correlation have been reported before in potatoes (Reddivari *et al.*, 2007; Al Meraj *et al.*, 2018) and papaya (Nieto-Calvache *et al.*, 2019) but not yet on banana. No correlation was found between carotenoid bioaccessibility and phenolic content, as both processes were expected to be unrelated (Saltveit, 2017).

Our study showed that the accessions Pisang Lidi, Pisang Keilyn, Jaribuaja, ThapMaeo, Pisang Liling, and Manzano have a TPCP almost as high as its TPCPL. The amounts of TPCP in

our report are more than three-fold higher than what has been previously reported in similar literature (0.91 g GAE/100g) (Balasundram *et al.*, 2006; Bennet *et at.*, 2011). We consider this fact of great importance, as accessions with high phenolic content in the pulp are deemed exceptional (Siduh & Zafar, 2019), and they could have a significant impact on banana biofortification programs for Phenolics and nutrition in general.

To our knowledge, this is the first study reporting the identification of banana germplasm with relatively high total phenolic content in the pulp. Given the importance of phenolics in human health, these findings open opportunities to characterize this material further, either for breeding purposes, to be used in the food or farmaceutical industry, or just to be directly consumed. This report could be further supplemented by including a characterization of a phenolic profile among these accessions. Previous studies (Kevers *et al.*,2007; Russel *et al.*,2009; Tsamo *et al.*,2015) indicate that phenolics such as: 6-gingerol, Sesamol, 3,4,5-trihydroxybenzoic acid, serotonin, tryptophan, trans-4-hydroxy-3-methoxycinnamic acid, malic acid, 3-hydroxy-4-methoxy cinnamic acid, and Dopamine, can be found in banana pulp and peel, suggesting a base for a profile characterization.

3.4.2 Total Sugar Content (TSC) variation and profile in banana pulp

Our study found that total sugar content (TSC) ranged from 0.02±0.027 g/100 g in Pisang Keilyn at stage A (unripe) to 76.14±4.7 g/100 g in Bri Bri at stage Ccn (fully ripe on the plant). By individual sugar, the average fructose content varied from 0 in several accessions at stage A to 18.15±1.94 g/100 in Kru at stage Ccn. The average glucose content ranged from 0 in several accessions to 18.05±1.7 g/100 in Kru at stage Ccn. Furthermore, sucrose has the highest average content across all accessions and maturation stages, ranging from 0.67±0.53 g/100 in Pisang

Ceylan at stage A to 59.83±1.5 g/100 in Red Macabu at stage Ccn. There was significant variation among the average glucose, fructose and glucose content in all accessions. However, there is also a significant interaction between TSC by type and the ripening stage for glucose and fructose. In comparison, the total sugar content is both accession and maturation dependent.

Several individual studies have confirmed that total sugar content and the individual sugar profile are accession-dependent in bananas, and they increase as the fruit ripens. Marriot *et al.* (1981) found that sucrose comprised more than 70% of the total sugars in fully ripe bananas and plantains and about 50% of the total sugars in overripe fruits, giving the fruit its characteristic sweet taste. Emaga *et al.* (2007) found that the soluble sugar content increased in different proportions in different varieties, which can be explained by the degradation of starch and the formation of free sugars under enzymatic action. Moreover, Adao & Glória (2005) reported increasing content of soluble sugars, from green to ripe bananas, ranging from 3.4% to 15.7–3.4%.

There is an intrinsic relationship between sugar and starch since sugar derived in the ripening process is the product of starch hydrolysis (Wills *et al.*, 1984). Our data shows that there is a significant negative correlation between TSC, sucrose content, and total and soluble starch content (R^2 0.46 $p>0.05$) in accessions ripened on the plant (Ccn). On the other hand, bananas ripened with exogenous ethylene (Ccn) showed a significant negative correlation between sucrose content and total starch (R^2 0.52 $p>0.05$) and resistant starch content (R^2 0.41 $p>0.05$). This result can be explained as banana matured with exogenous ethylene has a higher resistant starch content than those ripened in the plant. The starch hydrolysis in accessions ripened with exogenous ethylene is not yet completed, as Hakim *et al.* (2012) reported (Appendix G).

There were significant differences among ripening stages (ANOVA $p<0.05$) and ripening methods (paired T-test $p<0.05$), and such differences are independent of the accessions. Furthermore, TSC increased 32-fold from stage A to Stage Ccc, while TSC on average is 1.36 times higher in bananas at stage Ccn than the corresponding samples at stage Ccc.

The source-sink relationship theory may explain the higher sugar content of samples at stage Ccn than their counterpart at stage Ccc. Bananas ripening on the plant have a higher mobilization of photosynthates from the leaves to the fruit, while the bananas that were cut unripe can only convert to sugar the starch that has accumulated so far. Depending on the fruit biomass relative to the plant, bananas may take up to 80% of the photosynthates produced by the leaves, being sucrose 90% of them (Fischer *et al.*, 2012). Previous research has indicated that the amount of leaves on the plant during fruit maturation has an effect on the banana's final weight. Plants left with four or fewer leaves may produce fruit with up to 19% less weight. These results suggest that the source-sink relationship during fruit maturation and ripening has a profound impact on the fruit filling (Vargas *et al.*, 2009)

Jullien *et al.* (2001) found an intrinsic relationship between cell division and dried weight in the banana pulp. Fruit that has been severed from the bunch during maturation but before the end of cell division will have less dry weight and less metabolite accumulation. While the cell-filling rate seems to be more influenced by source-sink relationships, our study shows an average 63% increase in fresh weight in fruit ripened at stage Ccn than samples at stage Ccc. However, there is no noticeable difference in the average dry matter pulp weight in either ripening treatment, suggesting that most of the banana in this study was harvested uniformly, and the difference in fresh weight does not correspond to differences in tissue development.

Additionally, our results showed an average 48% increment of sucrose content in bananas ripened on the plant than their counterparts, along with the weight gain. This data suggest that banana kept on the plant, with no addition of exogenous ethylene, accumulated more sugar and possibly starch, among other metabolites, for much longer than banana harvested unripe and ripened with exogenous ethylene. Given that sucrose is an essential source of energy for metabolism, and that bananas make up to 25% of the carbohydrates consumption for some people from impoverished countries (Van Asten & Staver, 2012), small acreage farmers can take advantage of this knowledge to improve the nutritional content of banana by just delaying its harvesting.

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Chapter 4. Evaluation of global banana germplasm for Black Sigatoka Resistance, using classical and effector mediated resistance

4.1 Introduction

Banana production for export markets is generally intensive, with higher external inputs, such as pesticides and fertilizers that keep the soil fertile while decreasing the losses caused by pests and disease, and is often carried out as mono-crops plantations organized along agro-industrial lines. Due to its fruit characteristics, the Cavendish variety has become the preferred clon in agro-commercial plantations. However, this banana is very susceptible to the attack of pests such as nematodes (*Radophulus similis*) and diseases such as Black Sigatoka (*Pseudocercospora fijensis* Morelet) (CORBANA, 2011).

Moreover, banana mono-crops are characterized by a high density of plants with little to no genetic variation and poor biodiversity within the agro-ecosystem. All these characteristics put together tend to attract a wide range of pathogens, especially fungal infections, and at the same time, more pesticides are needed to keep such diseases under control. Over time the pathogens will develop chemical resistance to the pesticides, while the industry will create new pesticide formulations to fight back, producing a never-ending cycle of pesticide applications and pathogen mutations (Arias *et al.*, 2004).

Black Sigatoka, caused by the ascomycete fungus *P. fijiensis*, represents the most common and economically important disease in banana production around the globe (Friesen, 2016; Arias *et al.*, 2003). The pathogen destroys leaf tissue which reduces photosynthesis and affects plant growth and yield. This disease cause loss of at least 50% of net weight in the banana bunch, to up to 100% loss of fruit that meets commercial quality (length and thickness of the

fingers). Other challenges associated with Black Sigatoka symptoms are the premature ripening of fruits in the field or during transport to the final market destinations (Jimenez *et al.*, 2007; Martinez *et al.*, 2011).

Controlling Black Sigatoka in bananas adds a significant production cost and has a negative environmental impact. According to data reported in the Caribbean region, the application of fungicides can represent up to 80% of the total agrochemicals incorporated for the maintenance of the plantation, which translates into about 53 Kg of active ingredient per hectare and 27% of the total production cost (CORBANA, 2011).

The intensive use of pesticides, in addition to the negative environmental impact, has a detrimental effect on the health of plantation workers and neighboring communities (Martinez *et al.*, 2011). For this reason, there's a need to implement new methods to control the disease, such as the use of resistant varieties along with structural changes in banana plantations (Ortiz, 2002).

4.1.1 Description of the pathogen

The causal agent of Black Sigatoka (*Mycosphaerella fijiensis*) is an Ascomycete fungus, which reproduces sexually and asexually and has a hemibiotrophic life cycle. The asexual form (anamorphous) is called *Pseudocercospora fijiensis*. It can be found in the first lesions of the disease (pinches and stretch marks), in which there is a relatively low number of conidiophores just outside the stomata, mainly on the abaxial surface of the leaf. The conidia germinate during periods of high relative humidity (92-100% humidity) (Marín *et al.*, 2003).

The sexual form (teleomorphic) is named *Mycosphaerella fijiensis* and is the most important mode of reproduction since it produces many ascospores (Marín & Romero 1992). It is found in mature necrotic lesions. This form of the fungi will produce spermogonium and

spermatia, which move within the free water on the leaf's surface to join receptive hyphae that will develop into pseudothecium and produce ascospores (Marín *et al.*, 2003) (Figure 4.1).

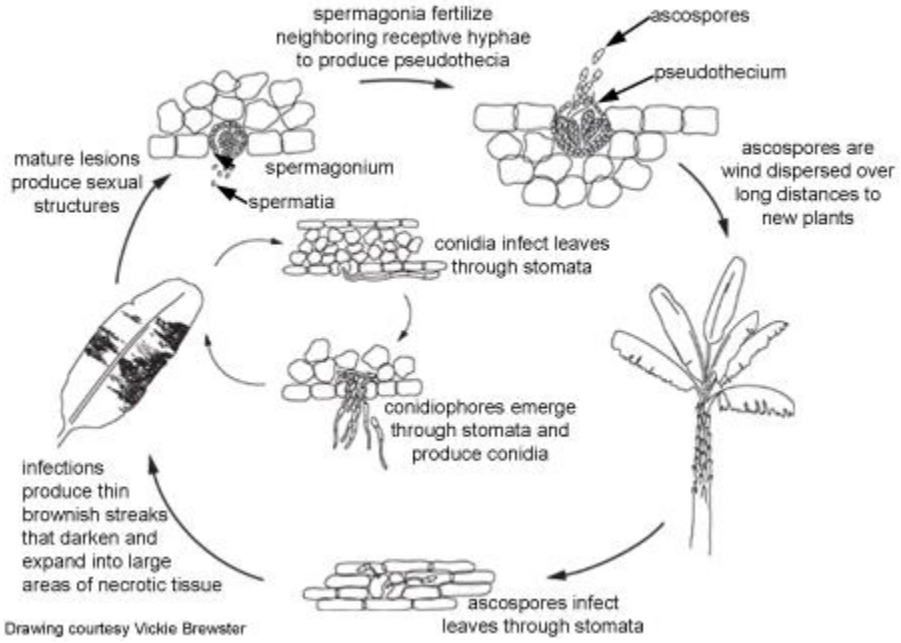

Figure 4.1. *Pseudocercospora fijiensis* life cycle (Bennet & Aresen, 2003).

The ascospores and the conidia constitute the dissemination media by which the pathogen spreads during the sexual and asexual stages (Marín *et al.*, 2003). However, *P. fijiensis* forms relatively few conidia, and ascospores are generally believed to be more crucial during the disease cycle because of its ability to germinate over a broader range of water potentials and temperatures than conidia (Bennett & Arneson, 2003; Trapero-Casas & Kaiser, 2007). The mature ascospores and conidia are released in the wind through rain and end up in the accumulated water on cigar leaf and the four youngest leaves (from top to bottom). Under these conditions, the spores germinate, producing hypha that grows into the stomata, starting the infection process (Bennett & Arneson, 2003; Churchill, 2011).

The severity of the infection can be modulated by the plant variety, its maturation stage, and the strength of the pathogen. The symptoms start as a white spot on the leaf, which develops into black spots that grow until it covers most of the leaf, killing it during the process. Under ideal weather conditions, the spores will germinate within six hours, reaching the inside of the leaf via stomata within 12 to 15 hours. The hyphae keep growing through the mesophyll layers and into the palisade tissue, covering the leaf within four to six weeks (Alvarez, 2015; Arango Isaza *et al.*, 2016; Rodriguez *et al.*, 2016).

Fouré (1984) developed a descriptive scale to determine the stage of infection into six phases. Although it has been modified, this scale is still in use to describe the Black Sigatoka infection in commercial plantations, and it includes the following stages:

- Stage 1: 1 mm long spots yellowish white to brown.
- Stage 2: Stripes or chlorotic striae of 3-4 mm in length per 1 mm wide, color brown.
- Stage 3: Streaks or stretch marks lengthen and expand without defined borders can reach up to 2 cm in length.
- Stage 4: Oval brown spots on the underside and black on the upper side of the leaf.
- Stage 5: Black spots surrounded by a black ring, sometimes a yellowish halo, and dry and half-sunken center.
- Stage 6: Large dry spots with a hollow center, brown in color, surrounded by chlorotic tissue (Alvarez *et al.*, 2015; Foure, Grisoni, & Zurfluh, 1984).

4.1.2 Natural resistance

The majority of banana cultivars available for commercialization are hybrids of two wild species: *M. acuminata* (A genome) and *M. balbisiana* (B genome). They belong to the sub-group Cavendish, which is susceptible to Black Sigatoka. Most banana cultivars represent somaclonal variants obtained through asexual propagation, and as a result, banana cultivars harbor low genetic variation (Bennett & Arneson, 2003).

Breeding strategies to control the disease vary from country to country. In contrast to Colombia, Costa Rica, or Ecuador, where large corporations (e.g., Dole, Chiquita) produce most of the product for trade, banana production in most countries is controlled by small-scale farmers for domestic consumption. For this reason, the production challenges associated with Black Sigatoka have become more of public concern rather than a market problem. Public research institutions (EMBRAPA, FHIA, CORBANA) have established breeding programs to develop Black Sigatoka resistant varieties. Most of this research is focused on traditional breeding rather than GMOs or chemical control (Cordoba & Jansen, 2014).

As a result of these efforts, some varieties somewhat resistant or highly resistant to Black Sigatoka have been developed in the last few years (Table H.1). However, these varieties do not have fruit characteristics that meet the commercial standards (production, quality of the fruit, plant structure, or flavor); thus, they are usually used for domestic consumption. A portion of this study aims to characterize the stability of Black Sigatoka resistance in some of these varieties and find new sources of resistance within a group of 18 edible banana accessions that could potentially be used in breeding programs.

4.1.3-Mechanisms of resistance and resistance genes

Understanding the interaction between *Musa spp* and *P. fijensis* is critical to finding strategies towards genetic resistance in the crop. Several defense mechanisms are known to be involved in Black Sigatoka resistance, including 1) physical barriers through lignin biosynthesis, 2) production of phytoalexins, and 3) proteins related to recognizing the pathogen and hypersensitive response (Hidalgo & Schneider, 2016).

The first line of defense is a physical barrier. Lignins are the second most accumulated biopolymer in plants and the most rigid material in the cell wall. It provides rigidity and is highly resistant to biodegradation. Although it has not yet been studied for its role in *P. fijensis* resistance, it has been shown to confer a certain degree of resistance to nematodes in bananas and has been theorized to maintain the physical integrity of the cell wall during pathogenic infections (Aguirre *et al.*, 2016; Freitas *et al.*, 2015; Suganthagunthalam *et al.*, 2014).

Phytoalexins are metabolites that accumulate at infection sites inhibiting the growth of fungi and bacteria, acting as antibiotics or antioxidants when the plant is under stress. Its role as a protectant has already been described in *Poaceas, Brassicaceae, Leguminosae, and Solanaceae,* among other important agricultural products (Singh & Chandrawat, 2017). Within this chemical family, phenylphenalenones have been found in leaves (with *P.fijensis* infection), roots (with *F. oxysporum* f. sp. cubense infection), and fruit peels (with *P. musae* infection) of *Musa* AAA spp. Ibota Bota, cultivar Yangambi, reafirming its role as a protectant in plant infections (Hidalgo *et al.*, 2016; Hölscher *et al.*, 2014; Otálvaro *et al.*, 2007).

Regarding the resistance genes, the effector-plant recognition complex from *P. fijensis* and banana and other genes related to hypersensitive response have been described. Several resistance genes associated with defense response to *P. fijensis* with the variety Calcutta IV have

been identified. Torres *et al.* (2012) observed rapid induction of the genes phenylalanine ammonia-lyase (PAL), peroxidase (POX), and b-1,3-glucanase within the first 72 hours after infection, all of them involved in hypersensitive response. Rodriguez-Cabal (2012) and Portal *et al.* (2011) have found at least eight Pathogenesis-related proteins (PR) which are induced in host plants after pathogen infection and are thought to have antifungal activity. Among those, PR4 and PR10 have been found to play a unique role in the defense response from Calcutta IV (Soares *et al.*, 2021).

Rodriguez *et al.* (2020) described at least 24 genes involved in the Jasonic Acid (JA) and ethylene (ET) pathways that are overexpressed in Calcutta IV after inoculation with *P. fijiensis,* while the same genes were either activated later or suppressed in Cavendish. Although these genes are not considered R-genes, JA and ET play a significant role in systemic acquired resistance (SAR) and Induced systemic resistance (ISR) (Andersen *et al.*, 2018).

Besides genes involved in the defense response, avirulent genes associated with the plant-pathogen interaction in *P. fijiensis* have also been identified. The CfAvr4 effector is a 135 amino acid protein (14.55 kDa) secreted by *C. fulvum* to the tomato apoplast. CfAvr4 is processed at its amino (N) and carboxyl (C) terminal by proteases from the same fungus and the host, producing a mature protein of 86 amino acids (9.56 kDa). CfAvr4 possesses a chitin-binding domain that binds to the fungal cell walls. Chitin is absent in plants and is therefore targeted by plant chitinases as part of the basal defense response (Joosten *et al.*, 1997; Grover, 2012; Van den Burg *et al.*, 2003; Chang & Stergiopoulos, 2015).

Stergiopoulos *et al.* (2010), using BlastP and bioinformatics tools, analyzed the genomes of *P. fijiensis, P. graminicola, S. nodorum, P. tritici-repentis,* and *A. brassicicola* in search of orthologous effectors of CfAvr4. In the genome of *P. fijiensis,* an orthologous protein was

identified as Mycfi 1: 87167. This protein was renamed PfAvr4, and its structural and amino acid sequence analysis showed that PfAvr4 has a 42% identity with CfAvr4. PfAvr4 is a 121 amino acid protein (approx. 13.03 kDa to 10.80 kDa), has a 21 amino acid signal peptide at its N-terminus, and a chitin-binding domain (Van den Burg *et al.*, 2004).

The response of Black Sigatoka resistant banana varieties to the PfAVR4 protein strongly suggests that it acts as an avirulence factor recognized by the plant. This interaction elicits hypersensitive response-like necrosis (Arango *et al.*, 2016). By monitoring *P. fijensis* PfAvr4 gene expression and its relative quantification using real-time PCR in banana leaf sections, it has been shown that the growth of *P. fijensis* and the PfAvr4 gene expression can be used to monitor the development of Black Sigatoka in the field (Rodriguez Garcia, 2016). *P. fijensis, P. eumusae, and P. musae* share common effectors; there are three paralogs of Ecp2 (i.e., Ecp2-1, Ecp2-2, and Ecp2-3) and two CfAVR4 homologs (Ecp6 and Avr4) Chang & Stergiopoulos, 2015).

The variety Calcutta IV (*M. acuminata* ssp. subsp. *burmannicoides* var. Calcutta IV) has often been regarded as the most resistant variety against Black Sigatoka. A recent study by Stergiopoulos *et al.* (2010) indicated that tomato plants inoculated with PfAVR4 developed HR-mediated necrosis and triggered the expression of the Cf-4 R-gene. Two studies carried in Calcutta IV showed that PfAVR4 could induce a hypersensitive response in banana (Arango Isaza *et al.*, 2016; Rodríguez-García *et al.*, 2016), suggesting that an R-gene/effector response was triggered. However, these studies have not evaluated the expression of candidate R-genes in resistant and susceptible plants in response to PfAVR4 inoculation.

To close this gap, this study aims to characterize the defense response in Black Sigatoka resistant and susceptible plants induced by introducing the protein PfAVR4 from *P. fijensis*. Such response will be described by measuring the expression levels of five genes, POX involved

in hypersensitive response (HR) and four R-genes (PR4, PR10, DRR1, and R4), in response to PfAVR4 in Calcutta IV and Cavendish.

4.2 Materials and methods

4.2.1 Banana *in vivo* propagation

Corms from Cavendish (Landrace Ecuadorian Dwarf – Super Green), Calcutta IV, Bri Bri, Pisang Awak, Pisang Pahan, and FHIA 25 were obtained from the Dole Food Company's banana collection located in Rio Frio, Costa Rica (10 ° 18'29.9" North and 83 ° 52'59,6").

The corms were grown for 4-6 months in the greenhouse at 28 ºC and gouged to induce auxiliary growth before being used for in vitro tissue culture.

The accessions Khai Nai On, Jaribuaya, Bungai, P. Keilyn, Kunan, P. Liling, P. Lidi were obtained from the ITC center from Bioversity International. These plants were directly used for *in vitro* propagation.

All accessions were propagated in vitro, transferred in soil substrates, and grown in a growth chamber at 28 ºC, 80% relative humidity, and 12 H of light cycle for eight weeks until ready for inoculation.

4.2.2 Banana *in vitro* propagation

Corms from mature banana plants were harvested, cleaned, peeled, and incubated in autoclaved water at room temperature for 3 hours. Each corm was disinfected in a 20% bleach solution for 30 minutes under constant movement, washed, and disinfected with a 70% EtOH for 30 more minutes. External tissue was sterilized using a flame from the gas burner before being placed in media.

The corms were further cut until the meristem was exposed, and each meristem was placed in growing media (2g/L phytagel, 30g/L sucrose, 4.43g/L MS, 4mg/L BAP, 1.6mg/L IAA, 80 mg/L adenine sulfate, 1% PPM) and incubated at 28 ºC with a 12H light cycle for 2-4 weeks. Grown-clean banana plantlets were transferred into rooting media (2g/L phytagel, 30g/L

sucrose, 4.43g/L MS, 1% PPM) and incubated at 28ºC with a 12h light cycle for four weeks until they were ready for acclimation and transfer to soil.

4.2.3 Field evaluations of Black Sigatoka

Field evaluations were performed at the Dole Food Company's banana collection in Rio Frio, Costa Rica (10 ° 18'29.9" North and 83 ° 52'59, 6'') in 2016, on 2+-year-old plants over the duration of a month with natural inoculation at plots that were maintained free of fungicides.

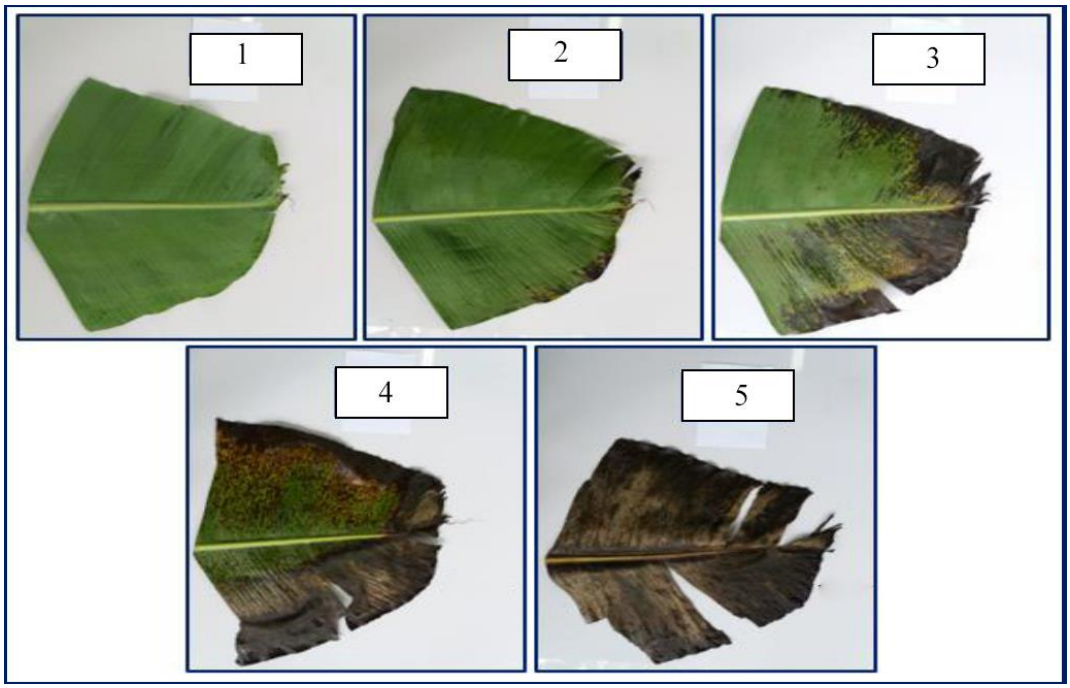

Figure 4.2. Scoring scale for Black Sigatoka disease progression on the field. Scale provided by Dole industries (Benavides, 2019).

Trained personal visually measured the number of lesions on leaf number one on the plant. The whole leaf area was divided into nine portions, and the upper-right portion was selected for analysis (Figure 3.1). A quantification method based on the determination of the Severity of the Disease (SE) proposed by Stover modified by Gauhl (Gauhl, 1994) was adapted. The scores were assigned based on the amount of lesion coverage (ALC) on that portion of the

leaf, one if the ALC ranged from 0 to20%; two, if the ALC ranged between 20 to 40%; three if the ALC ranged between 40 to 60%, four of the ALC ranged from 60-80%; and five if the ALC ranged from 80 to100% (Figure 3.1).

4.2.4 *P. fijensis* propagation

Banana leaves from Cavendish plants infected with Black Sigatoka were obtained from Costa Rica. Dried leaf sections were stapled on bond paper squares (10 x 12 cm) with the pseudothecia facing up and incubated in a plastic box with a wet paper towel for 48 hours.

The paper squares with the samples were placed in a beaker with autoclaved water for five minutes to hydrate the pseudothecia. The samples were then dried on paper towels and placed over Petri dishes with 3% water agar. The plates were inoculated at room temperature for 30 minutes for spore discharge.

The leaves were removed, and the plates were sealed and incubated at 28 ºC for 24 hours in order to induce spore germination. Using a fine syringe needle, 10 to 12 individual germinating spores were transferred to PDA dishes and incubated for ten days at 28 ºC under complete darkness. The agar with the fungi was cut into small plugs and placed in 5 ml of PDB with 50% glycerol, and stored at -80 ºC until used.

Five isolates (CR01-CR05) obtained from monospore cultures of *P. fijiensis* were grown on PDA plates for three weeks at 28 ºC under darkness. The mycelia were diced with a scalpel, macerated, and diluted with sterile water to make a slurry of inoculum. This isolate was used for downstream experiments because CR03 had a higher growth rate and produced more conidia (Ristaino, 2018; Noar, 2016).

4.2.5 *In vivo* plant inoculation

200 µl of inoculum of CR03 was incubated in modified V8 agar plates for one week under black, and white lights at 18 °C. 5 ml of sterile Tween 20 water were added per plate to collect the spore suspension. The spores were diluted to 0.5 X 10 4 per ml using Tween 20 water to spray plants.

25 ml of the spore suspension was sprayed per plant. Plants were kept at 28 ºC and 80% relative humidity, and 12 H light cycle and covered with transparent bags for one week. After bag removal, the plants were kept under the same conditions for three weeks until evaluation (Noar, 2016)

4.2.6 *In vitro* plant inoculation

200 µl of inoculum of CR03 was incubated in modified V8 agar plates for one week under black and white lights at 18 °C; 5 ml of sterile Tween 20 water were added to each plate to collect the spore in suspension. The spores were diluted to 0.5 X 10^4 per ml using Tween 20 water.

Leaves from 8 week grown banana plants were cut and cleaned with autoclaved water. 1.5 inches discs from the leaf center were cut, and four discs were placed in water agar plates with 1% Gibberellic Acid. Five 15 µL drops of the spore dilution were placed on each leaf disc. The plates were sealed and incubated at 28 ºC with a 12H light cycle for three weeks until evaluation.

4.2.7 Black Sigatoka disease evaluation

In the *in vivo* assay, the diseased leaves (12-week old plants, four weeks after inoculation) were scanned, and the lesion areas were measured by image analysis. The whole

leaves area was divided into three portions, and the middle portion was selected for analysis. The entire leaf area (except the edges) was selected for image analysis for the *in vitro* assay.

All the pictures in the analysis were scaled to the same size, the background was erased, and the color was standardized, using the software Photoshop 2020 ®. The treated images were uploaded into the R script PLIMAN (Olivoto, 2021). The percentage of diseased leaf area was calculated by dividing the total lesion area by the total leaf area of the portion scanned.

A score of one was assigned to the images with the least amount of lesion coverage (ALC), while five was assigned to the images with the highest ALC. The rest of the scores were then calculated by converting the ALC into the scoring system by using a linear equation. Data were analyzed with R studio. Analysis of variance was determined using the general linear model procedure, and means were separated with the least significance difference at P = 0.05. A T-test was used for the *in vivo* assay sample, and Tukey's HSD test was used for the *in vitro* assay samples.

4.2.8 PfAVR4 protein cloning and purification

The sequence for PfAVR4 protein was obtained from GenBank (KB446558.1). Primers were designed (Appendix I) and use to amplify the gene. PCR was conducted using the following conditions: Denaturation (95^{o}C, 3'0), Annealing (95^{o}C, 30";51^{o}C, 30", 68^{o}C, 45")X35, Extension (68^{o}C, 7'). An amplicon of the expected size, 421 bp, was obtained and cloned into pET-30 a (+) plasmid obtained from EMD Biosciences. 4 µL of the ligation buffer was kept on ice for 20 minutes and combined in a vial with 50 µl of DH5-α competent cells. The cells were heat-shocked for 40 seconds at 42 ^{o}C and immediately cooled in ice for 5 minutes.

The transformed DH5-α were added to a fresh Eppendorf tube containing 1 ml of LB media and incubated at 37 ºC and 200 rpm for one hour. After incubation, 100 µl of the cell prep was plated in LB-KAN (LB and 50 µg/mL of Kanamycin) plates and kept at 37 ºC overnight. Single-cell colonies were tested using PCR and replated in LB-KAN plates at 37 ºC overnight.

Plasmids from positive colonies were extracted using QIAprep Spin Miniprep Kit and transformed into the expression strain BL21-D3 using the same protocol. Colonies that tested positive by PCR were incubated for one hour at 37 ºC and 200 rpm in 1 mL of LB-KAN media and then mixed with 50% Glycerol to be stored at -80 ºC until further use. The plasmid was also sequenced for verification.

For expression, 100 µL of the LB-KAN-Glycerol stock was spread in LB-KAN (50 µg/mL) plated and incubated at 37 ºC overnight. After incubation, a single colony was incubated in a starter culture of 10 mL of LB-KAN at 37 ºC and 200 rpm for 5 hours. The starter culture was then transferred into 500 mL of LB-KAN and incubated at 37 ºC and 200 rpm until culture density reached 0.5-0.6 OD600. The culture was cooled down in an ice bath, and expression was induced by adding 0.25 mM of IPTG. The culture was incubated for 16 hours at 20 ºC and 200 rpm.

One liter of the induced culture was centrifuged at 4 ºC and 5,000 rpm for 20 minutes. The pellet was redissolved in 15 mL of Guanidinium Lysis buffer and one tablet of complete Mini Protease Inhibitor Tablets® (#23201100). The suspension was pressed three times through a french press at 4 °C and 10,000 psi and sonicated four times with 30-second pulses. The protein was purified following the protocol under hybrid conditions from the ProBond™ purification system (https://www.thermofisher.com/order/catalog/product/K85001#/K85001) and

desalted using Bio-Spin® Columns with Bio-Gel® P-6 (https://www.bio-rad.com/en-us/product/bio-spin-columns-with-bio-gel-p-6?ID=bc8c0603-5797-423c-bcbd-0cbc8e1463cd).

The induced and purified protein was tested for size using an SDS-page gel system, and the concentration was measured using the Pierce™ BCA protein assay kit. The protein was diluted to a 1 µg/ml concentration with water and stored at 4 ^{o}C overnight until used.

4.2.9 PfAVR4 inoculation

1.5-inch discs on the underside of the first and second leaf from 8-week grown banana plants were abraded by brushing 500 grit Silicon Carbide. Immediately after abrasion, 50 µL of the diluted protein solution was brushed onto the surface. The plants were kept at 28 ^{o}C, 80% relative humidity, with a 12h light cycle.

Discs were collected at 0.5 hours after inoculation (HAI), 1 HAI, 6 HAI, 12 HAI, 24 HAI, 48 HAI, and 72 HAI. Samples were also collected before inoculation and but not inoculated.

4.2.9 Resistance gene selection

Four R genes were selected for gene expression analysis. In response to Black Sigatoka, three R genes named PR1, PR4, and DR1 were previously identified as differentially expressed in Calcutta IV vs Cavendish/Williams. The sequence of the genes and the primers were obtained from (Rodriguez *et al.* 2016) (Appendix I).

DRAGO2 was used to predict R-gene in the *M. acuminata* genome (Osuna-Cruz *et al.*, 2018). DRAGO2 (http://www.prgdb.org/prgdb/genes) is a program that identifies plant resistance genes in a set of protein or nucleotide sequences based on the identification of conserved R-gene protein domains (e.g., LRR, NBS). The predicted gene set is attached in

Appendix K. Cf-4 protein sequence was then aligned against predicted R genes to identify four genes R1: GSMUA_Achr3T18840_001, R2: GSMUA_Achr3T25790_001, R3: GSMUA_Achr3T28580_001, and R4: GSMUA_Achr3T30680_001) as the most similar gene in the Calcutta IV genome (Appendix K).

4.2.10 RNA extraction and qRT-PCR

The samples collected from section 3.2.8 were frozen in liquid nitrogen and stored at -80 ºC for further RNA extraction. Total RNA was extracted from the samples following the protocol from Spectrum™- Plant Total RNA Kit (Sigma # STRN250) with On-Colum DNA digestion. RNA concentration and quality were measured at 260 nm using a NanoDrop 2000 UV spectrophotometer. The concentration for each sample was adjusted to 100 ng/µL. First-strand cDNA was synthesized following the protocol from SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen #18080-051) following the Oligo(dT) approach.

qPCR was performed on a LightCycler ®480 II machine from Roche. The cycle conditions were: activation (50ºC, 2'; 90ºC, 2'), amplification (95ºC, 15"; 55ºC, 15"; 72ºC, 1')X40, and dissociation (95ºC, 15"; 60ºC, 1'). The master mix formula used was Power Up™SYBR™ Green (Applied Biosystems # 25742). The primers used for the procedure are reported in Appendix I. The housekeeping gene used for all samples was the 26S ribosomal subunit, as recommended by Rodriquez (2016). For each sample, three biological replicates were analyzed with three technical replicates. The expression ratio was calculated using the ΔΔ-Cycle threshold (CT) method, geometric means, and log 2 conversion of the data was used for the statistical analysis of the expression (Pfaffl, 2001).

4.2.11 Statistical data analysis

All statistical analyses were performed using the software R Studio (R Core Team, 2014; RStudio Team (2020)), and figures were produced using the package ggplot2 (Wickham, 2009). Means were compared using a one-way analysis of variance (ANOVA) to determine the significant difference ($p < 0.05$) among accessions. In contrast, a T-test was used to determine any significant differences between resistant and susceptible accessions ($p < 0.05$).

4.3 Results

4.3.1 Field assessment, *in vivo* inoculation, and *in vitro* inoculation evaluation.

Field evaluation of Black Sigatoka banana is an arduous task to carry out as it is done by field workers based on their experience. However, this evaluation is continuously done at a commercial level to assess the severity of the disease and determine the pesticide needs in the plots.

Table 4.2. Black Sigatoka disease severity score in 18 banana accessions.

	Disease Severity		
Accession	**Field assessment**	***In vivo* inoculation**	***In vitro* inoculation**
Awak	N.E.	N.E.	2.83±0.38 ab
Baby Khai	4.25±0.80 ab	N.E.	N.E.
Bri Bri	2.75±0.27 abc	N.E.	3.00±0.37 ab
Bungai	2.25±0.09 c	N.E.	N.E.
Calcutta IV	2.00±0.00 c	1.00±0.29*	1.00±0.61 b
Cavendish	4.25±0.09 ab	5.00±1.37*	4.74±0.56 a
Clon Frances	2.50±0.18 bc	N.E.	N.E.
Ducasse	2.00±0.00 c	N.E.	N.E.
FHIA25	N.E.	N.E.	1.11±0.44 b
Jaribuaya	2.25±0.09 c	N.E.	1.23±0.40 b
Khai Nai On	4.50±0.18 a	N.E.	5.00±0.60 a
Kunan	2.25±0.09 c	N.E.	N.E.
P. Keilyn	2.75±0.27 abc	N.E.	N.E.
P.Ceylan	3.00±0.00 abc	N.E.	N.E.
P.Lidi	2.50±0.18 bc	N.E.	N.E.
P.Liling	2.50±0.18 bc	N.E.	N.E.
Pahang	2.75±0.09 abc	N.E.	1.41±0.33 b
Selangor	2.25±0.09 c	N.E.	N.E.

-N.E.: not evaluated

-An (*) indicates scores that were significantly different (Paired T-test $p<0.05$).

-Letters next to each score bar represent Tukey's HSD grouping.

-A score of one was assigned to the plant with the least amount of lesion coverage (ALC), while five was assigned to the plant with the highest ALC. The rest of the scores were then calculated by converting the ALC using a linear equation

Black Sigatoka symptoms were observed on all banana accessions evaluated. The symptoms ranged from stage two to the late necrotic stage five. Khai Nai On, Baby Khai, and Cavendish were the accessions most affected by the disease with a score of 4.50±0.18, 4.25±0.80, and 4.25±0.09, respectively. In contrast, the accessions Calcutta IV and Ducasse showed the lowest amount of lesion coverage (ALC) with a score of 2.00. Bungai, Kunnan, Jaribuaya, and Selangor show a high resistance level with a score of 2.25±0.09. The accessions Bri Bri (2.75±0.27), P Keilyn (2.75±0.27), P.Ceylan (3.00±0.00), and Pahang (2.75±0.09) showed a medium level of resistance (Table 3.2). Overall all the scores fall in between 2-3 and 4 and 5 (Table 3.2).

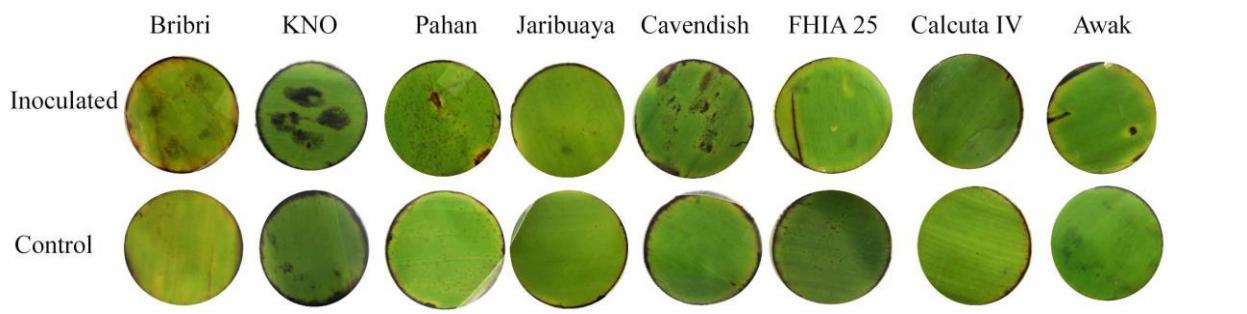

Figure 4.2. *In vitro* inoculation of seven banana accession inoculated with conidia from *P. fijensis*, four weeks after inoculation.

Based on the field trials, eight accessions were selected for the *in vitro* assay. Calcutta IV showed the lowest amount of necrotic lesions with an average score of 1.00 ±0.61, corresponding to an ALC of 16.16%±2.51. Similarly, FHIA 25 (1.11±0.44), Jaribuaya (1.23±0.40) (Figure H.5), and Pahan (1.41±0.33) (Figure H.7) showed high levels of resistance with an ALC that ranged from 16.63% to 17.89% (Table 3.2., Figure 3.2)

Khai Nai On (Figure H.6) and Cavendish (Figure H.3) showed the lowest resistance level with a score of 5.00±0.60 and 4.74±0.56, respectively. Such a score corresponds to an ALC that ranges from 31.69±2.33 to 32.79%±2.51 (Table 3.2., Figure 3.2).

The accessions Awak (2.83±0.38) (Figure H.8) and Bri Bri (3.00±0.37) (Figure H.1) scored medium levels of resistance with an ALC ranging from 23.76%±3.25 to 24.48%±1.56. All the controls for this assay were scored across accessions, and the analysis showed an ALC that ranges from 1.09%±0.24 to 2.83%±0.31 (Table 3.2., Figure 3.2).

Overall, the field data and the *in vitro* data show a coefficient of determination of 0.92 ($p<0.05$), indicating that both sets are highly correlated. At the same time, since Calcutta IV and Cavendish were consistently resistant and susceptible to Black Sigatoka, respectively, these two genotypes were used for the *in vivo* assay.

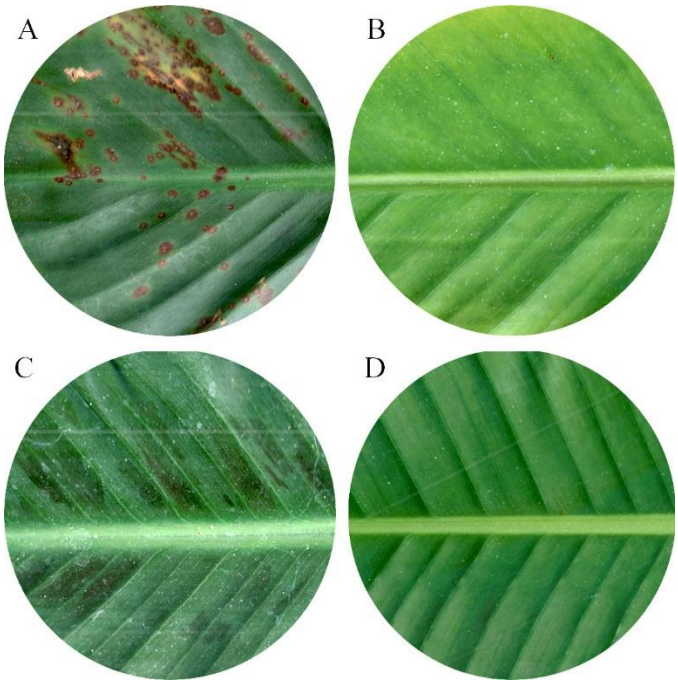

Figure 4.3. *In vivo* inoculation four weeks after P. *fijensis* inoculation. A) Cavendish inoculated, B) Calcutta IV inoculated, C) Cavendish non inoculated, D) Calcutta IV non inoculated

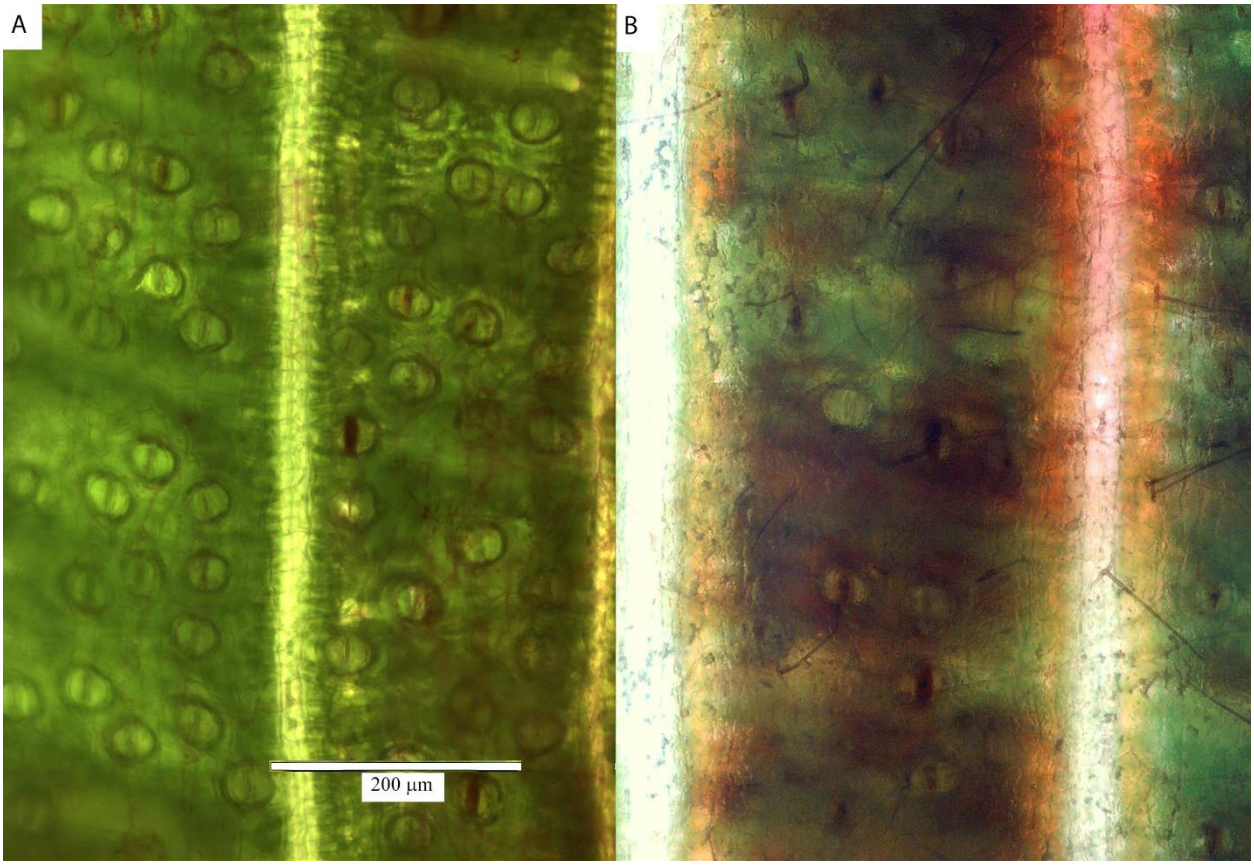

Figure 4.4. *P. fijensis* mycelia were growing in a Cavendish leaf four weeks after inoculation under 100 X amplification in a confocal microscope using Rose Bengal dye to create contrast. A) Leaf top face, B) Leaf bottom face.

As expected, Cavendish showed a higher ALC, scoring an average of 5.00±1.37. The necrotic and dead tissue areas among these plants range from 44.11% to 91.96%. For this accession, the control group (non inoculated plants) showed an average ALC of 2.11%±0.22 (Table 3.2, Figure 3.3 A). Calcutta IV showed the least amount of necrotic lesions with an average score of 1.00±0.29. The ALC ranges from 16.04% to 26.22% in all the samples, while the control (non inoculated plants) had an average of 1.38%±0.13 (Table 3.3, Figure 3.3 B).

Infection in Cavendish was later confirmed under the microscope by slicing tissue samples adjacent to the necrotic spots on the infected leaves. For visualization, Rose Bengal dye was used (Jackson & Johnson, 1988). *P.fijensis* mycelia were detected inside Cavendish

inoculated leaves (Figure 3.4). The same analysis was performed in Calcutta IV, and besides the fact that small lesions developed, no mycelia were detected.

Calcutta IV showed the highest resistance levels across the different tests, while Cavendish and Khai Nai On showed the highest levels of susceptibility.

4.3.2 Cloning, expression, and purification of PfAVR4

The *P.fijensis* isolates used to clone the sequence were the same isolates used for the *in vivo* and *in vitro* assays (CR03). The PCR amplification of the CDS of PfAvr4 from *P.fijensis* was cloned, purified, and ligated into the vector pET30-a using DH5-α cells. A 421bp PCR fragment was cloned, and a colony named here CR031 was used for the following analysis (Appendix I, Figure 3.5 A).

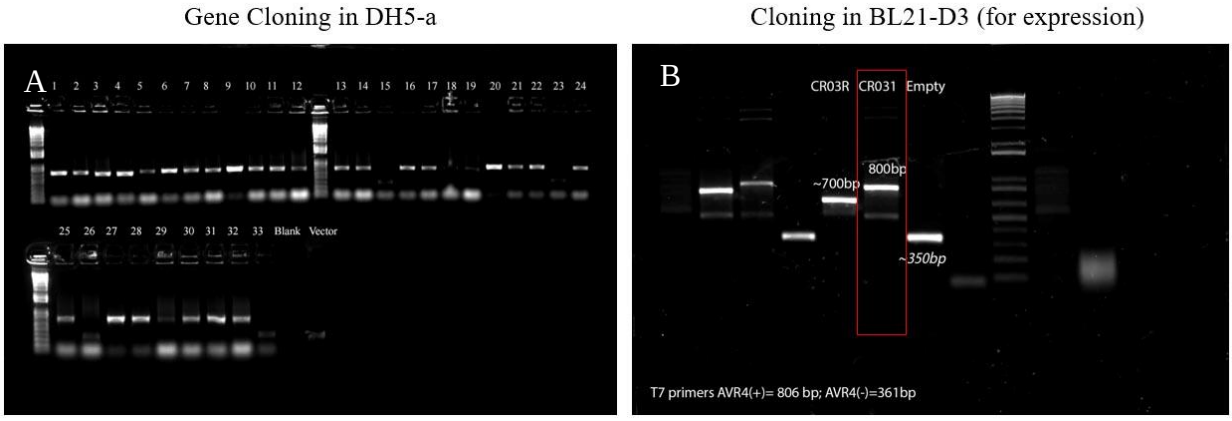

Figure 4.5. PfAVR4 gene fragment amplified by PCR. A) DH5-a colony PCR with PfAVR4 primers. The expected size is 421bp. B) pET30-PfAVR4 vector cloned in BL21-D3, the PCR fragment was amplified using T7 primers. The predicted size is 806bp.

CR031 was then cloned, amplified using the T7 primers, and sequenced to verify the integrity of the sequence and ORF. The T7 primers include the HIS-tag, the PfAVR4 sequence, and the T7 termination site from the vector for an expected size of 806 bp (Figure 3.5B,

Appendix I), .and its sequences were aligned with the known pET30-a-PfAVR4 nucleotide sequence using the Benchling software (https://benchling.com) (Figure J.1).

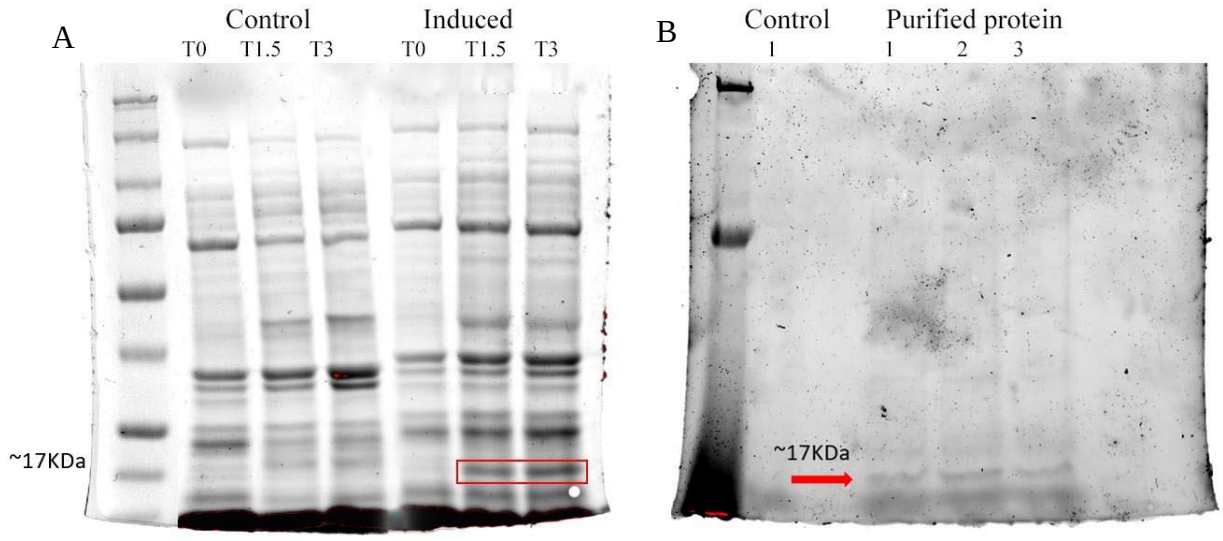

Figure 4.6. SDS page gel (12%) for protein extraction of PfAVR4 cloned in BL21-D3 cells. A) Full protein extract from induced and not induced cells at OD 0.5 before induction (T0), 8 hours after induction (T1.5), and 16 hours after induction (T3). B) Purified and desalted PfAVR4 protein.

The *E. coli* BL21-D3 strain was transformed following the protocol described in section 3.11. As the protein can be overexpressed in BL21-D3, it went through a cold induction for 16 hours. After extraction, the protein remains in the insoluble fraction of the extract (Figure 4.6 A).

To solubilize the protein, it was denatured in a urea solution and then refolded in a phosphate solution. Then the solution was desalted, and the protein was re-solubilized in water. The purified, desalted protein was visualized in a 12% SDS-PAGE gels. A band with the PfAVR4 expected protein size (~17KDa) was obtained (Figure 4.6 B).

4.3.3 PfAVR4 inoculation.

Previous experiments reported using PfAVR4 to produce a hypersensitive response (HR) in banana leaves where the effector was delivered by infiltration, a practice commonly used in plant pathology studies in tomato, potato, and tobacco (Arango Isaza *et al.*, 2016; Munoz, 2018). Those studies reported that using infiltration in banana leaves causes physical damage resulting in cuts and tears that limit the HR reaction to the damaged site (Arango *et al.*, 2016; Munoz, 2018)

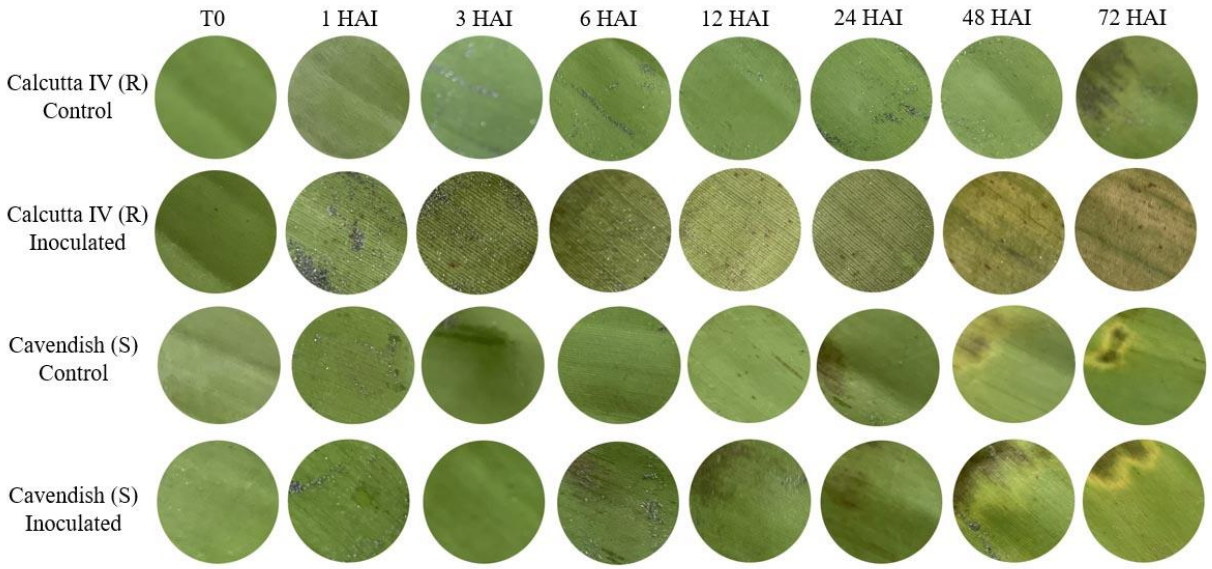

Figure 4.7. Hypersensitive response (HR) on banana leaves after PfAVR4 inoculation, at 0, 1, 3, 6, 12, 24, 48, and 72 hours after inoculation (HAI). The accessions used in this assay were Cavendish (Susceptible to Black Sigatoka) and Calcutta IV (Resistant to Black Sigatoka).

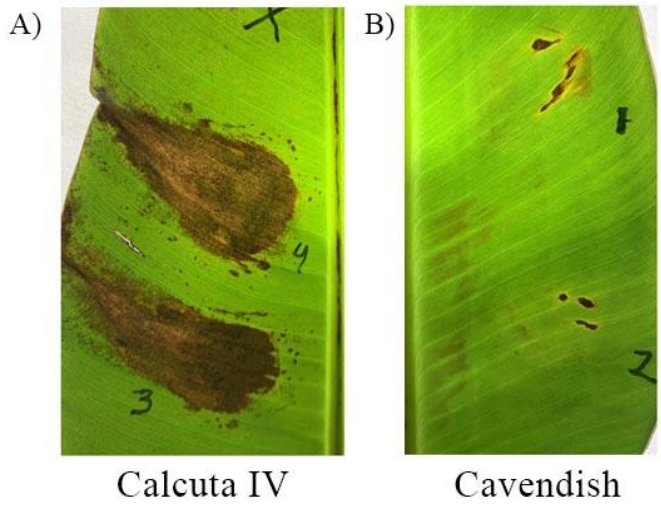

Figure 4.8. Hypersensitive response (HR) on banana leaves after PfAVR4 Inoculation at 96 hours after inoculation (HAI). A) Calcutta IV (Resistant to Black Sigatoka), B) Cavendish (Susceptible to Black Sigatoka).

For this reason, instead of using infiltration, in this experiment, micro-wounds were produced using an abrasive agent (silicon carbide); this method is commonly used in plant pathology to deliver viruses on plant leaves (Trigiano *et al.*, 2016). Several trials were attempted in order to find the optimal conditions for delivering the protein while minimizing physical damage to the leaves.

Following the *in vitro* and *in vivo* assay results, Cavendish was selected as the susceptible accession, while Calcutta IV was used as the resistant accession. All the plants used were tissue culture clones used for the field evaluation assay, similar to the material used for the *in vitro* and *in vivo* assays. The leaves selected for this experiment were the first two top leaves after the cigar leaf, on which ten 1.5” areas (circles) were abraded (five on each leaf), and the protein and the control solution were applied with a sterile brush.

Samples were collected at six-time points (0, 1, 6, 12, 24, and 48 hours after inoculation, HAI) by cutting 1.5” circles between the application area and the healthy tissue in the outward direction, which is the direction where we observed that the inoculum/response moved through the vascular tissue (Figure 4.8 A).

The brown coloration on the application area was associated with Hypersensitive Response (HR) necrosis, as noted in the previous studies from Arango Isaza *et al.* (2016) and Munoz (2018). Leaf color changes in the application area become noticeable between one and three HAI on inoculated Calcutta IV. It becomes darker and distinguishable from other lesions between three and 6 HAI. After 6 HAI, the brown coloration starts spreading towards the edge of the leaf, and in between 48 HAI and 96 HAI, the area where PfAVR4 was applied died and started drying out (Figure 4.7).

Only Calcutta IV inoculated with PfAVR4 showed HR-related symptoms. While some Cavendish plants and the Calcutta IV controls showed some lesions, these were associated with the mechanical damage caused by the abrasion. The main difference between both symptoms is that mechanical damage stays localized in the application area, the lesions are smaller than the application area, and they become apparent after 48 HAI (Cahil *et al.*, 2013). In HR-related necrosis, the damage covers the whole area of application and moves towards the edge of the leaf, and it becomes visible around 6 HAI (Balint-Kurti, 2019) (Figure 4.8).

4.3.4 Gene expression analysis

Since PfAVR4 from *P.fijensis* is known to produce HR-related symptoms in bananas resistant to Black Sigatoka (Arango Isaza *et al.*, 2016; Munoz, 2018) the effect of this protein in some of the already recognized R-genes was evaluated. For this study, the genes PR-4, PR-10, DRR1, and POX were selected.

At the same time, a homologous analysis was performed to identify possible R-genes in Calcutta IV based on the sequence of Cf4 from tomato. The analysis identified 28 homologs belonging to the nucleotide-binding site–leucine-rich repeat (NBS-LRR) family, recognized for its role in pathogen detection and infection triggered response (ETI) (DeYoung & Innes, 2006). Among the four genes, R1-R4, having the highest similarity, were tested (Appendix K).

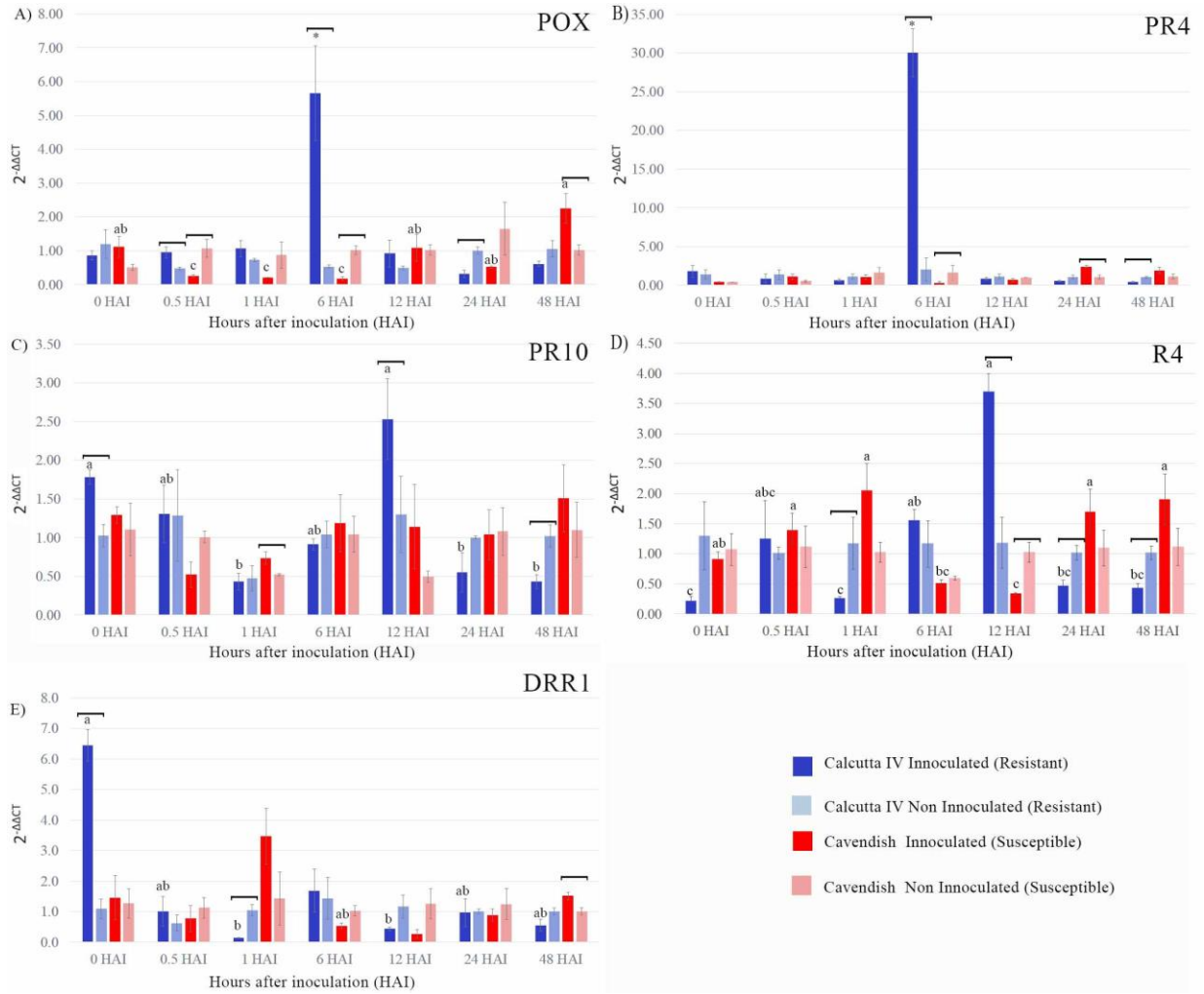

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Figure 4.9. Gene expression analysis for Calcutta IV and Cavendish plants inoculated with the effector protein PfAVR4 from *P. fijensis*, at 0, 0.5, 1, 6, 12, 24, and 48 hours after inoculation (HAI). A) POX (peroxidase), B) PR-4 (Pathogen Response 4, C)PR-10 (Pathogen Response 10), D) R4 (Cf4 Homologous), E) DRR1(Disease-Related Response). Letters on top of the bars represent Tukey's HSD grouping for time points within treatments. A (━) on top of two bars represents a group that is significantly different among themselves (T-test $p<0.05$). An (*) on top of a bar represents a significantly different sample from its group (Tukey's HSD).

For POX, a gene related to HR, its expression shows a spike on inoculated Calcutta IV at 6 HAI. Indeed, compared to all other samples and time points, the gene expression was

significantly upregulated (5.66±1.40 fold) at 6HAI. In Cavendish inoculated plants, POX expression was upregulated at 48 HAI (2.25±0.43) (Tukey's HSD, $p<0.05$) (Figure 4.9 A).

Among the PR (Pathogen Response) genes, the expression level of PR-4 was highest at 36 HAI (30.06±3.09), and its expression was significantly upregulated (Tukey's HSD, $p<0.05$) (Figure 4.9B). The gene was not differentially expressed at any other time point.

The expression level of PR-10 in inoculated Calcutta IV was upregulated at 0 HAI and 12 HAI (Tukey's HSD, $p<0.05$) and was significantly downregulated at 24 and 48 HAI (Tukey's HSD, $p<0.05$) (Figure 3.9 A) (Figure 4.9C).

Similar to PR10, DRR1 (Disease Resistance Response) was significantly upregulated 6.4±0.5 fold at 0HAI in inoculated Calcutta IV, in contrast to Calcutta IV control that remains close to 1 (1.1±0.3) (Tukey's HSD, $p<0.05$) (Figure 4.9 D). For both PR10 and DRR1 expression profiles is important to note that at 0 HAI, the protein has not yet been applied to any of the plants; therefore, such a response would be related to other factors than PfAVR4.

R4 was downregulated in Calcutta 4 inoculated plants at 0, 1, 24, and 48 HAI and upregulated at 12 HAI (3.69±0.30) (Tukey's HSD, $p<0.05$) (Figure 4.9 D).

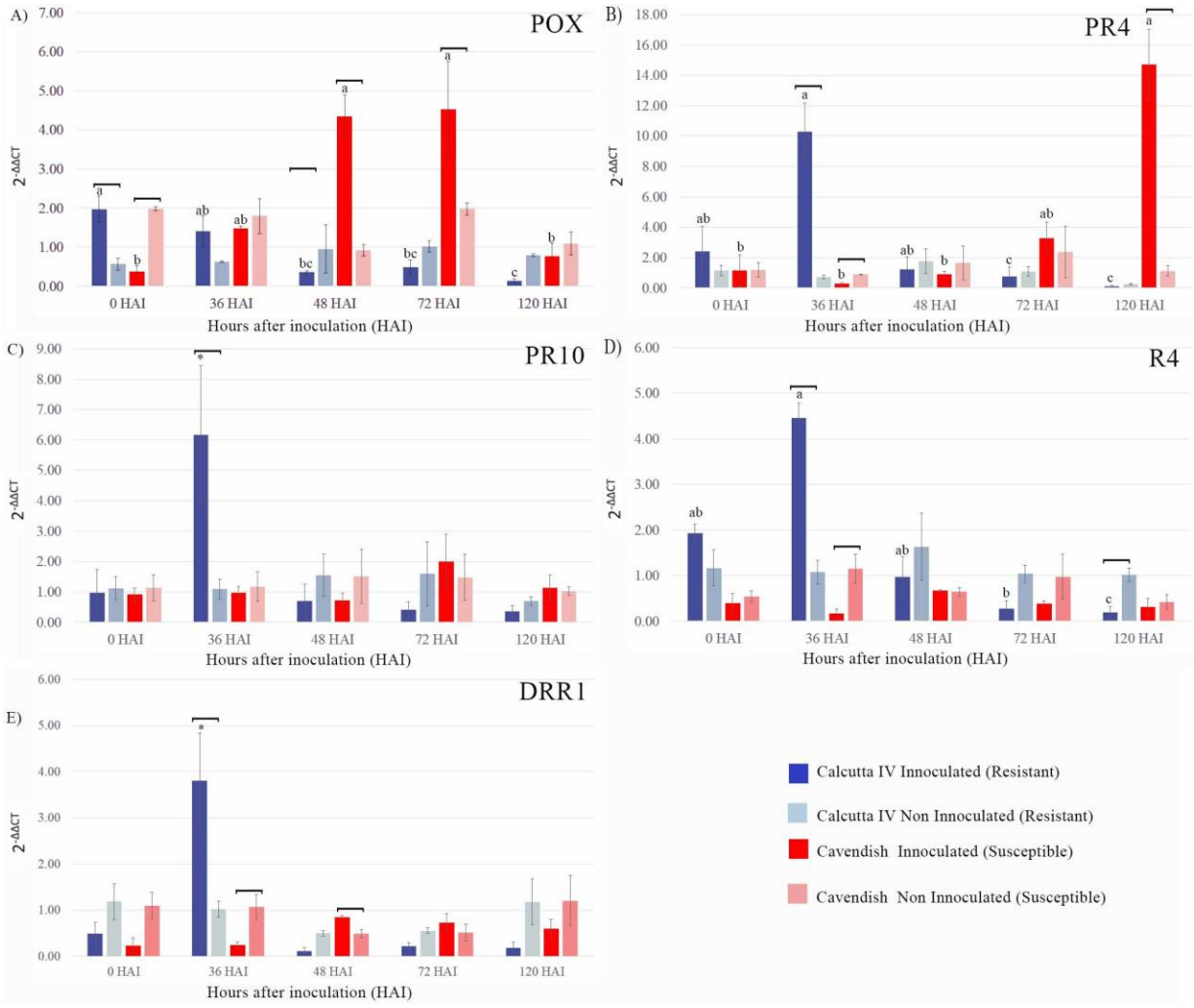

Figure 4.10. Gene expression analysis for Calcutta IV and Cavendish plants inoculated with conidia from *P. fijensis* at 36, 48, 72, and 120 hours after inoculation (HAI). A) POX (peroxidase), B) PR-4 (Pathogen Response 4, C)PR-10 (Pathogen Response 10), D) R4 (Cf4 Homologous), E) DRR1(Dissesase-Related Response). Letters on top of the bars represent Tukey's HSD grouping for time points within treatments. A (━) on top of two bars represents a group that is significantly different among themselves (T-test $p<0.05$). An (*) on top of a bar represents a significantly different sample from its group (Tukey's HSD).

For plants inoculated with *P.fijensis* conidia, POX expression was upregulated at 0 HAI and downregulated at 48 HAI in Calcutta 4 inoculated plants. The gene was also downregulated at 0 HAI and upregulated at 48, and 72 HAI in Cavendish inoculated plants (Figure 4.10 A)

(Tukey's HSD, $p<0.05$). PR4 was upregulated at 36 HAI in Cavendish inoculated plants and at 120 HAI in Cavendish inoculated plants (Figure 4.10 B) (Tukey's HSD, $p<0.05$).

PR-10 has upregulated at 36 HAI in Calcutta IV inoculated plants (Figure 4.10 C) and is not differentially expressed in any other conditions. Similarly, R4 and DRR1 have upregulated at 36HAI in Calcutta IV inoculated plants (Figure 3.10 D-E). R4 was also downregulated at 120 HAI in Calcutta 4 inoculated plants and downregulated at 36 HAI in Cavendish inoculated plants. In contrast, DRR1 was only downregulated at 36, and 48 HAI in Cavendish inoculated plants (Tukey's HSD, $p<0.05$).

Overall all four R genes were upregulated in Calcutta IV inoculated plants at 36 HAI, reaching maximum expression. There was also a relatively high level of expression of PR4 in Cavendish inoculated plants at 120 HAI. Compared with the PfAVR4 inoculation response, the maximum expression level of PR4, PR10, and R4 in Calcutta IV inoculated plants happened within 6 and 12 HAI, about 24 hours earlier than the conidia experiment.

4.4 Discussion

4.4.1 Accessions resistant to Black Sigatoka

A total of 18 accessions for the field experiment were evaluated and of these 16 accessions were part of the metabolite studies described in chapters one and two. Calcutta IV and Pahan were only used for the Black Sigatoka study since they are not edible bananas but are regarded as highly resistant to Black Sigatoka. The *in vitro* assay included six accessions evaluated in the field trials, plus FHIA 25 and Pisang Awak, which are considered resistant varieties. Furthermore, for the *in vivo* assay, only Calcutta IV and Cavendish were considered (Table 4.1)

From the field evaluations, Khai Nai On, Baby Khai, and Cavendish were scored highly susceptible (an average score higher than 4). Bri Bri, Pisang Keilyn, Pisang Ceylan and Pahang, were scored as moderately resistant (higher than 3 and lower than 4). Bungai, Kunnan, Jaribuaya, and Selangor were scored as resistant (higher than 2 lower than 3). Calcutta IV and Ducasse were scored as highly resistant (a score of 2 or lower). *In vitro* evaluations yielded similar results to field evaluations, with the exception of Jaribuaya which was scored as highly resistant. *In vivo* evaluation of Cavendish and Calcutta IV yielded scores similar to the other two evaluations.

Cavendish is one of the most studied accessions in banana, given that it is the golden standard in current commercial production (CORBANA, 2011). Most companies have developed their own clones that may differ in plant size, fruit production, or resistance to Black Sigatoka (Stover, 1987). In our case, the variety is called Super Green, which was developed from Ecuadorian dwarf (Cavendish cv. Grand Nain) and has been described in the literature as highly susceptible to black Sigatoka (Reyes-Borja, 2007; Arango Isaza *et al.*, 2016; Soares *et al.*, 2021). In the same fashion, Calcutta IV or *Musa acuminata* ssp. *Burmannica*, has been used in banana

breeding programs as the primary source of resistance to Black Sigatoka. Most of the resistant varieties developed by FHIA and IITA, have used Calcutta IV as the male parent in their breeding programs. It is also the recommended standard by INIBAP for studies un Black Sigatoka resistance (Carlier *et al.*, 2002, Vuylsteke, 1992, PROMUSA, 2021)

Two of the highly susceptible varieties are Khai Nai On, and Baby Khai. These accessions belong to the subgroup of *Musa* AA cv Khai (Perrier *et al.*, 2009) that originates in the northern part of Thailand. The fruit from these bananas is characterized for its sweetness, and this group is preferred in local markets in Asia pacific (MUSANET, 2121). In concordance with our data, a study by Nascimento *et al.* (2020) scored bananas in this group as highly susceptible.

From the accessions scored as moderately resistant, Pisang Keilyn is a popular dessert variety in Malaysia, and it has been reported not only resistant to Black Sigatoka but also Panama Disease. Unfortunately, this accession is very susceptible to Banana Stripped Virus (BSV) (Ploetz, 2007). Pisang Ceylan was reported resistant in this study, similar to the results found in Cameroon by Guzmán *et al.* (2019). In contrast to our findings, Kimunye *et al.* (2021) scored Pisang Ceylan and Pahan as highly resistant, but their scoring scale only includes three categories (resistant, moderate, and susceptible).

From our resistant and highly resistant group, most of these accessions have been previously described as resistant to Black Sigatoka: Awak (Kimunye *et al.*, 2021), Jaribuaya (Soares *et al.*, 2021), FHIA 25 (FHIA, 2021), and Ducasse (Gurmu *et al.*, 2017). In contrast, Kunnan and Selangor's accessions have been previously described as susceptible varieties (Jenny *et al.*, 2011; Kimunye *et al.*, 2021). No information was found for Bungai and Bri Bri.

Overall, our results using multiple methods of evaluations/environments confirmed previous results for most of the accessions evaluated here. Some discrepancy was observed (e.g.,

Jaribuaya) and could be explained by the different environmental conditions used in the studies. On the other hand, Bri Bri has not been previously evaluated in other studies for Black Sigatoka resistance. This accession, collected from the Bri Bri region in Costa Rica, its morphology resembles those from the edible diploid group, but its genome and classification are still unknown. Bri Bri has a high content of carotenoids, high levels of carotenoid bioaccessibility, high sugar content (chapters 2 and 3), and it is also moderately resistant to Black Sigatoka, making it a strong candidate for breeding programs or direct marketing.

Although breeding programs have already produced hybrids resistant to Black Sigatoka, it is essential to confirm their resistance level across multiple environmental conditions/experiments and identify new sources of resistance that also include numerous valuable traits. At least 10,000 banana accessions are stored in germplasm banks and collections worldwide (MUSANET, 2016); some of these accessions might have commercial quality characteristics and high nutritional value while resisting this disease. Studies such as ours provide essential information for decision-making in breeding and growing programs.

4.2 PfAVR4 effector protein from *P.fijensis* and its effect on Black Sigatoka resistant banana.

One of the primary defense mechanisms that the plant uses to fight pathogens is the gene-for-gene interaction. This mechanism involves a gene product of the pathogen (effector) interacting with a gene product from the host (receptor) in a specific manner. This interaction, referred to as recognition, promotes the programmed cell death on the site of the infection, stopping the pathogen from spreading further (Stunkenbrock, *et al.,* 2009). For example, the CfAVR4 effector functions as a virulence factor in *C. fulvum* species in the absence of the related Cf4 resistance proteins in the host. If Cf4 is present, an effector-triggered immunity is induced, resulting in HR necrosis (Stergiopoulos *et al.,* 2010). Multiple studies in resistant banana Calcutta IV indicated that a gene-for-gene mechanism is likely involved in the resistance to Black Sigatoka.

Over 30 R genes have been identified as differentially expressed in Black Sigatoka resistant plants such as Calcutta IV, and three of them named PR4, PR10, and DDR1 were confirmed across multiple studies (Kimunye *et al.*, 2021). None of these genes has been identified as orthologous to Cf4 (Thomma *et al.*, 2005), which is the R-gene that interacts with the PfAVR4 orthologous gene CfAVR4 in tomatoes. This would be expected since R genes evolve very fast compared to other genes with a more conserved specialized function (e.g., MYB transcription factors), especially when compared with very distantly related species such as banana and tomato (Mondal & Roy, 2018).

On the other hand, orthologues of CfAVR4 have been identified in Dothideomycete fungi, including the banana pathogen *P. fijiensis* (PfAvr4), the pine tree pathogen *D. septosporum* (DsAvr4), and the poplar pathogen *S. musiva* (SmAvr4). Interestingly, despite the

difference in sequence and pathogen-host, all of them have been proven to be recognized by Cf4 in tomato eliciting an HR response (Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012).

PfAvr4 and CfAvr4 have a common specificity for binding chitin, protecting the fungi from plant and microbial chitinases. This shared specificity suggests that the function of these effectors is likely involved in interactions with plants and other microorganisms (Kholer *et al.*, 2016). It has been recently discovered a PfAVR4 paralogue PfAVR4-2, both effectors have the same affinity for binding chitin, but instead, PfAVR4-2 binds to pectin in the cell wall of the host, loosening the plant cell structure facilitating the colonization of the fungi (Chen *et al.*, 2021).

Arango *et al.* (2016) infiltrated a concentrate of the culture medium of *P. pastoris* containing the recombinant protein PfAVR4 in Calcutta IV (resistant) and Cavendish var. Williams (susceptible) plants. The resistant plants developed HR symptoms. It was hypothesized that most likely, the resistant plants possess NB-LRR-like proteins of resistance against the causative agent of Black Sigatoka. However, since this study used whole culture media as inoculum, and the amount of protein administered was unknown, the effect of the PfAVR4 could not be separated from the entire pathogen proteome.

Munoz (2018) opted instead to use two purified versions of the protein: PfAvr4-I, which contains the signaling peptide at the N-terminus of the protein, and PfAVR4-M, without the signaling peptide. His work suggested that both proteins are biologically active, causing HR-like symptoms in Calcutta IV, and are consistent with the hypothesis that the presence of a cognate in the resistant plant recognizes PfAVR4 (Arango *et al.*, 2016). However, in his work, the author used physical cuts to deliver the protein in the leaves, causing necrotic spots in the susceptible plants and the controls masking the pure effect of the effector.

To complement these studies, we optimized an inoculation assay that would deliver only the PfAVR4 effector that would limit response not directly related to the plant-effector interaction (e.g., presence of salt or invasive damages of the leaves) that might cause interference in the expression of the genes. To achieve this, the effector was purified and desalted, and delivered to the plant by gentle abrasion on the leaves. As shown in Figure 3.8-B, the physical damage to the leaves was minimal. The brown coloration on the application area was associated with HR necrosis, as noted by Arango Isaza *et al.* (2016) and Munoz (2018). In our study, the brown coloration becomes to appear between 1 and 3 hours after inoculation (HAI) on inoculated Calcutta IV plants. Between 3 and 6 HAI, the coloration becomes darker, more defined, and spreads towards the edge of the leaf. After 48 HAI, the inoculation area dries (Figure 4.7).

Arango Isaza *et al.* (2016) observed HR-like necrosis on inoculated Calcutta IV plants at 96 HAI, while the spot became darker until 10 days after inoculation (DAI). On smaller plants, the earliest necrosis was observed at 10 DAI. Munoz (2008) observed HR-like necrosis on inoculated Calcutta IV plants starting at 24 HAI, but the necrosis was apparent between 74 and 96 HAI. The control plants showed HR-necrosis on different levels in both studies, either because of the delivery method or medium.

Our study observed HR-necrosis symptoms faster than the previous studies. Infiltration has a single entry point of application, while the microabrasion with silicon carbide used in our study produced multiple micro-cuts on the whole application area, allowing faster entry of the effector (Keppler *et al.*, 1990; Liu *et al.*, 2015). This observation and the fact that these experiments were conducted in different conditions could explain the faster HR response observed.

For the gene expression experiment, previously identified resistant genes in bananas were selected. The first gene, POX (peroxidase, or peroxidase reductase), has been previously described to have a role in defense response in Solanaceae, starting to upregulate immediately after bacterial infection (Prakasha & Umesha, 2016).

Torres *et al.* (2012) observed hypersensitivity-like reactions in Calcutta IV plants inoculated with *P. fijiensis,* where the enzymatic activity of POX (not its expression) was maximum at 24 HAI. Rodriguez *et al.* (2016) observed an increase of POX expression starting after inoculation with conidia and peaking at 12HAI in the resistant plants and a peak of POX expression in inoculated Cavendish var. Williams at 18 HAI, suggesting that POX response is delayed in the susceptible plants.

In our study, Calcutta IV inoculated with *P. fijensis* conidia, had POX expression higher at 36 HAI in Calcutta 4 resistant plants, but its expression was not significantly different from that of non-inoculated plants. In inoculated Cavendish, POX was upregulated at 48 and 72 HAI. Rodriguez *et al.* (2016) found that POX expression upregulates at was observed at 12 HAI. Compared to his work, the first samples were collected at 36 HAI in our study, and we may have missed its maximum expression level.

Interestingly, consistent with Rodriguez's study, we observed an overexpression of POX at 48 HAI and 72 HAI in the susceptible plants. Assuming a response in the resistant plant before 36 HAI, the response in the susceptible plants would be considered a delayed response. Overall, these observations suggest that banana HR resistance response activation is gene-dependent and time-dependent, as observed in other banana/pathogen interaction systems such as bacterial wilt (Tripathi *et al.*, 2019) and fusarium wilt (Li *et al.*, 2011).

For the in-vitro assay, in PfAVR4 inoculated plants, POX expression spiked at 6 HAI in Calcutta 4 resistant plants, corresponding to the initiation of the HR-related symptoms. No other plants in the study showed HR symptoms or a spike of activity in POX expression, confirming that the brown coloration observed was HR-related.

Accumulation of pathogenesis-related (PR) proteins during microbial infection or under abiotic stress conditions constitutes an integral component of innate immune responses in plants. PR proteins accumulate locally in the infected leaf and are also induced systemically (Negi & Ngapathi, 2016). They are associated with the trigger or systemic acquired resistance (SAR), and at least eight of these proteins have been identified in banana (Portal *et al.*, 2011; Van Loon & Van Strien, 1999). In our study, Calcutta IV plants inoculated with PfAVR, PR4 expression peaked at 6 HAI, corresponding to the beginning of the unmistakable appearance of the HR response. Calcutta IV plants inoculated with conidia had spikes at 36 HAI, and Cavendish inoculated with conidia started upregulating at 72 HAI, reaching a peak at 120 HAI. Rodriguez *et al.* (2016) observed a spike of PR-4 expression at 12 HAI for Calcutta IV and 24 HAI for Cavendish.

Both experiments showed that PR-4 has an inverted expression pattern; when the gene upregulates in Calcutta IV, it downregulates in Cavendish and vice-versa. The same trend was observed by Rodriguez *et al.* (2016) and Rodriguez *et al.* (2020). PR4 protein has been described as a chitinase (*Li et al.*, 2013), while PfAVR4 protects *P. fijiensis* from chitinases (Kholer *et al.*, 2016). As it would be expected, in both treatments (PfAVR4 and conidia), PR-4 is upregulated in the resistant plants while downregulated in the susceptible plants.

Expression levels of PR-10 in Calcutta IV plants inoculated with the effector steadily upregulate from 1 HAI to 12 HAI, then it downregulates towards 48 HAI. In Calcutta IV plants

inoculated with conidia, expression spikes at 36 HAI, and then it downregulates towards 120 HAI. Rodriguez *et al.* (2016) observed a spike of expression at 6 HAI that is kept up to 24 HAI, and then it drops at 72 HAI. PR10 proteins are transcriptionally responsive across biotic and abiotic stress (Negi & Ngapathi, 2016), which might explain the upregulation at 0 HAI in Calcutta IV from the effector group. Also, overexpression of PR-10 in bananas has conferred resistance to *F. oxysporum* sp. *cubense* TR 4 (Nadiya, 2017), confirming its role in defense against biotic stress. The upregulation of PR-10 in resistant plants in both treatment groups (12 HAI in PfAVR4 and 36 HAI in conidia) suggests that this protein has a role in the defense response against *P.fijensis*.

DDR1 expression significantly upregulates at 36 HAI in Calcutta IV inoculated with conidia and immediately drops at its lowest point at 48 HAI. No significant expression was found for Cavendish or plants inoculated with PfAVR4. Rodriguez *et al.* (2016) observed two spikes of upregulation at 6 HAI and 18 HAI on Calcutta IV inoculated plants. DRR1 has been correlated with lignin accumulation, cell wall thickening, and tolerance to abiotic stress in Arabidopsis (Yu *et al.*, 2021), which might explain the spike in the expression in resistant plants from the effector group at 0 HAI. Considering the inverse expression of DRR1 in the *in vitro* and in-vivo experiments in resistant Calcutta IV plants, our results suggest that this gene may not be directly involved in response to *P.fijensis*.

R4 expression in Calcutta IV plants inoculated with PfAVR4, expression upregulates at 12 HAI and immediately downregulates at 24 and 48 HAI. While in inoculated Cavendish plants, its expression upregulates at 24 and 48 HAI. For Calcutta IV inoculated with conidia, expression upregulates at 36 HAI. No significant changes in expression were observed in Cavendish.

R4 is a gene that belongs to the nucleotide-binding site–leucine-rich repeat (NBS-LRR) family, the most represented group of resistance genes (Marone 2013). It was predicted in banana from the tomato's Cf4 resistant gene sequence (E-value 3e-17 36.22% identity). The upregulation in expression in resistant plants and its downregulation in susceptible plants in both groups (PfAVR4 at 12 HAI and Conidia at 36 HAI) suggests a role of R4 in the resistance response to *P.fijensis*.

This study has reproduced some of the same resistance responses found in Calcutta IV inoculated with conidia by using only the PfVR4 effector. Most of the resistance responses in the conidia experiment occur close to 36 HAI, while most of the responses in the effector experiment occur between 6 and 12 HAI.

Some gene expression patterns among plants inoculated with Conidia and PfAVR4 were similar. For example, in both groups, PR-4 upregulates in Calcutta IV at the same time when it downregulates in Cavendish, indicating the effector might be able to trigger the defense response in resistant plants while suppressing it in susceptible plants. PR-10 in both groups experience a peak of upregulation (12 HAI for PfAVR4 and 36 HAI for conidia), and then it steadily downregulates.

Overall, these experiments confirmed that resistant plants but not susceptible plants inoculated with the PfAVR4 effector expressed a hypersensitive response (HR), compatible with the gene-for-gene resistance mechanism. PR4, PR10, and R4 expression levels were consistently upregulated at 6-36 hours post-inoculation with the PfAVR4 effector or conidia. In resistant plants inoculated with the PfAVR4 effector, a very high expression of PR4 was detected at 6 HAI, coinciding with the initial appearance of HR symptoms, including small necrotic lesions and upregulation of the peroxidase (POX). Taken together, our results suggest that PR4, which

was previously identified to be upregulated in resistant plants inoculated with *P. fijensis*, is a strong candidate for being the gene that recognizes the PfAVR4 effector, which triggers the HR response and ultimately confer resistance to Black Sigatoka. A genome-wide transcriptome analysis would represent the next step to understand better how these genes play a role in the resistance response to Black Sigatoka. Also, functional characterization of the PR4 gene by gene editing/transformation and/or a gene-to-gene interaction assay could complement these efforts.

In conclusion, the results of this study inform banana breeding and production programs about material and strategy to improve nutrient delivery, bioactive content, and Black Sigatoka resistance. In addition, it establishes the foundation to understand further the genetic and molecular mechanisms controlling these traits, which represents a critical step to developing an effective and sustainable crop improvement program.

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APPENDICES

Appendix A

Phenotypical characterization of 27 banana accessions

Table A.1 Phenotypical characterization of 27 banana accessions at three ripening stages (A, B, C) and two ripening methods at stage C, fully ripened in controlled conditions (Ccc) and fully ripened naturally on the plant (Cnc).

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Name	Ploidy	Market type	Maturation stage	Length (cm)	Weight (g)	Moisture content (%)
Baby Khai	AA	Dessert	A	11.47	71.29	72
			B		66.95	68
			Ccc		74.66	68
			Ccn		144.02	69
Bluggoe	ABB	Cooking	A	18.90	223.45	69
			B		207.75	69
			Ccc		216.60	67
			Ccn		430.39	69
Bri Bri	unknown	Dessert	A	11.50	56.53	71
			B		58.48	74
			Ccc		58.95	74
			Ccn		110.38	75
Bungai	AA	Dessert	A	15.40	66.37	69
			B		68.73	68
			Ccc		66.75	67
			Ccn		102.06	70
Cavendish	AAA	Dessert	A	23.20	186.23	81
			B		176.63	79
			Ccc		180.76	79
			Ccn		241.76	79
Clon Frances	AAA	Dessert	A	8.30	63.27	68
			B		58.64	69
			Ccc		61.76	72
			Ccn		102.77	74
Ducasse	ABB	Dessert	A	12.17	79.88	71
			B		72.70	73
			Ccc		72.84	69
			Ccn		140.90	69
FHIA18	AAAB	Dessert	A	16.07	157.79	73
			B		145.86	76
			Ccc		160.61	76
			Ccn		253.65	75

Table A.1 (Continued).

Name	Ploidy	Market type	Maturation stage	Length (cm)	Weight (g)	Moisture content (%)
Hua Moa	AAB	Plantain	A	19.27	289.58	71
			B		284.43	71
			Ccc		342.56	72
			Ccn		501.62	71
Jaribuaya	AA	Dessert	A	9.90	46.78	68
			B		50.32	69
			Ccc		49.66	70
			Ccn		85.80	69
KKB	AAA	Dessert	A	15.93	118.20	81
			B		123.67	78
			Ccc		120.33	78
			Ccn		260.04	78
Kru	ABB	Dessert	A	15.33	96.81	74
			B		99.95	76
			Ccc		91.82	76
			Ccn		144.07	79
Kunnan	AB	Dessert	A	15.35	119.12	73
			B		122.89	73
			Ccc		125.30	74
			Ccn		156.72	73
Latex Rojo	AAA	Dessert	A	17.50	102.17	76
			B		76.04	75
			Ccc		83.01	75
			Ccn		133.96	75
Manzano	AAB	Cooking	A	14.67	86.22	68
			B		90.56	70
			Ccc		85.61	69
			Ccn		204.46	72
OA511	unknown	Dessert	A	13.27	99.63	69
			B		97.00	69
			Ccc		88.21	70
			Ccn		205.80	70
P. Awak	ABB	Dessert	A	14.50	112.75	67
			B		98.64	65
			Ccc		109.38	65
			Ccn		214.98	69
P. Keilyn	AAB	Cooking	A	12.97	84.27	65
			B		88.61	64
			Ccc		86.20	66
			Ccn		133.69	68

Table A.1 (Continued).

Name	Ploidy	Market type	Maturation stage	Length (cm)	Weight (g)	Moisture content (%)
P. Ceylan	AAB	Cooking	A	17.00	148.49	72
			B		136.93	75
			Ccc		145.21	74
			Ccn		284.59	73
P. Lidi	AA	Dessert	A	12.17	37.66	69
			B		39.36	71
			Ccc		34.41	74
			Ccn		62.09	68
P. Liling	AA	Dessert	A	11.53	45.71	69
			B		48.19	70
			Ccc		44.18	71
			Ccn		76.51	70
Pitogo	ABB	Plantain	A	6.67	47.00	67
			B		44.63	66
			Ccc		45.50	67
			Ccn		55.73	68
Prata	AAB	Dessert	A	13.00	74.33	79
			B		70.67	77
			Ccc		70.52	77
			Ccn		77.81	76
Red Macabu	AAA	Dessert	A	16.90	85.90	72
			B		121.11	73
			Ccc		126.81	74
			Ccn		248.95	77
Selangor	AA	Dessert	A	17.33	122.37	73
			B		113.49	75
			Ccc		112.99	73
			Ccn		176.92	74
ThapMaeo	AAB	Cooking	A	15.67	124.00	66
			B		131.67	70
			Ccc		133.93	70
			Ccn		266.21	76
Tindok	AAB	Plantain	A	29.67	363.28	63
			B		357.08	63
			Ccc		387.35	64
			Ccn		446.97	64

Appendix B

Total carotenoid content (TCC) of 27 banana accessions

Table B.1. Total carotenoid content (mg/100g D.W.) of 27 banana accessions at ripening stage A.

Total carotenoid content at maturation stage A (unripe)											
Name	Lutein		α-carotene		β-carotene		Total Carotenoids		Total Carotenoids (RAE)		Tukey's HSD
Baby Khai	0.377	±0.15	0.316	±0.142	0.396	±0.184	1.856	±0.509	0.063	± 0.028	bcd
Bluggoe	0.064	±0.022	0.042	±0.017	0.188	±0.07	0.443	±0.126	0.019	± 0.003	bcd
Bri Bri	0.144	±0.024	0.373	±0.057	0.588	±0.157	1.758	±0.237	0.074	± 0.016	abcd
Bungai	0.146	±0.011	0.063	±0.009	0.264	±0.06	0.708	±0.072	0.029	± 0.002	bcd
Cavendish	0.126	±0.048	0.051	±0.018	0.035	±0.013	0.397	±0.082	0.026	± 0.008	bcd
Clon Frances	0.065	±0.028	0.016	±0.007	0.017	±0.006	0.181	±0.042	0.012	± 0.003	cd
Ducasse	0.031	±0.021	0.013	±0.011	0.043	±0.028	0.143	±0.069	0.011	± 0.005	d
FHIA18	0.038	±0.031	0.01	±0.01	0.016	±0.014	0.118	±0.06	0.019	± 0.006	bcd
Hua Moa	0.019	±0.002	0.346	±0.016	0.582	±0.022	1.395	±0.047	0.12	± 0.027	abc
Jaribuaya	0.128	±0.065	0.021	±0.013	0.027	±0.015	0.331	±0.097	0.016	± 0.002	bcd
KKB	0.088	±0.009	0.212	±0.044	0.341	±0.065	1.016	±0.132	0.052	± 0.017	bcd
Kru	0.148	±0.035	0.621	±0.162	0.591	±0.16	2.233	±0.35	0.125	± 0.049	ab
Kunnan	0.027	±0.007	0.004	±0.002	0.005	±0.001	0.074	±0.014	0.007	± 0.003	d
Latex Rojo	0.104	±0.048	0.191	±0.093	0.096	±0.056	0.717	±0.208	0.046	± 0.022	bcd
Manzano	0.01	±0.003	0.001	±0.001	0.007	±0.001	0.029	±0.002	0.006	± 0.003	d
OA511	0.02	±0.01	0.008	±0.005	0.013	±0.007	0.07	±0.021	0.022	± 0.011	d
P. Awak	0.077	±0.013	0.021	±0.005	0.089	±0.016	0.305	±0.038	0.009	± 0.003	d
P. Keilyn	0.339	±0.048	0.049	±0.018	0.047	±0.014	0.832	±0.077	0.018	± 0.003	bcd
P.Ceylan	0.028	±0.014	0.021	±0.01	0.043	±0.016	0.151	±0.04	0.013	± 0.007	bcd
P.Lidi	0.044	±0.006	0.004	±0.001	0.008	±0.001	0.104	±0.006	0.011	± 0.004	cd
P.Liling	0.028	±0.014	0.003	±0.002	0.005	±0.003	0.068	±0.018	0.008	± 0.007	d
Pitogo	0.018	±0.008	0.08	±0.041	0.495	±0.238	0.787	±0.333	0.149	± 0.034	abc
Prata	0.098	±0.023	0.063	±0.018	0.073	±0.024	0.406	±0.065	0.037	± 0.012	bcd
Red Macabu	0.075	±0.042	0.024	±0.01	0.018	±0.006	0.224	±0.056	0.025	± 0.008	bcd
Selangor	0.139	±0.069	0.04	±0.033	0.028	±0.022	0.406	±0.135	0.012	± 0.005	cd
ThapMaeo	0.043	±0.006	0.014	±0.005	0.029	±0.006	0.149	±0.015	0.014	± 0.004	bcd
Tindok	0.03	±0.006	0.552	±0.123	0.773	±0.166	2.079	±0.303	0.194	± 0.074	a

Table B.2. Total carotenoid content (mg/100g D.W.) of 27 banana accessions at ripening stage B.

Total carotenoid content at maturation stage B (semi-ripe)											
Name	Lutein		α-carotene		β-carotene		Total Carotenoids		Total Carotenoids (RAE)		Tukey's HSD
Baby Khai	0.346	±0.042	0.343	±0.067	0.497	±0.076	1.186	±0.181	0.076	± 0.007	abc
Bluggoe	0.11	±0.006	0.038	±0.004	0.173	±0.009	0.321	±0.01	0.028	± 0.004	c
Bri Bri	0.135	±0.016	0.447	±0.092	0.691	±0.162	1.273	±0.27	0.085	± 0.017	abc
Bungai	0.086	±0.016	0.046	±0.007	0.221	±0.053	0.353	±0.063	0.038	± 0.009	bc
Cavendish	0.242	±0.041	0.067	±0.009	0.067	±0.008	0.376	±0.064	0.011	± 0.004	c
Clon Frances	0.157	±0.026	0.039	±0.003	0.066	±0.005	0.262	±0.036	0.01	± 0.001	c
Ducasse	0.072	±0.017	0.032	±0.018	0.143	±0.026	0.247	±0.067	0.012	± 0.006	c
FHIA18	0.156	±0.005	0.047	±0.013	0.095	±0.025	0.298	±0.048	0.015	± 0.007	c
Hua Moa	0.083	±0.004	0.777	±0.053	1.368	±0.099	2.228	±0.171	0.089	± 0.029	abc
Jaribuaya	0.269	±0.068	0.029	±0.006	0.078	±0.026	0.376	±0.086	0.015	± 0.007	c
KKB	0.096	±0.022	0.257	±0.075	0.478	±0.159	0.831	±0.293	0.077	± 0.025	abc
Kru	0.244	±0.017	1.108	±0.175	1.092	±0.182	2.444	±0.348	0.11	± 0.02	ab
Kunnan	0.045	±0.019	0.03	±0.015	0.023	±0.013	0.098	±0.033	0.007	± 0.006	c
Latex Rojo	0.147	±0.065	0.491	±0.18	0.298	±0.134	0.936	±0.422	0.058	± 0.024	abc
Manzano	0.023	±0.006	0.006	±0.001	0.04	±0.006	0.069	±0.012	0.007	± 0.004	c
OA511	0.067	±0.034	0.034	±0.017	0.035	±0.018	0.136	±0.076	0.016	± 0.009	c
P. Awak	0.074	±0.019	0.018	±0.004	0.105	±0.007	0.197	±0.028	0.014	± 0.003	c
P. Keilyn	0.256	±0.013	0.019	±0.004	0.031	±0.004	0.306	±0.02	0.014	± 0.001	c
P.Ceylan	0.114	±0.042	0.138	±0.103	0.222	±0.124	0.474	±0.298	0.022	± 0.012	c
P.Lidi	0.182	±0.026	0.023	±0.003	0.076	±0.016	0.281	±0.025	0.015	± 0.008	c
P.Liling	0.028	±0.017	0.004	±0.002	0.009	±0.005	0.041	±0.025	0.011	± 0.008	c
Pitogo	0.059	±0.012	0.27	±0.028	2.001	±0.06	2.33	±0.041	0.095	± 0.053	abc
Prata	0.198	±0.064	0.164	±0.022	0.216	±0.047	0.578	±0.144	0.024	± 0.01	c
Red Macabu	0.205	±0.035	0.108	±0.07	0.147	±0.047	0.46	±0.101	0.026	± 0.014	c
Selangor	0.267	±0.055	0.07	±0.017	0.107	±0.034	0.444	±0.057	0.021	± 0.003	c
ThapMaeo	0.128	±0.008	0.039	±0.011	0.125	±0.005	0.292	±0.023	0.022	± 0.011	c
Tindok	0.122	±0.025	0.703	±0.109	1.07	±0.147	1.895	±0.276	0.121	± 0.007	a

Table B.3. Total Carotenoid content (mg/100g D.W.) of 27 banana accessions at ripening stage C, fully ripe in controlled conditions (Ccc)

Total Carotenoid content at maturation stage Ccc (fully ripe, controlled conditions)											
Name	Lutein		α-carotene		β-carotene		Total Carotenoids		Total Carotenoids (RAE)		Tukey's HSD
Baby Khai	0.349	±0.051	0.362	±0.079	0.52	±0.105	1.231	±0.213	0.086	± 0.019	abc
Bluggoe	0.106	±0.007	0.05	±0.021	0.212	±0.071	0.368	±0.099	0.022	± 0.009	bc
Bri Bri	0.11	±0.011	0.535	±0.188	0.851	±0.3	1.496	±0.532	0.089	± 0.025	abc
Bungai	0.107	±0.036	0.054	±0.007	0.261	±0.027	0.422	±0.043	0.032	± 0.007	bc
Cavendish	0.367	±0.029	0.141	±0.044	0.123	±0.036	0.631	±0.116	0.018	± 0.003	bc
Clon Frances	0.206	±0.003	0.052	±0.009	0.089	±0.015	0.347	±0.026	0.009	± 0.005	c
Ducasse	0.068	±0.014	0.024	±0.012	0.134	±0.01	0.226	±0.033	0.013	± 0.003	c
FHIA18	0.167	±0.021	0.063	±0.031	0.11	±0.035	0.34	±0.095	0.012	± 0.006	bc
Hua Moa	0.068	±0.002	0.778	±0.026	1.389	±0.046	2.235	±0.082	0.091	± 0.032	abc
Jaribuaya	0.261	±0.069	0.031	±0.004	0.092	±0.015	0.384	±0.06	0.018	± 0.006	bc
KKB	0.175	±0.015	0.381	±0.02	0.656	±0.023	1.212	±0.063	0.08	± 0.026	bc
Kru	0.263	±0.035	1.247	±0.254	1.267	±0.255	2.777	±0.561	0.128	± 0.03	ab
Kunnan	0.125	±0.01	0.042	±0.003	0.054	±0.011	0.221	±0.021	0.006	± 0.003	c
Latex Rojo	0.204	±0.017	0.615	±0.109	0.382	±0.071	1.201	±0.202	0.04	± 0.015	bc
Manzano	0.088	±0.018	0.03	±0.009	0.071	±0.01	0.189	±0.021	0.008	± 0.004	c
OA511	0.308	±0.056	0.128	±0.007	0.154	±0.009	0.59	±0.075	0.029	± 0.013	bc
P. Awak	0.065	±0.026	0.017	±0.006	0.096	±0.023	0.178	±0.061	0.017	± 0.003	c
P. Keilyn	0.395	±0.023	0.028	±0.005	0.033	±0.002	0.456	±0.03	0.02	± 0.004	bc
P.Ceylan	0.123	0.037	0.135	±0.093	0.256	±0.103	0.514	±0.265	0.034	± 0.014	bc
P.Lidi	0.171	0.01	0.023	±0.003	0.109	±0.019	0.303	±0.027	0.016	± 0.008	c
P.Liling	0.233	0.034	0.036	±0.005	0.103	±0.017	0.372	±0.024	0.018	± 0.008	c
Pitogo	0.066	0.007	0.229	±0.021	1.641	±0.286	1.936	±0.316	0.077	± 0.018	abc
Prata	0.326	0.002	0.298	±0.009	0.371	±0.007	0.995	±0.012	0.04	± 0.012	abc
Red Macabu	0.26	0.026	0.232	±0.062	0.233	±0.053	0.725	±0.162	0.056	± 0.04	bc
Selangor	0.135	0.019	0.052	±0.013	0.088	±0.03	0.275	±0.047	0.018	± 0.007	bc
ThapMaeo	0.143	0.018	0.045	±0.016	0.15	±0.023	0.338	±0.059	0.02	± 0.009	bc
Tindok	0.125	0.009	1.953	±0.039	2.794	±0.14	4.872	±0.21	0.173	± 0.07	a

Table B.4. Total Carotenoid content (mg/100g D.W.) of 27 banana accessions at ripening stage C, fully ripe naturally on the plant (Cnc).

Total carotenoid content at maturation stage Ccn (fully ripe on the plant)											
Name	Lutein		α-carotene		β-carotene		Total Carotenoids		Total Carotenoids (RAE)		Tukey's HSD
Baby Khai	0.272	±0.037	0.404	±0.08	0.675	±0.148	1.351	±0.29	0.064	± 0.006	bcdef
Bluggoe	0.106	±0.003	0.101	±0.002	0.325	±0.014	0.532	±0.021	0.031	± 0.005	cdef
Bri Bri	0.139	±0.015	0.622	±0.092	0.983	±0.103	1.744	±0.198	0.116	± 0.024	bcdef
Bungai	0.042	±0.01	0.07	±0.01	0.507	±0.028	0.166	±0.066	0.031	± 0.007	cdef
Cavendish	0.089	±0.017	0.113	±0.007	0.093	±0.005	0.295	±0.033	0.021	± 0.004	cdef
Clon Frances	0.087	±0.012	0.041	±0.008	0.084	±0.022	0.212	±0.045	0.018	± 0.001	ef
Ducasse	0.094	±0.017	0.043	±0.008	0.111	±0.017	0.248	±0.045	0.015	± 0.003	f
FHIA18	0.137	±0.017	0.093	±0.01	0.128	±0.015	0.358	±0.043	0.014	± 0.001	f
Hua Moa	0.044	±0.002	0.299	±0.013	0.553	±0.022	0.896	±0.043	0.125	± 0.031	abc
Jaribuaya	0.327	±0.006	0.058	±0.008	0.096	±0.014	0.481	±0.02	0.022	± 0.003	def
KKB	0.185	±0.037	0.503	±0.03	1.053	±0.035	1.741	±0.116	0.08	± 0.013	bcdef
Kru	0.275	±0.054	0.705	±0.099	0.689	±0.079	1.669	±0.130	0.133	± 0.027	ab
Kunnan	0.117	±0.048	0.072	±0.009	0.057	±0.011	0.246	±0.068	0.01	± 0.001	f
Latex Rojo	0.219	±0.043	0.57	±0.121	0.357	±0.092	1.146	±0.2	0.057	± 0.005	bcdef
Manzano	0.248	±0.047	0.057	±0.024	0.056	±0.015	0.361	±0.094	0.013	± 0.007	f
OA511	0.265	±0.036	0.25	±0.023	0.238	±0.021	0.753	±0.09	0.033	± 0.009	cdef
P. Awak	0.117	±0.017	0.065	±0.003	0.167	±0.007	0.349	±0.028	0.017	± 0.002	f
P. Keilyn	0.29	±0.026	0.012	±0.002	0.016	±0.001	0.318	±0.028	0.016	± 0.001	def
P.Ceylan	0.156	±0.032	0.183	±0.023	0.307	±0.02	0.646	±0.07	0.038	± 0.015	ef
P.Lidi	0.301	±0.035	0.103	±0.019	0.209	±0.042	0.613	±0.063	0.026	± 0.008	cdef
P.Liling	0.312	±0.028	0.079	±0.006	0.139	±0.015	0.53	±0.011	0.015	± 0.008	f
Pitogo	0.096	±0.011	0.157	±0.01	0.904	±0.065	1.157	±0.092	0.14	± 0.025	bcdef
Prata	0.341	±0.09	0.202	±0.04	0.237	±0.047	0.78	±0.199	0.044	± 0.013	cdef
Red Macabu	0.201	±0.03	0.478	±0.172	0.606	±0.233	1.285	±0.476	0.042	± 0.011	cdef
Selangor	0.193	±0.09	0.086	±0.019	0.077	±0.016	0.356	±0.133	0.015	± 0.005	cdef
ThapMaeo	0.154	±0.019	0.156	±0.02	0.267	±0.028	0.577	±0.081	0.021	± 0.005	f
Tindok	0.158	±0.034	0.869	±0.161	1.175	±0.137	2.202	±0.32	0.185	± 0.072	a

Appendix C

Carotenoid relative bioaccesibility, bioaccessible content, and starch content in banana

Table C.1. Relative bioaccessibility, bioaccessible content, and percent daily for 11 banana accessions at ripening stage Ccc (fully ripe, controlled condition).

Accession Name	Relative Bioaccessibility (%)		Bioaccessible content (mg/100g D.W.)		Bioaccessible content (RAE mg/100g D.W.)	%DV Standard portion[1]	%DV Single portion[2]
Baby Khai	0.27±0.1	bc	0.27±0.1	abc	0.082±0.01	4	2
Bri Bri	34.68±0.45	a	0.57±0.17	ab	0.11±0.028	4	2
Cavendish	29.79±3.91	ab	0.6±0.11	bc	0.034±0.004	1	1
Hua Moa	15.89±1.63	c	0.09±0.01	abc	0.167±0.004	7	18
KKB	25.35±3.61	abc	0.56±0.09	abc	0.087±0.002	3	3
Kru	20.75±3.14	bc	0.22±0.02	a	0.191±0.022	6	5
Latex Rojo	21.44±1.37	abc	0.77±0.05	abc	0.074±0.007	3	2
Pitogo	18.37±1.48	bc	0.27±0.01	abc	0.172±0.025	8	3
Prata	18.04±1.47	bc	0.46±0.06	bc	0.065±0.001	2	1
Red Macabu	13.14±2.6	c	0.24±0.02	c	0.045±0.005	2	2
Tindok	13.14±2.6	c	0.14±0.03	a	0.347±0.013	17	54

*Letters next to the numbers indicate Tukey's HSD grouping
1- Percent daily values (%DV) based on FDA recommended DV for Vitamin A (0.9 mg RAE) on a standard portion of a medium banana (Cavendish, 126g) for a 2000 calories diet (FDA, 2021).
2- Percent daily values (%DV) based on FDA recommended DV for Vitamin A (0.9 mg RAE) on a single portion (1 banana) for a 2000 calories diet (FDA, 2021).

Table C.2. Relative bioaccessibility, bioaccessible content, and percent daily values for 11 banana accessions at ripening stage Ccn (fully ripe on the plant).

Accession Name	Relative Bioaccessibility (%)		Bioaccessible content (mg/100g D.W.)		Bioaccessible content (RAE mg /100g D.W.)	%DV Standard portion[1]	%DV Single portion[2]
Baby Khai	17.57±2.77	a	0.25±0.01	bc	0.099±0.015	4	5
Bri Bri	32.96±6.76	ab	0.62±0.08	a	0.129±0.008	5	4
Cavendish	32.69±7.21	a	0.23±0.03	c	0.018±0.001	1	1
Hua Moa	9.9±6.06	b	0.04±0.01	c	0.071±0.002	3	11
KKB	13.07±0.65	ab	0.32±0.09	bc	0.138±0.004	4	9
Kru	16.21±1.65	ab	0.22±0.13	b	0.119±0.007	3	4
Latex Rojo	7.82±1.26	b	0.27±0.01	c	0.072±0.009	3	3
Pitogo	16.96±1.56	ab	0.21±0.04	bc	0.103±0.007	5	2
Prata	18.04±1.47	ab	0.11±0.02	bc	0.048±0.005	2	1
Red Macabu	10.53±2.13	b	0.16±0.04	bc	0.089±0.022	3	6
Tindok	10.52±2.13	b	0.29±0.18	bc	0.163±0.013	8	29

*Letters next to the numbers indicate Tukey's HSD grouping
1- Percent daily values (%DV) based on FDA recommended DV for Vitamin A (0.9 mg RAE) on a standard portion of a medium banana (Cavendish, 126g) for a 2000 calories diet (FDA, 2021).
2- Percent daily values (%DV) based on FDA recommended DV for Vitamin A (0.9 mg RAE) on a single portion (1 banana) for a 2000 calories diet (FDA, 2021).

Table C.3. Starch content for 11 banana accessions at stage Ccc (ripened with exogenous ethylene)

Accession Name	Soluble Starch (g/100g D.W.)	Resistant Starch (g/100g D.W.)	Total Starch (g/100g D.W.)
Baby Khai	38.95±7.99	10.57±1.46	49.52±6.54
Bri Bri	24.34±0.24	10.27±0.85	31.56±3.57
Cavendish	24.56±1.05	13.56±0.36	38.12±0.78
Hua Moa	27.76±1.03	12.84±3.12	40.6±3.41
KKB	27.82±2.84	13.29±0.59	41.1±3
Kru	34.86±2.12	9.82±1.33	44.68±1.28
Latex Rojo	26.49±1.03	5.02±1.06	31.51±1.08
Pitogo	17.54±0.79	14.09±1.31	31.63±0.91
Prata	32.03±6.66	9.2±3.82	41.24±3.45
Red Macabu	23.99±1.94	4.25±0.37	28.24±1.8
Tindok	23.87±2.18	12.97±0.39	36.84±1.81

Table C.4. Starch content for 11 banana accessions at stage Ccn (ripened on the plant)

Accession Name	Soluble Starch (g/100g D.W.)	Resistant Starch (g/100g D.W.)	Total Starch (g/100g D.W.)
Baby Khai	30.81±1.71	8.36±0.83	39.17±2.34
Bri Bri	15.29±1.39	8.97±1.42	24.26±2.51
Cavendish	26.14±1.62	5.49±0.79	31.63±2.16
Hua Moa	29.74±1.05	4.68±0.42	34.42±1.44
KKB	32.93±2.15	11.28±0.41	44.21±2.52
Kru	29.41±0.88	2.19±0.53	31.61±1.4
Latex Rojo	27.08±0.37	0.49±0.14	27.57±0.43
Pitogo	21.98±1.47	12.11±0.39	34.09±1.83
Prata	28.98±0.93	6.32±1.68	35.3±2.05
Red Macabu	16.25±1.43	3.28±2.22	19.53±0.8
Tindok	26.99±2.01	11.7±0.89	38.69±1.18

Appendix D

Total phenolic content of 27 banana accessions

Table D.1. Total phenolic content of 27 banana accessions at ripening stage A (unripe), expressed in galic acid equivalents (GAE)/100g D.W.

	Total Phenolic content at maturation stage A (unripe)			
Name	Total phenolic content in pulp (TPCP)	Tukey's HSD	Total phenolic content in peel (TPCP)	Tukey's HSD
Baby Khai	0.25±0.08	d	3.88±1.02	abc
Bluggoe	0.23±0.09	d	2.1±0.05	bcde
Bri Bri	0.15±0.03	d	2.23±0.2	bcde
Bungai	0.74±0.1	bcd	3.1±0.11	abcde
Cavendish	0.25±0.09	d	1.67±0.26	de
Clon Frances	0.44±0.16	d	2.49±0.47	abcde
Ducasse	0.57±0.19	cd	3.76±0.15	abcd
FHIA18	0.39±0.01	d	2.77±0.01	abcde
Hua Moa	0.08±0.01	d	1.62±0.21	e
Jaribuaya	2.61±0.57	a	2.78±0.19	abcde
KKB	0.69±0.05	cd	2.03±0.14	bcde
Kru	0.09±0.01	d	1.95±0.31	cde
Kunnan	0.66±0.15	cd	4.42±0.2	a
Latex Rojo	0.31±0.03	d	3.63±0.31	abcde
Manzano	0.95±0.51	bcd	2.65±0.12	abcde
OA511	0.86±0.43	bcd	3.98±0.12	abc
P. Awak	0.62±0.15	cd	3.14±1.26	abcde
P. Keilyn	1.33±0.72	abcd	2.2±0.36	bcde
P.Ceylan	0.24±0.02	d	1.92±0.21	cde
P.Lidi	2.57±0.21	a	2.81±0.38	abcde
P.Liling	2.04±0.39	abc	2.42±0.24	abcde
Pitogo	0.49±0.03	d	3.84±0.26	abc
Prata	0.51±0.07	d	4.51±0.11	a
Red Macabu	0.64±0.26	cd	2.73±0.32	abcde
Selangor	0.57±0.29	cd	4.09±0.16	ab
ThapMaeo	2.24±0.51	ab	2.45±0.11	abcde
Tindok	0.04±0.01	d	2.68±0.25	abcde

Table D.2. Total phenolic content of 27 banana accessions at ripening stage B (semi-ripe), expressed in galic acid equivalents (GAE)/100g D.W.

	Total Phenolic content at maturation stage B (semi-ripe)			
Name	Total phenolic content in pulp (TPCP)	Tukey's HSD	Total phenolic content in peel (TPCP)	Tukey's HSD
Baby Khai	0.79±0.54	de	3.8±0.91	abcdefg
Bluggoe	0.46±0.05	de	2.43±0.04	fg
Bri Bri	0.18±0.01	e	3.33±0.15	abcdefg
Bungai	0.86±0.04	cde	3.87±0.19	abcdef
Cavendish	0.46±0.03	de	3.11±0.15	bcdefg
Clon Frances	0.64±0.09	de	4.65±0.13	ab
Ducasse	0.36±0.09	de	3.77±0.16	abcdefg
FHIA18	0.26±0.06	e	2.47±0.52	fg
Hua Moa	0.11±0.02	e	2.17±0.16	g
Jaribuaya	2.39±0.52	ab	3.4±0.21	abcdefg
KKB	0.49±0.07	de	2.69±0.06	efg
Kru	0.09±0.01	e	2.4±0.32	fg
Kunnan	0.97±0.02	cde	4.78±0.35	a
Latex Rojo	0.25±0.04	e	4.46±0.13	abc
Manzano	1.7±0.23	abcd	3.12±0.19	bcdefg
OA511	1.3±0.13	bcde	4.19±0.23	abcde
P. Awak	0.85±0.23	cde	4.51±0.18	abc
P. Keilyn	2.24±0.92	abc	2.98±0.2	cdefg
P.Ceylan	0.4±0.14	de	2.47±0.11	fg
P.Lidi	2.99±0.16	a	3.4±0.35	abcdefg
P.Liling	1.27±0.25	bcde	3.29±0.02	abcdefg
Pitogo	0.34±0.1	de	4.36±0.31	abcd
Prata	0.3±0.03	de	4.78±0.11	a
Red Macabu	0.96±0.23	cde	3.23±0.58	abcdefg
Selangor	0.39±0.14	de	3.95±0.06	abcdef
ThapMaeo	1.08±0.05	bcde	2.79±0.1	defg
Tindok	0.12±0.06	e	3.03±0.29	bcdefg

Table D.3. Total phenolic content of 27 banana accessions at stage C, fully ripe in controlled conditions (Ccc) expressed in galic acid equivalents (GAE)/100g D.W.

	Total Phenolic content at maturation stage c (fully ripe in controlled conditions, Ccc)			
Name	Total phenolic content in pulp (TPCP)	Tukey's HSD	Total phenolic content in peel (TPCP)	Tukey's HSD
Baby Khai	0.38±0.14	c	2.45±0.64	bc
Bluggoe	0.36±0.05	c	2.37±0.13	bc
Bri Bri	0.15±0.02	c	3.06±0.29	abc
Bungai	0.79±0.12	bc	4.16±0.24	abc
Cavendish	0.46±0.04	c	3.26±0.08	abc
Clon Frances	0.75±0.09	bc	4.38±0.17	ab
Ducasse	0.16±0.1	c	2.92±0.41	abc
FHIA18	0.28±0.03	c	2.68±0.31	bc
Hua Moa	0.09±0.01	c	1.96±0.17	c
Jaribuaya	2.17±0.6	ab	3.28±0.21	abc
KKB	0.51±0.02	c	2.61±0.03	bc
Kru	0.1±0.02	c	2.66±0.47	bc
Kunnan	1.09±0.17	bc	3.84±0.4	abc
Latex Rojo	0.29±0.03	c	3.99±0.36	abc
Manzano	1.56±0.25	bc	2.97±0.29	abc
OA511	1.43±0.03	bc	3.71±0.61	abc
P. Awak	0.68±0.32	bc	3.62±1.05	abc
P. Keilyn	2.2±0.92	ab	2.73±0.19	bc
P.Ceylan	0.43±0.1	c	2.57±0.2	bc
P.Lidi	3.26±0.21	a	3.62±0.34	abc
P.Liling	1.49±0.15	bc	2.95±0.41	abc
Pitogo	0.48±0.1	c	4.1±0.5	abc
Prata	0.49±0.07	c	5.18±0.25	a
Red Macabu	0.63±0.44	bc	3.74±0.2	abc
Selangor	0.43±0.3	c	2.58±0.95	bc
ThapMaeo	1.03±0.71	bc	2.79±0.12	bc
Tindok	0.32±0.19	c	2.92±0.25	abc

Table D.4. Total phenolic content of 27 banana accessions at stage C fully ripe naturally on the plant (Ccn) expressed in galic acid equivalents (GAE)/100g D.W.

	Total Phenolic content at maturation stage C (fully ripe on the plant, Ccn)			
Name	Total phenolic content in pulp (TPCP)	Tukey's HSD	Total phenolic content in peel (TPCP)	Tukey's HSD
Baby Khai	0.14±0.04	b	1.42±0.13	def
Bluggoe	0.09±0.05	b	1.6±0.3	cdef
Bri Bri	0.11±0.02	b	1.76±0.38	bcdef
Bungai	1.13±0.77	a	3.62±0.97	abcd
Cavendish	0.19±0	ab	2.33±0.17	bcdef
Clon Frances	0.09±0.02	b	4±0.18	ab
Ducasse	0.02±0	b	2.82±0.38	abcdef
FHIA18	0.22±0.07	ab	2.56±0.25	bcdef
Hua Moa	0.06±0	b	1.6±0.07	cdef
Jaribuaya	0.3±0.05	ab	2.46±0.07	bcdef
KKB	0.14±0.04	b	1.26±0.09	f
Kru	0.08±0.01	b	1.35±0.4	ef
Kunnan	0.43±0.07	ab	3.67±0.38	abc
Latex Rojo	0.23±0.03	ab	3.54±0.58	abcde
Manzano	0.35±0.16	ab	2.02±0.1	bcdef
OA511	0.49±0.06	ab	2.99±0.02	abcdef
P. Awak	0.16±0.12	ab	2.8±0.26	abcdef
P. Keilyn	0.91±0.15	ab	2.13±0.31	bcdef
P.Ceylan	0.26±0.15	ab	2.51±1.19	bcdef
P.Lidi	0.3±0.09	ab	2.34±0.28	bcdef
P.Liling	0.3±0.11	ab	2.16±0.48	bcdef
Pitogo	0.1±0.05	b	3.06±0.1	abcdef
Prata	0.15±0.06	b	4.84±0.42	a
Red Macabu	0.22±0.08	ab	2.68±0.11	abcdef
Selangor	0.08±0.04	b	2.99±0.44	abcdef
ThapMaeo	0.43±0.33	ab	1.85±0.13	bcdef
Tindok	0.05±0.01	b	2.29±0.22	bcdef

Appendix E

Total sugar content of 27 banana accessions

Table E.1. Total sugar content (mg/100g D.W.) of 27 banana accessions at ripening stage A.

	Total sugar content (mg/100g D.W) at maturation stage A			
Name	Sucrose	Glucose	Fructose	Total Sugar
Baby Khai	0.96±0.12	0±0	0±0	1.18±0.12
Bluggoe	1.43±0.24	0±0	0±0	1.11±0.24
Bri Bri	6.21±4.25	1.09±1.01	1.09±1.01	0.21±6.24
Bungai	2.13±0.8	0.01±0.01	0.03±0.01	3.72±0.81
Cavendish	0.84±0.19	0±0	0±0	0.75±0.19
Clon Frances	1.12±0.25	0±0	0±0	1.1±0.25
Ducasse	2.97±2.29	0.05±0.05	0.04±0.04	0.56±2.39
FHIA18	1.34±0.41	0±0	0±0	2.15±0.41
Hua Moa	2.01±0.71	0±0	0±0	0.74±0.71
Jaribuaya	1±0.32	0±0	0±0	1.64±0.32
KKB	1.82±0.48	0.02±0.02	0.01±0.01	0.92±0.49
Kru	5.44±1.64	3.11±2.64	3.45±2.97	2.38±6.22
Kunnan	1.37±0.44	0±0	0±0	0.85±0.44
Latex Rojo	1.66±0.2	0.1±0.05	0.04±0.02	1.63±0.17
Manzano	0.9±0.06	0±0	0.09±0.01	0.9±0.05
OA511	1.48±0.53	0.01±0.01	0.07±0.03	0.42±0.57
P. Awak	0.99±0.4	0±0	0.02±0.02	0.71±0.42
P. Keilyn	1.17±0.57	0±0	0±0	0.02±0.57
P.Ceylan	0.68±0.53	0.33±0.25	0.39±0.38	1.99±0.55
P.Lidi	1.05±0.15	0±0	0.02±0.02	0.87±0.15
P.Liling	0.68±0.15	0±0	0±0	0.96±0.15
Pitogo	1.19±0.29	0.04±0.04	0.07±0.02	1.75±0.3
Prata	0.97±0.04	0±0	0±0	0.92±0.04
Red Macabu	1.53±0.26	0±0	0±0	1.8±0.26
Selangor	12.09±10.21	2.4±2.28	2.26±2.16	1.18±14.65
ThapMaeo	2.45±0.26	0.03±0.02	0.02±0.01	1.94±0.29
Tindok	0.9±0.04	0±0	0±0	0.91±0.04

Table E.2. Total sugar content (mg/100g D.W.) of 27 banana accessions at ripening stage B.

Name	Total sugar content (mg/100g D.W) at maturation stage B)				Tukey's HSD
	Sucrose	Glucose	Fructose	Total Sugar	
Baby Khai	25.32±10.14	4.46±1.96	3.62±1.87	61.31±13.96	abc
Bluggoe	12.19±2.78	4.63±0.88	4.66±0.95	17.01±4.55	bc
Bri Bri	42.56±6.23	10.54±3.11	10.17±2.81	77.59±12.05	a
Bungai	22.65±2.17	5.88±0.86	5.91±0.93	42.15±3.94	abc
Cavendish	17.09±0.78	3.43±0.2	3.28±0.08	23.13±0.79	abc
Clon Frances	22.11±1.04	3.03±0.24	2.89±0.26	28.65±1.48	abc
Ducasse	20.46±1.78	2.64±0.4	2.5±0.47	26.93±2.49	abc
FHIA18	24.88±4.75	12.91±4.6	12.93±4.58	24.58±13.89	ab
Hua Moa	13.34±2.66	0.5±0.11	0.4±0.09	8.75±2.85	bc
Jaribuaya	9.78±1.86	3.33±0.85	3.3±0.92	22.02±3.61	bc
KKB	19.46±9.03	10.49±5.25	10.31±5.18	60.75±19.37	abc
Kru	12.33±5.01	12.06±5.97	13.05±6.46	27.26±8.23	abc
Kunnan	20.01±1.22	3.21±0.03	3.01±0.01	27.18±1.19	abc
Latex Rojo	26.69±11.92	10.71±3.41	10.74±3.42	64.18±8.62	abc
Manzano	8.04±3.89	1.13±0.63	0.98±0.55	5.2±5.07	c
OA511	14.36±1.84	1.93±0.28	1.71±0.32	13.27±2.39	bc
P. Awak	17.74±1.78	2.62±0.42	2.51±0.51	24.45±2.59	abc
P. Keilyn	18.48±2.07	3.06±0.17	3.09±0.16	23.71±1.82	abc
P.Ceylan	11.6±2.77	3.44±0.49	3.5±0.53	23.84±3.67	bc
P.Lidi	8±1.07	2.63±0.19	2.39±0.24	13.12±1.49	bc
P.Liling	8.45±1.47	3.32±0.6	3.1±0.68	19.88±2.54	bc
Pitogo	22.12±2.55	5.83±1.73	6±1.84	39.25±5.78	abc
Prata	24.63±1.35	11.78±1.25	11.81±1	54.97±3.57	abc
Red Macabu	27.68±12.3	1.76±0.97	1.89±1.08	6.45±14.33	abc
Selangor	31.86±1.59	6.63±0.65	6.25±0.45	46.15±2.33	abc
ThapMaeo	17.4±2.61	2.48±0.74	2.2±0.75	17.66±3.97	bc
Tindok	19.22±1.45	7.85±0.67	7±0.12	35.01±1.65	abc

Table E.3. Total sugar content (g/100g D.W.) of 27 banana accessions at ripening stage Ccc.

Name	Total sugar content (g/100g D.W)at maturation stage Ccc				Tukey's HSD
	Sucrose	Glucose	Fructose	Total Sugar	
Baby Khai	24.8±6.32	9.25±0.81	8.58±1.05	51.67±4.52	bcd
Bluggoe	14.45±1.48	7.92±1.06	8.03±1.11	28.15±3.59	bcd
Bri Bri	46.17±1.28	13.45±1.94	13.23±2.09	78.71±4.58	a
Bungai	14.07±9.64	17.79±3.5	17.76±3.86	54.44±2.56	abc
Cavendish	23.15±2.23	4.9±0.38	4.89±0.28	27.31±2.83	bcd
Clon Frances	28.27±1.46	4.3±0.26	4.46±0.34	34.21±2	bcd
Ducasse	31.71±5.9	7.19±2.03	7.07±1.5	34.83±9.34	abc
FHIA18	23.74±1.63	14.64±1.64	14.44±1.68	45.83±3.63	abc
Hua Moa	27.97±6.71	3.52±0.76	3.69±0.85	18.68±8.3	bcd
Jaribuaya	17.98±4.46	7.57±1.73	7.57±1.57	37.61±7.66	bcd
KKB	22.81±0.61	12.13±0.89	12.31±0.8	50.31±2.05	abc
Kru	19.36±7.16	13.91±3.93	14.78±4.33	49.94±1.96	abc
Kunnan	30.95±1.04	9.04±0.72	8.7±0.95	44.2±2.3	abc
Latex Rojo	40.16±2.57	9.6±1.35	8.99±1.52	59.43±0.61	ab
Manzano	11.07±3.21	2.49±1.51	2.46±1.69	10.11±6.41	d
OA511	23.48±4.2	6.86±1.47	7.18±1.42	35.25±1.57	bcd
P. Awak	17.64±8.32	10.12±3.69	10.46±3.69	31.96±4.67	bcd
P. Keilyn	25.23±3.4	4.45±0.3	4.67±0.28	29.8±2.89	bcd
P.Ceylan	18.85±3.04	7.94±0.81	8.39±1.01	40.19±4.86	bcd
P.Lidi	25.43±6.02	9.97±0.28	10±0.11	37.07±6.23	abc
P.Liling	15.35±0.54	7.06±0.19	7.21±0.24	29.8±0.12	cd
Pitogo	25.5±1.5	8.13±1.82	8.63±1.86	47.22±3.02	bcd
Prata	17.33±6.39	17.59±3.76	17.74±4.12	54.5±4.42	abc
Red Macabu	36.07±13.28	2.98±1.19	3.36±1.28	10.98±15.75	bcd
Selangor	32.63±2.59	10.37±0.72	9.87±0.82	56.89±2.01	abc
ThapMaeo	33.42±2.5	6.74±0.25	6.12±0.34	45.59±1.99	abc
Tindok	19.94±0.8	8.66±0.48	9.08±0.49	39.17±0.86	bcd

Table E.4. Total sugar content (g/100g D.W.) of 27 banana accessions at ripening stage Ccn.

Name	Total sugar content (g/100g D.W)at maturation stage Cccn				Tukey's HSD
	Sucrose	Glucose	Fructose	Total Sugar	
Baby Khai	34.72±1.19	6.81±1.65	5.91±1.34	40.43±3.74	bc
Bluggoe	21.51±2.15	9.99±1.79	10.41±1.88	47.04±5.45	c
Bri Bri	44.17±3.86	13.44±0.66	13.04±0.61	76.14±4.7	ab
Bungai	36.47±0.79	11.17±0.14	10.57±0.17	59.17±0.48	abc
Cavendish	43.26±0.5	12.42±0.77	11.68±0.74	70.52±2.01	ab
Clon Frances	36.87±7.56	5.16±1.62	5.16±1.48	26.05±10.58	bc
Ducasse	41.81±1.76	7.87±1.37	7.57±1.15	52.11±2.95	abc
FHIA18	20.37±0.73	15.23±0.11	16.6±0.17	52.86±0.83	abc
Hua Moa	31.42±1.62	8.58±1.81	8.49±1.8	47.65±2.3	abc
Jaribuaya	35.03±0.51	7.48±0.51	6.95±0.57	47.28±1.47	abc
KKB	31.12±1	11.82±0.8	11.68±0.78	56.1±0.8	abc
Kru	32.35±3.23	18.05±1.7	18.15±1.94	65.69±1.48	ab
Kunnan	39.63±2.28	10.19±1.32	9.4±1.33	61.98±4.7	abc
Latex Rojo	41.65±5.79	15.32±2.3	15.79±2.65	71.11±0.96	a
Manzano	43.92±5.29	6.02±0.34	6.23±0.05	48.07±4.99	abc
OA511	38.14±4	5.38±1.42	5.32±1.03	38.82±6.23	abc
P. Awak	37.58±4.94	11.51±3.1	11.15±3.01	68.27±8.32	abc
P. Keilyn	43.53±3.19	5.13±0.58	5.67±0.64	45.62±4.39	abc
P.Ceylan	26.67±2.43	10.93±1.34	11.08±1.6	49.84±1.25	abc
P.Lidi	30.43±9.36	9.49±1.24	9.4±1.09	59.87±11.07	abc
P.Liling	36.77±1.79	10.13±1.81	9.98±1.94	47.79±5	abc
Pitogo	27.61±11.07	14.07±5.75	14.07±6.24	53.69±1.13	abc
Prata	39.63±1.97	12.9±1.76	12.36±1.91	65.33±2.12	abc
Red Macabu	59.83±1.5	4.56±0.81	5.1±0.72	65±2.79	ab
Selangor	37.49±3.59	9.9±0.54	9.34±0.45	56.66±4.31	abc
ThapMaeo	35.51±0.99	6.34±0.85	5.96±0.62	46.95±1.48	abc
Tindok	38.27±1.6	6.26±0.77	5.96±0.63	50.84±2.57	abc

Appendix F

NIR methodology for measuring TSC and sugar profile

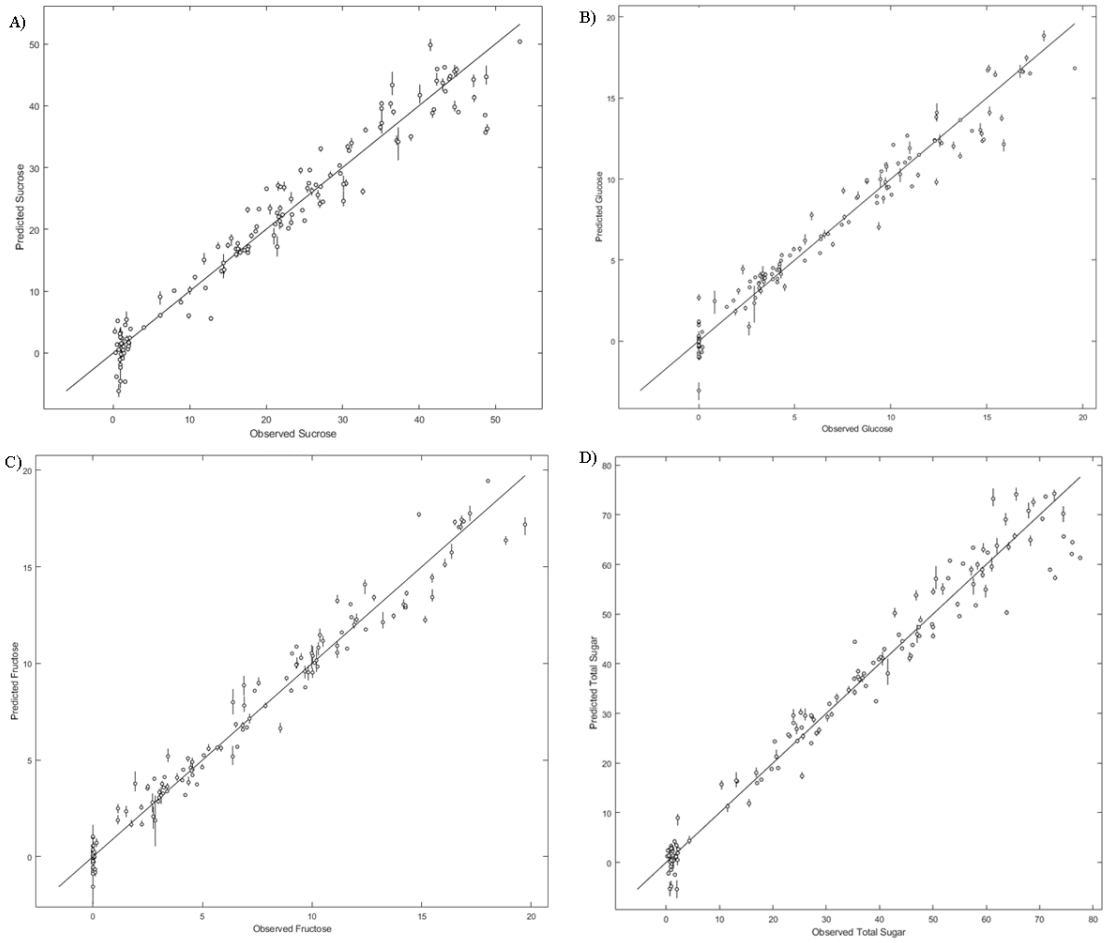

Figure F.1. Calibration and prediction results for determination of sugar in lyophilized banana powder. A) Sucrose, B) Glucose, C) Fructose, D) Total Sugar

The calibration models for sugar in 12 banana accession indicated a high correlation between NIR and the measured sugar content through HPLC. (Figure 7). The values of R^2 ranged from 0.94 in sucrose to 0.97 in fructose, and the model complexity was determined using external validation (independent dataset from calibration N= 276) is presented in Table 1. The

high model precision indicated that NIR is a reliable methodology to measure total sugar content and individual sugars in lyophilized banana powder.

Table F.1. Statistical parameters for NIR prediction models for sucrose, glucose, fructose, and total sugar content (TSC), in lyophilized banana powder

	Sucrose	Glucose	Fructose	TSC
RMSE	3.47	1.07	0.91	4.51
SE	3.48	1.07	0.91	4.52
Robust SE	3.17	0.99	0.85	3.44
RPD	4.36	5.15	6.13	5.34
R^2	94.80	96.23	97.34	96.50
Bias	0.11	0.02	-0.051	0.18
Y_{obs}	$0.978y_{pred}$+0.559	$1.005y_{pred}$-0.015	$0.994y_{pred}$-0.014	0.997_{ypred}+0.263
SDD	0.78	0.24	0.31	0.87

The application of near-infrared spectroscopy (NIR) is a rapid, non-destructive, and accurate methodology for quantitative analyses, chemical composition determinations, and qualitative analyses in agriculture, food, and other industries. It has provided a valuable tool for postharvest quality control and real-time quality monitoring during handling and processing (Paliwal, *et al.* 2006).

NIR methodology is based on a calibration model, which draws a mathematical relationship between the absorption spectra and the quality of interest in the object of study. The success of such a model requires spectra measurements from samples that most likely will include all variances in future prediction. Such variation might come from the physical attributes of the object of study to the mathematical handling of the data. Establishing a large sampling population that provide the most sources of variance will make this methodology more accurate (Cao, 2013).

Several studies have previously established NIR methods for measuring sugar in bananas. Tarkosova and Copikova, J. (2000) developed a methodology using fresh bananas (Cavendish at eight maturation stages. N=80) and HPLC data for measuring, glucose (R^2 = 0.97), sucrose (R^2 = 0.96), fructose (R^2 = 0.95) and total sugar (R^2 = 0.98). Other studies have utilized a similar approach but using fewer samples (Zude, 2003), or refractometer data rather than HPCL data (Liew & Lau, 2012; Subedi & Walsh 2011).

In contrast, our study developed a calibration curve from 12 different banana accessions at three maturation methods (A, B, and Ccc) and two maturation methods (Ccc and Ccn) using 144 different measurements for measuring glucose (R^2 = 0.95), sucrose (R^2 = 0.97), fructose (R^2 = 0.96) and total sugar (R^2 = 0.96) 94.80, and 1104 measurements for model validation. Our model has proven to effectively predict sugar content and sugar profile in different banana accession at different ripening levels.

Appendix G

Correlation matrix between sugar content and starch content

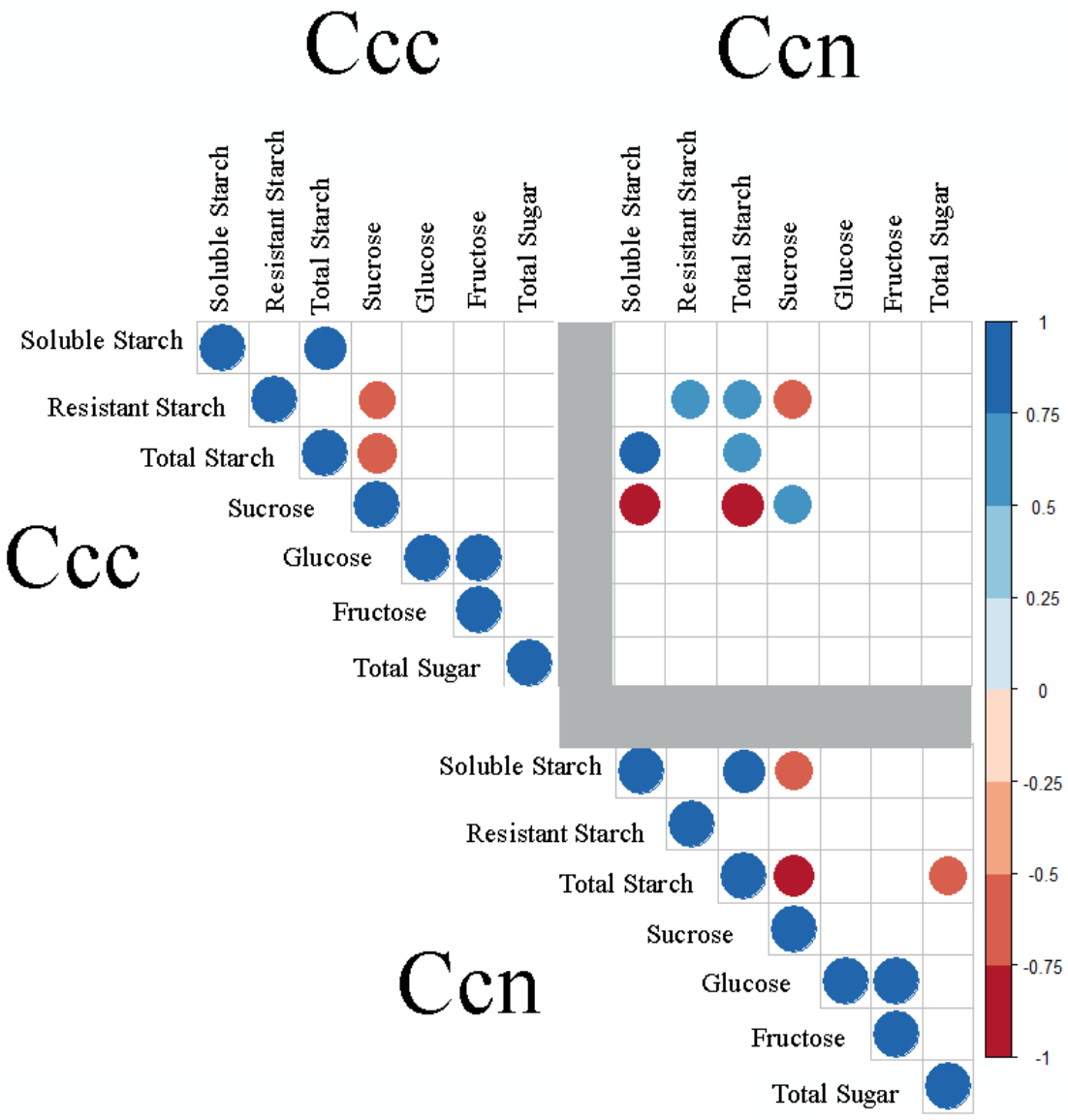

Figure G.1. Correlation matrix among starch (Soluble starch, Resistant starch, and Total Starch) and sugar content (Glucose, Fructose, Sucrose, and Total Sugar) in 27 banana accessions at three ripening stages (A, B, Ccc); and two ripening methods (fully ripe in controlled conditions -Ccc and fully ripe naturally on the plant -Cnc). Only those coefficients of determination statistically significant ($p<0.05$) are shown.

Appendix H

Banana accessions resistant to Black Sigatoka

Table H.1. Banana accessions recognized in published data as Black Sigatoka resistant

Musa genotype	Genomic group	Reference
Calcutta IV	AA	Soares *et al.*, 2020; Cruz-Cruz *et al.*, 2011
Orito	AA	Soares *et al.*, 2020
Pisang Awak	AA	Cruz-Cruz *et al.*, 2011
Pisang Liling	AA	Cruz-Cruz *et al.*, 2011
Birmanie	AA	Soares *et al.*, 2020
Krasan Saichon	AA	Soares *et al.*, 2020
Tuu Gia	AA	Soares *et al.*, 2020
Zebrina	AA	Soares *et al.*, 2020
118	AA	Soares *et al.*, 2020
DH-Pahang	AA	Soares *et al.*, 2020
Yangambi Km5	AAA	Soares *et al.*, 2020
Kiwangaazi (M9)	AAA	Soares *et al.*, 2020
Limeno	AAB	Soares *et al.*, 2020
NAROBan1	AAB	Soares *et al.*, 2020
NAROBan2	AAB	Soares *et al.*, 2020
NAROBan3	AAB	Soares *et al.*, 2020
NAROBan4	AAB	Soares *et al.*, 2020
Thap Maeo	AAB	Soares *et al.*, 2020
PV42-68	AAAB	Soares *et al.*, 2020
Pacovan Ken	AAAB	Soares *et al.*, 2020
Galil 18		Weber, Garruti, & Noroes, 2017
BRS Vitoria	AAAB	Soares *et al.*, 2020
BRS Japira	AAAB	Soares *et al.*, 2020
BRS Preciosa	AAAB	Soares *et al.*, 2020
BRS Garantida	AAAB	Soares *et al.*, 2020
BRS Tropical	AAAB	Soares *et al.*, 2020
BRS Platina	AAAB	Soares *et al.*, 2020; Weber, Garruti, & Noroes, 2017
BRS Maravilha	AAAB	Soares *et al.*, 2020; Weber, Garruti, & Noroes, 2017
FHIA 02	AAAB	Soares *et al.*, 2020
FHIA 18	AAAB	Soares *et al.*, 2020
FHIA 20	AAAB	FHIA, 2017
FHIA 21	AAAB	FHIA, 2017
FHIA 23	AAAB	FHIA, 2017
Yagambi	AAA	Cruz-Cruz *et al.*, 2011

Appendix H

Results from *in vitro* inoculation assay

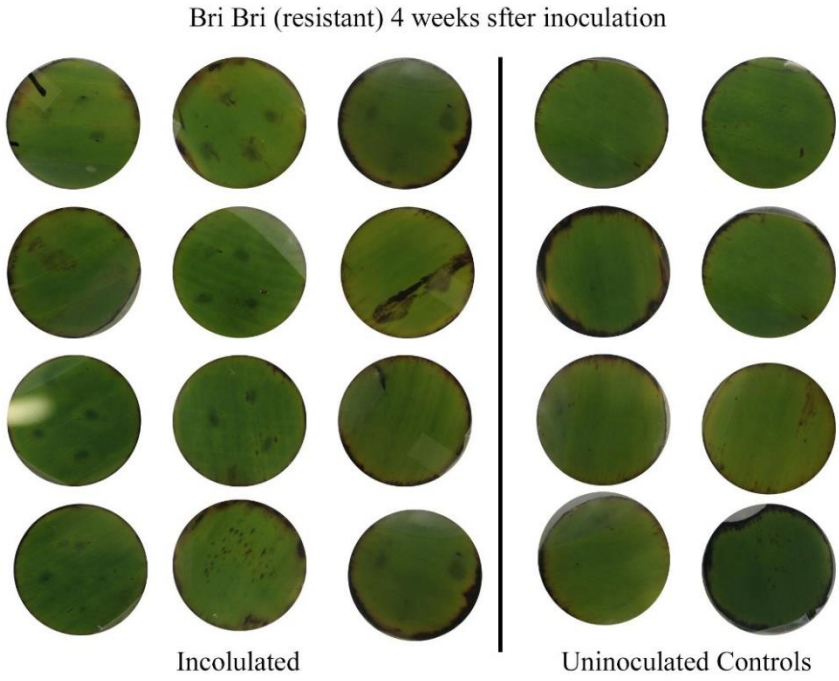

Figure H.1.*In vitro* Bri Bri leaves inoculated with *P.fijensis* conidia four weeks after inoculation**.**

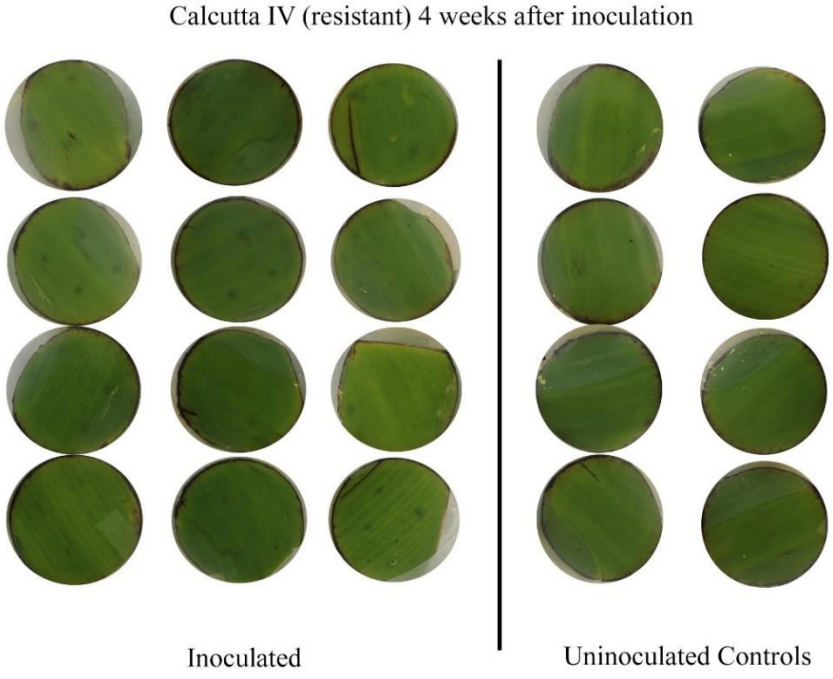

Figure H.2. *In vitro* Calcutta IV leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

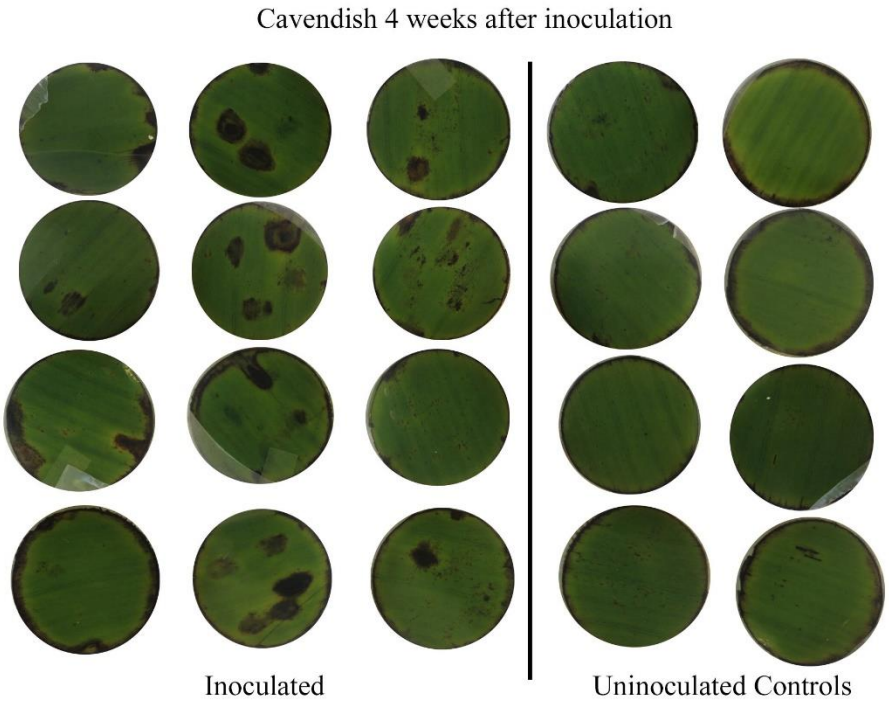

Figure H.3. *In vitro* Cavendish leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

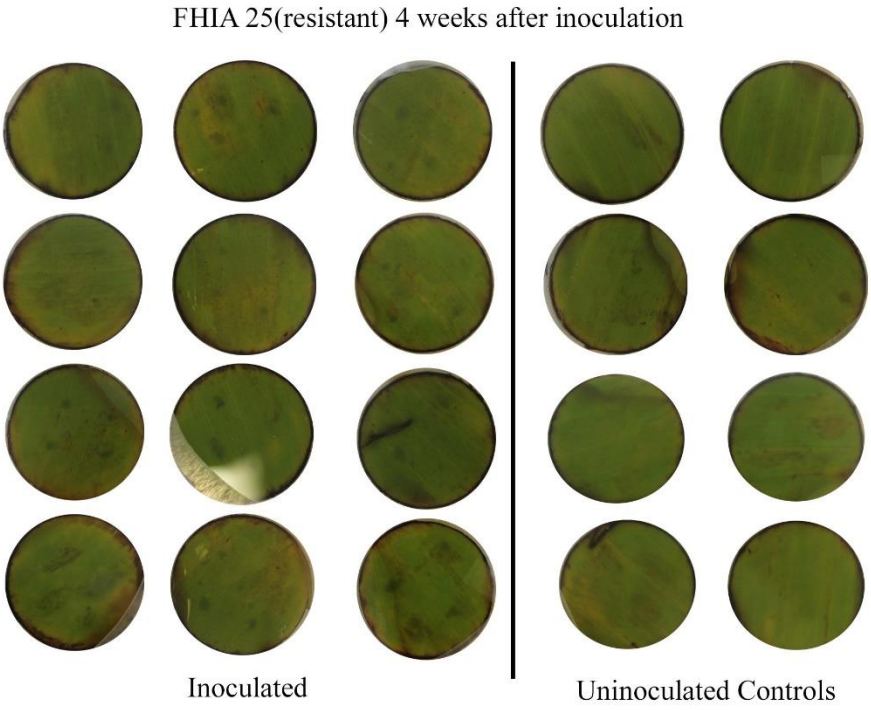

Figure H.4. *In vitro* FHIA 25 leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

Figure H.5. *In vitro* Jaribuaya leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

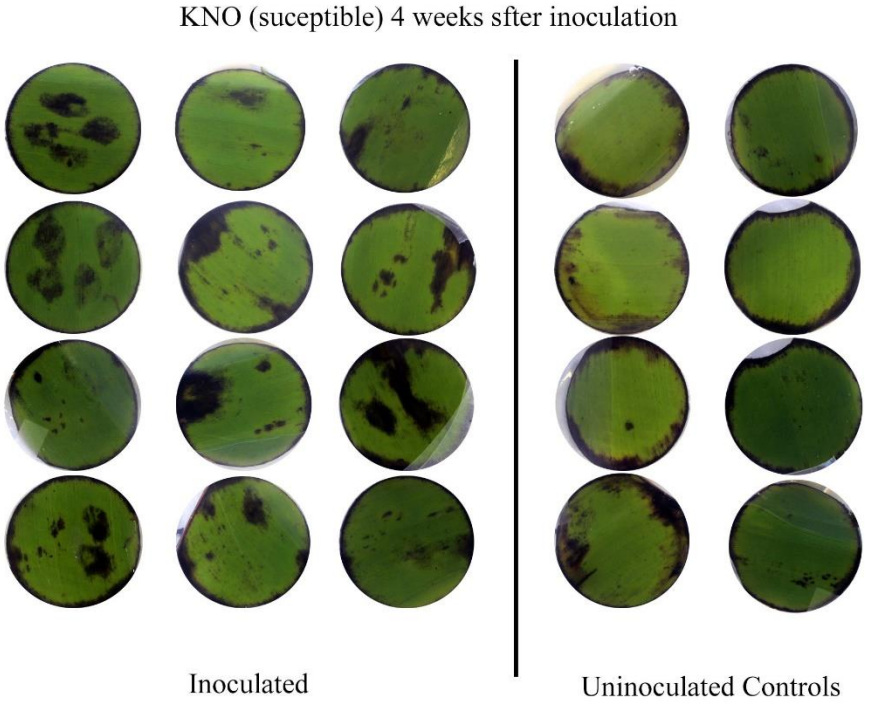

Figure H.6. *In vitro* KNO leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

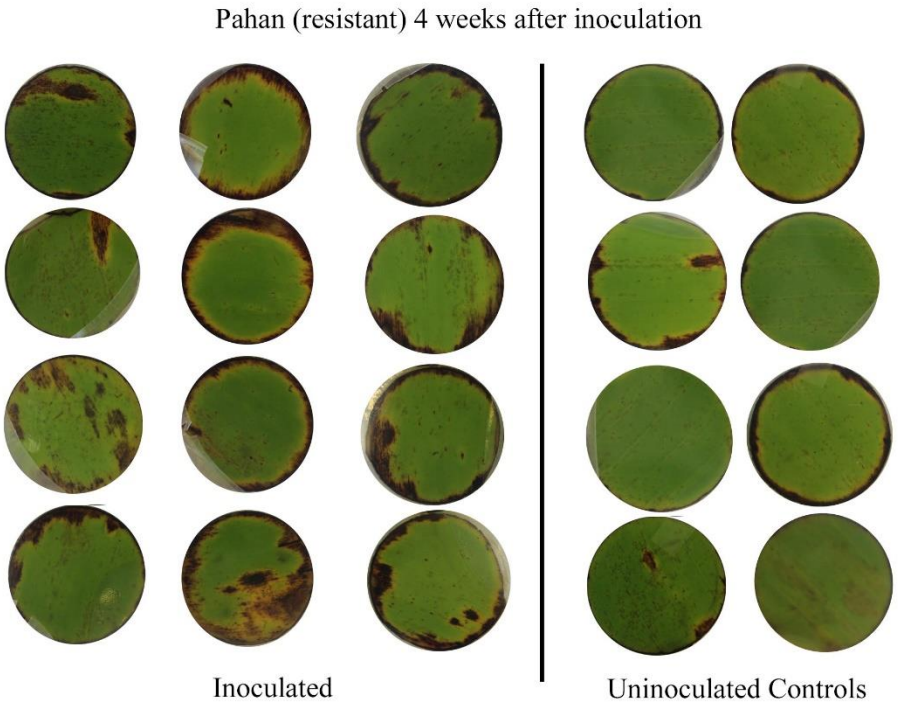

Figure H.7. *In vitro* Pisang Pahan leaves inoculated with *P.fijensis* conidia four weeks after inoculation.

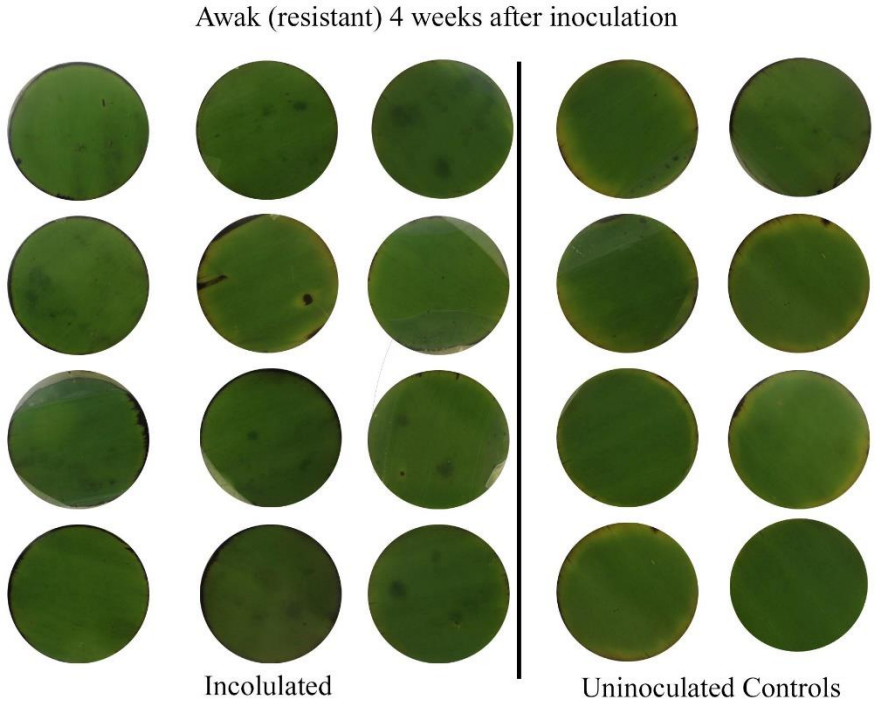

Figure H.8. *In vitro* Pisang Awak leaves inoculated with *P.fijensis* conidia four weeks after inoculation

Appendix I

Primers used for gene expression assay

Table I.1. Primer sequences used for gene expression on defense response

Gene	Function	Primer Sequence	Source
POX	Defense HR response	F>5'-AAGTCCACCTGCCCCAACG-3'	Rodriguez *et al.*,
		R> 5'-GCCTGCCGACCCATCCAACA-3'	2016
PR4	R-Gene	F>5'-TGGGACGCCAATAAGCCCCT-3'	Rodriguez *et al.*,
		R> 5'-TCCTGACCGTAGCCTGAGTTCC-3'	2016
PR10	R-Gene	F>5'-ACGGATGATGAAGTCGGTGGA-3'	Rodriguez *et al.*,
		R>5'-GACAAAGAGAGAGATGGCAGCA-3'	2016
DR1	R-Gene	F>5'-TCCTCTTTGCCGCTGTTGCCA-3'	Rodriguez *et al.*,
		R> 5'-TTGTTGCCCACCCAGATGCTCC-3'	2016
R4	Predicted R-Gene	F>5'-AACAGCCAAAAGCCAATCAC-3'	Primer Blast, NCBI
		F>5'-AACTGCACCAACCTCCAGTC-3'	
26s subunit	House Keeping	F>5'-ACATTGTCAGGTGGGGAGTT-3'	Rodriguez *et al.*,
ribosomal		R>5'-CCTTTTGTTCCACACGAGATT-3'	2016
PfAVR4	Cloning sequence	F>5'CTGATATCGGATCCGATGCTTTCAACT	Primer Blast, NCBI
	effector	ACG-3'	
		R>5'GTC GAC GGA GCT CGC TAT CCG	
		TGA TAT TT-3'	
T7	Cloning sequence	F>5'- TAATACGACTCACTATAGG-3'	Primer Blast, NCBI
	effector	R>5'- GCTAGTTATTGCTCAGCGG-3'	

Appendix J

Sequence alignment for pET30-a –PfAVR4

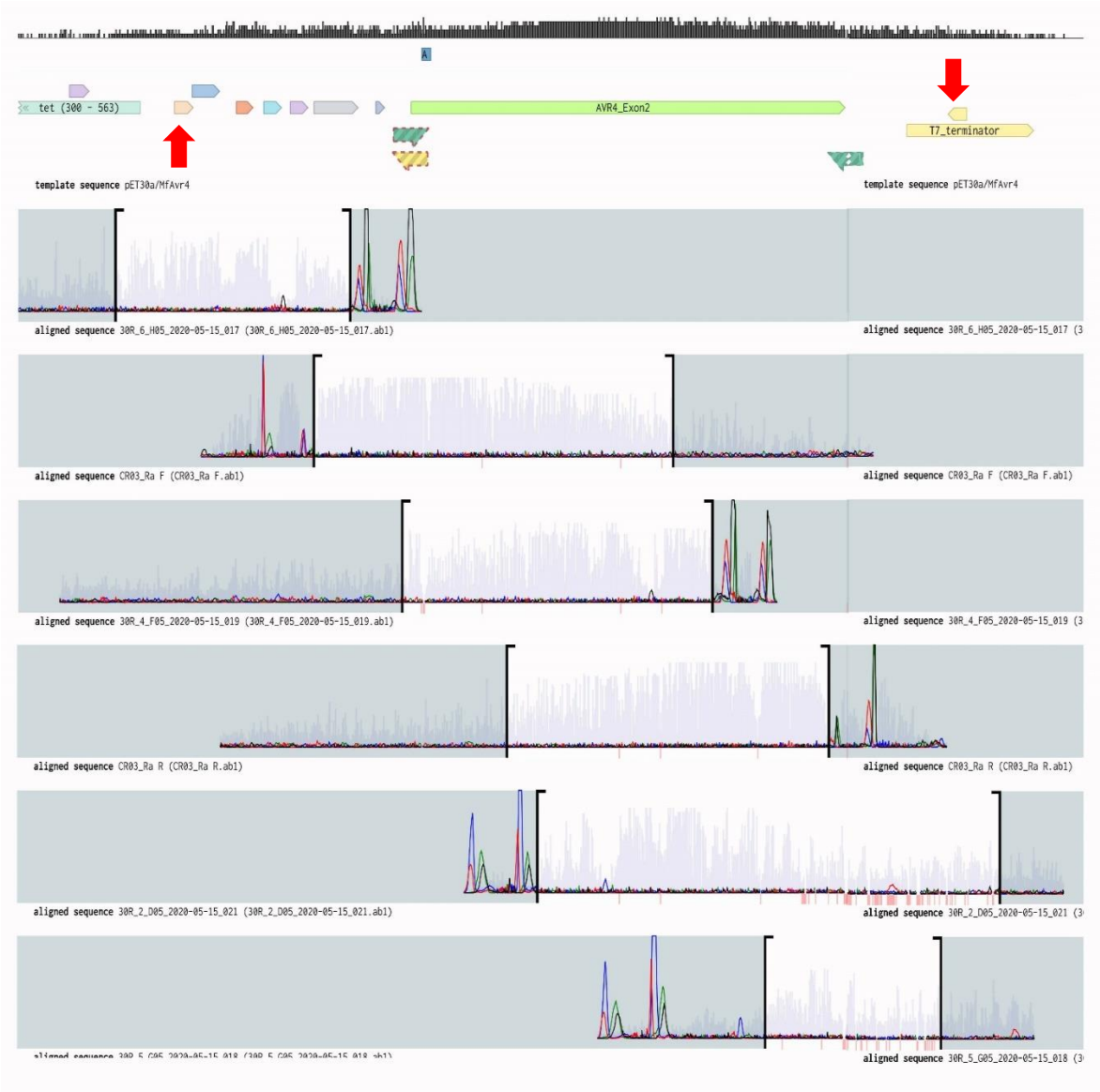

Figure J.1. Sequence alignment from the clon CR031 to the pET30-a –PfAVR4 sequence. The red arrows denote the position of the T7 primers that include the H-Tag, PfAVR4, and the T7 termination site.

Appendix K.

In silico resistance gene selection

Table K.1. DRAGO2 predicted R-genes for *M.acuminata* based on the sequence of Cf-4 gene from *S. lycopersicum*. (See http://www.prgdb.org/prgdb/genes).

Identifier	Pathogen Receptor Genes (PRGs) database name
R1	>GSMUA_Achr3T18840_001
R2	>GSMUA_Achr3T25790_001
R3	>GSMUA_Achr3T28580_001
R4	>GSMUA_Achr3T30680_001
R5	>GSMUA_AchrUn_randomT02770_001
R6	>GSMUA_AchrUn_randomT15510_001
R7	>GSMUA_AchrUn_randomT16540_001
R8	>GSMUA_AchrUn_randomT16570_001
R9	>GSMUA_AchrUn_randomT20550_001
R10	>GSMUA_Achr7T01190_001
R11	>GSMUA_Achr7T27510_001
R12	>GSMUA_Achr2T08610_001
R13	>GSMUA_Achr2T09550_001
R14	>GSMUA_Achr2T10780_001
R15	>GSMUA_Achr9T05950_001
R16	>GSMUA_Achr9T22170_001
R17	>GSMUA_Achr9T29150_001
R18	>GSMUA_Achr8T03860_001
R19	>GSMUA_Achr8T21630_001
R20	>GSMUA_Achr1T14260_001
R21	>GSMUA_Achr1T25250_001
R22	>GSMUA_Achr6T07980_001
R23	>GSMUA_Achr6T09270_001
R24	>GSMUA_Achr6T16840_001
R25	>GSMUA_Achr10T01500_001
R26	>GSMUA_Achr10T19720_001
R27	>GSMUA_Achr10T20250_001
R28	>GSMUA_Achr5T07830_001