PANAMA DISEASE IN BANANA

Spread, Screens and Genes

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Preface

I first became interested in bananas as a journalist, when I wrote a relatively short feature article about the fruit – and the danger it faced with Panama Disease – for a science magazine in the United States. At the time, I was looking for a book project, and as I studied bananas, I came to see them as a tool for a multidisciplinary explanation (and I'm being grandiose here) of *how the universe works.*

I'm almost serious. No matter what angle one approaches bananas from – scientific, historic, cultural, creative, or whatever else – the world's most beloved and popular fruit reveals itself to be complex, fascinating, even addicting.

As I fell down the banana rabbit hole (it was, and remains, a happy journey) I read dozens of books about the fruit, some more than a century old. Thousands of journal and newspaper articles also filled binder after binder, overflowing my hard drive. And it wasn't over. Once my book came out, I kept writing about bananas, publishing over 200 stories, appearing on television and radio, and ultimately embracing the sobriquet "Dan, the Banana Man."

I couldn't get enough bananas because people can't get enough bananas, whether they're bananas at their table or bananas on their mind. And one of the best things about my banana journey has been when I've found kindred banana spirits. I wasn't sure I'd discovered one when I first met Fernando Garcia during a visit to a banana research lab in the Netherlands, but my suspicion was confirmed when I looked at his twitter feed: Fernando wasn't just a dedicated banana scientist – he was a very funny and gifted banana cartoonist. That's a sure diagnostic of banana madness.

Such obsessiveness is admirable, but it is also important to remember that the obsession serves a critical cause: saving the fruit that millions of people rely on for their daily nutrition.

I'd like to say that the book you are about to read represents the culmination of Fernando's work. It is certainly fascinating, authoritative, and massive, and is the best modern overview of the fruit's life history and battle against Panama Disease that I've so far seen. But a "culmination" is an end, and I know that once one starts with bananas, there can be no end. That's something Fernando, I'm sure, is well aware of, and something you, reader, are about to discover.

- Dan Koeppel, author of the book "*Banana: The Fate of the Fruit that Changed the World"*
  Sandwich, New Hampshire, USA, February 2019

CHAPTER 1

General Introduction

Introduction

Etymologically, the word banana most likely originated when soldiers from the Middle East introduced the fruit to Africa around 650 BC. They named the fruit 'banan' which is the Arabic variation of the word for 'finger' (Koeppel 2008). Alternatively, it is claimed that the word banana originated from the West African Wolof word 'banaana', later incorporated into English through Spanish or Portuguese travellers. Although the fruit was most likely introduced in the Western world from Indian plantations by soldiers under the command of Alexander the Great (327 BC), early evidence suggests that inhabitants of Papua New Guinea already started to cultivate wild bananas 5,000 years B.C. (Price 1995, Denham *et al.,* 2003). Around the 1500s, bananas were brought to the Caribbean (likely the contemporary Dominican Republic) and rapidly spread all over the world. They were commercially cultivated and eventually exported (1870's) in unstoppable progress. Ultimately, bananas gained significant attention in the 20th century, when they became one of the most traded fruit crops (Koeppel 2008). From a botanical point of view, bananas are giant herbs, commonly up to 3 m tall, and no trees. They are perennial monocotyledonous herbs of the order Zingiberales, a sister group to the Poales, which also includes the cereals and have pseudostems that are formed by the tightly overlapping leaf sheaths. Hence, they do not have lignification or the secondary thickening of the stems that is characteristic of true botanical tree species (Tomlinson *et al.,* 1969). Carl Linnaeus developed the taxonomical genus *Musa*, which comprises all banana diversity. He assigned the Latin binomial name *Musa sapientum* to the cultivated dessert types and *Musa paradisiaca* (1753) to the cooking bananas (Cheesman 1948). Unknown to Linnaeus, the cooking and dessert bananas he had described are in fact hybrids and not two distinct species. Nevertheless, Latin binomial names are currently strictly designated to wild relatives, of which more than 180 have been classified to date (Lescot 2017). Bananas (including cooking bananas) belong to the family *Musaceae*, that besides the East-Asian genus *Musa* also includes the Asian and African genus *Ensete* and the genetically proximal Asian genus *Musella* (Perrier, 2011). Linnaeus was not the only one struggling with banana classification. This task has always been complex and also challenges contemporary botanists and breeders. In the last decades, banana classification has undergone a new nomenclature, taking both genetic and genomic aspects of the banana into account. Nowadays, the genus *Musa* is divided into four (or five) sections: *Eumusa* and *Rhodoclamys* with a base number of x =11 chromosomes and *Australimusa* and *Callimusa* with typically x =10 chromosomes. The majority of edible bananas is included in the *Eumusa* section (Dolezel and Bartoš 2005). The late Finnish botanist Markku Häkkinen recently proposed a simplification of *Musa* by just identifying the sections *Callimusa* and *Musa* (Häkkinen 2013). The cultivated hybrid bananas are also classified into genome groups and subgroups according to the relative contribution of their ancestral wild relatives. This classification proposed by Simmonds and Shepherd (1955) is currently the most common system to classify edible bananas. Their genome-based system also allows the classification of tetraploid bananas, which have essentially been developed by breeding for plant improvement purposes in different parts of the world.

Banana: from inedible wild plants to edible cultivars

Within the cultivated bananas, there are four known genomes represented by the letters A, B, S and T corresponding to the wild *Eumusa* species *Musa acuminata, M. balbisiana, M.*

schizocarpa and *M. australimusa*, respectively (d'Hont *et al.,* 2000). All edible bananas belong to the genus *Musa* and are hybrids, originating from natural and spontaneous inter and intra-specific crosses of the two diploid, seeded ancestors *M. acuminata* (genome-AA) and *M. balbisiana* (genome-BB). Southeast Asia is generally considered to be the main gene centre of banana, particularly Malaysia and Indonesia. However, the Indian subcontinent and Africa, specifically for the East African Highland bananas (EAHB), have been considered as secondary centres of diversification (Price 1995, Perrier *et al.,* 2011). The process of banana domestication started around 7,000 years ago in Southeast Asia (d'Hont *et al.,* 2012). In general terms, this process occurred by multiple spontaneous hybridizations between various species and subspecies. Domestication involved spontaneous seed abortion and selection of various seedless diploids and triploids along with parthenocarpy, which enabled vegetative propagation and wider(r) dissemination (d'Hont *et al.,* 2012) (Figure 1).

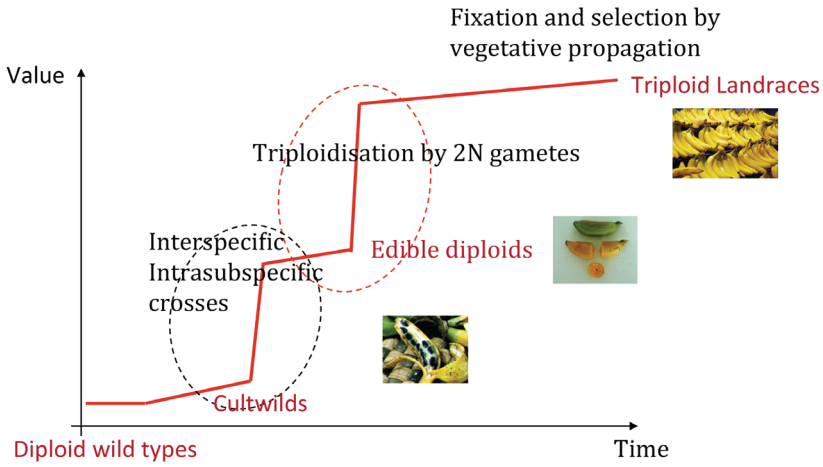

Figure 1. Representation of the discontinued evolution of banana adapted from Bakry and Horry (2014)

Recently, multidisciplinary studies unveiled new insights into the complex route of banana domestication and the geomorphological configurations that resulted in the contemporary range of banana cultivars (Perrier *et al.,* 2011). In short, domestication provided mankind with a suite of edible germplasm with AA, BB, AB, AAA, AAB, and ABB genome combinations, which all belong to the taxonomic family of the *Musaceae* and are available at many locations (Simmonds 1962, d'Hont *et al.,* 2000). Nowadays, more than 1,200 classified edible triploid varieties have been identified with potential for cultivation around the world. Additionally, 180 wild relatives and at least 50 tetraploids were created by breeding and are available in living collections or as cryopreserved specimens in gene banks (Lescot 2017) such as the International Transit Centre of Bioversity International in Leuven, Belgium. However, only a fraction of the global diversity is available in these repositories and likewise, only a limited fraction is globally consumed depending on taste and cooking preferences (e.g. Cavendish, Gros Michel, cooking bananas)

Crop and importance

Bananas, including dessert and cooking types, rank as the fourth most important food crop after rice, wheat and maize (Bakry *et al.,* 2009, Churchill 2011) they are also a significant source of income in nearly 135 producing countries. Dessert banana is a top global fruit crop with an annual production of more than 148 million tons, representing one of the most important commodities according to FAO statistics (FAOSTAT 2016). In Europe and the United States, bananas are mostly known as a fruit or snack (e.g., the globally cultivated Cavendish bananas are sold in every supermarket). However, bananas are versatile and can be cooked, roasted, fried or even brewed (Perrier, 2011). Therefore, banana production is considered as one of the greatest development opportunities for several agriculture-based economies with an estimated value of US$ 35.2 billion in the tropical world, where more than 80% fruit is produced for local consumption (Churchill 2011). In contrast to the above-described extensive genetic diversity of banana, global banana production relies on just a few clones from essentially three genomic groups that slightly differ from each other due to somaclonal variation (Bakry and Horry 2014). In fact, from the huge diversity only a few triploids (AAA, AAB, ABB) are responsible for nearly 75% of the global banana production, which is accounted for by Cavendish cultivars (~20 cultivars; 46%), plantains (~120 cultivars; 15%) and the in Eastern Africa highlands bananas (~150 cultivars; 39%) (Lescot 2017). Clearly, the dominance of Cavendish cultivars grown in global monocultures leaves the crops intensely vulnerable to disease outbreaks. As in any other crop, the diversity of harmful organisms co-evolving with bananas is huge (Maryani *et al.,* 2018). These include several biotic constraints such as fungi, viruses, bacteria, nematodes and insect pests (Wardlaw 1961, Thurston and Pennycook 1997, Jones 1999, Ploetz 2005a). The major fungal banana disease are the globally distributed Yellow and Black Sigatoka that are caused by *Pseudocercospora musae* and *P. fijiensis* (Morelet), respectively, as well as Panama disease also known as Fusarium wilt, which is caused by a range of *Fusarium* species (Maryani *et al.,* 2018) that threaten banana production (Butler 2013).

Panama disease, the banana nightmare

As there are no control options, Panama disease is considered to be the most important disease in banana (Ploetz 2000b, Ploetz *et al.,* 2015). It is extremely destructive and caused an epic epidemic in the 1900s that wiped out the “Gros Michel” based industry in Central America. The disease was first reported in Australia in 1876 (Bancroft 1876), but it is shown that the causal fungi originate from Southeast Asia, where it co-evolved with banana (Maryani *et al.,* 2018). To date, the disease is globally distributed destroying thousands of hectares of banana in tropical and subtropical countries. The abovementioned outbreak of Panama disease in Central and South America (Stover 1962, Ploetz 2006) forced the industry to deploy resistant cultivars of the Cavendish (AAA) subgroup. This allowed farmers to continue planting in infested soils. Since then, however, the new destructive Tropical Race 4 (TR4) know now as *Fusarium odoratissimum* has emerged, threatening Cavendish bananas and many other local varieties, in Southeast Asia (Ploetz 1994). Currently, this species is the major threat for international banana production as it has already spread to Africa (Ordóñez *et al.,* 2015) (Figure 2), where banana is a staple food, and may eventually cross to other vital banana producing areas such as Latin America which is a region of prime importance for the global export industry.

Figure 2. Distribution of the Tropical Race 4 (TR4) www.fusariumwilt.org

It is known that infected plant material, contaminated water and tools and/or machinery with attached infested soil are the main vectors for Panama disease dissemination, particularly in regions of vast monocultures (Stover 1962, Stover and Ploetz 1990). The infection of a plant begins with fungal hyphae entering the roots of a banana plant. Subsequent colonization in susceptible plants eventually causes occlusion of the xylem vessels, which is also due to the formation of gels and tyloses by the plant to restrict further ramification and cell collapse (Ghag *et al.,* 2015). This results in a reddish-brown discolouration of rhizomes and pseudostem vessels (Li *et al.,* 2012). Externally, plants exhibit progressive wilting with chlorotic bright yellow leaves that eventually collapse around the pseudostem along with occasional splitting of the pseudostem (Ploetz 2006) (Figure 3).

Infected plants often die before they produce bunches, hence Panama disease significantly reduces yields in affected fields (Stover and Ploetz 1990, Dita *et al.,* 2010). In addition, the pathogen produces persistent chlamydospores that contaminate soils for decades (Schippers and Van Eck 1981, Buddenhagen 2009). Statements of survival have been reported even in the absence of bananas and non-host weed species (Hennessy *et al.,* 2005). As mentioned above, Panama disease threatens the global Cavendish production, but also a wide variety of local cultivars destined for domestic markets, thereby reducing food security of economically vulnerable producers (Ploetz *et al.,* 2015). To date, no commercially accepted replacements for the Cavendish varieties are available and there are limited options for disease management. In general, chemical and physical control options are not available; hence, efforts have focused on cultural and quarantine strategies (Ploetz 2015).

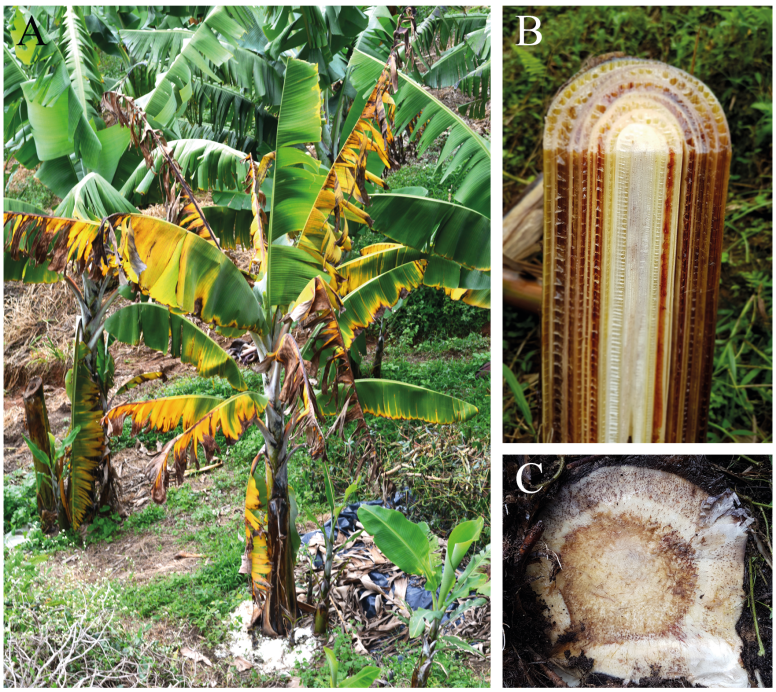

Figure 3. Characteristic external and internal symptoms of Panama disease in banana caused by *Fusarium* spp. External symptoms with extensive leaf chlorosis, foliar collapse and pseudostem breaking (A), internal symptoms, showing discoloration of the vascular system in the pseudostem (B) and corm (C)

The pathogen and the host

Agriculturally and economically, *Fusarium* is one of the most important fungal genera (Ploetz 2005b). Therefore, it was recently ranked fifth on a list of top fungal plant pathogens based on its scientific/economic importance (Ploetz 2005b, Dean *et al.,* 2012). In this taxon, *Fusarium oxysporum* is a species complex, which includes both non-pathogenic species as well as plant pathogens that cause vascular wilts, root rots, and damping-off in hundreds of host plants (Domsch *et al.,* 1980, Gerlach and Nirenberg 1982, Nelson and Toussoun, Meldrum *et al.,* 2012). Until now, over 150 host-specific forms of *F. oxysporum* known as formae speciales (ff. spp.) have been described (Baayen *et al.,* 2000, Hawksworth 2001) and each forma specialis (f. sp.) has the ability to infect a unique host plant species (Kistler 1997, Baayen *et al.,* 2000, Meldrum *et al.,* 2012). Initially, it was assumed that the members of a particular f. sp. are related to each other, and may have arisen from a common ancestor (Kistler *et al.,* 2001). However, this hypothesis was reconsidered based on the latest DNA sequence data that suggest a polyphyletic origin of the genus *Fusarium* (O'Donnell *et al.,* 1998, Ploetz 2005b, Lievens *et al.,* 2009). This includes the previously recognized *F. oxysporum* f. sp. *cubense* (*Foc*) infecting banana comprising a range of distant genotypes, so-called vegetative compatibility groups (VCGs) (Ordóñez *et al.,* 2015). Recently, however, Maryani *et al. (*2018) has taken the ultimate consequence of this situation and revised the nomenclature of all *Fusarium* species infecting banana.

Traditionally, *Fusarium* taxonomists used the shape of the macroconidia as an important basis for the classification of the various species and ff. spp. (Figure 4), but this became increasingly difficult because their conidia are mostly indistinguishable (Domsch *et al.,* 1980, Gerlach and Nirenberg 1982, Nelson and Toussoun 1983). Although teleomorphs of *Fusarium* spp. affecting banana have never been observed, the presence of mating type (*mat*) loci suggests the potential for sexual reproduction (Booth 1971, Koenig *et al.,* 1997). Therefore, it is assumed that these species exclusively reproduce clonally, although somatic fusion and heterokaryon formation could result in parasexual recombination and hence diversification (Taylor *et al.,* 1999). The ability of the previously recognized *Foc* strains to form heterokaryons enabled phenotyping for VCGs (Puhalla 1985, Correll 1991, Ploetz and Pegg 2000). Overall, however, *Foc* populations show a large gametic disequilibrium, which is indicative for non-random association of alleles and hence the absence of sex (Puhalla 1985, Kistler 1997, Koenig *et al.,* 1997). Recent genetic and molecular studies unveiled the evolutionary relationship between different clonal lineages of *Foc*, resulting in the identification of 24 VCGs (Meldrum *et al.,* 2012), with the greatest diversity in Asia. This is not surprising since this is the centre of diversity of banana and hence for the co-evolving *Fusarium* spp. (Maryani *et al.,* 2018). In any case, TR4 is only present in the recently identified new species *F. odoratissimum* with the unique VCG1213, whereas other physiological races are present in various other genotypes that are currently recognized as new *Fusarium* species (Brake *et al.,* 1990, Ploetz 1990, Moore *et al.,* 1993, Bentley *et al.,* 1998, Meldrum *et al.,* 2012, Maryani *et al.,* 2018).

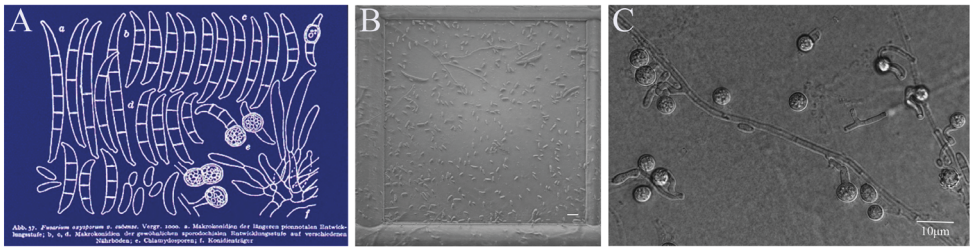

Figure 4. (A) classical cartoon from the monograph: 'Die Fusarien' by Wollenweber and Reinking (1935) showing the morphological characters of the propagules of the Panama Disease fungus hitherto known as *Fusarium oxysporum* f.sp. *cubense;* (B) micro- and macroconidia (C) chlamydospores.

Since the mid-1990s, the race nomenclature of the Panama disease fungus is defined by pathogenicity on a few banana and plantain accessions and so far resulted in the definition of four races (Table 1), Stover 1962, Persley and De Langhe 1987). Race 1 is pathogenic on 'Gros Michel' (AAA) as well as on the so-called 'Silk' (AAB) banana, 'Pisang awak' (ABB), 'Abaca' (AA), 'Maqueño' (AAB) and 'Pome' (AAB). Race 2 is compatible with genotypes of the Bluggoe (ABB) group, some tetraploid breeding genotypes and *Ensete* (Waite and Stover 1960).

Table 1. Response of clones of banana germplasm and *Heliconia* to the hitherto identified races of *Fusarium oxysporum* f. sp. *cubense.* Adapted from: *(Orjeda 1998)*

Reference cultivar		Response to *Foc*			
Cultivar/accession	**ITC[1]code**	**Race 1**	**Race 2**	**Race 3**	**Race 4[2]**
Gros Michel (AAA)	ITC1122	S	R	R	S
Bluggoe[3] (ABB)	ITC0643	R	S	R	ND
Cavendish[4] (AAA)	ITC0365	R	R	R	S
Heliconia spp.	NA	R	R	S	ND

[1] International Transit Center, Bioversity International, [2]Race 4 is further divided into Tropical (TR4) and Subtropical Race 4. [3]Bluggoe group 'Cachaco'; [4]Cavendish group 'Williams' or 'Grand Naine', S: Susceptibility, R: Resistance, ND: Not defined/not clear

Waite (1963) identified *Fusarium oxysporum* strains that affected several *Heliconia* spp. in the Americas, which suggested the presence of Race 3. Unfortunately, the original material is not maintained and hence, cannot be verified. Due to the fact that Race 3 does not affect banana, it is no longer considered to be part of the *Fusarium* spp. affecting banana, but rather should be considered as *F. oxysporum* f. sp. *heliconiae* (Moore *et al.,* 2001, Ploetz 2006).

Before 1990, isolates classified as race 4 only caused serious yield losses in Cavendish genotypes in the subtropical areas of Australia, the Canary Islands and Taiwan, particularly during substantial abiotic stress. However, the hope that the fungus would not regularly affect Cavendish bananas was shattered during the early 1990s (Ploetz 2005b), when the incidence and severity of Panama disease increased independent of abiotic stress and resulted in unanticipated yield loss in commercial Cavendish plantations in Southeast Asia, particularly in Taiwan (Ploetz 2000a, 2005a, 2006). Eventually, the causal agent was identified as a new race that was called TR4, whereas the milder variant that caused disease in the subtropics after abiotic stress was called Subtropical Race 4 (ST4). TR4 is extremely virulent and pathogenic on banana germplasm in tropical and subtropical environments, including many local plantain and cooking banana varieties (Ordóñez *et al.,* 2015).

However, the contemporary race concept is debatable and ambiguous. Undoubtedly, the small germplasm set as well as varying field conditions are -generally speaking- inadequate to precisely classify and describe pathogenic variation and it does not reflect the genetic variation in *Fusarium* spp. affecting banana. For instance, Ploetz (2005b) mentions isolates originating from East Africa and Southeast Asia that affect 'Gros Michel' and Bluggoe, but not Cavendish, which suggest a much larger pathogenic diversity.

The quest for resistance in banana breeding

Since its appearance, Panama disease has caused severe damage in a range of important cultivars all over the world (Shivas and Philemon 1996, Ploetz and Pegg 2000, Molina *et al.,* 2008). There is no doubt that the aggressive TR4 continues to disseminate to other areas and hence, it causes grave concerns in countries that depend on the Cavendish based export trade or have it as a staple food or an important ingredient of their daily diet.

Panama disease is a polycyclic disease, which together with the perennial nature of the crop complicates the implementation of control options. Despite the fact that researchers

have investigated countless methods to manage Panama disease (Ploetz 2015), it remains very complicated, even 50 years after its re-emergence. The development of molecular diagnostics has contributed to rapid alerts and quarantine implementation since TR4 surfaced and disseminated (Dita *et al.,* 2010, Paul *et al.,* 2011, Yip *et al.,* 2011, Dita *et al.,* 2013, Li *et al.,* 2013, Aguayo *et al.,* 2017). However, quarantine and exclusion by biological, cultural (physical) and chemical means are still the primary paths for control (Lakshmanan *et al.,* 1987, Davis *et al.,* 1994, Saravanan *et al.,* 2003, Cao *et al.,* 2005, Nel *et al.,* 2007, Kidane and Laing 2008, Yuan *et al.,* 2012, Zhang *et al.,* 2013). Alternative control methods such as "vaccines" are frequently advocated but have never significantly cured banana plants or contributed to successful disease control (Thakker *et al.,* 2013). Thus, apart from diagnostics that alerted stakeholders and enabled the implementation of quarantine measures, none of the other methods has significantly contributed to disease management in the field. As neither fungicides nor other measures were sufficiently effective for sustainable production of susceptible varieties in infested soils, resistance is the cornerstone for an environmentally sound disease management strategy (Ploetz 2005b) as demonstrated by the global roll-out of Cavendish to quench the race 1 epidemic. However, it is complicated to generate new varieties due to the low fertility of cultivated bananas, which is, therefore, a challenge for breeders. Cross breeding by hand pollination sometimes leads to irregular seed set (Bakry *et al.,* 2009, Bakry and Horry 2014). New triploids are usually the main objective of breeders as these results in cultivars with outstanding agronomical performance and they can be generated through "reconstructive breeding" by intercrossing diploids and diploid derived tetraploids.

Resistant hybrids have been developed in various breeding programs via pedigree breeding, but the commercial value of these new varieties was limited to a few niche markets, such as Goldfinger (FHIA01) in Australia and a few other FHIA hybrids in Latin America and the Caribbean (FHIA25, FHIA17, FHIA21, FHIA 20, FHIA01). In addition, somaclonal variation is being advocated as a way to generate varieties with improved resistance to Panama disease, but resistance levels are controversial and seem to be at most partial, which is not adequate for soil-borne diseases (Dale *et al.,* 2017a, Dale *et al.,* 2017b). Of course, there is abundant genetic variation for resistance available in indigenous and wild germplasm. For instance, the sequencing of the DH Pahang (*M. acuminata* ssp. *malaccensis* (AA)) and accompanying phenotyping revealed its resistance to TR4. These developments undoubtedly contribute to new insights and opportunities for developing new and resistant commercial banana varieties to eventually manage the new Panama disease epidemic (d'Hont *et al.,* 2012).

Selection of promising materials by breeders in almost all crops is currently accomplished in field and greenhouse trials. However, for banana selection programs such trials are only possible in TR4 infested areas (Hwang and Ko 2004). In practice, however, this is expensive, time-consuming and usually unreliable due to high variation of environmental conditions as well as unequal inoculum distribution (Mert and Karakaya 2003, Subramaniam *et al.,* 2006, Sutanto *et al.,* 2013). Greenhouse trials, on the other hand, were satisfactory and reproducible (Amorim *et al.,* 2009, Dita *et al.,* 2011, Li *et al.,* 2015, Zuo *et al.,* 2018), but to date, there is no consensus for banana phenotyping for TR4 or other races. Hence, several methods are used, including *in-vitro* assays, complex hydroponic systems as well as pot systems (Sun and Su 1984, Smith *et al.,* 1999, Mohamed *et al.,* 2001, Subramaniam *et al.,* 2006, Smith *et al.,* 2008, Wu *et al.,* 2010, Dita

et al., 2011). All these methods differ in terms of plant age, pre-treatment of plants, type of inoculum or substrate. This complicates data generation and comparison and thus efficiency and reliability, which hampers progress in the evaluation of germplasm, which is urgently required as an essential building block for banana improvement.

Scope of the thesis

The overall aim of this study is to address and investigate genetic diversity in banana for resistance to Panama disease. This required the development of several methods to efficiently collect and analyse the results of host – pathogen trials. These were then also used to monitor and validate the present dissemination of TR4 in an international context. Finally, the thesis provides a first insight into the interactions between the genetic diversity of *Fusarium* spp., causing Panama disease in banana. Taken together, the thesis presents a broad view on the perspectives for the genetic improvement of banana.

Chapter 1 provides a general introduction and addresses the history, biology and importance of banana as well as its role as a staple. Furthermore, the chapter provides an overview of the current problems in global banana production, with an emphasis on Panama disease and how this is perceived and dealt with by the various stakeholders. Finally, an outline of the objectives and the contents of the thesis is described.

Chapter 2 compiles and integrates different reports describing the dissemination of TR4, including the first outbreak outside Southeast Asia and the subsequent dissemination to the Middle East, the Indian subcontinent, Africa and the greater Mekong area.

Chapter 3 describes the development of a high-throughput method for Panama disease phenotyping in banana with potential application to other pathosystems. This chapter boils down to outlining an adequate protocol for studying banana-Panama disease interactions comprising a step-by-step process of inoculum production, inoculation methods, disease severity evaluation and data analysis.

Chapter 4 reports the phenotyping of a panel of traditional and commercial banana cultivars, including dessert and cooking types, with TR4 and race 1 strains. The results show the great genetic diversity for resistance to both strains of which resistance to TR4 is of particular interest in view of developing resistant bananas.

Chapter 5 is an overview of the resistance to Panama disease TR4 and race 1 in wild and edible banana diploids and their relatives. This germplasm has contributed to contemporary edible bananas varieties and breeding lines that dominate global banana production. The data are important for the design of new breeding programs aiming for a new and diverse portfolio of commercial banana varieties that meet market demands.

Chapter 6 describes an investigation of pathogenicity of the widest possible panel of *Fusarium* spp. towards the two banana varieties that dominated commercial banana production during the last 100 years. Both 'Grand Naine' and 'Gros Michel' are affected by multiple but different *Fusarium* species. This shows that the classical race concept is out-dated and needs a thorough revision.

Chapter 7 addresses an aspect of "probing" by investigating the effect of a pre-treatment with avirulent or non-pathogenic *Fusarium* species on the compatible interaction between TR4 and 'Grand Naine' bananas. Disease severities of plants probed with race 1 are significantly reduced and RNAseq data unveil which genes are involved in this process.

Chapter 8 is on the development of the first transgenic banana with resistance to TR4 by cloning a resistance gene from the wild diploid *M. acuminata* ssp. *malaccensis.* The gene was placed in the Cavendish 'Grand Naine' by *Agrobacterium tumefaciens* mediated transformation and the resulting plants were trialled for three years in a TR4 infested field in Lambells Lagoon, Northern Territory, Australia. While all controls were killed over time, the transgenic bananas remained healthy during the entire experiment. All laboratory trials confirmed the presence of TR4 in the affected plants, but no isolates were recovered from the transgenic plants. This demonstrates that transgenic bananas with resistance to TR4 can be used as a control strategy for Panama disease.

Chapter 9 is the final general discussion of the entire thesis and places all results in a context for the future sustainability of global banana production and the required research to achieve that goal.

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CHAPTER 2

Panama Disease in Cavendish Bananas and Beyond: From Regional Problem Towards a Global Pandemic

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Abstract

Bananas represent fruits that can be eaten fresh, but they are also cooked, fried or even brewed and are among the world's most important commodities and profitable crops. They are a staple food and source of income for nearly 400 million people in developing economies. The global trade revolves around a set of Cavendish clones with limited genetic diversity, which results essentially in a global monoculture that is a vehicle for the dissemination of pathogens to local and equally susceptible cultivars. Moreover, a number of domestic markets, such as those in China and India also largely depend on Cavendish bananas. Currently, global banana production is increasingly threatened by Panama disease or Fusarium wilt, which is caused by a suite of *Fusarium* species. Since its first description in 1870's, Panama disease has immensely affected yield production and developed into a major epidemic in the middle of the 20th century, which decimated the then popular 'Gros Michel' banana leading to staggering costs and societal impact. This left the industry with an engraved milestone of the "pre" and "post" Panama disease era, particularly since Cavendish bananas are resistant to the strains that caused this epidemic and hence, their expanded cultivation quenched it. Now, *Fusarium odoratissimum*, commonly named Tropical Race 4 (TR4), kills Cavendish and history repeats itself. To date, this pathogen has already spread across Southeast Asia, to the Middle East and the Indian subcontinent and even expanded into Africa and more recentlyl, the United Kingdom. In this chapter I merged the latest reports on the dissemination of TR4 and describe a combination of classical morphology and molecular techniques to diagnose new isolates as well as pathogenicity tests on Cavendish bananas under greenhouse conditions to fulfil Koch's postulates. This enabled us to alert on the presence of TR4 in new areas and additional DNA analyses underscored the likely origin of these incursions.

First Report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 associated with Panama Disease of banana outside Southeast Asia

Fusarium wilt or Panama disease of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is among the most destructive plant diseases (Ploetz 2006). Race 1 ravaged 'Gros Michel'-based export trades until the cultivar was replaced by resistant Cavendish cultivars. However, a new variant of *Foc*, tropical race 4 (TR4), was identified in Southeast Asia in 1992 and has spread throughout the region (Ploetz 2006). Cavendish clones, which are most important in subsistence and export production, are among the wide range of cultivars that are affected, and there is a huge concern that TR4 will move into Africa and Latin America, thereby threatening other vital banana-growing regions. In Jordan, Cavendish bananas are produced on 1,000-1,500 ha. in the Jordan Valley (ca. 32°N,35.5°E). In 2006, symptoms of Panama disease were observed and sampled for the isolation of *Foc.* On half-strength PDA amended with 100-ppm streptomycin sulfate, pale salmon-colored colonies with floccose mycelia developed consistently from surface-disinfested xylem. Single microconidia from these colonies were transferred to half-strength PDA, and conidia and mycelia from these monospore colonies were stored at -80°C in 15% glycerol. On banana leaf agar (Co $_{60}$-irradiated leaf tissue on water agar), isolates resembled *F. oxysporum* phenotypically by producing infrequent three- to five-celled macroconidia, copious, usually aseptate microconida on monophialides, and terminal and intercalary chlamydospores after two weeks (Leslie *et al.,* 2006). With nitrate-nonutilizing (nit) mutants and testers for different vegetative compatibility groups (VCGs), each of seven examined monospore isolates were placed in VCG 01213, which contains only strains of TR4 (Ploetz 2006) (Figure 1 left). Total DNA was extracted from six isolates and PCR analyses, which confirmed their identity as TR4 (Dita *et al.,* 2010) (Figure 1, right). Subsequently, one of the isolates (JV11) was analyzed for pathogenicity.

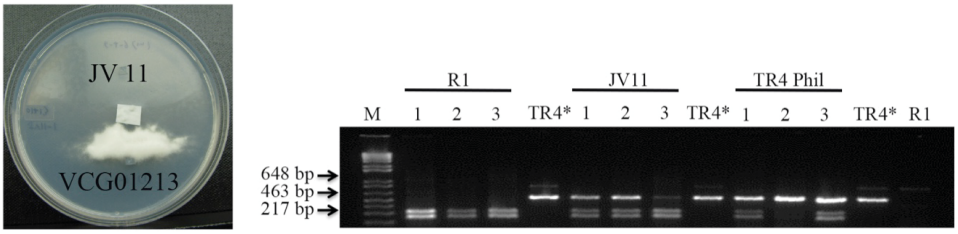

Figure 1. Identification of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) by multiplex PCR and Vegetative Compatibility Group (VCG) analysis. Left: identification of VCG01213 of *Foc* isolate JV11, isolated from infected Jordanian Cavendish banana plants. Right: amplicons of multiplex PCR on DNA from infected 'Grand Naine' plants tissues, four weeks after inoculation, using the elongation factor-1α (EF-1/EF-2) primer set as a internal control for amplifications, TR4 specific primers TR4 (*Foc* TR4-F/*Foc*TR4-R and the banana actin primers (*BanAct*-F/*BanAct*-r) as an internal control for banana DNA. Three panels each (Race 1, JV11 and TR4 from the Philippines) containing lanes 1-3 for infected roots, corm and pseudostem tissue from 'Grand Naine' plants, respectively. Panels are separated by positive controls of DNA from a pure culture of TR4* reference isolate II-5. Specific bands for TR4 (463 bp), elongation factor-1α (648 bp) and the banana actin gene (217 bp) are indicated on the left. M, molecular marker 1-kb DNA ladder plus.

Inoculum production and inoculation were according to (Dita *et al.,* 2010) by dipping (30 min.). Root-wounded 10-week-old plants of the Cavendish 'Grand Naine' in 2 L of spore suspension (1.0 x 10^6 spores/ml). Inoculated plants were then placed in sand in 3L pots

under 28°C, 70% relative humidity and a 16/8h light/darkness photoperiod. Sets of three plants were each treated with either JV11 or two TR4 controls (isolate II-5 and a strain isolated from an affected Cavendish plant in Mindanao, Philippines, both of which were diagnosed as TR4 by PCR and pathogenicity analyses. Control sets were either treated with race 1 (originating from Cruz das Almas, Bahia, Brazil see (Dita *et al.,* 2010), or water. After 2 weeks, plants inoculated with JV11 and TR4 controls produced typical symptoms of Fusarium wilt. After 4 weeks, tissue was collected from all plants and plated on Komada's medium. TR4 was directly confirmed by PCR (Dita *et al.,* 2010), either directly from symptomatic plants (JV11 and TR4 controls), or from isolates that were recovered from these plants (Figure 2). Nothing was reisolated from race 1 inoculated plants and water controls, which remained asymptomatic. This is the first report of TR4 affecting Cavendish outside Southeast Asia, is its northernmost outbreak, and represents a dangerous expansion of this destructive race. Currently, 80% of the Jordan Valley production area is affected by Fusarium wilt, and 20-80% of the plants are affected in different farms.

Figure 2. Biological confirmation of pathogenicity of *Fusarium oxysporum* f. sp. *cubense* collected from diseased 'Grand Naine' Cavendish banana plants in Jordan. A, inoculation of 'Grand Naine', superior, after inoculation; inferior, four weeks after inoculation with negative and positive controls as follow; A1: water, A2: race 1, A3: pure culture of JV11, A4: TR4 from Philippines A5: TR4 strain II-5. B, a close-up of *Foc* symptoms on 'Grand Naine' after inoculation with JV11. C, longitudinal-section of 'Grand Naine' corm four weeks after inoculation with JV11. D, a close-up of the infected veins in the pseudostem. E, Growth of *Foc* on Komada medium after re-isolation from 'Grand Naine' plants infected with *Foc* isolate JV11.

First report of *Fusarium oxysporum* f. sp*. cubense* tropical race 4 causing Panama disease in Cavendish bananas in Pakistan and Lebanon.

Panama disease of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), poses a great risk to global banana production. Tropical race 4 (TR4), which affects Cavendish bananas as well as many other banana cultivars (Ploetz 2006), was confirmed for the first time outside Southeast Asia in Jordan in 2013 (García-Bastidas *et al.,* 2014). In Pakistan, bananas are produced in the Sindh and Balochistan provinces (91% [31,000 ha] and 9% of the country's production, respectively). Symptoms of Fusarium wilt, including wilting of leaves and vascular discoloration in rhizomes and pseudostems, were first observed in 2012 in a 2-ha Cavendish plantation in Baoo Pooran (ca. 24°N, 68°E), Sindh province. By January 2014, approximately 121 ha were affected. In Lebanon, bananas are produced for local consumption and regional export, especially to Syria. Yellowing of leaves and internal vascular discoloration in the pseudostems were first observed in Cavendish plants in October 2013 in the Mansouri and Berghliyeh regions. Thus far, 1 ha has been affected. Infected, pseudostem tissue samples from Pakistan and Lebanon were processed for *Foc* isolation and characterization as described by García-Bastidas *et al. (*2014).

White colonies developed from the surface sterilized (70% ethanol) tissue on Komada's medium (Leslie *et al.,* 2006) and nine single microconidia isolates were generated, four from the Pakistan sample and five from the Lebanon samples and transferred to quarter-strength PDA. All isolates phenotypically resembled *F. oxysporum* (Leslie *et al.,* 2006) and were diagnosed as vegetative compatibility group (VCG) 01213 (Figure 3), which was confirmed by PCR (Figure 4), thereby corroborating that VCG01213 only represents TR4 strains (Ploetz 2006).

Subsequently, one of the isolates from Pakistan (Pak1.1A) and one isolate each from Mansouri (Leb1.1A) and Berghliyeh (Leb1.2C) in Lebanon were analyzed for pathogenicity. Inoculum production and inoculation were according to Dita *et al. (*2010) by dipping (30 min, 10^6 spores/ml) root-wounded 10-week-old Cavendish 'Grand Naine' plants, which were then placed in sand in 3L pots under 28°C, 70% relative humidity and a 16h diurnal light periods for 6 weeks. Sets of three plants were each treated with either Pak1.1A, Leb1.1A, Leb1.2C or TR4 (reference isolate II-5, which was diagnosed as TR4 by PCR and pathogenicity analyses, see (Dita *et al.,* 2010). Control sets were each treated with either *Foc* race 1 (Cruz das Almas, Brazil, (Dita *et al.,* 2010), or water. After four weeks, all plants inoculated with the isolates from Pakistan, Lebanon and TR4 (II-5) produced typical symptoms of Fusarium wilt.

After six weeks, internal symptoms were recorded (Figure 3) and tissue was collected from all plants and plated on Komada's medium. TR4 was confirmed by PCR from isolates that were recovered from all symptomatic plants. No isolates were recovered from plants infected with race 1 or the water controls, all of which remained asymptomatic.

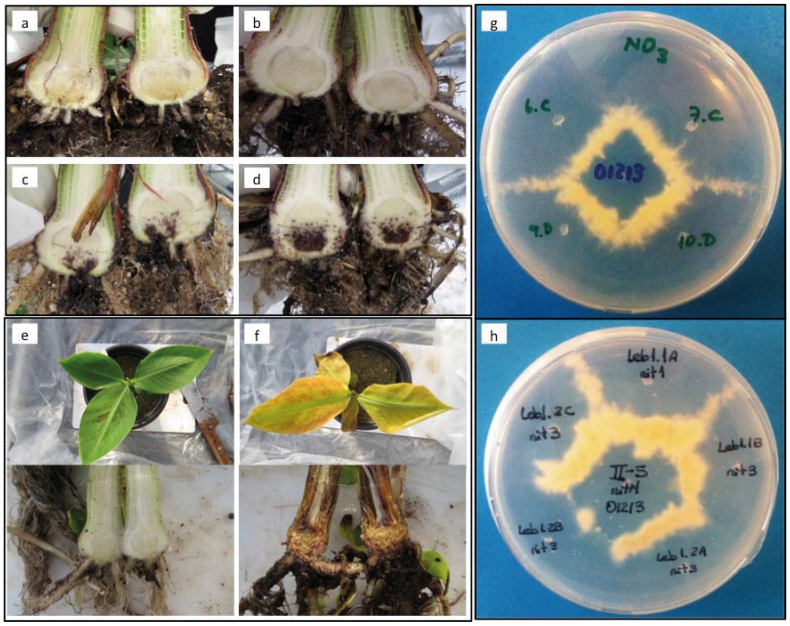

Figure 3: Biological confirmation of pathogenicity of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) isolated from tissues collected from Cavendish banana in Pakistan and Lebanon: (a) water control, (b) Race1 (originated from Cruz das Almas, Brazil), (c) Pakistan strain Pak1.1A, (d) TR4 (reference isolate II5/VCG 01213), (e) general appearance (upper panel) and longitudinal section of the rhizome (lower panel) of 'Grand Naine' Cavendish plants 6 weeks after (e) water control treatment, (f) inoculation with the Leb1.1A strain from Lebanon, and VCG tests with isolates from (g) Pakistan and (h) Lebanon confirming their identity as VCG 01213. Greenhouse tests for Pakistan and Lebanon were performed separately as samples were not received at the same time. Inoculations with additional *Foc* strains from Pakistan and Lebanon are not shown but caused similar symptoms as in panel (f).

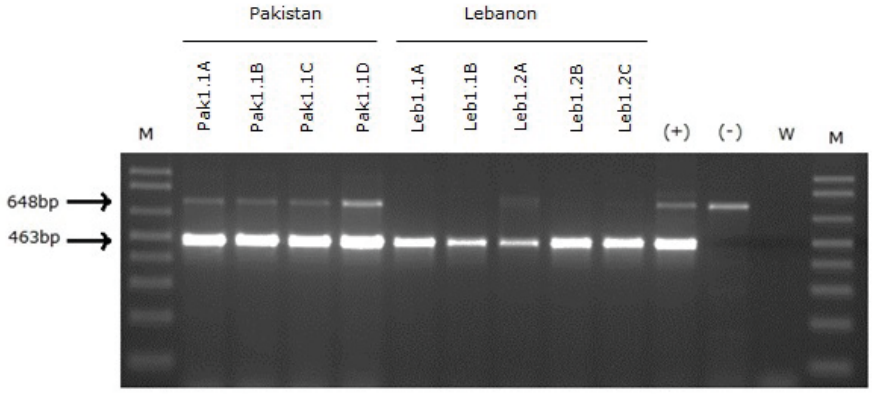

Figure 4. Identification of isolates of *Fusarium oxysporum* f. sp. *cubense* from Pakistan and Lebanon as Tropical Race 4 (TR4) with the PCR diagnostic of Dita *et al*. (2010). Specific DNA bands for *Foc*TR4 (463 bp) and elongation factor-1α (648 bp) are indicated on the left. (M) molecular marker 1-kb DNA ladder plus; (+) positive control TR4-II5; (-) negative control race 1; and (W) water.

Thus, we confirm the presence of TR4 in Pakistan and Lebanon and its continued expansion and distribution in Western Asia. Although comparatively limited production areas have been affected to date, increasing damage will undoubtedly occur in these countries in the near future.

New geographical insights of the latest expansion of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 into the Greater Mekong Subregion

Abstract

Banana is the most popular and most exported fruit and also a major food crop for millions of people around the world. Despite its importance, research into this crop is limited and hence, it suffers from major disease threats. One of those is Panama disease or Fusarium wilt. In the previous century Fusarium wilt wiped out the 'Gros Michel' based banana industry in Central America. Planting Cavendish bananas eventually quenched the epidemic. However, 50 years ago the disease recurred, but now on Cavendish bananas. Since then the disease has spread across Southeast Asia, to the Middle East and the Indian subcontinent and leaped into Africa. Here, we report the presence of *Fusarium* spp. Tropical Race 4 (*TR4*) in Cavendish plantations in Laos, Myanmar and Vietnam. A combination of classical morphology, DNA sequencing and phenotyping assays revealed a very close relationship between the TR4 strains in the entire Greater Mekong Subregion (GMS), which is increasingly prone to intensive banana production. Analyses of single-nucleotide polymorphisms enabled us to initiate a phylogeography of TR4 across three geographical areas - GMS, Indian subcontinent and the Middle East revealing three distinct TR4 sub-lineages. Collectively, our data place these new incursions in a broader agroecological context and underscore the need for awareness campaigns and the implementation of validated quarantine measures to prevent further international dissemination of TR4.

Keywords: Laos, Myanmar, Vietnam, China, Fusarium wilt, single nucleotide polymorphism (SNP), phytogeography, The Greater Mekong Subregion (GMS)

Introduction

Panama disease or Fusarium wilt is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and was first described in Australia in 1874 (Bancroft 1876). The fungus penetrates the roots and from there colonizes the vascular system of the banana plant. Together with the plant responses, this results in occlusion of the xylem vessels which causes wilting and eventually death of infected plants (Guo *et al.,* 2014). The decimation of susceptible 'Gros Michel' bananas that were grown in large-scale monoculture plantations in Central America during the 1900s earned Fusarium wilt its reputation as a pathogen of global significance. Losses of 'Gros Michel' were first recognized in Central America (Costa Rica and Panama) in 1890, and were soon reported in Africa, the Caribbean and South America (Ploetz 2015). The Fusarium wilt epidemic was caused by a set of *Foc* strains that are collectively called race 1 and decimated the large-scale monocultures of 'Gros Michel' on which the banana industry in America relied. No effective control methods were found other than replacing 'Gros Michel' with resistant Cavendish bananas in Central America during the 1960s. This replacement has been highly successful to quench the Fusarium wilt epidemic that was caused by *Foc* race 1 strains. Since then, Cavendish production expanded into large global monocultures, which are evidently prone to disease threats, including black Sigatoka and Panama disease (Ordóñez *et al.,* 2015b, Arango *et al.,* 2016, Diaz-Trujillo *et al.,* 2017). However, this has not resulted in global research efforts to neutralize these problems. Therefore, another genetic lineage of *Foc* (vegetative compatibility group (VCG) 01213), colloquially called Tropical Race 4 (TR4), which originates from Indonesia and affects many banana cultivars, including those belonging to the Cavendish group, has now developed into a global threat (Ordóñez *et al.,* 2015b). It has spread to five Asian Cavendish -producing countries and Australia, and was recently also discovered in the Middle East, the Indian subcontinent and Africa (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a, Ploetz *et al.,* 2015, Promusa 2016). Therefore, the global banana industry is under serious threat by this soil-borne fungal disease (Ploetz and Churchill 2009, Pocasangre *et al.,* 2009, Shabani *et al.,* 2014, Ordóñez *et al.,* 2015b) and its recent rapid spread has raised international concerns with regard to future food security in the tropics and sustainability of the international banana trade that is nearly exclusively based on Cavendish clones (d'Hont *et al.,* 2012, FAO 2014).

Currently, Cavendish clones comprise 15% of the global banana production but they are increasingly gaining importance for domestic markets. Presently, they occupy approximately 40% (Ploetz 2015) of the total global area. Clearly, this comes with a huge risk for a pandemic, as these clones are susceptible to TR4. The vegetative propagation of planting material and a lack of diversification efforts over the last century have increased the genetic vulnerability of the crop to unacceptable levels, which threatens food security. This urges for international, regional and local measures aimed at prevention and management of this destructive disease (Ploetz 2015).

The biological complexity of soil-borne diseases - with surviving propagules that remain viable for decades - and taking into account the historical track-record of *Foc* (Li *et al.,* 2013), demonstrates that disease management has proven to be difficult (Ploetz 2015). Hence, prevention is currently the major strategy to avoid new TR4 incursions. In 1967, TR4 surfaced in Taiwan, supposedly after introduction of infected plants from Sumatera, Indonesia (Su *et al.,* 1986, Hwang and Ko 2004). From there, it has disseminated likely

into the Chinese province of Fujian, and then gradually to Guangdong, Guangxi, Hainan and finally in 2009 to Yunnan (Sun 1978, Su *et al.,* 1986, Hwang and Ko 2004, Buddenhagen 2007, Qi *et al.,* 2008, Li *et al.,* 2013) The expansion of TR4 was facilitated by new large scale 'Cavendish' production practices across this area along with limited awareness and lacking quarantine measures. The cultivation of Cavendish now shifts to Laos, Myanmar, Vietnam and other countries in the Greater Mekong Subregion because of limited suitable land for banana production to meet the increasing market demand. During survey in Vietnam, Laos and Myanmar in March and May 2016 we observed the presence of Fusarium wilt in Cavendish plantations. Here, we provide details on the regional and international expansion of TR4, which is worrisome as it threatens both food security and the international trade (Ordóñez *et al.,* 2015b, Ploetz 2015, Mostert *et al.,* 2017a)

Materials and methods

Sample collection.

To investigate the presence of TR4 we sampled commercial Cavendish plantations in Laos, Myanmar, Vietnam and Yunnan during March and May 2016 (Table 1, Figure 5). Samples from Guangxi and Guangdong were collected during 2011-2014. Banana plants affected by Fusarium wilt which showed chlorosis older leaves or a skirt of dead leaves around the pseudostem were internally sampled. Discoloured vascular strands were collected from five plants at each location. Samples were wrapped in paper bags and maintained in a cool box until arrival in the laboratory.

Table 1. *Fusarium oxysporum* f. sp. *cubense* sampling code from Laos, Myanmar, Vietnam and the Chinese provinces Yunnan, Guangxi and Guangdong.

Sampling date	Site	Code	Cultivar	Location	Altitude (m)
2016-05-11	Laos	La-1	Brazilian	21°25′32 ″ N 101°11′2 ″ E	580
2016-05-11	Laos	La-2	Brazilian	21°25′33 ″ N 101°11′2 ″ E	600
2016-05-11	Laos	La-3	Brazilian	21°25′33 ″ N 101°11′2 ″ E	590
2016-05-11	Laos	La-4	Brazilian	21°25′33 ″ N 101°11′3 ″ E	590
2016-05-11	Laos	La-5	Brazilian	21°25′34 ″ N 101°11′2 ″ E	590
2016-05-10	Myanmar	My-1	Brazilian	21°24′ 4 ″ N 100°23′4 ″ E	500
2016-05-10	Myanmar	My-2	Brazilian	21°24′ 3 ″ N 100°23′6 ″ E	490
2016-05-10	Myanmar	My-3	Brazilian	21°24′ 3 ″ N 100°23′6 ″ E	490
2016-05-10	Myanmar	My-4	Brazilian	21°24′ 5 ″ N 100°23′4 ″ E	510
2016-05-10	Myanmar	My-5	Brazilian	21°24′ 6 ″ N 100°23′4 ″ E	490
2016-03-17	Vietnam	VN-1	Guijiao No 6	22°30′42 ″ N 104°2′31 ″ E	102
2016-03-17	Vietnam	VN-2	Guijiao No 6	22°30′41 ″ N 104°2′31 ″ E	104
2016-03-17	Vietnam	VN-3	Guijiao No 6	22°30′39 ″ N 104°2′32 ″ E	108
2016-03-17	Vietnam	VN-4	Guijiao No 6	22°30′39 ″ N 104°2′32 ″ E	90

Sampling date	Site	Code	Cultivar	Location	Altitude (m)
2016-03-23	Yunnan	YN-1	Nantianhuang	21°51′52 ″ N 100°56′17 ″ E	540
2016-03-23	Yunnan	YN-2	Nantianhuang	21°51′43 ″ N 100°56′13 ″ E	530
2016-03-23	Yunnan	YN-3	Nantianhuang	21°51′43 ″ N 100°56′13 ″ E	530
2016-03-23	Yunnan	YN-4	Nantianhuang	21°51′51 ″ N 100°56′23 ″ E	540
2016-03-23	Yunnan	YN-5	Nantianhuang	21°51′51 ″ N 100°56′23 ″ E	540
2012-02-16	Mengpeng, Yunnan	No. 3	Guijiao No 6	2l°30′32 ″ N 101°20′21 ″ E	550
2011-01-19	Puweng, Yunnan	No. 5	Brazilian	22°33′ 38 ″ N 101°23′37 ″ E	772
2013-11-24	Mengding, Yunnan	No. 6	Brazilian	23°28′34 ″ N 99°01′26 ″ E	450
2014-11-09	Wuming, Guangxi	No. 16	Guanfen No. 1	23°16′37" N 108°05'3" E	150
2015-08-07	Pubei, Guangxi	No. 33	Xigong	22°13'36" N 109°19'37" E	56
2015-08-07	Lingshan, Guangxi	No. 34	Xigong	22°09'59" N 109°13'40" E	112

Figure 5. Banana plants with Fusarium wilt symptoms in sampled 'Cavendish' plantations in Laos (A), Myanmar (B), Vietnam (C) and Yunnan (D).

Strain isolation and characterization.

The collected samples were processed for *Foc* isolation and characterization as described earlier (Dita *et al.,* 2010, García-Bastidas *et al.,* 2014). Half of the dried vascular strands

were placed on Komada medium (Leslie *et al.,* 2006) and the remaining part was used for DNA extraction to verify the presence of TR4 (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a). Once purified, single spore cultures were obtained, total DNA was isolated with the Wizard® Magnetic DNA Purification System for Food kit (Promega, Madison, USA) - following the manufacturer's instructions - for multiplex PCR analyses using diagnostic primers for TR4 as well as for elongation factor-1α internal controls (Dita et al., 2010); Amplicons were visualized on agarose gels (1.2%, Roche, Mannheim, Germany) using an UV illuminator (Herolab, Wiesloch, Germany). Subsequently, one positive TR4 strain for each country was phenotyped under greenhouse conditions (Unifarm, Wageningen, The Netherlands) following earlier reported protocols (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a) For each strain we used six highly susceptible 'Grand Naine' plants (biological replicates) for each isolate that were placed randomly in the greenhouse, along with the appropriate controls (negative: water and race 1 from Cruz das Almas, Brazil, positive: TR4 reference isolate II5/VCG01213). The inoculated plants and the controls were monitored weekly and final external and internal scoring was conducted seven weeks after inoculation by a team of three experimentators according to previously reported protocols (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a). Corm tissue of each plant was collected and plated on Komada medium for fungal isolation and subsequent PCR confirmation of TR4 as causal agent.

Sequence analyses of TR4 strains.

To determine the identity of the strains and their relationship with other strains, one TR4 strain from each country was arbitrarily selected for whole-genome sequencing at the Beijing Genome Institute (Hong Kong, China), using Illumina technology, yielding ~20 million cleaned reads (150 nt). To establish the phylogenetic relationship between the publically available *Fusarium oxysporum* f. sp. *lycopersici* isolate *Fol* 4287 (Ma *et al.,* 2010) and a range of *Foc* isolates (Table 2) we utilized the reference sequence alignment-based phylogeny builder (REALPHY; v. 1.11) (Bertels *et al.,* 2014). As previously described (Woudenberg *et al.,* 2015) for *Alternaria* genomes, Illumina generated short reads and sequence fragments (100 nt) derived from the previously assembled genomes (Fol4286 and TR4 II5) were mapped against the TR4 II5 reference genome using Bowtie2, followed by the extraction of high quality (default settings) polymorphic and non-polymorphic sites conserved in all analyzed isolates. The final pseudo-molecule was used to infer a maximum-likelihood phylogeny using PhyML with the generalized time reversible (GTR) nucleotide substitution model, and the robustness of the phylogeny was assessed using 500 bootstrap replicates.

Table 2. *Fusarium oxysporum* f. sp. *cubense* strains used for phylogenetic analysis.

***Fusarium* spp. isolate**	**Pathogenicity code**	**Origin**	**Source**
II-5	TR4	Indonesia	Dita *et al.,* 2010
NRRL36102	Race 1	Brazil	Dita *et al.,* 2010
B2	TR4	China	Guo *et al.,* 2014
Pak1.1A	TR4	Pakistan	Ordoñez *et al.*, 2015a
Phi2.6C	TR4	Philippines	Ordoñez *et al.*, 2015a
Leb1.2C	TR4	Lebanon	Ordoñez *et al.,* 2015a
JV11	TR4	Jordan	Garcia-Bastidas *et al.,* 2014
My-1	TR4	Myanmar	This work
La-2	TR4	Laos	This work

Fusarium spp. isolate	Pathogenicity code	Origin	Source
VN-2	TR4	Vietnam	This work

Single-nucleotide polymorphisms (SNPs) were identified using GATK v3.3.0 (DePristo *et al.,* 2011) by mapping short reads against the TR4 II5 reference using BWA-mem, and duplicate reads were marked using Picard tools. Genomic variants were identified using GATK HaploTypeCaller, and a joint variant call set was generated using GATK GenotypeGVCFs. Subsequently, SNP variants were selected and filtered to retain high quality SNPs. These were used to determine the relationship between TR4 isolates using a principle component analyses (PCA; R, adegenet package) and hierarchical clustering (UPGMA; R).

Results

Observation and sampling of Fusarium wilt in the Greater Mekong Subregion.

In Laos and Myanmar, the predominant banana variety encountered in the plantations was the Cavendish cultivar 'Brazilian', while in the northern part of Vietnam Cavendish selection 'Guijiao No. 6' was grown. Samples from Yunnan were collected in the Honghe and Xishuangbana prefectures in 2016 from the Cavendish cultivars 'Nantianhuang', 'Brazilian' or 'Guijiao No. 6'. Fusarium wilt was observed in all plantations (Figure 5). In total 19 samples were collected; five samples from cultivar 'Brazilian' in Laos and Myanmar, four samples from cultivar 'Guijiao No 6' in Vietnam and five samples from cultivar 'Nantianhuang' in Yunnan (Figure 5 and Table 1). Analyses of the samples resulted in 16 isolates of which 13 were identified as TR4 by diagnostic (463 bp) PCR reactions. The negative samples were positive for elongation factor-1α PCR (648 bp) and hence the DNA was present and of adequate quality. Positive controls yielded the diagnostic 463 bp PCR product and the negative controls did not show any DNA amplification (Figure 6).

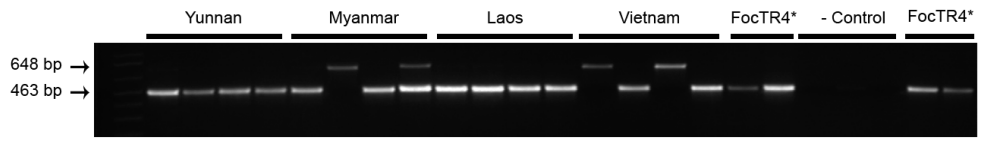

Figure 6. Identification of *Fusarium oxysporum* f. sp. *cubense* from samples derived from Yunnan, Myanmar, Laos and Vietnam as Tropical Race 4 (*TR4*) by PCR. Specific DNA bands for *TR4* (463 bp) and elongation factor-1α (648 bp) are indicated on the left. TR4-II5 was taken as positive control "TR4*"; The *Foc* Race 1 strain was used as negative control (- control) (Dita et al., 2010)

Phenotyping of TR4 isolates.

We selected one TR4 isolate from Vietnam, Yunnan, Myanmar and Laos for confirmatory phenotyping assays. Except the control plants inoculated with race 1 or untreated controls (water), all inoculated 'Grand Naine' plants showed typical external symptoms of Fusarium wilt starting from the fourth week after inoculation. The disease progressed steadily during incubation until plants were externally and internally scored for disease severity at seven weeks after inoculation. At that stage, all plants inoculated with TR4

diagnosed strains were completely decayed. The race 1 inoculated plants, however, were healthy and unaffected by *Foc*, similar as the water controls (Figure 7).

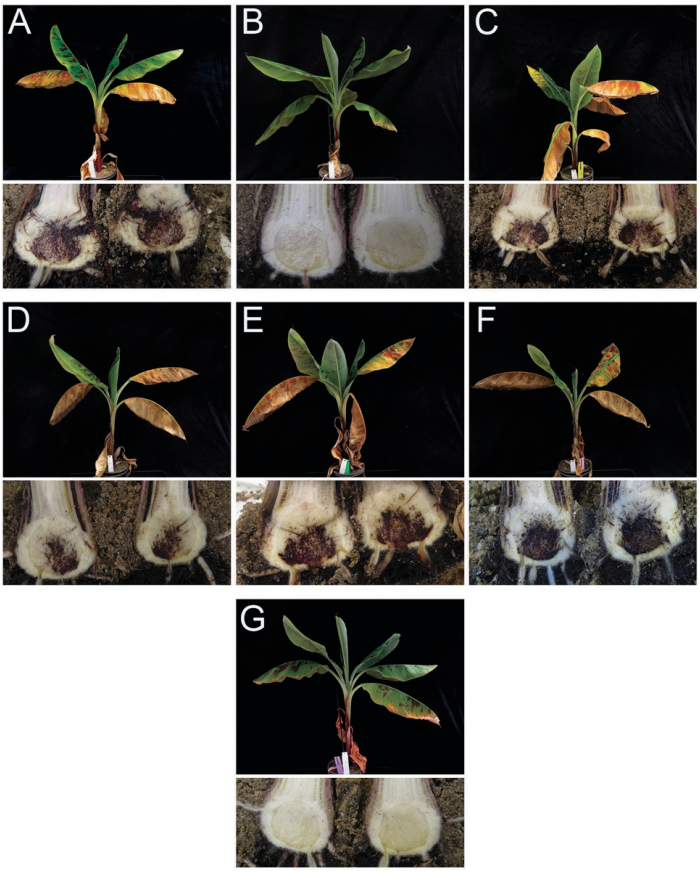

Figure 7. External foliar (top) and internal rhizome (bottom) symptoms of *Fusarium oxysporum* f. sp. *cubense* infection in 'Grand Naine' banana plants seven weeks after inoculation using isolates from Vietnam, Yunnan, Myanmar and Laos. A. TR4 (reference TR4, isolate II5 from Indonesia/VCG01213); B. race 1 (from Cruz das Almas, Brazil); C. *Foc* isolate from Xishuangbanna, Yunnan, China; D. *Foc* isolate from Myanmar; E. *Foc* isolate from Laos; F. *Foc* isolate from Vietnam and; G. Mock (water control).

All inoculated plants and controls were sampled for another round of verification. In contrast to the controls (water and race 1), all rhizomes from diseased plants enabled re-isolation of *Foc*. The resulting cultures showed typical *Foc* morphologies on Komada media (Figure 8) and subsequent TR4 diagnostic PCR tests were positive for all reisolated strains (not shown).

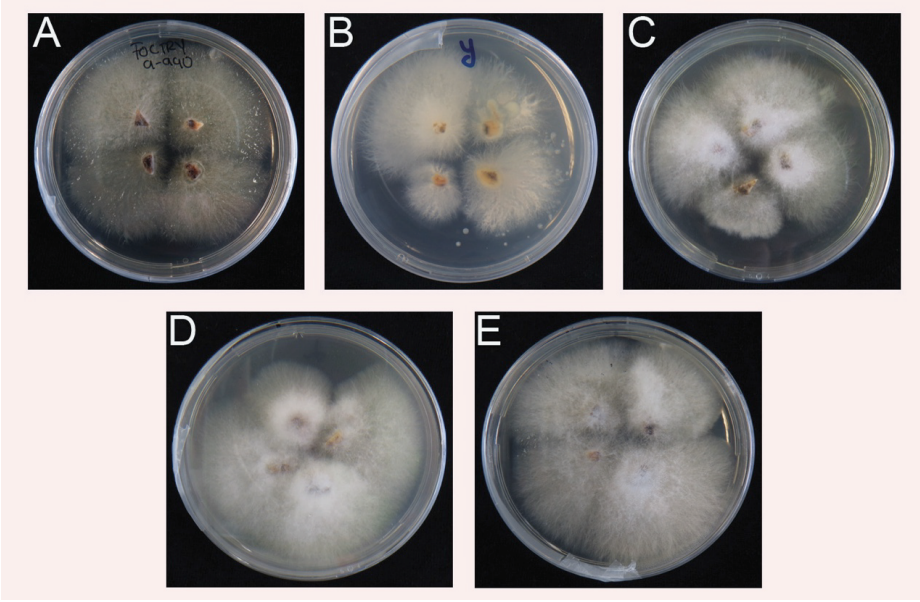

Figure 8. Re-isolation of *Fusarium oxysporum* f. sp. *cubense* TR4 from inoculated plants (see Figure 3). A-E from Indonesia, Yunnan, Myanmar, Laos and Vietnam, respectively. No positive isolates for TR4 were recovered from water and race 1 controls.

Sequence analysis of TR4 strains.

We used whole-genome sequencing of each representative TR4 strain for each GMS country and comparisons with the previously sequenced TR4 strains from recently reported incursions to study their genetic relatedness. The maximum-likelihood phylogeny of the genome sequences clearly confirmed that these strains belong to the TR4 genetic lineage (Ordóñez *et al.,* 2015b). Subsequent comparative analyses among the GMS TR4 strains and those from recent incursions in Jordan, Lebanon, and Pakistan as well as a Philippine TR4 strain revealed a total of 251 single nucleotide polymorphisms (SNPs) that were distributed across the genome (supplemental Figure 1, Supplemental Table 1). Subsequent principal component analyses (PCA; supplemental Figure 2) and hierarchical clustering revealed three geographically distinct groups of TR4 isolates, despite the overall limited amount of SNPs indicative of the clonality of TR4 strains. One group represents the GMS TR4 strains including the strain from Yunnan, China. A second group links the recent Pakistan incursion (Ordóñez *et al.,* 2015a) with the Philippine TR4 strain. The third group shows a strong similarity between the recent incursion of TR4 in Lebanon and Jordan (García-Bastidas *et al.,* 2014); some of the analyzed SNPs were indicative of inconsistencies based on the *Foc*-II5 reference genome. Therefore, we further filtered the SNPs more stringently, yielding a subset of 161 SNPs (Supplemental Table 1). Overall, the resulting PCA and the hierarchical cluster (Supplemental Figures 2 and 3) were nearly indistinguishable from the initial plots, thereby supporting the occurrence of three geographically distinct groups.

Discussion

This study provides the earliest collected records of TR4 in Vietnam and Laos and its first report in Myanmar. Recently, (Mostert *et al.,* 2017b) denied the presence of TR4 in Vietnam, Cambodia and Thailand based on samples that were collected a decade ago, but (Chittarath *et al.,* 2017, Hung *et al.,* 2017) confirmed it in Vietnam and Laos. Our genome analyses revealed a set of SNPs that we used to further analyse the genetic diversity of TR4 strains. Isolates from Vietnam, Laos and Myanmar are genetically closely related and resemble the TR4 strain from Yunnan. Furthermore, we demonstrate genetic association between the TR4 strains from Pakistan and the Philippines as well as between the strains from Lebanon and Jordan. Although TR4 is an asexually reproducing fungus which therefore shows a very strong linkage disequilibrium, clonal evolution does occur as evidenced by genetic clustering which enabled our biogeographical analysis (Tibayrenc and Ayala 2012). Recently, we demonstrated that the globally disseminating TR4 strain represents essentially a single clone (Ordóñez *et al.,* 2015b). Hence, it was difficult to unveil the origin of new incursions. However, we identified 251 high value SNPs that also after filtering elucidate basic associations between the here identified TR4 strains. Such analyses were recently also used to reveal the dissemination of the quarantine pathogen *Xylella fastodiosa* in olive trees (Loconsole *et al.,* 2014). Here, such a phylogeography approach provides initial evidence that TR4 in Laos, Vietnam and Myanmar was likely introduced from China. This supports the circumstantial evidence of ongoing TR4 epidemics on Cavendish plantations in these countries and adjacent Chinese provinces, which were developed by Chinese banana entrepreneurs. The SNP analyses also revealed that the TR4 strains from the Philippines and from Pakistan are closely related. Since TR4 was diagnosed in the Philippines in 2005 (Molina *et al.,* 2009) and is currently omnipresent in Mindanao, the recent incursion in Pakistan (Ordóñez *et al.,* 2015a) seems to originate from the Philippines. Similarly, the phylogeography data set indicates that the TR4 incursions in Lebanon and Jordan are associated (García-Bastidas *et al.,* 2014).

The introduction of large-scale Cavendish monocultures in the GMS resulted in displacement of local peoples, disputes on landownership and also resulted in a rapid decrease in forest area, which challenges ecological stability (Rerkasem *et al.,* 2009, Yoshida *et al.,* 2010, Friis and Nielsen 2016). We demonstrated that it also facilitated yet another expansion of TR4. The dissemination of TR4 in China upon the initial introduction from Taiwan is not well documented. Evidently, low awareness among banana growers and industry stakeholders has resulted in an almost unlimited movement of banana suckers, contaminated nursery soils, and farming equipment as well as the use of contaminated surface irrigation water. Our phylogeography approach indicates that these practices and the mobility of banana stakeholders may have contributed to the expansion of TR4 (Drenth and Guest 2016).

Similar to the previous Panama disease epidemic in 'Gros Michel' caused by *Foc* race 1 the lag phase of the current epidemic is substantial as the first occurrence of TR4 was observed 50 years ago in Taiwan (Su *et al.,* 1977, Su *et al.,* 1986, Hwang and Ko 2004). However, the track-record of Fusarium wilt epidemics in banana is unparalleled in botanical epidemiology (Ploetz 2015) and hence we should not underestimate the impact of the current TR4 pandemic on food and fruit production. Despite numerous efforts to alert and mobilize the banana sector for enhanced quarantine practices, we observe a continuous dissemination of TR4 (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a,

Ploetz 2015, Promusa 2016, Chittarath *et al.,* 2017, Hung *et al.,* 2017). High-resolution phylogeography may increase overall awareness and responsibility among banana stakeholders to prevent the further dissemination of TR4.

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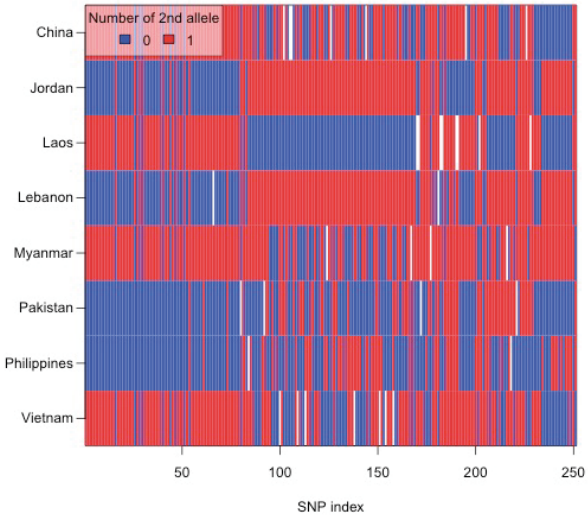
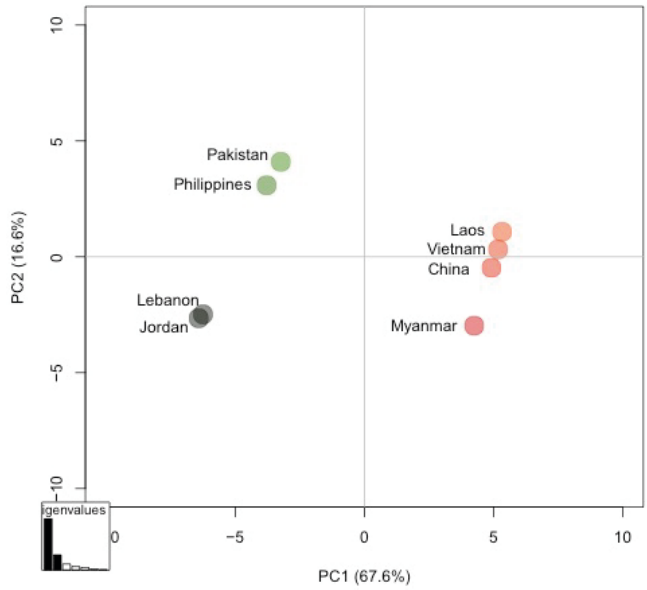
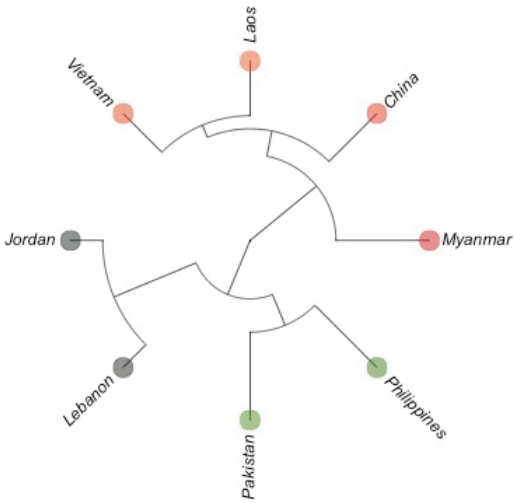


Supplemental Figure 1. Graphical representation of the distribution of 251 high quality SNPs over the genomes of the GMS TR4 strains.



Supplemental Figure 2. Principal component analysis plot based on 161 filtered high quality SNPs of TR4 isolates from Yunnan, Myanmar, Laos, Vietnam, Jordan, Lebanon, Pakistan, and Philippines.



Supplemental Figure 3. UPGMA tree based on 161 high quality SNPs over the genomes of the GMS TR4 strains.

Supplemental Table 1. Distribution of 251 high quality SNPs over the genomes of the GMS TR4 strains.

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.1	654887	G	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.1	1458700	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.1	1787865	C	T	FOIG_00628T4,FOIG_00628T1,FOIG_00628T2,FOIG_00628T3,FOIG_00628T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.1	2943895	A	G	FOIG_01024T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.1	3247785	C	T	FOIG_01141T3,FOIG_01141T2,FOIG_01141T1,FOIG_01141T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	27307	T	C	FOIG_01589T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	36211	C	A	FOIG_01593T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	430585	C	A	FOIG_01750T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	1264665	T	G	FOIG_02067T1,FOIG_02067T2,FOIG_02067T3,FOIG_02067T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	1352731	C	T	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.2	2122409	G	T	FOIG_02347T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.3	309536	T	C	FOIG_02508T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.3	371628	A	G	FOIG_02538T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.3	371629	G	T	FOIG_02538T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.3	1235003	T	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.4	513357	A	T	intergenic	Y	0	1	0	1	0	0	0	0
Supercontig_1.4	1121692	A	G	FOIG_03542T0,FOIG_03543T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.4	1875736	C	A	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.4	1884466	T	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.4	1978399	A	C	FOIG_03881T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.5	615490	G	A	FOIG_04148T2,FOIG_04148T0,FOIG_04148T3,FOIG_04148T1	Y	1	0	1	0	1	0	0	1

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.5	758313	C	T	FOIG_04200T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.5	850886	C	T	FOIG_04227T1,FOIG_04227T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.5	920213	C	T	FOIG_04254T0,FOIG_04254T1	Y	1	0	1	0	1	0	0	1
Supercontig_1.6	780057	C	T	FOIG_04908T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.6	1325607	A	G	FOIG_05103T3,FOIG_05103T0,FOIG_05103T1,FOIG_05103T4,FOIG_05103T2	Y	0	1	0	1	0	0	0	0
Supercontig_1.6	1514485	G	A	FOIG_05161T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.6	1692468	A	C	intergenic	Y	0	1	0	1	0	0	0	0
Supercontig_1.6	1702323	C	A	FOIG_05229T0,FOIG_05229T1	Y	1	0	1	0	1	0	0	1
Supercontig_1.7	1007173	C	G	intergenic	Y	0	1	0	1	0	0	0	0
Supercontig_1.8	94448	G	A	FOIG_05832T1,FOIG_05832T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.8	241008	T	C	FOIG_05880T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.8	1167657	T	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.8	1289418	T	C	FOIG_06248T1,FOIG_06248T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.8	1337175	C	A	FOIG_06270T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.9	1383195	C	A	FOIG_06887T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.10	1090138	G	A	FOIG_07284T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.11	742653	A	G	FOIG_07713T0,FOIG_07714T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.13	2588	A	G	FOIG_08364T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.13	1010783	G	A	FOIG_08801T0	Y	0	1	0	1	0	0	0	0
Supercontig_1.14	537513	A	G	FOIG_09012T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.15	347201	C	A	FOIG_09319T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.15	454571	C	T	FOIG_09372T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.16	515283	G	T	FOIG_09770T0	Y	0	1	0	1	0	0	0	0
Supercontig_1.16	782381	A	G	intergenic	Y	1	0	1	0	1	0	0	1

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.17	331553	C	A	FOIG_10058T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.18	12788	A	G	intergenic	Y	0	1	0	1	0	0	0	0
Supercontig_1.18	36789	G	A	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.18	362386	A	G	FOIG_10438T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.20	555994	A	G	FOIG_11109T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.21	143464	C	A	intergenic	Y	0	1	0	1	0	0	0	0
Supercontig_1.21	189518	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.22	186101	G	C	FOIG_11523T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.23	465787	A	T	intergenic	Y	1	1	1	1	1	1	1	0
Supercontig_1.24	443510	T	A	FOIG_12076T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.27	361683	A	G	FOIG_12607T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.28	343198	C	T	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.30	321518	T	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.32	6817	C	A	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.32	327846	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.32	371795	A	G	FOIG_13366T0	N	1	0	1	0	1	1	1	1
Supercontig_1.33	67285	C	T	FOIG_13391T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.33	258829	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.34	86818	C	A	FOIG_13549T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.34	299791	T	C	FOIG_13634T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.36	216436	C	G	FOIG_13836T0	Y	1	0	1	NA	1	0	0	1
Supercontig_1.38	2243	C	T	FOIG_14008T0,FOIG_14008T2,FOIG_14008T1,FOIG_14008T3	Y	1	0	1	0	1	0	0	1
Supercontig_1.38	45829	A	T	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.38	193989	G	C	FOIG_14105T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.39	227081	A	T	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.39	258411	G	A	FOIG_14277T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.40	232563	C	T	FOIG_14377T0	Y	1	0	1	0	1	0	0	1

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.40	250319	A	G	intergenic	N	1	0	1	1	1	1	1	1
Supercontig_1.40	262562	C	T	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.43	238722	C	T	FOIG_14722T0,FOIG_14722T1	Y	1	0	1	0	1	0	0	1
Supercontig_1.44	237782	A	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.46	35988	G	T	FOIG_14954T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.65	92080	G	A	FOIG_16094T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.66	59047	G	A	FOIG_16120T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.70	23683	G	A	intergenic	N	0	1	0	1	0	NA	0	0
Supercontig_1.70	24246	A	G	intergenic	N	1	1	1	0	1	1	1	1
Supercontig_1.70	26083	C	T	intergenic	N	1	1	0	1	1	0	1	1
Supercontig_1.70	32048	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.76	928	T	A	intergenic	N	1	1	0	1	1	0	NA	1
Supercontig_1.76	1155	A	G	intergenic	N	1	1	0	1	1	0	1	1
Supercontig_1.76	1191	T	A	intergenic	N	1	1	0	1	1	0	0	1
Supercontig_1.76	1364	G	A	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.76	1424	T	C	FOIG_16310T0	Y	1	1	0	1	1	0	0	0
Supercontig_1.76	1430	A	G	FOIG_16310T0	Y	0	1	0	1	1	0	0	0
Supercontig_1.76	1493	G	T	FOIG_16310T0	Y	1	1	0	1	1	0	0	0
Supercontig_1.76	1557	T	C	FOIG_16310T0	N	1	1	0	1	1	0	1	1
Supercontig_1.76	1595	C	T	FOIG_16310T0	N	1	1	0	1	1	NA	1	1
Supercontig_1.76	1654	C	T	FOIG_16310T0	N	0	1	0	1	1	1	0	1
Supercontig_1.76	1656	G	A	FOIG_16310T0	N	0	1	0	1	1	1	0	1
Supercontig_1.76	1766	T	C	FOIG_16310T0	Y	0	1	0	1	0	0	0	1
Supercontig_1.76	2063	G	A	intergenic	Y	0	1	0	1	0	1	1	0
Supercontig_1.76	2133	C	T	intergenic	Y	1	1	0	1	0	0	0	0
Supercontig_1.76	2152	T	G	intergenic	Y	1	1	0	1	0	0	1	0
Supercontig_1.76	2326	G	T	intergenic	Y	0	1	0	1	0	0	0	0

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.76	2448	T	G	intergenic	N	1	1	0	1	1	1	0	NA
Supercontig_1.76	2483	A	G	intergenic	N	1	1	0	1	1	1	0	1
Supercontig_1.76	2838	T	A	intergenic	Y	NA	1	0	1	0	1	0	0
Supercontig_1.76	3208	T	C	intergenic	N	1	1	0	1	1	1	1	0
Supercontig_1.76	3364	T	C	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.76	3956	T	C	intergenic	Y	NA	1	0	1	0	0	0	0
Supercontig_1.76	3965	G	T	intergenic	Y	NA	1	0	1	0	0	0	0
Supercontig_1.76	4176	G	A	intergenic	Y	0	1	0	1	1	1	0	0
Supercontig_1.76	4337	G	T	intergenic	N	1	1	0	1	1	0	1	1
Supercontig_1.76	4543	A	C	FOIG_16312T0	Y	0	1	0	1	0	0	0	NA
Supercontig_1.76	4630	A	G	FOIG_16312T0	N	1	1	0	1	0	1	0	1
Supercontig_1.76	4681	T	C	FOIG_16312T0	Y	1	1	0	1	0	1	0	0
Supercontig_1.76	4732	A	G	FOIG_16312T0	Y	0	1	0	1	0	1	0	0
Supercontig_1.76	4778	T	C	FOIG_16312T0	Y	0	1	0	1	0	0	1	NA
Supercontig_1.76	5167	G	A	intergenic	N	0	1	0	1	1	0	1	1
Supercontig_1.76	5417	C	A	intergenic	Y	0	1	0	1	0	0	1	1
Supercontig_1.76	5857	G	A	intergenic	N	1	1	0	1	0	1	1	0
Supercontig_1.76	5916	T	A	intergenic	Y	1	1	0	1	0	1	0	0
Supercontig_1.76	6054	C	T	intergenic	N	1	1	0	1	1	0	0	1
Supercontig_1.76	6442	T	C	FOIG_16313T0	Y	0	1	0	1	0	0	0	1
Supercontig_1.76	6481	T	G	FOIG_16313T0	Y	1	1	0	1	0	0	0	1
Supercontig_1.76	6568	C	T	FOIG_16313T0	N	1	1	0	1	1	1	0	0
Supercontig_1.76	6602	T	G	FOIG_16313T0	N	1	1	0	1	1	1	0	0
Supercontig_1.76	6658	C	T	FOIG_16313T0	Y	0	1	0	1	0	0	1	0
Supercontig_1.76	6742	T	C	FOIG_16313T0	Y	0	1	0	1	NA	0	1	0
Supercontig_1.76	6859	T	C	FOIG_16313T0	Y	1	1	0	1	1	0	0	0
Supercontig_1.76	6928	A	C	FOIG_16313T0	Y	NA	1	0	1	1	0	0	0

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.76	7774	C	T	FOIG_16313T0	Y	0	1	0	1	0	1	1	0
Supercontig_1.76	8448	C	T	intergenic	N	1	1	0	1	1	1	1	1
Supercontig_1.76	8842	A	G	FOIG_16314T0	Y	1	1	0	1	0	1	0	0
Supercontig_1.76	9131	T	A	FOIG_16314T0	N	1	1	0	1	1	0	1	0
Supercontig_1.76	9144	A	G	FOIG_16314T0	N	1	1	0	1	1	0	1	0
Supercontig_1.76	9199	A	G	FOIG_16314T0	N	1	1	0	1	1	0	1	0
Supercontig_1.76	9391	A	G	FOIG_16314T0	Y	1	1	0	1	0	0	0	0
Supercontig_1.76	9474	G	A	FOIG_16314T0	Y	0	1	0	1	0	0	1	0
Supercontig_1.76	10604	C	G	intergenic	N	0	1	0	1	0	1	1	1
Supercontig_1.76	11524	G	T	FOIG_16315T0	N	1	1	0	1	0	0	1	1
Supercontig_1.76	11566	C	T	FOIG_16315T0	N	1	1	0	1	0	0	1	1
Supercontig_1.76	11790	G	T	FOIG_16315T0	N	1	1	0	1	1	0	1	NA
Supercontig_1.76	11847	C	T	FOIG_16315T0	N	1	1	0	1	1	0	1	0
Supercontig_1.76	12165	C	T	FOIG_16315T0	Y	0	1	0	1	0	0	1	0
Supercontig_1.76	12296	C	T	FOIG_16315T0	Y	0	1	0	1	0	0	1	0
Supercontig_1.76	13808	T	C	intergenic	Y	1	1	0	1	1	0	0	0
Supercontig_1.76	14444	T	C	FOIG_16316T0	Y	0	1	0	1	1	0	0	0
Supercontig_1.76	14891	A	G	FOIG_16316T0	N	NA	1	0	1	1	0	1	0
Supercontig_1.76	15301	T	C	FOIG_16316T0	N	1	1	0	1	0	0	1	1
Supercontig_1.76	17607	A	G	FOIG_16317T0	Y	0	1	0	1	0	0	0	0
Supercontig_1.76	18313	T	C	FOIG_16317T0	Y	0	1	0	1	0	0	1	1
Supercontig_1.76	18398	G	A	FOIG_16317T0	N	1	1	0	1	1	0	1	1
Supercontig_1.76	18405	A	T	FOIG_16317T0	N	1	1	0	1	1	1	1	1
Supercontig_1.76	18561	A	G	FOIG_16317T0	N	1	1	0	1	1	1	1	1
Supercontig_1.76	18645	G	A	FOIG_16317T0	N	1	1	0	1	0	0	1	NA
Supercontig_1.76	18769	C	T	FOIG_16317T0	N	1	1	0	1	0	0	1	1
Supercontig_1.76	19470	G	A	intergenic	Y	0	1	0	1	0	0	0	0

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.76	20306	T	G	FOIG_16318T0	Y	1	1	0	1	0	0	0	NA
Supercontig_1.76	20603	T	G	FOIG_16318T0	N	1	1	0	1	1	0	0	1
Supercontig_1.76	20656	G	A	FOIG_16318T0	N	1	1	0	1	1	0	0	1
Supercontig_1.76	20663	T	C	FOIG_16318T0	N	1	1	0	1	1	0	0	1
Supercontig_1.76	20745	G	A	FOIG_16318T0	N	1	1	0	1	0	1	1	NA
Supercontig_1.76	22394	T	C	intergenic	Y	1	1	0	1	0	1	0	0
Supercontig_1.76	22424	A	G	intergenic	Y	0	1	0	1	1	1	0	0
Supercontig_1.76	22700	A	G	FOIG_16319T0	N	1	1	0	1	1	0	0	1
Supercontig_1.76	22708	C	T	FOIG_16319T0	N	1	1	0	1	1	0	0	1
Supercontig_1.76	22848	A	C	FOIG_16319T0	Y	0	1	0	1	0	1	0	1
Supercontig_1.76	22854	A	G	FOIG_16319T0	Y	0	1	0	1	0	1	0	1
Supercontig_1.76	23330	C	A	intergenic	N	0	1	0	1	1	1	0	1
Supercontig_1.76	25265	C	G	intergenic	Y	1	1	0	1	1	1	1	1
Supercontig_1.76	26728	T	C	FOIG_16320T0	Y	0	1	0	1	NA	0	0	0
Supercontig_1.76	27494	T	C	FOIG_16320T0	N	0	1	0	1	1	1	1	1
Supercontig_1.76	27596	T	G	FOIG_16320T0	Y	0	1	0	1	1	0	0	0
Supercontig_1.84	15215	G	C	FOIG_16465T0	Y	1	0	NA	0	1	0	0	1
Supercontig_1.84	27077	G	A	FOIG_16470T0	Y	1	0	NA	0	1	0	0	1
Supercontig_1.85	13391	A	G	intergenic	N	0	1	1	1	1	NA	0	1
Supercontig_1.85	13403	G	A	intergenic	N	0	1	1	1	1	0	0	1
Supercontig_1.85	24733	C	T	intergenic	N	0	1	1	1	1	0	0	0
Supercontig_1.85	24795	C	T	intergenic	N	1	1	1	1	1	0	1	0
Supercontig_1.85	24814	C	T	intergenic	N	1	1	1	1	1	1	0	0
Supercontig_1.85	24900	C	T	intergenic	N	1	1	0	1	NA	1	0	0
Supercontig_1.85	52471	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.86	2558	A	G	intergenic	N	1	1	1	1	1	0	1	1
Supercontig_1.86	19596	A	G	FOIG_16502T0	N	1	1	1	0	1	1	1	1

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.90	16313	A	T	intergenic	N	0	1	1	NA	1	1	1	0
Supercontig_1.91	14311	A	G	intergenic	Y	1	0	NA	0	1	0	0	1
Supercontig_1.91	19872	C	T	FOIG_16548T0	Y	1	0	NA	0	1	0	0	1
Supercontig_1.92	28236	C	T	intergenic	N	0	1	1	1	0	1	0	1
Supercontig_1.94	532	C	T	intergenic	Y	1	0	1	0	1	1	1	1
Supercontig_1.94	573	A	G	FOIG_16574T0	Y	1	0	1	0	1	1	1	1
Supercontig_1.94	12623	T	C	intergenic	Y	1	0	1	1	1	1	1	1
Supercontig_1.94	25913	T	G	FOIG_16582T0	Y	1	0	1	1	1	1	1	1
Supercontig_1.94	35440	C	T	intergenic	Y	1	0	1	0	1	1	1	1
Supercontig_1.94	37259	C	T	intergenic	Y	1	0	NA	1	1	1	1	1
Supercontig_1.94	37273	A	T	intergenic	Y	1	0	NA	1	1	1	1	1
Supercontig_1.99	26154	C	A	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.99	26251	T	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.99	26253	A	C	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.99	26389	T	C	intergenic	Y	NA	0	1	0	1	0	0	1
Supercontig_1.101	7813	C	A	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.101	7820	A	G	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.102	15860	A	G	intergenic	Y	1	0	1	0	1	0	0	1
Supercontig_1.102	19881	T	C	FOIG_16635T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.107	20397	C	T	intergenic	N	0	1	1	1	1	0	1	1
Supercontig_1.112	3316	A	G	intergenic	N	1	1	0	1	0	1	1	0
Supercontig_1.112	9359	T	A	FOIG_16669T0	N	1	1	NA	1	0	1	1	1
Supercontig_1.117	10380	A	G	intergenic	N	1	1	1	1	0	1	1	1
Supercontig_1.122	11518	C	T	FOIG_16695T0,FOIG_16695T1	Y	1	0	1	0	1	0	0	1
Supercontig_1.128	5352	G	T	FOIG_16710T0	N	0	0	1	0	1	1	1	1
Supercontig_1.128	7462	A	G	intergenic	N	1	1	0	1	0	1	1	1
Supercontig_1.128	7467	G	A	intergenic	N	1	1	0	1	0	1	1	0

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.128	7707	T	A	intergenic	N	1	1	0	1	1	1	0	1
Supercontig_1.128	7757	A	G	intergenic	N	1	1	0	1	1	1	0	1
Supercontig_1.128	7826	A	G	intergenic	N	1	1	0	1	1	1	0	1
Supercontig_1.128	7954	G	T	FOIG_16711T0	N	1	1	0	1	0	1	0	0
Supercontig_1.128	8613	T	C	FOIG_16711T0	N	0	1	0	1	0	1	0	1
Supercontig_1.128	8985	C	T	FOIG_16711T0	N	0	1	0	1	1	1	1	0
Supercontig_1.128	8998	T	G	FOIG_16711T0	N	0	1	0	1	1	1	0	0
Supercontig_1.128	9053	G	A	FOIG_16711T0	N	0	1	0	1	1	1	0	0
Supercontig_1.128	9137	A	G	FOIG_16711T0	N	0	1	0	1	NA	1	0	1
Supercontig_1.128	9346	C	A	FOIG_16711T0	N	0	1	0	1	0	1	1	1
Supercontig_1.128	10408	T	G	intergenic	N	1	1	0	1	1	1	NA	1
Supercontig_1.128	10414	C	G	intergenic	N	1	1	0	1	1	1	0	1
Supercontig_1.128	10713	A	G	intergenic	Y	0	1	0	1	0	1	0	0
Supercontig_1.144	9283	G	A	intergenic	N	0	0	1	0	1	NA	0	1
Supercontig_1.190	1642	T	C	intergenic	N	0	1	1	1	1	0	0	1
Supercontig_1.190	1678	A	G	intergenic	N	1	1	1	1	1	1	0	1
Supercontig_1.190	1834	C	T	intergenic	N	1	1	1	1	1	1	0	1
Supercontig_1.190	1952	A	G	intergenic	N	1	1	1	1	1	1	0	1
Supercontig_1.190	2003	A	G	intergenic	N	NA	1	1	1	1	1	0	0
Supercontig_1.190	2049	A	C	intergenic	N	1	1	1	1	0	1	0	0
Supercontig_1.190	2204	C	T	intergenic	N	1	1	NA	1	1	1	0	0
Supercontig_1.190	2462	A	G	intergenic	N	1	1	1	1	1	1	0	1
Supercontig_1.219	2379	T	C	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.219	2418	G	T	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.219	2456	T	C	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.219	2477	T	C	intergenic	N	0	0	1	0	1	0	0	1
Supercontig_1.264	262	G	A	intergenic	Y	0	1	0	1	1	0	1	0

Contigs	Position	Ref	Alt	Location	Retained after filtering	China	Jordan	Laos	Lebanon	Myanmar	Pakistan	Philippines	Vietnam
Supercontig_1.264	309	T	G	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	543	G	A	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	614	G	C	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	777	C	T	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	953	A	G	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1038	A	C	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1060	C	T	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1118	G	A	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1157	A	G	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	1556	C	T	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1585	G	A	intergenic	Y	0	1	0	1	1	0	0	0
Supercontig_1.264	1608	G	A	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1661	A	G	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.264	1888	A	G	intergenic	Y	0	1	0	1	1	0	1	1
Supercontig_1.264	2010	G	T	intergenic	Y	0	1	0	1	1	0	1	0
Supercontig_1.354	776	G	A	FOIG_16942T0	Y	1	0	1	0	1	0	0	1
Supercontig_1.360	199	C	T	FOIG_16945T0	Y	1	1	1	1	0	1	1	0

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CHAPTER 3

An Improved Phenotyping Protocol for Panama Disease in Banana

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Abstract

Fusarium oxysporum (*Fo*) belongs to a group of soil-borne hyphomycetes that are taxonomically collated in the *Fusarium oxysporum* Species Complex (FOSC), comprising many plant pathogens causing wilt diseases on a broad range of crops worldwide, including banana. The host range of each *Fo* is limited and defined by so-called formae speciales (ff. spp.). Hitherto, those infecting bananas were placed in the forma specialis *cubense* (*Foc*), despite their polyphyletic origin. Recently, however, these genetically different *Foc* lineages were consequently recognized as new *Fusarium* spp. that were placed in the Fusarium of Banana Complex (FOBC). Most species produce three types of spores: macroconidia, microconidia and the persistent chlamydospores that can contaminate soils for many years. Inoculum production has been an important bottleneck for efficient phenotyping due to the low or variable number of conidia and the elaborate laboratory procedures requiring specific infrastructure. Here, we report a rapid, simple and high-yielding spore production method for nine *F. oxysporum* formae speciales as well as the biocontrol species *Fo*47 and *Fo*618-12. For *Fusarium* spp. causing Fusarium wilt or Panama disease of banana, we used the protocol for four species comprising the recognized physiological races, including Tropical Race 4 (TR4). We subsequently tested the produced inoculum in comparative inoculation trials on banana plants to evaluate their efficiency. All assays resulted in typical symptoms within 10 weeks; significant differences in final disease ratings were observed and depended on inoculum concentration. Pouring inoculum directly onto banana plants showed the most consistent and reproducible results, as expressed in external wilting, internal discolouration and determined by real-time PCR assays on entire rhizomes. Moreover, this method allows the inoculation of 250 plants per hour by one individual thereby facilitating the phenotyping of large mutant and breeding populations.

Introduction

The genus *Fusarium* comprises many of the most important fungal plant pathogens. It is ranked fifth on a list of top fungal plant pathogens based on scientific and economic importance (Ploetz 2005b, Dean *et al.,* 2012). The *Fusarium oxysporum* species complex (FOSC) combines a morphologically diverse suite of species, including plant pathogens, saprophytes and even facultative human pathogens (Dean *et al.,* 2012). Many of the pathogens cause wilting diseases, root rots and damping-off in hundreds of plant species (Domsch *et al.,* 1980, Gerlach and Nirenberg 1982, Nelson and Toussoun, Meldrum *et al.,* 2012). Over 120 formae speciales (ff. spp.) have been described (Baayen *et al.,* 2000, Hawksworth 2001), each affecting one or a limited number of different host plant species. Differences in pathogenicity on specific host cultivars define physiological races among isolates, which has been studied intensively in some pathosystems (Kistler 1997, Baayen *et al.,* 2000, Takken and Rep 2010, Meldrum *et al.,* 2012).

Fusarium oxysporum f. sp. *cubense* (*Foc*) is the hitherto species name of strains infecting banana and causing Fusarium wilt, or Panama Disease. However, it has long been recognized that *Foc* has a polyphyletic origin (O'Donnell *et al.,* 1998, Ploetz 2005b, Lievens *et al.,* 2009), hence comprises a suite of genetically distinct lineages (Ordóñez *et al.,* 2015b). Therefore, Maryani *et al. (*2018) have recently revised the taxonomy of *Foc* and designated different species names to strains affecting banana and merged them into the Fusarium of Banana Species Complex (FOBC). The disease cycle of these *Fusarium* spp. starts with infection of the root system and subsequent colonization of the vascular tissue, leading to water stress, severe chlorosis and wilting (Beckman 1987, Ploetz 2015). Infected plants frequently die before they produce bunches, hence Fusarium wilt significantly reduces yields in infested fields (Stover and Ploetz 1990, Dita *et al.,* 2010). Strains associated to race 1 caused one of the worst botanical epidemics in history and decimated the commercial 'Gros Michel' banana-based industry in Central America in the 1950s (Ploetz 2005a). As a result, 'Gros Michel' was replaced with resistant and now globally cultivated Cavendish clones. Albeit that Cavendish quenched the race 1 driven epidemic, many regionally important banana varieties are still susceptible to these strains and succumb to the disease (Ploetz 2006). Meanwhile, another species, *F. odoratissimum* that uniquely comprises the so-called Tropical Race 4 (TR4), is sweeping through major production zones of Cavendish banana (Butler 2013, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a, Zheng *et al.,* 2018), thereby significantly affecting susceptible local and regionally important banana varieties destined for domestic markets (Ploetz 2015).

For most crops, host resistance is a cornerstone for sustainable disease management, usually achieved by intensive breeding programs. However, breeding for resistance in banana is limited and has not resulted in diversification. Cavendish is the cornerstone for the international trade and hence, TR4 threatens the entire global production. Improved banana varieties are mostly mutants of Cavendish clones that were selected in extensive field trials. These are time consuming, expensive and sometimes unreliable due to variable environmental conditions and unknown inoculum diversity and distribution (Mert and Karakaya 2003, Subramaniam *et al.,* 2006, Sutanto *et al.,* 2013). In contrast, greenhouse phenotyping facilitates higher throughput under controlled conditions with specific fungal genotypes, leading to more reproducible results, which accelerate breeding programs (Smith *et al.,* 2008). With the progress in high-throughput genotyping

methods, phenotyping has become a major bottleneck for plant improvement, particularly for perennial crops such as banana.

Hence, efficient inoculum production is the first critical factor in optimizing phenotyping protocols. For *Fusarium* spp. several protocols were developed or used for screening or tests (Sun and Su 1984, Adesemoye and Adedire 2005, Amorim *et al.,* 2009, Dita *et al.,* 2011, García-Bastidas *et al.,* 2014, Li *et al.,* 2015, Ordóñez *et al.,* 2015a), of which many are based on the use of commercial growth media but also natural sources such as beans (*Vigna radiata* L.) (Bai and Shaner 1996, Li *et al.,* 2001, Yuan and Zhou 2005, Mudge *et al.,* 2006, Amorim *et al.,* 2009, Dita *et al.,* 2010, Dita *et al.,* 2011, García-Bastidas *et al.,* 2014, Li *et al.,* 2015, Ordóñez *et al.,* 2015a). However, these methods cannot be upscaled to the large volumes of inoculum required for extensive phenotyping experiments (Burgess *et al.,* 1991, Leslie *et al.,* 2006), due to either large quantities of expensive culture media, costly infrastructure or labour intensity. These practical constraints have therefore contributed to manifold inoculation assays (Sun and Su 1984, Smith *et al.,* 1999, Mohamed *et al.,* 2001, Subramaniam *et al.,* 2006, Smith *et al.,* 2008, Wu *et al.,* 2010, Dita *et al.,* 2011), lacking uniformity, which complicates data comparison and interpretation. Thus, there is a need for a standardized and widely accepted phenotyping protocol to evaluate Panama disease resistance in banana.

Hitherto protocols facilitated the mere screening of approximately 15 plants per hour per person (php) (Dita *et al.,* 2010, Dita *et al.,* 2011, Ordóñez *et al.,* 2015a). Clearly, this hampers throughput and potential automation during phenotyping mutant panels or segregating populations that usually comprise hundreds or even thousands of plants. Here, we report the development of an optimized Mung bean-based *Fusarium* spp. inoculum production protocol that matches all the aforementioned constraints and enables the screening of up to 250 banana plants php. Moreover, its wide applicability was shown for other *Fusarium* spp. affecting different crops

Material and Methods

Fusarium spp. *and growth conditions*

In total, 11 genotypes of *Fusarium* spp. were tested, including the known races: Race 1 (R1), Race 2 (R2), Subtropical Race 4 (ST4) and TR4, as well as two well-known *Fo* biocontrol agents (Table 1). All strains are maintained in the Wageningen University and Research (WUR) collection and mostly originate from infected plant tissues. Strains were grown at 27-28˚C on potato dextrose agar (PDA; Sigma Chemical Co., St. Louis, Mo) for five days in the dark and plugs were then taken from the edge of the colony to inoculate liquid media.

Table 1. Origins and characteristics of the strains of *Fusarium* spp. used in this study.

***Fusarium* spp.**[1]	**Isolate Code**	**Host**	**VCG**[2]	**Experimental Code**	**Origin**
FOSC clade 4	CNPMF-008-01-R1	Banana	Unknown	R1	Cruz das Almas, Bahía, (Brazil)
Fusarium tardichlamydosporum	NRRL 25607	Banana	0124	R2	USA
Fusarium odoratissimum	II-5	Banana	01213	TR4	Indonesia
Fusarium phialophorum	ST4/98	Banana	0120	ST4	Canary Islands (Spain)
Fusarium oxysporum f. sp. *melongenae*	0-1877	Eggplant	n.a.	*Fom*	Israel
Fusarium oxysporum f. sp. *lycopersici*	40698	Tomato	n.a.	*Fol*	Netherlands
Fusarium oxysporum f. sp. *cepae*	Eza	Onion	n.a.	*Foce*	Australia
Fusarium oxysporum f. sp. *gladioli*	10194	Gladiola	n.a.	*Fog*	-
Fusarium oxysporum f. sp. *albedinis*	0-2015	Date palm	n.a.	*Foca*	Canary Islands (Spain)
FOSC Clade 3	Fo47	Biocontrol	n.a.	*Fo*47	France
-	Fo 618-12	Biocontrol	n.a.	*Fo*618-12	Netherlands

[1]Names adapted based on latest classification on Maryani *et al.* (2018). [2] VCG = vegetative compatibility group.

Plant material

in-vitro plants were obtained from various sources (Table S1), transferred to individual pots containing a standard soil from the WUR, Unifarm greenhouse facility (Swedish sphagnum peat 5%, grinding clay granules 41%, garden peat 5%, beam structure 4%, steamed compost 33%, PG-Mix-15-10-20- 12%) and then placed in an environmentally controlled greenhouse compartment (28±2˚C, 16h light, and ~85% relativity humidity). Plants were acclimatized under plastic for two weeks to facilitate high humidity and thereafter grown in the same greenhouse for ~2.5 months prior to inoculation. We used Cavendish 'Grand Naine' for all comparative analyses but added additional banana accessions with various levels of resistance to TR4 for validation.

Spore production

To determine the optimal conditions for conidia production we used the reference TR4 isolate II-5 (Dita *et al.,* 2010) as it is currently the most important threat to global banana production. Six sporulation media (SM) were prepared by autoclaving 500 ml water in 1 L Erlenmeyer flasks supplemented with 20 gr pre-boiled Mung beans, as in the original protocol (SM1; Bai et al., 1996), or 20 gr (SM2), 5 gr (SM3), 2 gr (SM4), 1 gr (SM5) or 0.5 gr (SM6) of fresh Mung beans, respectively. The Erlenmeyer's were closed with cotton plugs and sterilized at 120˚C for 20 min. Five mycelium plugs were taken from a freshly grown PDA plate and were transferred to the Mung bean media. The inoculated Erlenmeyer flasks were incubated on a rotary shaker at 130 rpm at 25±2˚C for a maximum of eight days. A 1 ml aliquot was taken under sterile conditions and passed through two layers of sterile cheesecloth to remove hyphal fragments at 1, 3, 6 and 8 days after inoculation (dai) for viability testing and spore quantification. A 10 µl sample of the remaining suspension was plated on a PDA plate and then incubated at 25˚C in the dark to germinate the spores after which the growing area was measured. The number of conidia was counted using Glastic Slides (Hycor Biomedical., CA, USA), photographs of each grid were taken with a light microscope (Zeiss Axiocam ICc3) and spores were counted manually, using a hemocytometer, with five repetitions per sample. All experiments were repeated twice. Eventually, the SM4 protocol was used for spore production of all other *Fusarium* spp. infecting banana (Table 1) and the spore concentration was quantified at 6 dai.

Chlamydospores of TR4 were produced following the protocol of Amorim *et al. (*2009) with minimal modifications. Briefly, plugs with mycelial growth of *Foc* were mixed with twice autoclaved substrate composed by sandy soil, corn powder and distilled water in 500 ml Erlenmeyer flasks. Then flasks were incubated at 25˚C for around 12 days. New autoclaved sandy soil was infected with substrate in a 1:12 ratio and then flasks with the mixture were incubated for six more weeks. Infected maize kernels were produced by inoculating 100 g of sterilized grains in Erlenmeyer flasks with five TR4 plugs derived from a fresh colony growing on PDA. Flasks were incubated at 25˚C in the dark for 10 days.

Inoculation and plant maintenance

Five phenotyping assays were compared: (i) dipping banana plants with trimmed roots in spore suspension and transplanting them into either non-sterilized soil or (ii) in

sterilized sand, (iii) uproot plants and transplant them into soil infested with chlamydospores, (iv) add TR4 colonized maize kernels to soil of potted plants or (v) pouring inoculum directly on the soil of potted plants. For the dipping method with repotting either in soil or sand, banana plants were uprooted and the root systems were rinsed with water and then trimmed to a third of the original mass (removing ~10 cm from the tip of the root) and left in water to avoid plant stress until inoculation before immersing them for 30 minutes in inoculum of various concentrations (10^3, 10^4, 10^5, 10^6 spores.ml^{-1}). For the chlamydospores method banana plants were transplanted in fresh soil that was mixed with approximately 10^4 chlamydospores.gram of soil^{-1} at various ratios (2,5; 5; 10 and 20 g.L^{-1}). For the maize kernel method 3, 5, 10 or 20 colonized grains were placed in two equidistant holes from the base of the banana plant. Finally, for the pouring method, 200 ml of inoculum with various concentrations (10^3, 10^4, 10^5, 10^6 spores.ml^{-1}) were directly added onto the pots. For all experiments, we selected five plants with six to seven leaves and 30-50 cm height which were maintained in a closed pot system to prevent inoculum leakage and cross-contamination (Mohamed *et al.,* 2001) and for each treatment non-inoculated Cavendish 'Grand Naine' banana plants were used as mock. The resistant accessions 'Pahang' and cv. Rose were included as negative controls.

Disease assessment

Upon infection, the plants were monitored and scored for disease symptoms and progress at weekly intervals. The latency period was set as the time elapsed between inoculation and the appearance of the first symptoms in three out of five plants. Plants were externally and internally inspected when totally decayed or when 75% of the leaves turned yellow or ultimately at 10 weeks after inoculation (wai). External symptoms - the percentage of chlorotic leaves - were scored following a 1 - 4 scale in which 1 = 0 > x ≤ 25%, 2 = 25 < x ≤ 50%, 3 = 50 < x ≤ 75%, and 4= 75 < x ≤ 100%. In addition, morphological changes of leaves and pseudostem splitting were recorded. For internal evaluation, plants were uprooted, cleaned and the rhizome (corm) of each plant was cut longitudinally. Disease severity was visually assessed following a 1 - 6 scale where 1 = No discoloration in the corm, 2 = isolated points, x < 5 %, 3 = 5 < x ≤ 30%, 4 = 30 < x ≤ 50%, 5 = 50 < x ≤ 90% and 6 = plant totally decayed x < 90%. To guarantee an accurate quantification of the affected and discoloured tissues we conducted image analyses (ImageJ 1.49, http://rsb.info.nih.gov/ij/) and disease indexes were calculated following McKinney (1923).

Finally, fungal biomass per individual corm was determined by qPCR. Corms were sliced into small pieces (3–5 cm) and collected in 50 ml tubes and freeze-dried for 48h (Epsilon 1-4 LSC, Christ GmbH, Germany). The remaining mass was weighed after which three chrome-steel beads (6,35 mm, Biospec) were added and the samples were ground using a conventional vortex (IKA Labortechnik, Staufen, Germany) until the material was homogenized (~1,5 minutes). Subsequently, 100 mg tissue was processed for DNA extraction using a Kingfisher robot (Thermo Labsystems, Oy, Finland) and the AGOWA Sbeadex® Maxi plant DNA isolation kit (LGC Genomics, location, Germany). Samples were mixed with 600 µl lysis buffer, bead beaded (Bertin technologies, Ampere montigny-le-Bretonneux, France) for 40 seconds and then incubated at 65°C for 15 minutes, followed by centrifugation for 20 minutes at maximum speed in an Eppendorf centrifuge (Eppendorf 5415 D, Germany) after which 200 µl of supernatant per sample was

collected and transferred to a deep 96-well plate containing 520 µl binding buffer following the manufacturer's instructions.

The total amount of genomic DNA was quantified by PicoGreen using 5 µl of the total DNA that was placed in a deep 96-well plate with 100 µl of 1x PicoGreen and 99 µl TE after which the samples were measured with Tecan Infinite M200Pro using Icontrol 107 software (US, Morrisville, NC). Verifications of TR4 infections were performed by multiplex PCR using the *EF* and *BanActin2* genes as internal controls (Dita *et al.,* 2010) and quantitative PCR (qPCR) by SYBR® Green technology using the commercial ClearDetections® TR4 molecular diagnostic kit (Clear® Detections, Wageningen, The Netherlands) in a real time PCR machine (ABI 7500, Thermo Fisher Scientific, USA). The amount of biomass was calculated as the amount of DNA.mg^{-1} dry weight based on the resulting Ct values using a 10-fold dilution series of TR4 genomic DNA as standard (1 ng – 1 fg).

Statistical analysis

The comparative inoculation experiment was set up following a factorial design with two factors: method and level of inoculations. Means of the percentages of chlorotic foliage, corm discoloration, disease index, corm dry weight and Ct values obtained by real time PCR were analysed by ANOVA and differences of the means of each variable were compared by using Fisher's test (LSD, p=0.005).

Results

Fusarium spp. *inoculum production depends on Mung bean medium composition*

Different amounts of Mung beans in gr per 500 ml water were tested in order to standardize the most suitable sporulation media (SM). Conidia production of TR4 commenced within 24 hours after inoculation of the media. All SM, except SM2 (20g), showed a significant increase of conidia compared to the control SM1 (original protocol) (Figure 1). After 24 hours, a steep increase on conidia production was observed in SM3 (5gr), SM4 (2gr) and SM5 (1gr), while a moderate increase was observed in SM6 (0,5 gr). In contrast, no conidia were observed for SM1 and SM2 at that time. During final quantification at 8 dai, the SM1 culture contained 4,5 x 10^6 conidia ml^{-1} but the highest concentrations were obtained in SM3 and SM4, with over 1.1 x 10^8 conidia ml^{-1} (Figure 1). Albeit that SM3 and SM4 excelled in spore production at 8 dai, the former was eventually chosen to avoid clogging of inoculum during filtering and dilution due to the higher content of mycelia in SM3.

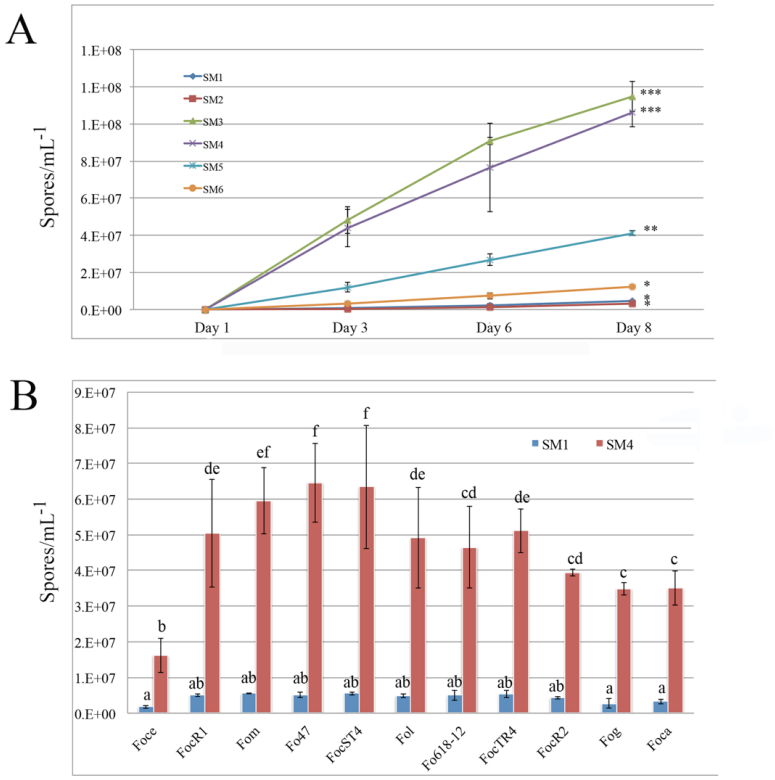

Figure 1. Conidia production of *Fusarium* spp. in sporulation media (SM). (A) Spore production over time for Tropical Race 4 (*F. odoratissimum* II-5) in SM1-SM6. (B) Spore production in SM1 and SM4 for 11 *Fusarium oxysporum* ff.spp. Spores were quantified after six days (n=3) and the experiment was repeated at least twice ($P > 0.05$, treatments with the same letters/symbol are not significantly different).

The new Mung bean protocol is applicable for other Fusarium spp.

To test whether a wider application of the SM4 protocol we tested other *Fusarium* spp. and two *Fo* biocontrol strains (Table 1) we compared spore production of SM1 (Figure 1B). All strains produced significantly more conidia in SM4 than in SM1 media. Despite the fact that *Foce* was the least productive (1.6 x 10^7 spores.ml^{-1}), SM4 enabled a 10-fold increase over SM1. The highest concentration of conidia was obtained with *Fom, Fo*47 reaching >6.0 x 10^7 spores.ml^{-1} (Figure 1), and TR4 produced more than 5.0 x 10^7 spores.ml^{-1} (Figure 1), which was sufficient to inoculate 250 plants per hour by one person (see Figure 2).

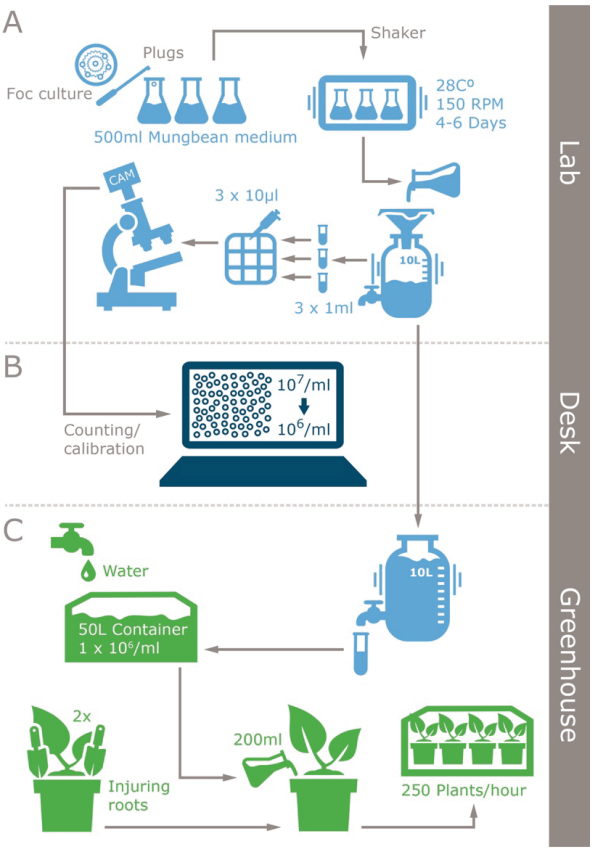

Figure 2. Infographic of the inoculum and inoculation for *Fusarium* spp. causing Panama disease in banana.

Comparison of inoculation assays and inoculum concentrations

We compared the five most commonly used inoculation methods and conclude that – contrary to all other methods – pouring inoculum enables the inoculation of many more plants. This is possible because the new inoculum production is easier, the phenotyping assay is faster, and the final disease index is proportionate with the applied inoculum concentration (Figures 3 and 4). Initial chlorosis/wilting was observed for the dipping and chlamydospores inoculation methods, as well as their un-inoculated controls but these recovered after two weeks suggesting that these effects resulted from root trimming. Depending on the method used, the latency period was between two and four (wai) but was shortest in plants that were inoculated with the highest inoculum concentrations and using inoculation methods that involved root trimming (e.g. dipping and chlamydospore methods). The latency period for the pouring method was approximately 3 wai at all inoculum levels, whereas the application of colonized kernels resulted in a significantly delayed latency period (Table S2). Plants challenged with high inoculum concentrations decayed 5 – 7 wai, except for the maize kernel treatment, which showed inconsistent values between low and high inoculum concentrations. All controls showed natural chlorosis and senescence and hence had score 1. Plants scored 2 once low inoculum concentrations were used for the dipping method (sand) and pouring

assays, as well as after using the maize kernels assay. The dipping method (sand) and pouring assays with 1.10^4 spores.ml^{-1} consistently scored 3, whereas all other treatments resulted in score 4 for foliage discoloration.

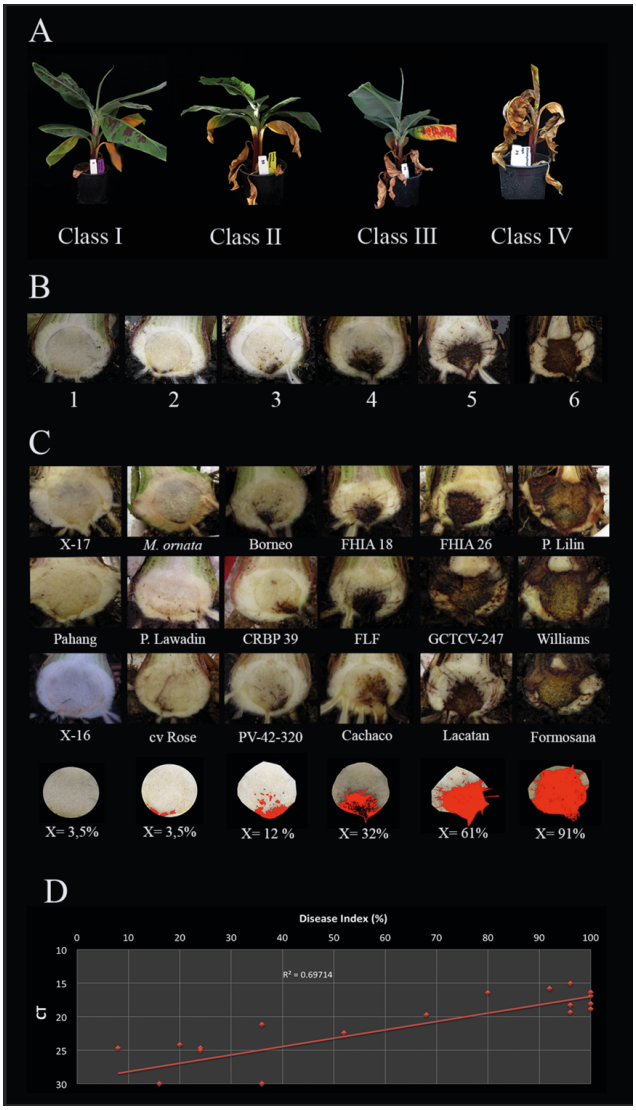

Figure 3. Panama disease progress incited by Tropical Race 4 (*Fusarium odoratissimum* isolate II-5). (A) Four class rating scale of leaf chlorosis : I = (0 > x ≤ 25%), II = (25 < x ≤ 50%), III = (50 < x ≤ 75%), and IV (75 < x ≤ 100%); (B and C) Internal severity levels of 18 banana accessions (Table S2) following the 1-6 scale of affected corm tissue at 10 weeks after inoculation and the accompanying percentages of affected tissue as calculated by ImageJ; (D) The correlation between qPCR quantification and Disease Index per corm with trendline and R value.

All inoculation assays resulted in internal corm discoloration, but affected areas differed significantly between the applied methods and inoculum concentrations (Figures 3 and 4; Table S2). Generally, higher inoculum concentrations resulted in higher disease

severities, except for the maize kernel treatment, where inoculum dosage and symptom development were not significantly correlated. For the other assays, all inoculum concentrations resulted in > 75% corm discoloration. The determined disease indices (DI) showed a range of values that divide the inoculation assays into three groups based on their severity (Figure 3; Table S2): maize kernel and dipping methods (10^3 spores.ml^{-1}) and dipping (sand) (10^3 and 10^4 spores.ml^{-1}) resulted in low severities with DI values between 0-50; pouring (10^5 spores.ml^{-1}) and chlamydospore (2,5g) treatments exhibited moderate severities with DIs between 50-80; all remaining treatments developed high severities with DIs between 80-100. Since the newly developed pouring assay displayed the widest variation in DI, we chose to validate this assay on 12 additional banana accessions with various levels of resistance to TR4 (Figure 3C). This enabled the ranking of these accessions by their DIs from immune to highly susceptible (Figure 3). Across all experiments, 'Grand Naine' plants inoculated with race 1 as well as 'Pahang' and 'cv Rose' inoculated with TR4 did not develop any external and internal symptoms, independent of the used inoculum concentrations. All water controls remained healthy (Figure 4).

Figure 4. External and internal symptoms of 'Grand Naine' at six weeks after inoculation with *Fusarium odoratissimum* strain II-5 (Tropical Race 4). The panels A to C show the results of different inoculation methods (top panels), (A) DM soil, (B) DM Sand, (C) pouring method and the controls (lower panels). Plants inoculated with chlamydospores (D) or Maize kernels (E), also develop similar symptoms but the latency period differs from conidial inoculations. Plants were challenged with the highest inoculum doses described for each method. Controls include 'Grand Naine' inoculated with race 1 using the dipping method (F) as well as Pahang (H) and cv. Rose (G). An untreated plant is shown in (J).

Additional corm analyses

Corm dry-weights correlated with DIs, except for the colonized maize kernels assay (Table S1). Lyophilizing complete corms was performed to avoid statistical errors in sampling and enabled the detection and quantification of TR4 biomass. Nearly all corms derived from infected plants tested positive for TR4 with conventional PCR (Dita *et al.,* 2010), except some dipping (sand) and pouring treatments (both 10^3 spores.ml^{-1}) and some of the pouring method (10^4 spores.ml^{-1}) replicates, which are all supposed to result from sampling errors. However, qPCR analysis confirmed the presence of TR4 DNA in all samples with average Ct values between 15,06 and 24,73, whereas all controls plants were negative (Table 2), but the correlation with DI was rather low (R^{-2}=0.697, Figure 3D).

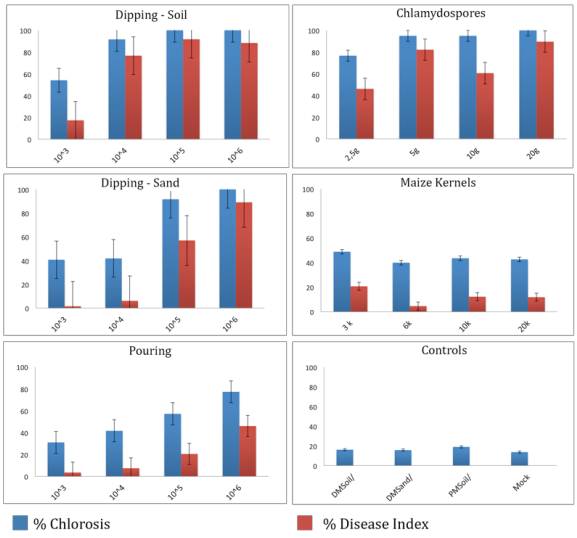

Figure 5. Dose-response disease indexes of 'Grand Naine' plants at final score (5 to 10 weeks after inoculation) with *Fusarium odoratissimum* II-5 (Tropical Race 4) using five inoculation methods. For each treatment the average result for four inoculum doses are expressed as percentage of foliar chlorosis and as the disease index at the time of scoring.

Discussion

The global dissemination of plant pathogens and pests is a serious concern for future food and feed production (Bebber *et al.,* 2014). Cereal plagues draw massive attention (Sun 1978, Bhattacharya 2017), but diseases in orphan crops usually pass unnoticed. Banana is no exception, as the occurrence of TR4 threatening Cavendish bananas in Taiwan was already noticed in the 1960s, published in 1978 (Sun 1978), but the occurrence in Jordan and other countries outside Southeast Asia drew eventually global attention (Butler 2013, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a). Since then, Panama disease is again considered a serious threat for global banana production, which results in an increased level of fundamental and applied research. Hence, there is an urgent need for reliable and standardized phenotyping protocols to seek banana accessions with adequate resistance and to verify the efficacy of control methods. Such assays should

enable high throughput data gathering, ideally of parallel screens using multiple *Fusarium* strains, thereby facilitating comparisons of data collected in different laboratories.

Here, we describe an inoculum production protocol that entails efficiency, by eliminating pre-boiling and pre-filtering steps prior to autoclaving, and by using just two grams of Mung bean seeds to produce up to $7{,}5.10^6$ spores.ml^{-1} in six days, irrespective of the *Fusarium* species. Comparable results were also obtained for other *Fo* species, including the biocontrol strains *Fo*47 and *Fo*618-12. Nout *et al.* (1987) reported the production of 5.10^4 to 3.10^6 spores.ml^{-1} for *Rhyzopus oligosporus* using 100 gr. L^{-1} of Mung bean sprouts and Bai and Shaner (1996) found *F. graminearum* condia concentrations to oscillate between 4,6 to $5{,}5.10^5$ spores. ml^{-1}. Jo *et al.* (2015) compared seven sporulation media for *Fo* f. sp. *niveum* for watermelon bioassays and reported that in Czapek-dox broth $4{,}0 .10^4$ spores.ml^{-1} were obtained, whereas a maximum production of $2{,}6.10^7$ spores.ml^{-1} was found in V8-juice broth. For race 1 (VCG01217), Subramaniam *et al. (*2006) reported a production of $1{,}4.10^5$ at 7 dai, $4{,}6.10^5$ at 21 dai and only $6.2.10^5$ spores.ml^{-1} four wai. The latter was confirmed as the production of SM1 resulted in $8{,}3.10^5$ spores.ml^{-1}. Thus, our improved protocol allowed us to produce on average 100x more spores than previously published methods, which will be beneficial for studying various *Fusarium* pathosystems and also boosts the production of biocontrol strains like *Fo*47 (Postma and Luttikholt 1996). The production of spores in all media was reproducible, although some variation was observed in the final number of spores, potentially due to variations in the prepared media or the origin of isolates (Oswald 1949, Nelson *et al.,* 1994). The produced conidia were infective across the tested bioassays. We observed that optimal conidia production was accomplished at six dai since mycelial formation at later stages complicated inoculum filtering and eventually resulted in lower recovered spore concentrations, thereby reducing the efficiency of the protocol.

After establishing the optimal inoculum production protocol, we evaluated different Panama disease phenotyping assays, including a new method in which a conidial TR4 suspension is directly poured onto the soil. This new method resulted in typical disease symptoms comprising wilting, chlorosis, malformation of the emerging leaf, pseudostem splitting as well as discoloration of the rhizome and in severe cases plant death, similarly to the effects of the other treatments and corresponding to results shown in other studies (Smith *et al.,* 2008, Amorim *et al.,* 2009, Dita *et al.,* 2009, Dita *et al.,* 2010, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a). However, external symptom development, such as chlorosis of the foliage, is an unreliable parameter for disease evaluation, despite that it is a direct indication for the pathogen's progress (Smith *et al.,* 2008, Wu *et al.,* 2010, Dita *et al.,* 2011, García-Bastidas *et al.,* 2014, Li *et al.,* 2015). Clearly, latency period depended largely on inoculum concentrations with the shortest period for high spore concentrations using the dipping method in soil, sand and in the chlamydospore method (~10 -15 dai), as reported before (Mohamed *et al.,* 2001, Smith *et al.,* 2008, Amorim *et al.,* 2009). However, apart from the required large inoculum volumes, the time-consuming root trimming provokes stress, which results in morphological changes, including atypical chlorosis that is easily confused with initial Panama disease symptoms. The observed chlorosis of older leaves during the first week was therefore attributed to plant stress. This was confirmed by the appearance of comparable symptoms for incompatible interactions such as race 1- 'Grand Naine' and TR4 – 'Pahang' and cv. Rose.

Different propagules and infectious structures revealed significant variation in DIs. Thus, infectivity depends on the composition of the inoculum, *i.e.* micro and macroconidia, chlamydospores or mycelium (Amorim et al., 2009; Smith 2008), which was also observed in the tomato – *Fo* f. sp. *lycopersici* pathosystem (Cal *et al.,* 1997). The dipping and chlamydospore methods invariably resulted in high disease severities at high inoculum concentrations, but for the maize kernel assay results were too variable across the used inoculum concentrations, probably due to the limited distribution of kernels/inoculum propagules in the pots and/or their position to nearby roots.

Inoculum concentrations affect the latency period, as observed by external discoloration of the foliage, as well as internal symptom severity. The most intense corm discoloration values and subsequent highest DIs were observed for the invasive methods (DI 80 to 100). Jo *et al.* (2015) found a DI ~90 in the susceptible watermelon cv. Sugar baby by using the dipping method in a concentration of inoculum of 1.10^6 spores.ml^{-1}. However, no significant differences were observed when inoculum concentrations were modulated between 1- 9.10^6 spores.ml^{-1}. In our trials, dipping methods produced very low corm severities (1,5 and 6.1%) and low DIs (8 and 24) at low inoculum concentrations (10^3 and 10^4 spores.ml^{-1}) and transplanting in sterilized sand, likely due to escapes, suggesting a minimal required inoculum concentration. However, transplanting in non-sterilized soil resulted in enhanced disease development (17,3 and 76% for 10^3 and 10^4 spores.ml^{-1}, respectively). Whether this is due to microorganisms present in untreated soil is unknown, which underscores the need for studies focusing on the role of the microbiome of the rhizosphere in *Fusarium* spp. -banana interactions (Köberl *et al.,* 2017)

Phenotyping protocols require efficiency, reliability and discriminative power. The invasive methods proved to be effective and reproducible, but require extensive plant pre-treatment, including root cleaning and trimming which may take up to 10 minutes per plant. Subsequently, plants must be immersed 30 minutes in large volumes of inoculum and then be transplanted to new pots. Albeit that chlamydospores are important under field conditions, their production takes up to three months and accurate quantification is challenging. Moreover, extra quarantine and safety steps are needed, as chlamydospores are extremely aggressive, even at low doses as observed in our trials. Hence, it is practically impossible to produce batches for individual *Fusarium* genotypes or species. Thus, these invasive methods hamper throughput required for large experiments, such as replicated interaction trials, segregating populations in genetic studies or the evaluation of mutant panels. We found that the pouring method enables the inoculation of 250 plants per hour, by a single individual, which is a huge improvement of throughput compared to any other method. Moreover, it produces dose dependent DIs, thereby providing an adequate discriminating protocol for disease ranking. An inoculum concentration of 1.10^6 spores.ml^{-1} is the most suitable and resulted in consistent data, which is a great advantage over erratic field trials. These may take over nine months before disease expression, due to the mostly unknown distribution of inoculum in the soil (Sun, et al. 1984). Current phenotyping assays often rely on visual (external) scoring, which is straightforward but has severe limitations. Here, we complemented such visual scoring with image analyses, which resulted in reproducible and objective data enabling standardization. The validation by real-time PCR for fungal biomass quantification in the corm showed a low correlation between the amount of fungal DNA and DI, which is most likely highly influenced by DNA degradation in the corm at late stages of infection. Therefore, we conclude that the DI, based on the quantification

of corm discoloration is the most reliable method to assess disease severities in banana – *Fusarium* interactions.

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Supplemental Table 1. Banana accessions that were evaluated in the comparative inoculation trials

Genotype	Genome composition	Source	Habitus
A-11	AA	CIRAD	Hybrid
Borneo	AA	Bioversity	Wild
IND 110 cv Rose	AA	CIRAD	Landrace
Musa ornata	AA	USDA-ARS	Wild
Pahang *M. acuminata* CMR	AA	CIRAD	Wild
Pisang lilin	AA	CIRAD	Landrace
Cavendish Grand Naine	AAA	Rahan Meristem	Cultivar
Cavendish- GCTCV-247	AAA	Queensland	Cultivar (somaclone)
Cavendish-Formosana	AAA	Queensland	Cultivar
Cavendish-Williams	AAA	Queensland	Cultivar
FHIA-25	AAA	Queensland	Hybrid
Lacatan	AAA	USDA-ARS	Cultivar
CRBP 39	AAAB	USDA-ARS	Hybrid
FHIA-18	AAAB	Queensland	Hybrid
FLF	AAAB	Queensland	Hybrid
PV-42-320	AAAB	USDA-ARS	Hybrid
Cachaco	AAB	Bioversity	Cultivar - Bluggoe
Pisang Lawadin	AAB	Bioversity	Landrace
X-17	ABA	CIRAD	Hybrid

Supplemental Table 2. Characteristics of the TR4 (*Fusarium odoratissimum)* – banana interaction experiments for each investigated inoculation method.

Method[1]	*Inoculum dose*	*LP*[2]	*FST*[3]	*Chlorosis*		*Corm Disc%*	*DI*[4]		*Corm dry Weight*[5]	*Ct values*
				%	*Class*		*%*	*Group*		
DM + Soil	1 x 10^3 spore ml^{-1}	3	10	54,1 B	III	17,3 A	36 BC	I	8,3 G6	21,19 DEF
	1 x 10^4 spore ml^{-1}	2	7	91,7 CD	IV	76,7 CD	96 E	III	2,1 ABCD	19,38 CDE
	1 x 10^5 spore ml^{-1}	2	5	100 D	IV	91,7 D	100 E	III	1,0 AB	18,77 BCD
	1 x 10^6 spore ml^{-1}	2	5	100 D	IV	88,3 D	100 E	III	0,7 A	18,61 ABC
DM + Sand	1 x 10^3 spore ml^{-1}	3	10	40,7 AB	II	1,5 A	8 A	I	6, 4 EFG	24,71 GH
	1 x 10^4 spore ml^{-1}	3	10	41,8 AB	II	6,1 A	24 AB	I	6,6 EFG	24,73 GH
	1 x 10^5 spore ml^{-1}	3	6	91,7 CD	IV	57 BC	92 E	III	2,6 D	15,81 AB
	1 x 10^6 spore ml^{-1}	2	7	100 D	IV	89,1 D	100 E	III	1,4 ABCD	16,98 ABC
PM + Soil	1 x 10^3 spore ml^{-1}	3	10	31,0 A	II	3,5 A	20 AB	I	7,3 FG	24,20 FGH
	1 x 10^4 spore ml^{-1}	3	10	41,6 AB	II	7,4 A	34 AB	I	6,6 EFG	25,05 H
	1 x 10^5 spore ml^{-1}	3	10	57,1 AB	III	20,5 A	62 CD	I	6,1 EFG	22,43 EFG
	1 x 10^6 spore ml^{-1}	3	6 - 10	77,3 B	IV	45,99 B	88 D	III	4,6 EFG	19,74 DE
CM + Soil	2,5 g / L^{-1} soil	2	6	76,7 C	IV	46,0 B	80 D	II	5,6 EF	18,29 ABC
	5 g / L^{-1} soil	2	7	95,0 D	IV	82,1 CD	96 E	III	2,2 BCD	16,44 ABC
	10 g / L^{-1} soil	2	8	95,0 D	IV	60,5 BC	96 E	III	2,5 CD	15,06 BCD
	20 g / L^{-1} soil	2	5	100 D	IV	89,5 D	100 E	III	1,0 ABC	15,32 BCD
KM + Soil	3 kernel/ L^{-1} soil	4	10	48,8	II	20,6	-	I	-	NI
	6 kernel/ L^{-1} soil	4	10	39,8	II	4,6	-	I	-	NI
	10 kernel/ L^{-1} soil	3	10	43,5	II	12,2	-	I	-	NI
	20 kernel/ L^{-1} soil	3	10	42,6	II	11,8	-	I	-	NI
Controls	DM/Soil/water	1	10	16,2	I	NA	-	-	8,3	-
	DM/Sand/water	1	10	15,7	I	NA	-	-	8,2	-
	PM/Soil/water	-	10	19,0	I	NA	-	-	7,7	-
	Mock[7]	-	10	13,7	I	NA	-	-	5,2	-

1. DM: dipping method soil, DMS: dipping method sand, PM: pouring method, CM: chlamydospore method, KM: kernel method. 2. Latency period (LP): week in which at least one plant started to show external symptoms. 3. Final sampling time (FST), weeks after inoculation in which half of the plants were harvested. 4. Disease Index (DI). 5. Corm dry weight of the entire corm in grams. 6. Significance, values within a column followed by the same letter were not significantly different according to LSD test at P=000.1. 7. Untreated plant. NA= not applicable; NI = not included; - not measured.

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CHAPTER 4

Resistance in banana to *Fusarium* spp. causing Panama Disease: Triploid accessions

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Abstract

Bananas belong to the genus *Musa* and comprise many wild seeded species as well as all manifold seedless and therefore edible dessert varieties that are eaten as fresh fruits besides the many varieties that are processed before consumption such as cooking, frying and brewing varieties. Therefore, bananas are undeniably among the most important fruits in the world and are a major staple food for millions of consumers in developing economies. Panama disease is one of the most important diseases of banana and is caused by a suite of soil-borne *Fusarium* fungi, previously known as *F. oxysporum* f. sp. *cubense* (*Foc*). The disease decimated the 'Gros Michel'-based industry in Central and South America in the early 20th century, which still is one of the most devastating botanical epidemics in history, with major societal implications. Eventually, the identification and subsequent adoption of the resistant Cavendish banana cultivars helped the industry to overcome the havoc. Fifty years ago, a new *Fusarium* species, which is pathogenic on Cavendish cultivars, surfaced in production fields in Asia. This new species comprising Tropical Race 4 (TR4), is responsible for a new Panama disease pandemic and is threatening the global banana industry once again. Despite TR4's well-documented effect on important cultivars in the Cavendish subgroup (e.g., 'Williams', 'Valery', 'Grand Naine'), little is known about how it affects other lesser-known triploid *M. acuminata* (AAA) cultivars or other prominent subgroups such as plantains (AAB) and cooking bananas (ABB). Therefore, an urgent need exists to phenotype a broad range of cultivated banana genetic resources with TR4 to possibly identify and deploy resistance. We screened 120 triploid banana accessions, representing a broad range of cultivated banana subgroups in greenhouse experiments. In addition, a subset of 36 accessions was also screened with race 1 isolates. We defined a disease index <25% as an adequate level of resistance thereby adopting the response of Cavendish bananas to race 1 as a reference score because these are successfully produced around the world even in heavily infested soils. The results showed a wide range of genetic variation for TR4 resistance, independent of the genomic composition or taxonomical position of the tested germplasm as well as among somaclones. However, we did not identify any resistance in the AAB sweet acid bananas, comprising Pome, Silk and Mysore Subgroups as well as Bluggoes. Seventeen (14%) accessions showed required levels of TR4 resistance and 26 out of the 36 accessions (70%) showed appropriate resistance to race 1. Our data provide a foundation for advanced genetic analyses of the identified sources of resistance and their deployment as parents in breeding programs aiming at developing new competitive banana varieties that meet consumer demands at domestic and international markets.

Introduction

Bananas belong to the genus *Musa* and are undeniably the most important freshly consumed fruits worldwide ((FAOSTAT 2016)/ *Banana consumption*). However, they are also a major staple food for millions of consumers in developing countries either as fresh fruits, or after processing through cooking, frying or brewing. As such, bananas are ranked as the eighth most important food crop worldwide and are the third major staple in many developing economies to sustain daily life (Ploetz and Pegg 2000). Bananas, including dessert and the abovementioned cooking types, are grown in more than 135 countries on five continents and in some places, they are the main component of the daily diet (Lescot 2004). Therefore, bananas are very important for food security next of being a cash crop for domestic and export markets and the gross production value reached more than 35.2 billion US$ from an overall yield of at least 145 million tonnes (FAOSTAT 2016)

The vast majority of current varieties originates from interspecific and/or intraspecific hybridization between *M. acuminata* (A genome) and *M. balbisiana* (B genome), two wild diploid (2n=2x = 22) species native to Southeast Asia (Perrier *et al.,* 2011). This hybridization resulted in the diversity of modern cultivated triploids, including *M. acuminata* triploid cultivars (AAA) such as 'Gros Michel' and the Cavendish varieties and interspecific *M. acuminata* x *M. balbisiana* triploids (AAB and ABB) comprising plantain and cooking bananas, respectively (Buddenhagen 1990, d'Hont *et al.,* 2000). Today, more than 80% of the global banana production relies on just a few triploid varieties in no more than four subgroups coming from at most seven to 14 meiotic events (Bakry and Horry 2014), mostly in the Cavendish and plantain subgroups (Lescot 2017). Most cultivated diploid, and hence parthenocarpic banana varieties have exclusively been vegetatively propagated. Therefore, the arisen phenotypic diversity is merely due to somaclonal variation (Bakry and Horry 2014), and the acquired differentiation leads rapidly to the identification of true subgroups. Varieties belonging to the same subgroups share the same sexual ancestor and have identical or very similar nuclear and cytoplasmic profiles (Faure *et al.,* 1993). Due to this narrow genetic base and combined with global monoculture practices the industry at large was and still is vulnerable to any changing climatic condition or emerging disease (Bakry and Horry 2014, Ordóñez *et al.,* 2015b)

Worldwide banana production is suffering from several constraints, and principal among them is Fusarium wilt (most popularly known as Panama disease). The disease is caused by a suite of soilborne *Fusarium* fungi formerly known as different genotypes or vegetative compatibility groups (VCGs) of *F. oxysporum* f. sp. *cubense* (*Foc*) (Ordóñez *et al.,* 2015b, Maryani *et al.,* 2018). Pathogenic diversity among these *Fusarium* spp. towards a set of banana accessions resulted in the assignment of several physiological races (Waite and Dunlap 1953, Hennessy *et al.,* 2005) and VCGs reflected the genetic proximity based on the formation of heterokaryons (Correll *et al.,* 1987). Three races (R1, R2, and R4) have been reported affecting edible banana cultivars (Ploetz 2006) and over 20 VCGs have been established to date (Fourie *et al.,* 2009), but whole genome sequencing as well as multilocus genotyping and genotyping by sequencing have resulted in additional genotypes that eventually have contributed to an entirely new nomenclature of the causal *Fusarium* spp. (Maryani *et al.,* 2018). Race 1 species caused one of the most destructive epidemics of all times in the previous century in 'Gros Michel' plantations in Central America (Stover 1962, Ploetz 2000). TR4 currently threatens the successive Cavendish

plantations of the large-scale export industry as well as those of small holder farmers that are also frequently planted to other locally preferred banana varieties (Ploetz *et al.,* 2015).

As with other races in the past, there is no doubt that the dissemination of TR4 will continue. For example, for many years TR4 was restricted to Southeast Asia and Northern Australia where it had caused considerable damage on Cavendish monocultures (Ploetz 2000). However, recent reports have shown its spread outside Southeast Asia (Butler 2013, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a) as well as its expansion inside Southeast Asia (Chittarath *et al.,* 2017, Hung *et al.,* 2017, Mostert *et al.,* 2017) but more advanced forensic studies also underscore the origin of several incursions in the Greater Mekong area (Zheng *et al.,* 2018). Since most of the global export banana industry production relies on clones which are genetically closely related to each other, the continued spread of TR4 seriously threatens the future of not only the banana industry but also small farmers that depend on it (Butler 2013, Lescot 2017). Characteristic symptoms of Panama disease include chlorosis of the foliage and wilting of petioles followed by eventual death of pseudostem and rhizome. In the later stages of infection, chlamydospores develop in infected tissues and they play an important role in long-term survival of the fungus (Waite and Dunlap 1953, Stover 1962, Ploetz 2007, Ploetz 2015). Since bananas are perennial crops and the soil-borne causal *Fusarium* spp. have a polycyclic lifestyle, disease management has essentially failed throughout history (Ploetz *et al.,* 2015). Of course, quarantine and exclusion are key in limiting pathogen spread, but Cavendish banana cultivars have shown for decades their productivity on race 1 infested soils due to their high level of resistance (Ploetz 2005, Ploetz 2015). However, phenotyping of banana germplasm is limited and hence the resistance of most cultivars, and many of their wild relatives, to the different Panama disease races is unknown and also poorly understood. For instance, there is no report on the genetic basis of Cavendish to race 1 and even genetic studies of resistance in fertile diploids has only started recently (Swarupa *et al.,* 2013, Fraser-Smith *et al.,* 2014, Zhang *et al.,* 2016, Araújo *et al.,* 2017, Li *et al.,* 2017). Resistance to TR4, however, has resulted in the identification and cloning of the first resistance gene and it transfer to Cavendish protected its collapse in a multiyear field trial (Dale *et al*., 2018). In addition, clonal variation resulted in substantial genotypic diversity in established Cavendish cultivars, plantains and cooking types (Hwang 1991, Vuylsteke *et al.,* 1991, Hwang and Ko 2004). However, the resistance of somaclonal variants have a partial nature and their quantitative response depends on the inoculum concentration of the pathogen (Ploetz 2015, Dale *et al.,* 2017). Moreover, their expansion often results in less attention for the required quarantine efforts and thereby enlarges the problem over time. Additionally, these somaclones often show inferior horticultural characteristics (Pillay and Tenkouano 2011, Ploetz 2015).

More than 1,200 banana cultivars are described of which most are naturally occurring diploid or triploid selections of *M. acuminata* or *M. acuminata* x *M. balbisiana* hybrids (Lescot 2017). The far majority of that germplasm has never been tested for resistance to race 1 or TR4. Here, we report on the phenotyping of a shortlist of banana germplasm, comprising the most important banana varieties/cultivars in three genomic groups (AAA, AAB, and ABB) with these races as a basis for advanced additional genetic studies and their exploration in breeding programs to extend the genetic diversity and hence, sustainability of global banana production.

Materials and methods

Plant material

The studied banana germplasm was obtained from a variety of institutions (Table 1) and comprised 120 triploid accessions divided over *M. acuminata* (AAA) and *M. acuminata* x *M. balbisiana* interspecific hybrids (AAB and ABB) representing 22 of the most well know subgroups (Table 2). Tissue culture plantlets of different accessions were obtained from different institutions and multiplied at the Laboratory of Plant Breeding at Wageningen University and Research, Wageningen, Netherlands (Table 2). Upon arrival, plants were removed from plastic containers and growth media was rinsed with water containing 1% hypochlorite and henceforward plantlets were acclimatized for ~2.5 months. Then plants were transferred directly to 1L pots and kept under greenhouse conditions at 26 ±2˚C and a relativity humidity of 85%, with a day length of 16 hours. 'Grand Naine' plants inoculated with TR4 and 'Gros Michel' inoculated with race 1 were included as positive controls, whereas water treated plants were used as negative controls.

Table 1. Institutions and international organizations that made banana accessions available for surveying resistance to Panama disease.

Donating organization	Country	Acronym	Internal code	Number of accessions
Bioversity International, Belgium	Belgium	Bioversity	ITC[1]	26
Agricultural Research Centre for International Development & Vitropic	France	CIRAD/VITROPIC	CIRAD	2
United States Department of Agriculture, Puerto Rico	Puerto Rico	USDA-ARS	TARS[2]	58
National Banana Corporation S.A.	Costa Rica	CORBANA	N.A[3]	1
Cultivos y Tecnología Agraria de Tenerife, S.A.	Spain	CULTESA	N.A	3
Cuban Plant Health Research Institute	Cuba	INISAV	N.A	4
Kenya Agricultural Research institute	Kenya	KARI	N.A	3
Queensland Department of Agriculture, Fisheries and Forestry	Australia	DAFF	DAFF	14
Rahan Meristem	Israel	NA	N.A	5
Texas A&M University	USA	TAMU	N.A	4

[1]International Transit Centre, [2]Tropical Agriculture Research Station [3] N.A. not applicable/unknown

Table 2. Genome composition, groups and subgroups included in this study disease Index column values with the different letter were significantly different), In total 120 accessions were tested representing 22 groups or/and subgroups belonging to the genome groups AAA, AAB, and ABB

Genome	Group/subgroup	Number tested accessions	DI % TR4
AAA	Cavendish	45	
	Mutika (EAHB)*	11	
	Gros Michel	9	63,79[a]
	Ibota	4	
	Red	4	

	Rio	1	
AAB	Plantain	9	
	Pome	6	
	Silk	3	
	Mysore	2	
	Iholena	1	49,92[b]
	Lawadin	1	
	Laknao	1	
	Maia Maoli/Popoulu	1	
	Pisang Kelat	1	
ABB	Bluggoe	8	
	Peyan	3	
	Pisang Awak	3	
	Pelipita	2	60,80[ab]
	Ney Mannan	2	
	Saba	2	
	Tiparod	1	

*East African Highland Bananas A: Genome A from *Musa acuminata* B: Genome B from *Musa balbisiana*

Statistical analysis

The experiments were carried out following a completely randomized block design. To avoid cross contamination, controls were physically separated. We tested at least three independent plants of each genotype. Final scores were calculated as Disease Index (DI) in percentages, which do not meet the requirements of normal distribution and equal variance of the data for comparison of the means. This was checked prior to the statistical analyses and did not influence the conclusions after using an ANOVA model with genome composition or subgroups as a main factor using Genstat 5 (Payne 1993)

Fungal strains and inoculation

Two *Fusarium* strains were used for the phenotyping and were obtained from the Wageningen University and Research collection. The TR4 strain was the reference *F. odoratissimum* II-5 strain originating from Indonesia. The race 1 strain originates from Brazil, where it was recovered from 'Maçã' (AAB, Silk subgroup) bananas and coded as CNPMF 000-8-01-R1 (FOSC). Both strains were grown following a new high throughput protocol developed by García-Bastidas *et al*. (Chapter 3). Inoculations were performed by pouring a 200 ml (10^6 conidia.ml^{-1}) spore suspension directly into the pot. Additionally, five *Fusarium* infected maize kernels were incorporated in the soil of each pot carrying one banana plant as suggested by Dita *et al.* (2010). Prior to the phenotyping screen both strains were confirmed as race 1 and TR4 by testing them on 'Gros Michel' (AAA, Gros Michel subgroup) and on 'Grand Naine' (AAA, Cavendish subgroup), respectively.

Disease assessment

The response of the germplasm was evaluated by scoring internal and external symptoms. Externally, plants were monitored for chlorosis and wilting as well as possible splitting of the pseudostem. Internally, plants were assessed by scoring the grade of

discoloured corm tissue at 8 to 13 weeks after inoculation (wai), when controls exhibited > 80% foliar chlorosis following García-Bastidas *et al*. (Chapter 3). Individual pictures of each plant and their affected rhizomes were taken to score the severity by calculating the quantitative area of discoloured corm tissue by Image J 1.49 (http://rsb.info.nih.gov/ij/) image analysis. We then used McKinney's Formula (McKinney 1923) to determine the DIs of each interaction, which were then used to divide germplasm as highly resistant (HR= DI < 5,0%); resistant (R= 5 < DI ≤ 24±1 %); moderately susceptible (MS=25 < DI ≤ 44±1 %); susceptible (S=45 < DI ≤ 64±1%); very susceptible (VS=65 < DI ≤ 84±1 %) or extremely susceptible (XS= DI ≤ 85%).

Post inoculation TR4 diagnostics

To confirm the presence of TR4 in inoculated plants, we randomly sampled the rhizomes, roots, pseudostems and petioles from inoculated plants and the controls during the final scoring. These samples were lyophilized and used in TR4 diagnostic as suggested by Dita *et al.* (2010) to confirm the presence of TR4.

Results

The phenotyping assays were performed over a period of four years and comprised 22 separate trials. They always included the references 'Grand Naine' and 'Gros Michel' inoculated with race 1 and TR4 along with water controls. These showed the typical differential responses in every experiment (Figure 1). Control plants never showed any external or internal symptoms and the susceptible responses of both cultivars were not significantly different Cavendish having a DI of 90% with TR4 and 'Gros Michel' had a DI of 78,8% with race 1.

However, both showed a significant difference with race 1 as 'Gros Michel' was extremely susceptible (DI=85.7%) whereas Cavendish is highly resistant (DI=6,4) (Figure 2). Moreover, the molecular diagnostic confirmed the presence of TR4 in all inoculated Cavendish controls, but no amplicon was observed in the water controls and after inoculation with race 1. This is an important observation as it is the reference for all other assays under greenhouse conditions. TR4 was successfully detected in the rhizome (including roots) of most of the other assayed genotypes and in most cases TR4 was also detected in the vascular system of the pseudostems and petioles, especially in genotypes with DIs > 50% (scores 4, 5, and 6). Nevertheless, we could not always confirm the presence of TR4 and in some cases we had to sample and re-isolate the fungus before confirmation.

We screened 120 triploid banana accessions, which were on average moderately to highly susceptible to TR4. Interestingly, the plantain subgroup (AAB) was significantly less affected than the AAA genotypes but did not differ from the ABB subgroup (Table 2). However, despite these overall observations, we also observed significant genetic diversity within these classes, which ranged from highly resistant to very or even extremely susceptible (Supplemental table 1).

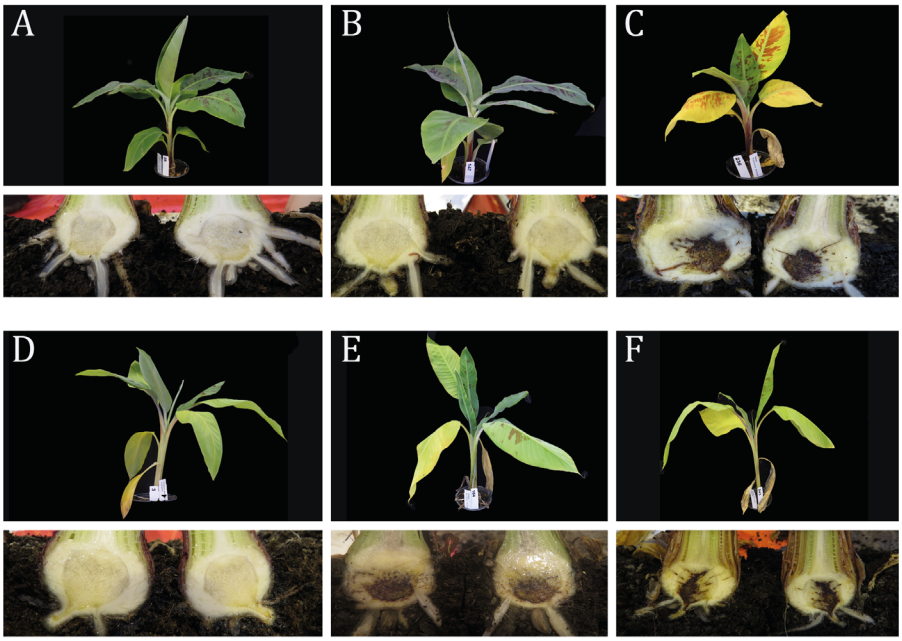

Figure 1. Differential responses of banana cultivars 'Grand Naine' and 'Gros Michel' after inoculation with race 1 isolate CNPMF 000-8-01-R1 and *F. odoratissimum* TR4 isolate II-5 at 10 weeks after inoculation. Top rows show foliar symptoms and bottom rows show cross sections of corms. A-C. Cultivar 'Grand Naine' water control (A) resistant response to race 1 (B) and susceptible response to TR4. D-F. Cultivar Gros Michel water control (D) susceptible responses to race 1 and TR4, E-F, respectively.

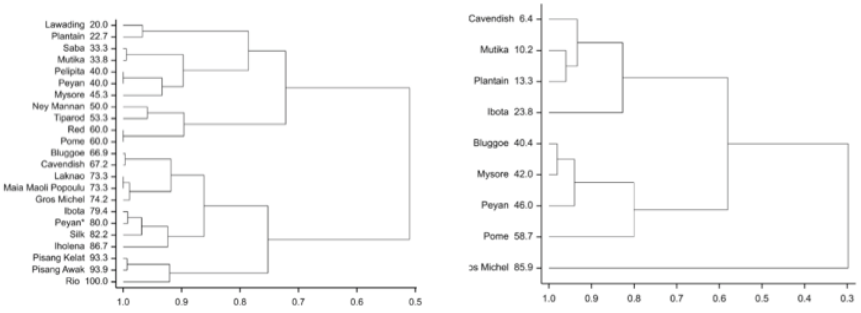

Figure 2. Cluster of subgroups according to their response to *F. odoratissimum* TR4 isolate II-5 (left) and race 1 isolate CNPMF 000-8-01-R1 values in percentages of Disease index

AAA bananas

Cavendish subgroup

All accessions were tested to race 1 with DIs < 17,5% (actual area of affected tissue <5%) (Table 2). However, 41 of the 45 tested accessions showed DI > 25% and hence were susceptible to TR4. Some accessions showed moderate susceptibility (e.g. 'Gal', 'GCTCV-

119', 'MA13' DI from 34 to 42,35%) but we never observed immunity to TR4 as was observed for race 1 (see Figure 3G and E). Nevertheless, some Cavendish clones such as 'Dwarf Cavendish', 'Dwarf Nathan' and two transgenic Cavendish lines were resistant to TR4 with DIs ≤ 24 (Figure 3).

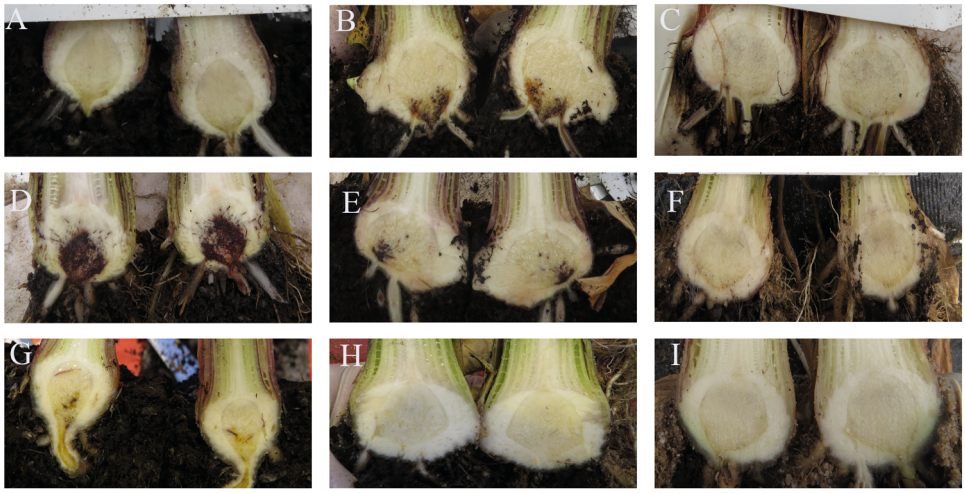

Figure 3. Differential responses of banana selection after inoculation with *F. odoratissimum* TR4 isolate II-5 at 10 weeks after inoculation. Pictures show cross sections of corms. (A), 'Dwarf Nathan'-AAA Cavendish-Somaclone; (B), GCTCV-AAA Cavendish-Somaclone; (C), 'Verdin' AAA-Red; (D), 'Nchumbahaka' AAA – 'Mutika' EAHB; (E) 'M13'-AAA Cavendish-Mutant; (F) 'Dwarf superplantain'-AAB plantain; (G), 'Gal' (AAA Cavendish-mutant); (H), 'Igitsiri (intuntu)'-AAA Mutika EAHB; (I), Congo 300-AAB plantain.

Mutika lujugira subgroup (EAHB)

The responses to TR4 of the 10 accessions representing this group varied from highly resistant to susceptible. For example, the clone 'Igitsiri-Intuntu' was highly resistant under our conditions (Figure 2), but 'Nchumbahaka' was very susceptible with a DI of 70% (Figure 3). In summary, we identified four accessions with resistance to TR4. Additional screens of six genotypes with race 1 showed that five accessions were resistant, but 'Mpologoma' showed a significantly higher DI with race 1 than for TR4 (Supplementary Table 1).

Gros Michel subgroup

The nine clones that belong to the Gros Michel subgroup were tested with TR4 and three accessions were also tested with race 1. No resistance was identified with either strain and DIs were always ≥ 60%.

Other AAA groups/subgroups

The remaining nine AAA accessions were at least susceptible, except for 'Verdin' that with a DI of 13,3% was categorized as resistant.

AAB bananas

Plantain subgroup

Out of the nine plantain varieties originating from Africa and Latin America, 'Congo -300' and 'Dwarf superplantain' (Figure 3F) were highly resistant and 'French dwarf' and 'Big Ebanga' were resistant. The remaining accessions reached maximum scores of moderate susceptibility to TR4, whereas additional screens with race 1 were always lower and ranged from highly resistant to resistant (DIs 4-18%).

Pome, Silk, Mysore and Lawadin subgroups

The remaining AAB accessions were moderately to extremely susceptible to TR4, except for 'Pisang Lawading' which was resistant with a DI of 20%. Three of these accessions were also moderately to very susceptible to race 1.

ABB bananas

Bluggoe subgroup

The eight Bluggoe accessions were susceptible to extremely susceptible to TR4 and only 'Cachaco', which is extensively cultivated in Latin America, is resistant to race 1.

Other ABBs

We observed a wide range of variation among the other accessions. Interestingly, we found that the accession 'Pisang Gajih Merah' is highly resistant to TR4 but is susceptible to race 1 (Figure 4) and the two tested Pelipita accessions showed DIs=13 (resistant) or DI=66 (very susceptible). A similar observation applies to accessions ('Blue Java' and 'Blue Torres Straight Island') of the 'Ney Mannan' subgroup that also showed contrasting DIs (20 vs. 80).

Figure 4. Differential responses of the accession 'Pisang Gajih Merah' after inoculation with (A) *F. odoratissimum* TR4 isolate II-5 (B) race 1 at 10 weeks after inoculation. (C) Control plant treated with water. Pictures show cross sections of corms.

Discussion

Food security is threatened by the increasing incidence of pests and pathogens (Gurr *et al.,* 2011, Bebber *et al.,* 2014, Fisher *et al.,* 2016). Despite immense progress and effort in crop protection, huge crop losses are recorded annually (Oerke 2006, Anten and Vermeulen 2016). The first Panama disease epidemic in 'Gros Michel' in the previous century was caused by at least six different *Fusarium* species that all are race 1 strains (Stover 1962, Maryani *et al.,* 2018). The underlying factor was the genomic uniformity of the vast monoculture plantations. Eventually, this epidemic that lasted for several decades was unstoppable and hence pushed the industry nearly to bankruptcy and had immense geo-political and societal implications (Ploetz 2015, Ploetz *et al.,* 2015). The discovery of Cavendish clones with resistance to the race 1 strains was initially welcomed with reluctance due to the required logistic modifications but turned out to be the only solution to stop the epidemic. Since then, these clones – including e.g. 'Lacatan', 'Valery', 'Williams', 'Poyo', and 'Grand Nain' - have become increasingly popular and currently dominate international and domestic markets (Lescot 2017). However, they essentially represent just one genotype that only differ from each other in plant height or bunch and fruit shape (Bakry *et al.,* 2009). However, besides these morphological differences, also dissimilar responses to plant disease were observed. Essentially, however, these small genetic differences did not cancel out overall genetic uniformity, which maintained the

genetic vulnerability of the banana sector at large for any newly upcoming threat. Indeed, several studies already confirmed the susceptibility of many banana varieties to TR4 and other *Fusarium* species or genotypes (Zuo *et al.,* , Liew 1996, Smith *et al.,* 1999, Wu *et al.,* 2010, Li *et al.,* 2015, Zuo *et al.,* 2018). These studies not only confirmed the overwhelming vulnerability of the commercial Cavendish cultivars to Panama disease caused by TR4, but also addressed the susceptibility of many local varieties and wild types that play an essential role in smallholder communities (Walduck and Daly 2007, Molina *et al.,* 2011, Riska and Hermanto 2012, Li *et al.,* 2015, Ploetz *et al.,* 2015). Therefore, surveying resistance to TR4 is not only important to identify potential replacements of the Cavendish clones, but primarily also to identify potential sources of resistance that can be unravelled and deployed in breeding programs.

Recent studies have shown the response to TR4 of several cultivars under greenhouse and field conditions (Li *et al.,* 2015, Zuo *et al.,* 2018). Nevertheless, despite these efforts, still little is known about the resistance across all different subgroups of the main cultivated and commercially available edible bananas, including dessert and cooking types. Additionally, exploring resistance to TR4 in germplasm derived from mutagenesis or genetic modification programs is unexplored. A drawback of many previous studies is that these were mostly executed under field conditions. Initially, greenhouse evaluations were therefore perceived as artificial, but are gradually also accepted for studying banana-*Fusarium* interactions due to efficiency and repeatability and hence reliability of the data. In contrast to field evaluations, bioassays under controlled conditions are rapid and less costly (Amorim *et al.,* 2009, Li *et al.,* 2015). Factors that generate obvious variation in the field do not occur under greenhouse conditions (Crouch *et al.,* 1999, Dita *et al.,* 2011, Zuo *et al.,* 2018). Our methodologies successfully generated reproducible data and varied from immune to extremely susceptible. Both strains produced the typical symptoms in compatible interactions and were in accord with other studies that were performed under *in-vitro* (Wu *et al.,* 2010) or greenhouse conditions (Smith *et al.,* 2008, Li *et al.,* 2015, Zuo *et al.,* 2018). As expected, most accessions were susceptible to TR4 but those that showed moderate susceptibility require further investigation. These may slow down the disease, but still enable fungal proliferation and hence do not stop it. Besides, deploying of moderately susceptible accessions by small holders that are often ignorant of the ongoing disease threats does not encourage the adoption of quarantine measures and hence, actively promoting such materials actually only worsens the situation, albeit that short-term successes may initially be observed (Dale *et al.,* 2017, Ploetz 2015)

Our study included 120 accessions that represent the currently commercially available banana cultivars, traditional landraces and a small representation of bananas genetically modified (GM). Thirty-seven accessions were also tested with race 1 and, as expected, more than 85% of the accessions were susceptible to TR4. In contrast, 70% of the suite of banana accessions were resistant to race 1 and since we used the reference controls ('Grand Naine' and 'Gros Michel' with TR4 and race 1) throughout the study, our data are reliable. However, we cannot extrapolate to other *Fusarium* spp. Clearly our data do challenge the current race concept as genetic variation was often encountered within subgroups, for example Bluggoe accessions are generally considered to be susceptible to race 2 strains (Ploetz 2006), but we clearly showed that some are also susceptible to race 1. In addition, we identified a few cases where accessions were susceptible to TR4, but resistant to race 1. Continued phenotyping will contribute to the identification of more

"differential" responses, but what is foremost required is unveiling the genetic basis of these responses, which will greatly contribute to gene discovery and hence deployment.

Musa acuminata triploids are considered to be generally susceptible to TR4 due to the susceptibility of the Cavendish clones and 'Gros Michel' (Stover and Waite 1960, Ploetz 1990). However, we showed that they clearly differ in their response to different *Fusarium* strains. As mentioned above, the limiting factor is the understanding of the underlying genetics as both groups are genetically closely related and are in fact siblings as they originate from a three-way cross between a *M. banksii* x *M. zebrina/M. microcarpa* parent and a *M. malaccensis* parent (Bakry and Horry 2014). In some cases, different *M. acuminata* donors have been suggested, but this has not been confirmed. Nevertheless, it is well known that edible bananas originate from two seeded ancestors (Perrier *et al.,* 2011), but the donors and possible speciation and domestication of the modern varieties might also result from other parental combinations. For example, recently, a gene that confers resistance to TR4 was isolated from a wild *M. acuminata* spp. *malaccensis* accession and was transferred into the Cavendish cv. Williams by *Agrobacterium tumefaciens*-mediated transformation (Dale *et al.,* 2017). In addition, this study suggests that this gene might already be present in the susceptible Cavendish varieties, but that its expression is attenuated, which clearly makes it a target for genome editing through CRISPR-Cas9 strategies (Bortesi and Fischer 2015)

Clonal variation has resulted in remarkable diversity, among the overall clonal banana varieties and have resulted in cultivars with improved agronomic traits such as dwarfism, shorter crop duration, higher yields and better organoleptic characteristics (Smith and Hamill 1993, Walther *et al.,* 1996). Recently, there is a strong focus on increasing disease resistance through similar processes, particularly to Panama disease (Hwang 1991, Toyoda *et al.,* 1991, Bhagwat and Duncan 1998, Hwang and Ko 2004, Saraswathi *et al.,* 2016). Again, the underlying processes are unclear but might be due to epigenetic regulation (Zuo *et al.,* 2018). To our knowledge, we are the first to identify existing Cavendish clones as resistant to TR4, such as 'Dwarf Cavendish' and 'Dwarf Nathan'. This demonstrates that other somaclonal strategies may lead to resistant germplasm. Indeed, somaclonal variants of Cavendish are being promoted as alternative for the regular Cavendish clones to stop the TR4 epidemic (Hwang 1991, Bhagwat and Duncan 1998, Hwang and Ko 2004), but grossly underestimate the epidemiological pitfalls of partially resistant germplasm to soil-borne disease in a perennial crop of which Panama disease in banana is the ultimate example (Ploetz 2007, Ploetz 2015). Moreover, they leave the overall genetic uniformity towards other diseases such as black Sigatoka untouched. Durable resistance improvement programs address these threats simultaneously. Taken together, we propose to use the current data as a platform to initiate the urgently required genetic studies to unravel the genetic basis of resistance to Panama disease, which will be a basis for progress and enhanced disease resistance in this globally important food crop.

Contrary to the opinion of a number of authors (Pegg and Langdon 1987, Smith *et al.,* 1999, Zuo *et al.,* 2018) immunity is an aspect of the banana-*Fusarium* pathosystem, which is also clearly exemplified by the Cavendish – race 1 interaction. In fact, this explains the historical efficacy of these clones to stop the race 1 epidemic, which is still epitomised by the fact that they can be grown in soils that are infested with race 1 strains in most producing countries. Also, contrary to previous suggestions (Molina *et al.,* 2014), we

show that EAHB are not generally resistant to TR4. As expected, they show genetic diversity. Hence, some clones are susceptible, and others are resistant. Our data show that 'Ingagara', 'Igitsire (intuntu)' and 'Kazirakwe' are resistant to TR4, which confirm the data of Zuo *et al* (2018) but contradict their claims as 'Inkira' and 'Mbwazirume' were susceptible under our conditions. These conflicting data likely result from different experimental conditions and procedures, underscoring the need for standardization for comparative reasons (García-Bastidas *et al*., Chapter 3). From that point of view, greenhouse trials are preferred over lengthy and costly field trials with their manifold environmental fluctuations. Hence, the results observed in the EAHBs are important and relevant since the incursion and likely expansion of TR4 across the continent. Therefore, these results call for a wider and rapid evaluation program of all African germplasm to anticipate on the upcoming threat of TR4. There are more than 160 clones of different identified plantains in the CARBAP field collection in Cameroon (the widest plantain collection in the world) and it is likely that it contains new sources for TR4 resistance.

Finally, our data show that the genome composition of bananas apparently cannot predict susceptibility to Panama disease. We identified resistant and susceptible accessions in virtually every subgroup. Interestingly, we also showed that race 1 resistance has a higher frequency across the tested germplasm, but we also identified some germplasm such as 'Pisang Gajih Merah' (ABB), which was immune to TR4 but susceptible to race 1, which was also observed under field conditions (Prof. Andre Drenth, University of Queensland, personal communication). Albeit a unique observation, it is principally of interest as it suggests differences in genetic control of resistance towards these two races and hence the potential to combine them in new germplasm. This is important, as resistance to race 1 strains is a prerequisite for modern banana production. Thus, the sooner these genes are identified the better it is to enhance and speed-up diversifying breeding efforts to eventually replace the dominating monocultures by a suite of competitive new banana varieties.

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Supplemental table 1. Passport information and disease response of banana (*Musa* spp.) in greenhouse phenotyping arrays with Tropical Race 4 (TR4) and race 1 (R1) of *Fusarium* spp. causing Panama disease. Accessions were clustered in groups in order to identify different responses among and within subgroups.

Provider	Provider Code	Group/Subgroup	Status	Accession name	Genome Formula	TR4		R1	
						DI%[1]	LR[2]	DI%[1]	LR[2]
USDA-ARS	TARS16525	Cavendish	Cultivar	Dwarf Cavendish	AAA	13,33	R	-	-
TAMU	BCL 161 #4	Cavendish	GMO	Grand Naine	AAA	20	R	-	-
TAMU	BCL 161 #5	Cavendish	GMO	Grand Naine	AAA	20	R	-	-
Queensland	n.a	Cavendish	Cultivar	Dwarf Nathan	AAA	24	R	12,5	R
Rahan	Gal	Cavendish	Mutant	Gal	AAA	28,57	MS	2	HR
Bioversity	ITC1442	Cavendish	Somaclone	GCTCV-106	AAA	34	MS	5,5	HR
CIRAD	n.a	Cavendish	Mutant	MA13	AAA	36	MS	16	R
Queensland	DAFF1860	Cavendish	Somaclone	GCTCV-119	AAA	42,35	MS	0	HR
USDA-ARS	TARS 17426	Cavendish	Cultivar	Guineo enano	AAA	53,33	S	-	-
Bioversity	ITC1329	Cavendish	Hybrid	Novaria	AAA	53,33	S	0	HR
Cultesa	n.a	Cavendish	Cultivar	Palmerita	AAA	54	S	4	HR
Cultesa	n.a	Cavendish	Cultivar	Ricasa	AAA	54	S	2	HR
Queensland	DAFF1191	Cavendish	Somaclone	GCTCV-215	AAA	56,67	S	17,5	R
Cultesa	n.a	Cavendish	Cultivar	Dwarf Cavendish	AAA	60	S	4	HR
USDA-ARS	TARS 17402	Cavendish	Cultivar	Dwarf Valery	AAA	60	S	-	-
USDA-ARS	TARS 17143	Cavendish	Cultivar	Enano Gigante	AAA	60	S	-	-
USDA-ARS	TARS 17395	Cavendish	Somaclone	Taiwanese somaclone	AAA	60	S	-	-
USDA-ARS	TARS 17414	Cavendish	Cultivar	Williams	AAA	70	VS	-	-
Queensland	DAFF1893	Cavendish	Somaclone	GCTCV-247	AAA	71,43	VS	-	-
USDA-ARS	TARS 17135	Cavendish	Cultivar	5-A	AAA	73,33	VS	-	-
USDA-ARS	TARS 17130	Cavendish	Cultivar	6-A	AAA	73,33	VS	-	-

Provider	Provider Code	Group/Subgroup	Status	Accession name	Genome Formula	TR4		R1	
						DI%[1]	LR[2]	DI%[1]	LR[2]
USDA-ARS	TARS 17159	Cavendish	Cultivar	8-A	AAA	73,33	VS	-	-
USDA-ARS	TARS 17167	Cavendish	Cultivar	9-A	AAA	73,33	VS	-	-
USDA-ARS	TARS 17163	Cavendish	Cultivar	Giant Governor	AAA	73,33	VS	-	-
USDA-ARS	TARS 17423	Cavendish	Cultivar	Volunteer Musa	AAA	73,33	VS	-	-
Rahan	n.a	Cavendish	Cultivar	Adi	AAA	76,92	VS	2	HR
USDA-ARS	TARS 17164	Cavendish	Cultivar	1-A	AAA	80	VS	-	-
USDA-ARS	TARS 17125	Cavendish	Cultivar	2-A	AAA	80	VS	-	-
USDA-ARS	TARS 17154	Cavendish	Cultivar	3-A	AAA	80	VS	-	-
TAMU	BCL 161 #3	Cavendish	GMO	Grand Naine	AAA	80	VS	-	-
TAMU	CED#3	Cavendish	GMO	Grand Naine	AAA	80	VS	-	-
USDA-ARS	TARS 17428	Cavendish	Cultivar	Monte Cristo Enano	AAA	80	VS	-	-
Rahan	n.a	Cavendish	Cultivar	Williams	AAA	80	VS	8	R
Queensland	DAFF1868	Cavendish	Cultivar	DPM25	AAA	83,33	VS	-	-
Queensland	DAFF1861	Cavendish	Hybrid	Formosana	AAA	85	VS	-	-
USDA-ARS	TARS 17158	Cavendish	Cultivar	Valery	AAA	86,67	XS	-	-
Queensland	DAFF1899	Cavendish	Cultivar	Williams	AAA	86,67	XS	-	-
USDA-ARS	TARS 17169	Cavendish	Cultivar	Ziv	AAA	86,67	XS	-	-
Queensland	DAFF349	Cavendish	Cultivar	Dwarf parfitt	AAA	90	XS	-	-
Rahan	n.a	Cavendish	Cultivar	Grand Naine	AAA	90	XS	10	R
USDA-ARS	TARS 18061	Cavendish	Cultivar	Johnson	AAA	90	XS	-	-
USDA-ARS	TARS 17168	Cavendish	Cultivar	Grand Naine	AAA	93,33	XS	-	-
Rahan	n.a	Cavendish	Cultivar	Jaffa	AAA	94,29	XS	6,67	R
Queensland	DAFF1876	Cavendish	Cultivar	CJ19	AAA	95	XS	-	-
Queensland	DAFF1888	Cavendish	Cultivar	Fai Palagi	AAA	95	XS	-	-
Bioversity	ITC0081	Mutika	Cultivar	Igitsiri (intuntu)	AAA	0	HR	-	-
Bioversity	ITC1355	Mutika	Cultivar	Kazirakwe	AAA	8,57	R	20	R
Bioversity	ITC0166	Mutika	Cultivar	Ingagara	AAA	10	R	6,67	R

Provider	Provider Code	Group/Subgroup	Status	Accession name	Genome Formula	TR4		R1	
						DI%[1]	LR[2]	DI%[1]	LR[2]
USDA-ARS	TARS 17409	Mutika	Cultivar	Ignamico	AAA	13,33	R	-	-
Bioversity	ITC0179	Mutika	Cultivar	inkira	AAA	28	MS	16	R
KARI	n.a	Mutika	Cultivar	Mbwazirume	AAA	33,33	MS	0	HR
KARI	n.a	Mutika	-	M9	AAA	40	MS	0	HR
USDA-ARS	TARS 17396	Mutika	Cultivar	Igcpoca	AAA	50	S	-	-
KARI	n.a	Mutika	Cultivar	Mpologoma	AAA	53,33	S	100	XS
Bioversity	ITC1546	Mutika	Cultivar	Kisukari usiniguse	AAA	65	S	18,33	R
USDA-ARS	TARS 17408	Mutika	Cultivar	Nchumbahaka	AAA	70	VS	-	-
USDA-ARS	TARS 17151	Gros Michel	Cultivar	4-R-2, 500	AAA	60	S	-	-
Bioversity	ITC0484	Gros Michel	Cultivar	Gros Michel	AAA	70	VS	100	XS
USDA-ARS	TARS 17152	Gros Michel	Cultivar	10-A	AAA	73,33	VS	-	-
USDA-ARS	TARS 17153	Gros Michel	Cultivar	Highgate	AAA	73,33	VS	-	-
USDA-ARS	TARS 17144	Gros Michel	Cultivar	Lacatan	AAA	73,33	VS	-	-
USDA-ARS	TARS 17141	Gros Michel	Cultivar	3-R-2, 500	AAA	75	VS	-	-
Corbana	n.a	Gros Michel	Cultivar	Gros Michel	AAA	78,75	VS	85,71	VS
USDA-ARS	TARS 17840	Gros Michel	Cultivar	Guaran enano	AAA	80	VS	-	-
Bioversity	ITC1122	Gros Michel	Cultivar	Gros Michel	AAA	84	VS	72	VS
INISAV	n.a	Ibota	Cultivar	Yangambi Km 5	AAA	53,33	S	20	R
Bioversity	ITC0662	Ibota	Cultivar	Khai Thong Ruang	AAA	77,5	VS	27,5	MS
Bioversity	ITC1336	Ibota	Cultivar	JD yangambi	AAA	86,67	XS	-	-
USDA-ARS	TARS 17844	Ibota	Cultivar	Yangambi	AAA	100	XS	-	-
USDA-ARS	TARS 17150	Red	Cultivar	Verdin	AAA	13,33	R	-	-
USDA-ARS	TARS 17392	Red	Cultivar	Cuban Red	AAA	73,33	VS	-	-
USDA-ARS	TARS 17178	Red	Cultivar	Morado enano	AAA	73,33	VS	-	-
USDA-ARS	TARS 17148	Red	Cultivar	Morado	AAA	80	VS	-	-
Bioversity	ITC0277	Rio	Cultivar	Leite	AAA	100	XS	-	-
USDA-ARS	TARS 16513	Plantain	Cultivar	Congo -300	AAB	0	HR	-	-

Provider	Provider Code	Group/Subgroup	Status	Accession name	Genome Formula	TR4		R1	
						DI%[1]	LR[2]	DI%[1]	LR[2]
USDA-ARS	TARS 16507	Plantain	Cultivar	Dwarf superplantain	AAB	0	HR	-	-
USDA-ARS	TARS 17825	Plantain	Cultivar	French dwarf	AAB	6,67	R	-	-
CIRAD	n.a	Plantain	Cultivar	Big Ebanga	AAB	16	R	4	HR
USDA-ARS	TARS 16514	Plantain	Cultivar	Plantain	AAB	33,33	MS	-	-
USDA-ARS	TARS 17813	Plantain	Cultivar	False-horn Dom. red	AAB	33,33	MS	-	-
INISAV	ITC1258	Plantain	Cultivar	Cemsa 3/4	AAB	36,67	MS	18	R
INISAV	ITC1165	Plantain	Cultivar	Curare	AAB	38	MS	18	R
USDA-ARS	TARS 17816	Plantain	Cultivar	French type Dominican red	AAB	40	MS	-	-
Bioversity	ITC0207	Pome	Cultivar	Prata	AAB	40	MS	44	MS
USDA-ARS	TARS 16522	Pome	Cultivar	Rajapuri	AAB	40	MS	-	-
USDA-ARS	TARS 17410	Pome	Cultivar	Señorita	AAB	40	MS	-	-
USDA-ARS	TARS 17378	Pome	Cultivar	Hy Brazilian	AAB	73,33	VS	-	-
Bioversity	ITC0125	Pome	Cultivar	Pome	AAB	80	VS	-	-
Bioversity	ITC0649	Pome	Cultivar	Foconah	AAB	86,67	XS	73,33	VS
Bioversity	ITC0348	Silk	Cultivar	Silk	AAB	73,33	VS	-	-
USDA-ARS	TARS 17136	Silk	Cultivar	Manzano	AAB	80	VS	-	-
Bioversity	ITC1275	Silk	Cultivar	Yangambi no. 2	AAB	93,33	XS	-	-
USDA-ARS	TARS 17171	Mysore	Cultivar	Mysore	AAB	26,67	MS	-	-
Queensland	DAFF1864	Mysore	Cultivar	Pisang ceylan	AAB	64	S	42	MS
USDA-ARS	TARS 17407	iholena	Cultivar	Tigua (PNG-265)	AAB	86,67	XS	-	-
Bioversity	ITC0449	Lawading	Cultivar	Pisang Lawading	AAB	20	R	-	-
USDA-ARS	TARS 16516	Laknao	Cultivar	Laknau P.I. 23472	AAB	73,33	VS	-	-
USDA-ARS	TARS 17824	M.M.Popoulu	Cultivar	Hua moa 'Popoulu'	AAB	73,33	VS	-	-

Provider	Provider Code	Group/Subgroup	Status	Accession name	Genome Formula	TR4		R1	
						DI%[1]	LR[2]	DI%[1]	LR[2]
USDA-ARS	TARS 17992	Pisang Kelat	Cultivar	Thousand fingers	AAB	93,33	XS	-	-
Bioversity	ITC0643	Bluggoe	Cultivar	Cachaco	ABB	48	S	8	R
USDA-ARS	TARS 18023	Bluggoe	Cultivar	Gipumgusi	ABB	53,33	S	-	-
USDA-ARS	TARS 18020	Bluggoe	Cultivar	Cacambou	ABB	60	S	-	-
INISAV	n.a	Bluggoe	Cultivar	Burro CEMSA	ABB	62,22	S	38,33	MS
Bioversity	ITC0364	Bluggoe	Cultivar	Silver Bluggoe	ABB	66,67	VS	-	-
USDA-ARS	TARS 17397	Bluggoe	Cultivar	Dwarf Orinoco	ABB	73,33	VS	-	-
Bioversity	ITC0767	Bluggoe	Cultivar	Dole	ABB	85	VS	75	VS
Bioversity	ITC0032	Bluggoe	Cultivar	Matavia	ABB	86,67	XS	-	-
Queensland	-	Peyan	-	Pisang Gajih Merah	ABB	0	HR	46	S
Bioversity	ITC0334	Peyan	Cultivar	Nzizi	ABB	80	VS	-	-
USDA-ARS	TARS 18024	Peyan*	Cultivar	Monthan MIA 34906	ABB	80	VS	-	-
Queensland	DAFF1897	Pisang Awak	Cultivar	Ducasse	ABB	86,67	XS	-	-
Queensland	DAFF1884	Pisang Awak	Cultivar	Dwarf Ducasse	ABB	95	XS	-	-
USDA-ARS	TARS 17149	Pisang Awak	Cultivar	Pisang Awak	ABB	100	XS	-	-
USDA-ARS	TARS 17818	Pelipita	Cultivar	Pelipita - Costa Rica	ABB	13,33	R	-	-
Bioversity	ITC0095	Pelipita	Cultivar	Pelipita	ABB	66,67	VS	-	-
Bioversity	ITC0361	Ney Mannan	Cultivar	Blue Java	ABB	20	R	-	-
USDA-ARS	TARS 18018	Ney Mannan	Cultivar	Blue Torres Straight Island	ABB	80	VS	-	-
USDA-ARS	TARS 17822	Saba	Cultivar	Cardaba - Honduras	ABB	33,33	MS	-	-
USDA-ARS	TARS 17173	Saba	Cultivar	Praying hands	ABB	33,33	MS	-	-
Bioversity	ITC0652	Tiparod	Cultivar	Kluual Tiparod	ABB	53,33	S	-	-

[1] DI = Disease index, [2] CR= Cultivar response

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CHAPTER 5

Resistance in Banana to *Fusarium* spp. causing Panama Disease: Diploid and Hybrid accessions

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Abstract

Fusarium spp. are the causal agents of Panama disease in banana. Despite the fact that banana is the global top fruit and is also a major staple food in many countries, the production largely relies on a few clones. This limited genetic stock is exploited in extensive monocultures around the world. Among these, the so-called Cavendish varieties, and many other regional varieties, are extremely susceptible to the Tropical Race 4 (TR4), which represents a new migrating species named *Fusarium odoratissimum.* This urges for the characterization of resistance to TR4 in contemporary banana germplasm, which is limited to just a few clones. Since the identification of new sources of resistance is essential for breeding programs, we phenotyped 122 genotypes - including 25 wilt types, 50 diploid landraces, and 47 breeding lines - under greenhouse conditions. We inoculated replicated trials with TR4 and with race 1, which caused the first Panama disease epidemic in 'Gros Michel' in the previous century. In total, 27 and 43 accessions showed adequate levels of resistance to TR4 and to race 1, respectivelly. These accessions are vital as potential new sources of resistance and can be incorporated into conventional and non-conventional breeding programs geared towards developing new cultivars that meet market demands and withstand Panama Disease.

Introduction

Banana (*Musa* spp.) is the top global fruit and a major staple in developing economies (Bakry *et al.,* 2009, Churchill 2011). Currently, the annual global banana production is larger than 145Mt representing a gross value of more than 35,2 billion US$ per year (FAOSTAT 2016). Hundreds of banana cultivars are currently cultivated around the world and 85% of the production is grown for local consumption (Churchill 2011). They are mostly cultivated for the fruit, which can be consumed fresh (many dessert cultivars) or after processing (cooked, fried, brewn). In traditional societies, also other parts of the plant, such as leaves, male buds and even the pseudostems, are also consumed (Bakry *et al.,* 2009). Cultivated bananas are giant monocotyledons herbs, mostly sterile and multiplied by vegetative propagation (Simmonds and Shepherd 1955). They belong to the family of the Musaceae, which is placed in the order of the Zingiberales that comprises the genera Ensete and Musa. The most striking difference between these two genera is that Ensete plants do not form suckers (Brewbaker and Umali 1956).

The genus *Musa* was originally divided into the sections *Callimusa* and *Australimusa* (2n= 20), *Rhodochlamys* and *Eumusa* (2n=22), which were recently merged into the sections *Musa* and *Callimusa* (Häkkinen 2013) and comprise more than 100 diploid, seminiferous, non-parthenocarpic species (Häkkinen and Väre 2008). The *Musa* section contains a dozen species, including *M. acuminata* (AA) and *M. balbisiana* (BB), which are the ancestors of several hundreds of modern diploid and polyploid cultivars that resulted from intra- and interspecific hybridizations (Simmonds 1962, Heslop-Harrison and Schwarzacher 2007). Currently, more than 2,000 different cultivars have been identified, which are divided into six genomic groups: AA, AAA, AB, AAB, ABB and ABBB (Lescot 2017). During the banana domestication process, seedless fruits were preferred which reduced gamete fertility and recombination (Heslop-Harrison and Schwarzacher 2007, Donohue and Denham 2009, Heslop-Harrison 2009, Perrier *et al.,* 2011, De Langhe *et al.,* 2015). Therefore, nearly 95% of contemporary banana production can be traced back to just a few genetic combinations, involving seven to 14 meiotic events and one single recombinant (Cavendish clones) represents 50% of the global (export and domestic) market (Bakry and Horry 2014). Clearly, edible bananas are characterized by a lack of genetic variation, except for somatic mutations or bud-sporting that generate phenotypic diversity among clones that share the same sexual ancestors (Perrier *et al.,* 2011). This turns a staple food for millions of people into an agronomically unique and extremely fragile production system (d'Hont *et al.,* 2012) that suffers from pest and diseases (Blomme *et al.,* 2011, Dubois and Coyne 2011).

The major fungal diseases threatening bananas are black Sigatoka, a foliar blight caused by *Pseudocercospora fijiensis* (Morelet) (Arango *et al.,* 2011, Blomme *et al.,* 2011, Arango *et al.,* 2016) and Panama disease, a vascular wilt that is caused by various *Fusarium* species (Maryani *et al.,* 2018). This disease caused havoc in the previous century by wiping out the 'Gros Michel' based industry in Central America (Carleton 1922, Stover and Waite 1960, Stover 1962, Ploetz 2000, 2005a, Marquardt 2001), which was then saved by the adoption of the resistant Cavendish clones (Ploetz 2005b). Ironically, this success contributed to the global expansion of Cavendish that now represents over 50% of the global production, but is also a vehicle for the global dissemination of a new aggressive species; *Fusarium odoratissimum,* representing the so-called Tropical Race 4 (TR4) that also kills manifold local cultivars (García-Bastidas *et al.,* 2014, Ordóñez *et al.,*

2015a, Chittarath *et al.,* 2017, Hung *et al.,* 2017, Mostert *et al.,* 2017, Maryani *et al.,* 2018, Zheng *et al.,* 2018). This threatens the future of the banana industry and millions of small farmers that depend on it (Butler 2013). Particularly, since breeding efforts have not resulted in varieties that can replace Cavendish and hence disease management focuses on prevention and after incursions of TR4 on quarantine and eradication (Ploetz 2015).

To date, there are no commercial fungicides on the market to control Panama disease, albeit that various control methods are being tested (Nel *et al.,* 2007, Nguyen *et al.,* 2018, Ploetz 2015). Nevertheless, as Cavendish has shown resistance to race 1 strains, disease resistance significantly contributes to sustainable disease management. Therefore, surveying resistance to TR4 in genetic resources is necessary and valuable for identifying germplasm that can potentially replace Cavendish or can be deployed in breeding programs. Clearly, there is an urgent need to broaden the genetic base of cultivated banana cultivars by innovative classical breeding, which captures the available genetic diversity for overall disease resistance and other agronomical characteristics, including productivity, taste and shelf life. However, the low fertility of cultivated bananas is a handicap for breeders (Bakry *et al.,* 2009, Bakry and Horry 2014), but some cultivars may produce seeds when hand pollinated (Menendez and Shepherd 1975, Stover and Buddenhagen 1986, Persley and De Langhe 1987, Buddenhagen 1990). The creation of diversity can be achieved by cross breeding towards new triploids that without exception excel over diploids in productivity and over tetraploids in postharvest and processing qualities (Persley and De Langhe 1987, Bakry *et al.,* 2009, Heslop-Harrison 2009, Amorim *et al.,* 2011, Tenkouano *et al.,* 2011, Bakry and Horry 2014). The generation of new triploids is mostly achieved by developing and using improved diploids to synthetize tetraploid hybrids which can be back-crossed with diploids to generate secondary triploids (Menendez and Shepherd 1975, Pillay and Tenkouano 2011).

Here, we identified new sources of resistance in a large number of accessions with a wide range of genetic diversity. In total, we screened 122 wild and cultivated banana accessions with TR4 and race 1 under greenhouse conditions to identify new sources of resistance for breeding programs. Moreover, we identified whether such resistances were transmitted to new hybrids developed in ongoing breeding programs that potentially replace susceptible varieties.

Materials and methods

Plant material

Tissue culture plantlets were obtained from eight institutions and international organizations (Table 1). Upon arrival, plants were multiplied and maintained in the laboratory of plant breeding at Wageningen University and Research (WUR). After developing a strong visible root system, usually three weeks after arrival, they were transferred to two pots containing standard soil from WUR UNIFARM greenhouse facilities (Swedish sphagnum peat 5%, grinding clay granules 41%, garden peat 5%, beam structure 4%, steamed compost 33%, PG-Mix-15-10-20- 12%) and hardened in a greenhouse at 26 ±2 ˚C and ~80% relativity humidity (RH) under 16 hours light. Henceforward, they were transplanted to new 1L pots in the aforementioned soil and placed in the same greenhouse at 85% RH for three months until phenotyping.

Table 1. Institutions and international organizations that made banana accessions available for surveying resistance to Panama disease.

Organization	Country	Acronym	Internal code
1. Bioversity International, Belgium	Belgium	Bioversity	ITC[1]
2. Agricultural Research Centre for International Development & Vitropic	France	CIRAD/VITROPIC	CIRAD
3. United States Department of Agriculture, Puerto Rico	Puerto Rico	USDA-ARS	TARS[2]
4. National Banana Corporation S.A.	Costa Rica	CORBANA	--
5. Cuban Plant Health and Research Institute	Cuba	INISAV	--
6. Kenyan Agricultural Research institute	Kenya	KARI	--
7. Queensland Department of Agriculture, Fisheries and Forestry	Australia	DAFF	DAFF
8. Rahan Meristem Ltd.	Israel	--	--

[1]International Transit Centre. [2]Tropical Agriculture Research Station.

The accessions represent a wide range of genetic diversity, from clones that are close to wild relatives (CRW) to edible diploids/landraces and developed hybrids. The 25 CWR mostly comprise *M*. *acuminata* and *M. balbisiana* accessions from the *Musa* section due to their phylogenetic relationships with cultivated banana varieties, but also include wild accessions from other sections including former *Rhodochlamys* and *Australimusa* sections. All accessions were arranged and presented according to Perrier *et al.* (2011) in Table 2.

In total, we tested 50 edible diploids which were also grouped as suggested by Perrier *et al.* (2011) and represent a wide range of varieties collected in Southeast Asia (from Papua New Guinea (PNG) to India). Most clones from PNG are classified as cooking types that contain a high proportion of *M. acuminta* ssp. *banksii* genetic background. The other edible diploids are dessert types, mainly from Indonesia, Malaysia, Thailand and India. Their genomic background is vast and diverse, mainly derived from one, two and four relative wild clusters (Table 3). Some of these clones have been and are currently used in resistance breeding programs.

Finally, we also phenotyped 47 hybrids from diverse research groups, breeding programs or private companies, comprising various ploidy levels and genome compositions (Table 4).

Inoculations and phenotyping

Three-months' old plants were randomly placed in the greenhouse and then inoculated with a the reference TR4 isolate (*F. odoratissimum* TR4, isolate II-5), originating from Indonesia. Additionally, various accessions were inoculated with a race 1 isolate originating from Brazil (CNPMF 000-8-01-R1).

Controls included the susceptible Cavendish 'Grand Naine' for TR4 and 'Gros Michel' for race 1 and the resistant Pahang (*Musa acuminata* spp *malaccensis).* As well as plants treated with water. Inocultion procedures were according to the protocol of García-

Bastidas *et al.* (Chater 3), complemented with five maize kernels colonized by TR4 or race 1 strains. External symptom development was monitored weekly and at seven to 13 weeks after inoculation (wai), plants were uprooted and individual corms were photographed and scored following the protocol of García-Bastidas *et al*. (Chapter 4).

Statistical analyses

All experiments were performed following a completely randomized block design. To avoid cross contamination between TR4 and race 1 treatments, plants were physically separated and placed on different tables in the greenhouse. Additionally, all statistical units (plants) were placed in individual pots in trays to prevent unintended movement of inoculum. Each genotype was tested at least three times. All statistical analyses were performed with the statistical package Genstat 5.3 (Payne 1993), using an ANOVA model with accession genome-type or subgroups as main factors.

TR4 molecular confirmation

During disease scoring we sampled the rhizomes (including roots), pseudostems and petioles of all TR4 inoculated plants and controls to confirm the presence of TR4 by PCR according to Dita *et al.* (2010).

Results

In total, 122 accessions of various ploidy levels and of different genomic composition were evaluated under greenhouse conditions for their response to TR4 and 86 were tested with race 1. Panama disease symptoms were observed six to ten weeks after inoculaton (wai), depending on the accession. Typical chlorosis, pseudostem splitting and cigar leaf abnormalities were displayed by susceptible genotypes. Controls such as 'Gros Michel' inoculated with race 1 (susceptible), 'Pahang' inoculated with TR4 and race 1 (resistant) and 'Grand Naine' inoculated with TR4 and race 1 (susceptible and resistant, respectively) exhibited the expected response with the selected strains, therby confirming the effectiveness of the phenotyping methodology. No symptoms were observed in all water controls confirming the absence of cross contamination among treatments. The vast majority of the 122 genotypes displayed general susceptibility with DIs ranking between 25 (MS) and 100 (XS). However, resistant genotypes were identified in each category of plants (CWR, landraces and hybrids). The highest level of resistance was observed in the CWR accessions where nine genotypes (36%) showed DIs between 5 and 25 (Tables 2 - 4 and Figure 1).

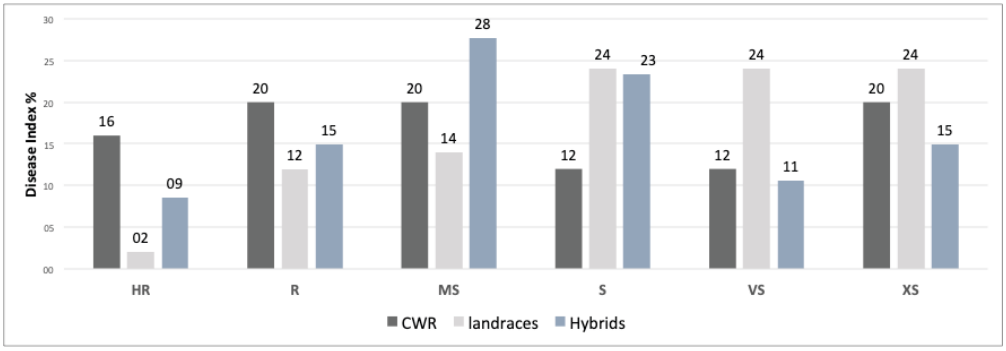

Figure 1. Phenotyping banana accessions with *Fusarium odoratissimum* Tropical Race 4. General responses of Close to Wild Relatives (CWR), landraces and hybrids are displayed as Disease Index (DI. Response classes are defined as highly resistant (HR), resistant (R), moderately susceptible (MS), susceptible (S), very susceptible (VS) and extremely susceptible (XS) with the number of tested accessions displayed over the bars.

Resistance of the close wild relatives (CWR)

We observed that the *M. balbisiana, M. acuminata* spp. *banksii*, and *M. acuminata* spp. *truncata* accessions were highly susceptible. However, the emphasis of this study on *M. acuminata* enabled us to conduct a detailed study on various genotypes of *M. acuminata*. subspecies. The four clones of *M. acuminata* spp. *banksii* (Table 2, cluster 1) were severely affected by TR4 and one was also susceptible to race 1. However, the clones in cluster 3, mostly originating from the northern territories of the species' diversification, showed variable degrees of resistance. 'Calcutta 4' is highly resistant and the *burmannica* and *siamea* genotypes also showed resistance. These original wild species are not subject to domestication and selection and originate from not anthropized habitats in northeast India, Myanmar, southern China and Thailand. Greater variation was observed in *M. acuminata* spp. *malaccensis* (cluster 2). The two Pahang clones tested in this study are highly resistant, but the 'Pisang serum', originating from Malaysia is susceptible to TR4. The androgenic double-haploid line DH Pahang (d'Hont *et al.,* 2012) is highly resistant to TR4, but it is not characteristic for the species. *M. acuminata* spp. *malaccensis* "nain" from a seedling in Guadeloupe is susceptible (Figure 2). Finally, the two *M. acuminata malaccensis* derivatives 'Pa (Musore) No. 2' and 'Pa (Musore) No. 3' which originate from Thailand (https://www.crop-diversity.org/mgis/) were also susceptible to TR4. Finally, the two *M. acuminata* accessions belonging to zebrina/microcarpa cluster 4 and originating from a seedling selected at CARBAP in Cameroon and from Kalimantan in Indonesia are also susceptible. Remarkably, the accessions from the sections *Australimusa* (now in *Callimusa*) as well as *Rhodochlamys* (now in *Musa*) showed an overall reduced susceptibility to TR4 and the ornamental *M. ornata* was resistant to TR4 showing a DI of 6.6.

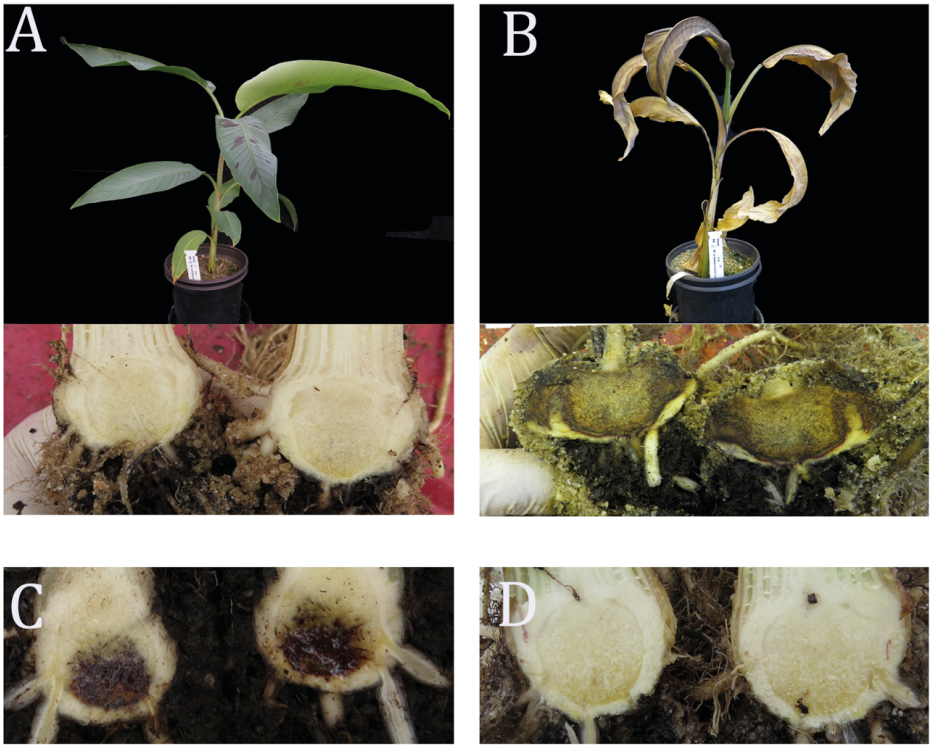

Figure 2. Diferential response of banana accessions from the subspecies *Musa acuminata* spp. *malaccensis* cluster 2 (A) *Musa acuminata* spp. *malaccensis* "nain" water control and (B) same accesion treated with *F. odoratissimum* TR4 isolate II-5 at 10 weeks after inoculation. (C) Pisang Serum displayng typical susceptitility and (D) Pahang exhibiting resistance.

Table 2. Passport information and disease response close wild relatives of banana (*Musa* spp.) in greenhouse phenotyping arrays with Tropical Race 4 (TR4) and race 1 (R1) of *Fusarium* spp. causing Panama disease.

Section	Species	Subspecies	Cluster	Name	Genome	Code	TR4		R1	
							DI[1]	RL[2]	DI	RL
Musa	*M. balbisiana*		-	Tani	BB	ITC1120	73.33	VS	-	-
			-	*M. balbisiana*	BB	USDA18021	93.33	XS	-	-
	M. acuminata	*banksii*	1	Waigu	AA	ITC0465	55	S	-	-
			1	A (3617 9)	AA	ITC0530	93.33	XS	-	-
			1	Paliama	AA	ITC0766	75	VS	-	-
			1	*M. acuminata* "Paliama"	AA	CIRAD	96	XS	80	VS
		malaccensis	2	*M. acuminata* spp. *malaccensis*	AA	Pahang	0	HR	5	HR
			2	Pisang Serum 400	AA	ITC1349	56	S	73.33	VS
			2	*M. acuminata* spp. *malaccensis* "Nain"	AA	CIRAD	100	XS	68	VS
			2	Pahang (CMR)	AA	CIRAD	0	HR	15	R
		malaccensis Derivative	2	Pahang DH	AA	ITC0609	0	HR	15	R
			2	Pa (Musore) No. 2	AA	ITC0668	32	MS	-	-
			2	Pa (Musore) No. 3	AA	ITC0406	30	MS	12.73	R
		truncata	2	Truncata	AA	ITC0393	67.5	VS	66.67	VS
		burmannica	3	Long Tavoy	AA	ITC0093	15	R	0	HR
		burmannicoides	3	Calcutta 4	AA	CIRAD	0	HR	20	R
		siamea	3	Khae (Phrae)	AA	ITC0660	25	R	-	-
			3	Pa (songkhla)	AA	ITC0408	22.5	R	3.33	HR
			3	Pa (Rayong)	AA	ITC0672	20	R	0	HR

Section	Species	Subspecies	Cluster	Name	Genome	Code	TR4		R1	
							DI[1]	RL[2]	DI	RL
		zebrina	4	Zebrina (G.F.)	AA	ITC0966	96	XS	26.67	MS
		microcarpa	4	Borneo	AA	ITC0253	34.29	MS	45	MS
Rhodochlamys*	*M. ornata*	--	-	Abaca Ai	OO	USDA18065	6.67	R	-	-
Australimusa**	*M. textilis*	--	-	Abaca Al	TT	na	40	MS	-	-
		--	-	*M. ornata*	TT	na	55	S	-	-
	M. peekelii	--	-	*M. peekelii* ssp. *angustigema*	PP	ITC0618	30	MS	-	-

[1] DI = Disease index, [2] RL= Response level

*Now included in the *Musa* section, **included in *Callimusa* section.

Edible diploid landraces

The landrace panel represents a wide range of geographical origins from East Asia (East New Britain, PNG) to West India. It also covers almost all the genetic diversity of edible diploids, including those that produced natural triploid varieties. The edible AA diploid varieties are distributed over 13 clusters as suggested by Perrier *et al*. (2011). Within clusters, diploid clones derived from others by vegetative propagation where placed in the same subgroup. Sixteen clones, mostly from PNG are considered as cooking types whereas the remainig 33 cultivars are dessert types, originating from the more occidental part of Asia and East Africa. The response to TR4 is diverse and ranked from extremely susceptibility to immunity (Figure 3). For example, the accession 'Morong princesa' (Buaya cluster) is very resistant whereas 'Pisang Lilin' (Khai Cluster) and 'Safet' Velchi (Ney Poovan cluster) are extremely susceptible. This wide variation in response was also observed in the screens with race 1.

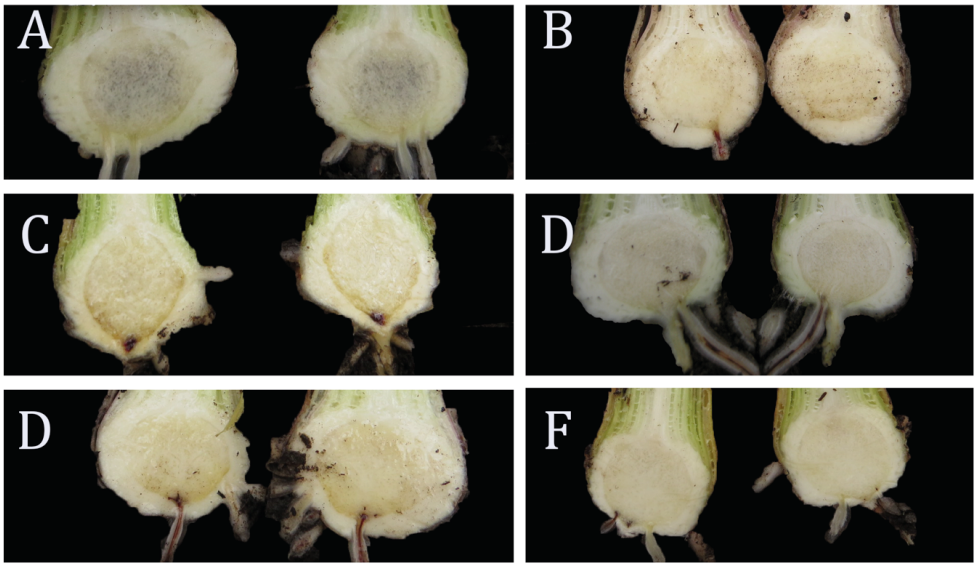

Figure 3. Different resistant responses of banana accesion after inoculation with *Fusarium odoratissimum* TR4 isolate II-5 at 10 weeks after inoculation. Pictures show cross sections of corms. (A) 'Morong princesa'; (B) 'Uwati'; (C) 'NBA-14'; (D) 'Manang'; (E) 'Paka'; (F) 'Tjau Lagada'.

Among the cooking bananas, which all originate from PNG, except Guyod that is of Philippine origin, the 'Uwati' and 'NBA14' clones were resistant (DI < 20%) to TR4 and race 1, including 'Djum Tau' which showed a differential response as it was susceptible to TR4. This overall susceptibility aligns with the responses of the CWR *M. acuminta* spp. *banksii* accessions from the same region, which were also susceptible. In general, our results show that the genetic diversity of dessert bananas is wider than that of cooking bananas, but only four clones are resistant (DI < 15%) to TR4; 'Morong Princesa', 'Manang' (CIRAD), cv. Rose (Cirad and ITC0712) and 'Tjau Lagada' and belong to different clusters of the 33 phenotyped accessions. At cluster level, no resistant clones to TR4 were

identified in the Mshare, Tongat and Figue clusters, of which the latter contains the sucrier subgroup (Disease index varying between 48 to 96%) and albeit that 'Pisang Berlin' and 'Pisang Oli' were immune to race 1 (DI=0). In the Mshare cluster, the resistance of 'Paka' that was derived from a cross between a true Mshare and a CWR (Perrier *et al.,* 2018), is higher than the susceptible parent, but otherwise, all these clones except 'Mjenga Gros Michel' and 'Pisang Tongat' are moderately or highly susceptible to race 1. The different clones of the Buaya cluster originated from vegetative propagation of a common susceptible ancestor, hence comprise a set of somaclones of which 'Morong Princesa' is highly resistant to TR4. Furthermore, the number of accessions with resistance to TR4 was highest in the Khai cluster. Four of the eight clones tested showed a high level of resistance. In addition to the likely identical AAcv Rose and cv. Rose clones, there are at least three additional accessions with resistance to TR4 and all accessions were resistant to race 1, except 'Pisang Pipit' (DI 80%). Finally, the AB clones were susceptible to both TR4 and race 1.

Table 3. Passport information and disease response of edible diploid bananas (*Musa* spp.) in greenhouse phenotyping arrays with Tropical Race 4 (TR4) and race 1 (R1) of *Fusarium* spp. causing Panama disease.

Common Name	Genome	Cluster	Location	Type	Code	TR4		Race 1	
						DI[1]	RL[2]	DI	RL
Wiliman	AA	Katual	Papua New Guinea - West	cooking	ITC0949	53.33	S	-	-
Katual Vunalir	AA		New Britain East (PNG) - Rabaul	cooking	USDA17412	55	S	-	-
Tomolo	AA		Papua New Guinea	cooking	ITC1187	93.33	XS	-	-
Guyod	AA	Guyod	Philippines - Mindanao	cooking	ITC0299	44.71	MS	73.33	VS
Gorop	AA		New Britain East (PNG) - Kokopo	cooking	ITC0778	70	VS	80	VS
NBA 14	AA	Bagul	Papua New Guinea	cooking	ITC0267	24	R	16	R
Sowmuk	AA		Papua New Guinea	cooking	ITC0266	53.33	S	40	MS
Djum Tau	AA		Papua New Guinea -Tembutkin	cooking	ITC0292	60	S	10	R
Kuspaka	AA		Papua New Guinea - Kabiufa	cooking	ITC0840	60	S	80	VS
Odwa	AA		Papua New Guinea - West -Kiunga	cooking	ITC0889	65	S	80	VS
Adina	AA		Papua New Guinea - Muyua Island	cooking	ITC0893	80	VS	80	VS
Uwati	AA	Galeo	New Britain East (PNG) - Kokopo	cooking	ITC0373	10	R	10	R
Pitu	AA		New Britain East (PNG) - Kokopo	cooking	ITC0294	36	MS	60	S
Galeo	AA		Papua New Guinea	cooking	n.a	72	VS	85	VS
Kwaro	AA	Beram	Papua New Guinea - West	cooking	ITC0943	82.86	VS	66.67	VS
Fu Des	AA		Papua New Guinea - North West	cooking	ITC0939	93.33	XS	80	VS
Morong Princesa	AA	Buaya	Malaya	dessert	ITC0310	4	HR	-	-
Not named	AA		Malaya	dessert	ITC0318	33.33	MS	-	-
Saing Todloh	AA		Malaya	dessert	ITC0316	42.86	MS	72	VS
Pisang Jari Buaya	AA		Malaya	dessert	ITC0312	68	VS	71.11	VS
Tuu Gia	AA	Tuu Gia	Vietnam	dessert	17382	45	MS	-	-
Paka	AA	Mshare	East Africa	dessert	ITC0320	26.67	MS	-	-

Common Name	Genome	Cluster	Location	Type	Code	TR4		Race 1	
						DI[1]	RL[2]	DI	RL
Akondro Mainty	AA		East Africa - Madagascar	dessert	ITC0281	40	MS	53.33	S
Mjenga "Gros Michel" Diploide	AA		East Africa - Pemba	dessert	ITC1253	67.5	VS	0	HR
Mshare Mshia Wa Komba	AA		East Africa - Mayotte	dessert	Mayo 11	86.67	XS	80	VS
Mshare Mshia Wa Komba	AA		East Africa - Mayotte	dessert	Mayo 18	90	XS	73.33	VS
Pu-te La-Bum	AA		Malaya - Borneo	dessert	ITC0446	60	S	40	MS
Pisang Tongat	AA	Tongat	Indonesia	dessert	ITC0063	70.91	VS	21.82	R
Pu-te Wey	AA		Malaya - Borneo	dessert	ITC0447	93.33	XS	40	MS
Pisan Jaran	AA		Indonesia - Java	dessert	n.a	48.89	S	60	S
Inarnibal	AA		Philippines	dessert	ITC1149	53.33	S	-	-
Terema	AA		Papua New Guinea - East Goroka	dessert		53.33	S	20	R
Pisang Berlin	AA		Indonesia - Java	dessert	ITC0611	64	S	0	HR
IND077	AA	Figue	Indonesia - Java	dessert	IND 077	70	VS	40	MS
Pisang Mas	AA		Malaya - Selangor	dessert	ITC1403	80	VS	-	-
Pisang Oli	AA		Indonesia - Java	dessert	ITC1157	85	VS	0	HR
Ngu	AA		Vietnam - North Hanoi	dessert	ITC1358	86.67	XS	-	-
Niño Común	AA		USA Puerto Rico	dessert	17379	85	VS	-	-
Datil La Lima	AA		USA Puerto Rico	dessert	17413	96	XS	-	-
Manang	AA		Philippines - Camerines Del Sur	dessert	n.a	7.27	R	6.67	R
cv. Rose	AA		Indonesia - Java	dessert	IND 110	11.11	R	0	HR
AA cv. Rose	AA		Indonesia - Java	dessert	ITC0712	13.33	R	0	HR
Tjau Lagada	AA	Khai	Indonesia	dessert	ITC0090	15	R	10	R
Pisang Pipit	AA		Indonesia - Java	dessert	ITC0685	80	VS	88	XS
Pisang Buntal	AA		Malaysia - Borneo Sarawack	dessert	ITC0480	90	XS	-	-
Pisang Madu	AA		Malaysia - Borneo Sarawack	dessert	n.a	94.29	XS	11.11	R

Common Name	Genome	Cluster	Location	Type	Code	TR4		Race 1	
						DI[1]	RL[2]	DI	RL
Pisang lilin	AA		Malaya	dessert	n.a	100	XS	10	R
Kunnan	AB	Kunnan	India - Kerala	dessert	ITC1034	53.33	S	-	-
Safet Velchi	AB	Ney Poovan	India	dessert	ITC0245	100	XS	-	-
Vunapope	AB	-	New Britain East (PNG) - Kokopo	cooking	ITC0990	88	XS	100	XS

[1] DI = Disease index, [2] RL= Response level

Hybrids

The investigated hybrids originate from different breeding strategies, one being essentially based on the high heritability of characters (pedigree breeding) and the other based on the combination of two distant genomes to maximize heterozygosity in a triploid hybrid (reconstructive breeding). Eleven (25%) out the 47 parental lines and final hybrids displayed acceptable levels of resistance for TR4 and 56% to race 1 under greenhouse conditions (Figure 1).

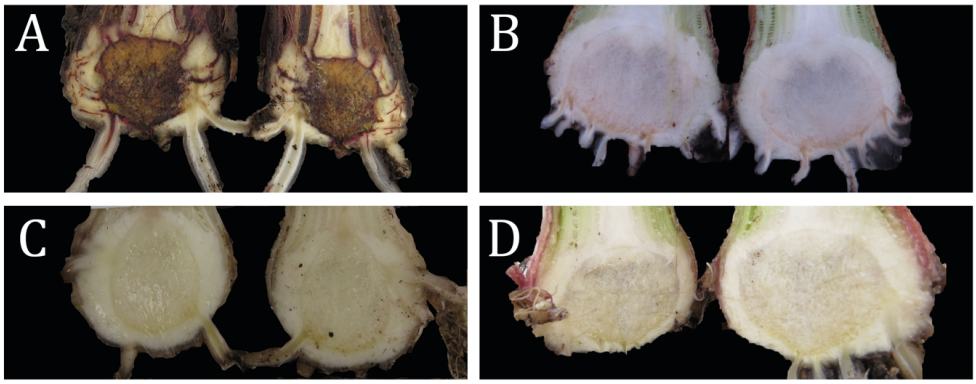

Figure 4. Examples of resistant banana hybrids compared to the susceptible Cavendish 'Grand Naine' (A). Pictures show cross sections of corms after inoculation with *F. odoratissimum* TR4 isolate II-5 at 10 weeks after inoculation (B) 'Carbap A11'; (C) 'TMPx 1621-1' and (D), 'Flhorban X17'.

Tetraploid plantain derived hybrids (AAAB - pedigree breeding)

Four clones were resistant 'FHIA-04', 'FHIA-21', 'TMP x 1621-1' and 'Carbap 832' and have the susceptible 'Yangambi Km5' and the resistant 'Calcutta 4' clone as male parent (Cal4), the level of resistance of the other male parents (M53, SH-3437, SH-3142) is unknown.

Tetraploid Pome derived hybrids (AAAB – pedigree breeding)

Out of the nine tested FHIA and EMBRAPA selections, 'FHIA-18-High Noon' and 'FHIA-18-FLF' - selected bud-sports from the 'FHIA-18' hybrid – showed some levels of resistance to race1, but are susceptible to TR4. Unfortunately, the male parents were not characterized for their resistance in this sample of hybrids.

Tetraploid AAAA hybrids (pedigree breeding)

The resistance to TR4 in the four AAAA hybrids ranked from resistant ('FHIA-02'), to moderately susceptible ('FHIA-17' and 'FHIA-23') to susceptible ('SH-3436'). The resistance level of 'FHIA-02' clearly differentiates this clone from the others dessert FHIA hybrids. 'FHIA-17' and 'FHIA-23' are resistant to race 1.

The other hybrids of the 'pedigree breeding' approach come from various combinations and are always the result of many recombinations that are difficult to interpret in terms of character transmission. Hence, our results support a more directed use of these genotypes in crossbreeding or for direct use. Some clones showed very promising responses to TR4. The 'Carbap A11' (Figure 4) and 'SH-3217' hybrids remained unaffected to TR4 and race 1, but none of the secondary triploids (AAA, AAB) evaluated in this study showed resistance to TR4, including the 'Carbap K74' hybrid that was derived from a cross between two resistant parents; the aforementioned highly resistant diploid 'Carbap A11' hybrid and the resistant 'Carbap 832' tetraploid AAAB hybrid.

New triploids

We screened 16 new triploid hybrids in this study. Out of the nine AAA hybrids, two were highly resistant ('Flhorban 931', 'Flhorban PRAM01') and two were resistant ('Flhorban 938', 'Flhorban 940'). The others ranged from moderately to highly susceptible. Furthermore, out of the six AAB hybrids, only 'Flhorban X17' was highly resistant to TR4 (Figure 4 D). Finally, the synthetic triploid BBB ('Flhorban J24') and the doubled-diploid interspecific elite parental line 'Kunnan T' were very susceptible to both TR4 and race 1.

Table 4. Passport information and disease response of banana hybrids (*Musa* spp.) in greenhouse phenotyping arrays with Tropical Race 4 (TR4) and race 1 (R1) of *Fusarium* spp. causing Panama disease.

Origin	Name	Putative Genome	Method*	Type	Background/Pedigree	TR4		R1	
						DI[1]	RL[2]	DI%	RL
CARBAP	Carbap A11	AA	P	diploid Elite line	CRBP60 (Mbai - AAB) x M53 (AA) x Tomolo	0	HR	0	HR
	Carbap 39	AAAB	P	tetraploid hybrid	French Clair (French Plantain AAB) x M53	33,33	MS	-	-
	Carbap 832	AAAB	P		Amou (French Plantain - AAB) x Yangambi Km 5 (AAA)	12,5	R	6,7	R
	Carbap K74	AAB	P	secondary triploid	Hyb832 (AAAB) x A11 (AA)	45	MS	30	MS
FHIA	SH-3217	AA	P	diploid Elite line	SH-2095 x SH-2766	21,82	R	14,5	R
	FHIA-02	AAAA	P	tetraploid hybrid	Williams (Cavendish -AAA) x SH-3393	23,33	R	28,9	MS
	FHIA-17	AAAA	P		Highgate (Gros Michel - AAA) x SH-3362	40	MS	8	R
	FHIA-23	AAAA	P		Highgate (AAA, Gros Michel) x SH-3362	32	MS	20	R
	SH-3436	AAAA	P		Highgate (AAA, Gros Michel) x SH-3142 (SH-1734 x SH-P. Jari buaya)	46,67	S	28,9	MS
	FHIA-01	AAAB	P		Prata Ana (Pome - AAB) x SH3142 (SH1734 x Pisang Jari Buaya)	73,33	VS	-	-
	FHIA-18	AAAB	P		Prata Ana (Pome - AAB) x SH3142 (SH1734 x Pisang Jari Buaya)	64	S	20	R
	FHIA-18-High Noon	AAAB	P		Prata Ana (Pome - AAB) x SH3142 (SH1734 x Pisang Jari Buaya)	90	XS	-	-
	FHIA-18 - FLF	AAAB	P		Prata Ana (Pome - AAB) x SH3142 (SH1734 x Pisang Jari Buaya)	53,33	S	-	-
	SH-3641	AAAB	P		Prata Ana (Pome- AAB) x SH-3393 (SH-3142 x SH-3217)	56	S	29,1	MS
	SH 3640	AAAB	P		Prata Ana (Pome- AAB) x SH-3393 (SH-3142 x SH-3217)	71,43	VS	14	R
	SH-3656	AAAB	P		Prata Ana (Pome- AAB) x SH-3393 (SH-3142 x SH-3217)	86,15	XS	16,9	R
	FHIA-04	AAAB	P		AVP-67 (French plantain AAB) x SH-3437 (AA)	15,56	R	6,7	MS
	FHIA-20	AAAB	P		AVP-67 (French plantain AAB) x SH-3437 (AA)	28	MS	4	HR

Origin	Name	Putative Genome	Method*	Type	Background/Pedigree	TR4		R1	
						DI[1]	RL[2]	DI%	RL
	FHIA-26	AABB	P		Pisang Awak (Pisang Awack - ABB) × SH-3437	57,33	S	80	VS
	FHIA-03	AABB	P		SH-3386 ((Gaddatu x BB) x SH-2471) x SH-3320	37,78	MS	52	S
	FHIA-25	AAB	P	secondary triploid	SH-3648 (AABB) x SH-3142	33,68	MS	10	R
	SH-3748	AAB	P		SH-3648 (AABB) x SH-3437 (SH-2989 x SH-3217)	40	MS	66,7	VS
CIRAD	IRFA 905	AA	R	diploid Elite line	Guyod x Galeo	33,33	MS	3,3	HR
	Fhlorban 931	AAA	R	triploid hybrid	Manang (AA) x IRFA903 T (AAAA)	2,5	HR	8	R
	Flhorban 916	AAA	R		Manang (AA) x P Lilin T (AAAA)	60	S	8	R
	Flhorban 918	AAA	R		P. Madu (AA) x IDN110 T (AAAA)	96	XS	32	MS
	Flhorban 920	AAA	R		Khai Nai On (AA) x IDN110 T (AAAA)	86,67	XS	0	HR
	Flhorban 924	AAA	R		IDN110 T (AAA) x Khai Nai On (AA)	35	MS	20	R
	Flhorban PRAM 01	AAA	R		IND110 T (AAAA) x Pisang Madu (AA)	4	HR	4	HR
	Flhorban 925	AAA	R		P. Madu (AA) x IDN110 T (AAAA)	96,67	XS	0	HR
	Flhorban 938	AAA	R		Manang T (AAAA) x IDN110 (AA)	15	R	20	R
	Flhorban 940	AAA	R		P. Madu (AA) x Pisan Lilin T (AAAA)	22	R	12	R
	Flhorban X17	ABA	R		Kunnan T (AABB) x IDN110 (AA)	2,5	HR	28,6	MS
	Flhorban L9	ABA	R		Kunnan T (AABB) x IDN110 (AA)	50	S	30	MS
	Flhorban M9	ABA	R		Kunnan T (AABB) x IDN110 (AA)	57,14	S	0	HR
	Flhorban L16	AAB	R		*M. malaccensis* nain (AA) x Kunnan T (AABB)	86,67	XS	80	VS
	Flhorban U15	AAB	R		*M. malaccensis* nain (AA) x Kunnan T (AABB)	55	S	35	MS
	Flhorban U16	AAB	R		*M. malaccensis* nain (AA) x Kunnan T (AABB)	64	S	64	S
	Flhorban J24	BBB	R		Lal Velchi T (BBBB) x P. Batu (BB)	80	VS	72	VS
	Kunnan T	AABB	P	tetraploid elite line	Double-diploid from Kunnan	80	VS	100	XS
EMBRAPA	PA 03-22#	AA	P	diploid Elite line	Pacovan (AAB) x Cal4 - 'Synthetic hybrid'	46,67	S	-	-
	PV 42-320	AAAB	P	tetraploid hybrid	pacovan (AAB) x M53 (AA)	40	MS	-	-
	PV 42-81	AAAB	P		pacovan (AAB) x M53 (AA)	40	MS	-	-
NARO	M9 (kari)	AAA	P	triploid hybrid	Pacovan (AAB) x Cal4 - 'Synthetic hybrid'	40	MS	0	HR

Origin	Name	Putative Genome	Method*	Type	Background/Pedigree	TR4		R1	
						DI[1]	RL[2]	DI%	RL
IITA	TMB2x 9128-3	AA	P	diploid Elite line	plantain-derived diploides(TMP2x)	93,33	XS	-	-
	TMP x 1621-1	AAAB	P	tetraploid hybrid	Obino l'Ewaï (French plantain - AAB) x Calcutta 4	13,33	R	-	-
USDA	2390	AAAA	-	unknown	Synthetic hybrid	80	VS	-	-

[1] DI = Disease index, [2] RL= Response level

*P = Pedigree method; R = Reconstructive method.

Discussion

Despite the enormous diversity of the *Musa* genus comprising more than 1,200 banana cultivars with potential for cultivation and a suite of approximately 150 classified wild species, contemporary global production balances on a few fragile clones, representing more than 80% of the market (Lescot 2017). The most prominent clones are the Cavendish cultivars as well as A/B interspecific triploids that make up approximately 40% of the global production and >95% of the entire export trade (Baurens *et al.,* 2018). They surfaced during the first Panama disease epidemic in the previous century that decimated the then favoured 'Gros Michel' variety, which succumbed to race 1 strains of the causal *Fusarium* spp. across Central and Latin America (Ploetz 1994, Ploetz 2005a). The resistance level to race 1 strains in Cavendish is excellent and allowed their production on even the most contaminated soils (Buddenhagen 1990, Ploetz 2005a). This boomed the industry and expanded commercial banana production into many other areas, including major production zones in Southeast Asia, until now despite their overall susceptibility to the black Sigatoka fungus that can only be controlled by weekly fungicide applications (Churchill 2011). Upon the early observations that Cavendish cultivars were affected by Panama disease in Taiwan (Su *et al.,* 1977, Su *et al.,* 1986), their monocultures around the world became a vehicle for rapid and almost global dissemination of these new *Fusarium* spp. causing Panama disease, that was recently identified as *Fusarium odoratissimum* (Maryani *et al.,* 2018) and is colloquially called Tropical Race 4 (TR4) (Ploetz 2015). TR4 is not only killing Cavendish but is also very pathogenic to many local cultivars (Walduck and Daly 2007, Wu *et al.,* 2010, Molina *et al.,* 2011, Riska and Hermanto 2012, Li *et al.,* 2015, Zuo *et al.,* 2018). The imminent expansion of Panama disease outside Southeast Asia raised a major concern as it threatens other production zones that are either important for staple food production, such as East Africa, or major export markets, such as Central and Latin America (Butler 2013).

The raised concerns on the sustainability of global banana production call for new initiatives and data gathering on resistance in existing germplasm. Indeed, disease evaluations for resistance to Panama disease have been conducted in greenhouse and field trials (Sun and Su 1984, Mert and Karakaya 2003, Adesemoye and Adedire 2005, Subramaniam *et al.,* 2006, Amorim *et al.,* 2009, Dita *et al.,* 2011, Sutanto *et al.,* 2013, García-Bastidas *et al.,* 2014, Li *et al.,* 2015, Ordóñez *et al.,* 2015a) but the throughput and hence the number of assessed accessions is low and the methodologies varied, disabling comparative analyses (Sun and Su 1984, Smith *et al.,* 1999, Mohamed *et al.,* 2001, Subramaniam *et al.,* 2006, Smith *et al.,* 2008, Wu *et al.,* 2010, Dita *et al.,* 2011). Evidently, greenhouse phenotyping is advantageous over field screens as they are cheaper, have a higher throughput and avoid many unknown factors that affect repeatability, such as unknown inoculum in the soil and genotype by environment interactions (Brake *et al.,* 1995, Amorim *et al.,* 2009, Zuo *et al.,* 2018). For many banana accessions, the response to TR4 is unknown, as they have never been exposed to the pathogen. We, therefore, set out to conduct a series of repeated greenhouse trials, using newly developed and reliable protocols to survey resistance to TR4 and race 1 strains in a suite of shortlisted banana accessions that are important for breeding and cultivation. Our experiments resulted in clear characteristic Panama disease symptoms such as wilting and corm discolouration similar to field observations and were in accord with previous studies (Amorim *et al.,* 2009, Dita *et al.,* 2011, Li *et al.,* 2015). The controls, incompatible and compatible interactions between Cavendish and race 1 and TR4, respectively, were solid references

throughout these multiyear trials, along with compatibility between 'Gros Michel' and TR4 and R1 and clean water controls.

Despite the century-old havoc caused by Panama disease (Marquardt 2001) and the fact that the resistance in Cavendish clones to race 1 strains saved the industry as well as maintained the livelihoods of millions of smallholders, not a single resistance gene to this devastating disease was ever identified. Only recently, Dale *et al.* (2017a) cloned the RGA2 locus from the wild ancestor *Musa acuminata* ssp. *malaccensis.* This gene protected Cavendish to TR4 in transformants that were trialled for several years in the field in Australia. Interestingly, all Cavendish clones, except a few somaclones that are partially resistant, are very susceptible to TR4 (García-Bastidas *et al*. Chapter 4), and also carry RGA2, but the expression is too low. This could clearly be a target for genome editing, which would improve the resistance to TR4 in these clones. However, this would not expand the required genetic diversity to improve the sustainability of commercial banana production. Therefore, it is necessary to survey resistance to Panama disease in banana. Here, we screened germplasm with two strains representing TR4 and race 1. The former represents a single clone that globally disseminates (Ordóñez *et al.,* 2015b, Zheng *et al.,* 2018), but the latter results cannot be generalized as race 1 strains belong to at least six different *Fusarium* species (Maryani *et al.,* 2018)

In a nutshell, our data demonstrate that only a fraction of the tested germplasm has the required level of resistant to TR4. Out of the interactions with 25 CRW diploids, 50 landraces and 47 new hybrids, only four, one and four showed HR responses, respectively. This is the ultimate level of resistance, similar to the incompatible Cavendish/race 1 combination that shows durable resistance in the field at manifold locations. Here it is important to realize that the compatible TR4 strain is not a mutant of race 1 strains but represents the new migrating species *F. odoratissimum* (Maryani *et al.,* 2018). These HR responses are typical for the highly resistant (DH) Pahang genotypes of *M. acuminata* ssp*. malaccensis,* which is in accord with earlier observation by d'Hont *et al.* (2012), and 'Calcutta 4' that is also known for its resistance to *P. fijiensis* (Arango *et al.* 2016). 'Pahang' has been reported as resistant by Ridley in Malaysia since 1894 (Simmonds 1956). Albeit that 'Pahang' has an excellent level of resistance to TR4, this should not be generalized to the species as a whole as demonstrated by the susceptible response of Pisang Serum 400 and the extremely susceptible response of *M. acuminata* ssp*. malaccensis* "Nain". This might be obvious for many other crops, in banana responses are erroneously, but frequently broadened to the species level. This however, also depends on how species or accessions have been maintained. Clones that were introduced as suckers maintain their genetic make-up, but others such as CWR were introduced as seeds and hence show segregating phenotypes as observed by Fraser-Smith *et al. (*2014) with a *M. acuminata* ssp. *malaccensis* accession originating from Indonesia. However, other species such as *M. acuminata* ssp. *banksii* and *M. acuminata* ssp. *siamea* are hermaphroditic species and consequently homozygotes that also can show diverse phenotypes (Horry *et al.,* 2007). In our study, most *M. acuminata* ssp. *banksii* accessions were at least susceptible to TR4, although 'Paliama' was resistant to race 1. Likewise, by testing just two *M. balbisiana* accessions, which turned out to be susceptible, we obviously cannot predict the response of the species that has a vast distribution area spanning from Eastern India and China to the Philippines.

It is also of interest that the landrace 'Morong Princesa' in the Buaya cluster showed an HR response, which is in accord with (Zuo *et al.,* 2018), despite the fact that its parent and

sister somaclones are moderately to highly susceptible, thereby demonstrating that generating somaclonal variants is a potential way to obtain resistance to Panama disease (Hwang 1991, Khayat *et al.,* 1997, Hwang and Ko 2004, Khayat *et al.,* 2004, Khayat and Ortiz 2011, Huang *et al.,* 2012). This is currently important as in Southeast Asia the so-called Giant Cavendish Tissue Culture Variants (GCTCV) somaclones are recommended as the solution for TR4 (Molina *et al.,* 2011). However, these clones have not shown the required level of resistance, at least in our trials as well as under field conditions (Dale *et al.,* 2018), and are at least moderately susceptible. Moreover, the conflicting results with these clones may also depend on the geographical location and mostly unknown natural inoculum (Simmonds 1956, Stover and Waite 1960, Rowe and Rosales 1996, Silva *et al.,* 2001, Buddenhagen 2009, Zuo *et al.,* 2018). Replacement of Cavendish by these somaclones in Mozambique has not resulted in the expected control of Panama disease (see: https://www.norfund.no/investeringsdetaljer/matanuska-africa-article12030-814.htm). Moreover, swaps to somaclones without an integrated eradication, exclusion and cultural practices program contribute to further dissemination of the disease as most farmers abandon quarantine practices once these clones are planted. Finally, these strategies are entirely focused on controlling Panama disease, which is just one of the important fungal threats in banana cultivation. From that point of view transgenic, cisgenic and genome editing strategies as well as somaclones are just addressing a single biotic threat (Novak *et al.,* 1990, Novak *et al.,* 1995, Hwang and Ko 2004, Roux 2004, Dale *et al.,* 2017a, Dale *et al.,* 2017b)). To our knowledge, these strategies have not resulted in any clones with improved resistance to black Sigatoka, which is a much more complex system due to the wide genetic diversity of the fungal population (Carlier *et al.,* 2002, Arango *et al.,* 2016), in contrast to *Fusarium* populations that are asexual and hence largely clonal (Taylor *et al.,* 1999, Leslie *et al.,* 2006, Ordóñez *et al.,* 2015b).

Merging HR and R classes increases these numbers to 9/25, 7/50 and 11/47 different levels of resistance to TR4 in CRW, landraces and hybrids, respectively. These include the cv. Rose accessions, also known as IND110, a genotype that is known in Indonesia as 'Rejang' which is widely distributed as a weedy plant and consumed and was never observed to be affected by Panama disease in Indonesia, despite the wide presence of TR4 (Maryani *et al.,* 2018). This material has also been used in ongoing breeding programs of CIRAD. Several of the tested hybrids that showed resistance to TR4, such as 'TMP x 1621-1' (AAAB), 'Fhlorban PRAM01' (AAA), 'Fhlorban 931' (AAA), 'Fhlorban 938' (AAA), 'Fhlorban 940' (AAA) and 'Fhlorban X17' (ABA) have 'Manang', IND110 or 'Calcutta 4' in their genetic background. Similar levels of resistance were observed in tetraploid accessions derived from crosses with French plantain, which showed resistance to both TR4 and race 1 (García-Bastidas *et al*. Chapter 4), such as 'CARBAP 832' (AAAB) and 'FHIA-04' (AAAB).

We consider that our greenhouse trials have been performed under defined and stable experimental conditions. However, data sets generated from adult plants in the field, may be after several generations, can easily differ from tests on younger plants (Whiley *et al.,* 1998). This is common in other pathosystems where even adult plant resistance genes have been identified, such as to cereal rusts (Chartrain *et al.,* 2005, Kema *et al.,* 2018, Saintenac *et al.,* 2018). There, however, this is based on solid genetics and this is entirely missing in banana research. Hence, any claim on resistance or different phenotypes under varying conditions should not be used to denounce data generated by others but should be taken as a basis for extended genetic studies to identify and map genes which then can be followed by expression analyses. Thus, observations of Vakili (1965) on differential

responses towards race 1 in *M. balbisiana* at different growth stages of the plant is not uncommon in other crops. Comparison of our data with those of Li *et al.* (2015) and Zuo *et al.* (2018) showed that the data are usually in accord with our observations. Nevertheless, Li *et al.* (2015) observed that several CRW showed different responses in comparative greenhouse and field trials. Similarly, Zuo *et al*. (2017) showed that plants with MS scores in the greenhouse were resistant in the field. In addition, several accessions that were resistant in Zuo *et al*. (2017) were susceptible in our trials, including 'Tuu Gia', 'Pisang lilin', 'Pisang jari buaya', Zebrina and 'Pisang Berlin' whereas others were susceptible, but scored MS in our experiments (e.g. FHIA- 17, IRFA905, Abaca). We consider that these differences are mostly due to different experimental protocols, different plant ages, environmental conditions and unknown inoculum sources and concentrations in field trials. For instance, germplasm that was scored as susceptible to race 1 in our trials, such as 'Flhorban 918', 'Flhorban L9', 'Flhorban, J24' and 'Kunnan T' have been maintained in the field in Guadeloupe without any sign of Panama disease. The source of inoculum in this field, however, is certainly different from the race 1 strain that we used in the greenhouse trials. This strain was isolated from an ABB 'Maça' in Cruz das Almas, Brazil and is used in the routine phenotyping of the Embrapa breeding program (Dita *et al.,* 2010). It is very pathogenic on 'Gros Michel', but it does not cause any disease on Cavendish, even under high pressure of inoculum. Hence, it is considered as race 1, but its genetic make-up is different from all other tested *Fusarium* genotypes and species (Ordoñez *et al.,* 2018). These observed differences call for a wide adoption of validated protocols to enhance data sharing and comparison García-Bastidas *et al*. (Chapter 3).

Our analyses have identified valuable sources of resistance to TR4 in diploid wild species and landraces. Deploying such resources is required in banana breeding to broaden the genetic base of commercial varieties. This will contribute to a much more sustainable future of the crop. Although banana breeding is considered difficult, it is possible (Menendez and Shepherd 1975, Stover and Buddenhagen 1986, Persley and De Langhe 1987, Buddenhagen 1990), and it is certainly not the only crop where the timelines are long compared to e.g. perennials and tree breeding (van der Vossen 1985, Rajanaidu *et al.,* 2000, Namkoong *et al.,* 2012). Moreover, the use of wide genetic resources has contributed to a rich diversification in many other crops albeit that linkage drag also resulted in undesirable traits (Sadowski and Kole 2016). In tomato, for example, *Solanum pimpinellifolium* has shown to be an important source of fruit quality (Voorrips *et al.,* 2000, da Costa *et al.,* 2013) and disease resistance (Sharma *et al.,* 2008). Wide crosses programs have resulted in numerous new genes for wheat breeding (see: https://shigen.nig.ac.jp/wheat/komugi/genes) (Wulff and Moscou 2014). In coffee, the diploid species *Coffea canephora* carries genes for resistance to *Hemileia vastratix*, the coffee leaf rust fungus and has been used to introduce these genes into the cultivated species *C. arabica* L (Lashermes *et al.,* 2000, Herrera *et al.,* 2002). Hence, using the identified resources in modern banana breeding programs using the latest genetic technologies to support selection and eventually deploying new genes in improved varieties is the way forward. Our data provide a first attempt to identify and characterize such valuable resources for future banana breeding.

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CHAPTER 6

The Pathogenicity of Genetically Diverse *Fusarium* species causing Panama Disease in Banana

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Abstract

Fusarium wilt caused by *Fusarium* spp. previously known as *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is one of the most devastating fungal diseases on bananas. *Foc* belongs to the *F. oxysporum* species complex (FOSC) and is composed of genetically diverse isolates, grouped into vegetative compatibility groups (VCGs). Based on pathogenicity towards a set of banana cultivars, *Foc* strains are classified into races. However, race designations are often solely based on the origin from which a particular *Foc* strain was sampled, without phenotyping for pathogenicity on other cultivars. In this study, the pathogenicity of 22 *Foc* VCGs was explored on 'Gros Michel' and 'Grand Naine', two iconic banana cultivars from the *Musa* AAA group, under greenhouse conditions. Additionally, the virulence of *Foc* isolates, popularly known as tropical race 4 (TR4, unified in VCG01213), was tested on 'Grand Naine' plants. Genetically diverse *Foc* genotypes caused distinct pathogenic responses on 'Gros Michel' and 'Grand Naine' cultivars. 'Gros Michel' was generally more susceptible to various VCGs than 'Grand Naine'. All VCG01213 isolates, regardless of the year of isolation and country of origin, were highly infectious on both 'Gros Michel' and 'Grand Naine', underpinning the risk for disease outbreaks in banana plantations that are planted to these cultivars. The plant responses of both cultivars were indistinctively associated with *Foc* VCGs from clade 1 and 2 of the FOSC and did not frequently correspond to their related *Foc* races. These findings show that the current race concept does not accurately reflect the virulence of the diverse *Foc* genotypes on bananas.

Introduction

Food security is challenged by emergent fungal diseases that decimate yields, causing widespread malnutrition and starvation (Gurr *et al.,* 2011). Bananas (*Mus*a spp.) are among the most important food and fruit crops, representing a major commodity for numerous agro-based economies worldwide and an important staple food in Asia and Africa (Frison and Sharrock 1998, Aurore *et al.,* 2009, FAOSTAT 2016). In 2014, world banana production reached 78.8 million tonnes, with India contributing 38% of produced bananas (Lescot 2017). Edible bananas result from interspecific and intra-specific hybridization of the two-founding species: *Musa acuminata* (AA) and *Musa balbisiana* (BB) (Simmonds and Shepherd 1955). They have evolved through a selection of diploid and triploid seedless clones that were reproduced over centuries via asexual vegetative propagation. Therefore, edible bananas are often sterile or parthenocarpic (Perrier *et al.,* 2011). Nevertheless, the genetic and phenotypic diversity is enormous, with hundreds of banana cultivars largely destined for domestic markets (FAOSTAT 2016, Lescot 2017). To classify this diversity, the international standard is to refer to the genus name *Musa,* followed by a code denoting the genome group and ploidy level, the subgroup name (if any) and lastly the popular name of the cultivar, for example *Musa* AAA (Cavendish subgroup) 'Grand Naine' (Robinson and Saúco 2010). Despite the immense genetic diversity of banana germplasm, the export trade relies on a few dessert or sweet cultivars from the *Musa* AAA group (Robinson and Saúco 2010, Perrier *et al.,* 2011). Among them are 'Gros Michel' and 'Grand Naine' cultivars, two economically important dessert bananas in the Gros Michel and Cavendish subgroups, respectively (Robinson and Saúco 2010). The Cavendish subgroup accounts for more than 40% of total banana production around the globe (Ploetz 2015), with 'Grand Naine' as one of the major constituents across the world (Robinson and Saúco 2010). From the plant pathology perspective, such monoculture production systems are easily jeopardized by disease and pest threats (Ploetz 2015). For instance, by the 1960s, the dominating export 'Gros Michel' cultivar was nearly wiped out during a Panama disease epidemic. This disease that is also known as Fusarium wilt, is caused by the pathogen previously known as *Fusarium oxysporum* f. sp. *cubense* (*Foc*), which comprises a set of genetically diverse clonally propagating strains (Boehm *et al.,* 1994, Koenig *et al.,* 1997, Bentley *et al.,* 1998, Bogale *et al.,* 2006, S Groenewald *et al.,* 2006, Susan Groenewald *et al.,* 2006, Fourie *et al.,* 2009), is considered one of the most devastating fungal diseases of bananas (Ploetz 2015). The pathogen initially infects the root system and reaches the rhizome causing internal tissue discolouration. Later, the pseudostem xylem vessels get clogged due to deposition of callose, formation of tyloses and gels, accumulation of phenolics and intense mycelium and microconidia production. As a result, the transport of nutrients and water is impeded (Ghag *et al.,* 2015, Ploetz 2015). Under natural conditions, external symptoms appear relatively late, often six months after the initial infection, showing chlorosis of older leaves that progress to the younger leaves until the whole plant wilts and dies.

In 1910, *Foc* was isolated for the first time from wilted banana plants in Cuba (Smith, 1910). Since then, *Foc* strains have been recovered from nearly all banana-growing regions in the world. These strains are commonly characterized by vegetative compatibility, also known as heterokaryon compatibility. It is a naturally occurring characteristic of many fungi, governed by a specific set of nuclear loci known as *het* or

vic-loci (Puhalla 1985, Correll 1991). Compatible fungal isolates share an allele at the *vic* locus enabling them to form a stable hyphal fusion, resulting in heterokaryon formation, and such compatible isolates are then assigned to the same vegetative compatibility group (VCG)(Correll 1991, Leslie *et al.,* 2006). Until now, 24 VCGs have been recognized for *Foc,* from VCG0120 through 0126 and 0128 through 01224 (Moore *et al.,* 1993, Bentley *et al.,* 1995, Katan and Di Primo 1999, Ordóñez *et al.,* 2015b, Ploetz 2015). Additionally, since some isolates from different VCGs frequently form stable heterokaryons, three VCG complexes have been proposed, namely 0120/15, 0124/5/8/20 and 01213/16 (Ploetz 2006). The VCG01213/16 complex is considered as a single group (01213) composed of genetically similar isolates (Bentley et al., 1998; Ordóñez et al., 2015). More recently, VCG01211 and 01222 were included in the 0120/15 and 0124/5/8/20 complexes, respectively, based on VCG cross-compatibility (Mostert *et al.,* 2017)

The *Foc* strains belong to the *F. oxysporum* species complex (FOSC) that is composed of four main clades (1, 2, 3 and 4) containing cosmopolitan non-pathogenic and several pathogenic strains that are morphologically indistinguishable (O'Donnell *et al.,* 1998, Baayen *et al.,* 2000, Fourie *et al.,* 2009, Fourie *et al.,* 2011). With the advent of molecular anaylses, the reported *Foc* genotypes have been grouped into clade 1 and 2. In these clades, *Foc* isolates within the same VCG and VCG complexes showed a high level of genetic similarity (Bentley et al., 1998; Boehm et al., 1994; Bogale et al., 2006; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997). Given that to date no sexual cycle was reported for *Foc* (Fourie et al., 2009), this genetic similarity suggests a clonal reproductive behavior (Bentley et al., 1998; Boddy, 2016; Fourie et al., 2009). Only for *Foc* isolates from the VCG0124/5/8/20 complex, some degree of recombination, related to parasexual events, has been reported (Taylor *et al.,* 1999).

Despite, extensive progress in the molecular differentiation of *Foc* isolates, little advance has been made in analysing the pathogenicity towards banana. Traditionally, *Foc* strains have been classified into three races: 1, 2 and 4, depending on field responses of a set of banana cultivars (Stover 1962, Armstrong and Armstrong 1968). Race 1 is virulent to 'Gros Michel' and also affects 'Maqueño', 'Pisang Awak', Pome and Silk. Race 2 affects Bluggoe and other cooking bananas. Lastly, race 4 is the most damaging since it affects race 1 and 2 susceptible cultivars as well as Cavendish bananas (Ploetz, 2015a). Race 4 is further subdivided into subtropical race 4 (ST4) and tropical race 4 (TR4). ST4 strains cause disease in Cavendish plantations in subtropical regions under abiotic stress such as low temperatures (Ploetz, 2006). TR4, however, is highly pathogenic and infects bananas irrespective of environmental conditions (Buddenhagen 2009). Generally, race 1 and 2 (VCGs 0124, 0125, 0128, 01217 and 1218) reside in clade 2 of the FOSC, and have been mostly recovered from bananas containing a B genome (*M. acuminata* and *M. balbisiana* hybrids), while race 4 strains (VCGs 0120/15, 0121, 0122, 0126, 0129, 01211, 01213/16 and 01214) are mostly placed in clade 1 and were recovered from bananas containing A genomes (*M. acuminata* related) (Bentley et al., 1998; Boehm et al., 1994; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997). Despite these general trends, exceptions with dissimilar pathogenic profiles occur. For instance, VCG0124, commonly related to race1 and 2, caused a disease outbreak in a commercial Cavendish plantation in India (Thangavelu and Mustaffa 2010). Thus, presently a single VCG can be related to multiple races.

Since *Foc* race designation is commonly defined by the banana host cultivar, we decided to scrutinize the pathogenicity of 22 *Foc* VCGs towards 'Gros Michel' and 'Grand Naine', thereby estimating their potential risk for current banana production. The former cultivar dominated the export trade in the previous century but was wiped out by race 1 strains. The latter currently dominates the trade market and represents 40% of the global production but is extremely susceptible to TR4. Therefore, this banana cultivar is a vehicle for the rapid expansion of Fusarium wilt in global Cavendish production (Butler 2013, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a, Zheng *et al.,* 2018). Additionally, geographically diverse VCG01213 isolates were tested on 'Grand Naine' plants to determine variation in TR4 aggressiveness. Our findings challenge the *Foc* race concept, by showing that the current definition inadequately reflects *Foc* virulence on banana cultivars.

Materials and Methods

Plant material

Tissue culture plantlets of 'Gros Michel' and 'Grand Naine' were transferred upon arrival to small pots containing standard soil (Swedish sphagnum peat 5%, grinding clay granules 41%, garden peat 5%, beam structure 4%, steamed 140 compost 33%, PG-Mix-15-10-20- 12%) from the UNIFARM greenhouse facility of Wageningen University & Research, Wageningen, The Netherlands and maintained for two weeks at 28 ±2˚C and ~100% relative humidity (RH) to acclimatize. Thereafter, they were transplanted to 2 L pots with the same soil and maintained for approximately three months at the same temperature, RH (~80%) and a 16/8 diurnal schedule until inoculation.

Inoculum preparation

Fusarium isolates were transferred to potato dextrose agar (PDA, Difco™, USA) and incubated at 25˚C. After five days, four circular plugs (~5 mm diameter) from the edge of the colony of each isolate were incubated together with sterile maize kernels on Petri dishes for five days at 25˚C (Dita et al., 2010; Lichtenzveig et al., 2006). Likewise, five additional circular plugs were inoculated into a sterile mung bean media for inoculum production (10^6 conidia.ml^{-1}) following the protocol of García-Bastidas *et al.* (Chapter 3).

Plant inoculation and disease assessment

Three-month-old 'Gros Michel' and 'Grand Naine' plants were tested under the aforementioned greenhouse conditions. Per *Foc* isolate representing each of the 22 VCGs (Table 1), six plants per banana cultivar were inoculated following the pouring method developed by García-Bastidas *et al.* (Chapter 3) supplemented with placing five infested maize kernels in each pot and six control plants were treated only with water. To test potential differences in aggressiveness of the VCG01213 isolates (Table 1), we inoculated five 'Grand Naine' plants with each isolate and five control plants were inoculated with either race 1 or water. After inoculation, plants were maintained in the greenhouse at the aforementioned conditions, but at a slightly higher RH of 80% until scoring and were monitored weekly for disease development and progress. Final scoring was conducted at nine weeks after inoculation (wai) according to the protocol of García-Bastidas *et al.* (Chapter 3) with slight modifications depending on disease development. We also scored

secondary symptoms such as pseudostem splitting at the base of the plants and the presence of stunted new leaves.

Recovery and confirmation of inoculated genotypes

During final scoring, we collected rhizome tissue for each *Foc* isolate to fulfill Koch´s postulates. Samples were surface sterilized starting with an initial washing step using tap water followed by an immersion in 70% alcohol for 5 minutes (min). Thereafter, they were rinsed with sterile water for 5 min and dried on filter paper. Subsequently, two pieces (~5 x 2 mm^2) of rhizome tissue per plant were plated on ¼ enriched PDA amended with streptomycin (1 g/L) and incubated for seven to 10 days at 25˚C in darkness. Growing strains were identified by PCR and/or VCG testing. For PCR diagnostics, mycelium was transferred to 2 mL Eppendorf tube and freeze-dried (Epsilon 1-4 LSC, Christ GmbH, Germany) prior to DNA extraction. VCG testing was performed on for every strain recovered from the inoculated plants, except for VCG01213 (see below section).

Table 1. *Fusarium oxysporum* f. sp. *cubense* isolates included in this study.

VCG	Race[2]	Clade[3]	Isolate code	Host name	Host genotype	Country	Year of isolation	Provider
0120	ST4, R1	1	*Foc*ST498	Dwarf Cavendish	AAA	(Canary Islands) Spain	1998	Julio Hernandez, Spain
0121	ST4, TR4	1	NRRL36102	Cavendish	AAA	(Taiwan) China	n.d.	K. O'Donnell, USA
0122	R2, TR4	1	NRRL36103	Cavendish	AAA	Philippines	n.d.	K. O'Donnell, USA
0124	R1, R2	2	NRRL36105	Bluggoe	ABB	Honduras	n.d.	K. O'Donnell, USA
0125	R1, R2	2	NRRL36106	Lady finger	AAB	Australia	n.d.	K. O'Donnell, USA
0126	R1, ST4	1	NRRL36107	Maqueno	AAB	Honduras	n.d.	K. O'Donnell, USA
0128	R1, R2	2	NRRL36111	Bluggoe	ABB	Australia	n.d.	K. O'Donnell, USA
0129	R1, ST4	1	NRRL36110	Mons	AAA	Australia	n.d.	K. O'Donnell, USA
01210	R1	1	*Focu*7	Apple	AAB	(Florida) USA	n.d.	M.J. Daboussi, Université Paris – Sud, France
01211	ST4	1	NRRL36109	SH 3142	AA	Australia	n.d.	K. O'Donnell, USA
01212	n.d.	2	NRRL36108	Ney Poovan	AB	Tanzania	n.d.	K. O'Donnell, USA
01213/16	TR4	1	24662	Cavendish	AAA	Australia	1999	A. Drenth, Australia
01213[1]	TR4	1	II5	Pisang Manurung	AAB	Indonesia	n.d.	C. Kistler, USA
01213	TR4	1	*Foc*.T105	Cavendish	AAA	(Taiwan) China	n.d.	P.F.L. Chang, Taiwan
01213	TR4	1	JV11	Cavendish	AAA	Jordan	2006	R.C. Ploetz, USA
01213	TR4	1	Leb1.2C	Cavendish	AAA	Lebanon	2013	M.Y. Akkary, Debbane Freres, Lebanon, N. Ordóñez[4], The Netherlands
01213	TR4	1	Mal123	Cavendish, Williams	AAA	Malaysia	n.d.	W. O'Neil, Australia
01213	TR4	1	Pak1.1A	Cavendish	AAA	Pakistan	2012	Hadi Bux Laghari, Pakistan, N. Ordóñez[4], The Netherlands
01213	TR4	1	Phi2.6C	GCTCV218	AAA	Philippines	2013	L.M. Bacus, The Philipines, N. Ordóñez[4], The Netherlands
01214	R2	2	NRRL36113	Harare	ABB	Malawi	n.d.	K. O'Donnell, USA
01215	ST4, R1	1	NRRL36112	Cavendish	AAA	South Africa	n.d.	K. O'Donnell, USA

VCG	Race[2]	Clade[3]	Isolate code	Host name	Host genotype	Country	Year of isolation	Provider
01217	R1	2	Mal43	Pisang Rastali	AAB	Malaysia	1995	A. Drenth, Australia
01218	R1	2	NRRL36120	Kluai Nam Wa	ABB	Thailand	n.d.	K. O'Donnell, USA
01219	n.d.	1	Indo25	Pisang Ambon	AAA	Indonesia	1993	A. Drenth, Australia
01220	R4	2	24218	Cavendish	AAA	Australia	1993	A. Drenth, Australia
01221	n.d.	2	NRRL36118	Kluai Nam Wa	ABB	Thailand	n.d.	K. O'Donnell, USA
01222	n.d.	2	NRRL36117	Pisang Awak Legor	ABB	Malaysia	n.d.	K. O'Donnell, USA
01223	n.d.	2	NRRL36116	Pisang Keling	AAB	Malaysia	n.d.	K. O'Donnell, USA
01224	n.d.	2	NRRL36115	Pisang Ambon	AAA	Malaysia	n.d.	K. O'Donnell, USA
n.d.	R1	3	*Foc*_R1	Silk	AAB	Brazil	2008	M. Dita and C. Waalwijk, The Netherlands

[1]Only the II5 isolate (VCG01213) was used for the assay with multiple VCGs. [2]Race related (race 1, R1; race 2, R2 and race 4, R4) to each VCG according to previous reports (Boehm et al., 1994; Koenig et al., 1997; Bentley et al., 1998; Groenewald et al., 2006; Fourie et al., 2009; Fraser-Smith et al., 2013). [3]The selected isolates were previously analysed and grouped into the three clades of the *Fusarium oxysporum* species complex described in O'Donnell et al. (1998). [4]Providers sent tissue samples to our facilities and the strain isolation was performed at Wageningen University & Research. n.d. stands for "not determined".

DNA isolation and molecular identification

The DNA isolation was carried out using a Kingfisher robot (Thermo Labsystems, Oy, Finland) using the AGOWA Sbeadex® Maxi plant DNA isolation kit from LGC Genomics (Germany) according to the manufacturer's instructions. Samples were mixed with 600 µl of lysis buffer and homogenized for 40 seconds at 5800 rpm in the homogenizer Precellys® (Bertin Technologies, France). Subsequently, they were incubated at 65˚C for 15 min. Supernatants (200 µl) were recovered after centrifugation for 20 min at 13,000 rpm and transferred to a deep well plate containing 520 µl of binding buffer. The process was finished according to the manufacturer's protocol using the KingFisher technology (ThermoFisher Scientific, USA). The total amount of genomic DNA was quantified using Quant-iT™ Picogreen® dsDNA Reagent and Kit (Life Technologies, USA), according to the manufacturer's instructions. The fluorometric measurements were performed using Tecan Infinite® M200 PRO monochromator (Tecan, Männedorf, Switzerland) using Icontrol 107 software (US, Morrisville, NC). DNA samples were adjusted to 5 ng/µL and stored at −20˚C until use. All DNA samples were assessed using the *F. oxysporum* primers (Edel *et al.,* 2000), using an annealing temperature of 62˚C (van Brunschot, 2006). Subsequently, the TR4 diagnostic primers (Dita et al., 2010) were used to verify VCG01213 isolates. PCR products (10 µL) were visualized on a 1.5% agarose gel to check for predicted size products.

Vegetative compatibility confirmation

Testing of vegetative compatibility was performed on all recovered *F. oxysporum* strains, except for VCG01213. Single-spore cultures were generated and then, *nit* (NO_3-non-utilizing) mutants were obtained by incubating them for 7-14 days at 25˚C in darkness on minimal medium (MM) amended with 1.5-2% $KClO_3$. The *nit* mutants were characterized as *nit1*, *nit3* or NitM following the Leslie & Summerell (2006) protocol. Pairing tests were run on MM for 7-14 days to check compatibility with NitM testers of corresponding inoculated VCG's, using at least two independent *nit1* and/or *nit3* mutants. Compatible isolates were scored based on their ability to form heterokaryons.

Results

Distinct responses to genetically diverse VCGs

The tested VCGs represent the widest genetically diverse *Foc* panel which is distributed over clades 1 and 2 of the FOSC (Bentley et al., 1998; Boehm et al., 1994; Bogale et al., 2006; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997). All strains elicit distinct responses in both banana cultivars, with 'Gros Michel' being, in general, more susceptible to various VCGs (Figure 2). The typical leaf chlorosis was observed for all inoculated plants including the controls (Table 2). Usually, high percentages of chlorosis were accompanied by pseudostem splitting and stunted new leaves, particularly in 'Gros Michel', inoculated with VCGs 0120, 01213, 01218 and 01219 compared to 'Grand Naine' plants and the controls (Figure 1), but the overall

Table 2. Phenotyping *Fusarium oxysporum* f. sp. *cubense* (*Foc*) on the banana varieties 'Grand Naine' and 'Gros Michel'. Internal and external disease scores are shown for each *Foc* isolate and the disease index has been calculated for each interaction.

Fusarium isolate	'Gros Michel'				'Grand Naine'			
	External symptom Chlorosis		Internal symptom Rhizome discoloration		External symptom Chlorosis		Internal symptom Rhizome discoloration	
	Average discoloration (%)	Class rate scale	Average scale	DI (%)	Average discoloration (%)	Class rate scale	Average scale	DI (%)
VCG0120	80.2	IV	3.7	53.3	43.5	II	3.2	43.3
VCG0121	61.9	III	3.0	40.0	29.7	II	2.5	30.0
VCG0122	61.1	III	2.8	36.7	39.7	II	2.7	33.3
VCG0124	60.5	III	2.5	30.0	27.9	II	3.2	43.3
VCG0125	67.0	III	3.3	46.7	24.1	I	1.0	0.0
VCG0126	66.6	III	1.8	16.7	31.7	II	1.3	6.7
VCG0128	59.8	III	2.2	23.3	28.8	II	2.0	20.0
VCG0129	50.9	III	1.8	16.7	29	II	1.2	3.3
VCG01210	52.1	III	1.7	13.3	22.1	I	1.2	3.3
VCG01211	68.6	III	3.0	40.0	34.1	II	3.2	43.3
VCG01212	66.9	III	4.0	60.0	33.1	II	1.3	6.7
VCG01213	77.3	IV	4.0	60.0	58.3	III	4.0	60.0
VCG01214	60.2	III	2.3	26.7	22.1	I	2.8	36.7
VCG01215	71.8	III	2.5	30.0	60.5	III	2.7	33.3
VCG01217	64.8	III	3.2	43.3	38.3	II	1.2	3.3
VCG01218	74.9	IV	4.7	73.3	25.4	II	1.7	13.3
VCG01219	75.9	IV	3.3	46.7	33.1	II	1.2	3.3
VCG01220	63.2	III	2.7	33.3	52.5	III	3.3	46.7
VCG01221	67.7	III	3.2	43.3	27.3	II	2.3	26.7
VCG01222	71.6	III	3.3	46.7	29.5	II	1.7	13.3
VCG01223	64.9	III	4.5	70.0	19.7	I	2.0	20
VCG01224	55.6	III	3.2	43.3	26.2	II	1.2	3.3

Fusarium isolate	'Gros Michel'				'Grand Naine'			
	External symptom Chlorosis		Internal symptom Rhizome discoloration		External symptom Chlorosis		Internal symptom Rhizome discoloration	
	Average discoloration (%)	Class rate scale	Average scale	DI (%)	Average discoloration (%)	Class rate scale	Average scale	DI (%)
Control	47.4	II	1.0	0.0	26.3	II	1.0	0.0

In terms of clade classification of the tested *Foc* isolates, nine out of 10 isolates from clade 1 and all clade 2 isolates caused severe disease symptoms in 'Gros Michel', while for 'Grand Naine' cultivar, six isolates from each clade elicited moderate rhizome discoloration (Figure 2A). The cross-compatible *Foc* isolates from the VCG0120/11/15 complex produced moderate disease levels in each cultivar, except for VCG0120 that was particularly severe on 'Gros Michel'. All isolates from the VCG0124/5/8/20/22 complex inflicted moderate severities in 'Gros Michel', but induced low to moderate severities in 'Grand Naine'. In terms of race classification commonly designated to the tested *Foc* VCGs (Table 1), race 1 strains were generally very pathogenic for 'Gros Michel' but avirulent on 'Grand Naine', whereas ST4 and TR4 strains were particularly pathogenic towards 'Grand Naine' (Figure 2C). However, we also observed that some race 1 isolates caused only limited disease in 'Gros Michel' (VCG01210), but moderate severities on 'Grand Naine' (VCG0124 and 0128), whereas some race 4 isolates caused limited disease levels in 'Grand Naine' (VCG0126 and 0129). Unexpectedly, the race 2 isolate (VCG01214) cause moderate disease levels in both 'Gros Michel' and 'Grand Naine'. Lastly, the undefined VCGs 01212, 01219 and 01221-01224 were categorized as race 1 (01212, 01219, 01222 and 01224) and race 4 (01221 and 01223), based on the current *Foc* race concept.

Figure 1. External symptoms of Fusarium wilt recorded under greenhouse conditions: (A) leaf chlorosis, (B) stunted new leaf and (C) splitting on the base of the pseudostem (Photos FA García-Bastidas).

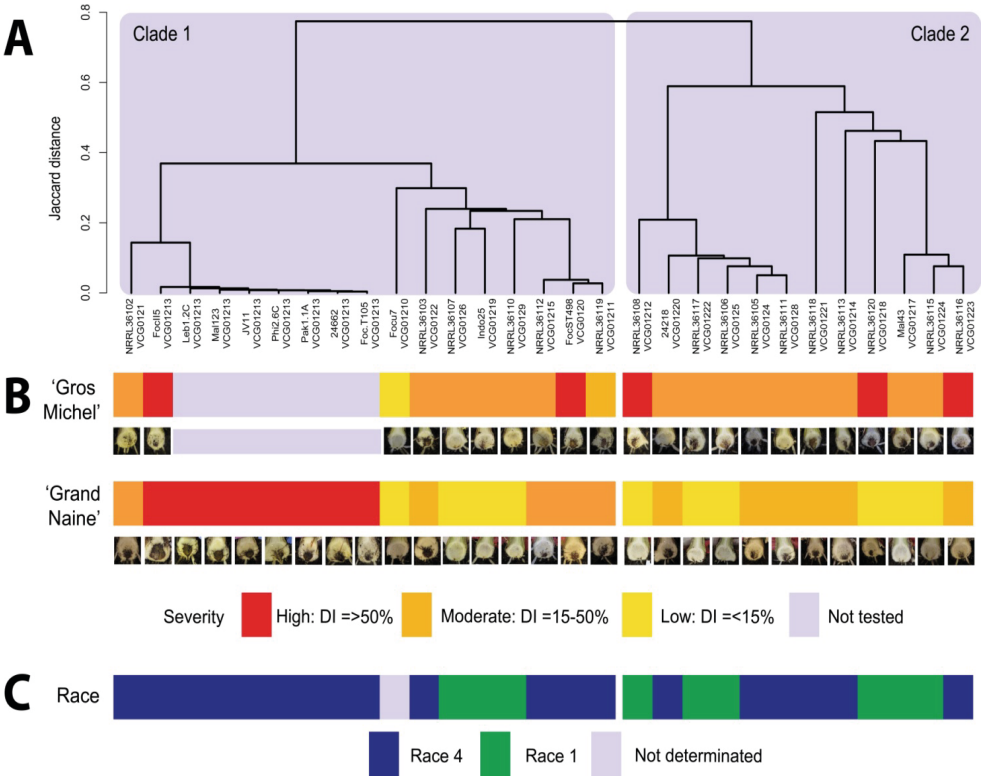

Figure 2. Disease severity caused by 22 vegetative compatibility groups (VCGs) of *Fusarium* spp., including multiple isolates belonging to VCG01213, on 'Gros Michel' and 'Grand Naine' banana cultivars. (A) The dendrogram represents the genetic diversity of the tested *Foc* isolates based on DArTseq markers (adapted from Ordóñez *et al.,* 2015). (B) Categorization of disease severities (high, moderate and low) on 'Gros Michel' and 'Grand Naine' as shown by the different levels of rhizome discoloration. (C) Race designation based on the current *Foc* race concept.

Quantitative variation among tropical race 4 isolates on 'Grand Naine'

The eight geographically diverse VCG01213 isolates representing TR4 caused severe symptoms in 'Grand Naine' (Fig 2 B). During the final scoring at nine wai, chlorosis of the foliage was >40%, while the controls showed 6.7-13% (Table 3). The isolates from Lebanon and Pakistan seemed more aggressive (>80%) followed by isolates from Indonesia, Jordan and Malaysia (60-66.7%). Such quantitative variation was also observed for the internal symptoms. DIs varied between 56 and 88% (Table 3), with highest DI values (>80%) for isolates from Indonesia, Lebanon and Pakistan. All controls (water and 'Grand Naine' with R1) remained healthy with no internal symptoms.

Table 3. Phenotyping aggressiveness levels of *Fusarium oxysporum* f. sp. *cubense* VCG01213 isolates on banana 'Grand Naine'. Internal and external disease scores are shown for each isolate and the disease index has been calculated for each interaction.

Isolate code	VCG	External symptoms Leaf yellowing		Internal symptoms Rhizome discoloration	
		Average discoloration (%)	Class rate scale	Average Scale	DI (%)
Leb1.2C	01213	86.7	IV	5.4	88.0
Mal123	01213	66.7	III	4.6	72.0
Phi2.6C	01213	43.0	II	4.4	68.0
JV11	01213	66.7	III	4.8	76.0
Pak1.1A	01213	80.0	IV	5.0	80.0
II5	01213	60.0	III	5.2	84.0
Foc.T105	01213	40.0	II	3.8	56.0
24662	01213	46.7	II	3.8	56.0
Foc_R1	n.d	6.7	I	1.0	0.0
Water	-	13.0	I	1.0	0.0

Recovery of inoculated fungal genotypes associated with severity groups

Rhizome samples from all interactions were collected and analysed. For samples from interactions with high DIs, mycelium rapidly developed on PDA and isolate identities were confirmed by VCG analysis or molecular diagnostics. Similarly, we retrieved *Foc* isolates from interactions with moderate DIs (<37%), but none from 'Grand Naine' plants inoculated with strains representing VCG0122, 01214, 01221 and 01223 and neither from any interaction with low DIs. The few developing colonies from these isolations did not morphologically resemble *F. oxysporum* strains and also tested negative with *F. oxysporum* primers (Edel et al., 2000). Finally, all samples from the controls did not result in any fungal colonies on PDA (Figure 3).

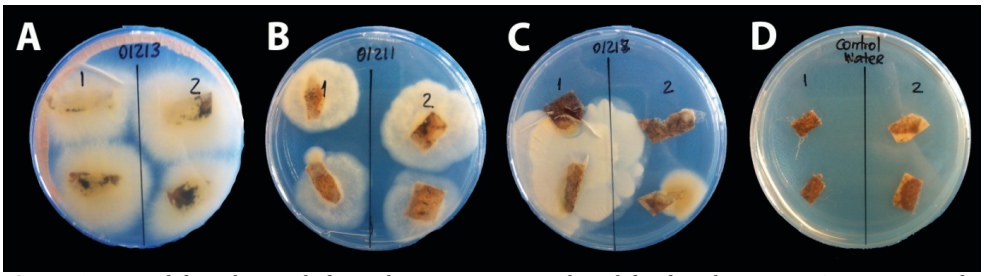

Figure 3. Typical fungal growth from rhizome tissue incubated for five days on PDA, originating from plants with (A) high, (B) moderate and (C) low disease severities. (D) No fungal growth was observed from rhizomes of the control plants.

Discussion

Plant diseases affect human wellbeing by provoking serious agricultural and economic losses, as those exemplified by for instance late blight disease on potatoes and Karnal bunt on wheat (Anderson *et al.,* 2004). Nowadays, crop-destroying fungi account for

perennial yield losses of ~20% worldwide, with a further 10% loss postharvest (Fisher et al., 2018). In bananas, the race 1 outbreak of Fusarium wilt caused havoc in Latin America, wiping out 'Gros Michel' plantations that caused an estimated loss of $400 million up to 1960s (Ploetz 2005). Despite the importance of Panama disease, only a handful of studies addressed pathogenicity of *Foc* strains to banana germplasm(Ribeiro and Hagedorn 1979, Liew 1996, Wu *et al.,* 2010, Li *et al.,* 2015, Ploetz 2015, Zuo *et al.,* 2018), and even fewer determined *Foc* races in inoculation trials (Araújo et al., 2017; Thangavelu et al., 2012). Instead, *Foc* races are traditionally designated by the banana variety from which they are isolated, mostly under field conditions. Therefore, pathogenic specialization of *Foc* VCGs is unclear, since pathogenicity is not systematically assessed by inoculating various banana cultivars with a suite of *Foc* isolates. Moreover, mechanisms of host resistant are not considered under the current *Foc* race concept. In other pathosystems such trials have been the basis for resistance discovery research. Cereal rusts have been threatening global wheat production for decades, and detailed phenotyping trials have eventually resulted in the identification of effectors (Chartrain *et al.,* 2005, Kema *et al.,* 2018, Saintenac *et al.,* 2018), which are currently being used in advanced breeding programs and *Phytophthora* research has benefited enormously from phenotyping data in the discovery of *RXLR* effectors (Anderson et al., 2015). Therefore, we considered that such a plant-pathogen matrix would be foundational for understanding the banana–*Fusarium* pathosystem and decided to test all the known *Foc* VCGs on 'Gros Michel' and 'Grand Naine', two important banana varieties that dominated the banana trade for the last century and historically are considered race 1 and race 4 differential cultivars, respectively (Ploetz, 2005; Ploetz, 2015). Contrary to field screening, our assays were conducted under controlled conditions, that provide comparable data and different responses are assumed to reflect genetic differences between the *Foc* isolates. In general, the infection with 22 genetically diverse *Foc* VCGs resulted in distinct responses, with 'Gros Michel' being more susceptible to the majority of VCGs. The *Foc* isolates related to clade 1 and 2 of the FOSC indistinctly inflicted a moderate or high rhizome discoloration to 'Gros Michel' and 'Grand Naine'. This finding is in accordance with phylogenetic studies suggesting that *Foc* pathogenicity towards a certain banana cultivar is a polyphyletic trait (Fourie *et al.,* 2009, Fourie *et al.,* 2011, Fraser Smith and Aitken 2011, Fraser-Smith *et al.,* 2014), and challenges the observation that genotypes in clade 1 are usually associated with Fusarium wilt on Cavendish cultivars and clade 2 to 'Gros Michel' (Bentley et al., 1998; Boehm et al., 1994; Fourie et al., 2009; Groenewald et al., 2006; Koenig et al., 1997). *Foc* VCGs are phylogenetically distant genotypes with multiple evolutionary origins (O'Donnell et al., 1998), exempting isolates within VCG complexes such as VCG0120/11/15 and 0124/5/8/20/22 complexes that are genetically related (Bentley *et al.,* 1995, Bentley *et al.,* 1998, Fourie *et al.,* 2009, Fourie *et al.,* 2011, Ordóñez *et al.,* 2015b, Maryani *et al.,* 2018). In our study, isolates from the VCG0120/11/15 complex caused similar rhizome discoloration on both banana cultivars, but those from the VCG0124/5/8/20/22 complex differentially affected 'Grand Naine' plants. Despite their relative close genetic distance, isolates from this VCG complex are not regarded as clonal. Instead, parasexual recombination is suggested to occur among isolates of this VCG complex (Taylor et al., 1999). Such genetic differences might explain the diverse responses of 'Grand Naine' to these slightly diverse genotypes. Conversely, the genetically similar VCG01213 were equally highly pathogenic to both banana cultivars regardless of the year of isolation or geographical origin, despite quantitative variation in aggressiveness towards 'Grand Naine' (DI from 56% to 88%). This was recently also confirmed by Maryani et al. (2018) who studied pathogenicity of

a suite of Indonesia TR4 isolates. Our data show that despite the limited single nucleotide polymorphisms (SNPs) that geographically diversified these VCG01213 isolates (Zheng et al., 2018), the overall sequence identity (~0.01% SNPs) (Ordóñez et al., 2015) was reflected in their high pathogenicity in the current trials. These results indicate that phenotyping trials in the banana–*Fusarium* pathosystem should consider testing multiple strains from each VCG. However, the current methodologies, despite recent improvements García-Bastidas *et al. (*Chapter 3), constrains throughput. Therefore, future phenotyping should ideally be based on effector screens, which will revolutionize the understanding of pathosystem, as was also shown in many other pathosystems (Mes *et al.,* 1999, Anderson *et al.,* 2004, Chartrain *et al.,* 2005, Kema *et al.,* 2018, Saintenac *et al.,* 2018)

The effect of varying environmental conditions on *Foc* race classification was primarily associated with unexpected disease development in Cavendish cultivars by other *Foc* lineages than VCG01213 (Bentley et al., 1998; Boehm et al., 1994; Fourie et al., 2009; Fraser-Smith et al., 2013; Groenewald et al., 2006; Koenig et al., 1997; Mostert et al., 2017). As a result, other VCGs such as 0121 (Aguayo *et al.,* 2017) and 0122 (Fraser-Smith *et al.,* 2014) have also, but incorrectly, been regarded as TR4s. In our study, 11 *Foc* VCGs caused moderate rhizome damage in 'Grand Naine' plants. Such isolates might be potentially harmful, particularly once unfavourable soil and environmental conditions predispose 'Gros Michel' and 'Grand Naine' (Pegg *et al.,* 1995, Shivas and Philemon 1996, Deltour *et al.,* 2017) but should never be considered as TR4. Also, the concentration and distribution of *Foc* inoculum in the soil might determine disease severity. Banana cultivars that were symptomatic under greenhouse conditions remained unaffected in field conditions, due to a lower concentration of inoculum under natural conditions (Li et al., 2014). Clearly, varying genetic background of banana germplasm also affects disease development. In our study, VCGs associated with race 1 caused moderate to high severities in 'Gros Michel', except for VCG01210 isolate *Focu*7. This isolate was originally recovered from the so-called apple banana cultivar (*Musa* AAB group), and might not affect 'Gros Michel' (AAA), given their different host genotype. Similarly, VCGs associated with race 4 affected 'Grand Naine', except for isolates from VCG0126 and 0129, which were recovered from Cavendish plants (Fraser-Smith et al., 2013). However, this is no guarantee that such isolates also are pathogenic on 'Grand Naine', as shown in our experiments. The Cavendish subgroup belongs to the *Musa* AAA group with a large phenotypic diversity as a result of selection and random somatic variation that can influence diverse responses to the same pathogen (Hwang and Ko 2004, Robinson and Saúco 2010).

Taken all together we conclude that the genetic diversity of the tested *Foc* panel underlies the diverse responses of 'Gros Michel' and 'Grand Naine'. Their responses were indistinctively associated with VCGs from clade 1 and 2 of the FOSC and did not frequently correspond with their assumed *Foc* races. Hence, the current race concept requires significant revision based on extended isolate-germplasm evaluations along with genetic and genomic analyses as in for instance the tomato–*F. oxysporum* f. sp. *lycopersici* pathosystem where a range of effector and resistance genes were identified and cloned (Mes *et al.,* 1999, Catanzariti *et al.,* 2015, Van Dam *et al.,* 2016). From that perspective, the understanding of the banana–*Fusarium* pathosystem is at its very early stage. However, the recent discovery and cloning of the first resistance gene (Dale *et al.,* 2017a, Dale *et al.,* 2017b) is an important step forward that should be extended to formal

genetics for gene discovery in the host as well as further investigations to understand the complexity of *Fusarium* pathogens in bananas (Maryani *et al.,* 2018). Our studies contribute to that aim and should eventually result in enhanced disease control.

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CHAPTER 7

Induced Resistance to Panama disease Tropical Race 4 after Priming Cavendish Bananas with Avirulent *Fusarium* spp.

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Abstract

The Cavendish cultivars saved the global banana industry from race 1 (R1) strains of various *Fusarium* species causing Panama disease in the then prime banana 'Gros Michel'. As a result, their cultivation expanded globally and currently represents approximately 50% of the production area and dominates the export trade. However, since the so-called Tropical Race 4 (TR4), a genotype of the new *F. odoratissimum,* emerged in the 1960s as a new pathogen. Since then TR4 has spread almost globally, causing havoc in Cavendish plantations as well is manifold local varieties that are primarily grown by small holders. Presently, there is not a commercially available replacement for the Cavendish clones and hence, control strategies have to be developed and implemented to manage Panama disease. Here, we used RNAseq analyses to study whether triggering incompatibility by inoculations with R1 and various *F. oxysporum* formae speciales causing disease in other crops, protects Cavendish from the pathogenic TR4. Therefore, we conducted challenge experiments comprising pretreatments with R1 and other *Fusarium* spp. prior to inoculations with TR4 at various timescales and *vice versa.* Indeed, we observed that pre-treatments with R1 significantly reduced the disease development of TR4. However, inoculations with the other non-pathogenic *Fusarium* spp. did not protect Cavendish from TR4. In addition, we observed that this priming effect was stable at various nutritional conditions and varying pH conditions. Unique transcripts were identified during the initial stages of individual infections with TR4 and R1 as well as in combined treatments. The data support the hypothesis that activating the resistance to R1 in Cavendish bananas affects TR4 development and provide a first insight of gene expression during the interaction between various *Fusarium* spp. and banana.

Introduction

Banana (*Musa* spp.) is the fourth most important crop after rice, wheat, and corn (Heslop-Harrison and Schwarzacher 2007, FAOSTAT 2016). Bananas and plantains are a major staple food crop in many producing countries with an annual production of approximately 145 million tons in 2016 (FAOSTAT 2016). Most cultivated bananas are derived from inter and intra specific hybridizations between two wild seeded diploid ancestors, *Musa acuminata* (AA, 2n=22) and *M. balbisiana* (BB, 2n=22) (Price 1995, Heslop-Harrison and Schwarzacher 2007) such as plantains (AAB) and cooking bananas (ABB). The popular dessert bananas such as 'Gros Michel' (from the 18e century to mid-1998) and 'Cavendish' clones are sterile triploids (AAA). The latter dominate global production and trade as 40% of the global banana production relies on these clones (Lescot 2017). Particularly, after the demise of 'Gros Michel' plantations in Central and Latin America (Stover and Ploetz 1990, Ploetz 2005a) due to Panama disease or Fusarium wilt, as Cavendish varieties are resistant to the *Fusarium* species that caused this epidemic in the previous century. Currently, however, Panama disease is once again the biggest threat of the industry since Cavendish bananas and manifold local varieties succumb to a strain of *Fusarium odoratissimum.* This is a new species that is indigenous across Indonesia and disseminates around the world (García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a, Chittarath *et al.,* 2017, Hung *et al.,* 2017, Maryani *et al.,* 2018, Zheng *et al.,* 2018) and is colloquially called Tropical Race 4 (TR4) (Ploetz 1994). Previously, Panama disease was supposed to result from genotypes of *F. oxysporum* f.sp. *cubense* (*Foc*), but recent taxonomical revisions have concluded that, as expected from the polyphyletic nature of *Foc* (Koenig *et al.,* 1997, O'Donnell *et al.,* 1998, Baayen *et al.,* 2000, Ordoñez *et al.,* 2018) these actually represent a suite of new *Fusarium* species (Maryani *et al.,* 2018). The nomenclature of the recognized races is based on specific interactions incurred by these *Fusarium* spp. on a small set of banana varieties (R.H. Stover 1962, Armstrong 1981, Ploetz 2015). Race 1 (R1) is pathogenic to 'Gros Michel' and 'silk' among others, has different genetic origins and was responsible for the devastating epidemic that caused the collapse of the 'Gros Michel' based banana industry in the 1950's (Ploetz 2005c). Race 2 (R2) infects genotypes of the Bluggoe group and other members with an ABB genome (Stover 1962, Ploetz and Correll 1988, Brake *et al.,* 1990, Ploetz 1990, Moore *et al.,* 1991, Moore *et al.,* 1993, Meldrum *et al.,* 2012). Race 4, however, attacks the R1 resistant cultivars from the Cavendish group as well as other banana genotypes susceptible to R1 and R2. Race 4 strains are currently divided into subtropical race 4 (STR4) that affects Cavendish under specific abiotic constraints such as water logging (Ploetz and Correll 1988, Brake *et al.,* 1990, Ploetz 1990, Moore *et al.,* 1991, Bentley *et al.,* 1995, Pegg *et al.,* 1995, Shivas and Philemon 1996, Groenewald *et al.,* 2006, Meldrum *et al.,* 2012) and TR4. In the absence of genetics, however, this nomenclature is ambiguous and needs a thorough revision.

The collapse of Cavendish plantations (Ploetz 2005b, Butler 2013) calls for new resistant varieties and sustainable disease management strategies. However, little is known about sources of resistance and commercially acceptable TR4 resistant banana varieties are currently unavailable. Recently, Garcia-Bastidas *et al*. (Chapters 4 and 5) have surveyed resistance in hundreds of banana accessions under greenhouse conditions and concluded that only a fraction showed the required level of resistance to TR4 (See Chapters 4 and 5). However, the genetic make-up of these accessions is unknown and the first resistance gene to Panama disease was only discovered and deployed recently (Dale *et al.,* 2017).

This situation urges for exploration of alternative strategies to rapidly reduce the dissemination of TR4. Induced systemic resistance (ISR) and systemically acquired resistance (SAR) are common response pathway of plants to non-pathogenic strain of microorganisms like bacteria, fungi, and viruses as well as host–incompatible nematodes (Fuchs *et al.,* 1997). The resistance can be either localized or systemic resulting in a reduced susceptibility of the entire plant to subsequent pathogen infections (Matta 1989). Induced resistance also triggers several defence mechanisms like the production of antimicrobial compounds, the formation of reactive oxygen species (ROS) and synthesis of pathogenesis-related (PR) proteins (Ahuja *et al.,* 2012). Naturally induced SAR and ISR have been found in several plant species, however, it has been reported more frequently in dicots than in monocot plants (Hammerschmidt and Kuć 1982, Hammerschmidt *et al.,* 1982, Kroon *et al.,* 1991, Mandeel and Baker 1991, Hervás *et al.,* 1995, Wei *et al.,* 1996, Fuchs *et al.,* 1997, Harvás *et al.,* 1997, Pieterse *et al.,* 1998, Fuchs *et al.,* 1999, Heil and Bostock 2002, Silvar *et al.,* 2009). Just a few studies describe ISR to nematodes (Vu *et al.,* 2006), the banana bunchy top virus (Kavino *et al.,* 2007, Harish *et al.,* 2008, Kavino *et al.,* 2008) and *Fusarium* strains (Thangavelu *et al.,* 2003). More recently, Wu *et al.* (2013) showed the effect of a non-pathogenic strain as activator of SAR on Cavendish *in-vitro* plants. Thus, the knowledge of ISR in banana is very limited, particularly in the Banana-*Fusarium* pathosystem.

We explored the potential of SAR and ISR by treating Cavendish banana plants with an avirulent R1 *Fusarium* strain as well as nonpathogenic *F. oxysporum* formae speciales to prevent or slow down pathogenesis of a virulent TR4 strain. Additionally, we determined the effects of abiotic stresses on cross protection and performed a transcriptome analysis to study gene regulation in the initial stages of infection upon inoculations with individual TR4 and R1 strains as well as various challenge inoculation with both strains.

Materials and Methods

Plant Material

Experiments were performed under greenhouse conditions in the facilities of Unifarm at Wageningen University & Research, The Netherlands. Tissue cultured Cavendish 'Grand Naine' plants were obtained from Rahan Meristem Ltd. (Western Galilee, Israel), and then acclimatized by growing for approximately trhee months until they were 30 cm in height and had 5-6 true leaves. Controlled greenhouse conditions 28±2˚C, 16h light, and ~85% relativity humidity for hardening plants. Subsequently conditions for plant development were adjusted to 26 ±2 ˚C and ~80% relativity humidity (RH) under 16 hours light. Henceforward, they were transplanted to new 1L pots in sterilised river sand and placed in the same greenhouse at 85% RH for three months until phenotyping.

Fungal isolates

Nine *Fusarium* strains were used (Table 1), including a R1 strain from Brazil that is used for routine phenotyping in the Embrapa breeding program in Cruz das Almas. This strain was isolated from an ABB-'Maça' banana, and according to the latest information has an unknown genotype (Maryani *et al.,* 2018, Ordoñez *et al.,* 2018) as it differs from all earlier described vegetative compatibility groups (Bentley *et al.,* 1995, Katan and Di Primo 1999, Moore *et al.,* 2001, Dita *et al.,* 2010, Ploetz 2015). In addition, we used a R2 strain

originating from an ABB-Bluggoe banana and is currently known as *F. tardichlamydosporum* and the reference *F. odoratissimum* TR4 strain II5 (http://www.broadinstitute.org/). Finally, we used four *Fusarium oxysporum* formae speciales that are non-pathogenic on banana as well as two well-known biocontrol strains (Table 1). To obtain fresh mycelium, isolates of each strain were grown at 25 ± 2°C on potato dextrose agar (PDA) for 4-5 days in the dark and then 5 mm plugs were taken from the edge of a colony to inoculate liquid Mung bean media prepared according to García-Bastidas et al. (Chapter 3). Final inoculum was prepared by filtration through cheesecloth and adjustment to 10^6 conidia.ml^{-1}.

Co-inoculation of banana plants

Challenge experiments were carried out with a nonpathogenic or incompatible strain as an inducer and TR4 as the challenge strain (Table 2). Banana plants were uprooted and cleaned by rinsing the roots with water. They were then immersed in the inoculum for 30 min. followed by immersion in a TR4 spore suspension for 30 min. Thereafter, the plants were potted into sterile sand in 2L pots. To measure the duration of induced resistance by R1 the challenge inoculation with TR4 was conducted at 0,5h, 3h and 1, 2, 5 and 10 days after R1 inoculation. Controls included the same number of plants inoculated with TR4 and mocks treated with water. Controls included (i) plants that were only inoculated with TR4 or (ii) with the non-pathogenic strains or (iii) exposed to a reverse experiment where TR4 inoculation was followed by R1 or non-pathogenic *Fusarium* strains as challengers.

Co-inoculations under different abiotic conditions

We also tested whether induced resistance upon inoculations with R1 was maintained under different abiotic conditions. Therefore, after inoculations as describe above, plants were transferred to a light textured (2% clay) and medium fertile (soil organic matter 2.6%; pH 5.2; CEC 16mmol g^{-1}) soil with either pH 6.0 or pH 5.2 and were supplemented with three levels of nitrogen (0g, 0,28 and 0,85 g/plant/week). pH levels were generated by applying calcium carbonate ($CaCO_3$) and nitrogen levels were obtained through weekly fertilization with ammonium nitrate ($N_2H_4O_3$).

Table 1. Origins and characteristics of the strain of *Fusarium* spp. used in this study.

***Fusarium* spp.**[1]	**Isolate Code**	**Host**	**Experimental Code**	**Origin**
FOSC clade 4	CNPMF-008-01-R1	Banana	R1	Cruz das Almas, Bahía, (Brasil)
Fusarium tardichlamydosporum	NRRL 25607	Banana	R2	USA
Fusarium odoratissimum	*Foc*II-5	Banana	TR4	Indonesia
Fusarium oxysporum f. sp. *melongenae*	0-1877	Eggplant	*Fom*	Israel
Fusarium oxysporum f. sp. *lycopersici*	40698	Tomato	*Fol*	Netherlands
Fusarium oxysporum f. sp. *cepae*	Eza	Onion	*Foce*	Australia
Fusarium oxysporum f. sp. *gladioli*	10194	Gladiola	*Fog*	-
FOSC Clade 3	Fo47	Biocontrol	*Fo47*	France
-	Fo618-12	Biocontrol	*Fo618-12*	Netherlands

[1]Names adapted based on latest classification on Maryani *et al.,* 2018.

Disease assessment and statistical lay-out

Plants were monitored weekly for Panama disease development and a final evaluation was performed when progression of wilting stopped in the TR4 inoculated controls. Final scoring included external and internal evaluation of the disease progress according to Garcia-Bastidas *et al*. (Chapter 3) comprising foliar chlorosis, pseudostem splitting and deformation of new leaves. Internally, the infected area of the corm was photographed, and symptoms were quantified by ImageJ analyses and scored using disease indices (DIs) according to Garcia-Bastidas *et al*. (see also Chapters 3, 4 and 5). All experiments were arranged in a completely randomized block design with at least two replicates. Data were subjected to analysis of variance and least significant differences (LSD, p=0.05) were calculated to determine the effects of the treatments. The experiment involving abiotic factors was performed in a complete factorial analysis with four replicates and individual pots as experimental units (Supplemental Table 1). Data were collected on pseudostem diameter (Dia), Foliage (FA), dry weight (DW) and plant height (He). Pseudostem diameter was selected as the best plant growth variable for data analyses. The temporal challenge experiments were scored at six weeks after inoculation, corms were evaluated, and DI was calculated.

Analysis of the transcriptome of the interaction between R1 and TR4 on 'Grand Naine'

Complete rhizome (corms and roots) samples of the inoculated plants and controls were taken at each time point for RNA isolation. Rhizomes tissues were flash frozen in liquid nitrogen and maintained at -80C in a conventional freezer until the sampling of the last experiment. For total RNA isolation, 100 g of rhizome were ground with pestle and mortar in liquid nitrogen and the RNeasy® Qiagen kit (Chatsworth, CA, USA) was used following the manufacturer's instructions. RNA concentration was measured with the Kit Quant-iT® RiboGreen® (Invitrogen, Karlsruhe, Germany) in a Tecan® infinite M200 microplate reader (Salzburg, Austria) (ex. 490nm, em. 530nm). RNA was also isolated from pure colonies of TR4 and R1 taken from PDA plates as *in-vitro* controls. Samples were sequenced at the cluster Applied Bioinformatics-Bioscience of WUR after library preparation with Illumina TruSeq® stranded mRNA, followed by sequencing on a HiSeq 2500 machine (125 bp pair end).

Transcriptome assembly and analysis

We compared the transcript levels between the roots inoculated with the TR4 and R1 at time 0 (0.5h), 3h, 1 and 2 days post inoculation using the mock inoculation treatment as control. RNASeq read abundances per sample were summarized for banana transcripts based on the V2.0 annotation, using Kallisto v0.42.4 (Bray *et al.,* 2016). On average 72% of the RNAseq reads per sample could be used to estimate transcript abundances. Transcriptome analysis was performed using R software (Team 2013). Principal component analysis was performed using regularized Log transformed TPM (Transcripts Per Kilobase Million) values as implemented in the DESeq2 package (Love *et al.,* 2013) and differential gene expression between individual conditions was analyzed using the same package. Only genes with a +/-2 log-Fold Changes (*LogFC*) and adjusted p-value of 0,01 (padj) were considered. Genes were grouped in four main clusters according to their functional annotations retrieved from the Gene Ontology and KEGGs databases. Hierarchical clustering analysis of the differential gene expression was performed using

MultiExperimentViewer version 4.8.1., based on read counts and FPKM values (Pearson Correlation, average linkage clustering, Howe *et al.* (2011). Hierarchical clustering was performed using Multiple Array Viewer Software (version 4.8.1; http://www.tm4.org/mev.html).

Results

The incompatible interaction between Cavendish 'Grand Naine' and R1 incurs induced resistance to TR4

In these trials we investigated the potential antagonistic effect of different incompatible interactions between 'Grand Naine' and a series of non-pathogenic *Fusarium oxysporum* formae speciales and the so-called R1 and R2 isolates on subsequent treatments with TR4. No infection was observed at six weeks after inoculation (wai) with the non-pathogenic strains or with the banana pathogenic strains (Figure 1). However, severe symptoms were observed after inoculating with the compatible TR4 strain as well as with a R2 strain (Tables 1 and 2; Figure 1). Thus, all *Fo* strains were potentially suitable for the induction of hypothetical cross-protection with the exception of the so-called *Foc* Race 2.

Table 2. Disease development (DI) of Cavendish "Grand Naine" plants upon inoculation with various *Fusarium* spp. and the effect of subsequent challenge inoculations with TR4 and R1 strains of *Fusarium* spp. that cause panama disease at six weeks after inoculation.

Treatments	Mock[1]	TR4	R1	R2	*Fom*	*Fol*	*Foce*	*Fog*	*Fo47*	*Fo618*
DI [2]	0,0	100	5,0	60	0,0	20	0,0	0,0	0,0	0,0
DI + TR4[3]	-	-	20	75	60	60	50	50	95	80
DI TR4 + R1[4]	-	-	95	-	-	-	-	-	-	-

[1]water control. TR4: Tropical Race 4, R1: Race 1, R2: Race 2, Fom: *Fusarium oxysporum* f.sp. *melongenae*, Fol: *Fusarium oxysporum* f.sp. *lycopersici*, Foce: *Fusarium oxysporum* f.sp. *cepae*, Fog: *Fusarium oxysporum* f.sp. *gladioli*. [2]Disease index of inoculated plants with an individual strain, calculated as described in Materials and Methods, [3]Disease index of co-inoculated plants first with the nonpathogenic strain and then with TR4, [4]Disease index of co-inoculated plants first with the pathogenic TR4 and then with the R1.

We tested the antagonistic capacity of these strains on TR4 by a range of challenge inoculations. After three wai, leaf chlorosis and wilting were observed in all treatments, except in the R1 treatment and the controls (Table 2; Figure 1). At six wai, all plants had developed severe disease symptoms, but the plants that were pre-treated with R1 lacked visible external symptoms in any of the replicates, which obviously was also reflected in the calculated DIs (Table 2). All DIs exceeded 50%, showing intense corm discoloration, severe chlorosis and wilting and other typical symptoms such as pseudostem splitting and deformed new leaves (Figure 2), but R1 pre-treated plants had a DI of 20%. The reverse treatment (first TR4 and then R1) did not result in reduced DIs, which was expected, but plants pre-treated with R2 also showed high DIs (60-75%) (Figure 1G). Finally, all plants that developed symptoms were positive for the TR4 molecular diagnostic (data not shown), but the corms from plants that were pre-treated with R1 and subsequently with TR4 tested negative.

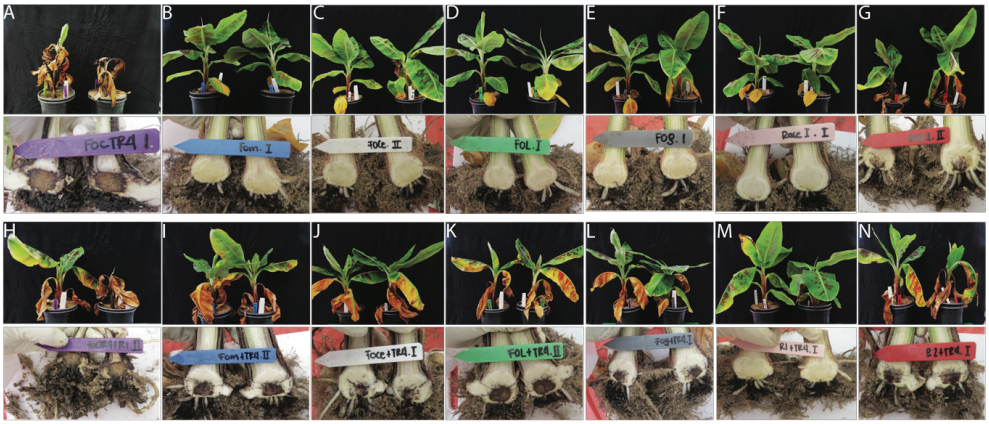

Figure 1. Phenotyping challenge inoculation on Cavendish 'Grand Naine'. (A-G) Individual inoculations and external and internal evaluation at six weeks after inoculations with (A) *Fusarium odoratissimum* TR4, (B) *F. oxysporum* f. sp. *melongenae*, (C) *F. oxysporum* f. sp. *cepae*, (D) *F. oxysporum* f. sp. *lycopersici*, (E) *F. oxysporum* f. sp. *gladioli*, (F) *Fusarium* R1, (G) *F. tardichlamydosporum* R2. (H-N) Challenge inoculations. (H) First inoculation with TR4 followed by challenging with R1. (I-N), first inoculation as per (B-G) followed by a challenge with TR4.

The experiment to test the duration of priming with a R1 pretreatment prior to TR4 inoculation showed that extensive reduction of disease development was still observed when TR4 challenge inoculations were performed 10 days after priming (Figure 2).

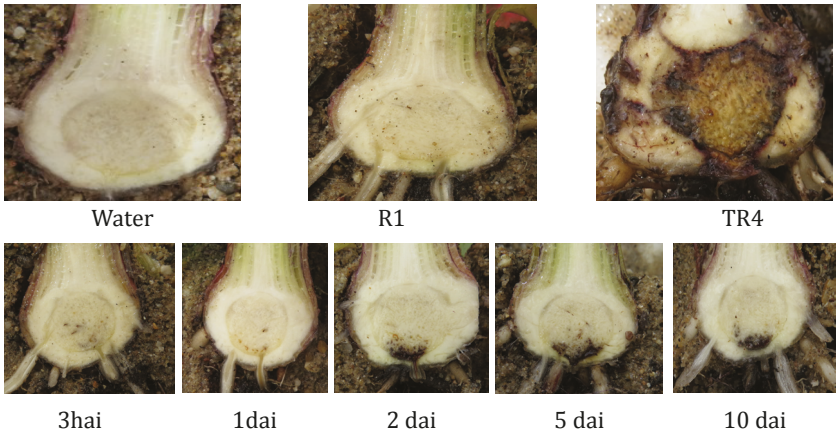

Figure 2. Durability of induced resistance in Cavendish banana cv. Grand Naine after inoculating with *Fusarium* R1. Cross sections of corms at six weeks after inoculation. Top row: controls water, R1 and *F. odoratissimum* TR4. Bottom row: results after challenging with TR4 3 hours and 1, 2, 5 and 10 days after R1 inoculations.

Abiotic conditions do not affect the induced resistance incurred by R1

We observed significant reductions in disease severity upon pre-treatments with R1 and subsequent inoculations with TR4. Here, we tested whether abiotic factors, such as soil

pH and nitrogen (N) levels, influence such responses. Irrespective of the applied pH and N modulations, the induced resistance by pre-treatments with R1 reduced the disease severity incurred by subsequent TR4 inoculations (Figure 3). At six wai the DIs of R1 pre-treated plants was always lower than those that were only inoculated with TR4, with mean values of 20 and 96, respectively (Table 3). It was also obvious that soil pH had an effect in disease development for the R1 and R1 + TR4 treatments (Table 4). Under high pH levels the disease severity was significantly lower in the R1 and R1 + TR4 treatment, with DIs of low:high= 1.68 : 41.66 and 8.43:31.67, respectively (Table 4). Similar trends were observed for leaf chlorosis, stem diameter, foliar area and dry weight. Nutrition, however, did not affect the DI, except for the R1 treatment where the DI was significantly lower under low N. Evidently, N did significantly affect plant growth as measured by plant height, but significant differences in stem diameter were only observed in the TR4 treatment, which result from increasing disease severity under high N levels (Table 5).

Table 3. The effect of *Fusarium* inoculations on the development of Cavendish 'Grand Naine'.

Treatment	Chlorosis (%)	Score	DI[1]	Height (cm)	Dia[2] (mm)	Foliage (cm^2)	DW[3]
Control	27,82^{a}	1^{a}	00,1^{a}	16,0^{a}	24,29^{c}	1592^{c}	313,7^{c}
R1	46,13^{b}	2^{b}	20,0^{b}	21,83^{b}	21,45^{b}	1183^{b}	257,3^{b}
R1 + TR4	46,72^{b}	2^{b}	21,67^{b}	23,60^{c}	22,34^{b}	1166^{b}	286,7b^{c}
TR4	92,06^{c}	5,8^{c}	96,65^{c}	25,05^{d}	15,08^{a}	552^{a}	138,3^{a}

[1]DI = disease index, [2]Dia = pseudostem diameter (mm), [3]DW = dry weight (g).

Table 4. The effect of soil acidity on Panama disease development in Cavendish 'Grand Naine' six weeks after inoculating with *Fusarium* R1, *F. odoratissimum* TR4 and challenge inoculations (R1 + TR4)

Treatment	pH[1]	Chlorosis (%)	Score	DI[2]	Height (cm)	Dia[3] (mm)	Foliage (cm^2)	DW[4]
Water	high	20,32^{a}	1^{a}	00,1^{a}	25,0^{d}	1621^{c}	337,0^{de}	24,79^{c}
	low	35,32^{b}	1^{a}	00,1^{a}	25,0^{d}	1563^{c}	290.3^{d}	23,80^{c}
R1	high	32,32^{ab}	1^{a}	1,68^{a}	25,5^{d}	1921^{d}	327,8^{de}	24,61^{c}
	low	59,53^{c}	3^{b}	41,66^{b}	18,12^{bc}	445^{a}	186,7^{bc}	18,29^{b}
R1 + TR4	high	27,58^{ab}	1^{a}	8,34^{a}	27,49^{e}	2019^{d}	373,0^{e}	25,51^{c}
	low	65,87^{c}	2,5^{b}	31,67^{b}	19,71^{c}	314^{a}	200,4^{c}	19,17^{b}
TR4	high	89,68^{d}	5,7^{c}	98,31^{c}	14,65^{a}	819^{b}	143,9^{ab}	14,56^{a}
	low	94,44^{d}	5,9^{c}	94,68^{c}	17,35^{b}	286^{a}	132,7^{a}	15,59^{a}

[1]Low pH is ~5, high pH is ~ 6, [2]DI = disease index, [3]Dia = pseudostem diameter, [4]DW = dry weight (g).

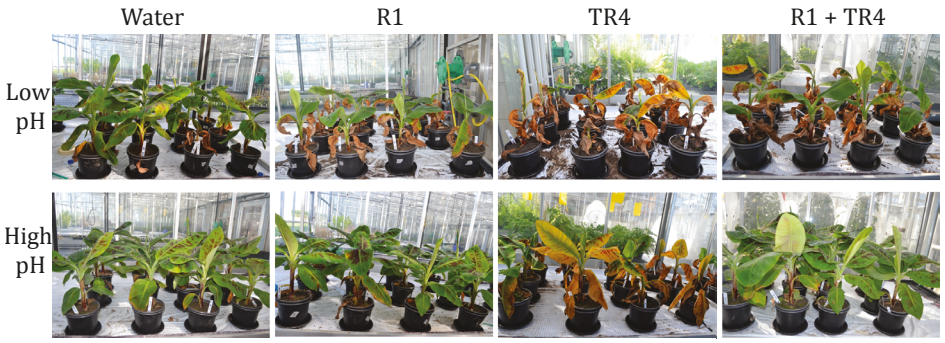

Figure 3. Phenotyping Cavendish banana 'Grand Naine' with *Fusarium* R1, *F. odoratissimum* TR4 and a challenge inoculation (R1 + TR4) at two soil acidity levels and three nutritional (N) levels. External appearance at six weeks after inoculation at low pH (~5) (top row) and at high pH (~6) (bottom row).

Table 5. The effect of different nitrogen (N) levels on Panama disease development in Cavendish 'Grand Naine' six weeks after inoculation with *Fusarium* R1, *F. odoratissimum* TR4 and challenge inoculations (R1 + TR4)

Treatment	N[1]	DI[2]	Height (cm)	Diameter (cm)
Water	Low	00,1^{a}	22,95^{de}	23,46^{cd}
	Mid	00,1^{a}	25,82^{f}	24,96^{d}
	High	00,1^{a}	26,38^{f}	24,47^{d}
R1	Low	5,01^{ab}	19,90^{c}	21,64^{c}
	Mid	30,0^{d}	24,66^{ef}	21,10^{c}
	High	30,0^{d}	20,9^{cd}	21,60^{c}
R1 + TR4	Low	25,0c^{d}	21,50^{cd}	21,64^{c}
	Mid	20,0^{cd}	24,19^{ef}	31,86^{c}
	High	15,0^{bc}	25,11^{ef}	23,56^{cd}
TR4	Low	97,48^{e}	17,0^{b}	16,55^{b}
	Mid	97,48^{e}	16,50^{ab}	15,90^{d}
	High	97,48^{e}	14,50^{a}	12,78^{a}

[1]Low, medium and high N levels are 0, 0.28 and 0.85 g/plant/week, [2]DI – disease: plant height, [3]Pseudomstem diameter.

Analysis of the transcriptome of the interaction between R1 and TR4 on 'Grand Naine'

We generated in total 317,020,712 RNAseq reads from three biological replicates of infected root tissues of the various treatments. Principal component analyses of regularized log transformed TPM values revealed minimal variability between replicates of individual samples (Supplemental Figure 1). Differentially expressed genes were observed in comparison with the water controls at the different time points. In general terms a higher number of transcripts was up or down regulated during the initial states of the infection for all the interactions, but numbers stabilized after one day (Supplemental Figure 2). Overall, 2162, 1754 and 2015 genes were differentially expressed in the plant when inoculated with race 1, TR4 and the R1 + TR4 challenge inoculation, respectively. In total, 207 genes were differentially up-regulated in plants

inoculated with TR4, while only 79 genes were down-regulated. After R1 treatment, 99 genes were up-regulated and 205 genes were down regulated. The challenge inoculations showed that in total 284 and 240 genes were up- and down-regulated, respectively (Figure 4). In total 1,023 genes showed alteration in all three treatments, but only 39 and 429 were common between the TR4 and R1+TR4 and the R1 and R1+TR4 treatments, respectively (Figure 4).

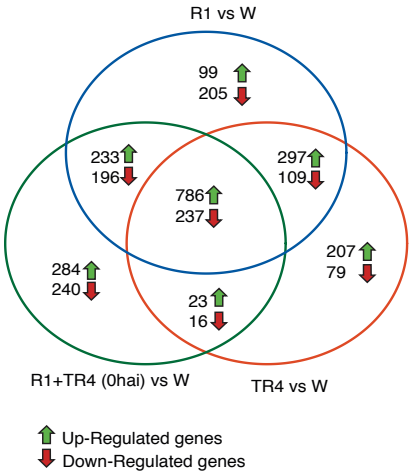

Figure 4. The number of upregulated and downregulated genes upon inoculating Cavendish 'Grand Naine' with *Fusarium* R1, *F. odoratissimum* TR4 and challenge inoculations (R1 + TR4) compared to the water control. The Venn diagram shows the total number of differentially expressed genes as well as the genes that are similar between the various treatments. Red arrows and green arrows indicate upregulated and downregulated gene numbers, respectively.

To identify genes that are expressed upon the priming, we focused on different classes of genes including pathogenesis related, hormone signalling and receptor like genes. In general, most genes were similarly regulated for R1, TR4 and the R1 + TR4 interaction up to the first day, but thereafter the expression of the majority of genes was not different from the controls. Among the aforementioned groups of upregulated genes, we identified genes encoding a thaumatin like protein, a chitinase-6 like, an aquaporin, an endoglucanase-9 and β- 1-3 endoglucanase like proteins as well as several peroxidase like and phenylalanine ammonia lyase like proteins (Figure 5). The chitinase-6 like and the β-1-3 endoglucanase like proteins were up regulated across all treatments up to 2 dpi. These data suggest that defence responses were similarly induced in all treatments. Interestingly, two homologues of the resistance gene analogue *RGA2* and one homologue of *RGA4* genes were up regulated in all treatments until 2 dpi.

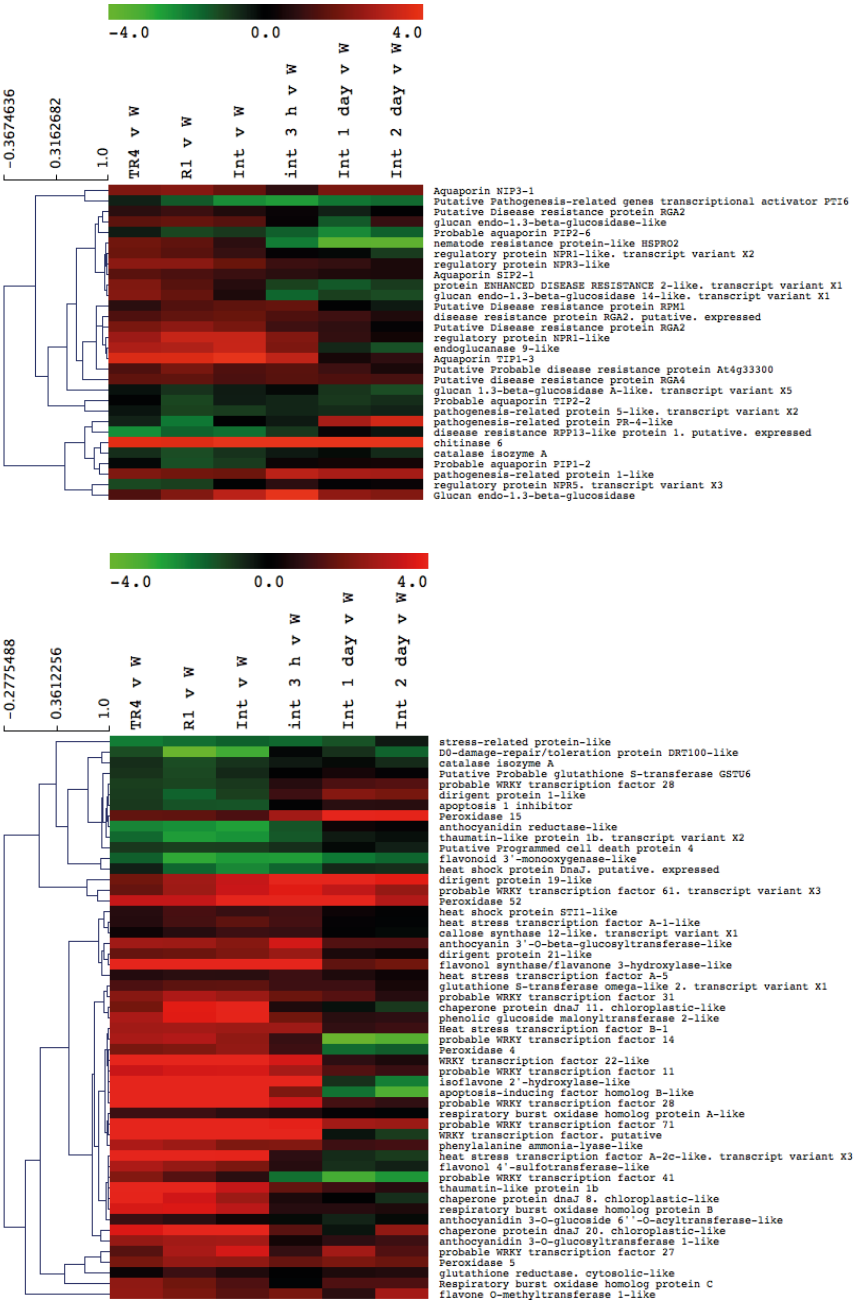

Figure 5. Transcriptional analyses of the interactions between Cavendish ' Grand Naine' and *Fusarium* R1, *F. odoratissimum* TR4 and the challenge inoculation R1 + TR4. Hierarchical clustering analysis of representative potential pathogenesis related genes (top figure) and hypersensitive response genes (lower figure). The heat plots represent significant ($P < 0.05$) average expression differences between the water control and TR4, R1 and R1 + TR4 at 0, 3 hours, 1 day and 2 days after inoculation. Bars represent upregulated genes (red) and downregulated genes (green).

We also searched for clues whether and if so, signaling pathway are involved in the induced resistance response. Until 3 dpi several hormone pathways were induced in the R1, TR4 and R1 + TR4 treatments, but the ethylene response pathway seemed to be most prominently up regulated involving several genes such as the *ERF018-like* ethylene-responsive transcription factor and the *AP2/ERF* transcription repressor as well as the *ACC synthase* gene. Interestingly, also several gibberellin synthesis associated genes were strongly up regulated including a *gibberellin 2-beta-dioxygenase 1-like* gene and the gibberellin receptor *GID1C like* (Figure 6). Finally, we identified up regulation of a jasmonate receptor (coronatine- insensitive protein) and *a* jasmonate O-methyltransferase-like protein in the jasmonate response pathway and *NPRace 1-like* regulatory protein (Ma06_t02690.1) as well as a *nudix hydrolase 8-like* gene (Ma10_t21290.1) in the salicylic acid pathway.

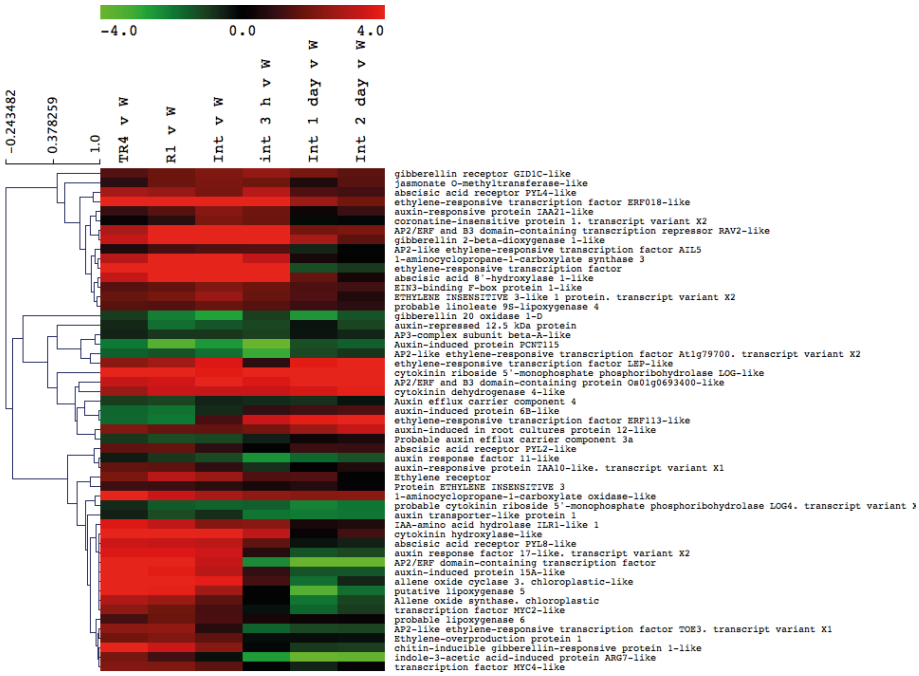

Figure 6. Transcriptional analyses of the interactions between Cavendish 'Grand Naine' and *Fusarium* R1, *F. odoratissimum* TR4 and the challenge inoculation R1 + TR4. Hierarchical clustering analysis of representative hormone response pathway. The heat plots represent significant ($P < 0.05$) average expression differences between the water control and TR4, R1 and R1 + TR4 at 0, 3 hours, 1 day and 2 days after inoculation. Bars represent upregulated genes (red) and downregulated genes (green).

Discussion

Contemporary banana production is threatened by the disseminating *F. odoratissimum* TR4 strain that kills Cavendish varieties as well as many local cultivars (Marquardt 2001, Butler 2013, Ploetz 2015, Ploetz *et al.,* 2015). As such, it is another demonstration of a new fungal species that causes havoc, similar to recently described amphibian fungal pathogens (O'Hanlon *et al.,* 2018). Taking into account previous experiences with Panama disease (Marquardt 2001), it is not an exaggeration to claim that TR4 threatens food security in many countries and has to potential to seriously affect global Cavendish

production. Despite this experience, the overall response to TR4 is very similar to the previous strategies to manage the Panama disease epidemic in 'Gros Michel' to R1 strains (Marquardt 2001, Ploetz 2015). As many agroecological approaches have not shown enduring efficacy, we focus in this study on the potential of induced resistance. In other crops, such as melon, tomato and chickpeas, priming has shown potential to protect plants to different strains of *Fusarium* spp. causing wilts (Biles and Martyn 1989, Kroon *et al.,* 1991, Hervás *et al.,* 1995, Fuchs *et al.,* 1997, Harvás *et al.,* 1997, Getha *et al.,* 2005, Mauch-Mani *et al.,* 2017). For banana such studies have not been undertaken. We considered that utilizing the incompatible response of 'Cavendish' cultivars to R1 that endures already for decades despite cultivating them on highly infested soils (Ploetz 2005b), might be a potential way to reduce the impact of TR4. Indeed, in all our trials (Garcia et al., 2018) we never observed symptoms after inoculating 'Grand Naine' with R1, even in extreme tests with high inoculum doses on injured roots, thereby confirming observations in other studies (Sun and Su 1984, Su *et al.,* 1986, Fernández-Falcón *et al.,* 2004, Amorim *et al.,* 2009, Dita *et al.,* 2010, Costa *et al.,* 2015).

As expected, we observed a compatible interaction with TR4, but the moderately susceptible response with R2 (Ploetz and Correll 1988) was unexpected, and even more as Moore *et al.* (1993) consider this particular strain to be R1. Hence, it is not always clear which species actually causes damage in plantations. For instance, it is still unknown which *Fusarium* species was responsible for the epidemic in 'Gros Michel' (Marquardt 2001, Ordóñez *et al.,* 2015b, Maryani *et al.,* 2018). The first original reports indicate that germplasm susceptible to R1 was resistant to R2 and *vice versa* (Stover 1962). Our R2 strain was isolated from symptomatic Bluggoe accessions (ABB), but has also been identified on Silk bananas (AAB) as well as 'Pisang Nangka' (AAA) and Inarnibal (AA) (Moore *et al.,* 1991, Ploetz *et al.,* 1999, Ploetz 2005b, Ghag *et al.,* 2015). Therefore, Ordóñez et al. (2018, see also Chapter 6) considered that the current race nomenclature of banana infecting *Fusarium* spp. requires a thorough revision as multiple species can infect 'Cavendish' varieties under greenhouse conditions. Clearly, various genotype x environment interactions complicate race identification, which warrants greenhouse trials under repeatable and controlled conditions. It is well known that avirulent *Fusarium* genotypes are able to cause disease on Cavendish cultivars during particular abiotic conditions. For instance, Costa *et al. (*2015) isolated several normally avirulent strains from diseased-like plants under severe drought. However, none of these isolates caused disease under optimal greenhouse conditions and they tested negative with a molecular diagnostic.

Across all our experiments we consistently showed that pre-treatments with R1 significantly reduced the levels of infection caused by subsequent TR4 inoculations. Moreover, the effect was maintained under different pH and nutrition levels and endures for at least 10 dai with R1. We, therefore, consider R1 as a potential inducer of systemically acquired resistance to TR4, which warrants further and more details analyses. Very similar observations were reported in other systems involving viral diseases and other fungal diseases (Ross 1961, Maurhofer *et al.,* 1994, Martínez-Hidalgo *et al.,* 2015, Bisen *et al.,* 2016, Elsharkawy *et al.,* 2018). Cross protection against *F. oxysporum* has been extensively reported in several hosts, with different activators including *Verticilium* ssp., saprophytes or even other *Fo* formae speciales. Several studies have confirmed the reduction of disease severity caused by *Fusarium* after plants were pre-treated with a non-pathogenic organism (Biles and Martyn 1989, Kroon *et al.,* 1991,

Getha *et al.,* 2005). Isolates of *F. oxysporum* have been investigated on cucumber, chickpea, and several other hosts affected by Fusarium wilts (Ogawa and Komada 1985, Paulitz and Baker 1987, Biles and Martyn 1989, Alabouvette 1990, Kroon *et al.,* 1991, Mandeel and Baker 1991, Alabouvette *et al.,* 1993, Hervás *et al.,* 1995, Harvás *et al.,* 1997). In general, strains mediating induced resistance are usually closely related to the pathogenic strains (Alabouvette 1990, Alabouvette *et al.,* 1993). The most effective inducers of resistance often belong to the same fungal species or forma specialis (Davis 1967, Biles and Martyn 1989, Fuchs *et al.,* 1997). For the banana *Fusarium* – banana pathosystem, Wu *et al.* (2013) observed that *in-vitro* plants of the Cavendish 'Brazil Xiangjiao' were resistant to TR4 after challenging with R1. Also, in our study, cross protection was only induced by R1 pre-treatments, as simultaneous inoculations of R1 and TR4 rapidly developed disease, similar to TR4 controls or to a TR4 inoculation followed by a treatment with R1. This suggests that protection does not result from antagonism or competition between the two strains.

Previously, three hypotheses were proposed as possible mechanisms of cross protection; competition for nutrients, competition for infection sites at the root surface and induction of a systemic host resistance response (Schneider 1984, Mandeel and Baker 1991, Alabouvette *et al.,* 1993). Our data show that for the *Fusarium* – banana pathosystem cross protection is the most likely explanation since no positive effect was observed neither with the other formae speciales nor with the biocontrol strains *Fo*47 (FOSC clade 3) and *Fo*618-12. Thus, despite the fact that these strains did not affect banana, except for small lesions incurred by *Fog,* they did not reduce the susceptibility to TR4*.* It is well known that various *Fusarium oxysporum* ff. spp. have a narrow host range and usually infect a single host (Armstrong and Armstrong 1968, Vakalounakis 1996, Punja and Parker 2000, Cohen *et al.,* 2015). Our trials have shown, that the induced resistance to TR4 is exclusively related to R1 treatments, which apparently trigger the required resistance pathways upon inoculation. Moreover, none of the abiotic modulations affected the overarching induction of resistance by R1. Initially, R1 caused severe symptoms at low pH (also in the R1 + TR4 trial), but after some weeks plants recovered, and hence the symptoms were most likely resulting from infection attempts of R1 under abiotic stress, which is also observed under field conditions (Pegg *et al.,* 1995, Molina *et al.,* 2007, Visser *et al.,* 2010).

Abiotic conditions affect Panama disease development. Groenewald *et al.* (2006) showed that high soil pH reduced the incidence of Fusarium wilt in banana, while the source of nitrogen fertilizer also affected disease development. In general, pH is an important soil quality indicator in banana plantations and higher productions are obtained at higher soil pH (Delgado *et al.,* 2010). In our trails, pH did not affect plant development in the controls, but plants were significantly taller and had bigger diameters after R1 inoculations at high pH, compared to low pH, which was maintained after the challenge inoculations with TR4. Nutritional levels contributed to plant development as the mid to high N levels resulted in significantly taller and thicker plants, except for the TR4 inoculated plants that rapidly succumbed. However, the DIs of R1 treated plants were also higher at these nutritional levels, but after TR4 inoculations they lowered again. From these experiments, it can be concluded that pH is the overruling factor for disease development. This has been confirmed for banana (Domínguez *et al.,* 2001) and other pathosystem in low soil pH (Höper *et al.,* 1995, Rengel 1999, Persson and Olsson 2000, Marschner 2012) as well as at high soil pH (Duffy *et al.,* 1997, Oyarzun *et al.,* 1998, Harrison and Shew 2001). We

observe some modifying effects of N levels, but only in the trials involving R1. This complies with field data accumulated during the previous Panama disease epidemic in 'Gros Michel' (Waite and Stover 1960, Stover 1962). The reduced disease levels at lower pH could be due to varying bacteria population in soil, even under greenhouse conditions, or priming nutritional effects in the rhizosphere such as a decrease of micronutrients that are essential to growth, sporulation as well as the virulence of *Fusarium* pathogens (Jones *et al.,* 1975, Woltz and Jones 1981, Jones *et al.,* 1989). In conclusion, we have shown that the induction of resistance could be deployed for enhanced Panama disease management. However, we cannot use the R1 strain in non-indigenous countries due to phytosanitary regulations. Moreover, upscaling our experiments to field applications are impossible. Hence, future studies should unveil the underlying mechanism of cross protection in order to have a handle on the phenomenon for in-field application as one of the manifolds contributing factors to disease management in Cavendish plantations affected by TR4. These should include trials to identify whether living inoculum is necessary for cross protection compared to the merely physical presence of R1 studies (Huertas-González *et al.,* 1999) and also address the effects to other pathogens and pests as induced resistance is rather nonspecific (Ross 1961, Kuć 1982, Matta 1989, Heil and Bostock 2002, Choudhary *et al.,* 2007, Mauch-Mani *et al.,* 2017).

Finally, our transcriptome analyses are a first approach to understand the molecular mechanisms involved in the R1 induced cross protection to TR4. In general, the number of differentially expressed transcripts was in accord with similar studies as well as those in other pathosystems (Li *et al.,* 2012, Wang *et al.,* 2012, Zhang *et al.,* 2013, Guo *et al.,* 2014). Defence related genes such as a chitinase, thaumatin, and peroxidases were clearly induced in the R1 and R1 + TR4 and TR4 treatments. This suggests that systemic induced resistance is the most probable mechanism involved in cross protection in the *Fusarium* - banana pathosystem and that the time of induction is critical for achieving protection since simultaneous R1 and TR4 treatments did not reduce disease development. Our data have shown that after 1-day R1 induced responses that are difficult to cope with by TR4, even after 10 days. Our data suggest that the ethylene, jasmonic acid and gibberellin synthesis pathways might be crucial for protecting cv. Grand Naine plants from TR4 upon R1 induced resistance. This complies with the observations of Wu *et al.* (2013) in their *in-vitro* pathosystem model where they identified systemic expression of PR genes regulated by these pathways, suggesting that several pathways could be involved in the resistance response. In addition, they observed that the total salicylic acid content in roots of banana plantlets increased after leaf inoculation with R1, suggesting cross talk between the different pathways.

Peraza-Echeverria *et al.* (2008) assessed the expression of resistance gene analogues (RGAs) by RT-PCR in banana leaf and root tissues. *RGA1*, *3* and *5* showed a constitutive expression profile in both resistant and susceptible plants to TR4, whereas no expression was detected for *RGA4*. In contrast, *RGA2* expression was exclusively observed in plants with resistance to TR4. *RGA2* is a non-TIR-NBS-LRR plant disease resistance protein that is involved in resistance to TR4 in several banana accessions, including *M. acuminata* spp. *malaccensis* (Dale et al., 2017, see also Chapter 8). In our experiments, we observed upregulation of *RGA2* like genes in all treatments. Hence, *RGA2* seems also to be involved in the resistance to R1, but as shown previously, is crucial for resistance to TR4, which was proven by transferring the gene from *M. acuminata* spp. *malaccensis* to Cavendish and extensive field trials (Dale *et al.,* 2017).

Taken together, we have shown that R1 is an important inducer of resistance to TR4 in Cavendish 'Grand Naine' plants. However, R1 is still a major concern in many banana producing regions and hence not suitable as a biocontrol agent. Moreover, application of other R1 strains in our trials did not incur the same levels of induced resistance to TR4 (not shown). Hence, it seems that the utilized R1 strain from Brazil has this unique capacity, which warrants further investigation and characterization to understand the underlying mechanisms of induced resistance to TR4 in order to evaluate whether these can be used as a component of overall Panama disease management. Fundamental understanding of these mechanisms could also apply to other banana varieties affected by other Panama disease associated *Fusarium* spp., which are important for domestic markets. Foremost, however, is the need for diversity and hence the identification of resistance genes that provide durable resistance to this devastating disease.

Acknowledgements

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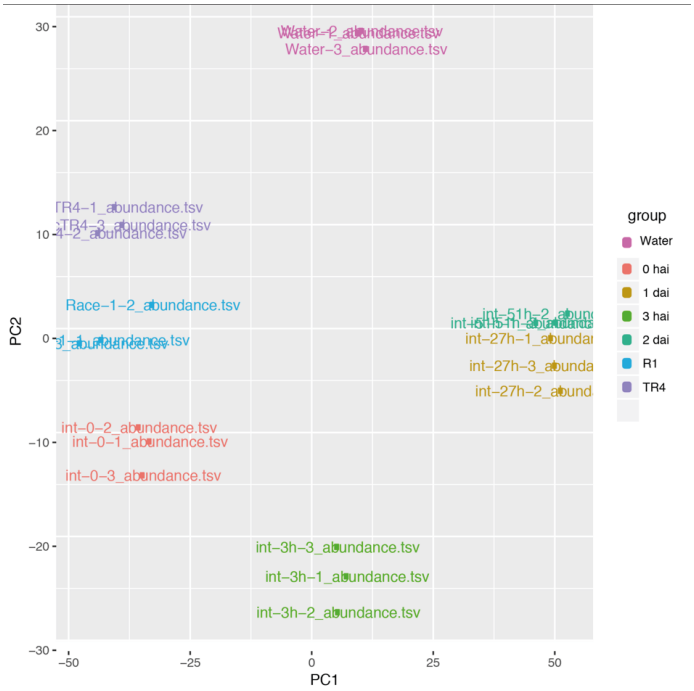
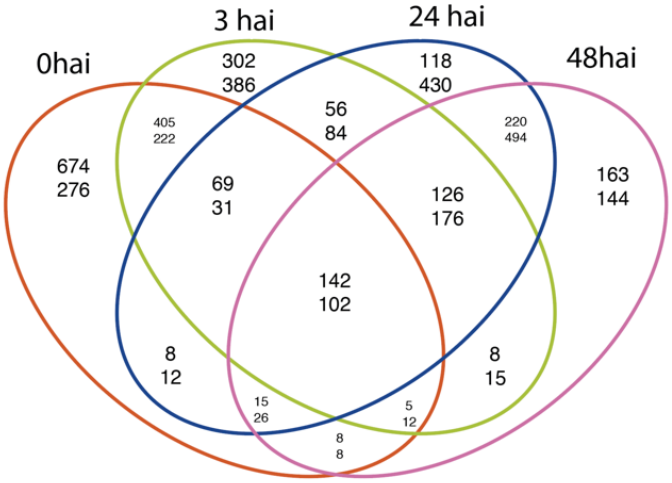
Supplemental Table S1. The effect of soil acidity and nitrogen (N) levels on panama disease development in Cavendish 'Grand Naine' six weeks after inoculation with *Fusarium* R1, *F. Odoratissimum* TR4 and challenge inoculation (R1 + TR4)

Treatment	pH	N	Chlorosis (%)	DI[1]	Height (cm)	Diameter (cm)	Foliage (cm^2)	Biomass (g)
Water	~ 5.1	Low	25	0	23,3	24,0	1422,6	293,8
		Mid	27	0	25,8	23,8	1259,0	291,2
		High	52	0	25,8	23,7	1574,0	291,6
	~6,1	Low	28	0	22,3	23,0	1523,8	311,7
		Mid	21	0	25,9	26,1	1878,0	364,3
		High	12	0	27,0	25,2	1460,4	335,2
Race 1	~ 5.1	Low	39	10	17,8	18,7	414,4	189,7
		Mid	68	55	20,7	18,1	336,4	211,3
		High	73	60	15,9	18,0	281,2	159,2
	~6,1	Low	21	0	22,0	24,6	1937,5	344,4
		Mid	30	5	28,6	24,1	1950,4	333,0
		High	45	0	26,0	25,2	1875,0	306,1
Race 1 + TR 4	~ 5.1	Low	61	30	20,1	19,6	278,9	193,5
		Mid	75	40	19,1	18,0	253,6	195,9
		High	62	25	19,9	20,0	346,6	211,7
	~6,1	Low	19	20	22,9	23,7	1868,5	384,7
		Mid	31	0	29,3	25,7	2111,6	389,4
		High	33	5	30,4	27,1	2075,4	344,8
TR4	~ 5.1	Low	100	100		16,9	77,8	102,7
		Mid	88	90	14,0	13,9	117,4	167,6
		High	96	95	18,0	15,9	148,1	127,6
	~6,1	Low	84	95	14,0	15,7	726,6	151,1
		Mid	96	100	17,5	16,9	1123,1	177,1
		High	89	100	11,0	11,0	442,8	103,5

[1]DI = disease index



Supplemental Figure 1. Principal component analyses of regularized log transformed TPM values of replicates of individual samples.



Supplemental Figure 2. The number of upregulated and downregulated genes upon inoculating Cavendish 'Grand Naine' with *Fusarium* R1, *F. odoratissimum* TR4 and challenge inoculations (R1 + TR4) at different time points compared to the water control. The Venn diagram shows the total number of differentially expressed genes as well as the genes that are similar between the various treatments. Number on top indicates upregulated while numbers below indicate downregulated gene numbers.

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CHAPTER 8

Transgenic Cavendish Bananas with Resistance to Panama Disease Tropical Race 4

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Abstract

Banana (*Musa* spp.) is a staple food for more than 400 million people. Over 40% of world production and virtually all the export trade is based on Cavendish banana. However, Cavendish banana is under threat from a virulent fungus, *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) for which no acceptable resistant replacement has been identified. Here we report the identification of transgenic Cavendish with resistance to TR4. In our 3-year field trial, two lines of transgenic Cavendish, one transformed with RGA2, a gene isolated from a TR4-resistant diploid banana, and the other with a nematode-derived gene, *Ced9*, remain disease free. Transgene expression in the RGA2 lines is strongly correlated with resistance. Endogenous RGA2 homologs are also present in Cavendish but are expressed tenfold lower than that in our most resistant transgenic line. The expression of these homologs can potentially be elevated through gene editing, to provide non-transgenic resistance.

Introduction

Fusarium wilt or Panama disease is a devastating disease of bananas. In the first half of last century, it caused one of the most serious plant disease epidemics in history. During that period, *F. oxysporum* f. sp. *cubense* (*Foc*), the fungus responsible for Fusarium wilt, caused a major epidemic in commercial banana plantations in South and Central America in the then dominant export cultivar 'Gros Michel' (Ploetz 2015). This epidemic was caused by *Foc* race 1 and led to the almost complete replacement of 'Gros Michel' with Cavendish, which is resistant to *Foc* race 1. Cavendish now accounts for >40% of world's banana production and completely dominates the banana export market, which amounts to 15% of world production. Despite this, *Foc* race 1 continues to cause significant disease in a wide range of other locally produced and traded banana cultivars (Karangwa *et al.,* 2016). *Foc* invades through the roots before entering the corm and pseudostem where it causes extensive necrosis leading to plant death (Stover 1962). The fungus is disseminated in infested soil, infected planting material and water including irrigation water and floods, and can remain in the soil for >40 years (Ploetz 2015). In the early 1990s, another form of *Foc*, Tropical Race 4 (TR4) was recognized in Southeast Asia (Ploetz 2006), which differed from *Foc* race 1 in that it infects and kills Cavendish as well as a number of other important race 1-resistant cultivars. *Foc* TR4 now devastates Cavendish plantations in Indonesia, Malaysia, China, the Philippines, Australia, and Mozambique. It continues to move internationally with recent reports of its spread into Jordan, Pakistan, and Lebanon (García-Bastidas *et al.,* 2014, Ploetz 2015) and it is highly likely that the fungus and the disease will continue to spread particularly in south and Southeast Asia. Of the banana-producing continents, only the Americas have yet to record TR4. The disease now poses a very significant threat to commercial banana production worldwide and, together with race 1, severely limits the number of banana cultivars suitable for either large scale or smallholder production.

There is no effective chemical control for TR4 (Ploetz 2006) and efforts to contain the disease through inter- or intra-national quarantine have clearly been ineffective as evidenced by the continued spread between continents, countries, and regions. Locally, efforts such as destruction of infected plants, isolation of infested areas, and disinfection of vehicles, machinery, and clothing are likely, at best, to slow the rate of spread. Although somaclonal variants of Giant Cavendish (Giant Cavendish Tissue Culture Variants (GCTCVs)) with varying levels of tolerance to TR4 have been generated in Taiwan through tissue culturing (Hwang and Ko 2004), these are considered a short-term solution to disease control at best due to lack of immunity and undesirable agronomic traits (Ploetz *et al.,* 2015). The lack of effective TR4 control measures and the devastating impact of the disease make the deployment of resistance genes an obvious and attractive strategy. Here we report the generation and field-trialling of transgenic Cavendish banana plants and the identification of lines with robust resistance to TR4.

Results

Generation and characterization of transgenic banana plants

We have previously shown that the *Ced9* anti-apoptosis gene derived from the nematode *Caenorhabditis elegans* can confer resistance to *Foc* race 1 in transgenic Lady finger bananas (Paul *et al.,* 2011). We have also previously isolated resistance gene analog 2 (*RGA2*), a putative nucleotide-binding and leucine-rich repeat (NB-LRR)-type resistance (R) gene, from a seedling of *Musa acuminata* ssp. *malaccensis* with resistance to TR4 (Peraza-Echeverria *et al.,* 2009). Sequence analysis of this gene (Ploetz *et al.,* 2015) revealed a close phylogenetic relationship to the NB-LRR type genes, including *I2* (Ori *et al.,* 1997) and *Fom-2* (Joobeur *et al.,* 2004), which have been shown to encode Fusarium wilt resistance in tomato and melon, respectively. Therefore, we constructed both *Ced9*- and *RGA2*-derived transgene expression cassettes (Figure 1a, b) where *Ced9* was under the control of the maize polyubiquitin promoter (Ubi-P) and *RGA2* was under the control of the *Agrobacterium nopaline synthase* promoter (Nos-P). These cassettes were transformed separately into the Cavendish 'Grand Nain' (GN). After screening primary transformants by PCR, five *RGA2* lines (RGA2-2, 3, 4, 5, and 7) and five *Ced9* lines (Ced9-17, 19, 21, 22, and 26), together with untransformed controls, were selected for Southern analysis. Each of the *RGA2* lines contained multiple transgene copies in addition to three endogenous *RGA2* homologs present in untransformed control plants (Figure 1c). The five *Ced9* lines contained between one and many transgene copies with no endogenous homologs identified (Figure 1d).

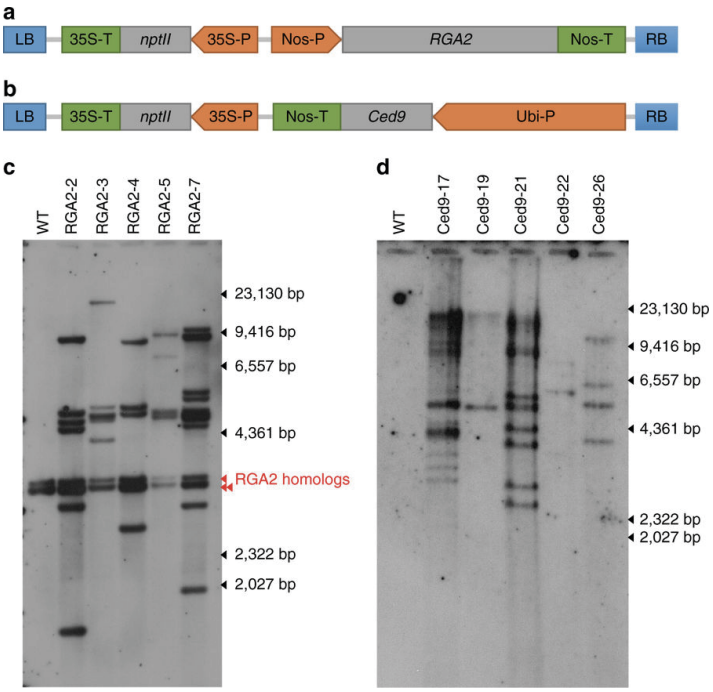


Figure 1. Transgene expression cassettes and Southern analysis of selected transgenic lines. **A** RGA2 and **b** Ced9 expression cassettes. LB, left border; RB, right border. Determination of transgene copy number in **c** *RGA2* and **d** *Ced9* transgenic banana lines by Southern blot analysis. Genomic DNA from WT, *RGA2* and *Ced9* lines was digested with *Hin*dIII and *Xma*I, respectively. DNA molecular weight marker II (Roche) reference is indicated on the right-hand side

Field assessment of transgenic banana plants

To determine whether these transgenes could confer TR4 resistance, we assessed the ten independent transgenic lines (RGA2-2, 3, 4, 5, and 7; Ced9-17, 19, 21, 22, and 26), along with five additional PCR-positive transgenic lines but for which Southern analysis had not been done (RGA2-6; Ced9-10, 15, 23, and 31), for resistance in a field trial over a 3-year period. The trial site was a commercial banana plantation in the Northern Territory of Australia where TR4 has become endemic and where Cavendish banana plants had previously been devastated by the disease. Non-transgenic controls included in the trial were the TR4-susceptible Cavendish cultivars GN and Williams, in addition to cultivars GCTCV 218 and DPM25 ('Dwarf Parfitt Mutant'). 'GCTCV 218' is a variant of Giant Cavendish selected in Taiwan for its tolerance to TR4 (Hwang and Ko 2004), whereas 'DPM25' is a ɣ-irradiated selection of Cavendish cultivar 'Dwarf Parfitt', which has resistance to another race of *Foc*, subtropical race 4 (STR4) (Smith *et al.,* 2006). The trial location has a tropical climate with about 90% of its annual rain usually falling during the wet season (November–April). The trial was planted during the wet season in early 2012, according to a randomized design, and run for 3 years, as banana is a perennial crop. To increase the inoculum pressure, infected plant material was buried between each plant.

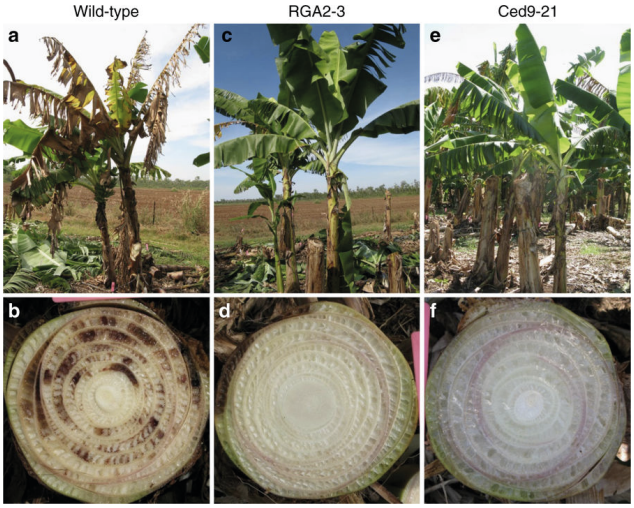


Figure 2. Characteristic symptoms of *Foc* TR4 in susceptible and resistant banana. External symptoms and reddish-brown internal vascular discoloration of *Foc* TR4 in infected WT Cavendish **a** and **b** compared with resistant transgenic lines RGA2-3 **c** and **d**, and Ced9-21 **e** and **f**

The trial was regularly inspected for plants showing typical TR4 symptoms such as wilting and/or leaf yellowing (Figure 2a). The pseudostems of symptomatic individuals were further examined for the presence of the reddish-brown vascular discoloration characteristic of TR4 infection (Figure 2b). The disease status of the plants, based on this visual assessment, was recorded at regular intervals in both the wet and dry (May–October) seasons (Figure 3a, b and Table 1). For the final assessment, the pseudostems from all of the surviving plants were scored for vascular discoloration and a selection of samples taken and tested for TR4 by a combination of fungal isolation and PCR-based assays (Leslie *et al.,* 2006, Dita *et al.,* 2010) Of the 118

samples, 107 were from plants showing no vascular discoloration and all tested negative for TR4. The 11 remaining samples came from plants with vascular discoloration and, of these, 10 tested positive for *Foc* TR4. This confirmed that vascular discoloration in the trial was more than 99% accurate as a diagnostic marker for infection by TR4.

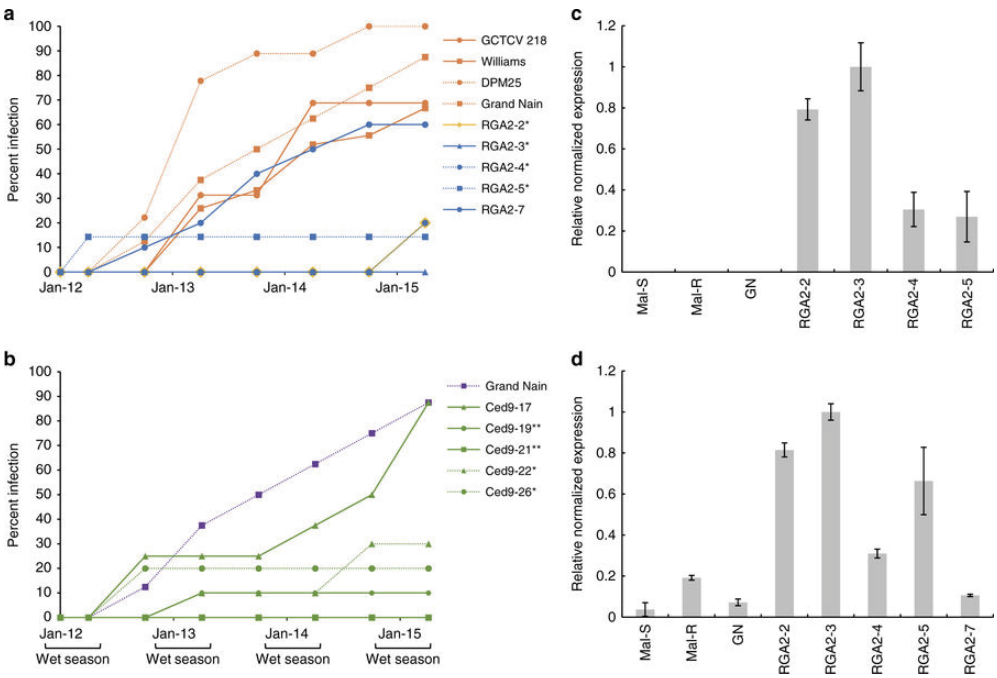


Figure 3. Disease incidence and gene expression analysis. **a, b** Levels of Foc TR4 infection in selected transgenic and WT banana plants throughout the 3-year field trial. **a** WT and RGA2 lines, and **b** WT and Ced9 lines. Wet seasons (November–April) are indicated. The number of biological replicates (*n*) of each independent line at the start of the trial is provided in Supplementary Table 1. Data points are percentage of biological replicates infected at time of assessment. *$0.01 < p < 0.05$, **$0.001 < p < 0.01$ at trial end (Tukey's HSD test). **c, d** Analysis of *RGA2* mRNA expression levels in transgenic and WT banana plants. **C** Analysis of transgene (*RGA2*-Nos) expression levels using primers designed to amplify a 96 bp fragment spanning the *RGA2* transgene/Nos terminator junction. **d** Analysis of *RGA2*transgene and endogenous mRNA expression levels using primers designed to amplify a 92 bp fragment of both the *RGA2* transgene and *RGA2* endogenous sequences. All values are normalized expression levels expressed relative to line RGA2-3. WT GN; TR4-susceptible *M. acuminata* ssp. *malaccensis* (Mal-S) and TR4-resistant *M. acuminata* ssp. *malaccensis* (Mal-R). A single biological replicate was analysed with three technical replicates ($n = 3$). Data are mean ± SEM

Table 1. 'DPM25', 'Dwarf Parfitt Mutant'; GCTCV, Giant Cavendish Tissue Culture Variants; GN, 'Grand Nain'; HSD, honest significant difference; TR4, tropical race 4. Data are percentage of biological replicates infected at time of assessment. *0.01 < p < 0.05, **0.001 < p < 0.01 at trial end (Tukey's HSD test).

Line	Number of plants in the field (n)	Chronological incidence of TR4 infection (percent of symptomatic plants)							
		Jan 2012	Apr 2012	Oct 2012	Apr 2013	Oct 2013	Apr 2014	Oct 2014	Apr 2015
GCTCV 218	16	0	0	0	31.3	31.3	68.8	68.8	68.8
Williams	27	0	0	0	25.9	33.3	51.9	55.6	66.7
DPM25	9	0	0	22.2	77.8	88.9	88.9	100	100
GN	8	0	0	12.5	37.5	50	62.5	75	87.5
RGA2-2	10	0	0	0	0	0	0	0	20*
RGA2-3	8	0	0	0	0	0	0	0	0*
RGA2-4	10	0	0	0	0	0	0	0	20*
RGA2-5	7	0	14.3	14.3	14.3	14.3	14.3	14.3	14.3*
RGA2-6	6	0	0	16.7	50	50	50	50	66.7
RGA2-7	10	0	0	10	20	40	50	60	60
Ced9-10	8	0	0	12.5	25	37.5	37.5	37.5	37.5
Ced9-15	10	0	0	0	10	10	20	20	50
Ced9-17	8	0	0	25	25	25	37.5	50	87.5
Ced9-19	10	0	0	0	10	10	10	10	10**
Ced9-21	9	0	0	0	0	0	0	0	0**
Ced9-22	10	0	0	0	10	10	10	30	30*
Ced9-23	10	0	0	0	10	10	10	10	20
Ced9-26	10	0	0	20	20	20	20	20	20*
Ced9-31	10	0	0	10	10	10	20	20	20

By the end of the trial, between 67 and 100% of all control plants were either dead or infected and all had displayed vascular discoloration. In general, disease developed earlier in the non-transgenic control plants, increased by about 20% per year, and was considerably faster during the wet seasons (Figures. 3 a,b and Table 1). DPM25, which is noted for its resistance to *Foc* STR4 (Smith *et al.,* 2006), appeared to be the most susceptible cultivar with all of its replicates becoming infected within 2.5 years, whereas the reportedly TR4-tolerant 'GCTCV 218' (Hwang and Ko 2004) was as susceptible as the Cavendish 'Williams'. However, there was no statistical difference in the total proportion of symptomatic infected plants between any of the controls compared with the reference control, GN (Tukey's honest significant difference (HSD) test, $p > 0.05$). In contrast, 30% or fewer of plants of four of the five characterized RGA2 lines and four of the five characterized Ced9 lines were symptomatic during the 3-year trial. Symptom development in the four RGA2 lines, RGA2-2, RGA2-3, RGA2-4, and RGA2-5, was significantly lower than that in GN (Tukey's HSD test, $0.01 < p < 0.05$), whereas RGA2-7 (60%) was not. Similarly, symptom development was significantly lower in the four Ced9 lines, Ced9-22, and Ced9-26 (Tukey's HSD test, $0.01 < p < 0.05$), and Ced9-19 and Ced9-21 (Tukey's HSD test, $0.001 < p < 0.01$) than GN, whereas Ced9-17 (87.5%) was not. Lines RGA2-3 and Ced9-21 appeared to be immune to TR4, as no internal (vascular discolouration) or external symptoms of the disease were observed in any plant throughout the 3 years (Figures. 2 c–f, Figures. 3 a, b, and Supplemental Table 1).

Analysis of gene expression levels

To investigate the basis of the resistance, and because TR4 is a soil-borne pathogen, roots from the Ced9 and RGA2 lines were assayed for transgene expression. Quantitative analysis was only done on RGA2 lines, because, as a banana-derived gene, *RGA2* was considered to be the most suitable transgene for deregulation. Reverse transcriptase PCR (RT-PCR) showed that all five Ced9 lines expressed the transgene (Supplementary Figure 1). For the RGA2 lines, quantitative RT-PCR (qRT-PCR), initially with primers that amplified the *RGA2* transgene only, was used to assess expression levels and a strong correlation was observed between *RGA2* expression level and the degree of TR4 protection (Figure 3c). Subsequently, qRT-PCR was repeated but with primers that would amplify both the *RGA2* transgene and endogenous *RGA2* in GN (Figure 3d). Again, the most resistant transgenic line (RGA2-3) was the highest expressor of RGA2 mRNA, whereas the other three TR4-resistant lines, RGA2-2 (20% infection), RGA2-4 (20% infection), and RGA2-5 (14.3% infection), also showed moderate to high levels of RGA2 expression. The most susceptible line RGA2-7 (60% infection) had the lowest expression levels, which were about tenfold less than RGA2-3. The level of expression in the highly susceptible Cavendish cultivar GN (87.5% infection) was also about tenfold lower than line RGA2-3. The observed inverse correlation between the proportion of infections and the expression level of *RGA2* in non-transgenic GN and the *RGA2* transgenic lines was statistically significant (Pearson's correlation = −0.86, $t = -3.37$, df = 4, $p = 0.028$; Spearman's rank correlation = −0.90, $S = 66.45$, $p = 0.015$). This inverse relationship persisted when only the RGA2 transgenic lines were considered but the correlation was no longer statistically significant (Pearson's correlation = −0.85, $t = -2.811$, df = 3, $p = 0.067$; Spearman's rank correlation = −0.82, $S = 36.42$, $p = 0.089$).

Interestingly, RGA2 expression in TR4-resistant wild diploid *M. acuminata* ssp. *malaccensis* was more than fivefold greater than in its susceptible sibling but more than fivefold less than RGA2-3. Fortunately, enhancing levels of RGA2 or Ced9 expression does not appear to have a detrimental impact on bunch size in the transgenic lines. A visual rating of the size of available mature bunches (Supplementary Table 2) based on the number of fruit hands per bunch showed no statistical difference between healthy control and transgenic GN ($\chi^2 = 31.7$, df = 28, $p = 0.29$).

Possible resistance mechanisms and future research

These results indicate that the Cavendish cultivar GN possesses endogenous *RGA2* loci, which are not sufficiently expressed to confer TR4 protection. Given the close relationship of GN and the other Cavendish cultivars Williams, GCTCV 218, and DPM25, it is probable that the latter clones also possess *RGA2* loci. Increasing RGA2 levels by transgenesis may reduce TR4 infection, possibly through an R-gene-like cascade pathway. The gene(s) responsible for the Foc TR4 resistance in *M. acuminata* ssp. *Malaccensis* have not been identified but *RGA2* is a possible candidate. It seems highly unlikely that the observed resistance to TR4 in our *RGA2* lines is due to somaclonal variation, because somaclonal variants that exhibit even tolerance to TR4 are rare, yet we identified four out of five independent *RGA2* lines with resistance. Southern blot analyses indicated that even the highly susceptible GN contains three *RGA2*-like sequences (Figure 1c). Cloning and analysis of these *RGA2*-like sequences confirmed the presence of three *RGA2* homologs, which were between 98.6 and 98.7% homologous with *RGA2* at the nucleotide level resulting in between 28 to 32 amino acid changes (Figure 4). Further analysis indicated that these were three alleles of a single copy *RGA2* homolog. To determine whether low expression levels of the homologs, amino acid differences, or a combination of the two renders the three *RGA2* homologs ineffective, GN has been transformed with each homolog, under the control of the Nos promoter, and the response of the transgenic lines to TR4 will be assessed in future field trials. The mode of action of *Ced9*-mediated TR4 resistance in transgenic bananas is also unclear. Functioning as an anti-apoptosis gene, it may be preventing fungal-induced cell death and contributing to the maintenance of organelle homeostasis.

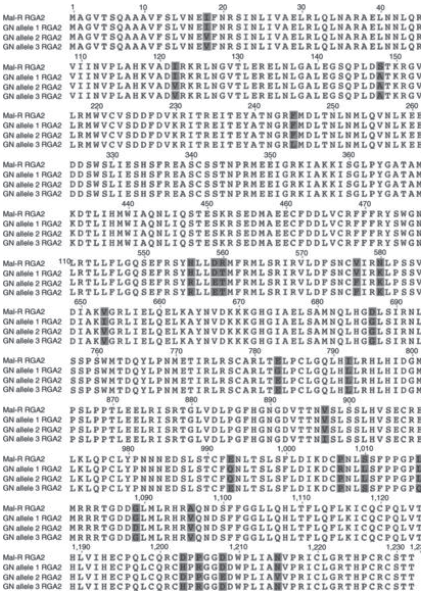


Figure 4. Protein sequence alignment of the *RGA2* transgene sequence from TR4-resistant *M. acuminata* ssp. *malaccensis* (Mal-R) and the three consensus *RGA2* homologous sequences from WT GN. Amino acid differences are highlighted

Previously, transgenic resistance to *Foc* race 1 in banana has been reported from glasshouse testing using either anti-apoptosis genes (Paul *et al.,* 2011, Ghag *et al.,* 2014b) or RNA interference targeting essential Foc genes (Ghag *et al.,* 2014a). This is the first report of Fusarium wilt resistance in transgenic bananas in the field. It is a very significant step toward averting the collapse of Cavendish-based banana export production and at the same time protecting a major subsistence crop. We are about to commence another more expansive field trial containing our RGA2 lines, as well as many more new Cavendish (cultivars Williams and GN) lines transformed with *RGA2*, to identify lines that are suitable for progression through to commercial release. Further, the discovery that Cavendish encodes three *RGA2* homologs provides the exciting prospect of using gene-editing technology to generate TR4 resistance by enhancing the expression of these endogenous genes with or without also editing the coding sequence.

Methods

Plant transformation and characterization

Binary vectors containing either the C. elegans gene Ced9 under the control of a maize Ubi-P and a cauliflower mosaic virus 35s terminator (35S-T) (Paul et al., 2011) or the M. acuminata ssp. Malaccensis RGA2under the control of the Agrobacterium tumefaciens Nos-P and terminator sequences (Echeverria 2007) were used in this study *M. acuminata* Cavendish cv. GN (AAA subgroup) embryogenic cell suspensions were prepared from immature male flowers and transformed using the centrifugation assisted *A. tumefaciens*-mediated method (Khanna *et al.,* 2004). Following selection, plants derived from single embryos were regenerated and were screened for the

presence of the respective transgene by PCR using specific primers. A total of six *RGA2* and nine *Ced9* lines were generated, and up to 10 replicates of each transgenic line was multiplied in tissue culture for field analysis. Both the number of transgenic lines and the number of replicates per line permitted in the field trial was limited by the license conditions (DIR 107) imposed by the Office of the Gene Technology Regulator (OGTR). Plants derived from non-transformed cell suspensions were also generated as controls. Before field planting, tissue-culture plants were acclimatized in a secure shade house over a 3-month period by which time they had reached a height of ~35 cm.

Field trial design

The field trial was conducted on a commercial banana plantation site located at Lambells Lagoon, Northern Territory, Australia. The site had previously been used to grow Cavendish banana plants and has a history of high incidence of TR4 infection. The field trial comprised two plantings, the first in January 2012 and the second in May 2012. Owing to the seriousness of the threat of TR4 to the Australian banana industry, it took 8 years from the initial generation of the transgenic lines to identify a suitable field trial location and obtain permission from the plantation owner, the biosecurity regulators in both Queensland and the Northern Territory, and the OGTR to conduct this trial. Unfortunately, the trial was only conducted for 3 years, not the intended 5 years, because of a forced quarantine termination order due to another disease.

Transgenic lines were planted in a randomized design, with rows containing blocks of ten transgenic plants and each block separated by four non-transgenic control plants. The control plants included cell line control plants (cv Cavendish selections GN and/or Williams) from QUT in addition to tissue culture plants of 'Williams', 'GCTCV 218' and 'DPM25' supplied by Mission Beach Tissue Culture Nursery, Queensland. To increase the level and uniformity of inoculum pressure, a small segment of pseudostem taken from TR4-infected banana plants growing outside the trial site was buried between each plant. When a banana is planted, the plant crop, a pseudostem comprising the petioles of leaves grows from the basal corm with the vegetative meristem remaining at the base of the pseudostem. When flowering is initiated, the meristem is pushed up through the center of the pseudostem, the fruit bunch emerges through the top of the pseudostem and develops through to maturity. After the bunch is harvested, this initial pseudostem dies back and another new pseudostem, known as the first ratoon, grows from a different meristem on the corm. This process can be repeated indefinitely. The trial consisted of the plant crop and up to three ratoon crops.

Assessment of symptoms and bunch size

Plants were regularly assessed over three crop cycles (~3 years) for the development of characteristic external symptoms of Fusarium wilt disease (Ploetz 2015). The pseudostem of plants exhibiting typical external symptoms was inspected to confirm the presence of characteristic internal reddish-brown vascular discoloration associated with infection by *Foc*. If a plant shows the characteristic external symptoms of wilting and/or yellowing of their leaves, it almost invariably develops severe vascular necrosis followed by death of that pseudostem. In some instances, the symptomatic pseudostem would die but an apparently healthy ratoon pseudostem would grow from the basal corm. The first observation of symptoms was recorded for each plant; however, ratoons from diseased

plants were allowed to develop naturally until the completion of the trial period. At the completion of the trial period, all plants were inspected for external and internal symptoms. The presence of the internal vascular discoloration, which is highly characteristic of *Foc* TR4 infection, was assessed in all remaining plants by cutting the pseudostem of all plants ~0.5 metres above ground level. Where vascular discoloration was observed, a pseudostem sample of ~3 cm × 1 cm was taken from the leading edge of the discoloration. Where no vascular discoloration was observed, a similar sample was taken from an equivalent area. Pseudostem samples from 118 plants were collected and were sent by airfreight to Wageningen UR, Plant Sciences Group, The Netherlands, for confirmatory diagnosis of *Foc* infection.

To avoid any bias, all plants in the trial were assigned a unique Field Trial Number which contained no identifying information. Further, the assessment of disease symptoms during the 3-year trial period was done independently by the farm manager (MS) who has more than 20 years' experience managing a TR4-infested commercial banana farm. At the completion of the trial, assessment for internal and external symptoms was done by two of the authors (JD and RH). At various times during the trial, the size of mature fruit bunches on healthy transgenic and non-transgenic plants was visually assessed and rated into three categories, <6, 6–8, or >8 hands per bunch.

Diagnosis of Foc infection

The pseudostem samples were used for a combination of fungal isolation and molecular diagnostic analyses by PCR (Leslie *et al.,* 2006, Dita *et al.,* 2010) and quantitative PCR (qPCR kit: "*Foc* TR4 DNA identification by Real-Time PCR, Clear Detections", The Netherlands). For DNA isolation from plant samples, small pieces of pseudostem tissue were selected and subsequently lyophilized in an Epsilon 1-4/2–4 LSC plus freeze dryer (Martin Christ) for 3 days. For a subset of 49 samples that were collected from the above-mentioned materials, 2– 4 pieces of the same tissue were sterilized with 1% hypochlorite, rinsed with Milli-Q water, dried in filter paper, and placed on Komada medium for fungal re-isolation (Dita *et al.,* 2010). After 5 days, emerging Foc colonies were sampled for PCR analysis (Dita *et al.,* 2010), as well as qPCR. Total plant and fungal DNA was extracted using the Sbeadex maxi plant Kit (LGC Genomics). DNA extractions from all plant and fungal samples were independently repeated at least twice. Analytical PCRs were also technically repeated twice. qPCR was conducted in a total volume of 20 µL reaction mixture containing 10 µL of Clear Detections PCR mix, 2 µL of Foc TR4 primer set, 3 µL of PCR enhancer, 0.2 µL of ROX reference dye II (Takara), 3 µL of DNA template, and Milli-Q water in a 7500 Real-Time PCR system (Applied Biosystems). Thermal cycling conditions for amplification were an initial enzyme activation at 95 °C for 3 min, followed by 35 cycles each consisting of 95 °C for 10 s, 63 °C for 60 s, and 72 °C for 30 s. Finally, for the PCR melt curve 0.2 – 0.5 °C, steps at 72 – 95 °C were included. The amplification results were analysed with 7500 Real-Time PCR software v 2.3 (Applied Biosystems).

Transgene expression analysis

Owing to quarantine restrictions, we were unable to transport samples from banana plants within Australia for analysis. Therefore, transcript analyses were done using samples taken from the original mother plants stored in tissue culture at QUT, Brisbane, Australia. Tissue culture plants of wild-type (WT) TR4-susceptible and resistant *M.*

acuminata ssp. *malaccensis* were used as controls (Walduck and Daly 2007, Peraza-Echeverria *et al.,* 2008).

Total RNA was extracted from 200 mg of root tissue using a protocol (Valderrama-Cháirez *et al.,* 2002) that was modified by increasing the tissue: extraction buffer ratio to 8, including a centrifugation step (18,000x*g* for 5 min) before the initial solvent extraction and the omission of phenol from all extraction steps. RNA (3 µg) was DNase-treated using an RQ1 RNase-free DNase Kit (Promega) and DNA-free RNA samples (1.8 µg) were reverse-transcribed to complementary DNA using an oligo(dT)18 primer and the GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions. Subsequently, cDNA samples were diluted either 1:10 (v/v) or 1:8 (v/v) in RNase-free water before use in RT-PCR and qRT-PCR, respectively. To ensure complete removal of potential gDNA contamination in our samples before RT-PCR and qPCR, total RNA, DNase-treated RNA, as well as cDNA was tested by PCR using the cyclophilin (*CYP*) housekeeping gene primers (Supplementary Table 3).

Reaction mixes for RT-PCR contained 1 × GoTaq Green master mix (Promega), 0.25 µM of each primer (Supplementary Table 3), diluted cDNA (2 µL), and nuclease-free water in a final volume of 20 µL. Thermal cycling conditions included a 2 min denaturation step at 94 °C followed by 35 cycles of 94 °C for 20 s, 55–62 °C (primer dependent) for 30 s, and 72 °C for 1 min Kbp^{-1} of expected amplicon size, and a final extension at 72 °C for 5 min.

qRT-PCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the SYBR Green I technology. Per 10 µL reactions, 2.5 µL of diluted cDNA was added to 1 × GoTaq qPCR Master Mix (Promega) premixed with primers (Supplementary Table 3) at a final concentration of 0.2 µM. The following amplification program was used: Hot-Start polymerase activation at 95 °C for 2 min, followed by 45 cycles of 10 s denaturation at 95 °C and 30 s annealing/extension at 60 °C. At the end of each run, a dissociation curve was produced from 65–95 °C, to confirm the specificity of the amplicon from each primer set. A standard curve of eight serial two-fold dilutions of cDNA was used to determine the qPCR efficiency of each of the primer sets used (Rasmussen 2001). All PCR reactions displayed a correlation coefficient $R^2 > 0.98$ and efficiencies >99% (Supplemental Figure 2). All samples were analyzed in triplicate and each run included triplicate non-template control reactions for each of the primer sets used on that run. In addition, selected samples from each run were electrophoresed through 2% agarose gels to validate production of a single amplicon.

Relative expression levels were calculated using CFX Manager 3.1 (Bio-Rad) software and the ΔΔCT method (Schmittgen and Livak 2008). Ct data obtained from target gene of interest were normalized using Ct values from the two stable reference genes *CYP* and ribosomal protein *S2* (*RPS2*) and expressed relative to the values of line RGA2-3. All primers were designed using the Primer3Plus freeware (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Southern blot analysis

For determination of transgene copy number integration by Southern analysis, total nucleic acid was extracted (James *et al.,* 2011) from banana leaf tissue, treated with RNAse A, and 15 µg aliquots of genomic DNA were digested overnight with 20 U of

restriction enzyme *Hin*dIII or *Xma*I (New England Biolabs) overnight at 37 °C. Digested DNA was electrophoresed through 0.9% agarose gels, transferred to a nylon membrane (Roche), and UV cross-linked. Gene-specific probes were amplified by PCR using Taq DNA polymerase (Sigma-Aldrich) in reaction mixes containing the appropriate primers (Supplementary Table 3), 2 ng of plasmid template and DIG PCR labelling mix (Roche). Hybridization of the probe was done under standard conditions (Sambrook and Russell 2001) and detection was achieved using CDP-star (Roche), as per the manufacturer's instructions. X-ray films (Fuji) were exposed for up to 1 h depending on signal intensity and developed manually.

Isolation and analysis of endogenous Cavendish RGA2 homologs

The entire open reading frame of the endogenous Cavendish *RGA2* sequence was amplified from total nucleic acid prepared (James *et al.,* 2011) from leaf tissue collected from WT GN plants. Primers *RGA2geneF* (5′-ATGGCTGGTGTCACATCACAGGCAG-3′) and *RGA2geneR* (5′-TCAGGTGGTGCTACAGCGACATGG-3′) were designed based on the *M. acuminata* ssp. *malaccensis RGA2* sequence (Echeverria 2007). PCR was carried out using either GoTaq Long Master Mix (Promega) or Expand Hi-Fidelity Enzyme Mix (Roche). GoTaq Long PCR mixtures contained 20 µL 2× GoTaq Long Master Mix, 10 ρmol of each primer, 0.5 µL TNA extract, and 17.5 µL water. Cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 50 °C for 15 s, 65 °C for 8 min, and a final extension at 72 °C for 10 min. PCR using Expand was carried out according to the manufacturer's instructions, with cycling as above except for extension carried out at 68 °C. PCR products were analysed on 1% agarose gels and visualized using SYBR safe DNA gel stain (Thermo Fisher Scientific). PCR products of the expected size were excised from gels, ligated into pGemT Easy (Promega), and heat-shock transformed into competent *Escherichia coli* XL-1 Blue cells (Invitrogen). Blue/white selection was used to identify putative recombinant colonies, with six clones derived from each PCR amplification selected and grown in overnight cultures. Plasmid DNA was purified using the Wizard *Plus* SV Miniprep DNA purification system (Promega) and digested using *Not*I to identify clones with inserts of the expected size. Plasmids were then sequenced using the BigDye Terminator mix v3.1 (Applied Biosystems) according to the manufacturer's instructions, with sequence reads generated at the Queensland University of Technology Molecular Genetics Research Facility. Specific primers were used to completely sequence the inserts in both directions. Raw sequence reads were compiled into full-length sequences using the VectorNTI Advance V11 software (Life Technologies) program ContigExpress, whereas sequence alignments were carried out using AlignX.

Consensus sequences of the WT GN *RGA2* homologs were compared with the *M. acuminata* spp. *malaccensis RGA2* sequence, to determine the level of nucleotide and amino acid sequence similarity.

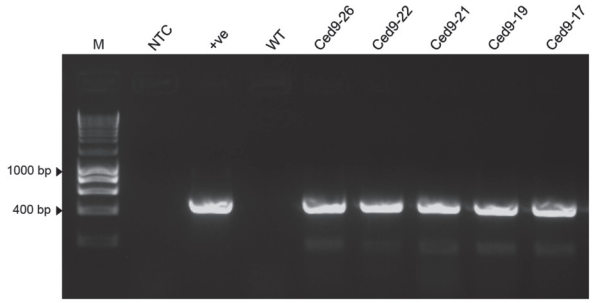
Statistical analysis

Differences in total proportions of infected plants between the treatment and control groups, and between lines, were assessed using single and mixed effects generalized linear models and corresponding analyses of variance, assuming a binomial response and with the respective addition or subtraction of 0.5 for 0 or total infections. Pairwise comparisons between treatments and lines, adjusted for multiple comparisons, were

evaluated using Tukey's HSD test. Model fit was assessed using a χ^2-test of deviance and the Akaike information criterion (AIC). The equivalence of ratings of fruit bunches from control, RGA2 lines and the Ced9 lines as number of hands in the bunch (<6, 6–8, and >8) was assessed using a χ^2-test of homogeneity. The hypothesis of a (linear) relationship between the proportion of infections and the expression level for each of the RGA lines and the GN control line was assessed using parametric (Pearson) and nonparametric (Spearman) correlations, and the associated tests. Statistical significance was asserted at the 5% level ($p < 0.05$). The analyses were undertaken in R using base statistical functions and the packages lme4, lmer, and multcomp.

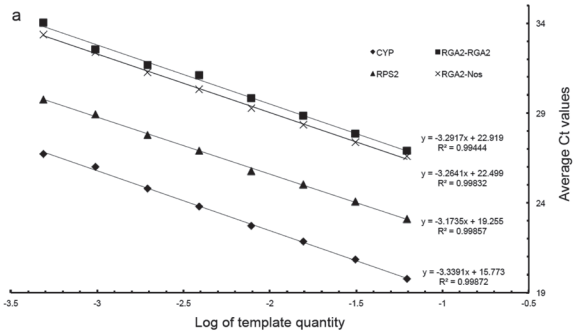
Data availability

The authors declare that all data supporting the findings of this study are available within the article and/or its Supplementary Information file. All relevant data are also available from the authors upon request.



Supplemental Figure 1. Reverse transcriptase-PCR analysis of Ced9 mRNA expression in selected lines. No-template control (NTC); plasmid DNA positive control (+ve); wild-type (WT) and DNA molecular weight marker HyperLadder I (Bioline) (M).

a



b

Target gene	Efficiency calculation	Efficiency (%)
RGA2	1+10^(-1/-3.2917)	101.3
RGA2-Nos	1+10^(-1/-3.2641)	102.5
CYP	1+10^(-1/-3.3391)	99.3
RPS2	1+10^(-1/-3.1735)	106.6

Supplemental Figure 2. Efficiency of primer pairs used for quantitative reverse transcriptase-PCR. **a**. Dose response curve of primer pairs used to amplify each target gene. **b**. Calculation of PCR efficiency for each primer pair.

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CHAPTER 9

General Discussion

Banana is without any doubt one of the world's most important crops. It is cultivated in more than 135 countries (Arango *et al.,* 2011) and is the most popular and cultivated fruit, followed closely by watermelon and apples. Including cooking bananas, more than 149 million metric tons were produced, and more than 20 million metric tons were exported in 2016 (FAOSTAT 2016). Bananas are a major, multibillion-dollar export commodity forged primarily in developed countries (Churchill 2011). Interestingly and despite having existed for more than two centuries the banana bonanza reached its peak during the last 100 years, primarily because of "banana barons" such as the United Fruit Company and Standard Fruit (now Chiquita and Dole, respectively) that figured out how to grow and transport a highly perishable but profitable exotic fruit for a minimal investment (Marquardt 2001, Koeppel 2008). Since then, bananas are ubiquitous fruits that can be found everywhere and can be eaten in a variety of ways (Ploetz 2005b). Bananas is also a key ingredient in many developing economies and a source of income for subsistence farmers especially in Southeast Asia, Africa and Latin America (Karamura *et al.,* 2013), hence, playing an important role in food security. The banana empire has been founded in a series of uneasy very well documented historical events (Koeppel 2008, Chapman 2014). Interestingly, from a genetically point of view, the banana production system is weakly founded and thus, fragile (Bakry *et al.,* 2009), for all modern cultivars are derived from limited genetic stocks, which make them vulnerable to many abiotic and biotic stresses (d'Hont *et al.,* 2012).

Over the last century the industry has relied on monocultures of nearly identical genotypes (Bakry *et al.,* 2009). Initially, the industry was based on the exploitation of the cultivar 'Gros Michel' and after the first Panama disease epidemic it shifted eventually to the production of another group of genetically identical cultivars; the so-called Cavendish subgroup. These varieties were used to overcome the Panama disease problem because they are resistant to the causative so-called race 1 strains of various *Fusarium* spp., which were responsible for the 'Gros Michel' debacle. Since then, banana production is based on monocultures of a few cultivars that slightly differ from each other (Bakry and Horry 2014) and are channelled for export or for local consumption. Half of the contemporary banana production relies on these Cavendish somaclones derived from the single triploid genotype that was found in a tropical forest and barely survived a range of botanical gardens and is extremely difficult to improve by classical breeding (d'Hont *et al.,* 2000, Bakry *et al.,* 2009, Arango *et al.,* 2011).

'Gros Michel' succumbed in the past to one of the biggest botanical epidemics in the agronomical history underscoring the risk of plant disease epidemics and systems based on monocultures (Anderson *et al.,* 2004, Gurr *et al.,* 2011, Dean *et al.,* 2012). This event epitomizes the best example of a recipe for disaster. Unfortunately, the history of 'Gros Michel' seems to presently repeat itself, for Cavendish clones that are susceptible to the new *Fusarium odoratissimum* tropical race 4 (TR4) strain, which originates from Indonesia and disseminates across continents thereby threatening the global banana industry (See Chapter 2; Maryani *et al.,* 2018, Ordoñez *et al.,* 2018). This thesis provides long awaited basic knowledge on the spread of Panama disease, on the required tools for discovery research, the potential for genetic improvement and crucial genes that play an important role during the pathogenesis of banana.

Surviving Panama disease: a century's tale

The first serious Panama disease incursion was caused by so-called race 1 strain of the fungus until recently known as *Fusarium oxysporum* f. sp. *cubense.* Recent taxonomical revisions showed that race 1 strains comprise several genotypes that represent different *Fusarium* species (Maryani *et al.,* 2018, Ordoñez *et al.,* 2018). These race 1 strains devastated the industry in the previous century and prompted the search for solutions at all levels (Stover 1962). This epidemic indirectly pushed the beginning of the breeding program in central America (Shepherd *et al.,* 1994). However, the subsequent implementation of Cavendish cultivars as the solution to mitigate the race 1 driven epidemic, paradoxically, indirectly caused the stagnation of implemented research (Ploetz 2005b). Moreover, a new threat, the foliar blight called black Sigatoka, which is caused by the ascomycete *Pseudocercospora fijiensis* (Arango *et al.,* 2016) surfaced and most research groups turned towards it (Stover and Dickson 1976, Stover 1980). During the first years of the Panama disease epidemic, several efforts targeted the development of bananas with resistance to race 1 (Stover and Buddenhagen 1986, Daniells *et al.,* 1995, Rowe and Rosales 1996). However, the subsequent expansion, popularization and dominance of Cavendish clones, rolled over academic concerns of monocultures. Hence, the need for genetic diversity was neglected, and despite the more than 1,200 cultivars, current production is based on no more than four groups that dominate the international market (Bakry and Horry 2014, Lescot 2017).

Nowadays, there is a big concern of the loss of biological diversity at a global scale. Monoculture entails growing the same crop on the same land repeatedly. This practice has shown to be effective for many years, as it provides financial and organizational benefits, such as mechanization, uniformity, fertilization and production. In monocultures, farmers specialize on production and the complexity of marketing is reduced (Power and Follett 1987). However, there is a substantial disadvantage due to the selection pressure that is exerted by these vast areas of genetic uniformity, which cause ecological and economic instability by facilitating the appearance of diseases and pests. It has been shown over and over again that the use of single resistance genes in a monoculture provides a strong selection pressure for new pathogen races that circumvent resistance, which are therefore often short lived (de Vallavieille-Pope 2004).

However, TR4 did not arise from such a process, but originates from the center of origin in Southeast Asia where co-evolutionary forces have resulted in a wide diversity of the *Musa* host as well as of manifold pathogens, including *Fusarium* spp. causing Panama disease (Volkaert 2009, Maryani *et al.,* 2018). Being confronted with a vast global monoculture, TR4 evidently threatens global banana production (Butler 2013). The speed of the epidemic is slow, but accelerates, similar to the previous epidemic caused by race 1 in Central America (Marquardt 2001). Cavendish has been affected by Panama disease in Southeast Asia for more than 70 years (Su *et al.,* 1977, Su *et al.,* 1986). No accurate official data exist, but we estimate that the TR4 affected area already has passed 100,000 ha. (Li *et al.,* 2013). Ploetz (2005a) estimated that the damage caused by race 1 in 'Gros Michel' exceeds 2.6 billion dollars. The responses to the previous and current epidemic are surprisingly similar (Marquardt 2001). In Southeast Asia the quest for "virgin" fields in combination with less susceptible germplasm and physical measures to reduce the inoculum, have allowed the co-existing with the disease (Ploetz 2015). Under these conditions of risk, banana growers have continued production by expansion

policies of Cavendish and other local varieties that are presumable susceptible to TR4. However, the lack of awareness and quarantine measures as well as the rapid distribution of the pathogen contextualizes once more the risk of a high-level pandemic in the coming years (Chapter 2).

The unstoppable monster

The dissemination of pathogens and pests on a global scale undoubtedly puts the future of food production at risk (Bebber *et al.,* 2014). Black Sigatoka and TR4 represent the largest constraints threatening banana production (d'Hont *et al.,* 2000, Marin *et al.,* 2003, Butler 2013). TR4 was first identified in Taiwan killing Cavendish bananas and causing severe damage in 1967 (Sun 1978). However, it was only diagnosed as a recurrence of Panama disease in the early 1990s when it caused severe damage in plantations in Lampung (Sumatra, Indonesia) and peninsular Malaysia. It was described as a new variant of *Fusarium oxysporum* f. sp. *cubense* (Foc) the previous name of the causal agent of Panama disease, that recently has undergone significant taxonomical revisions (Ploetz 1994, Maryani *et al.,* 2018). Later, between 1997 and 1999 it caused epidemics in the Northern territory of Australia. In 2004 severe incursions of banana plantations in the Guangdong province in southern China raised the alarms about the problem in other areas such as the Philippines where it was identified in 2006 (Molina *et al.,* 2007). Before the start of this thesis, these were the only official reports of the Panama disease situation. For some reason there was at that moment not a big concern, but the interest in Panama disease surfaced again as "a new strain was infecting Cavendish" again. However, it was only the appearance of the disease outside Southeast Asia - in Jordan (García-Bastidas *et al.,* 2014)- that triggered attention and alerted the sector. It was also the northernmost outbreak and represented a dangerous expansion of TR4. Currently, more than 80% of the Jordanian production is affected by TR4, which was likely also the origin of the expansion into Lebanon (García-Bastidas *et al.,* 2014).

This first report of TR4 outside southeast Asia gained a lot of attention, and the media coverage catapulted the interest into Panama disease. In addition, news sharing at social media platforms has also become a phenomenon of increasing social, economic and political importance because individuals can participate in news production and diffusion to large global virtual communities (Gil de Zúñiga *et al.,* 2012, Lee and Ma 2012). The fact that there is an imminent risk stalking the most popular fruit concerned society, which has driven communities to action and evaluate the problem from their own perspectives. This again proves how plant diseases can have a massive effect on local and regional communities and banana stakeholders along the logistic chain. However, despite the global alert that surfaced after the Jordan incursion, which changed the perception of TR4 from a regional issue to a developing global problem, the expansion of TR4 did not cease (see Chapter 2).

Before starting this thesis, TR4 was reported in China, Taiwan, Indonesia, Malaysia, the Philippines and the Northern Territory of Australia (Pin *et al.,* 1996, Davis *et al.,* 2000, Qi 2001, Walduck 2002, Molina *et al.,* 2007, Qi *et al.,* 2008, Buddenhagen 2009, Hermanto *et al.,* 2009). Since the incursion in Jordan, a significant number of papers and newsfeeds predicted a grim future for banana since its expansion outside Southeast Asia continued, particularly after the discovery in Mozambique, which was the first incursion of TR4 in the African continent (Butler 2013, Kema and Weise 2013, Ordóñez *et al.,* 2015a, Ordóñez

et al., 2015b). After all, it accelerated fundamental and applied research with a large number of publications appearing over the last couple of years. The improved monitoring of TR4 dissemination encouraged or strengthened the development and implementation of contingency plans in a number of countries, particularly in Latin America. Since we started research into Panama disease, the dissemination of TR4 is much better documented (Li *et al.,* 2013, García-Bastidas *et al.,* 2014, Ordóñez *et al.,* 2015a, Chittarath *et al.,* 2017, Hung *et al.,* 2017, Mostert *et al.,* 2017, Zheng *et al.,* 2018). Over the last six years the number of countries with documented TR4 presence increased from six to 17 (www.promusa.org). The advent of a new molecular diagnostic (Dita *et al.,* 2010) combined with classical morphology, DNA sequencing and phenotyping assays immensely facilitated the diagnosis of suspicions samples as well as the surveillance and monitoring of the disease in new areas. Now we know that the global dissemination of TR4 is caused by a single clone (Ordóñez *et al.,*2015b). Six years ago, it was difficult to unveil the origin of new TR4 incursions. Presently, the resolution of the used technology and bioinformatic pipelines enabled us to draw phylogeographic inferences on the origin of incursions (Zheng, García-Bastidas et al. 2018). Strains from Laos, Vietnam, and Myanmar were associated with Chinese strains likely due to banana expansion from the Yunnan province. In addition, we were able to link a TR4 incursion in Pakistan to the Philippines and those in Jordan and Lebanon to each other (Zheng *et al.,* 2018). Very similar approaches which used on next generation sequencing (NGS) data also traced the origin of *Xylella fastidiosa* in olive trees (Loconsole *et al.,* 2014).

We should not underestimate the impact of the current TR4 dissemination. Despite the media coverage on prevention and the efforts of the banana sector for enhancing quarantine practices, new incursions are to be expected inside and outside Southeast Asia. The experience is that expansion and dissemination are difficult to stop. Among the management strategies proposed by Ploetz (2015) prevention is currently the major strategy to avoid new TR4 incursions. However, it is important to develop a global map, as well as diagnostic tools for other *Fusarium* spp. and genotypes. Most importantly, however, there is a need for the identification of resistant banana germplasm to combat the disease. Our work significantly extends earlier reports (Liew 1996, Smith *et al.,* 2008, Amorim *et al.,* 2009, Li *et al.,* 2015, Zuo *et al.,* 2018, see also Chapters 4 and 5) although to date, there is no conventional breeding program with a focus on TR4 resistance (Ploetz 2005b, Ploetz 2015). Therefore, evaluation of the global banana germplasm has to intensify, as host resistance is the foundation for sustainable disease management. The history of Cavendish production on race 1 infested soils around the world has amply proven the value of this strategy to a level that is unique in agriculture. Therefore, we have also addressed the mechanism behind this resistance and discovered that induction of resistance with race 1 protects Cavendish from TR4 up to 10 day after this treatment with race 1 (Chapter 7). Although, this is insufficient as a potential protection under field conditions the mechanism is intriguing and potentially can help to manage the disease better. Encouraging examples have recently been published where induced resistance was applied under field conditions in canola (Walters *et al.,* 2013). This suggests that field implementation of our findings might also contribute to improved disease control. Moreover, we identified expression of *RGA2* in inoculated plants, which separately has shown that its transformation to Cavendish rendered it resistant to TR4 in a lengthy field trial (Dale *et al.,* 2017a)

Screens for genes

Host resistance is the basis for sustainable disease management. Identifying it requires reliable and efficient spore production and inoculation protocols to systematically evaluate resistance and rapid plant selection. However, banana breeding experiences complicated biological facts (Bakry *et al.,* 2009). While wild types are diploid and contain seeds, cultivars are in general sterile triploids that produce fruits by parthenocarpy and are multiplied vegetatively (Simmonds and Shepherd 1955, Shepherd 1994, Crouch *et al.,* 1999). Plant breeders recognized the potential of useful sources of genetic variation in the centre of origin and tests to evaluate traits such as resistance have been carried out under greenhouse and field conditions (Amorim *et al.,* 2009). However, field screens for resistance in the *Fusarium* – banana pathosystem, despite reported efficacy (Li et al 2015, Hwang and Ko 1987), are time consuming, expensive and usually unreliable due to G x E interactions and unequal inoculum distribution (Subramaniam *et al.,* 2006, Amorim *et al.,* 2009, Sutanto *et al.,* 2013). Zuo *et al.* (2018) also conclude that disease resistance can be affected by multiple endogenous genetic and environmental factors, including plant genotype, nutrient application and temperature. Amorim *et al.* (2009) affirm that the rate of escape is still a problem in field nurseries as at least 10% of the susceptible inoculated plants remained unaffected. Similar inconsistences were observed by (Zuo *et al.,* 2018) when comparing susceptible accession in the greenhouse that remained unaffected in the field, but they also mention the required labor and space for field screens in banana. Recently, phenotyping automation and imaging have paved the way for high throughput studies (Cobb *et al.,* 2013), and semi-automated systems were successfully applied to investigate various components of plant growth and development that can be used to tackle basic resistance questions when combined with genetic mapping strategies (Famoso *et al.,* 2010, Cobb *et al.,* 2013). For the *Fusarium* - banana pathosystem, such automated platforms are not yet available, mainly due to the considerable size of banana plants, which hampers automation. Therefore, greenhouse screens under controlled conditions and following established protocols are necessary and have also been used successfully (Sun and Su 1984, Mohamed *et al.,* 2001, Smith *et al.,* 2008, Amorim *et al.,* 2009). Such greenhouse tests for banana are frequently considered as an initial step due to space limitations, required controlled growth conditions, the throughput of inoculations and the risk of contamination once multiple strains are being used (Amorim *et al.,* 2009, Li *et al.,* 2015, Zuo *et al.,* 2018). Indeed, there are critical factors that must be considered such as plant age, inoculum type and concentration and methods that vary from *in-vitro* evaluations in flasks (Wu *et al.,* 2010) to complex hydroponic systems (De Ascensao and Dubery 2000, Susan Groenewald *et al.,* 2006, Van den Berg *et al.,* 2007), and many different pot techniques and substrates (Matsumoto *et al.,* 1995, Subramaniam *et al.,* 2006, Weber *et al.,* 2007, Smith *et al.,* 2008, Amorim *et al.,* 2009, Li *et al.,* 2015, Zuo *et al.,* 2018). In conclusion, all aforementioned methods have proven their efficacy but share the drawback of extensive plant pre-treatment. Therefore, consensus on phenotyping in the *Fusarium* – banana pathosystem would enable comparison of data sets of different groups that now frequently frustrate exchange and require additional tests. For example, Zuo *et al.* (2018) report that AAA 'Inkira' (ITC0179) and 'Mbwazirume' are resistant while they were classified as moderately susceptible under our conditions. These differences remain unexplained, but we attribute them to differences of inoculation and evaluation protocols, plant origin, and possible somaclonal variation during multiplication. This underscores the need for international and agreed standards for phenotyping, including a trustworthy scale for scoring disease severity in the

Fusarium – banana pathosystem under natural (field) and controlled conditions (greenhouse), similar to standards that are already applied for decades in cereal phenotyping (James 1971).

For many years there has been controversy on the type of inoculum structures to use for banana bioassays. Most researchers use conidia (Sun 1978, Sun and Su 1984, Mohamed *et al.,* 2001, Smith *et al.,* 2008, Amorim *et al.,* 2009, Wu *et al.,* 2010, Wu *et al.,* 2013) in concentrations that differ from 1.10^3 to 1.10^7 conidia.ml^{-1}. Others have focused on the use of the persistent chlamydospores either alone or in combination with macro and microconidia (Amorim *et al.,* 2009). We used several inoculum methods and concentrations and identified correlations between dosage and either infection or latency period. We showed that the efficacy of the pouring method depends basically on the volume and inoculum concentration. Contrary to other *Fusarium* – host model systems, banana rhizomes are considerable larger than for instance tomato, *Arabidopsis* or melon and therefore higher inoculum concentrations are required. Chlamydospores are effective and a major source of inoculum in the field, but it is not easy to standardize their production in the laboratory. The commonest substrate to produce chlamydospores is often a soil/sand mixture and corn flour, but it also facilitates the development of micro and macroconida (Amorim *et al.,* 2009). Therefore, it is in many cases difficult to compare the efficacy of inoculum sources. Moreover, plant age is frequently a diversifying issue between many studies ranging from *in-vitro* to three-month old plants, which raises a question about susceptibility in unmatured plants (Purrington 2000, Smith *et al.,* 2008, Zuo *et al.,* 2018). Vakili (1965) showed the differences between plantlets and mature *M. balbisiana* plants and Zuo *et al.* (2018) assume that older plants have enhanced immune responses due to cell wall enforcement contributing to resistance. In Chapter 3, we provide a step-by-step protocol that enables the simultaneous evaluation of thousand plants and conclude that the selection of the method usually relies on the number of plants to be screened. Indeed, breeding programs require the evaluation of thousands of genotypes with diverse pathogen strains. Hence, our developed method excels over the aforementioned protocols. We inoculate banana plants at the 5–6 leaf stage with a healthy root system without minimum pre-treatment and simply add 200 ml of a spore suspension with a concentration of 1.10^6 spore.ml^{-1}. This method offers several advantages, including rapid disease onset, consistent development and clear classification of disease symptoms enabling categorization of plants in response classes - which often varies between groups - reproducibility and an overall shorter timeline than all other methods. Moreover, it is less laborious and enables the simultaneous inoculation of several *Fusarium* genotypes without risking cross contamination. In conclusion, our method meets the requirements of sound phenotyping, which includes simplicity, speed and flexibility but also sensitivity, precision and accuracy (see details in Chapters 4 and 5). However, much of the abovementioned diversity in protocols is actually due to a lack of genetics in the *Fusarium* - banana pathosystem. The used fungi are clonal and genetic studies in banana are virtually absent, thereby disabling time proof of correct phenotyping or appropriated genotyping. Hence, the proof of our methods lies in genetic studies to map and clone resistance genes (Ahmad *et al.,* in prep).

The quest for a new Cavendish

The Irish botanist Charles Telfair (1778 - 1833) would have never imagined that the Chinese bananas he collected in the early 1820's and distributed later would have such an impact on the banana history 200 years later. Those plants that were later named Cavendish in honour of the Devonshire's Duke were most likely Telfair's clones that he and others helped to distribute around the world (Reynolds 1927, Simmonds 1966, Price 1995, Koeppel 2008). At some point those Cavendish clones saved the banana industry after the dreadful impact of Panama disease on 'Gros Michel' in the mid-1950s (Ploetz 2005a, b). Cavendish clones - such as 'Dwarf Cavendish', 'Grand Naine, 'Valery', 'Lacatan', 'Williams' - where rapidly adopted due to their acceptable agronomical characteristics but particularly because of their resistance to race 1. Contrary to expectations the *Fusarium* spp. associated with race 1 have until now not circumvented the resistance of Cavendish clones, which have been grown successfully for decades in the very same soils that knocked down 'Gros Michel'. This is an exceptional example of durable resistance (Johnson 1984, McDonald and Linde 2002). Despite the exploitation of Cavendish cultivars for more than 70 years, little or nothing is known about the underlying genetic factors that protect Cavendish to race 1. Apart from very few experiments performed by Beckman and Halmos (1962) and some recent reports (Fernández-Falcón *et al.,* 2004, Li *et al.,* 2012), agreed that *Fusarium* rapidly colonizes during the first hours after contact and subsequent colonization ramifies tissue of susceptible cultivars such as 'Gros Michel', but in Cavendish physical barriers impede the progress of the disease. Some authors have identified race 1 strains in Cavendish tissues, which can be explained by endophytic behavior (See Chapter 6)**.** The success of Cavendish reduced traditional breeding efforts to develop new race 1 resistant varieties (see Chapter 5). Hence, poor understanding is a significant handicap in contemporary breeding efforts focusing on TR4. Rebuilding Cavendish is impossible, yet several breeding efforts try to generate look-alikes of this successful variety rather than developing new excelling germplasm (See Chapter 4; (Ploetz 2015). The latest embraced solution to TR4 is a characteristic "easy-fix", comprising so-called Giant Cavendish Tissue culture variants (GCTCVs) that are considered as promising TR4-resistant alternatives in Southeast Asia and elsewhere. Historically, tissue culture has been considered as a method of cloning a particular favorite genotype (Larkin and Scowcroft 1981) assuming that the generated plants are exact copies of the parental plant. Hence, phenotypic off-types were usually discarded and considered to result from unworthy epigenetic events. In recent years those events have been considered a rich and novel source of variation and have been amply and deployed in several crops such as sugar cane, potato, tobacco, rice, oats, barley and pelargonium (Larkin and Scowcroft 1981). Hence, tissue culture variants in banana, primarily developed at the Taiwan Banana Research Institute (Hwang and Ko 2004) fit this development and indeed, some of these variants have demonstrated to be less susceptible to TR4 and could be a temporary solution for the problem of individual farmers in the Philippines (Molina *et al.,* 2011). In this thesis somaclones from the TBRI, CIRAD and Rahan Meristems also showed reduced susceptibility and might be considered for further analysis in the field. In the case of the CGTCVs however, their agronomical performance has shown to be inferior compared to the commercial Cavendish cultivars (Ploetz 2015) and we expect similar behavior for other somaclones that were investigated in this research. Stability and field performance of these clones under TR4 infection pressure in various environments needs to be scrutinized as G x E interactions confirm that their performance is not stable. Moreover, the underlying factors causing

reduced susceptibility should be further addressed as chromosome breakage and fusion, various translocations, deletions and inversions have been observed in plants derived from tissue culture in other crops (Foroughi-Wehr and Mix 1979, Orton 1980). The almost reckless promotion and embracing of somaclones as the solution for TR4, however, is denying fundamental epidemiological facts indicating that reduced susceptibility only postpones the tipping point of banana decline, as was also experienced in Mozambique.

The receipt of somaclones however, surfs on the failure of classical breeding to develop and market new banana varieties that outcompete Cavendish clones. Therefore, non-conventional approaches such as *in-vitro* selection, mutation breeding (Matsumoto *et al.,* 1995, Bhagwat and Duncan 1998) genetic transformation (Paul *et al.,* 2011, Dale *et al.,* 2017a, Dale *et al.,* 2017b) and protoplast fusion (Matsumoto *et al.,* 2002) receive more attention, particularly since banana is a sterile crop. Sophisticated genetic techniques have been developed to incorporate useful genes into commercial varieties, (Larkin and Scowcroft 1981). Until recently, however, not a single gene for resistance to Panama disease was identified let alone transferred to Cavendish. The use of biotechnology as an integrated part of a breeding program emerged in the 1980's. Several groups have focused on mutagenesis and the selection of somaclonal variants for improved resistance to Panama disease and various other agronomical traits (Novak *et al.,* 1990, Vuylsteke *et al.,* 1991, Novak *et al.,* 1995, Walther *et al.,* 1996, Bhagwat and Duncan 1998, Brar and Jain 1998, van Harten 1998, Hwang and Ko 2004, Jain and Maluszynski 2004, Khayat *et al.,* 2004, Roux 2004, Kulkarni *et al.,* 2006, Bakry *et al.,* 2009). Yet, the diversity of resistance in triploid sterile bananas, including Cavendish types, is low as over 90% was susceptible to TR4, but all genotypes were resistant to race 1. The few accessions with resistance to TR4 included merely those obtained through alternative technologies. For example, Dwarf Cavendish (Smith *et al.,* 2006) was generated by gamma irradiation and was previously reported as resistant to the so-called subtropical race 4, *Fusarium* spp. that infect Cavendish under abiotic stress. Indeed, over the last 15 years genetic technologies have significantly advanced, with contemporary CRISPR-Cas9 promises (Bortesi and Fischer 2015) that were once at the horizon and now a reality, even in humans (Le Page 2018). In this thesis we identified at least two transgenic lines with resistance to TR4 (see Chapters 4 and 8). Genetic modification by addition of non-host genes, such as the apoptosis related *Ced9* gene from *Caenorhabditis elegans* have provided resistance to TR4 in Cavendish bananas under greenhouse conditions. Similar results were obtained for other cultivars tested with other *Fusarium* spp. (Vishnevetsky *et al.,* 2011, Kovács *et al.,* 2013, Rustagi *et al.,* 2015). However, none of these studies meet the results of Dale *et al.* (2017a) who showed stable resistance to TR4, originating from the wild progenitor of edible banana *M. acuminata* spp*. malaccensis* to Cavendish in multiyear field trails. Clearly, legislation revolving around genetically modified crops and societal responses are crucial for their acceptance, apart from the fact that these techniques repair problems rather than contribute to overall sought-after genetic diversity to quit risky monoculture agriculture.

Exploring alternatives: Musa diversity and the potential for breeding

Bananas originated in Southeast Asia as wild seminiferous plants. Southeast Asia still holds a large diversity due to constant natural crosses and human selection (Lescot 2017). Banana domestication started most likely 7,000 years ago during a process that involved hybridizations between diverse species and subgroups promoted by human migration (Perrier *et al.,* 2011, d'Hont *et al.,* 2012). The process of domestication of edible bananas (sweet and cooking) is non-linear but has four independent stages that involved selection of parthenocarpic clones, selection for gametic sterility, with the subsequent selection of triploid plants for a final enhancement of phenotypic diversity via vegetative propagation (Simmonds and Shepherd 1955, Bakry *et al.,* 2009, Bakry and Horry 2014, Kema and Drenth 2018). The genus *Musa* is divided into seminiferous species with edible and inedible fruits and parthenocarpic varieties with fleshy seedless fruits. The Eumusa section to which most bananas belong, includes the species *Musa acuminata* (A genome) and *M. balbisiana* (Genome B), both of which contributed to the origin of modern cultivated varieties. Currently, more than 1,200 varieties, including natural landraces as well as breeding products (see Chapters 4 and 5), have been classified. Global banana production relies on some of these clones (Lescot 2004, Perrier *et al.,* 2011). Likewise, 180 wild accessions (see Chapter 5) have been reported. This last group is very important as a reservoir of interesting traits, including different levels of resistance to pests and diseases (Li *et al.,* 2015). Another group comprises the diploid cultivars (290), which are grown in different places but especially in Southeast Asia, where they originated (Lescot 2017). The triploid group (AAA, AAB and ABB) with at least 650 cultivars includes dessert and cooking varieties. Their genetic diversity is essentially due to mutations and many centuries of selection. "Cavendish" for example comprises at least a suite of 40 cultivars distributed around the world. We observed substantial differences in levels of resistance between and among these groups despite their limited genetic diversity (Bakry *et al.,* 2009).

Diversity, in the form of crop genetic diversity, polycultures and landscape heterogeneity, each at various temporal scales, has been effectively used to control the spread and damage caused by pests and diseases in agroecosystems (Hajjar and Hodgkin 2007). The reason why Panama disease ravaged 'Gros Michel' and hit the industry was the absence of host resistance and diversity (Marquardt 2001). Even though Cavendish rescued the industry, increasing genetic diversity is an important factor in crop production. There are at least twenty-five varieties of water melon and more than 100 apple varieties that are commercialized and found in supermarkets (Elzebroek and Wind 2008). For bananas only one or a few cultivars are commercially available, particularly in Western markets. There is a need to abandon fragility and broaden the availability of edible bananas. At the start of this research program, the response of the global *Musa* collection to TR4 was unknown. Therefore, we selected a shortlist of cultivars and breeding lines and evaluated them for their response to TR4 and race 1, which may provide new opportunities for the banana market. Similar to the controls - Cavendish inoculated with race 1 - plants categorized with score 1 showed no disease symptoms and no fungal growth, confirmed by negative molecular of TR4 in corms. Similar to previous research (Li *et al.,* 2015, Zhang *et al.,* 2018, Zuo *et al.,* 2018), we identified several diploids including cultivated as well as wild types, with resistance to TR4 as well as race 1. In all these cases responses were immune, once considered as impossible for the *Fusarium* - banana pathosystem (Pegg and Langdon 1987, Smith *et al.,* 1999) and more recently by Zuo *et al.* (2018), despite the

durable resistance of Cavendish clones to race 1 strains in practice as the cornerstone of the contemporary banana industry, which is based on that very fact.

We also decided to evaluate other important groups of the *Musa* diversity, including the East African Highland Bananas (EAHB), which are used for cooking and brewing. We show that at least four of the Mutika landraces were resistant to TR4 and two were moderately susceptible, which is in accord with previous studies. For example, Zuo *et al,* (2018) reported that 'Ingagara' (ITC0166), 'Igitsire (intuntu)' (ITC0081) and 'Kazirakwe' (ITC1355) are resistant to TR4. These limited data should be expanded by rigorous evaluations of African germplasm as TR4 landed in Mozambique and the chance for further dissemination to other areas is immense. Overall, we proved that the resistance to TR4 or race 1 is not correlated with genome composition. For instance, we found AAA genotypes with resistance to TR4 despite the fact that it is commonly believed that these are susceptible to TR4 (Chapters 4 and 5). Our data provide opportunities for long-term efforts to diversify the crop as well as short-term solutions as many of the resistant genotypes are actually traditional landraces that produce edible bananas, particularly for EAHBs and plantains, which are important staple foods in Africa and Latin America, respectively.

New and unknown risks

TR4 will undoubtedly continue its dangerous expansion. As mentioned before, during the last 10 years the number of new TR4 reports passed from six to 17 new locations. Even though the current focus is on TR4, the pathogenicity of the other *Fusarium* spp. and genotypes is uncertain and has not been deeply explored. Most field and greenhouse tests have focused on a few genotypes that underrepresent the overall fungal genetic diversity (Groenewald *et al.,* 2006, Li *et al.,* 2013, Araújo *et al.,* 2017). This likely results from the fact that other genotypes have barely been associated with Cavendish production. Only Thangavelu and Mustaffa (2010) and Magdama and Jimenez-Gasco (2015) have shown that other *Fusarium* spp. or genetic lineages were associated with Panama disease in Cavendish. Since *Fusarium* spp. associated with Panama disease are quarantine organisms in all banana-growing areas, verification of these observations or claims is complicated or impossible due to the lack of access to hosts plants or fungal cultures. Hence, exchange of materials and sound identification – which calls for extended molecular diagnostics for other *Fusarium* spp. and genotypes causing Panama disease in banana – is crucial for progress. Our program facilitated exchange as bananas are not grown in The Netherlands and the *Fusarium* spp. causing Panama disease are not subject to the require quarantine procedures. Along with the current work, this enabled developing important data bases (Maryani *et al.,* 2018, Ordoñez *et al.,* 2018) that can be shared in joint efforts to manage Panama disease in banana, but also demonstrated that the current race concept, which is older than 50 years (Stover and Waite 1960), needs revision and modernization to enable high-throughput phenotyping based on the crucial proteins involved in pathogenesis (Vleeshouwers and Oliver 2014). We phenotyped 'Gros Michel' and 'Grand Naine' with 22 genotypes of nine *Fusarium* spp. causing Panama disease. Based on the *Fusarium* phylogeny it was suggested that clade 1 strains are associated with *Fusarium* spp. causing disease in Cavendish cultivars, while clade 2 contains species causing disease in 'Gros Michel' (Bentley 1998, Boehm 1994, Fourie 2009, Groenewald 2006, Koening 1997). However, we did not observe such a phenomenon under our conditions as there was no big difference between these clades

on these cultivars, which might confirm that the pathogenicity on specific cultivars has a polyphyletic origin (Fourie *et al.,* 2009, Fraser Smith and Aitken 2011, Fraser-Smith *et al.,* 2014).

Taken together, this thesis contributes to a further understanding of the *Fusarium* – banana pathosystem and has delivered important facts, methods and genes that can be deployed towards a more sustainable Panama production.

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Summary

In this thesis the problem of Panama disease in banana is investigated, primarily focusing on the developing pandemic, the necessity to discover genetic diversity for resistance and its potential for sustainable disease management. In ***Chapter 1*** the subject is introduced by describing the origin of the crop and its historical track record towards an important food crop for millions of people in developing economies and Western markets. The threat of Panama disease or Fusarium wilt is explained in a historical context, thereby asking the question why so little has been learnt from the previous epidemic? Details of the host taxonomy and the *Fusarium* species that cause Panama disease are described and the potential of plant breeding to sustainably manage the problem are discussed. The chapter ends with the scope of the thesis and a short overview of the subsequent chapters. ***Chapter 2*** is a typical example of a repetitive history: how the first Panama disease epidemic was quenched, by cultivating Cavendish bananas, which currently facilitates a new pandemic. It describes the importance of the crop for millions of people as a global commodity as well as for regional markets both as fresh fruits and as a staple food. Since the global trade revolves around Cavendish clones, these susceptible varieties are a vehicle for the dissemination of the deadly *Fusarium odoratissimum* Tropical Race 4 (TR4) that also affects numerous local varieties. To date, this pathogen has already spread across Southeast Asia, the Middle East and the Indian subcontinent and also expanded into Africa. The chapter ends with an overview of the latest reports on the dissemination of TR4 and describes a combination of classical morphology, molecular techniques to diagnose new isolates and pathogenicity tests under greenhouse conditions. Finally, the data not only alerted the global banana community on the ongoing and upcoming threat of TR4, but DNA analyses also underscored the likely origin of new incursions, which adds another layer of responsibilities of stakeholders. ***Chapter 3*** describes the development of a rapid and efficient spore production protocol, which facilitated a new high throughput phenotyping assay. This is important to standardize methodological procedures that contribute to comparative data sets that are generated under greenhouse conditions. Contrary to previous protocols, the new method is efficient and enables inoculating 250 plants by one person per hour. Interestingly, the new method not only maximizes spore production of *Fusarium* spp. affecting banana, but also of other *Fusarium* species that infect other crops and non-pathogenic biocontrol species. The assays resulted in typical symptoms within 10 weeks after inoculation and significant differences in final disease ratings were observed, depending on the applied inoculum concentration. Eventually, pouring inoculum directly onto the potting soil of banana plants showed the most consistent and reproducible results, as expressed in external wilting, internal discoloration and determined by real-time PCR assays on entire rhizomes. This method allows the phenotyping of large mutant and breeding populations. In ***Chapter 4*** the focus is on phenotyping a large number of banana accessions with TR4 as well as a smaller number of accessions with a race 1 strain. In total, we screened 120 triploid banana accessions, representing AAA, AAB and ABB clones comprising representatives of all major subgroups. We defined a disease index <25% as an adequate level of resistance, thereby adopting the response of Cavendish bananas to race 1 as a reference score because these are successfully grown around the world in heavily infested soils. In conclusion, TR4 resistance showed to be independent of the genomic composition or taxonomical position of the tested germplasm. Cavendish somaclones showed quantitative variation for resistance to TR4, but never scored better than

moderately susceptible. We did not identify any resistance in the AAB sweet acid bananas, comprising Pome, Silk and Mysore subgroups as well as Bluggoe accessions. In total 17 (14%) accessions showed the required level of TR4 resistance and 26 out of the 36 accessions (70%) showed appropriate resistance to race 1. These data are a foundation for advanced genetic analyses of the identified sources of resistance and their potential deployment in breeding programs that should deliver new competitive banana varieties that meet consumer demands at domestic and international markets. ***Chapter 5*** continues with the description of a similar screening of 122 diploid accessions, including 25 wild types, 50 cultivars and 49 breeding lines for resistance to TR4 as well as to race 1. In this group, 28 showed adequate levels of resistance to TR4. The results represent new short-term opportunities of hybrids and landraces as well as potential new sources of resistance for both TR4 and race 1. These genotypes can be incorporated into conventional and nonconventional breeding programs in order to develop new cultivars that can contribute to manage Panama disease. ***Chapter 6*** continues with phenotyping, but now we screened the present genetic diversity of *Fusarium* spp. affecting banana on the cultivars 'Grand Naine' and 'Gros Michel'. Albeit that *Fusarium* spp. are classified into physiological races, designations of such races are often solely based on the origin from which a particular strain was sampled, without phenotyping for pathogenicity on other cultivars. In this chapter, the pathogenicity of 22 known genotypes of nine *Fusarium* spp. was explored under greenhouse conditions. Additionally, we also evaluated the genetic diversity of pathogenicity towards 'Grand Naine' in a suite of diverse TR4 strains originating from diverse locations. We observed that genetically diverse *Fusarium* spp. caused distinct pathogenic responses on 'Gros Michel' and 'Grand Naine' cultivars. 'Gros Michel' was generally more susceptible to various species than 'Grand Naine'. All TR4 isolates, regardless of the year of isolation and country of origin, were highly infectious on both 'Gros Michel' and 'Grand Naine', underpinning the risk for disease outbreaks in banana plantations that are planted to these cultivars. The plant responses of both cultivars were indistinctively associated with *Fusarium* spp. from clade 1 and 2 of the *Fusarium oxysporum* species complex (FOSC) and did not frequently correspond to their related races. These findings show that the current race concept does not accurately reflect the virulence of the diverse *Fusarium* spp. on bananas. In ***Chapter 7*** a series of challenge experiments is described to investigate possible induced resistance mechanisms. We studied the possibility to induce protection to TR4 by co-inoculating or pre-inoculating with nonpathogenic or incompatible strains of *Fusarium*. We found that treatment with race 1 is able to protect Cavendish 'Grand Nain' plants from infection by TR4 up to 10 days after the initial inoculation. None of the other *Fusarium* species tested in this study was able to induce this response. We also isolated RNA from infected corms and sequenced it and found that unique transcripts were identified. Defense related genes such as a chitinase, thaumatin and peroxidases were clearly induced in the race 1 and race 1+TR4 and TR4 treatments. This suggests that systematically induced resistance is the most probable mechanism involved in cross protection in the *Fusarium* - banana pathosystem. The data also suggest that the ethylene, jasmonic acid and gibberellin synthesis pathways might be crucial for protecting cv. Grand Naine plants from TR4 upon race 1 induced resistance. Finally, we observed that the expression of resistance gene analogue RGA2, was exclusively observed in plants with resistance to TR4. RGA2 is a non-TIR-NBS-LRR plant disease resistance protein involved in resistance to TR4 in several banana accessions, such as *Musa acuminata* spp. *malaccensis.* We observed upregulation of RGA2 like genes in all treatments. Therefore, RGA2 seems also to be involved in the resistance to race 1. However, it is crucial for

resistance to TR4, which was proven by transferring the gene from *M. acuminata* spp. *malaccensis* to Cavendish (see chapter 8). In ***Chapter 8*** we describe the cloning of the aforementioned RGA2 gene from the wild diploid banana *M. acuminata* spp. *malaccensis* and transfer to the Cavendish 'Williams' through *Agrobacterium tumefaciens* mediated transformation. This provided adequate levels of resistance to TR4 in a three-year field trial. Other plants were genetically modified to carry a nematode-derived gene, *Ced9*, and also remained disease free for the entire period. Transgene expression in the RGA2 lines is strongly correlated with resistance. Endogenous RGA2 homologs are also present in Cavendish but are expressed tenfold lower than in the most resistant transgenic line. The expression of these homologs can potentially be elevated through gene editing, thereby providing non-transgenic resistance to TR4. Finally, ***Chapter 9*** is an overarching general discussion that places all the generated data in a broader context and concludes that there is plenty genetic diversity that can be used to diversify banana production. Breeding programs have to be developed that capture the genetic diversity and translate it to commercially viable and diverse new varieties that meet producer and consumer demands and claims that the times of monoculture are over for the welfare of producers and their families.

Samenvatting

In dit proefschrift wordt het onderzoek naar het probleem van panamaziekte in banaan beschreven, met een focus op de zich ontwikkelende pandemie en de noodzaak om genetische diversiteit van resistentie te ontdekken vanwege het potentieel om bij te dragen aan een duurzame ziektebeheersing. In ***Hoofdstuk 1*** wordt het onderwerp geïntroduceerd met een beschrijving van de oorsprong van het gewas en de historische lijn naar een belangrijk voedingsgewas voor miljoenen mensen in ontwikkelende economieën en westerse markten. De bedreiging van panamaziekte, of Fusarium verwelkingsziekte, wordt uitgelegd in een historisch context, waarbij de vraag wordt gesteld waarom er zo weinig is geleerd van de vorige epidemie. Details van de taxonomie van de waardplant, de *Fusarium* soorten die panamaziekte veroorzaken worden beschreven en de potentie van plantenveredeling om het probleem duurzaam aan te pakken worden besproken. Het hoofdstuk wordt afgesloten met een overzicht van het proefschrift en een kort overzicht van de volgende hoofdstukken. ***Hoofdstuk 2*** is een typisch voorbeeld van een zich herhalende geschiedenis: hoe de eerste panamaziekte epidemie uitdoofde door de teelt van Cavendish, nu echter de ontwikkeling van een pandemie faciliteert. Het beschrijft het belang van het gewas als handelswaar –als fruit en basisvoedsel - voor miljoenen mensen en internationale en regionale markten. Omdat de wereldwijde handel zich concentreert op Cavendish klonen vormen deze vatbare rassen een vehikel voor de verspreiding van het dodelijke tropische fysio 4 (TR4) van *Fusarium odoratissimum,* die ook vele lokale rassen aantast. Momenteel is deze stam wijdverspreid in heel zuidoost Azië en heeft hij zich ook uitgebreid naar het Midden-Oosten, het Indisch subcontinent en Afrika. Het hoofdstuk eindigt met een overzicht van de laatste rapporten over de verspreiding van TR4 en beschrijft een combinatie van klassieke morfologische studies, moleculaire technieken en pathogeniteitstoetsen in de kas om nieuwe isolaten van de stam te identificeren. Tenslotte hebben deze data niet alleen de wereldwijde bananensector gealarmeerd ten aanzien van de zich ontwikkelende en opkomende bedreiging door TR4, maar onderstrepen DNA-analysen ook de herkomst van nieuwe isolaten. Dit legt een nieuwe verantwoordelijkheid bij de sector. ***Hoofdstuk 3*** beschrijft de ontwikkeling van een protocol om snel en efficiënt sporen van de schimmel te produceren. Dit heeft de ontwikkeling van een fenotyperingsmethode met een grote doorstroomsnelheid gefaciliteerd. Dit is belangrijk om gebruikte methoden te standaardiseren waardoor in de kas verkregen datasets vergeleken kunnen worden. In tegenstelling tot protocollen die eerder werden gebruikt is het met de nieuwe methode mogelijk om per persoon 250 planten per uur te inoculeren. Naast de *Fusarium* soorten die banaan aantasten is het met de nieuwe methode ook mogelijk de sporenproductie te maximaliseren van *Fusarium* soorten die andere gewassen aantasten of die als biologische bestrijders worden gebruikt. De toetsen resulteerden binnen 10 weken na inoculatie in de typische panamaziektesymptomen waardoor significante verschillen in de uiteindelijke waarnemingen werden vastgesteld, mede afhankelijk van de gebruikte sporenconcentraties. Tenslotte, blijkt het gieten van inoculum op de grond van opgepotte bananenplanten de consistentste en herhaalbare resultaten op te leveren bij het bepalen van externe verwelking, interne verkleuring en kwantitatieve PCR van de rizomen. Deze methode is daarom geschikt om grote mutanten- en veredelingspopulaties te fenotyperen. In ***Hoofdstuk 4*** ligt de focus op het fenotyperen van een groot aantal bananensoorten met TR4 en een kleiner aantal met fysio 1. In totaal werden 120 triploïde bananensoorten onderzocht die AAA, AAB en ABB klonen uit alle taxonomische geledingen vertegenwoordigden. Wij definieerden een ziekte index van

<25% als een adequaat niveau van resistentie, waarbij we de reactie van Cavendish op fysio 1 als referentie gebruikten omdat deze bananenrassen over de hele wereld succesvol worden geteeld op zwaar besmette gronden. Concluderend hebben wij vastgesteld dat resistentie tegen TR4 voorkomt in elke taxonomische groep. Cavendish mutanten -zogenaamde somaclones – vertoonden kwantitatieve variatie voor resistentie tegen TR4 maar waren altijd minstens matig vatbaar. Wij hebben geen enkele AAB zoetzure banaan - bekend als Pome, Silk en Mysore – of Bluggoe accessie geïdentificeerd met resistentie tegen TR4. In totaal waren er 17 (14%) soorten met het benodigde niveau van resistentie tegen TR4 en 26 van de 36 soorten (70%) hadden een vereist niveau van resistentie tegen fysio 1. Deze data zijn het fundament voor verder genetisch onderzoek van deze geïdentificeerde bronnen van resistentie en hun potentieel gebruik in veredelingsprogramma's die uiteindelijk nieuwe competitieve rassen moeten opleveren die tegemoetkomen aan de wensen van consumenten in regionale en internationale markten. ***Hoofdstuk 5*** continueert deze aanpak met de beschrijving van de fenotypering van 122 diploïde soorten - inclusief 25 wilde soorten, 50 cultivars en 49 veredelingslijnen – voor resistentie tegen TR4 en fysio 1. In deze groep waren er 28 soorten die een vereist niveau van resistentie tegen TR4 hadden. De resultaten vertegenwoordigen nieuwe korte-termijn mogelijkheden voor de teelt van hybriden en landrassen evenals hun gebruik als potentiele nieuwe bronnen van resistentie tegen zowel TR4 als fysio 1. Ze kunnen worden gebruikt in conventionele en vernieuwende veredelingsprogramma's die gericht zijn op het ontwikkelen van nieuwe rassen die kunnen bijdragen aan de beheersing van panamaziekte. ***Hoofdstuk 6*** gaat verder met de beschrijving van de fenotypering van de bekende bananenrassen Grand Naine en Gros Michel met de beschikbare genetische diversiteit van *Fusarium* schimmels die panamaziekte veroorzaken. Ofschoon *Fusarium* soorten worden geclassificeerd in fysiologische rassen (fysio's), zijn de beschrijvingen van deze fysio's meestal slechts gebaseerd op de herkomst van de bananananensoort vanwaar een dergelijke stam werd geïsoleerd, zonder enige vorm van fenotypering op andere rassen. In dit hoofdstuk is de pathogeniteit van 22 genotypen van negen verschillende *Fusarium* soorten getoetst onder kasomstandigheden. Daarnaast hebben we ook de genetische diversiteit van een diverse serie TR4 isolaten van verschillende herkomst op Grand Naine onderzocht. We hebben waargenomen dat genetisch diverse *Fusarium* soorten duidelijke symptomen veroorzaakten in beide rassen. Gros Michel werd in het algemeen door meerdere soorten aangetast en was ook vatbaarder dan Grand Naine. Alle TR4 isolaten, onafhankelijk van hun isolatiejaar of land van herkomst, waren zeer infectieus op beide rassen, daarmee het risico onderstrepend van plantages waar alleen deze rassen worden verbouwd. De reactie van deze rassen was zonder uitzondering verbonden met *Fusarium* soorten uit de groepen 1 en 2 van het *Fusarium oxysporum* soorten complex (FOSC) en correspondeerde regelmatig niet met de veronderstelde panamaziekte fysio's. Deze bevindingen laten zien dat het huidige fysio concept van *Fusarium* soorten die panamaziekte veroorzaken in banaan niet voldoet. In ***Hoofdstuk 7*** wordt een serie experimenten beschreven waarmee het bestaan van geïnduceerde resistentie tegen TR4 wordt onderzocht. We bestuurden dit door behandelingen met niet compatibele of niet pathogene *Fusarium* soorten die voorafgaand of tegelijkertijd met TR4 werden toegepast en hebben vastgesteld dat fysio 1 in staat is om Grand Naine wel tot 10 dagen na behandeling te beschermen tegen TR4. Geen een van de andere *Fusarium* soorten in dit onderzoek bleek in staat tot een dergelijke response. We hebben vervolgens uit de geïnfecteerde stambases RNA geïsoleerd en gesequenced waardoor wij unieke genen identificeerden. Genen die betrokken zijn bij de afweer, zoals chitinasen, thaumatine en peroxidasen, werden

duidelijk geïnduceerd door behandelingen met fysio 1, TR4 en de fysio 1 + TR4 combinatie. Dit suggereert dat systemisch geïnduceerde resistentie het meest voor de hand ligt als mechanisme voor de kruisbescherming in het *Fusarium* – banaan pathosysteem. De data suggereren ook dat de ethyleen-, jasmonzuur- en gibberelline synthese routes worden aangeschakeld na behandeling met fysio 1 en mogelijk cruciaal zijn om Grand Naine te beschermen tegen TR4. Tenslotte, hebben we ook vastgesteld dat de expressie van resistentiegenanalogen (RGAs) exclusief was voor soorten met resistentie tegen TR4. RGA2 is een non-TIR-NBS-LRR resistentie-eiwit tegen plantenziekten dat in diverse bananensoorten, zoals *Musa acuminata* spp. *malaccensis*, betrokken is bij de resistentie tegen TR4. We hebben waargenomen dat de expressie van RGA2 in alle behandelingen toeneemt en daarom lijkt RGA2 ook betrokken te zijn bij de resistentie tegen fysio 1. Echter, het is cruciaal voor de resistentie tegen TR4 wat bleek uit experimenten waarin het gen uit *M. acuminata* spp. *malaccensis* in Cavendish werd gezet (zie ook hoofdstuk 8). In ***Hoofdstuk 8*** wordt beschrijven hoe het hiervoor genoemde RGA2 gen werd geïsoleerd uit de wilde diploïde bananensoort *M. acuminata* spp. *malaccensis* en in het Cavendish ras "Williams" werd gezet door middel van een op *Agrobacterium tumefaciens* gebaseerd transformatie protocol. Dit leverde adequate resistentieniveaus op tegen TR4 in een driejarige veldproef. Andere planten werden getransformeerd met een gen uit een aaltje, het *Ced9* gen, en vertoonden ook een vergelijkbare resistentie gedurende dit veldexperiment. De expressie van het RGA2 gen is sterk gecorreleerd met resistentie tegen TR4. Endogene RGA2 homologen zijn ook aanwezig in Cavendish, maar hun expressie is tien keer lager dan in de transgene lijnen. De expressie van deze homologen kan mogelijk worden verhoog door middel van genoom editing. Dit kan niet-transgene resistentie tegen TR4 opleveren. Tenslotte biedt ***Hoofdstuk 9*** een overkoepelende algemene discussie die alle data in een bredere context plaatst en concludeert dat er voldoende genetische diversiteit beschikbaar is die gebruikt kan worden om de bananenproductie te diversifiëren. Veredelingsprogramma's moeten worden ontwikkeld die deze genetische diversiteit aangrijpen en vertalen naar commercieel levensvatbare nieuwe rassen die voldoen aan de wensen van de producent en de consument. Hiermee claimt het proefschrift dat de tijden van de grote monoculturen voorbij zijn ten bate van de producenten en hun families.

Resumen

En esta tesis se aborda el problema de la enfermedad del Mal de Panamá en banano también conocida como marchitamiento por Fusarium. La tesis se enfoca principalmente en el estudio desarrollo de esta pandemia y la necesidad de explorar la diversidad genética para la resistencia y su potencial para el manejo sostenible. En el ***Capítulo 1***, se describe el origen y el desarrollo histórico un cultivo vital para millones de personas en economías en desarrollo y mercados occidentales. La amenaza de la enfermedad del mal Panamá se explica en un contexto histórico, haciendo la pregunta ¿por qué se ha aprendido tan poco de la pandemia anterior? Se describen los detalles de la taxonomía del huésped y la especie *Fusarium*, el agente causal de la enfermedad y se analiza el potencial del fitomejoramiento para solucionar el problema de manera sostenible. El capítulo finaliza con el alcance de la tesis y una breve descripción de los capítulos siguientes. El ***Capítulo 2*** es un ejemplo típico de una historia repetitiva: se discute cómo la primera pandemia de la enfermedad del Mal de Panamá fue controlada de una manera que facilitó la nueva pandemia. Describe la importancia del cultivo para millones de personas como un producto global, así como para los mercados regionales de fruta fresca y alimento básico. Dado que el comercio global gira en torno a los clones Cavendish, estas variedades susceptibles son un vehículo para la diseminación de la agresiva raza 4 tropical – *Fusarium odoratissimum* (R4T) que también afecta a numerosas variedades locales. Hasta la fecha, este patógeno ya se ha extendido por el sudeste asiático, el Medio Oriente, el subcontinente de la India y también se ha expandido a África. El capítulo finaliza con una descripción general de los últimos reportes sobre la diseminación de R4T y describe el uso de una combinación de morfología clásica y técnicas moleculares para diagnosticar nuevos aislados y pruebas de patogenicidad en bananos tipo Cavendish en condiciones de invernadero. Los datos obtenidos no solo alertaron a las partes interesadas sobre la amenaza de R4T, sino que los análisis de ADN también resaltaron el posible origen de nuevas incursiones. El ***Capítulo 3*** describe el desarrollo paso a paso de un nuevo, rápido y efectivo protocolo para el fenotipado de germoplasma de banano, lo cual contribuye a la estandarización de los procedimientos metodológicos que contribuyen al análisis de datos comparativos que se generan en condiciones de invernadero. A diferencia de los protocolos anteriores, el nuevo método es altamente eficiente y permite inocular más de 250 plantas/persona/hora. Convenientemente, los nuevos métodos no solo maximizan la producción de esporas de las especies de *Fusarium* spp. que afectan al banano, sino también a otras especies de *Fusarium* que infectan otros cultivos y algunas especies no patogénicas que son comúnmente utilizadas como agentes biocontroladores. Los ensayos dieron como resultado síntomas típicos dentro de las 10 semanas posteriores a la inoculación y se observaron diferencias significativas en las calificaciones finales de la enfermedad, según la concentración de inóculo aplicada. Finalmente, el método propuesto en esta tesis mostró resultados consistentes y reproducibles, expresados en marchitamiento externo, decoloración interna y determinados mediante ensayos de PCR en tiempo real en rizomas completos. Este método permite el fenotipado de grandes poblaciones de mutantes y variedades mejoradas. En el ***Capítulo 4*** la atención se centra en el fenotipado de un gran número de accesiones de banano con R4T, así como un número menor de accesiones con la raza 1. En total, se seleccionaron 121 accesiones de banano triploides, que representan los clones AAA, AAB y ABB que comprenden representantes de todos los subgrupos. Definimos un índice de enfermedad <25% como un nivel adecuado de resistencia, adoptando así la respuesta de los bananos Cavendish contra la raza 1 como limite de

referencia ya que Cavendish se cultivan con éxito en todo el mundo en suelos altamente infestados con esta raza. En conclusión, la resistencia de R4T demostró ser independiente de la composición genómica o posición taxonómica del germoplasma analizado. Los somaclones de Cavendish mostraron una variación cuantitativa de la resistencia a R4T, pero nunca obtuvieron mejores puntajes que moderadamente susceptibles. No identificamos ninguna resistencia en los bananos ácidos dulces AAB, que comprenden los subgrupos Pome, Silk y Mysore, así como las accesiones del grupo Bluggoe. En total, 17 (14%) accesiones mostraron los niveles requeridos de resistencia a R4T y 26 de las 37 accesiones (70%) mostraron resistencia adecuada a la raza 1. Estos datos son una base para los análisis genéticos avanzados de las fuentes de resistencia identificadas y su implementación potencial en programas de reproducción que deberían ofrecer nuevas variedades competitivas de banano que satisfagan las demandas de los consumidores en los mercados nacionales e internacionales. El ***Capítulo 5*** continúa con la descripción de un análisis similar de 122 accesiones diploides, que incluyen 25 tipos silvestres, 50 cultivares y 49 híbridos. Entre este grupo, 28 mostraron niveles adecuados de resistencia a R4T. Los resultados representan nuevas oportunidades a corto plazo de híbridos y variedades locales, así como nuevas fuentes potenciales de resistencia tanto para R4T como para la raza 1. Estos genotipos pueden incorporarse en programas de mejoramiento convencionales y no convencionales para desarrollar nuevos cultivares que puedan contribuir al manejo de la enfermedad. El ***Capítulo 6*** continúa con la fenotipificación, pero ahora analizamos la diversidad genética actual de *Fusarium* spp. que afectan al banano en los cultivares 'Grand Naine' y 'Gros Michel'. A pesar de que *Fusarium* spp. se clasifican en razas fisiológicas, las designaciones de tales razas a menudo se basan únicamente en el origen del cual se tomaron muestras de una cepa particular, sin fenotipado para patogenicidad en otros cultivares. En este capítulo, la patogenicidad de 22 *Fusarium* spp. Fue explorado en condiciones de invernadero. Además, también evaluamos la diversidad genética en la patogenicidad hacia ´Grand Naine' de un conjunto de diversas cepas de R4T que se originan en diversos lugares. Observamos que genéticamente diversas *Fusarium* spp. causaron distintas respuestas patógenas en los cultivares 'Gros Michel' y 'Grand Naine'. "Gros Michel" era generalmente más susceptible a varias especies que 'Grand Naine'. Todos los aislamientos de R4T, independientemente del año de aislamiento y el país de origen, fueron altamente infecciosos tanto en 'Gros Michel' como en 'Grand Naine', lo que apuntala el riesgo de brotes de enfermedades en las plantaciones de banano que se siembran en estos cultivares. Las respuestas de las plantas de ambos cultivares se asociaron indistintamente con *Fusarium* spp. del grupo (clade) 1 y 2 del complejo de especies de *Fusarium oxysporum* (FOSC) y no correspondían con frecuencia a sus razas relacionadas. Estos hallazgos muestran que el concepto de raza actual no refleja con precisión la virulencia de las diversas especies de Fusarium sobre los plátanos. En el ***Capítulo 7*** se describen una serie de experimentos de para investigar posibles mecanismos de resistencia inducida. Se estudió la posibilidad de inducir protección a R4T mediante co-inoculación o pre-inoculación con cepas no patógenas o incompatibles de *Fusarium* spp. Descubrimos que el tratamiento con la raza 1 puede proteger a las plantas Cavendish 'Grand Naine' de la infección por R4T hasta 10 días después de la inoculación inicial. Ninguna de las otras especies de Fusarium probadas en este estudio pudo inducir esta respuesta. RNA procedente de cormos infectados se utilizó para secuenciación vía RNASeq. Se encontraron transcriptos únicos sobre-regulados, incluyendo genes relacionados a mecanismos de defensa de la planta tales como quitinasas, taumatinas y peroxidasas, las cuales fueron claramente inducidas en los tratamientos con R1, R4T y R1

+ R4T. Esto sugiere que la resistencia sistémica inducida es el mecanismo mas probable en la protección cruzada en el patosistema *Fusarium* -banana. Adicionalmente se pudo concluir que después de 1 día, R1 indujo respuestas que redujeron la patogenicidad de R4T incluso hasta después de 10 días. Los resultados sugieren que las rutas de síntesis del ácido jasmónico/etileno, y giberelinas serían de crucial importancia para la protección de cultivares 'Grand Naine' del ataque de R4T después de la inducción generada por la R1. Finalmente observamos que la expresión de RGAs (Genes análogos de resistencia), particularmente RGA2 fue exclusivamente observado en plantas con resistencia a R4T. El RGA2 no es típico TIR-NBS-LRR pero esta asociado a la resistencia a R4T en varios genotipos de banano, incluyendo *Musa acuminata* spp*. malaccensis.* Pudimos observar que el RGA2 fue sobre expresado en todos los tratamientos, por este motivo, RGA2 lo que sugiere que podría estar involucrado en la resistencia inducida por la R1. Es importante anotar que se ha demostrado que la expresión de RGA2 induce resistencia a R4T, lo cual ya ha sido probado mediante la trasformación genética de Cavendish bananas utilizando dicho gen proveniente de la accesión silvestre *Musa acuminata spp. malaccensis* (ver capítulo 8). En el ***Capítulo 8***, El gen mencionado anteriormente se clonó y se transfirió a Cavendish 'Williams' a través de la transformación mediada por *Agrobacterium tumefaciens* que proporcionó niveles adecuados de resistencia a R4T en un ensayo de campo de tres años. Otras plantas se modificaron genéticamente con un gen derivado de nematodos, *Ced9*, y también se mantuvieron libres de enfermedad durante todo el período. La expresión del transgén en las líneas RGA2 está fuertemente correlacionada con la resistencia. Los homólogos de RGA2 endógenos también están presentes en Cavendish, pero se expresan diez veces por debajo que en la línea transgénica más resistente. La expresión de estos homólogos puede elevarse potencialmente a través de la edición de genes, para proporcionar resistencia no transgénica a R4T. Finalmente en el ***capítulo 9*** es una discusión general que pone todos los datos generados en un contexto amplio y concluye que existe una gran diversidad genética que se puede utilizar para diversificar la producción de bananos. Deben desarrollarse programas de mejoramiento que capturen la diversidad genética y la traduzcan a nuevas variedades comercialmente viables y diversas que satisfagan las demandas de los productores y consumidores quienes además afirman que los tiempos de los monocultivos han terminado para el bienestar de los productores y sus familias.

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I would also like to thank all my colleagues and fellow students in the Laboratory of Plant Breeding and Phytopathology. In particular Ali and Francine and especially my colleagues in the writing room with whom I spent the last months of my PhD at WUR. Thanks, Chara, Jinling, Malaika and Aranka. Thanks to my colleagues Sijun, Michael and Harold, for their contribution to the Mekong paper.

Of course, to colleagues and friends from the Wageningen Plant Research Biointeractions and Plant Health business unit whom I admire and respect a lot and with whom I shared unforgettable moments. The list is very long but I would like to thank especially Marga, Els V, Cees and Odette. You were crucial during the development of my thesis but also made my days at Bioint. Thanks, Odette, Marga and Els for your advice and help in the lab

but special thanks for listening and for great the great -non-scientific- conversations. Els thanks for always providing me what I needed to complete my experiments even when I asked for weird things like mung beans from Indonesia, kilos of popcorn, 12 knives and a machete. Cees, thanks for your strictness and support with the strains I used in my research work but also for the great time at Martina Franca, a fantastic place where I discovered the great human being you are.

I am enormously grateful to Mrs. Margriet Huisman - de Lange and Mrs. Marion Notermans. Without your experience and collaboration in the logistics of many fundamental aspects outside and within my scientific area my work would not have been possible. Margriet was like a 2nd mother when I came to the Netherlands and I will never forget that. She guided me and helped me so much during the first difficult months in the shivering cold Netherlands. Likewise, Marion, thanks for making my life easier and take responsibility for all those terrible tasks I didn't want to do. You never complained and were always there with your kindness and a beautiful smile. Thanks to Willem-Stol and Cor for the nice semi-Spanish conversations and for your nice words and encouragements. Dear Peter, Willem Jan, Carolien, Stefan, Jose, Petra, Jan, Trudy, Balázs, Bram, Anne, Helen, Yvonne, Marc, Ilse, Pieter, Marjon, Theo, Carin, Els N, Joeke, Mirjam, Jolanda, Rob, Gerrie, and Patricia thanks for your direct and indirect contribution to my PhD.

During my study time I was fortunate to share the office with great people from whom I learnt a lot, not only in the academic world but also about culture and life. Thanks, Amir Mirzadi, for building a great friendship. Thanks for the nice movie recommendations but also for giving me the pleasure of being your paranynph. Thanks, Lamia, Cauca, Einar and Pablo for your help and feedback and sincere friendship. Thanks to you Cauca for being essential and very helpful during the first period of adaption. Without you everything would have been more difficult. Thanks for bringing me to the Action to buy cheap but necessary stuff to survive my first winter and for giving me access to internet when my connection crashed during that terrible first month at Haarweg.

During my time at WUR, so many people passed by our office, thanks to all of them. It was really a pleasure to meet you and learn from you. Being at Bioint was one of the greatest experiences of my life. All the science would not have been possible without the students. Their help and interest have been a most important component of my thesis. These include the students that I supervised directly but also all the students that were supervised by my colleagues and were part of the group. Thanks for letting me be part of your team. Let me tell you that the learning process was reciprocal, and I will keep you in my heart forever. Thanks to all of you, Ruud, Yolande, Wu, Marlene, Giulio, Isabel, Kevin, Richard, Saiful, Joris, Hayet, Elina, Katialena, Iris and especially to Valerie, Denja, Sina, Lex, Blanquita, Nico, Sabine and Pauline. With you I have a beautiful friendship that remains after years. I would love to say something about each of you but that would take me pages and pages. Believe me, you are the most important and precious part!

During this journey, I met very important scientists, professors and professionals in all fields from whom I received valuable feedback and from some of you, life lessons. I would like to thank Richard Visser for helping me every time I asked for help, Paul Keizer for your valuable contribution with the statistical analysis in most of my chapters, Kerry O'Donnell, Miguel Dita, Nicolas Roux, Brian Irish, Andre Drenth, Bart Thomma, Martijn

Rep, Xavier Perrier, Christophe Jenny, Antonio DiPietro, Anker Sørensen, Hanneke Hermans, Rene Hofstede, and Gonny Van Hal. Thanks for the inspiration and feed-back on my work. It is gratifying to hear nice words from people like you.

Thousands and thousands thanks to Eli Kayhat whom I respect and admire infinitely, your humility, passion and scientific-business vision are admirable. Thanks for the unconditional support to our projects and my project in particular. For making me feel an important person every time we meet. Thank you for the trust you have placed in me and for entrusting me with important innovative projects of which I am proud. I hope we can share a scientific publication in the future, as has been our intention for a long time.

I cannot forget James Dale whom I admire and respect as top-of-the-world banana scientist along with Fred Bakry and Eli Khayat. Thank you for the trust placed in me. I am so happy that our joint work resulted in a major scientific article. I never forget the day that we met WUR and you commented on my results and how pleased you were. You cannot imagine how happy I felt. Receiving compliments from a scientist of your category is an important encouragement and was priceless for my motivation and confidence. Surely, we will see each other in the future and seek new collaborations.

I would like to also acknowledge Randy Ploetz, our reference for Panama disease! Thanks a lot for your feedback, for sharing your knowledge and also for your friendship and trust. It was a real pleasure to share my first banana paper with you but also sharing time together in all the INREF and other banana meetings and conferences. Due to your collaboration we were able to start the screening of the banana germplasm, as you put me in touch with Brian Irish at the USDA-ARS Puerto Rico who provided immediate access to the USDA banana collection. I also hope we will continue our collaboration and maybe share a “balut”.

I would like to thank the scientific and technical staff of Augura, Asbama, ICA and Corpoica in Colombia. Special thanks to Danilo Sáchez, Anuar Escaf, Jorge Vargas, Karen Cuello, Gabriel Elejalde, Yancy Martínez and Catalina Quintero. Thanks for your invaluable contributions, for trust in our work and for bringing us to Colombia to establish a strong collaboration that will for sure lead top research in the coming years.

Communication and audiovisuals have been part of my life. However, I stopped it for some time until I came to Wageningen, where my interest was triggered again and used the opportunities. I would like to thank Erik Toussaint and Pauline Poelarends at the communication department of WUR. Thanks for your enthusiasm and taking into account my ideas and for making me part of your team sometimes. Due to you I became more professional in science communication. I wholeheartedly thank Pauline Robert, the most creative student I ever had. You were like a mirror in which I saw myself when I was younger! Thanks for encouraging me to do more audio-visuals and for helping me to start again with my drawings. It was a difficult time for you, but we were a great team. Thanks also to Eva Meijer who was my student but also an inspiration to make better pictures and more artistic stuff. Thanks also for helping me with the cover of this book.

I would like to express my appreciation to Dan Koeppel, thanks for writing that fantastic book, thanks for the inspiration and thousand thanks for writing the preface of my thesis. It is an honour that you are part of it.

Thanks, Linda, Marijn and Helena for trusting me and taking the challenge to work with me in the Resource Magazine. Thanks for making another dream coming true. Thanks, Stephan Brusche AKA @iSteef, banana artist for embracing my ideas for the Science meets art event in Impulse at WUR. Your positive attitude is an inspiration for people to use social media in a creative way.

This work would not have been possible without the contribution of the Unifarm personnel. I am infinitely grateful to Andre, Bertus, Pauline, Sean, Bert, Aart, Erik and Marteen. Thank you for your cooperation and for following my ideas, crazy ideas sometimes, to achieve the results of me and my fellow students and colleagues.

Last but not least I thank my family and friends. My mother has been the engine of my life, and I am sure she is very proud and likely happier than anybody else at this moment. After the passing of my father when my brother and I were just infants, her only mission was to dedicate her life to us, and she did a good job. My mother is an admirable person who against all odds stayed by herself and provided (with difficulties) all necessities for us to grow personally and professionally. Thanks to my brother. You have been a constant support and anchor during my entire life. You are the best example of a good brother.

Very special thanks to my great paranynphs, I couldn't have chosen better. Both of you represent a lot in my life at WUR. Giuliana you were my student for almost a year, and we worked so hard with all those mutants from Rahan. Complete days and sometimes you had to do it alone although it was not your responsibility. You never complained and you did it just because that's how you are; a responsible and good friend. Thanks for being there in good and bad moments. Likewise, Iris without you the screening, the backbone of this thesis and such an important part of the entire banana program, would not have been possible. Your hard work, organization and tidiness are admirable. You also never complained, and you did it all with a fantastic smile. Thanks for your teamwork, help and also for being my friend and supporter of my "art expressions".

Thanks to all my new friends I made in the Netherlands. Especially to those that I got to know during the difficult first years as a student. Thanks to Thijs and his winter-jacket for saving my life during the winter as well as for traveling to get our traditional burger/ribs dinners. Ramon Bonet gracias tío por los incontables coffee breaks e interesantes conversaciones, likewise to all my international friends at WUR and Wageningen. Thanks to my roommates at Haarweg where I experienced what a student corridor is. It was a difficult time in the beginning, but you guys made it a lot easier. I thank especially Arthur, Klaas, David, Maddie, Lisa, Ismael, Thom, Ivar, Wouter, David, Mathias and Christoph. The days at Haarweg were amazingly good and the basis for discovering my way and stay in Wageningen city.

Thanks to my new colleagues at Keygene for the support and for being here!

Thanks to my girlfriend. Thanks to all my friends in Colombia, the Netherlands and globally through my social media.

About the Author

Fernando Alexander García-Bastidas was born on May 14th, 1982 in San Juan de Pasto, Colombia where he finished his primary and higher education. He then went for five years to the University of Nariño to obtain his BSc in agronomic engineering and completed a thesis on the identification of *Meloidogyne* nematodes in tree tomato (*Solanum betaceae*) and naranjilla (*S. quitoense*), two important local crops. After this, he started an internship in the department of plant physiology of the National Coffee Research Center (Cenicafe) in Manizales, Colombia. He was in charge of the project to determine the factors of degreening in Tangelo (*Citrus reticulate x Citrus paradise*) and tangerine (*Citrus reticulate*), a project that was funded by the fruit producers of the area. Before his graduation he was hired at Cenicafe and joined the breeding program from 2005 to 2012. He specialized in molecular breeding and developed the first steps of marker-assisted selection for resistance to the coffee leaf rust fungus *Hemileia vastatrix*. As agronomist, he was also in charge of the coffee experimental plantations in the field. From 2009 to 2012 while working at Cenicafe he started a MSc degree in Plant Biotechnology with a specialization in plant breeding at the National University of Colombia in Medellin. He was a Thesis Award laureate, the highest distinction for outstanding academic and research work, with his thesis entitled "Identification and characterization of molecular markers associated with *Coffea canephora* P. in advanced F_5 lines of *Coffea arabica*". During the same period, he was appointed as lecturer at the University of Santa Rosa, Pereira, Colombia. From 2013 to 2017 he continued his studies as PhD at Wageningen University and Research in the INREF program under the supervision of prof. Gert H.J. Kema. During this period, he was also involved in several additional research projects and supervised BSc and MSc students. In 2017 he was appointed as a postdoc and was in charge of a project on the evaluation and selection of promising banana mutants with (partial) resistance to Panama disease caused by Tropical Race 4. In 2018 he was appointed as postdoc at Keygene in a collaborative project with MusaRadix B.V. on establishing a new banana research and breeding program. Fernando has been very active in communication activities during his personal and professional life. At Cenicafe he was a photographer, filmmaker and cartoonist for colleagues and currently he produces science related vlogs for the Resource Magazine of Wageningen University and Research.

Scientific production

* Authors with same contribution

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The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Fernando Alexander García-Bastidas**
Date: **19 March 2019**
Group: **Plant Sciences Group**
University: **Wageningen university & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
The Genetic Basis of Resistance to Panama Disease in Banana	10 May 2013	1,5
► **Writing or rewriting a project proposal**		
The Genetic Basis of the Resistance to Panama Disease in Banana	12 Nov 2013	6,0
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		7,5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
EPS PhD Student Days "Get2Gether", Soest, NL	28-29 Jan 2016	0,6
8th European Plant Science Retreat, Barcelona, Spain	20-23 Jun 2016	1,2
► **EPS Theme Symposia**		
EPS theme 2 "Interactions between Plants and Biotic Agents", Amsterdam, NL	25 Feb 2014	0,3
EPS theme 4 "Genome Biology", Wageningen, NL	03 Dec 2014	0,3
EPS theme 2 "Interactions between Plants and Biotic Agents", Utrecht, NL	20 Feb 2015	0,3
EPS theme 2 "Interactions between Plants and Biotic Agents", Leiden, NL	22 Jan 2016	0,3
► **Lunteren days and other National Platforms**		
Annual Meeting "Experimental Plant Sciences ", Lunteren, NL	14-15 Apr 2014	0,6
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	13-14 Apr 2015	0,6
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	11-12 Apr 2016	0,6
29th Meeting of the Fusarium working group of the KNPV, Utrecht, NL	26 Oct 2014	0,3
30th Meeting of the Fusarium working group of the KNPV, Utrecht, NL	26 Oct 2015	0,3
31th Meeting of the Fusarium working group of the KNPV, Utrecht, NL	25 Oct 2016	0,3
32th Meeting of the Fusarium working group of the KNPV, Utrecht, NL	25 Oct 2017	0,3
► **Seminars (series), workshops and symposia**		
Seminar: Dr. André Drenth, Associate Professor at The University of Queensland	06 Jan 2013	0,1
Seminar: Prof. K. Saito, "Metabolomics based functional genomics-from Arabidopsis to crops and medicinal plants"	08 Apr 2013	0,1
Seminar: Dr. Miguel Dita, Bioversity International	16 Apr 2013	0,1
Seminar: Dr. Kerry O'Donnell, USDA	18 Apr 2013	0,1
EPS Flying Seminar: Dr. Howard S. Judelson, Department of Plant Pathology and Microbiology, University of California, USA	07 May 2013	0,1

	Seminar: Prof. Brian Staskawicz, "Effector-targeted breeding for durable disease control of Xantomonas diseases in Tomato and Cassava"	21 May 2013	0,1
	Seminar: "Plant metabolomics, applications in physiology and pathology, past, present and future", Dr. Will Alwood	13 Dec 2014	0,1
	EPS Flying Seminar: Dr. Gero Steinber, University of Exceter, United Kingdom	05 Jan 2015	0,1
	Banana Day Wageningen University, INREF project, Wageningen, NL	18 Nov 2014	0,3
	EPS symposium: "Omics Advances for Academia and Industry: Towards True Molecular Plant Breeding", Wageningen, NL	11 Dec 2014	0,3
►	**Seminar plus**		
	Seminar: Dr. André Drenth, Associate Professor at The University of Queensland (WUR)	06 Jan 2013	0,1
	Seminar: Dr. Miguel Dita, Bioversity International (WUR)	16 Apr 2013	0,1
	Seminar: Dr. Kerry O'Donnell, USDA	18 Apr 2013	0,1
	EPS Flying Seminar: Dr. Gero Steinberg, University of Exceter, United Kingdom	05 Jan 2015	0,1
►	**International symposia and congresses**		
	INREF Workshop 2: "Can the spread of Panama disease in banana be managed? Finding multi-level solutions for a global problem", Davao, Philippines	06-07 Feb 2014	0,6
	International meeting in Colombia " The Spread of Panama Disease in Banana: Past, Present and Future", Medellin, Colombia	16-17 Jun 2014	0,6
	V Reunión Técnica Bananera de Colombia (V Technical Banana Meeting of Colombia), Santa Marta, Colombia	13-14 Aug 2015	0,6
	I Coloquio Técnico Bananero (I Technical Banana Colloqium), Santa Marta, Colombia	19 Nov 2015	0,3
	INREF Workshop 4 Homestead Fl, USA	18 Apr 2016	0,3
	VI congreso internacional sobre banano CORBANA y XXI Reunión Internatcional ACORBAT (ACORBAT/Corbana international Banana Congress), Miami, USA	19-22 Apr 2016	1,2
	VI Reunión Técnica Bananera de Colombia (VI Technical Banana Meeting of Colombia), Santa Marta, Colombia	06-07 Oct 2016	0,6
	Asbama Banana Forum, Santa Marta, Colombia	29-30 Nov 2017	0,6
	International Congress of Plant Pathology (ICPP), Boston, USA	29 Jul-3 Aug 2018	1,8
►	**Presentations**		
	Talk: Panama disease in banana, INREF Workshop I, Ede, NL	23-25 Sep 2013	1,0
	Talk: International Stakeholder workshop Davao, Philippines	03-09 Feb 2014	1,0
	Talk: 28th Meeting of the Fusarium working group of the KNPV, Utrecht	30 Oct 2013	1,0
	Talk: EFS13/13th European Fusarium Seminar, Martina Franca, Italy	10-14 May 2015	1,0
	Talk: VI Reunión Técnica Bananera de Colombia	06-07 Oct 2016	1,0
	Talk: I Coloquio Técnico Bananero, Santa Marta, Colombia	19 Nov 2015	1,0
	Talk: Foro Bananero, Asbama, Colombia	24 Nov 2016	1,0
	Talk: Keynote speaker Asbama Banana Forum, Colombia	29 Nov 2017	1,0
	Talk: ICPP satellite meeting: "Fusarium wilt disease of banana", Boston, USA	28 Jul 2018	1,0
	Poster: INREF Program project 2, Workshop 4 Homestead Fl, USA	18 Apr 2016	1,0
	Poster: VI congreso internacional sobre banano CORBANA y XXI Reunión Internatcional ACORBAT, Miami, USA	19-22 Apr 2016	1,0
	Poster: 8th European Plant Science Retreat	20-23 Jun 2016	1,0
►	**IAB interview**		
►	**Excursions**		
	CIRAD, Plant Breeding Group, Montpellier, France	13-15 Jul 2013	0,9
	Rijk Zwaan Company, Fijnaart, NL	27 Sep 2013	0,2
	Subtotal Scientific Exposure		27,5

3) In-Depth Studies	*date*	*cp*
► **EPS courses or other PhD courses**		

	Bioinformatics - A User's Approach, Wageningen, NL	26-30 Aug 2013	1,5
	An Introduction to Mass Spectometry-Based Plant Metabol§omics, Wageningen, NL	09-13 Dec 2013	1,4
	Bioinformatics Applied to Musa Genome, Montpellier, France	18-22 Nov 2013	1,5
	Microscopy and Spectroscopy in Food and Plant Sciences, Wageningen, NL	06-09 May 2014	0,6
	System Biology: Statistical Analysis of ~Omics Data, Wageningen, NL	15-19 Dec 2014	1,5
	Genome Assembly, Wageningen, NL	28-29 Apr 2015	0,6
	The power of RNA - Seq, Wageningen, NL	10-12 Feb 2016	0,8
►	**Journal club**		
	Literature discussion group at Plant Interactions and Health	2013 - 2018	1,5
	Phytopathology Friday meeting (weekly)	2014 - 2018	1,5
►	**Individual research training**		
	Guest Researcher CIRAD, Plant breeding Group, Montpellier, France	12-16 Feb 2018	0,9
	Subtotal In-Depth Studies		11,8

4) Personal Development		*date*	*cp*
►	**Skill training courses**		
	The Essentials of Scientific Writing & Presenting, Wageningen, NL	29 Jun-8 Jul 2015	1,2
	Reviewing a scientific paper, Wageningen, NL	05 Mar 2015	0,1
	Project and time Management, Wageningen, NL	Apr - May 2015	1,5
	How to write a world class paper, Wageningen, NL	17 Oct 2013	0,2
	Mobilising your Scientific Network, Wageningen, NL	13-20 Oct 2015	1,0
	Writing Grant Proposals, Wageningen, NL	22-24 Sep 2015	2,0
	Efficient Writing Strategies, Wageningen, NL	Sep-Oct 2015	1,3
	Scientific Writing, Wageningen, NL	Sep-Nov 2015	1,8
	Scientific Publishing, Wageningen, NL	15 Oct 2015	0,3
	Career Assessment, Wageningen, NL	19 Jul 2016	0,2
►	**Organisation of PhD students day, course or conference**		
	Organisation of the international meeting in Colombia " The Spread of Panama Disease in Banana: Past, Present and Future", Medellin, Colombia	16-17 Jun 2014	1,5
	Banana Day Wageningen University, INREF project, Wageningen, NL	18 Nov 2014	1,5
	Organisation of Workshop on Panama Disease diagnostics ICA, Medellin - Colombia	10-14 Oct 2016	0,0
	Organisation of Workshop on Panama Disease diagnostics ICA, Atlantico - Colombia	21-23 Nov 2016	0,0
	Organisation of the event "Science & Art: a joint attempt to save the Banana", Wageningen, NL	08 Feb 2018	0,0
►	**Membership of Board, Committee or PhD council**		
	Subtotal Personal Development		12,6

TOTAL NUMBER OF CREDIT POINTS*	**59,4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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