FUSARIUM WILT OF BANANA, A RECURRING THREAT TO GLOBAL BANANA PRODUCTION

EDITED BY: Gert H. J. Kema, Andre Drenth, Miguel Dita, Kees Jansen, Sietze Renze Vellema and Jetse Stoorvogel

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FUSARIUM WILT OF BANANA, A RECURRING THREAT TO GLOBAL BANANA PRODUCTION

Topic Editors:
Gert H. J. Kema, Wageningen University and Research, Netherlands
Andre Drenth, The University of Queensland, Australia
Miguel Dita, Bioversity International (Italy), Italy
Kees Jansen, Wageningen University and Research, Netherlands
Sietze Renze Vellema, Wageningen University and Research, Netherlands
Jetse Stoorvogel, Wageningen Environmental Research, Netherlands

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EDITORIAL
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Editorial: Fusarium Wilt of Banana, a Recurring Threat to Global Banana Production

Gert H. J. Kema[1], André Drenth[2]*, Miguel Dita[3], Kees Jansen[4], Sietze Vellema[5] and Jetse J. Stoorvogel[6]*

[1] Laboratory of Phytopathology, Wageningen University, Wageningen, Netherlands, [2] Queensland Alliance for Agriculture and Food Innovation, Centre for Horticultural Science, The University of Queensland, Brisbane, QLD, Australia, [3] Alliance Bioversity International and CIAT, Cali, Colombia, [4] Rural Sociology Group, Wageningen University, Wageningen, Netherlands, [5] Knowledge, Technology and Innovation Group, Wageningen University, Wageningen, Netherlands, [6] Soil Geography and Landscape Group, Wageningen University, Wageningen, Netherlands

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Editorial on the Research Topic

Fusarium Wilt of Banana, a Recurring Threat to Global Banana Production

THE TOPIC

This Research Topic contains a selection of papers dealing with Fusarium wilt of banana (FWB), also known as Panama disease, that investigate (i) the epidemiology, distribution, infection biology, and diversity of the pathogen, (ii) management practices, and (iii) ways to identify and screen for resistance. The Research Topic arose from the increasing spread and the growing global impact of FWB, affecting a wide range of banana production systems worldwide.

During the inception of this Research Topic an increased understanding of genetic diversity of *Fusarium oxysporum* f.sp. *cubense* (Foc), traditionally considered as the causal agent of FWB, emerged and showed that Foc comprises several different Fusarium species (Ploetz, 2006; Maryani et al., 2019). The so-called Tropical Race 4 (TR4) was found to be genetically distant from other FWB causing species and was described as *Fusarium odoratissimum* (Maryani et al., 2019).

Different strains of this Fusarium species have affected banana production worldwide. Prior to the 1960s, the spread of FWB was primarily caused by so-called Race 1 strains that caused severe losses in the production and export trade in Latin America, which was based almost entirely on the highly susceptible cultivar Gros Michel. The failing management of FWB in Gros Michel eventually convinced the export companies to convert the business to resistant Cavendish cultivars.

TR4 first emerged in Southeast Asia (Ploetz, 1990) and its current rapid spread was analyzed by Ordonez et al. (2015). Subsequent studies showed that the TR4 strain is extremely virulent toward many banana cultivars, including Cavendish cultivars grown in large-scale monoculture plantations for export markets and many banana varieties important for food security and domestic consumption. There are no readily available solutions to manage this disease. Moreover, this global threat connects export trade, strongly dependent on the susceptible Cavendish cultivars, to local production systems wherein a range of banana varieties contributing to food security are also impacted.

This Research Topic aims to provide a platform for information exchange and knowledge sharing. The contributions demonstrate an active research community in search of effective control of FWB. Taken together, the papers provide an overview of our current understanding of the

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****Correspondence:***
André Drenth
a.drenth@uq.edu.au
Gert H. J. Kema
gert.kema@wur.nl

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biology and epidemiology of TR4, its management and how integrated and innovative solutions are required and need to be embraced by all stakeholders in an effort to build a sustainable banana industry for the future.

BACKGROUND

Bananas evolved in Southeast Asia and are globally the most traded fruit, currently grown throughout the (sub-)tropics. Annual global production of banana and plantain combined is 155 million metric tons (FAOSTAT 2020; data for 2018). Over 400 million people rely on bananas and plantains for food security and for income. Banana and plantains are consumed as fruits or as a starchy food staple and are an important ingredient of local diets. Other uses include; beer brewing, packaging and food wrapping, fibers for clothing and handicrafts, transport pallets and animal feeds and dried sheaths for binding ropes, thatching materials, mulching material and traditional medicines.

Banana production takes place under diverse agro-ecological and social-economic conditions where disease pressure varies significantly. Approximately 84% of the crop is cultivated by smallholders and delivered to domestic markets. The international trade represents about 16% of the global banana production consisting of just over 25 million metric tons which are exported from tropical areas to mainly countries in the temperate zones (FAOSTAT 2020; data for 2018). The latter bananas are almost exclusively of the "Cavendish" variety grown in monoculture in large plantations for export. Cavendish cultivars are also important for domestic markets and represent ∼50% of the global banana production. They are resistant to Race 1 strains, but susceptible to TR4. The threat posed by Race 1 was countered by replacing the susceptible Gros Michel with the resistant Cavendish, particularly in export-oriented production systems in tropical lowlands.

Refrigerated transport enabled the export industry to be developed in the 20th century based on the banana variety Gros Michel. This hardy and highly popular variety however turned out to be susceptible to FWB Race 1. After invading Central America Race 1 continued to spread to other banana growing countries and destroyed Gros Michel plantations putting pressure on the export industry in Central and South America (Simmonds, 1966). Despite large scale and expensive efforts to manage FWB in large monoculture plantations, effective control was never achieved. For the first half of the 20th century the industry tried to hold on to "Gros Michel" by shifting cultivation to escape the pathogen with great environmental and socio-economic consequences (Marquardt, 2001; Soluri, 2005). The banana export industry only gradually adopted Cavendish as a replacement for Gros Michel as it required changes to the logistic supply chain. The introduction of cardboard boxes enabled shipment of the more fragile and easily bruised Cavendish fruit, and improved temperature control through refrigeration and artificial ripening, enabled delivery of bananas to Western markets in an acceptable quality. The change in variety as response to Race 1 necessitated major adjustments in logistics and marketing, but the large-scale and uniform production systems based on one single cultivar remained.

Since the 1960's global banana production has expanded significantly due to increased global demand. Cavendish turned out to be highly productive in intensively managed plantations and acceptable to international and domestic markets resulting in contributing approximately 50% of global production and 99% of export markets. The downside of relying on a single cultivar at a global scale is genetic vulnerability which has become evident by the rapid spread of Race 1, and black leaf streak disease, or black Sigatoka (Marin et al., 2003; De Bellaire et al., 2010) in Central and South America and more recently by the emergence and dissemination of TR4. The TR4 epidemic started in the 1960s in Taiwan, and apparently expanded into South East Asia and China and subsequently emerged in the Middle East, Africa, the Indian subcontinent, and most recently in Colombia (García-Bastidas et al., 2020). The spread of TR4 around the world has increased significantly and causes severe damage to the highly susceptible "Cavendish" cultivars planted in huge monocultures as well as backyard gardens.

THE SCOPE OF THE RESEARCH TOPIC

The papers in this Research Topic cover a wide range of issues in an effort to capture the biology and epidemiology of the pathogen (Zheng et al.; Dita et al.; Warman and Aitken; Liu et al.; Pegg et al.), the impact and management of the disease (Montiflor et al.; Carvalhais et al.; Bubici et al.; Staver et al., 2020), identification and screening for resistance (Chen et al.; García-Bastidas et al.) and the socioeconomic approaches to engage all stakeholders in coordinated efforts to contain the spread and to exchange knowledge under circumstances of uncertainty and unfamiliarity (Montiflor et al.; Staver et al., 2020).

The export sector represents the more salient part of the worldwide banana sector; however, the consequences of TR4 for banana producers, traders and consumers in local food provisioning systems is also severe. Therefore, the Research Topic aims to connect the two distinct domains, of export trade and local food security (Oosterveer et al., 2014). This multiplicity enables spread of the pathogen and complicates tailoring of disease management to different circumstances. Yet, the truly global nature of TR4 may be conducive to linking the resources and knowledge of the export-oriented industry with the problem-solving capacities and livelihood strategies of banana producers in diverse agro-ecological and socio-economic contexts.

THE RESEARCH PAPERS

Infection and Spread of the Pathogen

The paper by Pegg et al. deals with the epidemiology of FWB. The paper provides a complete introduction to the FWB problem and this Research Topic. The authors review the body of evidence with regard to the origin, spread and infection of the pathogen and the mechanisms of colonization of the banana plant leading to expression of disease symptoms. The authors outline the evidence for the survival of TR4 in the soil either

as chlamydospores or as an asymptomatic endophyte in a wide range of different non-host plants. Issues which prevent effective management of FWB, are discussed in detail. Some crucial points, such as the long incubation period, are highlighted which substantiate the observation that a lack of symptoms may not be a good indication of the presence or absence of the pathogen. In fact, it may be several years before the pathogen present in the soil infect the banana roots and FWB occurrence become evident.

Several fundamental epidemiological issues are brought to the forefront including the long established observation that all infections by Fusarium originate from the roots (Wardlaw, 1961). Banana root exudates stimulate the germination of chlamydospores present in the soil and the initial advancement of the pathogen through the roots is slow but once the pathogen enters the pseudostem it can spread rapidly through the formation of microconidia in the xylem vessels. The authors hypothesize that when Fusarium microconidia in the xylem vessels are confronted with a perforation plate they germinate and the resulting mycelium grows through the perforation plate to form microconidia at the other side, progressing the colonization of the pseudostem. In banana varieties resistant to FWB the defenses of the host arrest pathogen colonization in the rootlets, roots or at the root base while in susceptible plants the colonization of the xylem continues unabated. Symptoms expressed as wilting are a result of impaired water movement due to vascular clogging, significantly reducing the transpiration levels. In the final phase of colonization, the pathogen moves from the xylem into the parenchyma and cortex of the plant where an abundance of chlamydospores and conidia are produced in the degrading plant tissue. The paper also gives a pertinent overview of the areas that are insufficiently covered in contemporary research efforts such as; completing the disease cycle, investigating in detail the host pathogen interaction, development and application of effective containment measures, detection of affected plants, destruction of infected plant material, soil treatments to reduce the inoculum load, the process of colonization of the pseudostem and the nature of resistance in Cavendish to Race 1.

Warman and Aitken utilize in their paper a combination of a Subtropical Race 4 strain (SR4) labeled with Green Fluorescent Protein (GFP) and confocal microscopy to visualize pathogenesis in a compatible interaction (Warman and Aitken). Their results show that colonization of the roots and pseudostem is quite advanced before external symptoms of disease become apparent. This GFP study confirms the direct penetration of the epidermal cells by the pathogen in the root tips followed by intercellular growth along the elongation zone and the initial colonization of the xylem vessels in asymptomatic fashion. The mechanisms of the movement of the pathogen throughout the pseudostem remains unresolved. Colonization of the pseudostem is followed by colonization of the leaf sheaths and production of chlamydospores in symptomatic plants. The authors provide some evidence that senescent leaf sheaths may be an important source of inoculum, particularly since decaying leaves also show hypha appearing from stomata on the outside of leaf sheaths, which could result in aerial dissemination of the pathogen and underscores the relevance of leaves in disease epidemiology and management.

The genetic differentiation of Fusarium species causing wilt in bananas is poorly understood and an analysis among Race 1, SR4 and TR4 reveals some interesting findings (Liu et al.). Evidence that R1 has a highly diverse genetic background while TR4 shows a monophyletic origin which was identified previously (Ordonez et al., 2015) is corroborated in this study. The evolutionary characteristics of 12 Fusaric Acid Biosynthesis (FUB) genes and three common household genes are analyzed. The authors find a significantly higher level of recombination among the FUB genes compared to the three common household genes which led them to suggest that horizontal gene transfer plays a role in the evolution of *Fusarium* species causing wilt in bananas confirming previous reports (Ma et al., 2013; Mehrabi et al., 2017; Czislowski et al., 2018). FUB genes are postulated by the authors to be functionally relevant and the presence of negative selection on these genes provides circumstantial evidence that Fusaric acid may play a role as a virulence factor associated with symptoms expression. However, thus far sexuality in Fusarium species causing wilt in bananas is hitherto unknown, and hence, alternative hypotheses need to be tested for the occurrence of recombination in this pathogen (Drenth et al., 2019) and mechanisms of horizontal gene transfer need to be elucidated to gain an understanding of the evolution of new strains and/or species (Zheng et al.).

Containment, Control, and Coordination

The important question of how much yield loss is avoided by preventing the entry and delaying the spread of the TR4 is addressed by Staver et al. (2020). They show that resources allocated to reducing the spread of TR4 make a very good investment and back-up earlier findings which enumerated the exclusion benefits of keeping TR4 out of Australia (Cook et al., 2015). The authors specifically highlight the danger of touted, but yet unproven, successes such as the use of somaclonal variants and genetic solutions through gene-editing. They argue that such claims may even be a contributing factor to further spread of the disease as they may put the investment in exclusion and containment at risk. The paper clearly shows that investment aimed at reducing the impact of TR4 provides highly positive results. Different scenarios such as improved exclusion, integrated crop and disease management, breeding resistant cultivars and developing genetically modified resistant banana cultivars all significantly reduce future yield losses and provide a high internal rate of return on investment. The paper provides compelling evidence for increased research concerning the management of TR4 in bananas.

Effective implementation of quarantine and containment measures depends on early detection of the pathogen through reliable diagnostics. In addition to morphological identification and vegetative compatibility analyses several molecular diagnostic assays are routinely used to identify TR4. However, since there are multiple races and species of Fusarium causing wilt in a wide range of banana varieties producing similar disease symptoms, a high level of specificity of these tests is paramount. Evidently, one cannot differentiate a Race 1 infection from a

TR4 infection in a banana variety susceptible to both and one should not rely on visual symptoms only. To overcome this dilemma Carvalhais et al. developed a molecular diagnostic assay based on Secreted in Xylem (SIX) genes which can reliable detect FWB strains and species, and is able to reliable identify a Race 1, Subtropical (SR4) or TR4 strain. Another important aspect of this paper is the rigor in which the validation was conducted to develop a specific, sensitive and robust diagnostic assay. Reliable detection of different races and species causing FWB enables informed decision making and suitable control measures by banana industry stakeholders in case of an invasion.

Expansion of the geographic distribution of TR4 has been rapid and the disease has spread across Southeast Asia, the Middle East, Africa and more recently to South America (García-Bastidas et al., 2020). Although the pandemic of TR4 is caused by a single clone there are small differences appearing among strains of this clone due to mutations. In a detailed analysis using DNA sequencing to identify single nucleotide polymorphisms (SNP) among TR4 strains, the occurrence of slightly different lineages was demonstrated. Zheng et al. provide a detailed insight into the phylogeography of TR4 by comparing strains collected from various invasions around the world. Using this technology the authors demonstrate that strains from recent incursions in Vietnam, Laos, and Myanmar are genetically similar to the one from Yunnan, China. They also show similarity of strains between the Philippines and Pakistan and a close link between the strains in Lebanon and Jordan. This study shows that genomic analyses of the pathogen can identify individual strains which then can be used to shed light on the origin and distribution of TR4 around the world.

The global distribution of R1 and TR4 and the importance of research on surveillance, exclusion and containment to slow down and limit the spread of TR4 is emphasized in the review of Dita et al. It highlights that research on cropping systems is needed to increase the durability of new resistant clones. The authors outline that Race 1 susceptible varieties such as "Prata" and "Gros Michel," still can be profitably grown in some regions if combined with rigorous cultural practices. To do this, cultural practices need to significantly reduce the inoculum load in the soil. Hence, information on pathogen survival in the soil and the infection process becomes highly relevant as previously outlined (Warman and Aitken; Pegg et al.). Soil management, including both biotic and abiotic factors, to control FWB is discussed in detail. Factors affecting symptom expression such as weather, temperature, developmental stage of the plant and aspects of different soil components are outlined. However, despite considering all the cultural practices available to us today the authors conclude that control of FWB has not been sufficiently achieved in large scale monoculture systems based on Gros Michel or Cavendish using practical biological, chemical or cultural methods. It is clear that host resistance to FWB has an important contribution to make in sustainable cultivation of bananas.

Resistance to TR4

Although it is now well understood that "Cavendish" cultivars are susceptible to TR4, it has also become apparent that resistance occurs in other *Musa* germplasm. The paper by Chen et al. describes resistance to TR4 in the diploid AA banana subspecies *Musa acuminata* spp. *malaccensis* and *M. acuminata* spp. *burmannica*. Some polyploid FHIA hybrids, such as FHIA-18 and FHIA-25 are reported to be highly resistant to TR4. Their data reveals that there are different resistance mechanisms present across the 34 *Musa* cultivars they tested and that the resistance response also depends on the inoculum load. They furthermore observe that the resistance response mainly takes place in the rhizome and the outcome of this interaction plays an important role in inhibiting the fungus from colonizing the rest of the banana plant.

The success of plant breeding is largely defined by our ability to select for traits of interest. Therefore, the paper by García-Bastides et al. is fundamentally important as it describes the development of a standard operational procedure to screen germplasm for resistance to TR4, including an inoculum production protocol and additional methodologies underpinning a "high throughput phenotyping system." In addition, the paper describes how visual scoring is complemented with more objective data capture involving image analysis, contributing to overall reproducibility. A glasshouse-based screening method under controlled conditions using specific pathogen genotypes enables reproducible results in a short period of time, thereby speeding up progress in breeding (Smith et al., 2008; Rebouças et al., 2018).

Biological Control

In the absence of a market acceptable banana variety with a high level of resistance to TR4 it is not surprising that many efforts have been directed toward finding an effective biological control agent. An overview of articles investigating the prospects of biological control is contributed by Bubici et al. Although there are many studies showing promising leads with efficacy under controlled environmental conditions, none have been taken to the next step to achieve effective control of TR4 in the field. The paper outlines many of the knowledge gaps which exists in this area and the authors make a point that biocontrol should not be considered as an independent tool, but rather as a module of an integrated management framework. Indeed this links back to the paper by Dita et al. who state that a number of integrated cultural practices are required to significantly reduce the inoculum load in the soil to allow Cavendish production in TR4 infested soils.

Human Factor

Efforts aimed at reducing the impact of any disease are not merely technical but also have substantial social dimensions. The paper by Montiflor et al. looks at the TR4 problem through a different lens and underscores the need to involve all stakeholders in order to contain the disease. The authors argue that efforts should move beyond technical issues and look into how all industry stakeholders in the periphery of the growers, such as industry organizations, researchers, extension workers, exporters and traders, can be involved to partake in the solution (Vellema and Jansen, 2018). The authors investigate issues related to decision making, shared responsibilities and look at how tasks are coordinated and

shared at local levels, thereby highlighting the importance of cross-sector collaboration in responding to disease outbreaks and the development of effective partnerships to mobilize local action.

IN CONCLUSION

The emerging theme of this Research Topic is the reminiscence of the decline of "Gros Michel" due to FWB Race 1 and the current threat of TR4 to global banana cultivation overly dependent on Cavendish cultivars. TR4 affects Cavendish cultivars grown in large scale export-oriented plantations and confronts major banana companies with a recurrent disease problem. However, TR4 also impacts food security and local trade because it affects many other common banana varieties grown for domestic markets. Quarantine and containment may have reduced the spread of TR4 in some regions at a local scale but did not prevent international nor intercontinental dissemination (Drenth and Kema, in press). The application of cultural and biological control options can slow down the development of epidemics, but alone do not provide effective control, thereby merely contributing to gain time. The only long-term option proposed in several papers in this Research Topic is to deploy new varieties with effective disease resistance. This option quenched the Race 1 epidemic once Cavendish replaced Gros Michel. Hence, finding productive and consumer accepted varieties to substitute Cavendish is a key component of strategies to enhance resilience of the export-oriented banana industry as well as many local and regional production systems aimed at domestic markets. Research on genetics, breeding and selection for disease resistance should be accompanied by diversification of the genetic foundations of the banana industry and be combined with other types of solutions, such as soil management or integrating banana with other crops. Changes in the banana industry to control FWD require the involvement of local and international industry stakeholders and consumers to reduce the current extreme level of genetic vulnerability and avoid making the same mistakes once more.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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New Geographical Insights of the Latest Expansion of *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 Into the Greater Mekong Subregion

Si-Jun Zheng[1,2†], Fernando A. García-Bastidas[3,4†], Xundong Li[1†], Li Zeng[1], Tingting Bai[1], Shengtao Xu[1], Kesuo Yin[1], Hongxiang Li[5], Gang Fu[6], Yanchun Yu[7], Liu Yang[8], Huy Chung Nguyen[9], Bounneuang Douangboupha[10], Aye Aye Khaing[11], Andre Drenth[12], Michael F. Seidl[4], Harold J. G. Meijer[3] and Gert H. J. Kema[3,4*]*

[1] Agricultural Environment and Resources Institute, Yunnan Academy of Agricultural Sciences, Kunming, China, [2] Bioversity International, Kunming, China, [3] Wageningen University and Research, Wageningen Plant Research, Wageningen, Netherlands, [4] Wageningen University and Research, Laboratory of Phytopathology, Wageningen, Netherlands, [5] Xishuangbanna Dai Autonomous Prefecture, Plant Quarantine and Protection Station, Jinghong, China, [6] Institute of Microbiology, Guangxi Academy of Agricultural Sciences, Nanning, China, [7] Institute of Tropical and Subtropical Industry Crops, Yunnan Academy of Agricultural Sciences, Kunming, China, [8] Institute of Biotechnology, Guangxi Academy of Agricultural Sciences, Nanning, China, [9] Plant Protection Research Institute, Vietnam Academy of Agricultural Sciences, Hanoi, Vietnam, [10] Ministry of Agriculture & Forestry, National Agriculture & Forestry Research Institute, Horticulture Research Center, Vientiane, Laos, [11] Biotechnology Research Department, Mandalay, Myanmar, [12] Centre for Horticultural Science, The University of Queensland, Brisbane, QLD, Australia

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 and the presence of serious disease threats, research into this crop is limited. 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. The epidemic was eventually quenched by planting "Cavendish" bananas. However, 50 years ago the disease recurred, but now on "Cavendish" bananas. Since then the disease has spread across South-East Asia, to the Middle-East and the Indian subcontinent and leaped into Africa. Here, we report the presence of *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (Foc 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 Foc 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 Foc TR4 across three geographical areas—GMS, Indian subcontinent, and the Middle East revealing three distinct Foc 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 Foc TR4.

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

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***Correspondence:**
Si-Jun Zheng
sijunzheng63@163.com
Gert H. J. Kema
gert.kema@wur.nl

[†] These authors have contributed equally to this work.

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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 (Ordoñez et al., 2015b; Arango Isaza et al., 2016; Diaz-Trujillo et al., 2018). 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 (Foc TR4), which originates from Indonesia and affects many banana varieties, including those belonging to the "Cavendish" group, has now developed into a global threat (Ordoñez 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; Ordonez 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, 2011; Pocasangre et al., 2011; Shabani et al., 2014; Ordoñez 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 ∼40% (Ploetz et al., 2015) of the total global area. Clearly, this comes with a huge risk for a pandemic as these clones are susceptible to Foc 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 Foc TR4 incursions. In 1967, Foc TR4 surfaced in Taiwan, supposedly after introduction of infected plants from Sumatera, Indonesia (Su et al., 1977; 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 et al., 1978; Su et al., 1986; Hwang and Ko, 2004; Qi et al., 2008; Buddenhagen, 2009; Li et al., 2013). The expansion of Foc 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 a 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 Foc TR4, which is worrisome as it threatens both food security and the international trade (Ordonez et al., 2015a; Ploetz et al., 2015; Mostert et al., 2017).

MATERIALS AND METHODS

Sample Collection

To investigate the presence of Foc TR4 we sampled commercial "Cavendish" plantations in Laos, Myanmar, Vietnam, and Yunnan during March and May 2016 (**Table 1**, **Figure 1**). Samples from Guangxi and Guangdong were collected during 2011–2014. Banana plants affected by Fusarium wilt which showed yellowing older leaves or a skirt of dead leaves around the pseudostem were internally sampled. Discolored 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.

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 and Summerell, 2006) and the remaining part was used for DNA extraction to verify the presence of Foc TR4 (García-Bastidas et al., 2014; Ordonez 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 Foc 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,

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

Sampling date	Site	Code	Variety	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
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	N0. 16	Guangfen 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

one positive Foc TR4 strain for each country was phenotyped under greenhouse conditions (Unifarm, Wageningen, The Netherlands) following earlier reported protocols (García-Bastidas et al., 2014; Ordonez et al., 2015a). For each strain we used six highly susceptible "Grand Naine" plants (biological replicates) that were placed randomly in the greenhouse, along with the appropriate controls (negative: water and Foc Race 1 from Cruz das Almas, Brazil, positive: Foc 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; Ordonez et al., 2015a). Corm tissue of each plant was collected and plated on Komada medium for fungal isolation and subsequent PCR confirmation of Foc TR4 as causal agent.

Sequence Analyses of Foc TR4 Strains

To determine the identity of the strains and their relationship with other strains, one Foc 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 *F. 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 Foc TR4 II5) were mapped against the Foc 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.

FIGURE 1 | Banana plants with Fusarium wilt symptoms in sampled "Cavendish" plantations in Laos **(A)**, Myanmar **(B)**, Vietnam **(C),** and Yunnan **(D)**.

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

***Fusarium oxysporum* f.sp. *cubense* 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	Ordonez et al., 2015a
Phi2.6C	TR4	Philippines	Ordonez et al., 2015a
Leb1.2C	TR4	Lebanon	Ordonez et al., 2015a
JV11	TR4	Jordan	García-Bastidas et al., 2014
My-1	TR4	Myanmar	This work
La-2	TR4	Laos	This work
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 Foc 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 Foc 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" variety "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" varieties "Nantianhuang," "Brazilian" or "Guijiao No. 6." Fusarium wilt was observed in all plantations (**Figure 1**). In total 19 samples were collected; five samples from variety "Brazilian" in Laos and Myanmar, four samples from variety "Guijiao No 6" in Vietnam and five samples from variety "Nantianhuang" in Yunnan (**Table 1**, **Figure 1**). Analyses of the samples resulted in 16 isolates of which 13 were identified as Foc 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 2**).

Phenotyping of Foc TR4 Isolates

We selected one Foc TR4 isolate from Vietnam, Yunnan, Myanmar, and Laos for confirmatory phenotyping assays. Except the control plants inoculated with Foc 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

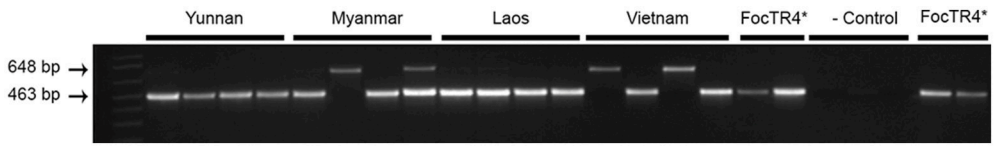

FIGURE 2 | Identification of *Fusarium oxysporum* f.sp. *cubense* from samples derived from Yunnan, Myanmar, Laos, and Vietnam as Tropical Race 4 (Foc TR4) by PCR. Specific DNA bands for Foc TR4 (463 bp) and elongation factor-1α (648 bp) are indicated on the left. TR4-II5 was taken as positive control "Foc TR4*"; The Foc Race 1 strain was used as negative control (- control) (Dita et al., 2010).

during incubation until plants were externally and internally scored for disease severity at seven weeks after inoculation. At that stage, all plants inoculated with Foc TR4 diagnosed strains were completely decayed. The Foc Race 1 inoculated plants, however, were healthy and unaffected by Foc, similar as the water controls (**Figure 3**).

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 4**) and subsequent Foc TR4 diagnostic PCR tests were positive for all reisolated strains (not shown).

Sequence Analysis of Foc TR4 Strains

We used whole-genome sequencing of each representative Foc TR4 strain for each GMS country and comparisons with the previously sequenced Foc TR4 strains from recently reported incursions were performed to study their genetic relatedness. The maximum-likelihood phylogeny of the genome sequences clearly confirmed that these strains belong to the Foc TR4 genetic lineage (Ordoñez et al., 2015b). Subsequent comparative analyses among the GMS Foc TR4 strains and those from recent incursions in Jordan, Lebanon, and Pakistan as well as a Philippine Foc TR4 strain revealed a total of 251 single nucleotide polymorphisms (SNPs) that were distributed across the genome (**Supplemental Table 1**, **Supplemental Figure 1**). Subsequent principal component analyses (PCA; **Figure 5**) and hierarchical clustering revealed three geographically distinct groups of Foc TR4 isolates, despite the overall limited amount of SNPs indicative of the clonality of Foc TR4 strains. One group represents the GMS Foc TR4 strains including the strain from Yunnan, China. A second group links the recent Pakistan incursion (Ordonez et al., 2015a) with the Philippine Foc TR4 strain. The third group shows a strong similarity between the recent incursion of Foc TR4 in Lebanon and Jordan (García-Bastidas et al., 2014; Ordonez et al., 2015a). Some of the analyzed SNPs were indicative of inconsistencies based on the FocII5 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**, **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 Foc TR4 in Vietnam and Laos and its first report in Myanmar. Recently, Mostert et al. (2017) denied the presence of Foc 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 analyze the genetic diversity of Foc TR4 strains. Isolates from Vietnam, Laos, and Myanmar are genetically closely related and resemble the Foc TR4 strain from Yunnan. Furthermore, we demonstrate genetic association between the Foc TR4 strains from Pakistan and the Philippines as well as between the strains from Lebanon and Jordan. Although Foc 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 Foc TR4 strain represents essentially a single clone (Ordoñez 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 Foc TR4 strains. Such analyses were recently also used to reveal the dissemination of the quarantine pathogen *Xylella fastidiosa* in olive trees (Loconsole et al., 2014). Here, such a phylogeography approach provides initial evidence that Foc TR4 in Laos, Vietnam, and Myanmar was likely introduced from China. This supports the circumstantial evidence of ongoing Foc 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 Foc TR4 strains from the Philippines and from Pakistan are closely related. Since Foc TR4 was diagnosed in the Philippines in 2005 (Molina et al., 2009) and is currently omnipresent in Mindanao, the recent incursion in Pakistan (Ordonez et al., 2015a) seems to originate from the Philippines. Similarly, the phylogeography data set indicates that the Foc 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 demonstrate that it also facilitated yet another expansion of Foc TR4. The dissemination of Foc TR4 in China upon

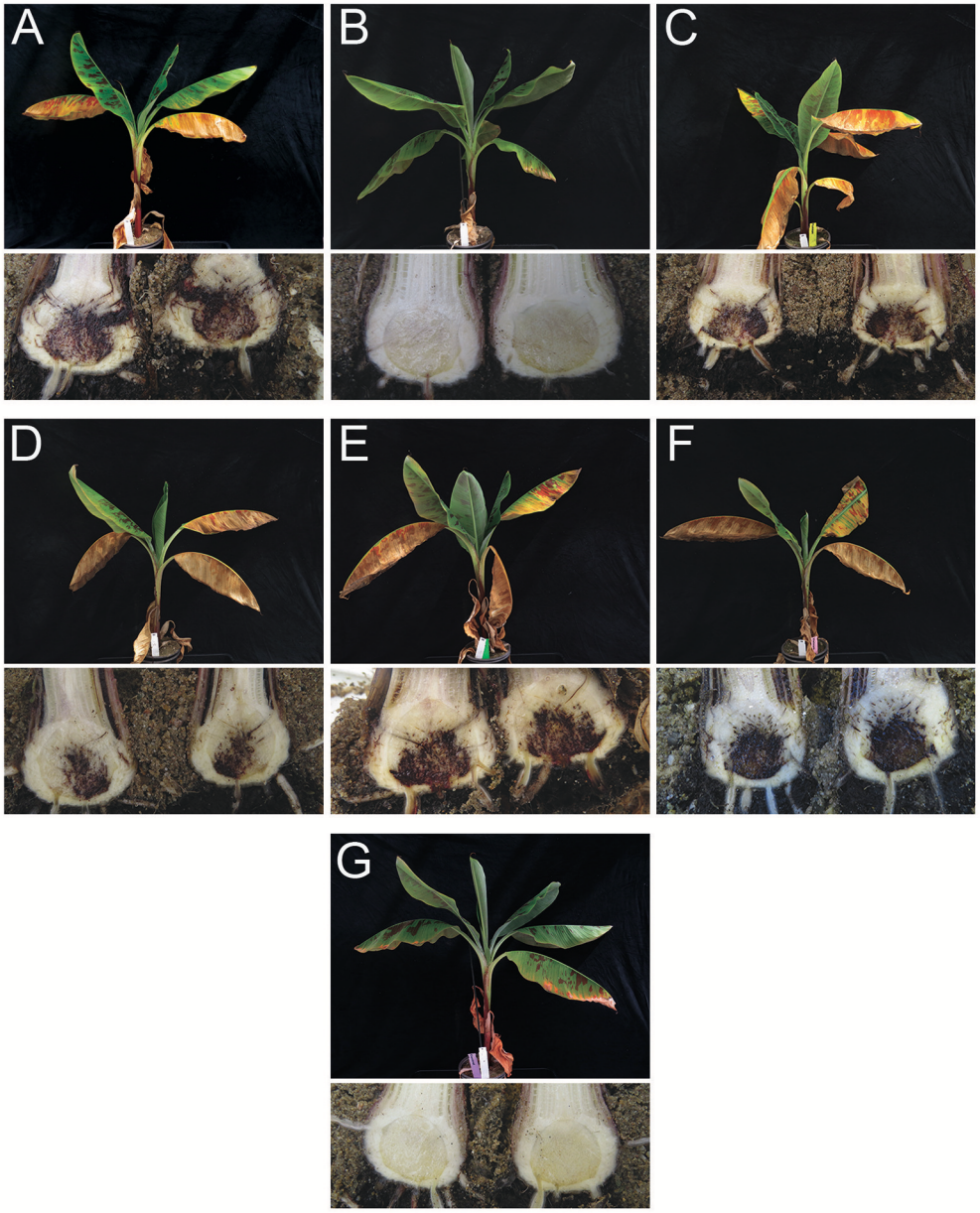

FIGURE 3 | External foliar (top) and internal rhizome (bottom) symptoms of *Fusarium oxysporum* f.sp. *cubense* (Foc) infection in "Grand Naine" banana plants 7 weeks after inoculation using Foc isolates from Vietnam, Yunnan, Myanmar, and Laos. **(A)** Foc TR4 (reference Foc TR4, isolate II5 from Indonesia/VCG01213); **(B)** Foc 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).

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 Foc 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 Foc TR4 was observed 50 years ago in Taiwan (Su et al., 1977, 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 Foc TR4 pandemic on food and fruit production. Despite numerous efforts to alert and mobilize the banana sector

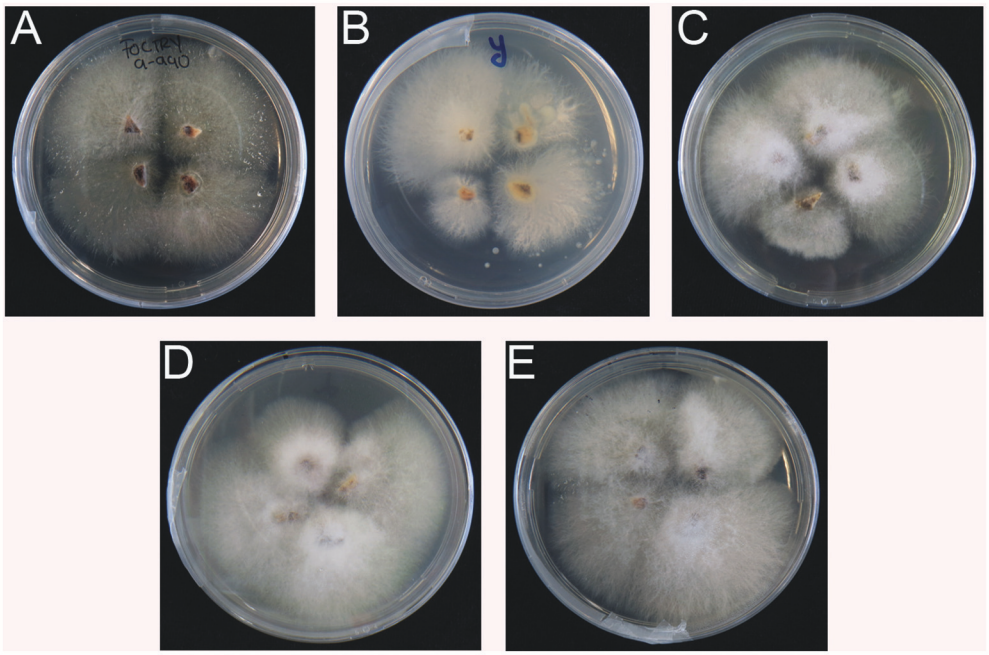

FIGURE 4 | Re-isolation of *Fusarium oxysporum* f.sp. *cubense* TR4 from inoculated plants (see **Figure 3**). **(A–E)** from Indonesia, Yunnan, Myanmar, Laos, and Vietnam, respectively. No positive isolates for Foc TR4 were recovered from water and Foc Race 1 controls.

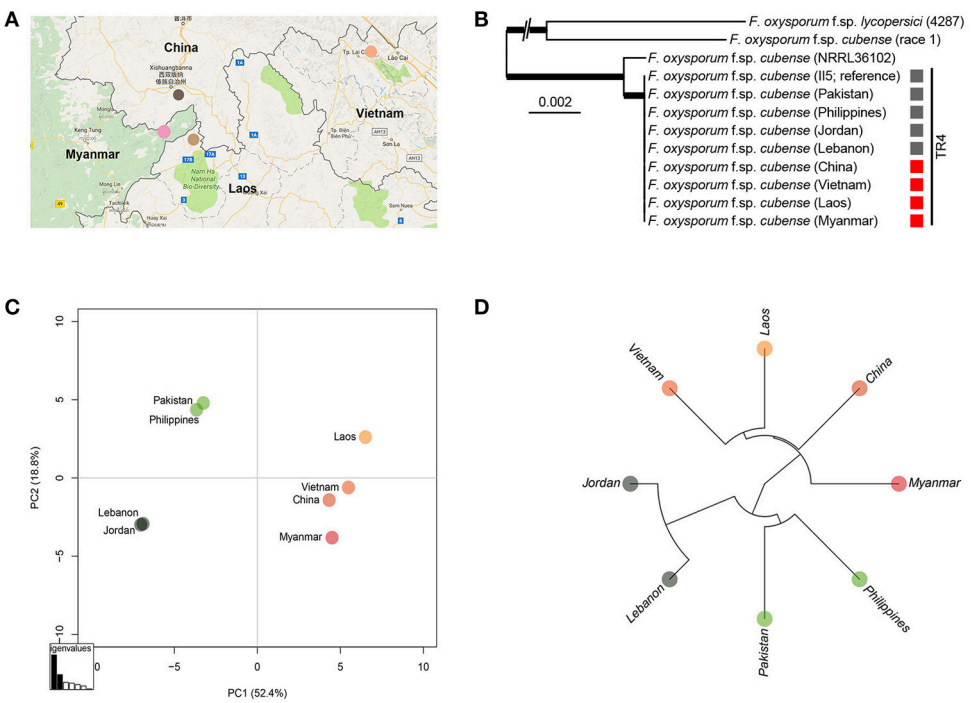

FIGURE 5 | Sequence analysis of *Fusarium oxysporum* f.sp. *cubense* (Foc) isolates from Yunnan, Myanmar, Laos, and Vietnam and alignments with other Foc isolates from Jordan, Lebanon, Pakistan, and Philippines. **(A)** Colored dots represent sampling areas in China, Laos, Myanmar, and Vietnam; **(B)** Isolates from different countries; **(C)** Principal component analysis plot based on 251 high quality SNPs and; **(D)** UPGMA tree of Foc TR4 isolates.

for enhanced quarantine practices, we observe a continuous dissemination of Foc TR4 (García-Bastidas et al., 2014; Ordonez et al., 2015a; Ploetz et al., 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 Foc TR4.

AUTHOR CONTRIBUTIONS

S-JZ: Conceived the experiments, collected the samples, analyzed the data, wrote the paper; FG-B: Analyzed the samples, wrote the paper; XL, LZ, TB, SX, KY, HL, YY, and LY: Collected the samples, analyzed the data; GF: Collected the samples, analyzed the data, wrote the paper; HN, BD, and AK: Ensured visits to banana farms, collected samples, organized permits; AD: Provided training, analyzed the data, wrote the paper; MS and HM: Analyzed the data, wrote the paper; GK: Conceived the experiments, organized the sampling, analyzed the data, wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00457/full#supplementary-material

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

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

Supplemental Figure 2 | Principal component analysis plot based on 161 filtered high quality SNPs of Foc 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 Foc TR4 strains.

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REVIEW
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Fusarium Wilt of Banana: Current Knowledge on Epidemiology and Research Needs Toward Sustainable Disease Management

Miguel Dita[1,2]*, *Marcia Barquero*[2,3], *Daniel Heck*[4], *Eduardo S. G. Mizubuti*[4] and *Charles P. Staver*[2]

[1] Embrapa Mandioca e Fruticultura, Bahia, Brazil, [2] Bioversity International, Montpellier, France, [3] Institute of Environment, Natural Resources and Biodiversity, Universidad de León, León, Spain, [4] Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Brazil

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***Correspondence:**
Miguel Dita
miguel.dita@embrapa.br

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Banana production is seriously threatened by Fusarium wilt (FW), a disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). In the mid-twentieth century FW, also known as "Panama disease", wiped out the Gros Michel banana industry in Central America. The devastation caused by *Foc* race 1 was mitigated by a shift to resistant Cavendish cultivars, which are currently the source of 99% of banana exports. However, a new strain of *Foc,* the tropical race 4 (TR4), attacks Cavendish clones and a diverse range of other banana varieties. *Foc* TR4 has been restricted to East and parts of Southeast Asia for more than 20 years, but since 2010 the disease has spread westward into five additional countries in Southeast and South Asia (Vietnam, Laos, Myanmar, India, and Pakistan) and at the transcontinental level into the Middle East (Oman, Jordan, Lebanon, and Israel) and Africa (Mozambique). The spread of *Foc* TR4 is of great concern due to the limited knowledge about key aspects of disease epidemiology and the lack of effective management models, including resistant varieties and soil management approaches. In this review we summarize the current knowledge on the epidemiology of FW of banana, highlighting knowledge gaps in pathogen survival and dispersal, factors driving disease intensity, soil and plant microbiome and the dynamics of the disease. Comparisons with FW in other crops were also made to indicate possible differences and commonalities. Our current understanding of the role of main biotic and abiotic factors on disease intensity is reviewed, highlighting research needs and futures directions. Finally, a set of practices and their impact on disease intensity are discussed and proposed as an integrative management approach that could eventually be used by a range of users, including plant protection organizations, researchers, extension workers and growers.

Keywords: *Musa* spp, panama disease, *Fusarium oxysporum* f. sp. *cubense*, integrated pest management, epidemiology

INTRODUCTION

Bananas, the world's most important fruit in terms of production volume and trade (FAOSTAT, 2017) and among the world's top 10 staple foods, is seriously threatened by Fusarium wilt (FW). The disease, considered one of the most destructive banana diseases in history (Stover and Simmonds, 1987), is caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) W.C. Snyder & H.N. Hansen (hereafter referred as *Foc).* A soil-borne pathogen with an extremely long residence time in soil, *Foc* infects the xylem, induces wilt and kills banana plants (Stover R., 1962). The pathogen was first reported in Australia (Bancroft, 1876; Ploetz and Pegg, 1997) and has been spreading globally with the informal exchange of planting material and the movement of spore-bearing soil (Ploetz, 2015a,b). The expansion of export banana in large monocropped fields in the Americas from the early 1900's was based on practices favorable to disease spread. By the mid-twentieth century the banana industry in the Americas, based in the susceptible cultivar Gros Michel (AAA) was in crisis due to FW (Stover R., 1962; Ploetz, 2005). A highly successful shift to FW-resistant cultivars of the Cavendish (AAA) subgroup, contributed to the expansion of Cavendish production for both national and export markets, but also resulted in a dramatic decline in FW research. The appearance of a new race of *Foc*, to which Cavendish and many other cultivars are highly susceptible, has generated a global concern and new demands for solution-oriented research on FW of banana. New resistant cultivars are not foreseen in the short-term, although clonally selected varieties with partial resistance have shown some promise (Hwang and Ko, 2004). Thus, research on epidemiology-based management programs has again become a high priority, which this review proposes to address.

Foc, a highly variable pathogen, is comprised of different evolutionary lineages (O'Donnell et al., 2009). At least 24 vegetative compatibility groups (VCGs) are known to date in *Foc* (Ploetz and Correll, 1988; Fourie et al., 2011; Mostert et al., 2017), which can affect *Musa acuminata*, *M. balbisiana*, *M. schizocarpa,* and *M. textilis* (Musaceae: Zingiberales; Ploetz, 2015b). Different races of the pathogen are identified based on the pathogenicity to reference host cultivars: race 1 (R1) affects Gros Michel (AAA) and Manzano/Apple/Latundan (Silk, AAB); race 2 (R2) affects cooking bananas of the Bluggoe (ABB) subgroup and race 4 (R4) affects all cultivars in the Cavendish (AAA) subgroup in addition to those susceptible to R1 and R2 (Waite and Stover, 1960; Su et al., 1986). A pathogen population causing FW in *Heliconia* spp., was described as race 3, but it is no longer considered as part of *Foc* (Ploetz, 2005).

Fusarium wilt epidemics occurring in Gros Michel, the original export banana, were attributed to *Foc* R1 (Stover R., 1962). The substitution of Gros Michel by Cavendish cultivars, resistant to *Foc* R1, though at high economic costs, solved the problem (Ploetz, 2006) and since then the banana export industry has expanded rapidly based on Cavendish. However, when cultivated under seasonal abiotic stresses (mainly low temperature) in subtropical regions such as South Africa, the Canary Islands and parts of Australia, Cavendish cultivars were susceptible to *Foc* R4 (Su et al., 1986; Ploetz and Pegg, 1997).

Until 1989, *Foc* was only reported affecting Cavendish in subtropical regions. However, a new variant that severely affects Cavendish cultivars in the tropics was reported in 1990 (Ploetz and Pegg, 2000; Ploetz, 2006). To discriminate *Foc* populations that only affect Cavendish in the subtropics from the populations that affect Cavendish in the tropics, two divisions of *Foc* R4 were created: Subtropical race 4 (SR4) and tropical race 4 (TR4; Ploetz, 2006). While *Foc* SR4 causes disease in Cavendish only in the subtropics, *Foc* TR4 is pathogenic under both tropical and subtropical conditions (Buddenhagen, 2009; Mostert et al., 2017). In addition, different VCGs (0120, 01201, 01202, 01209, 01210, 01211, 01215, 0120/15; 0129/11) have been associated with *Foc* SR4, while only one VCG (01213/16) to *Foc* TR4 (Buddenhagen, 2009; Mostert et al., 2017).

For more than 20 years, *Foc TR4* was restricted to East and parts of Southeast Asia and the Northern Territory of Australia, but recent reports confirmed its presence in Jordan, Oman, Mozambique (2013), Lebanon, Pakistan (2015) (García-Bastidas et al., 2014; Ordoñez et al., 2015), Vietnam (Hung et al., 2018), Laos (Chittarath et al., 2018), Myanmar (Zheng et al., 2018), and Israel (Maymon et al., 2018). In Australia, *Foc* TR4 was reported in the Northern Territory since 1997 (Bentley et al., 2001; Conde and Itkethley, 2001), but new outbreaks were reported in Queensland in 2015 (O'Neill et al., 2016). There are also informal reports that *Foc* TR4 is also present in India (**Figure 1**).

In 28 years *Foc* TR4 has been moved by planting material, soil vectored by workers, vehicles and planting materials, irrigation, and floodwaters resulting in the loss of hundreds of thousands of hectares of Cavendish production across China and into northern Southeast Asia and in the Philippines and Indonesia. This on-going dissemination of *Foc* TR4 and the impact caused in Cavendish in the region highlight the major threat that this race represents both to countries where the disease is already present and still spreading and those still TR4-free (**Figure 1**).

Although *Foc* TR4 has been highly associated with Cavendish monoculture, this pathogen also affects many other cultivars important for food security and income generation (Hermanto et al., 2011; Mostert et al., 2017). Producers facing TR4 and even producers of key cultivars for national markets susceptible to R1, face the spread and build-up of FW and resulting threat to income. In practice, cultivars like Gros Michel, Pome, Bluggoe, Pisang Awak and many South Pacific plantains, continue to be grown and marketed through two mechanisms. The production and supply of susceptible banana varieties is mainly due to movement of banana growers and establishment of new fields in clean areas.

The frequent claim that effective management of FW can only be achieved with resistant cultivars is partly due to the success of Cavendish for *Foc* R1. Chemical, biological and cultural practices have always been downplayed as not effective (Ploetz, 2015b). However, quantitative resistance linked to cultural and biological practices have been effective in different scenarios allowing farmers to grow susceptible varieties. In Brazil, for instance, Prata-type cultivars have high market value and are susceptible to FW, but combining resistance with cultural

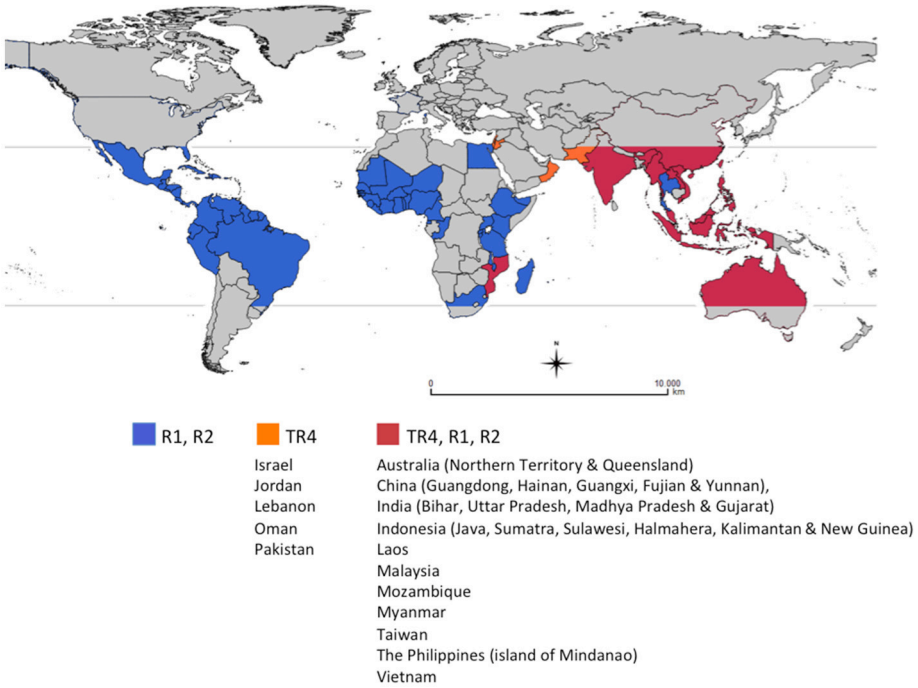

FIGURE 1 | Global distribution of races of *Fusarium oxysporum* f. sp. *cubense (Foc)*, causal agent of Fusarium wilt of banana. This map considers producing countries with presence or absence of a given race of *Foc* and does not represent banana-producing areas by countries. R1: Race 1, R2: Race 2, TR4: Tropical race 4. Races 1 (R1) and 2 (R2) are widely distributed in banana producing countries affecting local varieties (see introduction for more details). Subtropical race 4 was not included as it corresponds to *Foc* populations present in subtropical producing areas in Australia, Brazil, Canary Islands, China, South Africa and Taiwan, causing intermittent yield losses in Cavendish cultivars. For more information on the distribution of TR4 see http://www.promusa.org/Tropical+race+4+-+TR4#Distribution.

practices growers can cope with the disease and make the banana crop profitable (Haddad et al., 2018). Growers in Colombia have also adopted a two-cycle strategy to produce Gros Michel, rotating production before pathogen inoculum builds up in the soil.

To date, most elements of the strategy to manage *Foc* TR4 are based on limited research conducted to understand epidemics caused by R1. The replacement of Gros Michel by a fully resistant cultivar, partially removed FW from the "list of banana constraints" and consequently research efforts on this disease in most countries practically stopped.

With the threat of *Foc* TR4, research efforts have revived. Many reviews cover general aspects of history, pathogen biology, epidemiology and management options (Ploetz and Pegg, 1997, 2000; Ploetz, 2005, 2006, 2015a,b; Daniells, 2011; Ghag et al., 2015). These sources are quite useful to understand the historical and basic epidemiological aspects of FW and how the banana industry has been impacted. In this review we summarize the current knowledge of FW epidemiology, such as pathogen survival, dispersal, and host invasion. Most importantly, we identify knowledge gaps and research questions on how to live with the spread of *Foc* TR4 and other races of *Foc*. The impact of biotic and abiotic factors and crop management practices on FW intensity are discussed. Finally, a set of practices and their impact on disease intensity are discussed and proposed as an integrative management approach.

PATHOGEN SURVIVAL

Most species of the *F. oxysporum* complex, including *Foc*, are able to survive in the absence of it primary host, mainly in the form of the thick-walled survival spores, chlamydospores (Stover R., 1962; Leslie and Summerell, 2006). Chlamydospores are resistant to desiccation, resilient in unfavorable environmental conditions (Stover R., 1962), and may survive in the soil for more than 20 years (Stover R., 1962; Buddenhagen, 2009). Contrary to what is commonly assumed, chlamydospores of *Foc* are constantly produced once the host is invaded even before external symptoms are visible (Li et al., 2011), and not just after the death of the banana plant.

The capacity of *Foc* to colonize and grow saprophytically in debris increases chlamydospore production and contributes to increased pathogen persistence in the soil (Stover and Waite, 1960; Stover R., 1962). In addition to chlamydospores, long-term survival of *Foc* is promoted through the infection of weeds and non-economic host plants (hereafter collectively referred to as weeds). Studies carried out in America and Australia revealed that some weed species can be colonized by *Foc* without visible symptoms. *Foc* R1 was found in *Paspalum fasciculatum*, *Panicum purpurescens*, *Ixophorus unisetus* (Poaceae), and *Commelina diffusa* (Commelinaceae) in Central America (Waite and Dunlap, 1953), while *Foc* SR4 was reported in *Paspalum* spp. and *Amaranthus* spp. (Amaranthaceae) in Australia (Pittaway et al.,

1999). *Foc* TR4 was found in roots of *Chloris inflata* (Poaceae), *Euphorbia heterophylla* (Euphorbiaceae), *Cyanthillium cinereum* and *Tridax procumbens* (Asteraceae), growing in infested banana plantations in Australia (Hennessy et al., 2005). In all cases, weeds did not show external symptoms resembling FW. These findings suggest that *Foc* may be able to survive as endophyte in other hosts and when bananas are replanted in the area, weed hosts can act as FW inoculum reservoir.

The impact of weed management practices on *Foc* dispersal and survival needs further attention. Cover crops are commonly recommended in banana plantations as a soil-health practice to control weeds and nematodes, prevent soil erosion, and to reduce within field spread of FW (Charles, 1995; Fongod et al., 2010; Duyck et al., 2011; Djigal et al., 2012; Pattison et al., 2014; Tardy et al., 2015). However, no studies were found which investigate the potential role of cover crops used in banana as secondary hosts of *Foc*.

Little is known about the potential of *Foc* detected in weeds to infect banana, the infection process and related soil and abiotic factors that affect the efficacy of this potential source of inoculum, albeit the large impact these issues may potentially have for the epidemiology and management of FW. The life cycle of *Foc* in weeds or in alternative hosts appears not to be the same as in banana because no disease symptoms are observed, but what kind of *Foc* structures are present inside an alternative host? Are chlamydospores produced in these weeds or are they only produced when these plants die? Does chemical (herbicides) or mechanical (mowing) weed management stimulate the production of chlamydospores, as empirically claimed when banana plants are eliminated with glyphosate? Can chlamydospores originating from weedy vegetation survive in the soil and infect banana plants again? While sound scientific-based information is not available, the recommendation for using cover crops and weed management strategies in *Foc*-affected areas should consider its potential role as *Foc* propagators. Unfortunately, a drawback to answer these research questions is the lack of effective diagnostic tools with high resolution to take into account the worldwide diversity of *Foc*. Once these tools are available, relevant knowledge on these epidemiological aspects in different environments and *Foc* populations can be generated.

INFECTION PROCESS, PLANT-PATHOGEN INTERACTION AND DISEASE DEVELOPMENT

In the soil, the different *formae specialis* of *F. oxysporum* show negligible capacity for self-movement or growth without a host tissue (**Figure 2A**; Rekah et al., 1999). *Foc* seems to be no exception, even though no solid experimental data to support this claim were found. The structures of *Foc* remain dormant until stimulated to germinate by host or non-host root exudates or by the direct contact with susceptible root tissues (**Figure 2B**; Stover R., 1962). Conidia and hyphae of *Foc* can be seen adhered to root surfaces at 1 or 2 days post inoculation (Guo et al., 2015; Li et al., 2017). Hyphae can grow along the grooves at junctions between epidermal root cells and colonize the root surface (Guo et al., 2015). Infection takes place through secondary or tertiary feeder roots, but not through the main root, unless the central core is exposed directly to pathogen structures (Trujillo and Snyder, 1963). Penetration can occurs either directly or through wounds (**Figure 2B**) and no true appressoria or penetration pegs have been observed (Li et al., 2011, 2017). Hyphae become swollen at penetration site, but return to their normal size afterwards (Li et al., 2011). Occasionally lesions develop at the site of initial infection, but more often they are located near the root base (Rishbeth, 1955).

Even when the pathogen successfully attaches its structures to the roots, most of the infection attempts seem to be blocked by the host (Underwood, 2012). The cell wall can be a source of nutrients for pathogens as well as a barrier to access a most suitable environment to their survival (Cantu et al., 2008). Plant pathogens must pass this barrier to complete the infection and interact with the host (Underwood, 2012). The cell wall is composed of complex polysaccharides (cellulose, hemicellulose, or pectin) with associated proteins and aromatic compounds (Caffall and Mohnen, 2009). Some of the major groups of cell wall pectins could be digested by a series of cell wall-degrading enzymes secreted by hemibiotrophic pathogens, such as pectin methylesterases (PMEs), polygalacturonases and polymethylgalacturonases (Cantu et al., 2008). In response to *Foc* infection, the cell wall of banana roots trigger a series of coordinated responses, such as pectin methylesterification (Ma et al., 2013) and changes in the spatial distribution and abundance of hydroxiproline-rich glycoproteins, PMEs and pectins (Fan et al., 2017; Wu et al., 2017), which have direct impact on the host resistance. Other defenses in banana against *Foc* are incited by salycilic acid and jasmonic acid/ethylene. Both pathways may involve systemic acquired resistance (Wu et al., 2013), DNA methylation (Luo et al., 2016), and changes in the expression of genes related to pathogenesis, transcription factors, hypersensitive reaction, and synthesis of phytoalexins (Bai et al., 2013; Li et al., 2013; Niu et al., 2018). Genes related to the cell wall biosynthesis (lignification or degradation) can also be involved in the resistance of banana against *Foc* (Bai et al., 2013; Niu et al., 2018).

Fusarium oxysporum f. sp. *cubense* has been considered a necrotrophic or hemibiotrophic pathogen. Apparently, *Foc* needs to interact during part of its life cycle with living plant cells. In addition to the strategies described in the previous paragraph, *Foc* evolved to have a diverse array of proteins that determine infection capacity in bananas, similar to *Fol* in tomato (Takken and Rep, 2010).

Differences in the composition of *SIX* (secreted in xylem) homologues genes, involved in pathogenicity of *F. oxysporum* (Rep et al., 2004; Meldrum et al., 2012), were also observed in *Foc* races. While a *Foc* R4 strain presented homologues of *SIX1*, *SIX7* and *SIX8*, only *SIX1* was present in a *Foc* R1 strain (Meldrum et al., 2012). An additional study showed that *Foc* TR4 had three copies of *SIX1* gene (*Six1a-Six1c*), one copy of *SIX2*, *SIX6* and *SIX8,* while only one copy of *SIX1* and *SIX6* were observed in *Foc* R1 (Guo et al., 2014). Recently, using a whole-genome sequencing approach, Czislowski et al. (2017) studied 23 VCGs of *Foc* and

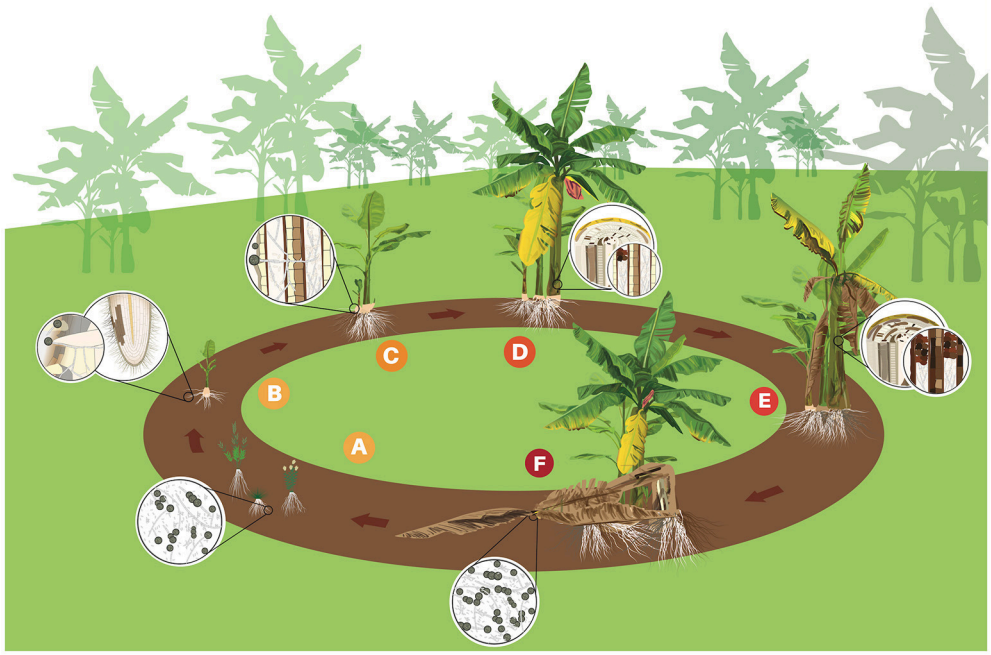

FIGURE 2 | Life cycle of *Fusarium oxysporum* f. sp. *cubense (Foc)* in banana. **(A)** Spores (micro and macro conidia and chlamydospores) rest in the soil or on alternative hosts such as weeds. **(B)** Chlamydospores germinate stimulated by root exudates and the germ-tubes penetrate banana roots. **(C)** *Foc* grows through the cortex to the epidermis and mycelium invades the vascular system. **(D)** Conidia and chlamydospores are constantly produced in the vascular tissues. Conidia are rapidly distributed through the plant via transpiration system. Mycelium and gum blocks the vascular tissues and first symptoms of yellowing are observed in the older leaves. **(E)** *Foc* colonizes and destroys more vascular tissues provoking intense wilting. **(F)** Infected plant dies and the follower plant (daughter), which was contaminated by the mother plant through vascular connection, shows initial symptoms. Mother plant eventually falls down and disease cycle starts again.

identified seven *SIX* genes (*SIX1*, *SIX2*, *SIX6*, *SIX8*, *SIX9*, *SIX10,* and *SIX13*). Strains belonging to R1 and R2 generally share the same profile of *SIX*. In contrast, strains of SR4 and TR4 presented more diverse profiles (Czislowski et al., 2017). The homologues, *SIX1* and *SIX9* were conserved in all VCGs, while no *SIX* genes were observed in a non-pathogenic *F. oxysporum* strain analyzed (Czislowski et al., 2017).

Transcriptome analysis revealed more virulence-associated genes up-regulated in *Foc* R4 than in R1, suggesting a more active pathogenesis in R4 interactions (Guo et al., 2014, 2015). While the role of *SIX* genes in pathogenicity against banana needs to be further demonstrated, the differences between *Foc* TR4 and R1 regarding composition and copy numbers of *SIX* genes seems to be a plausible hypothesis for their differential virulence patterns. However, comparisons between *Foc* R1 isolates regarding *SIX* genes need to consider that they can belong to different VCGs and lineages in spite of being classified as the same race. Czislowski et al. (2017) observed a discordant pattern among the evolution of *SIX* genes and housekeeping genes (*EF-1α*, *RPB1,* and *RPB2*) and also showed strong evidences of horizontal gene transfer (HGT) of *SIX* genes in *Foc*. Findings supporting HGT were recently reported when sequences of *IGS* and *EF-1α* were analyzed. One of the sequences belonging to pathogenic isolates, were identical to the largest group of local nonpathogenic individuals, while all pathogenic isolates had identical sequences of *SIX1* genes (Deltour et al., 2018).

Once penetration occurrs and *Foc* overcomes the first host barriers, the pathogen produces thickened hyphae and microconidia. The thickened hyphae then develop into chlamydospores in intra- and intercellular spaces (Li et al., 2011). A few hyphae can be seen in xylem vessels and growing in the root cortex before 10 days after inoculation (Li et al., 2011, 2017; Xiao et al., 2013). A hyphae network develops in the intercellular spaces along the junctions of root epidermal cells and also could be observed in xylem of the rhizome after some days (Li et al., 2011; Guo et al., 2015). Once *Foc* reaches the vascular zone of the lateral roots, the rhizome infection will occur (**Figure 2C**). *Foc* colonization of the rhizome vascular bundles occludes the vessels interfering with nutrient uptake and upward water transport to the pseudostem and leaves (Li et al., 2017). Rhizome infection is the most important step to disease development (Li et al., 2017). Once the rhizome was colonized the infection becomes systemic reaching the pseudostem (**Figures 2D,E**). According to Xiao et al. (2013), a large amount of hyphae is observed in the pseudostem at 17 days after the inoculation (dpi) and the plant may die at 24 dpi.

The infection process investigated under controlled conditions seems to reflect what happens in the field, but disease development and symptom expression may vary depending on

different factors (see **Factors driving disease intensity**) such as cultivar resistance. Under natural conditions, the disease is mostly perceived at flowering (**Figure 2D**). However, highly susceptible cultivars in the presence of high inoculum pressure may show external symptoms as early as 3 months after planting. Quick disease development has been observed in *Foc* R1 in Silk (AAB) in Brazil, *Foc* R2 in Bluggoe (ABB) in Nicaragua and *Foc* TR4 in Cavendish (AAA) in Taiwan. Apparently, under natural conditions, most conidia and chlamydospores produced by *Foc* return to the soil when the plant dies (**Figure 2F**). Chlamydospores may remain dormant or in secondary hosts for several years (**Figure 2A**) or start a new disease cycle (**Figure 2A**) immediately when favorable conditions and a susceptible host are available (Stover R., 1962). The perennial monocrop system used for banana is propitious for continuous FW cycles and an increasing inoculum build up.

PATHOGEN DISPERSAL

Apparently, *Foc* does not spread in soil by active vegetative growth as other soil-borne pathogens, such as *F. solani*, *F. pallidoroseum, Rhizoctonia* sp. and *Pythium* sp. (Stover and Waite, 1960; Stover R., 1962; Trujillo and Snyder, 1963). Dispersal mainly takes place by passive movement of pathogen propagules at short and long distances, from farm to farm or other locations locally or between countries or continents. Long distance dispersal is mainly due to anthropogenic-related factors, while dispersal at short distances may be associated to both anthropogenic and natural factors, such as water runoff, animal movement or spore-bearing soil.

The few studies on the spatial dynamics suggest that randomly distributed infected plants can be found in the field at the onset of the epidemics, but thereafter the main process for disease dissemination in the field is plant-to-plant movement, which ultimately leads to the aggregated pattern commonly seen in many areas (Meldrum et al., 2013). As a soil-borne pathogen, agents capable of moving soil particles and spores in the soil, including water, contribute significantly to pathogen spread. The movement of plant parts, including planting material, also appears to have a prominent role. A summary of main dispersal agents is detailed in **Figure 3** and discussed below.

Plant Materials

Once a plant is infected and colonized by *Foc*, production of chlamydospores in the root system, rhizome, and pseudostem is triggered (Li et al., 2011). Pathogen structures have also been found in the petiole and the pathogen DNA was also detected in leaves (Lin et al., 2009). Once a *Foc*- infected plant completes its cycle or is killed by *Foc*, these structures also die. While the aboveground parts and the rhizome can be readily destroyed, the roots remain in the soil. Therefore, if *Foc* was actively growing and colonizing the root system, spores could remain in the soil and become part of the arsenal of pathogen inoculum. Studies testing the role of banana roots on short-distance dispersal of *Foc* are needed to elucidate the possible involvement of banana roots as inoculum reservoir. These findings may have direct impact on crop and disease management.

Infected mother plants are able to transfer fungal structures to the suckers, which typically remain symptomless due to the long latent period of the disease (Stover R., 1962). Therefore any infected plant material, including symptomless suckers, is a potential inoculum source and can also disperse the pathogen. Planting material has been (and continue to be) one of the most important factors for FW dissemination (Ploetz, 2005; Dita et al., 2010; Pérez-Vicente et al., 2014). Additionally, banana leaves and pseudostem are frequently used for wrapping or packing banana. Infected plants used in compost preparation result in contaminated substrate which is readily transported from place to place.

As yet, no scientific evidence has been published of dispersal of *Foc* in banana fruits, but better understanding of this risk is required. A recent study related to the presence of *Foc* TR4 in the Middle East included the analysis of this possibility (Ploetz et al., 2015). A publication from Australia indicates that *Foc* could move as both symptomless infection of the vessels in fruit crowns, and in pieces of infected leaf trash associated with fruit shipments from The Philippines (Commonwealth of Australia, 2004). In summary, the movement of plant parts either to be used as propagation material or as a result of agricultural practices can imply significant risks at local, regional, and continental levels and should be strictly controlled in *Foc*-affected areas.

Water

Heavy rainfall hits the soil and drops containing contaminated fragments of infected plant materials and soil particles can splash causing short-distance *Foc* dispersal. Likewise, runoff flow may carry soil and plant parts and contaminate new clean areas, drainage canals, irrigation reservoirs and rivers. Once water coming from contaminated sources is used in irrigation or naturally reaches *Foc*-free areas, the pathogen spreads rapidly, and efficiently into and between banana fields. For instance, the rapid spread of *Foc* TR4 in China has been frequently associated to both infected planting material and irrigation water from the Pearl River (Xu et al., 2003). In areas naturally predisposed to flooding, the risk is even higher. Natural phenomena such as hurricanes and typhoons can boost *Foc* dispersion not only by direct spreading of *Foc* to the banana plantations, but also by contaminating irrigation water sources as recently observed in the Philippines (Trueggelmann, Unifrutti/Bloom Agro, *personal communication*). In this sense, an indirect effect of water might also occur when tissue-culture (TC) plants under acclimatization in greenhouses are irrigated with *Foc*-contaminated water. These TC-plants, though symptomless, might be infected and effectively spread the pathogen to the field. Therefore, TC-plants providers should enforce a strict quality control of water sources. This should be not only applicable for water, but also for TC-plant substrates as they could also be *Foc*-contaminated. Therefore, the whole acclimatization process, including water and substrate quality, should be reviewed to certify TC-planting material is *Foc*-free.

FIGURE 3 | Factors associated to pathogen spreading in Fusarium wilt epidemics in bananas. First incursion (upper left). Vehicles (middle). Planting material (upper right). Animals (upper left). Workers (bottom left). Water (in blue). These factors may operate separately or in association to disperse *Fusarium oxysporum* f. sp. *cubense* structures short or long distances.

Soil and Substrates

Dispersal of *Foc* in soil is associated with vectors of soil movement. Roots or other debris of infected plants, water, wind, insects, animals, tools, machinery tires, and other equipment can carry soil particles containing *Foc* propagules. Studies in other pathosystems indicate that *F. oxysporum* does not move significant distances in soil without the presence of a host roots, plant materials or other dispersal agents (Trujillo and Snyder, 1963; Rekah et al., 1999). Recent research has estimated the amount of *Foc* TR4 spores in soil samples from the Philippines and demonstrated that it is possible to detect the presence of significant inoculum levels in soil adhered to shoes after a short field visit (Gert Kema, *personal communication*). Substrates, organic amendments or even plants other than bananas may disperse *Foc*. For instance, in the coffee zone of Central America where bananas are associated with agroforestry coffee, *Foc* R1 is dispersed with coffee seedlings prepared with *Foc*-infected substrates. The growing trade of substrates, including those with coconut fibers produced in Asia, is potentially risky for the dispersal of *Foc* at transcontinental levels. Further studies of population dynamics of *Foc* in the soil at different layers are necessary to better understand how the pathogen survives, spreads, and infects plants in substrates.

Wind

The hypothesis that winds accompanied by rains can disperse *Foc* has not yet been confirmed. In dry regions, wind carrying contaminated dust particles could also be a dispersal agent of *Foc*. While aboveground sporulation has not yet been reported in banana, it is necessary to consider this epidemiological factor. Field-based studies on the aerobiology of FW are urgently needed to verify not only the formation of external spores, but also its mode of infection. The use of spore traps was useful to capture airborne conidia of *F. oxysporum* f. sp. *radicis-lycopersici* (*Forl*) and understand its epidemiology in tomato (Rekah et al., 2000). A similar approach could be used to assess the airborne spread of *Foc* and its role on FW epidemiology. In fact, aerial conidia production has been already reported in other soil-borne *formae speciales* of *F. oxysporum,* such as *F. oxysporum* f. sp. *lycopersici* (tomato; *Fol*), *F. oxysporum* f. sp. *cucumerinum* (cucumber) and *F. oxysporum* f. sp. *basilici* (basil; *Fob*). The capacity of airborne spores to cause above ground infections is either unclear or controversial. Experimental inoculation of stem wounds with conidia of *F. oxysporum* f. sp. *cucumerinum* failed to establish infections on cucumber, suggesting that airborne spores are deposited on the substrate surface and infection occurs primarily through the root (Scarlett et al., 2015). In contrast, infection through leaf wounds by airborne propagules of *Forl* in tomatoes and *Fob* in basil have been reported (Rekah et al., 2000).

While the production and dispersal or aerial inoculum of *Foc* under field conditions, as well as its potential to infect banana through above ground tissues need to be further investigated, heavy winds, either associated or not with rain, can effectively transport contaminated soil particles and plant debris from infested to disease-free areas. Therefore, wind should be considered as a dispersal agent when analyzing FW epidemics.

Animals

A range of vertebrate and invertebrate animals may inhabit banana plantations. These animals can transport soil particles from infested to *Foc*-free areas. Banana weevil borer, *Cosmopolites sordidus* (Germar; Coleoptera: Curculionidae), widespread in banana plantations, is the most important insect pest of bananas and plantains (Gold et al., 2001) and recent studies in Australia demonstrated the presence of viable spores on their exoskeletons (Meldrum et al., 2013). Although this finding means that *C. sordidus* may be a carrier of *Foc*, further studies are needed to determine whether weevils are effective vectors of *Foc*. *Metamasius hemipterus* L. (Coleoptera: Curculionidae), a false weevil frequently found in banana fields might also have a role on *Foc* dispersion, but no study was found on this subject. In smallholder farming systems or plantations close to human settlements, domestic animals could also contribute to farm-to-farm dissemination of FW. For example, since the detection of *Foc* TR4, rigorous campaigns to reduce feral pig populations have been implemented in the Tully Valley, Australia, as these animals are recognized as a serious vector of soil-borne fungal diseases (Biosecurity of Queensland, 2016). The potential role of subterranean vertebrate pests as *Foc* disseminator has not been studied, for example, pocket gopher, one of the most important vertebrates in banana fields in Central America (Monge-Meza, 2011). In addition, the putative role of other insect vectors needs further attention. For instance, fungus gnats (*Bradysia* spp.) are vectors of *Fusarium* spp. in greenhouses and nurseries in ornamentals (Gullino et al., 2015) and tomatoes (Gillespie and Menzies, 1993). Likewise the implication of shore flies (*Scatella stagnalis*) in the transmission of *Fol* and *Forl* to tomato has been reported (Matsuda et al., 2009). Finally, as a biosecurity rule, mainly for quarantine-declared areas, fencing preventing the transit of animals and the other means of dissemination need to be implemented to reduce risks.

Anthropogenic Factors

Humans play a key role dispersing plant pathogens and *Foc* is not an exception. The current globalized world increases movement of goods, people and trades. Therefore, the risk of introducing *Foc* through anthropogenic factors is clearly high. Rapid dispersal of *Foc* by asymptomatic, but infected, suckers as planting material was crucial for the spread of *Foc* R1 that devastated Gros Michel bananas in the past century (Stover R. H., 1962; Ploetz and Pegg, 2000; Pérez-Vicente, 2004). Likely, suckers are the main factor involved in *Foc* dispersal between nearby farms. Recent transcontinental jumps of *Foc* TR4 from Southeast Asia to Africa or Middle East suggest that anthropogenic factors may be involved. Either the introduction of infected planting material or boots or tools contaminated with *Foc*-infested soil may have been responsible for this process (Ploetz et al., 2015). All farm equipment, clothes, footwear, tools, containers, etc., which had been used in *Foc*-infested areas could transport and spread the pathogen into disease-free areas. The aforementioned is also applicable at farm scale. Unfortunately, in most cases growers do not prevent disease dissemination on a daily basis, either by lack of knowledge or by the lack of capacity or resources. Once *Foc* reaches a disease-free farm, epidemics may quickly develop. Similar dynamics of spread, seen with *Foc* R1 in the last century, are being repeated today for *Foc* TR4 (Ordoñez et al., 2015; Ploetz et al., 2015).

FACTORS DRIVING DISEASE INTENSITY

Diverse biotic and abiotic factors may accelerate or slow down *Foc* infections and consequently FW epidemics. Obviously, host resistance and pathogen aggressiveness are two key factors driving epidemics. Unfortunately, comparative studies considering the diversity of both *Foc* and banana germsplasm, in the same environmental conditions and production systems are scarce.

BIOTIC FACTORS

Nematodes

As a soil-borne pathogen that can penetrate the host by wounds, any external factor promoting root damage may facilitate *Foc* infections. In this sense, attack of plant parasitic nematodes may boost FW epidemics in banana. However, in spite of the importance of the burrowing nematode (*Radopholus similis*) as a banana constraint, studies to assess the interaction between *R. similis* and *Foc* at field level are limited, probably due to the fact that *R. similis* became a serious problem only after the introduction of Cavendish cultivars, which are resistant to *Foc* R1. Under greenhouse conditions (Somu, 2012; Dinesh et al., 2014) the combined inoculation of *R. similis* and *Foc* increased the incidence and severity of FW. However, this positive interaction between these pathogens was not observed in another greenhouse study (Chaves et al., 2014). The intensity of FW in Gros Michel bananas was not influenced when co-inoculated with *R. similis* and *Foc* R1. However, co-inoculated plants showed a significant root weight reduction compared with plants only inoculated with *Foc* or *R. similis*. The high susceptibility of Gros Michel to *Foc* R1 may have prevented detection of additional effects. Therefore, the analysis of the role of nematodes in FW epidemics should also consider cultivar resistance to *Foc*. Until more data are available on *Foc–R. similis* interaction at field level, the impact of nematodes should not be ignored as they can cause severe damage in banana. Production areas where both *Foc* TR4 and *R. similis* are present offer good opportunities to better understand the role of this nematode on FW epidemics in banana at field level. Possible interactions of *Foc* with other genera of nematodes should also be examined. In some banana production areas in Brazil, *Meloidogyne*, *Helicotylenchus*, *Rotylenchus*, and *Pratylenchus* are more prevalent and destructive than *R. similis* (Almeida et al., 2018). Larger populations of *Pratylenchus* spp., migratory endoparasites, were found in areas highly affected by FW. Almeida et al. (2018) suggest that nematode wounds to the roots may facilitate infection and colonization of *Foc*. Studies in other crops show that nematodes can boost FW epidemics. Races 1 and 2 of *F. oxysporum* f. sp. *vasinfectum* (*Fov*) are particularly devastating in cotton when the root-knot nematode, *M. incognita*, is present (Garber et al., 1979). In the absence of nematodes, these *Fov* races cause mild disease (Jorgenson et al., 1978). Likewise, root-knot nematodes increase severity of FW

F. oxysporum f. sp. *niveum* (*Fon*) in watermelon (Martyn, 2014) and the tobacco cyst nematode (*Globodera tabacum*), has also been associated with higher severity of FW (*F. oxysporum* f. sp. *nicotianae*) in tobacco (LaMondia, 2015).

Weevils

C. sordidus and *M. hemipterus*, may also act as a predisposing factor for FW epidemics, but, as with the nematode-*Foc* interaction, this arthropod-*Foc* interaction also needs further analysis beyond the general assumption that weak plants are more susceptible to *Foc*. Comparative analysis between FW-infested and FW-free areas regarding population density and rhizome damages could help to better understand the influence of weevils attack on FW in banana. In addition, the putative role of weevils on *Foc* dissemination may also impact epidemic as previously discussed.

Soil and Plant Microbiome

Since soil suppressiveness was conceived and documented, soils with an active and functionally diverse microbiota are assumed to have a higher capacity to suppress FW (Doran et al., 1996). In contrast, soils with poor biological activity and unbalanced food web would be more conducive. A suppressive soil is that in which the pathogen does not cause high levels of disease or no disease occurred, even in the presence of the pathogen, a susceptible host and appropriate environmental conditions. On the other hand, in a conducive soil even low levels of pathogen inoculum can cause serious damages.

In the last five years, with the advent of more powerful tools to evaluate soil microbiome and its functional diversity, significant progress on banana-*Foc* interaction with the microbiome has been made. Shen et al. (2015) studying suppressive soils to FW of banana in the Hainan Island, China, found higher richness and diversity indices as well as more operational taxonomic units in the suppressive than in the conducive soils. *Chthonomonas* spp., *Pseudomonas* spp. and *Tumebacillus* genera were significantly enriched in the suppressive soil. Likewise, a study also performed in Hainan by Xue et al. (2015), identified *Bacillus* spp. as the most dominant bacterial group isolated in a *Foc*-suppressive soil followed by *Rhizobium, Bhargavaea, Pseudolabrys*, and *Sinorhizobium*. Recently, Köberl et al. (2017) studying healthy versus *Foc*-infected banana plants in Central America, found higher richness and diversity of *Gammaproteobacteria* in healthy plants. In addition, healthy plants also revealed an increase in potentially plant-beneficial *Pseudomonas* and *Stenotrophomonas* species.

As previously stated *Foc* may be dispersed through infected planting material. Therefore, the use of TC-planlets is recommended to reduce this risk. However, TC-plantlets have been shown to be more susceptible to FW than conventional suckers (Smith et al., 1998). One hypothesis to explain this behavior is that during the TC-process beneficial microorganisms are removed, leaving plantlets more vulnerable. Lian et al. (2008) demonstrated that *Bacillus* spp. and *Pseudomonas* spp. were induced upon *Foc* inoculation and were able to colonize and increase the protection of bananas roots against this pathogen. Thus, the soil and plant microbiome may affect FW epidemics either by creating *Foc*-suppressive environments in the soil or by hindering host penetration and colonization. The identification of key microorganisms is proposed as a first step to rebuild the microbiome of TC-banana plants prior to planting, not only to improve defense responses against *Foc* (Forsyth et al., 2006; Weber et al., 2007), but also against nematodes (Vu et al., 2006) and to promote plant growth (Ting et al., 2012).

As aforementioned, there are several factors that might reduce or promote FW in banana including the resistance level of the cultivars, which was not discussed in this section. *Foc*-banana is a complex and multifactorial interaction. Thus, factors like pH, P-content or N-sources may reduce FW intensity in some cases, but only slightly affect disease intensity in other situations such as when affected by a virulent *Foc* variant, in the presence of nematodes in high numbers or even in an environment that favors *Foc* dispersion. There is an urgent need for research in these different aspects of the interactions related to FW epidemics in banana.

Finally, the impact of the environment should not be ignored. For instance, why do some *Foc* populations (*Foc* SR4) only affect Cavendish clones in the subtropics? A common assumption is that cold reduces banana plant defenses (Moore et al., 1993; Ploetz et al., 2015), but which defense genes are affected? What is the role of other factors, such as the decomposition rate of organic matter? Are antagonist soil microorganisms less competitive seasonally in the subtropics? Is the microbiome functional diversity compromised over the cold season in the subtropics? Answering these questions might help a better understanding of FW epidemics of banana both in tropical and subtropical conditions.

ABIOTIC FACTORS

Physical and chemical soil characteristics can also influence disease intensity, making the soil suppressive.

Physical Properties

The physical structure of soils has been associated with FW in banana, but so far comparative studies are scarce. In general, well-drained and aerated soils are assumed to reduce FW by improving root development and microbial activity (Stover and Simmonds, 1987). In contrast, soils with high levels of compaction and lower aeration may favor FW. However, physical indicators distinguishing conducive and suppressive soils to *Foc* need to be better understood. Studies conducted by Domínguez et al. (2001) in the Canary Islands indicated that higher values of water-stable aggregates were associated with conducive soils, whereas high clay content was consistently higher in suppressive soils. On an agroforestry farm in Brazil, higher clay content was correlated with higher suppressiveness of soil patches to *Foc*, while sand and silt with conduciveness (Deltour et al., 2017). In contrast, in India, sandy loam or sandy clay loam types of soils with low bulk density are more suppressive to FW of banana, while clay soils with high bulk density are more conducive (Felcy-Navajothy et al., 2012). Recent studies comparing clean and infested areas reported an association between higher values of soil penetration resistance and soil density with higher FW

intensity in banana. Although studies in different sites have generated contrasting results as seen above, the role of soil physical properties should continue to receive attention by both researchers and growers.

Chemical Properties

Synthetic fertilizers have impacted global agriculture since the green revolution and the banana crop is not an exception. The source and levels of these compounds can not only influence yield, but also the intensity of diseases (Huber and Watson, 1974) and in the case of soil-borne pathogens like *Foc* this interference may be more complex because of the direct impact on the pathogen habitat. Soil pH, which is influenced by many factors, is a fundamental variable. Higher levels of FW in banana are consistently associated with lower pH values (Domínguez et al., 2001; Nasir et al., 2003; Deltour et al., 2017). In fact, practices that reduce soil pH values, such as the application of urea and ammonium as sources of nitrogen (N) have been historically associated with severe epidemics of FW in bananas (Sequeira, 1958; Stover R., 1962; Nasir et al., 2003). However, questions on whether lower pH values cause a shift in the soil microbiome, interfering with plant resistance or enhancing pathogen virulence have been raised. In this scenario the type of N source, ammonium or nitrate, plays a fundamental role. Nitrate (NO_3^-) generally increases the pH near the rhizosphere, whereas ammonium (NH_4^+) reduces it. It is also generally assumed that ammonium applications boosts FW epidemic, whereas nitrate reduces it (Mur et al., 2016). However, the differential effects of these N sources on FW are not solely due to the impact on soil pH. According to Dong et al. (2016), nitrate contributes to increase the lignin content in banana after *Foc* infection and also improves the absorption of resistance-related nutrients thereby maintaining a higher photosynthetic rate and high disease resistance. In contrast, ammonium keeps the lignin content relatively stable and does not improve nutrient uptake. In addition, the effect of urea and other ammonium-based sources of N on increasing FW might also be related to citrate regulation (Wang et al., 2016). These authors associated the regulation of citrate exudation with the increase of FW intensity in cucumber. Nitrate significantly suppressed the disease compared with ammonium. Interestingly, in the ammonium-treated plants, citrate enhanced pathogen spore germination and penetration, increasing both disease incidence and pathogen population (Wang et al., 2016). While the impact of these N sources in the microbiome remains to be better understood in banana, the hypotheses that ammonium also suppresses population density of bacteria in soils deserves more attention. In summary, the available data so far indicate that lower pH values and ammonium-based sources of N increase FW in banana. However, changing pH on soils suppressive to FW in banana had little effect on disease severity (Peng et al., 1999). Therefore, the relation of N (sources and levels) and pH need to be addressed in management strategies (see **Management practices oriented to soil health and suppressiveness**).

Phosphorous (P) has been shown to be very important on root development and consequently may have direct implications on soil-borne pathogens like *Foc*. Comparative analyses of infested and clean areas in Brazil revealed that low soil P availability was associated with high FW incidence (Furtado et al., 2009). Interestingly, higher P levels were also correlated with FW suppression in banana fields in China (Shen et al., 2015). As P plays an important role in root development in banana, ensuring adequate P over the crop cycles (mainly at planting and in management of ratoon suckers) seems essential for better FW management.

Banana is a highly potassium-demanding crop, but consistent data on the role of potassium (K) on FW intensity, other than K-deficient plants are more susceptible to diseases, is lacking. In other crops, such as cotton, oil palm, tomato and muskmelon, relevance of K in reducing FW has been documented (Perrenoud, 1977). Long-term experiments to decipher the role of K on FW in bananas need to be conducted, as high K levels are always recommended at flowering, when, coincidentally, FW symptoms are more evident.

Applications of calcium (Ca) and magnesium (Mg) seem to reduce FW in banana and the effects are commonly associated to increasing pH values. However, adding $CaCO_3$, $Ca(OH)_2$ or $CaSO_4$ to the soil reduced the germination of chlamydospores and FW severity in banana without changing soil pH (Peng et al., 1999). In addition, Furtado et al. (2009) also found that Ca and Mg levels in the soil were significantly lower in banana areas affected by FW when compared with healthy sites, without any relation with pH.

Silicon (Si) application can also reduce FW symptoms in greenhouse conditions (Fortunato et al., 2012a). The reduced intensity of FW in Si-treated plants was correlated with higher concentrations of hydrogen peroxide (H_2O_2), total soluble phenolics and lignin-thioglycolic acid derivatives and greater activities of enzymes, like as phenylalanine ammonialyases, polyphenoloxidases, peroxidases, β-1,3-glucanases, and chitinases (Fortunato et al., 2012b). While a clearer effect of Si on the reduction of FW at field levels remains to be demonstrated, the application of Si should be considered for bananas at pre-planting as it can improve both the nutrient balance and boost plant defenses against pathogens (Wang et al., 2017).

The role of micronutrients in promoting or reducing FW in banana is less clear and in some case contradictory. According to Domínguez et al. (2001), available iron (Fe) is more abundant in soils where FW of banana is more severe in Canary Islands. The same authors suggested that higher content of Fe might promote *Foc* spore germination and increase disease severity. However, adding Fe-EDDHA (Fe 6%) to the soil reduced germination of *Foc* and FW severity in banana experiments conducted in Australia (Peng et al., 1999). The potential role of siderophore-producing bacteria, such as *Pseudomonas* spp., to reduce chlamydospores germination of *F. oxysporum* f. sp. *cucumerinum* has also been reported (Simeoni et al., 1987).

Fusarium wilt appeared to be more severe in bananas growing under Zn deficient conditions (Borges-Pérez et al., 1983, 1991). A possible role of Zn on improving tylose formation was suggested (Borges-Pérez et al., 1983). However, experiments conducted with *Foc* SR4 in Canary Islands failed to show a response (Hecht-Buchholz et al., 1998). These authors suggest that alterations in

the ultrastructure of chloroplasts and mitochondria may be the link between Zn deficiency and FW intensity.

In general macro- and micro-nutrient deficiency or inadequate use could be linked to high FW intensity, but also to many other diseases (Mur et al., 2016). Isolating single nutrient responses or interactions in fields with patchy *Foc* presence and variable soil biotic conditions remains a challenge.

OPTIONS TO MANAGE FUSARIUM WILT OF BANANAS

Considering the epidemiological aspects of *Foc* and the perennial and monoculture nature of most banana plantations, it is evident that FW management is not simple, unless a resistant and commercially accepted cultivar is available. The use of resistant cultivars is frequently stated as the only effective measure to manage this disease (Ploetz, 2015a,b). However, resistant cultivars might not match market's demands and resistance may be overcome by new pathogen strains, as is the case of Cavendish and *Foc* TR4. Unfortunately, the "dogma" on the effectiveness of cultivar resistance as the only plausible option to manage FW in banana may lead to insufficient emphasis on exclusion, biosecurity, soil management, as well as, innovative alternative options to reach integrated and long-term disease management approaches. Some of these measures can avoid or delay disease epidemics, reduce disease intensity and also enhance yields. Below we discuss a set of field options to manage FW of banana from exclusion to integrated disease management approaches, considering not only aspects of FW epidemiology, but also practices to enhance crop production.

Exclusion

Pathogen exclusion is a key measure to manage plant diseases, particularly those that do not occur in a given area, such as is currently the case with *Foc* TR4 in Latin America and the Caribbean (LAC), a large part of Africa and even in countries in Asia where TR4 has recently been detected. Therefore, major emphasis must be given to preventive measures at plot, farm, country, regional, and continental levels to avoid the entrance of pathogens. Exclusion has gained increasing interest to prevent or delay the entrance of the highly destructive *Foc* TR4. In this sense in LAC, the potential impact of *Foc* TR4 has been discussed in many technical events and action plans have been proposed to avoid the entry of this strain (Dita et al., 2013). However, exclusion and quarantine measures are extremely dependent on diagnostic tools, awareness, preparedness, readiness and a legal framework supported by National and Regional Plant Protection Organizations (N/RPPOs).

Different diagnostic methods to identify *Foc* TR4 (VCG 01213/16) are currently available and have supported the decision-making process of plant protection officers worldwide. Some of these methods are specific for VCG 01213/16, which is the strain globally recognized as *Foc* TR4 (Dita et al., 2010; Zhang X. et al., 2013). However, other tools detect more than one VCG. For instance, the methods described by Lin et al. (2009, 2016) react with eight different VCGs, in addition to 01213/16 (Dita et al., 2010). The method proposed by Aguayo et al. (2017) detects VCG 01213/16, but also VCG 0121. While VCG 0121 and 01213/16 are genetically related, it is important to consider that VCG 01213/16 is currently the only widely recognized as *Foc* TR4, and more importantly, the only one officially listed as a quarantine pest by many N/RPPOs worldwide. In this sense, regulatory agencies need to verify carefully each diagnostic tool and conceive the diagnostic as a process with different steps that starts in the field (adequate samples, expertise on the disease) and is later complemented with different laboratory techniques as described by O'Neill et al. (2016).

In spite of the importance of *Foc* TR4, FW epidemics in banana are not caused only by this race. There are non-TR4 strains currently causing serious epidemics and yield losses in Nicaragua, Peru and Brazil. Exclusion should also be implemented within country in these situations. Unfortunately, rapid and efficient tools to detect these strains are still missing. A generic diagnostic method to detect any banana-pathogenic strain of *Foc,* independently of the VCG to which it belongs, would improve biosecurity and quarantine measures to support epidemiological studies and consequently management tactics. While these diagnostic tools are not available, biosecurity measures should be implemented from country borders to the farm gates and should go further, not only targeting *Foc* TR4, but other pests and diseases of socio-economic relevance.

Certification agencies will play an important role to reduce the entrance and spread of *Foc* TR4 with a requirement for biosecurity measures at farm level as recently implemented by Global Gap (https://www.globalgap.org/uk_en/for-producers/globalg.a.p.-add-on/tr4-biosecurity/).

Eradication of Infected Plants and Pathogen Containment

Many practices to eradicate *Foc* have been tested, but there are no reports on complete elimination of the pathogen. Therefore, once *Foc* is established in a field, management strategies must be focused on avoiding the spread of the pathogen to disease-free areas and decreasing the level of inoculum in the infested area. Destruction of infected plants to reduce inoculum build-up and prevent pathogen spread is a fundamental starting point. Efficient inoculum reduction of *Foc* has been achieved by treating infected plant materials with urea under anaerobic environments (Biosecurity of Queensland, 2016).

Even as infected plants are being destroyed, the analysis of risk of pathogen movement into new areas should be detailed (see **Pathogen dispersal**). Strict biosecurity practices at the farm's gate and around the complete perimeter are essential to lock down contaminated areas. The following steps can support containment efforts: (1) Minimize the access of outsiders to farms and packing houses with zero access to areas where risk is highest, (2) Shoes and tools used by employees and visitors should remain in the farm, (3) Implement practices to minimize movement of soil and water from contaminated areas, (4) Foot and vehicle baths with proper disinfectants available on strategic points in the banana plantation and packinghouses; (5) Build capacities on disease diagnostic, epidemiology and biosecurity among all

persons associated with farm, and (6) Maintain communication channels with biosecurity officers and NPPOs to report any new suspicious plant or unexpected violation of containment.

Resistant Cultivars

Once FW is established in the area, the use of resistant varieties is the most effective means to manage this disease. The resistance of Cavendish to *Foc* R1 has had an enormous impact on the banana industry contributing to near complete Cavendish dominance in export trade. This resistance has been effective for about 50 years and it is an iconic case of resistance durability in crops to pathogens. In general, resistant cultivars are effective for less than 10 years (Johnson, 1984; McDonald and Linde, 2002). To date, there are no commercial cultivars resistant to *Foc* TR4 with similar levels of resistance of Cavendish to *Foc* R1.

To better understand the levels of resistance and its implication on FW management of bananas, three terms, considering the presence of *Foc* and favorable environmental conditions, are discussed below.

Complete Resistance

This definition, also called, qualitative resistance, is illustrated by the Cavendish (AAA)-*Foc* R1 interaction. *Foc* R1 does not cause any physiological disturbance, does not affect yield and there is no increase in inoculum levels in the field.

Intermediate Resistance

Also referred to as quantitative resistance, cultivars with intermediate resistance show less severe symptoms or damage than susceptible varieties when grown under similar environmental conditions and inoculum pressure. The Prata (AAB)–*Foc* R1 interaction can illustrate this definition. In this case, the inoculum density might be driving the intensity of FW. Prata can resist the infection by *Foc* R1 only up to certain levels of inoculum density. Under environmental conditions and management practices favorable to *Foc* (see **Factor driving disease intensity**) FW and yield losses will increase gradually.

Susceptibility

This definition can be illustrated with different interactions, such as Gros Michel-*Foc* R1, Cavendish-*Foc* TR4 and Silk-*Foc* R1. Severe epidemics of *Foc* TR4 in Cavendish and *Foc* R1 in Silk are often recorded and plantations may be totally destroyed in <2 cropping cycles. In these varieties, *Foc* infects the host and causes serious physiological disturbances and yield losses. Inoculum levels in the soil increase dramatically preventing new plantations of the same cultivar from being re-established in the same area.

Ideally, **complete resistance** should be used. If complete resistance is available, then cultural practices are mainly oriented to increase yield and to control other pests and diseases as has been the case of Cavendish plantations in Asia before the emergence of *Foc* TR4. However, after the emergence of *Foc* TR4, several Cavendish somaclones called Giant Cavendish Tissue Culture Variants (GCTCV) developed in Taiwan (Hwang and Ko, 2004) with **intermediate resistance** to *Foc* TR4 have been planted. Two clones of the first generation of GCTCV, GCTCV-118 and GCTCV-119, and recurrent selection based on the first somaclones, resulted in GCTCV-218 and GCTCV-219 which are being used to mitigate losses caused by *Foc* TR4 in Taiwan and the Philippines and have recently been planted in Mozambique.

Reduction in FW intensity using these somaclones in infested areas where regular Cavendish varieties cannot be grown has been widely communicated in international meetings. Peer-reviewed publications on the status and dynamic of soil inocula and disease incidence in commercial banana fields planted with these somaclones are not available yet. However, as mentioned, resistance to *Foc* TR4 is not **complete**, but **intermediate** (Hwang and Ko, 2004). Therefore, depending on inoculum pressure diseased plants can be observed as early as the first crop cycle. In all cases management practices, such as early detection and destruction of infected plants and a set of biosecurity measures for containment are recommended (see **Integrated management practices**).

Intermediate resistance has an enormous value (Corwin and Kliebenstein, 2017), mainly when no resistant commercial cultivars are available. However, the long crop cycle ($>$ 12 months) and perennial nature of bananas cultivation, bring additional implications for epidemiology and management, especially for growers used to a totally resistant variety, as Cavendish to *Foc* R1. Therefore, having certain levels of FW incidence in these Cavendish somaclones or in any other variety with intermediate resistance requires special attention to: (1) increasing inoculum levels of the pathogen in early infected patches, (2) pathogen dispersion from highly contaminated to less contaminated areas in the same farm and (3) pathogen dispersal from infested to disease-free areas. In fact, survival and inoculum buildup of *F. oxysporum* have been already reported in resistant varieties of other crops. For instance, *Fov,* can survive in the roots of resistant cotton cultivars, contributing to the maintenance of inoculum levels in the field (Cianchetta and Davis, 2015). Resistant tobacco varieties, while not exhibiting wilt disease symptoms, increased or maintained populations of *F. oxysporum* f. sp. *nicotianae* (*Fon*; LaMondia, 2015) and in lettuce, *F. oxysporum* f. sp. *lactucae* (*Fola*) can also colonize resistant lettuce cultivars (Scott et al., 2014). Thus, even when planting a resistant genotype, efforts in exclusion, eradication, containment, and integrated disease management need to be ongoing and more intensive with time.

Management Practices Oriented to Soil Health and Suppressiveness

Based on current understanding of FW epidemiology, management practices oriented to soil health and suppressiveness, such as crop rotation, the use of cover crops, application of organic amendments and biocontrol agents, as well as, the use of appropriate inorganic fertilizers and agronomic practices (see **Factors driving disease intensity**) can help suppress *Foc* inoculum, reduce disease intensity and enhance yields. The challenge is to identify the most effective practices for the pathosystem of a soil-borne pathogen with long-term survival capacity affecting a perennial crop with continuous cycles of flowering and harvesting. Promising components in FW management are emerging, although integrated field-scale validation is still incipient (**Table 1**).

TABLE 1 | Soil management practices and their effectiveness to control *Fusarium* wilt of banana under field conditions.

Management practice	Observed effects	Country	Genotype	Foc race*	References
Crop rotation and interplanting (*Manihot esculenta*)	Reduction of disease incidence. Low (less than 5%) incidence was maintained over three cropping cycles.	Indonesia	Cavendish (AAA)	TR4	Buddenhagen, 2009
Crop rotation (*Allium tuberosum*)	Reduction of disease incidence (up to 97 %) and improved crop value (up to 86 %). Antifungal volatiles released by *A. tuberosum* were associated with pathogen suppression.	China	Brazil (Cavendish, AAA)	TR4	Huang et al., 2012; Zhang H. et al., 2013
Crop rotation (*Ananas squamosa*)	Reduction of disease incidence up to 60% when compared with maize. Reduction of *Foc* abundances in the soil. Higher abundances of *Acidobacteria*, *Planctomycete* and *Chloroflexi* observed positively corresponded to *Foc* reduction.	China	Brazil (Cavendish, AAA)	TR4	Wang et al., 2015
Cover crop (*Arachis pintoi*)	Reduction of disease intensity by 20%. Increased the bunch weight in the second crop cycle.	Australia	Ducasse (Pisang awak, ABB)	R1	Pattison et al., 2014
Organic amendments and bio-organic fertilizers	Different organic amendments (cattle manure compost, pig manure compost Chinese medicine residue compost, bio-organic fertilizer -BIO) were compared with general operation control during one cropping cycle. Plants treated with BIO showed the lower disease incidence (20%) when compared with the control (38%). Pig manure showed highest incidence values. BIO improved soil microbial communities.	China	Brazil (Cavendish, AAA)	TR4	Shen et al., 2013
Bioorganic fertilizers	Continuous application of a bioorganic fertilizer (BIO) reduced the disease incidence (15%) when comparing with the control (40 %) over three cropping cycles. BIO also increases yields (up to 24%) and enriched culturable bacteria (*Firmicutes, Gammaproteobacteria* and *Actinobacteria*), potentially associated with pathogen suppression.	China	Brazil (Cavendish, AAA)	n.d.	Fu et al., 2016
Application of microorganisms	A set of 10 isolates of non-pathogenic *Fusarium oxysporum* (np*Foxy*) to banana reduced significantly the intensity of the disease in the greenhouse bioassays. When these isolates were tested in the field no disease reduction was observed. No disease reduction was observed with use of *Pseudomonas fluorescens* WCS 417 alone or combined with np*Foxy*.	South Africa	Cavendish (AAA)	SR4	Belgrove et al., 2011
Application of botanical formulations and biocontrol agents	Two botanical fungicides (Wanis 20 EC and Damet 50 EC), two, *P. fluorescens* strains (1, Pf1) and *Bacillus subtilis* (TRC 54) were tested individually and in combination under greenhouse and field conditions. Combined application (Wanis 20 EC + Pf1 + TRC 54) reduced disease incidence under greenhouse (64%) and field (75%) conditions.	India	Rasthali (Silk, AAB)	R1	Akila et al., 2011

* *Foc, Fusarium oxysporum f. sp. cubense; n.d., not determined.*

Crop Rotation

Widely used to manage soil-borne diseases in annual crops, crop rotation may be an option in some situations for a perennial crop like banana. Diversified farms may have access to different crops for crop rotation and intercropping as a strategy to manage FW. In this sense, crops with immediate market opportunities, such as cassava (*Manihot esculenta*), pineapple (*Annanas squamosa*) or plant species with different uses or purposes like Chinese leek (*Allium tuberousum*), have been used with different levels of success (Buddenhagen, 2009; Huang et al., 2012; Zhang H. et al., 2013; Wang et al., 2015; **Table 1**). However, in other cases crop rotation has not resulted in positive results. The use of velvet bean and sorghum did not contribute to reduce FW intensity as much as that obtained when sugarcane was used as succession crop (Sequeira, 1958). Later, sugarcane was used in combination with fallow with better results (Sequeira, 1962). However, in Taiwan, sugarcane was not recommended as a long-term strategy (Hwang, 1985). Crop rotation might have a direct effect lowering the *Foc* inoculum in the soil by creating a suppressive environment, an indirect effect by reducing or eliminating symptomless weed hosts or both. In most cases, the mechanism involved in the suppression of *Foc* has only been partially elucidated (**Table 1**; Huang et al., 2012; Zhang H. et al., 2013; Wang et al., 2015). Finally, it is important to consider the ability of *Foc* to colonize other crops. This capacity has been reported for other *forma speciales* of *F. oxysporum*, such as *Fov,* which in addition to cotton, can also infect soybean, flue-cured and burley tobacco, okra, alfalfa and lupine (Cianchetta and Davis, 2015), *Fola,* which colonizes three crops commonly grown in rotation with lettuce: broccoli, cauliflower and spinach (Scott

et al., 2014) and *Fon*, that infects both tobacco and sweet potatoes (LaMondia, 2015).

Cover Crops

The use of cover crops (also called ground cover or cover plant species) is considered a good agricultural practice for banana production and can help to manage weeds, pests, and diseases and also increase yield (Djigal et al., 2012; Pattison et al., 2014). Cover crops can be used before planting as green manure, as inter-planting or even as perennial species cultivated with banana. There are few studies available that assessed the effects of cover crops on FW. Pattison et al. (2014) found that Pinto peanut (*Arachis pintoi*) as ground cover reduced the intensity of FW in Ducasse bananas (Pisang awak, ABB) by 20% (**Table 1**). In addition, authors described a positive effect on yield through the increment of the bunch weight. In other FW pathosystems, such as in watermelon (Himmelstein et al., 2014) and cucumber (Klein et al., 2011), this practice has been successfully used. However, in other cases no positive effects have been observed (Njoroge et al., 2008). Similarly to crop rotation, cover crops can affect physical, chemical, and microbiological soil parameters and are also influenced by environment. Therefore, the selection of appropriate species or varieties, the planting rate and management need to be determined experimentally in each situation. One should always keep in mind that some cover species can eventually act as a host to *Foc* (see **Pathogen survival**).

Organic Amendments

Organic matter management is essential for soil health and suppressiveness (Noble, 2011; Larkin, 2015). Although organic matter can be added through crop residues and cover crops, off-field sources, such as organic amendments (OAs) are particularly important as they can be enriched with specific microorganisms (Noble, 2011; Hadar and Papadopoulou, 2012; Zhang et al., 2014). In addition, OAs can be applied at different dosages and in target sites, such as disease hotspots. Yogev et al. (2006) showed that composts based on plant-waste residues suppressed diseases caused by four different *formae speciales* of *F. oxysporum: melonis, basilici, radicis-lycopersici,* and *radicis-cucumerinum.* However, there are significant differences between banana and these annual crops, not only in terms of cropping cycle, but also in the amount of secondary inoculum produced per area. An infected banana plant may produce substantially more secondary inoculum than these annual crops. Therefore, the level of intervention to suppress *Foc* inoculum with application of OAs may need to be greater and integrated with other management practices such as the use of beneficial and antagonist microorganisms. In this sense, the susceptibility of *F. oxysporum* to competition for nutrients in the soil (Hadar and Papadopoulou, 2012) may facilitate its suppression if good competitors are in place. For instance, Fu et al. (2017) reported the suppression of FW in banana by the continuous use of an organic fertilizer. However, the effect of OAs on disease suppression may also be linked to biological control. In fact, the suppressive effect of OAs has increased by adding microorganisms (**Table 1**). Although the application of OAs is generally assumed to be beneficial to soil health, results can be variable and dependent on many factors (Bonanomi et al., 2010). This practice may also have risks as sometimes quality control of products is lacking (Hadar and Papadopoulou, 2012). For instance, the application of chicken manure increased the incidence of FW of banana in greenhouse experiments (Pittaway et al., 1999; Nasir et al., 2003). This pattern has been also observed in field conditions in Brazil, especially when using chicken manure without proper decomposition. The negative effect of chicken manure has been attributed to increased root damage, lower soil pH and the N source, which acted as predisposing factors (Nasir et al., 2003). In general, the application of composts alone often results in inconsistent levels of disease control (Lang et al., 2012). Therefore, further enrichment of composts with target microorganisms to produce the so-called, bioorganic fertilizers have been tested with promising results (Huang et al., 2011; Lang et al., 2012; Qiu et al., 2012; Zhang et al., 2014).

Biological Agents

The potential of beneficial microorganisms to control FW in banana has been studied since 1945 (Thaysen and Butlin, 1945; **Table 1**). Particular efforts have been made exploring the potential of non-pathogenic *F. oxysporum* (np*Foxys*) and *Trichoderma* spp. (Fravel et al., 2003; Forsyth et al., 2006; Belgrove et al., 2011). However, in spite of np*Foxys* being commonly found associated with banana plant (as endophytes) and in the soil, large scale use as a biocontrol agent to manage FW (or other diseases) should be carefully evaluated as some strains can increase FW disease (Forsyth et al., 2006) and horizontal gene transfer may also occur transforming np*Foxys* into pathogens (Ma et al., 2010).

Promising results have been more frequently reported in recent years (**Table 1**), but no single biological product can be recommended for widespread use to control FW of banana. Many factors could be responsible for the historical lack of success of biological agents to control FW in banana (and other crops). The pursuit of a biopesticide approach where single or strain mixtures are directly applied in the soil certainly underestimates the complexity of soil-pathogen-plant interactions. Managing the soil microbiota has an enormous potential to control soil-borne diseases and suppressive soils may hold key answers to better understand and explore microbes and develop efficient management tools (Weller et al., 2002). New and powerful approaches, such as metagenomics, have provided significant advances in understanding functional plant and soil microbiome and identifying promising microbes (Mendes et al., 2015; Shen et al., 2015; Cha et al., 2016). For instance, studies carried out in affected and unaffected soils showed that suppressive sites harbor unique microorganism communities with higher richness and diversity (Shen et al., 2015; Köberl et al., 2017). While applying "suppressive" organisms as bio-pesticide has largely failed (Mazzola and Freilich, 2017), the combination of target microorganisms with organic amendments has shown better results (**Table 1**). Shen et al. (2015) found that *Acidobacteria* was more abundant in suppressive soils, while *Bacteroidetes* were more abundant in conducive soils. *Bacillus* was the most abundant genera in the suppressive soil and a *B. amyloliquefaciens* strain (NJN-6) isolated from the suppressive soil showed significant inhibition of *Foc in vitro*.

Later, this strain was combined with compost to produce a bioorganic fertilizer, which has shown positive results to reduce epidemic caused by *Foc* TR4 in China (Shen et al., 2013; Xue et al., 2015). In addition, the continuous application of this bioorganic fertilizer changed the composition of the rhizosphere microbial community by increasing bacterial diversity (Fu et al., 2016, 2017). The success of biocontrol agents appears to rely on enhancing microbe persistence and activity (to reduce or avoid successive introductions of target microbes) and recovering the functional diversity of the local (indigenous) microbiota (physical and chemical properties improved). Strategies should rely on a community-oriented approach rather than on a single or a few target microorganisms. Meeting these conditions depend on many factors and require the integration of different tactics as discussed above. Developing disease suppressive and healthy soils takes time and, unfortunately, many farmers operate based on rapid response solutions like the application of pesticides, herbicides or highly soluble fertilizers. However, the benefits of management practices oriented to soil health accumulate across successive years increasing control of pests and diseases and enhancing productivity (Fu et al., 2016).

Integrated Practices

Integrated pest management (IPM) is a sound way to understand and manage the complexity of agro-ecosystems. For a soil-borne pathogen, integrated soil management is an important dimension of IPM in the context of integrated cropping systems management. Nevertheless, identifying the alternative practices to be used and their integration is not an easy task, mainly for those in the forefront of commercial plantations. For instance, control options based on fungicides (Nel et al., 2007) and resistance inducers (Borges et al., 2003, 2004) have been effective *in vitro* and in greenhouse conditions. Field validation is still pending and should address not just short-term disease response, but also impacts on the community of soil and rhizosphere organisms and plant microbiome. This approach needs to be tested broadly in different contexts, taking into account the status of FW (all races), the cultivar diversity and the cropping systems in the geographical area of concern.

Four different scenarios can be considered at farm level: (a) FW is not present (**Figure 4A**), (b) *Foc* is a quarantine pest and the first incursion has been detected (**Figure 4B**), (c) FW is already established, but with low levels of incidence (**Figure 4C**), and (d) FW intensity is high, the disease is evenly distributed in the plantation and new infected plants are periodically detected (**Figure 4D**). Considering these different scenarios, management practices and their integration may vary, but some of the practices, such as those aiming at exclusion, pathogen containment and suppression and soil management are applicable to all scenarios.

Exclusion is the preferred management practice, although its success depends on many off-farm factors. Growers need to implement all possible practices to avoid the entrance of any *Foc* strains (or other pests) into their properties. To support these strategies, *ex-ante* and tailored Pest Risk Analyses need to be in place. If eventually an outbreak of FW is detected, then a plan aimed at plant eradication and pathogen containment needs to be activated. Continuous monitoring for early detection is essential. To reach that level capacity building on disease identification and diagnostic is overarching. For instance, how fast can a *Foc* TR4-free country, unequivocally, diagnose, and report a new outbreak of TR4? When detected, are all the players synchronized and resources available to perform plant eradication and pathogen containment? Are national contingency plans available? Are NPPOs connected with independent growers and growers associations to carry out a concerted contingency plan? These questions, among other elemental aspects of biosecurity, need to be clearly answered to guarantee the success of pathogen containment if the first incursion of *Foc* TR4 is detected (**Figure 4B**).

Once the disease is present (**Figure 4C**), the use of resistant cultivars (if available) and tactics to suppress the pathogen and boost plant defenses are fundamental. At the same time exclusion and containment should not be neglected as they contribute to further pathogen dispersal and disease intensity, which builds up based on inocula in the soil. Eventually, FW epidemics reach levels where disease management is economically impracticable (**Figure 4D**). In that situation, plot eradication and crop rotation are inevitable, unless resistant cultivars are available. Finally, management tactics should not only focus on FW, but also sustainable productivity and follow the principle of a continual improvement process.

GENERAL RECOMMENDATIONS

Choice of Plots

Fields already infested with FW must be avoided unless a completely resistant cultivar will be planted. The selected area should have adequate drainage. If not, practices to improve drainage need to be implemented. Soil analyses must be performed and nutrients and especially pH must be corrected accordingly.

Soil pH

Soil with pH values from ranging 5.6 to 6 are recommended. Values for base saturation of Cationic Exchange Capacity (CEC) values must be at least 70%. Special attention must be paid to P, Ca and Mg content.

Source of N

For applied nitrogen, the use of nitrate sources (i.e., $CaNO_3$) is recommended instead of urea or other ammonium (NH_4) sources.

Bioorganic Fertilizer

Application of organic matter (5 t/ha) supplemented with beneficial microorganisms (i.e., *Trichoderma* spp. and *Bacillus* spp.) is strongly recommended. See options of bio-fertilizers in **Table 1**.

Cover Crops

The use of cover or inter-planting crops is especially useful to manage weeds and improve soil health (Pattison et al., 2014).

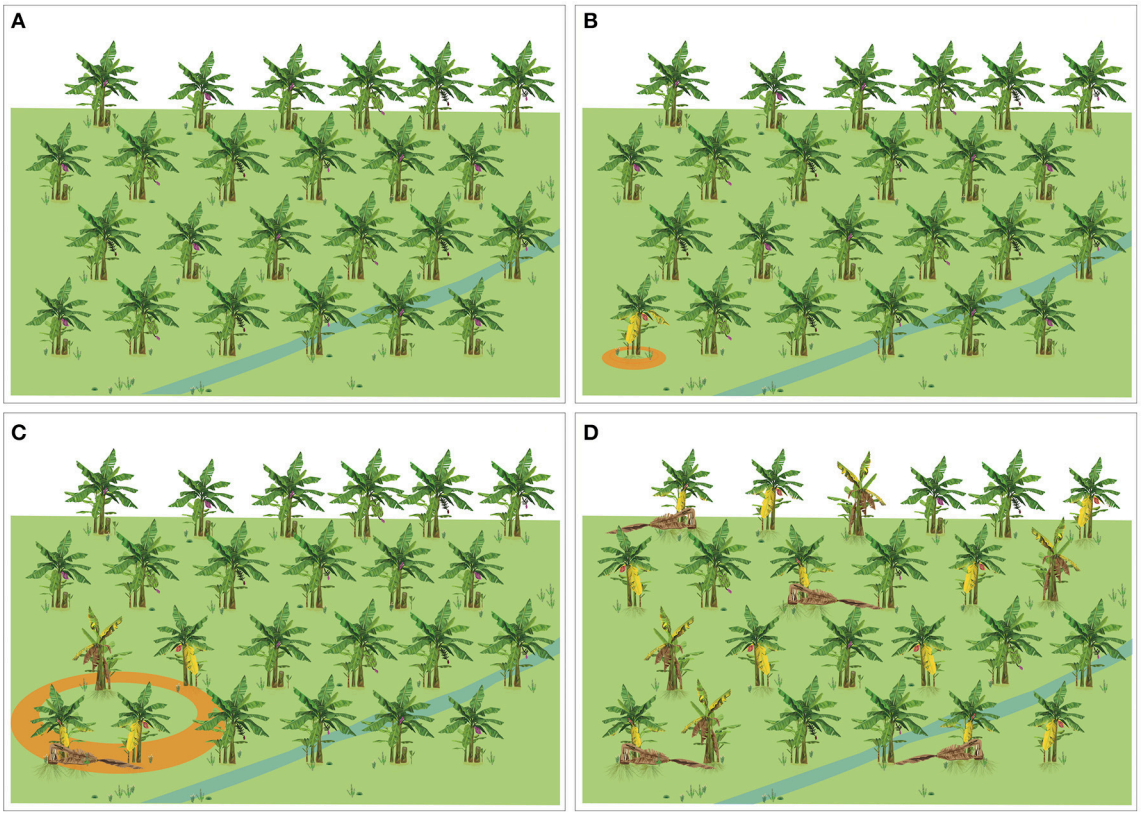

FIGURE 4 | Diagrammatic representation of four different scenarios of Fusarium wilt occurrence in a banana plantation. **(A)** The disease is not present. **(B)** The disease is a quarantine pest and the first incursion was detected. **(C)** The disease is established, but with a patchy distribution and low levels of incidence. **(D)** The disease is evenly distributed at high levels of incidence.

Planting Material

The use of disease-free certified planting material is recommended. Tissue-culture planting material should be well hardened with strong and healthy root systems. Rebuilding the plant microbiome both with endophytes and rhizosphere microorganisms by using beneficial microbes when available has proven to improve the plantlets performance against FW in the field.

Disease Monitoring

Field workers should be well trained on recognizing FW-infected plants at early stages and the risks of spreading the pathogen. Plantations should be monitored periodically to identify suspicious plants. Once FW is confirmed, the eradication of the whole mat is recommended. If *Foc* TR4 is a regulated pest and a suspicious plant is diagnosed as positive, Plant Protection officers should be contacted.

SUMMARY POINTS AND FUTURE ISSUES

More support to breeding programs is required to enhance both the identification and utilization of new resistant genotypes. These efforts should bring together new technologies such as gene editing and high throughput phenotyping and take advantage of the current knowledge generated for "conventional" approaches. Generating improved diploids has taken a considerable amount of time and has a remarkable value on understanding FW resistance. The so-called New Generation of molecular breeders might be forgetting fundamental knowledge and putting all efforts on new tools ignoring fundamental cornerstones.

Urgent need for exclusion and biosecurity measures with special attention to the strict use of disease-free planting material and prevention of the movement of contaminated soil and water. This need to be seen from farm gates to transcontinental levels and should not only consider TR4, but also more virulent *Foc* populations across countries. "Farmers and scientists cannot afford to be complacent just because they are growing a so-called resistant variety. Quarantine and clean planting material still need to be strongly adhered to" (Daniells, 2011).

The huge variability of *Foc* and its ability to mutate should not be ignored. Therefore the pathogen populations need to be monitored continuously to identify and manage new and more virulent strains.

The benefits of practices oriented to soil health such as organic amendments, cover crops and biological agents for FW control are incremental and cumulative. They are generally slower acting than other chemicals, but may last longer. These practices might

not only reduce FW, but also improve the control of other pest and diseases and enhance productivity (Pattison et al., 2014; Haddad et al., 2018). However it is important to keep in mind that the success of these practices appears to be genotype-dependent and linked to the resistance level to FW.

Strategies for integrated management once the disease is present should consider both boosting plant defenses and suppressing *Foc* propagules in the soil. Practices that reduce the ability of the host to express the genetic potential and respond properly to the pathogen invasion may increase disease severity even if other management practices to suppress the pathogen are in place (Nasir et al., 2003).

AUTHOR CONTRIBUTIONS

MD designed and conceptualized the structure of the work, wrote and reviewed the manuscript and supervised the design of figures. MB carried out a literature review and wrote initial drafts of some sections. DH carried out a further literature review, reviewed some sections and verified references. EM and CS completed critical reviews of organization, argumentation and writing of the manuscript.

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The Movement of *Fusarium oxysporum* f.sp. *cubense* (Sub-Tropical Race 4) in Susceptible Cultivars of Banana

*Noeleen M. Warman[1,2] and Elizabeth A. B. Aitken[1]**

[1] School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD, Australia, [2] Department of Agriculture and Fisheries, Queensland Government, Dutton Park, QLD, Australia

Fusarium wilt, caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*), is one of the most important and destructive diseases in banana crops worldwide. There have been numerous studies into the infection process of this soil-borne pathogen; however, the extent of research into the movement of the pathogen through the rhizome and into the rest of the plant is limited. Furthermore, little is known about the movement of the pathogen once it reaches the aerial components of the plant. A strain of *Foc* sub-tropical race 4, genetically transformed with green fluorescent protein (GFP) gene, was used to monitor the movement of the pathogen through two susceptible cultivars, Cavendish 'Williams' (*Musa* AAA) and Lady Finger (*Musa* AAB). Visualization of the pathogen *in planta* demonstrated its presence in the roots, the rhizome and in the outer leaf sheaths of the pseudostem prior to the appearance of external symptoms. Within the non-senescing leaf sheaths, the migration of *Foc* was confined to the xylem vessels; this included those where the vascular tissue was visibly discolored, as well as those, which were apparently healthy. As senescence of leaf sheaths occurred, chlamydospores developed within the gas spaces, while formation of sporodochia, and hyphal growth were apparent on the outer surface of senescing leaf sheaths. These results generate a greater understanding of the epidemiology of *Foc*, providing much needed knowledge to assist in the future management of Fusarium wilt incursions, as well as enhancing protocols for ongoing on-farm hygiene and biosecurity.

Keywords: *Fusarium oxysporum* f.sp. *cubense*, banana, GFP, Fusarium wilt, epidemiology

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****Correspondence:***
Elizabeth A. B. Aitken
e.aitken@uq.edu.au

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INTRODUCTION

Banana (*Musa* spp.) is one of the most important food crops in the world, with many developing countries relying on the fruit as a staple food (Roux et al., 2008; Ploetz, 2015). Banana is grown in approximately 120 tropical and subtropical countries, for both local and export markets (Roux et al., 2008). Up to 40% of global banana production is reliant on the Cavendish subgroup (*Musa* AAA); this includes both domestic markets and the vast majority of export markets. As a consequence of low genetic diversity in banana production pest and disease pressure is one of the major limiting factors worldwide (Ghag et al., 2015). Of these diseases, Fusarium wilt, also known as Panama disease, caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (E.F. Smith) Synder and Hansen

(*Foc*), continues to be the greatest threat to global banana production (Ploetz, 2015; Wen et al., 2015). In the mid-20th century, global production of Gros Michel (*Musa* AAA), the former export cultivar of trade, succumbed to Fusarium wilt; the causal agent has since been described as *Foc* race 1 (Stover, 1962; Ploetz, 2005). However the replacement cultivar, Cavendish, is now under threat.

Fusarium oxysporum f.sp. *cubense* is classified into four races, based on the host range of cultivars on which they cause disease, but there are at least 24 vegetative compatibility groups (VCG) within the different races (Mostert et al., 2017). The three main races affecting dessert banana include: *Foc* race 1 which causes disease on the cultivar Gros Michel as well as Lady Finger; *Foc* race 2, which affects the same cultivars as race 1 but also the cultivar Bluggoe; and race 4, which causes disease on most cultivars including Cavendish (Ploetz, 2015). Race 3 was at first described as a *Musa* infecting strain but has since been noted to only infect *Heliconia* spp. Initially, race 4 was known only to affect Cavendish cultivars in subtropical areas, where the relatively cooler temperatures are thought to increase Cavendish susceptibility (Ploetz, 2006). However, by the early 1990s, Cavendish cultivars in tropical areas of Southeast Asia began to succumb to Fusarium wilt, and hence, the realization of a "Tropical" race 4 of *Foc* (Ploetz, 2015). Since then different VCGs have been used to distinguish between sub-tropical race 4 (SR4) (VCGs 0120,0129,01211, and 01215) and tropical race 4 (TR4) (VCG 01213-01216) (Fourie et al., 2011). In Australia, Cavendish is the largest grown commercial crop of banana with the majority of production centered in north Queensland[1]. *Foc* TR4 was first identified in Australia in the Northern Territory in 1997 (Condé and Pitkethley, 1999). In 2015, the first ever incident of *Foc* TR4 in Queensland was detected in the Tully region, resulting in regional biosecurity monitoring, and enforcement (O'Neill et al., 2016). With the first wave of Fusarium wilt of banana in the mid-20th century, Stover (1972) then suggested that the only method of controlling the dissemination and subsequent infections by *Foc* in banana was by the quarantine or exclusion of infected properties or by planting non-host crops or cultivars. The same would appear to apply to *Foc* TR4.

Previous research into the infection process of *Foc*, using isolates transformed with the jellyfish green fluorescent pigment (GFP) gene, demonstrated the movement of the pathogen from the soil and into the roots and rhizome (Li et al., 2011, 2017). However, only a limited body of research has been conducted demonstrating the continued movement of the pathogen from the rhizome, through the pseudostem, and into the rest of the plant. Using scanning electron microscopy allowed VanderMolen et al. (1977) to observe the movement of *Foc,* along with the formation of the vascular gels and tyloses, in the xylem vessels. In doing so they found the naturally occurring end walls or perforation plates of the xylem strands somewhat inhibited the movement of the pathogen through the vessels. Microconidia trapped at these plates eventually germinated and were able to penetrate the perforation plate, and so continue to progress through the plant. Beckman (1964) used microscopic red vinyl particles to imitate microconidia and also extrapolated that conidia would be trapped at the scalariform vessel endings. Xiao et al. (2013) used a race 4 isolate of *Foc*, genetically transformed with GFP, to observe the movement through banana plants, tracking it from the roots through to the pseudostem. Using small plants and a large inoculum load, they found that *Foc* traveled through the roots, rhizome, and up the pseudostem, even reaching the outside of the pseudostem, by 24 days post inoculation. At this point the plants were showing severe symptoms or necrosis. Observations of *Foc* in the leaf blades of the plant or development of the long lived chlamydospores were not reported in the study by Xiao et al. (2013).

As a soil borne pathogen, *Foc* persists in the soil on decayed host plant material or as chlamydospores in the absence of a suitable host. Stover (1972) and Ploetz (2006) suggested the chlamydospores are able to remain dormant and viable for up to 30 years in soil. These melanised resting spores germinate in the presence of root exudates from a favorable host. Chlamydospores of other *formae speciales* of *Fusarium oxysporum* have also been reported to cause increased disease severity in their respective crops when compared to microconidia as an inoculum source (Couteaudier and Alabouvette, 1990; Cal et al., 1997).

In the absence of any current commercially viable cultivars resistant to *Foc* TR4 and lack of any effective fungicides, the only means of control is avoidance through quarantine and good hygiene practices. For such, a comprehensive understanding of the epidemiology of *Foc* is necessary. Knowledge of the movement of the pathogen within the plant tissues and its potential to persist in the plant debris is vital to ensure appropriate biosecurity strategies are deployed. The aim of this study was to assess the movement of *Foc* SR4 through entire susceptible banana plants, monitoring its growth in both healthy and senescing sections of the plants. For this purpose, a GFP transformed strain of *Foc* SR4 (*Foc* GFP) was used; quarantine restrictions prohibited the use of *Foc* TR4. Also investigated was the timing and tissue type in which the long-lived chlamydospores were produced.

MATERIALS AND METHODS

Planting Material

Two susceptible banana cultivars were used in this experiment, Cavendish 'Williams' (*Musa* AAA) and Lady Finger (*Musa* AAB). Tissue culture plants were deflasked and placed into steam sterilized UQ23 soil mix [70% composted pine bark 0–5 mm, 30% coco peat (coir)] and kept under 12 h fluorescent light for approximately 30 days. Plants were repotted into 140 mm (1.3 L) pots, using steam sterilized UQ23 soil mix and transferred to a containment glasshouse at the St Lucia Campus of The University of Queensland, Brisbane, where they were maintained at 26°C in a natural day light cycle between the months of September to April. For each of the eight time treatments (see below), three replicate plants of each cultivar were prepared as well as three replicates of both cultivars for the controls. Each pot was contained inside a double layer of autoclave bags to contain the genetically transformed *Foc*. To prevent waterlogging, the plants

[1]https://abgc.org.au/our-industry/key-facts/

were monitored daily and watered as necessary. Four weeks after repotting, the plants were inoculated as described below. At this stage, the plants had four to five green leaves.

Inoculation

An isolate of *Foc* SR4 (BRIP 23598, VCG 0120) which had been previously transformed with GFP by Forsyth (2006) was used. The isolate was regenerated on full strength potato dextrose agar (PDA) containing 100 mg L^{-1} of Hygromycin B and incubated for 7 days at 25°C. The millet seed inoculation method was adapted from Smith et al. (2008). Millet seed (*Pennisetum glaucum* (L.) R. Br.) was prepared by soaking overnight, drained, then autoclave sterilized twice at 121°C for 20 min. Following sterilization, each 100 g of the millet was inoculated with a 5 mm round plug of PDA containing *Foc* GFP. The control millet was left uninoculated. The inoculated millet was placed in an incubator set at 25°C for 2 weeks. In preparation for plant inoculation, the millet was ground using a mortar and pestle, and 15 mL of millet was spread around the base of the banana plants and covered with sterile UQ23 mix soil. The plants were not watered until the day after inoculation to allow the *Foc* GFP to colonize. Uninoculated millet was ground and distributed onto the control plants in the same method as for the *Foc* GFP millet.

Plant Sampling and Confocal Microscopy

Every 10 days post inoculation (dpi) for a period of 80 days, six Lady Finger and six Cavendish plant were harvested (three controls and three inoculated plants). Individual plants were assessed for external symptoms, which included leaf yellowing, pseudostem splitting, changes in leaf formation such as choking or stunting, petiole collapse, or leaf wilting and skirting of lower necrotic leaves. Each symptom was recorded as 1 for present and 0 for absent. Samples from the entire plant (**Figure 1**) were observed using confocal microscopy. The pseudostem was segmented into 5 cm pieces from the rhizome upward. These segments included the necrotic outer leaf sheaths as well as the healthy leaf sheaths.

The rhizome and roots were gently shaken to remove loose soil, then rinsed under running water to dislodge any excess soil. Rhizome disease severity ratings were conducted as outlined by Carlier et al. (2003) and described in **Table 1**.

Transverse and longitudinal sections were hand-sectioned using a double-edged razor blade. Each section was placed onto a microscope slide with drops of water and covered with a glass cover slip. A Zeiss 700 Laser Scanning Microscope was used to perform confocal microscopic examinations equipped with filter blocks with spectral properties matching those of GFP (excitation/emission = 488/555 nm).

Xylem Fluid Extraction and Examination

Prior to total dissection at each of the time points, inoculated banana plants were dissected in two places; first directly under the throat (**Figure 1** zone D), and second, at the soil level (**Figure 1** zone F) approximately 2 cm above the rhizome. The initial flow of laticifer sap (latex) was extracted using a 3 mL syringe and placed on a microscope slide with a cover slip. The cut banana tissue was wiped clean using a paper towel sprayed with 70% ethanol and left to sit for 60 min to allow xylem fluid, uncontaminated by latex, to accumulate on the cut surface (David Turner, University of Western Australia and Ken Pegg, Queensland Department of Agriculture and Fisheries Pers.Comm.). After this time, a second extraction was taken and placed onto a microscope slide. The xylem fluid samples were then examined using the confocal fluorescence microscopy to assess for the presence of *Foc* GFP.

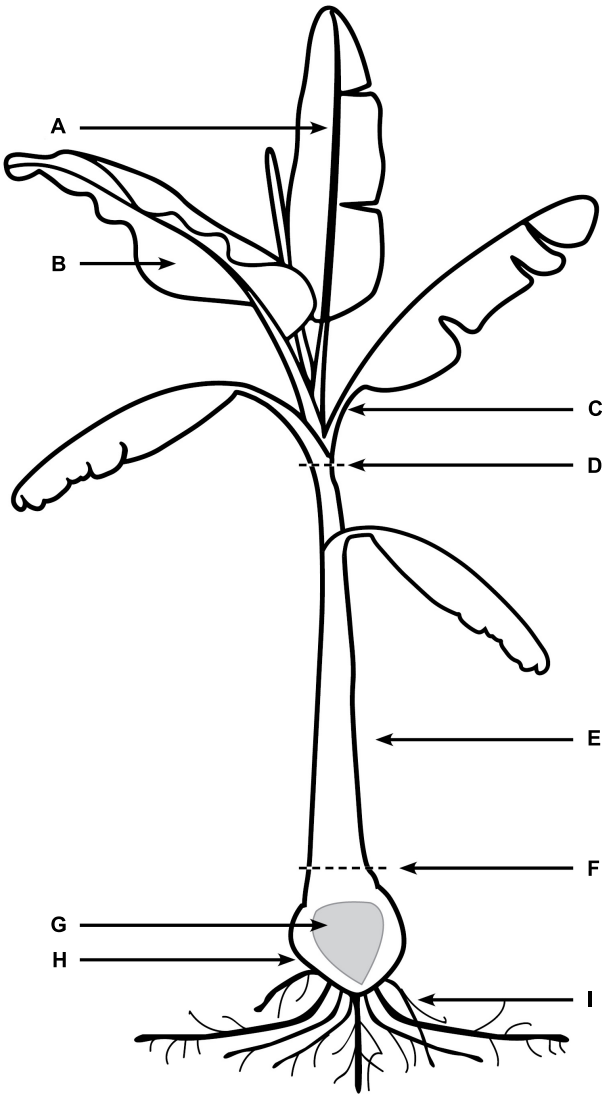

FIGURE 1 | Areas of banana plant used for observation of *Foc* GFP under confocal microscopy. **(A)** Midrib of leaf blade. **(B)** The leaf blade. **(C)** Petiole of the leaf blade. **(D)** Throat of the plant (area of new leaf emergence. **(E)** Leaf sheaths of the pseudostem, including attached senescing sheaths. **(F)** Area of pseudostem emergence above the soil. **(G)** Central cylinder of rhizome. **(H)** Cortex of rhizome. **(I)** Roots including rot tips and sections close to rhizome.

TABLE 1 | Disease severity rating scale for internal symptoms present in rhizome of banana plants caused by *Fusarium oxysporum* f.sp. *cubense* (Carlier et al., 2003).

Rating	Description of symptom
1	Clean rhizome with no evidence of vascular discoloration
2	Isolated points of vascular discoloration
3	Up to 33% of vascular tissue exhibiting discoloration
4	Between 33 and 66% of the vascular tissue discolored
5	Greater than 66% of the vascular tissue discolored
6	Total discoloration of vascular tissue

RESULTS

Disease Development

The first external symptoms of disease occurred in inoculated Cavendish at 20 dpi with the yellowing of the lowest old leaves. By 40 dpi the majority of Cavendish plants were expressing disease symptoms including yellowing of foliage, splitting pseudostem, changes to emerging leaves, wilting foliage and skirting of lower leaves. Whereas these same disease ratings across all inoculated Lady Finger plants occurred at approximately 60 dpi.

With regard to internal symptoms, at 30 dpi, Cavendish plants had developed a mean rhizome disease severity rating of 4, showing 33–66% of rhizome discoloration (**Figure 2**). At the same time point, Lady Finger rhizomes were displaying only isolated points of slight discoloration. By the end of the assessments (80 dpi), there was no difference in the rhizome disease severity ratings of the Cavendish and Lady Finger cultivars, with all plants displaying 100% rhizome discoloration.

Control plants displaying low level of rhizome discoloration in **Figure 2** may be related to potential cross contamination in the glasshouse environment.

Time Course of Infection as Observed by Confocal Microscopy

Substantial colonization of the roots by *Foc* GFP was observed using the confocal microscope from 10 dpi on both Lady Finger and Cavendish plants. At this time point, all plants were both externally and internally visually symptomless. As time progressed, hyphae were present in the intercellular spaces above the apical meristem of the root tip and in the elongation zone (**Figures 3A,C,D**). This was also observed when there were no external disease symptoms present in the inoculated plant (**Figures 3B,G**). Hyphae were also observed in the newly forming xylem vessels in the root zone of maturation. Chlamydospores were noted on the outside of the root tip and among the root hairs. With root tips that showed sign of decay, a hyphal network was apparent covering the entire tip (**Figure 3F**). In the portions of the senescing roots closest to the rhizome, hyphae were observed intercellularly and were not confined by the xylem (**Figure 3E**). Macroconidia were also present, appearing attached to the outside of the decaying root tips (**Figure 3H**) which was observed mainly in the oldest primary roots. In general, the majority of roots assessed showed some level of colonization by the pathogen.

In the inoculated Cavendish plants, the *Foc* GFP appeared to progress internally through the vascular system at a greater rate than that in the Lady Finger plants. At 10 dpi, the fungus was observed to have colonized the roots, rhizome and lower pseudostem of the Cavendish plants, while this did not occur until 20 dpi in the rhizome and 40 dpi in the lower pseudostem of the Lady Finger plants.

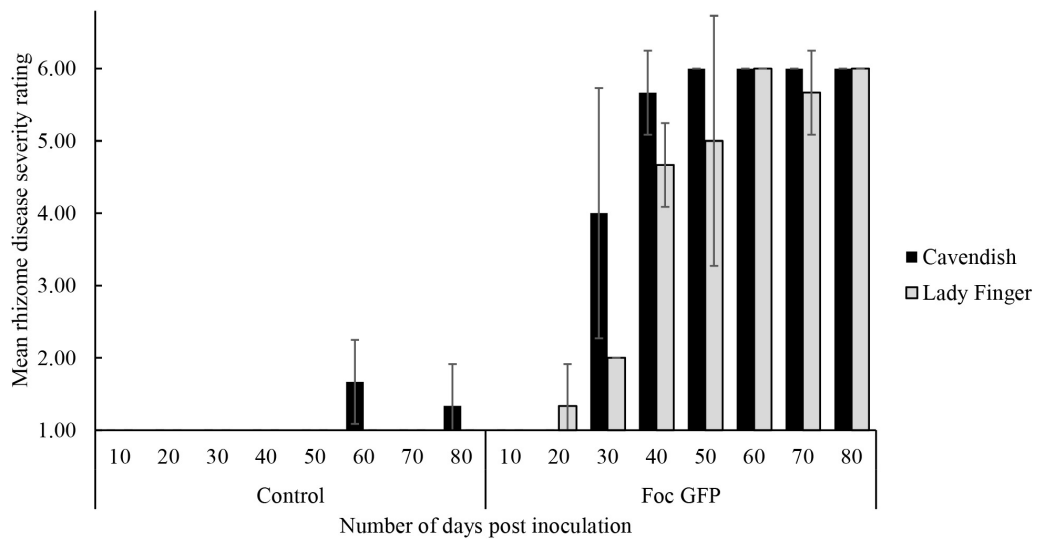

FIGURE 2 | Internal symptoms as mean rating of disease severity, according to the scale in **Table 1**, in the rhizomes of Cavendish and Lady Finger banana plants recorded in days post inoculation (dpi) with *Foc* GFP infested millet or uninoculated control millet. At each time point, three plants of each treatment were sampled. Data are shown as mean ± standard deviation of the three independent replications ($n = 3$).

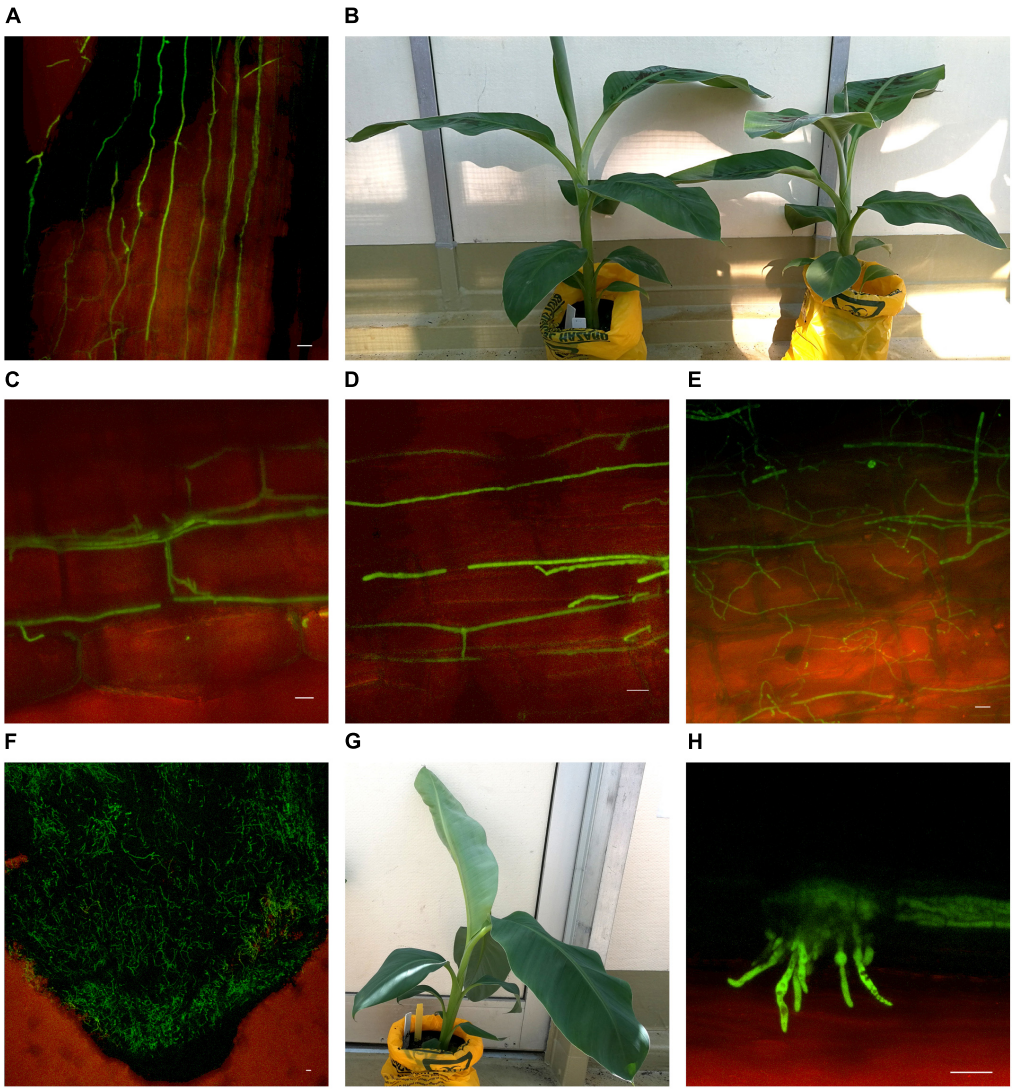

FIGURE 3 | Confocal microscopy highlighting the presence of fluorescing *Fusarium oxysporum* f.sp. *cubense* in different locations in the roots of Cavendish and Lady Finger banana plants. **(A)** Sample from a Cavendish root 20 dpi, showing hyphae apparently progressing through the intercellular spaces in the elongation zone above the root tip. **(B)** Cavendish plants at 20 dpi showing no symptoms of Fusarium wilt, plant on right inoculated with *Foc* GFP and plant on left inoculated with sterile millet. **(C,D)** A Cavendish root 50 dpi, showing hyphae present in the intercellular spaces between cortical cells. **(E)** A sample from a Lady Finger plant at 70 dpi, showing a decaying root with mycelial growth unconfined and throughout the cortical tissue. **(F)** Lady Finger roots 20 dpi, showing a mycelial network covering entire root tip. **(G)** Lady Finger plant at 20 dpi inoculated with *Foc* GFP and not showing symptoms of Fusarium wilt. **(H)** A Cavendish root sample 30 dpi, with macroconidia forming on the outside of root surface. Scale bars represent 20 μm.

Movement through the pseudostem occurred first in the outer leaf sheaths and seemed to progressively move from the rhizome to inner sheaths over subsequent time periods. In both cultivars, as vascular discoloration occurred in green leaf sheaths, *Foc* GFP was observed confined to the xylem vessels as single hyphal strands, mycelium, and/or microconidia (**Figure 4**).

As outer leaf sheaths and leaves began to senesce (from 30 dpi to 50 dpi in Cavendish and Lady Finger, respectively), hyphae were observed in the gas spaces of the leaf sheaths and no longer confined to the xylem vessels. At the later stage of disease development, mycelium was observed protruding from stomata in the leaf sheaths and proliferating on the outside of the sheath (**Figures 5A,B**). At this point, an abundance of macroconidia were arising from apparent sporodochia (**Figures 5C,D**).

Chlamydospore development also occurred, both on the outer surface of the leaf sheath and internally, in the gas spaces (**Figure 6**). No chlamydospore development was observed in green leaf sheaths of either Lady Finger or Cavendish plants. Additionally, there were no noted chlamydospores on the inside of intact yet discolored xylem vessels.

By 30 dpi and 50 dpi in Cavendish and Lady Finger plants, respectively, *Foc* GFP was observed in the midrib of the leaves

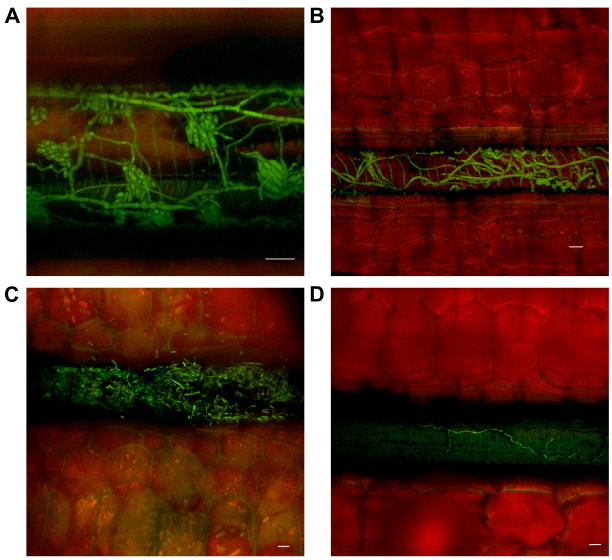

FIGURE 4 | Presence of fluorescing *Fusarium oxysporum* f.sp. *cubense* in the vascular system of the pseudostem of Lady Finger and Cavendish banana plants. **(A)** Lady Finger pseudostem 70 dpi at 10–15 cm above the rhizome showing microconidia formed on the end of monophialides. **(B)** Cavendish pseudostem 80 dpi at 10–15 cm above rhizome showing multiple strands of hyphae confined within a xylem vessel. **(C)** Lady Finger pseudostem 80 dpi at 10–15 cm above rhizome with germinated microconidia apparent within the xylem vessel. **(D)** Lady Finger pseudostem 40 dpi at 10–15 cm above the rhizome showing a single strand of hypha confined to a xylem vessel. Scale bars represent 20 μm.

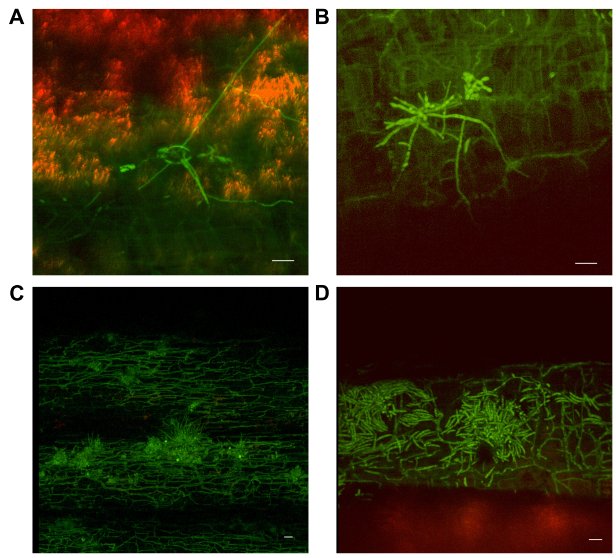

FIGURE 5 | Presence of fluorescing *Fusarium oxysporum* f.sp. *cubense* on the outer surface of dead or senescing leaf sheaths on Cavendish banana plants. **(A)** 80 dpi at 15–20 cm above the rhizome shows hyphae emerging from stoma on the outside of the leaf sheath. **(B)** 70 dpi at 0–5 cm above the rhizome showing hyphae and apparent sporodochia emerging from stoma on the outside of a leaf sheath. **(C)** 50 dpi at 10–15 cm above the rhizome showing mycelium present on the outer leaf sheath surface and sporodochia. **(D)** 40 dpi at 10–15 cm above the rhizome, prolific macroconidia presence on the outer leaf sheath surface. Scale bars represent 20 μm.

above the throat of the plant. The leaves of the Cavendish (**Figure 7A**) displayed no chlorosis nor showed any other symptoms typically associated with *Foc*, however, the Lady Finger leaves were distorted (**Figure 7C**). At this stage the pathogen was confined to the xylem vessels within the midrib (**Figures 7B,D**). Both mycelium and microconidia were produced continuously in the midrib until the final harvest (80 dpi) (**Figures 7E,F**). There was no evidence of *Foc* GFP observed on the leaf blades (lamina) throughout the experiment.

Assessment of Xylem Fluid and Laticifer Sap With *Foc* GFP Inoculated Plants

The xylem fluid and laticifer sap extraction procedures used in this experiment provided only eight positive samples overall from both the Lady Finger and Cavendish banana plants with a total of 84 samples viewed at the different time points (data not shown). Evidence of *Foc* GFP within the sap samples was observed as microconidia and these were observed sporadically within the eight samples. The initial laticifer sap extraction from both the high or low extraction points in the pseudostem were the only samples to provide evidence of *Foc* GFP at a rate of approximately 2–5 conidia per extraction. No *Foc* GFP was observed in the xylem fluid samples. Additionally, several plants failed to produce either xylem fluid or laticifer sap when sections of the pseudostem were removed.

DISCUSSION

Green fluorescent protein-traced pathogens have been widely used to study infection processes through numerous species and their hosts (Lagopodi et al., 2002; Vallad and Subbarao, 2008; Lü et al., 2014; Yuan et al., 2014). Observing the pathogen which has been transformed with GFP has the distinct advantage of being able to provide a visual analysis of the spore development stages of the pathogen *in planta*. The results of the confocal microscopy in this study provided important information regarding the development of chlamydospores in the pseudostem of the plant, as well as evidence of the pathogen moving through the plant prior to the occurrence of external symptoms. The latter has particular relevance for the management and containment of *Foc* in the field. Similarities between the two cultivars assessed included the confinement of the pathogen to xylem vessels while the leaf sheath was healthy and intact, as well as the movement of the pathogen to the outer surface of decaying leaf sheaths. This is the first known study detailing this progression of *Foc* within entire susceptible Cavendish and Lady Finger banana plants; albeit confined within a glasshouse setting.

The movement of *Foc* through the roots in this study was similar to that previously observed with *Foc* GFP by Li et al. (2011) and Xiao et al. (2013). Chlamydospores and microconidia were observed to germinate around the root tip and among root hairs, prior to penetrating the epidermal cells and moving through the elongation zone intercellularly. However, this study

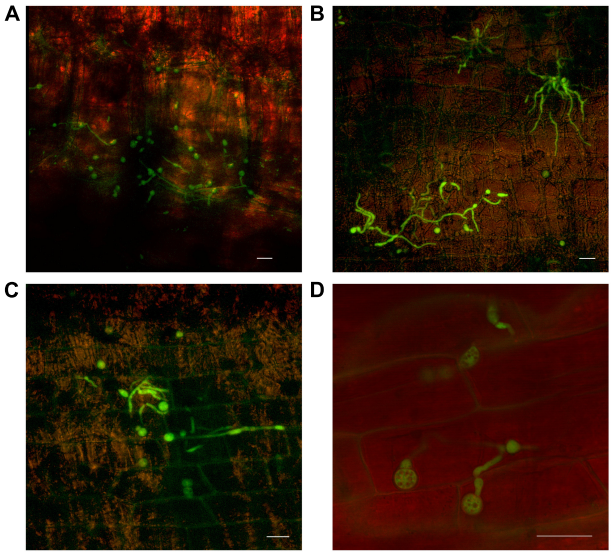

FIGURE 6 | Confocal microscopy highlighting the development of chlamydospores of fluorescing *Fusarium oxysporum* f.sp. *cubense* in decaying leaf sheaths of Lady Finger and Cavendish banana plants. **(A)** Cavendish pseudostem at 40 dpi at 0–5 cm above the rhizome showing chlamydospores present in the gas spaces of leaf sheath. **(B,C)** Cavendish pseudostem at 80 dpi at 5–10 cm above the rhizome showing chlamydospores present on the outside of the leaf sheath. **(D)** Lady Finger pseudostem at 70 dpi at 0–5 cm above rhizome showing the chlamydospores present in decaying leaf sheath. Scale bars represent 20 μm.

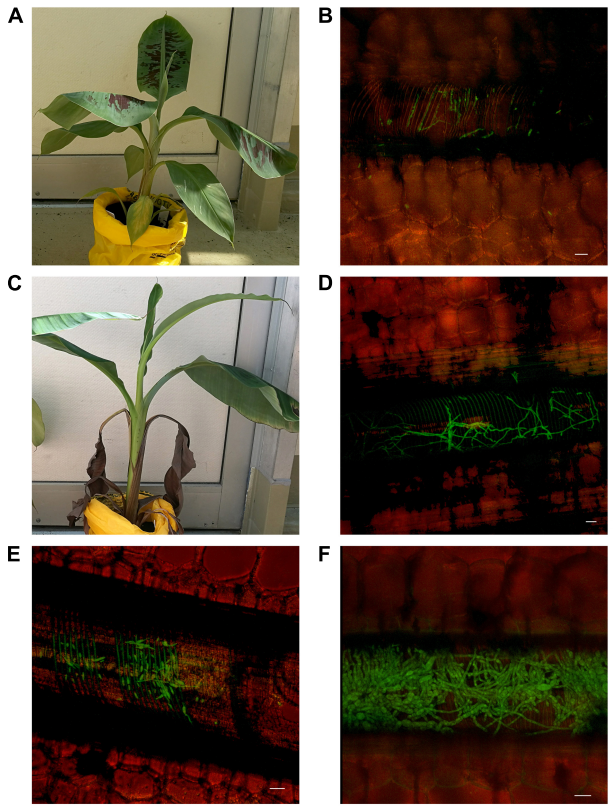

FIGURE 7 | Presence of fluorescing *Fusarium oxysporum* f.sp. *cubense* in the mid rib of leaves above the throat of Lady Finger and Cavendish banana plants. **(A,B)** Cavendish plant 30 dpi inoculated with *Foc* GFP showing limited symptoms of Fusarium wilt with hyphae and microconidia confined to non-discolored xylem vessels of midrib above the throat. **(C,D)** Lady Finger plant 50 dpi inoculated with *Foc* GFP showing hyphae confined to discolored xylem vessel in the midrib of leaf above the throat. **(E)** Cavendish leaf 50 dpi with germinated microconidia confined to xylem vessel. **(F)** Lady Finger leaf 60 dpi, with extensive mycelium appearing to block the xylem vessel. Scale bars represent 20 μm.

differed from Li et al. (2011) in that no intracellular movement or intracellular reproduction of the pathogen was observed within the roots. These differences may be attributed to the age of the plants when inoculated, banana cultivars assessed, inoculation methods or indeed the use of different fungal isolates; in the current study a sub-tropical race 4 isolate belonging to VCG 120 was used. Intracellular and intercellular movement of the pathogen have, however, been observed using other *formae speciales* of *F. oxysporum* transformed with GFP when assessed on tomato (*Solanum lycopersicum* L.) and strawberry (*Fragaria ananassa* Duch.) (Lagopodi et al., 2002; Yuan et al., 2014).

The various *formae speciales* of *F. oxysporum* are generally regarded as hemi-biotrophs, where the initial infection occurs as a biotroph, and switches to a necrotroph as the plant defense system reacts to the biotrophic invasion (Thaler et al., 2004; Bhadauria et al., 2009). Observations of the movement of the pathogen through the intercellular spaces of the root epidermal cells is characteristic of a biotrophic pathogen and has been noted in the infection process of other *Fusarium* species (Lagopodi et al., 2002; Yuan et al., 2014). Consequently, differences in the potential of strains of *F. oxysporum* to grow inter or intracellularly during different stages of the infection process may be highly relevant and worthy of further investigation.

The increasing abundance of *Foc* observed on the outer leaf sheaths as they senesced demonstrates the saprophytic ability of the pathogen to grow continuously on decayed plant material. These decaying outer sheaths were also a location where a high production of chlamydospores was observed. De-leafing is a common practice on banana plantations and is used as a cultural method of controlling banana leaf spot diseases such as black and yellow Sigatoka caused *Pseudocercospora fijiensis* (Morelete) and *Pseudocercospora musae* (Zimm.), respectively (Henderson et al., 2006). This study provides evidence that leaves removed from plants infected with *Foc*, but not yet showing significant symptoms, may later contribute to an increase in the inoculum levels found in the soil. Additionally, the growth of *Foc* on the outside of the leaf sheaths may also provide additional inoculum for aerial dissemination of the pathogen by human or animal/insect influence.

The movement of *Foc* to the outside of the leaf sheath via stomata has seldom been reported. Brandes (1919) noted sporodochia emerging through the stomata on the leaf bases, most commonly at the point where the sheath moves away from the pseudostem. The same development was observed in this study with both sporodochia and mycelia protruding from

stomata. A similar study using *Foc* GFP conducted by Xiao et al. (2013) noted *Foc* on the outside of leaf sheaths, however, there was no evidence to show it moving through the stomata. In the study by Xiao et al. (2013), the pathogen was shown to move intercellularly through the decaying leaf sheaths and was observed there 24 days post inoculation. The use of a different isolate, potentially one that was *Foc* TR4, may have resulted in the rapid progression of disease development in their study, resulting in necrotic plants at 24 dpi. Additionally, implementing a bare root dip as the inoculation method may have allowed symptom development and pathogen movement to progress at a faster rate than seen in this current experiment using millet inoculum.

Identifying whether it was microconidia or hyphae that were responsible for the spread of the fungus throughout the pseudostem could not be determined in this study. In some areas, microconidia were observed prior to any signs of hyphae, while at other times, single or multiple strands of hyphae were observed without the presence of any microconidia.

Contrary to observations made by Beckman and Halmos (1962) and Beckman et al. (1962), the pull of microconidia in the transpiration stream and their subsequent trapping in the vessel ends of the vascular elements was not observed. The method of growing the plants axenically and directly inoculating cut roots with a microconidia suspension may have attributed to the observations of Beckman and Halmos (1962) and Beckman et al. (1962), bypassing the infection process undertaken naturally in a soil environment.

The use of *Foc* GFP revealed specific details, highlighting the actual progression and etiology of the pathogen *in planta*. The use of the *Foc* GFP demonstrated the movement of the pathogen from the initial infection of the roots, through the rhizome, into the pseudostem and to the leaves at the top of the plant. Several key findings were observed through this process. The movement of the pathogen to the outer surface of senescing or decayed leaf sheaths, followed by the substantial production of macroconidia and chlamydospores, has serious implications regarding the pathogens potential spread. However how this relates to mature plants in field conditions requires further investigation. Future studies into possible air borne or vectored spread of inoculum and the potential for the pathogen to infect a healthy plant via aerial inoculation are required. Additionally, the production of chlamydospores in and on these outer leaf sheaths increases the risk of long-lived resting spores returning to the soil through cultural methods such as de-leafing. Identification of the progress of the pathogen into the pseudostem prior to external symptom development also provides vital information to assist in the advancement of monitoring and containment protocols currently in place where Fusarium wilt threatens banana production. This study has provided a comprehensive visualization of the movement of the pathogen through banana plants.

AUTHOR CONTRIBUTIONS

NW conducted the experimental procedure including glasshouse studies and confocal microscopy; analyzed the results and wrote the majority of the manuscript. EA planned the research, reviewed the results, and edited the manuscript.

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REVIEW
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Biological Control Agents Against Fusarium Wilt of Banana

Giovanni Bubici[1]*, *Manoj Kaushal*[2], *Maria Isabella Prigigallo*[1], *Carmen Gómez-Lama Cabanás*[3] and *Jesús Mercado-Blanco*[3]

[1] Consiglio Nazionale delle Ricerche (CNR), Istituto per la Protezione Sostenibile delle Piante (IPSP), Bari, Italy, [2] International Institute of Tropical Agriculture (IITA), Dar es Salaam, Tanzania, [3] Department of Crop Protection, Institute for Sustainable Agriculture (CSIC), Córdoba, Spain

In the last century, the banana crop and industry experienced dramatic losses due to an epidemic of Fusarium wilt of banana (FWB), caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 1. An even more dramatic menace is now feared due to the spread of *Foc* tropical race 4. Plant genetic resistance is generally considered as the most plausible strategy for controlling effectively such a devastating disease, as occurred for the first round of FWB epidemic. Nevertheless, with at least 182 articles published since 1970, biological control represents a large body of knowledge on FWB. Remarkably, many studies deal with biological control agents (BCAs) that reached the field-testing stage and even refer to high effectiveness. Some selected BCAs have been repeatedly assayed in independent trials, suggesting their promising value. Overall under field conditions, FWB has been controlled up to 79% by using *Pseudomonas* spp. strains, and up to 70% by several endophytes and *Trichoderma* spp. strains. Lower biocontrol efficacy (42–55%) has been obtained with arbuscular mycorrhizal fungi, *Bacillus* spp., and non-pathogenic *Fusarium* strains. Studies on *Streptomyces* spp. have been mostly limited to *in vitro* conditions so far, with very few pot-experiments, and none conducted in the field. The BCAs have been applied with diverse procedures (e.g., spore suspension, organic amendments, bioformulations, etc.) and at different stages of plant development (i.e., *in vitro*, nursery, at transplanting, post-transplanting), but there has been no evidence for a protocol better than another. Nonetheless, new bioformulation technologies (e.g., nanotechnology, formulation of microbial consortia and/or their metabolites, etc.) and tailor-made consortia of microbial strains should be encouraged. In conclusion, the literature offers many examples of promising BCAs, suggesting that biocontrol can greatly contribute to limit the damage caused by FWB. More efforts should be done to further validate the currently available outcomes, to deepen the knowledge on the most valuable BCAs, and to improve their efficacy by setting up effective formulations, application protocols, and integrated strategies.

Keywords: *Musa acuminata*, *Fusarium oxysporum* f. sp. *cubense*, Panama disease, soil microbiota, beneficial microorganisms, biocontrol

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Miguel Dita,
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Sijun Zheng,
Yunnan Academy of Agricultural Sciences, China

***Correspondence:**
Giovanni Bubici
giovanninicola.bubici@cnr.it

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INTRODUCTION

Among plant pathologists, everybody knows the story of Fusarium wilt of banana (FWB), also known as Panama disease. Until the 1950s, the cultivar Gros Michel had dominated the panorama of cultivated banana worldwide. The global banana production was threatened by a destructive soil-borne fungus, namely *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (**Figure 1**). Fortunately, a resistant cultivar was identified, the "Cavendish." Hence, due to its resistance to the races 1 and 2 of *Foc* (*Foc* R1 and R2), "Cavendish" was used to replace universally the "Gros Michel," which unique fruit flavor has become virtually a distant memory. The global banana industry was saved until the 1990s, when a *Foc* strain virulent on "Cavendish" emerged, the race 4 (R4). First identified in Taiwan, *Foc* race 4 (R4) rapidly spread to South East Asian countries (e.g., Indonesia, Malaysia, and the Philippines), China, northern Australia, India, Pakistan, Middle East countries (e.g., Jordan, Israel, and Lebanon) and Africa (Mozambique) (Vézina, 2018a). *Foc* R4 strains are further separated into tropical race 4 (TR4) and subtropical race 4 (STR4), based on the evidence that the latter group necessitates predisposing factors, such as low temperatures, to cause the disease. The race 4 affects not only "Cavendish," but also R1- and R2-susceptible varieties, while R1 affects "Gros Michel," "Silk" and "Pisang Awak," and R2 affects "Bluggoe" (Ploetz and Pegg, 2000). The race 3 (R3), which infects *Heliconia* spp., is no longer included in the *Foc* species (Ploetz, 2005). Currently, *Foc* TR4 is present in 19 of the 135 countries producing bananas (Dusunceli, 2017; Zheng et al., 2018), and its alarming spread has gained a remarkable interest from world media and cultural community (Butler, 2013; Gittleson, 2018).

Nowadays, bananas (including plantains and other cooking bananas) are the most produced fruit on the Earth (148 million tons produced in 2016 in 135 countries) and provide a staple for some 400 million people worldwide (Dusunceli, 2017). Its production and cultivated lands have progressively increased over the years (FAO, 2018b). Under the climate change scenario, the temperature increase will make conditions more favorable for banana production in the subtropical and tropical highlands. Land area suitable for bananas is estimated to augment 50% by 2070 (FAO, 2016). Currently, Cavendish varieties cover ca. 40% of the global production (the export trade accounts for 15% of the global production), and may be well the only bananas present on supermarket shelves of non-producer countries, because they dominate the export trade for a 12 billion dollars affair (FruiTrop, 2018). Therefore, it is clear that the economic impact of a second *Foc* epidemic would be more dramatic than the first one. As demonstrated in the past for the *Foc* R1 plague, the host genetic resistance is the better way to tackle the virulent strain. So far, however, no commercial varieties displaying an effective resistance against *Foc* R4 are available. Several research projects (Kema, 2018) and international initiatives, such as Promusa (Van den Bergh et al., 2018), World Banana Forum (Liu and Prada, 2018), the African Consortium for TR4 (Viljoen et al., 2018), and the four banana regional research networks MUSALAC, Innovate Plantain, BARNESA, and BAPNET (Bioversity International, 2018) have been started with the aim to save this crop. Lastly, the research project "Microbial Uptakes for Sustainable Management of Major Banana Pests and Diseases (MUSA)" (Horizon 2020 framework), in which we are involved, aims at improving the sustainable protection of banana crops from three major biotic constraints: FWB, nematodes, and banana weevil. The MUSA project holistically encompasses integrated pest management methods based on microbial consortia and available banana and enset germplasms, including newly developed elite hybrids (Ciancio, 2017).

FIGURE 1 | Typical symptoms of Fusarium wilt on a banana plant cv. Pequeña Enana (or "Dwarf Cavendish"; AAA genome) in Tenerife.

Biological control has gained great interest in the last years in many pathosystems, including *Foc*/banana. This has been mainly due to the large input of pesticides, which cause economic, environmental and safety concerns. Biological control must not be a strategy limited to organic farming (adopted in 1% of banana cultivated lands) (Liu and Prada, 2018), but included within integrated disease management frameworks implemented in agricultural systems. Historically, biological control has suffered from inconsistent results over the seasons and the environments, most likely due to interacting variables (e.g., environmental, genetic, physiological, etc.) present in any given agro-ecosystem, and that are not fully understood or difficult to control. Factors affecting the biocontrol efficacy of FWB have been summarized by Guo et al. (2013).

In the present article, we report the impact of FWB in different continents and the overall disease management strategies with special regard to biological control. Thus, we report a comprehensive literature review of the biological control agents (BCAs) used against *Foc*, we also analyze critically the most relevant results achieved, and identify gaps in our current knowledge of this control strategy in order to (i) show the actual potential of biological control of FWB, and (ii) foster new research lines based on currently-available powerful

technologies that will aid in developing novel and more effective biocontrol tools.

DISEASE IMPACT ON BANANA PRODUCTION …

… in Asia and Australia

Since 1967, the pathogen has caused severe damage in Taiwan, the major exporter of banana to Japan till that time, and the lands cultivated with banana have passed from 50,000 ha in the 1960s to about 6,000 ha in the 2000s. Banana plantations have been also decimated in Indonesia and Malaysia in the early 1990s. The spread of *Foc* TR4 has aggravated the condition in Asia (Molina et al., 2009). In the Philippines, *Foc* has been present since 1970, but TR4 has been detected in 2006. Then, hectares of banana plantations have been abandoned by farmers because of *Foc* TR4, resulting in an annual loss of 3 billion dollars, and approximately 66,000 families lost their livelihood (Molina et al., 2009). As a consequence, in 2014 the Federation of Cooperatives in Mindanao (FEDCO) advised banana growers to shift to oil palm in fields compromised by *Foc*, a crop suitable for the Philippines lands and with increasing market demand (Carillo, 2014). During a survey in 2006, about 6,700 ha of banana plantations were found severely affected by the pathogen in the Guangdong province, southern China (Yi et al., 2007), and in 2012 an extensive damage was also observed around the Guangxi's capital and the Hainan island (Farquhar, 2012). India is the largest producer of bananas in the world with 28.6 million tons produced annually (FAO, 2018b), 70% of which is of the Cavendish cultivar Grand Naine (AAA genome group). There, six vegetative compatibility groups (VCGs) of *Foc* have been found, and disease severity has been as high as 80–90% on susceptible cultivars such as "Silk" (AAB), "Ney Poovan" (AB), "Pisang Awak" (ABB), "Pome" (AAB), "Bluggoe" (ABB), "Monthan" (ABB), and "Mysore" (AAB) (the latter was resistant to *Foc*, but then found infected by VCG 0124/0125) (Mustaffa and Thangavelu, 2011). *Foc* TR4 has been first found in 2015 in the state of Bihar, in the northeastern part of the country, and then detected in the states of Uttar Pradesh, Madhya Pradesh, and Gujarat, on the west coast. It has been officially confirmed only in 2017 (Damodaran et al., 2018). It has been estimated that *Foc* TR4 can inflict losses for 500 billion Indian rupees (ca. 7 billion American dollars) to the country's banana industry (Kulkarni, 2018).

The most recent records of TR4 have come from Jordan, Lebanon, Pakistan, Laos, Vietnam and Myanmar (Molina et al., 2009; Zheng et al., 2018). In 2016, two TR4 outbreak areas were identified in Israel, where the affected farms were promptly fenced in, and diseased plants destroyed. In May 2018, the Israel's National Plant Protection Organization officially declared the TR4 eradicated from Israel (EPPO Reporting Service, 2018). Although the public declaration, scientists know that *Foc* cannot be eradicated from the soil.

FWB in Australia has been reviewed previously by Pegg et al. (1996). Australia was the continent where *Foc* was reported for the first time (Bancroft, 1876), whereas *Foc* TR4 has been reported in the Northern Territory since 1997 (Bentley et al., 2001; Conde and Pitkethley, 2001). Later, in Queensland, three TR4 spots were identified between 2015 and 2018. In Australia, the severe biosecurity regulations have been very effective, and have made the *Foc* TR4 spread extremely slower than elsewhere. In September 2018, Biosecurity Queensland in partnership with Biosecurity Solutions Australia has announced the development of a certification system for TR4-infested farms that meet the requirements for severe food safety and biosecurity standards (Northern Queensland Register, 2018). The lack of resistant cultivar has drastically reduced the banana production in Australia, with a commercial industry loss above 90% and an augmented price of bananas in domestic markets (Cook et al., 2015).

… in Latin America and the Caribbean

Latin America and the Caribbean (LAC) support 28% of the global banana production and provide more than 80% of the banana exports (FAO, 2018b). According to Dita et al. (2013), FWB in LAC impact on certain production systems more than others. Particularly affected systems are the monocultures such as "Prata" (AAB) in Brazil, "Isla" and "Palillo" (AAB, Pacific plantain) in Peru, and the banana plantations intercropped with coffee and cocoa. Due to the *Foc* R1 epidemic, not only "Gros Michel" (AAA) was replaced with "Cavendish" (AAA), but also "Prata Ana" (AAB) (in Brazil) and apple banana (AAB). The cooking banana "Bluggoe" (ABB) was replaced with "Pelipita" (ABB), which is also resistant to Moko (*Ralstonia solanacearum* race 2). With the "Cavendish" advent, FWB disappeared as a problem for the trades (Buddenhagen, 1990), while the black leaf streak (or black Sigatoka, caused by *Mycosphaerella fijiensis*) assumed the primary importance (Ploetz, 2015a). Then, the rapid spread of *Foc* TR4 has recovered the importance of FWB, though black leaf streak remains of high economic relevance, especially in tropical regions. At present, the banana industry in LAC is almost totally based on Cavendish varieties, and the limited production for national markets is based on apple banana, plantain and "Gros Michel." In countries of LAC, *Foc* TR4 is not present, therefore the pathogen exclusion by means of strong quarantine procedures is compulsory. Farmers, technicians, and politicians of LAC became sensitized of the FWB menace from the past, and they continue to become aware thanks to dissemination and technical events, workshops and meetings (Clercx, 2013; FAO, 2018c). An awareness campaign has been launched by a consortium of institutes in order to emphasize the importance of quarantine measures in preventing the entrance of *Foc* TR4 into LAC (Pocasangre et al., 2011).

… in Africa

Banana has been present on the continent for over 1400 years (Blomme et al., 2013). It is an important staple food for African people, as in countries such as Uganda, Rwanda, and Cameroon per capita consumption exceeds well 200 kg of bananas, so providing up to 25–35% of the daily nutrient intake. About 70–80% of banana production is consumed locally (FAO, 2018a; Viljoen et al., 2018).

Foc race 1 has first been reported from West Africa in 1924, and then from Tanzania in 1951 (Blomme et al., 2013; Viljoen et al., 2018). Vegetative compatibility groups and phylogenies of *Foc* populations in Africa have been extensively studied (reviewed by Blomme et al., 2013). Until the year 2000, FWB was reported in two out of the six production areas of South Africa: Kiepersol and southern KwaZulu-Natal. In KwaZulu-Natal, the disease appeared in 1940 and spread to Kiepersol with infected plant material, where it resulted in a 30% loss of banana plantations during the 1990s. At that time, only *Foc* STR4 occurred in South Africa (Viljoen, 2002). In Uganda, the major producer of cooking bananas worldwide with 3.7 million tons in 2016 (FAO, 2018b), FWB is less relevant than weevil [*Cosmopolites sordidus* (Germar)] and Xanthomonas wilt (*Xanthomonas campestris* pv. *musacearum*) and is present at altitudes above 1,300 m (Vézina, 2018b). With the occurrence in one farm in northern Mozambique, *Foc* TR4 officially entered the African continent in 2013, although it was likely an earlier presence (Butler, 2013). There, *Foc* TR4 destroyed about one million plants at a rate of approximately 15,000 plants per week (Jansen, 2017). After the detection of *Foc* TR4 in the six farms of Metochéria (about 1,500 ha cultivated with banana), the access of people and the movement of farm personnel and international staff have been restricted and controlled in order to avoid further spread of the pathogen (Viljoen et al., 2018).

The "Cavendish" varieties are not widely grown in Africa (about 10% of the region's production), whereas plantains (AAB) predominate in West Africa (71% of the production) with about 100 varieties, and highland banana (AAA; East Africa Highland Banana or EAHB) and other cooking types (ABB) predominate in East Africa (71% of production) with more than 50 varieties (FruiTrop, 2018; Viljoen et al., 2018). "Cavendish" was used in countries like Kenya to replace the susceptible varieties "Kampala" ("Gros Michel") and "Bokoboto" ("Bluggoe," ABB), susceptible to *Foc* R1. Besides these exceptions and few others (Sebasigari and Stover, 1988; Rutherford, 2001), African germplasm is largely resistant to *Foc* R1 and R2, but its reaction to *Foc* TR4 is still unknown. This scenario would make the *Foc* TR4 diffusion subtler than in a "Cavendish" monoculture, since new TR4 spots could be undetected properly or assumed to be caused by already established races but not TR4 (Vézina, 2018c). In addition, the fact that sucker-derived plant material is still used more than tissue culture plants in Africa (Dubois et al., 2013; Niere et al., 2014) may contribute to exacerbating the problem.

… in the Canary Islands

Banana is the most important intensive agricultural crop in the Canary Islands, an archipelago located in the Atlantic Ocean between 27° 37′-29° 25′ N and 13° 20′-18° 10′ W. The orography and pedological conditions have conditioned banana cultivation in these islands, shaping a particular landscape of terraces built up over volcanic stones and debris. Banana farms are usually small and about 80% of them have <1 hectare (Instituto Canario de Calidad Agroalimentaria, 2018). To check historical and current figures on banana production and cultivated area per island and municipalities, interested readers can consult the Canary Islands government's website (Gobierno de Canarias, 2018). Cultivar "Pequeña Enana" (or "Dwarf Cavendish," AAA) is overwhelmingly predominant in the islands, with a minor and more recent presence of "Gran Enana" (or "Grande Naine" or "Grand Nain"; AAA) as well as local selections from "Pequeña Enana" like "Gruesa," "Brier," and "Negrita" (Azkolain Olaondo, 2016). Banana production in the archipelago is highly technified with practices such as artificial soil preparation, use of *in vitro* propagated plants from selected cultivars, modern fertigation approaches and extended use of greenhouse cultivation systems (Azkolain Olaondo, 2016). In Tenerife, Fusarium wilt was detected for the first time in the 1920s (Blomme et al., 2013). It is currently present in any area of the archipelago where banana is cultivated, showing an incidence of affected plants ranging from 2 to 12%. In some cases, however, incidence has been reported to be much higher causing more than 30% of crop loss in specific spots (Rodríguez Serrano, 2012). Studies carried out to determine the racial structure and pathogenicity of *Foc* isolates obtained from infected plants and soils of "Dwarf Cavendish" and "Grande Naine" plantations pointed to the fact that there are no differences among pathogen populations (Regalado Guijarro and Hernández Hernández, 1998). The presence and virulence of *Foc* STR4 have been well determined in Tenerife, and this race has also been claimed to be responsible for Dwarf Cavendish infections occurring in Gran Canaria island (Ploetz et al., 1990; Domínguez-Hernández et al., 2008).

OVERALL CONTROL STRATEGIES CURRENTLY ADOPTED

Available and currently-implemented measures to control FWB have been recently compiled (Ploetz, 2015b; Dita et al., 2018; Siamak and Zheng, 2018), and comprehensive information are also available in web portals such as World Banana Forum (Clercx, 2013) and Promusa (Van den Bergh et al., 2018). Previously, other authors have also produced similar reviews (e.g., Figueroa, 1987; Jeger et al., 1996; Wui, 2000; Murray, 2001; Pocasangre et al., 2011; Pérez-Vicente, 2015).

Foc is particularly difficult to control for a number of reasons: (a) it is a soil-borne fungus with a long survival in the soil (more than 20 years), even in the absence of plant hosts (Stover, 1962; Buddenhagen, 2009), or within alternate hosts which do not necessarily show disease symptoms (Waite and Stover, 1960; Pittaway et al., 1999; Hennessy et al., 2005); (b) being a vascular pathogen, it escapes the contact with the control means (e.g., non-systemic fungicides, non-endophytic BCAs, etc.) once it penetrates into the plant; (c) it can be spread by banana vegetative propagation material, soil vectored by workers and machinery, irrigation water, etc.; and (d) the banana monoculture, especially Cavendish varieties in the case of *Foc* TR4, facilitates the pathogen spread.

Overall, fungicides have provided unsatisfactory control levels. *In vitro*, toxicity against *Foc* has been proved for phosphonate, ambuic acid, organotin mandelates, carbendazim, carboxin, propiconazole, benomyl, and difenoconazole (Davis et al., 1994; Davis and Grant, 1996; Li et al., 2001; Araujo et al., 2004; Somu et al., 2014). *In planta*, only a few research

articles have reported a significant disease control by using fungicides (e.g., carbendazim) (Lakshmanan and Selvaraj, 1984; Eswaramurthy et al., 1988; Roy et al., 1998) or resistance inducers (e.g., indoleacetic acid and menadione sodium bisulphite) (Fernández-Falcón et al., 2003; Borges et al., 2004). Until now, however, these pot-experiments have not been validated under field conditions.

In the lack of highly effective control means, like available sources of host genetic resistance, plant diseases are usually managed by integrated frameworks, with an emphasis in preventive measures. This is particularly true for soil-borne diseases like FWB and Verticillium wilts, whose causal pathogens cannot be eradicated once contaminate the soil. With few context-specific differences, integrated disease management strategies of diverse vascular diseases have much in common (Bubici and Cirulli, 2008; Cirulli et al., 2010; López Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012).

In the case of FWB, Dita et al. (2018) stressed that the implementation and integration of some disease management practices may vary according to four main farm-level scenarios, which indeed correspond to four stages of the disease epidemic: (i) absence of *Foc*, (ii) first incursion of *Foc*, (iii) low FWB prevalence, and (iv) high FWB prevalence (**Figure 2**). In the absence of a devastating pathogen like *Foc*, it is imperative to adopt exclusion measures to prevent the pathogen entrance, both at farm level using proper practices adopted by the personnel, and at the regional or national level using legal initiatives such as quarantine, certification, etc. This is the case of *Foc* TR4 in LAC and other countries where this race is not yet present. At the first incursion of *Foc*, exclusion methods still have great importance, but containment measures must be rapidly initiated and scrupulously applied. Farms in countries where *Foc* TR4 has been detected recently would be in this second situation (e.g., Mozambique, Lebanon, Pakistan, Israel, Laos, Vietnam, and Myanmar). Once *Foc* is established, exclusion tactics make no longer sense, but containment measures must be implemented, and integrated disease management can be adopted under low disease pressure. With high disease prevalence, containment measures are obviously not effective, and integrated disease management may be questionable.

Thus, the use of resistant varieties is the only way for successfully confronting FWB. As mentioned above, this was already experienced for the *Foc* R1 epidemic. On the other hand, Dita et al. (2018) have reported two examples where the integrated disease management against *Foc* R1 allows some farmers to grow the susceptible varieties "Prata"-type, in Brazil, and "Gros Michel," in Colombia.

Unfortunately, no commercial varieties displaying high resistance to *Foc* TR4 coupled with good agronomic traits and fruit characteristics are yet available. However, the research on breeding for resistance has been particularly active and, besides conventional breeding and screening of genotypes, several mutant, and transgenic bananas have been developed (**Figure 3**). Resistance sources to *Foc* TR4 have been found in banana wild relatives, especially *M. basjoo* and *M. itinerans*, but also in *M. yunnanensis*, *M. nagensium*, *M. ruiliensis,* and *M. velutina*, whereas higher disease intensity was observed in *M. balbisiana* and *M. acuminata* subsp. *burmannica* (Li et al., 2015). Within *M. acuminata*, the genotype DH-Pahang, which genome was sequenced, has been found resistant to *Foc* TR4 (D'Hont et al., 2012; Zhang et al., 2018). Other promising varieties resistant to *Foc* TR4 are being tested under field conditions. Some of them were developed by the Fundación Hondureña de Investigación Agrícola (FHIA), and others by the Taiwan Banana Research Institute (TBRI). Other resistant genotypes have been identified within the world's largest collection of bananas, owned by the International *Musa* Germplasm Transit Centre, which is managed by Bioversity International and hosted by the Katholieke Universiteit Leuven in Belgium (Vézina, 2018a). The so-called "Giant Cavendish" tissue-culture variants (GCTCV) have been selected for the resistance against *Foc* TR4 by the Taiwan Banana Research Institute (TBRI). Four GCTCVs and three important Philippine local varieties were assayed over two cropping seasons in a heavily *Foc*-infested field in the southern Philippines. While the commercially-grown varieties "Grand Naine" and "Lakatan" showed disease incidence up to 92%, the GCTCV varieties were largely resistant, with a disease incidence of 0–8%. Moreover, "Saba" (ABB) was completely resistant in the two seasons (Molina et al., 2016a). The TR4-susceptibility of African banana germplasm, generally known to be resistant to *Foc* R1, has been started to be evaluated. A collection of 14 genetically diverse EAHB and plantain varieties were evaluated in fields of China and the Philippines and found all resistant with disease incidence as low as 0–5%, except EAHB "Ibwi" which showed a disease incidence of 32% (Molina et al., 2016b).

When resistant varieties are not available, the management of FWB relies on an integrated approach. In the Canary Islands, for example, where FWB is caused by *Foc* STR4 and has a low prevalence, farmers try to contrast the pathogen by a number of palliative practices, sometimes based on empirical knowledge or deduced from basic research (Rodríguez Serrano, 2012; López-Cepero et al., 2014).

Soil amendment with calcium hydroxide or agricultural lime is practiced at the base of both diseased plants and symptomless neighboring ones with the aim to increase the pH and thus hinder *Foc* proliferation. The pH increase, however, may have deleterious effects on the physicochemical properties of soil, but probably the technicians and farmers choose the lesser evil. It has been observed that soil applications of $CaCO_3$, $Ca(OH)_2$, $CaSO_4$, or Fe-EDDHA reduced *Foc* conidia germination and FWB severity, though the calcium amount was insufficient to change the soil pH. Nevertheless, this observation was not considered conclusive enough by the authors (Peng et al., 1999). A higher FWB incidence has been associated with low soil pH (Alvarez et al., 1981), but such observation has not been reproduced experimentally (da Silva Junior et al., 2000). Also, ammonia fumigation and biofertilization have been reported to reduce FWB incidence in a pot-experiment, with concurrent increases in soil pH, nutrient contents, and beneficial microbial community (Shen et al., 2019). Although no clear experimental evidence exists about the effectiveness against FWB of raising soil pH, it is known that *Foc* prefers low pH.

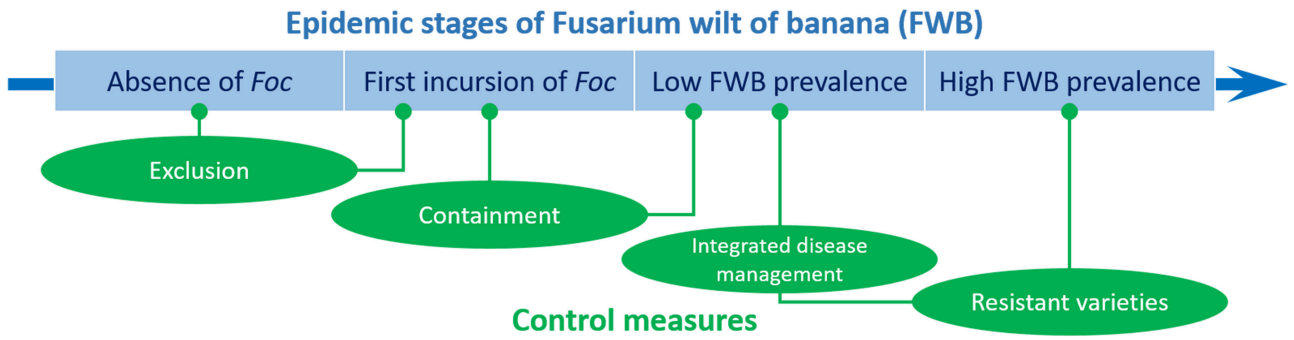

FIGURE 2 | Control strategies of Fusarium wilt of banana at different epidemic stages. In the absence of *Fusarium oxysporum* f. sp. *cubense* (*Foc*), exclusion measures must be used to prevent the pathogen entrance. At the first incursion of *Foc*, containment measures must be rapidly initiated. Once *Foc* is established, containment measures must be continued, and integrated disease management can be adopted. With a high disease prevalence, the use of resistant varieties is the only way to successfully combat FWB.

For instance, a semi-selective agar medium widely used for isolation and enumeration of *Foc* from soil must be adjusted to pH 3.8–4.0 to favor *Foc* and hamper the growth of other fungi (Komada, 1975).

Adequate irrigation and fertilization regimes are also important to combat FWB. Waterlogging and acidification of nutritive solutions are generally avoided, and monthly treatments with zinc sulfate recommended. Stimulated by practical evidence that indoleacetic acid sprays combined with zinc supply reduced FWB, Hecht-Buchholz et al. (1998) observed ultrastructural changes of chloroplasts and mitochondria in banana leaves associated with the zinc-deficiency and, thus, with the FWB reduction. In a later work, the same research team confirmed the involvement of zinc nutrition in FWB development. Plants fertilized with a Zn-deficient solution were all diseased, whereas only 25% of plants treated with a normal zinc solution showed FWB symptoms (Fernández-Falcón et al., 2004). Overall, zinc seems to have a detrimental effect on several species of *Fusarium*, as observed in *F. moniliforme* (reduced fusarin C biosynthesis), in *F. oxysporum* f. sp. *radicis-lycopersici* (suppressed fusaric acid production) and *F. verticillioides* (reduced fumonisin production) (Jackson et al., 1989; Duffy and Défago, 1997; Savi et al., 2013). Similarly, silicon can decrease the intensity of several crop diseases (Datnoff and Rodrigues, 2015), including FWB (Fortunato et al., 2012b). This element may accumulate in the plant cell walls and act as a mechanical barrier, while it can also induce the phenylpropanoid pathway (e.g., lignin) and activate defense-related enzymes (e.g., chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase, glucanase, peroxidase, polyphenoloxidase, etc.) (Smith et al., 2005; Fortunato et al., 2012a, 2014). Besides zinc and silicon, several other micro-nutrients like boron, iron, copper, and sodium, are known to affect FWB development (Qi et al., 2008; Sanjeev and Eswaran, 2008; Ji et al., 2012).

In the Canary Islands, it is also recommended (i) to increase the soil organic matter content above 3%, (ii) to avoid mulching for favoring soil aeration, (iii) to avoid high plant densities, and (iv) to apply plant resistance elicitors among other measures. Such practices arise from technical reports and, sometimes, from scientific articles (Alvarez et al., 1981; Aguilar et al., 2000; da Silva Junior et al., 2000; Rodríguez Serrano, 2012; López-Cepero et al., 2014). To the best of our knowledge, no detailed, long-term scientific studies have been conducted on the actual outcome of most of these practices, although they seem to help farmers to control FWB in a reasonable way.

Recently, Azkolain Olaondo (2016) investigated the use of biofumigation and soil solarization to manage FWB on a commercial farm in La Gomera island. However, results showed no differences in FWB incidence and severity among the treatments used (sheep manure or packing debris vs. control), although slight growth promotion was observed compared to the control. Moreover, solarization did not improve biofumigation treatments. An early trial from South Africa reported soil solarization as ineffective against FWB (Herbert and Marx, 1990). Nevertheless, in Indonesia, soil mulching with transparent polyethylene plastic for 10 months provided a 60% disease control until 14 months after planting. This treatment was superior to crop rotation with maize and bare soil treatment in terms of disease control and *Foc* TR4 suppression in the soil (Hermanto et al., 2012).

Crop rotation has provided attractive results in some cases. Rotation with rice reduced FWB to 8.1–17.6% after 1 year and to 0.8–6.3% after 2.5–3 years, compared to an initial level of 30–50%. *Foc* R4 population in the top layer of soil (20 cm) was undetectable after the treatment (Hwang, 1985). Furthermore, rotation with Chinese leek (*Allium tuberosum*) reduced FWB incidence and severity by 88–97% and 91–96%, respectively, and increased yield by 36–86% (Huang et al., 2012). *In vitro* assays showed that crude extracts of Chinese leek inhibit *Foc* R4 growth, suppress conidia proliferation and germination, and inhibit the activity of two cell wall degrading enzymes produced by *Foc*, polygalacturonase, and cellulase (Huang et al., 2012; Yang J. et al., 2015). Aqueous leachates and volatiles from Chinese leek exhibited strong inhibitory activity against *Foc*. Five volatiles including 2-methyl-2-pentenal and four organosulfur compounds (dimethyl trisulfide, dimethyl disulfide, dipropyl

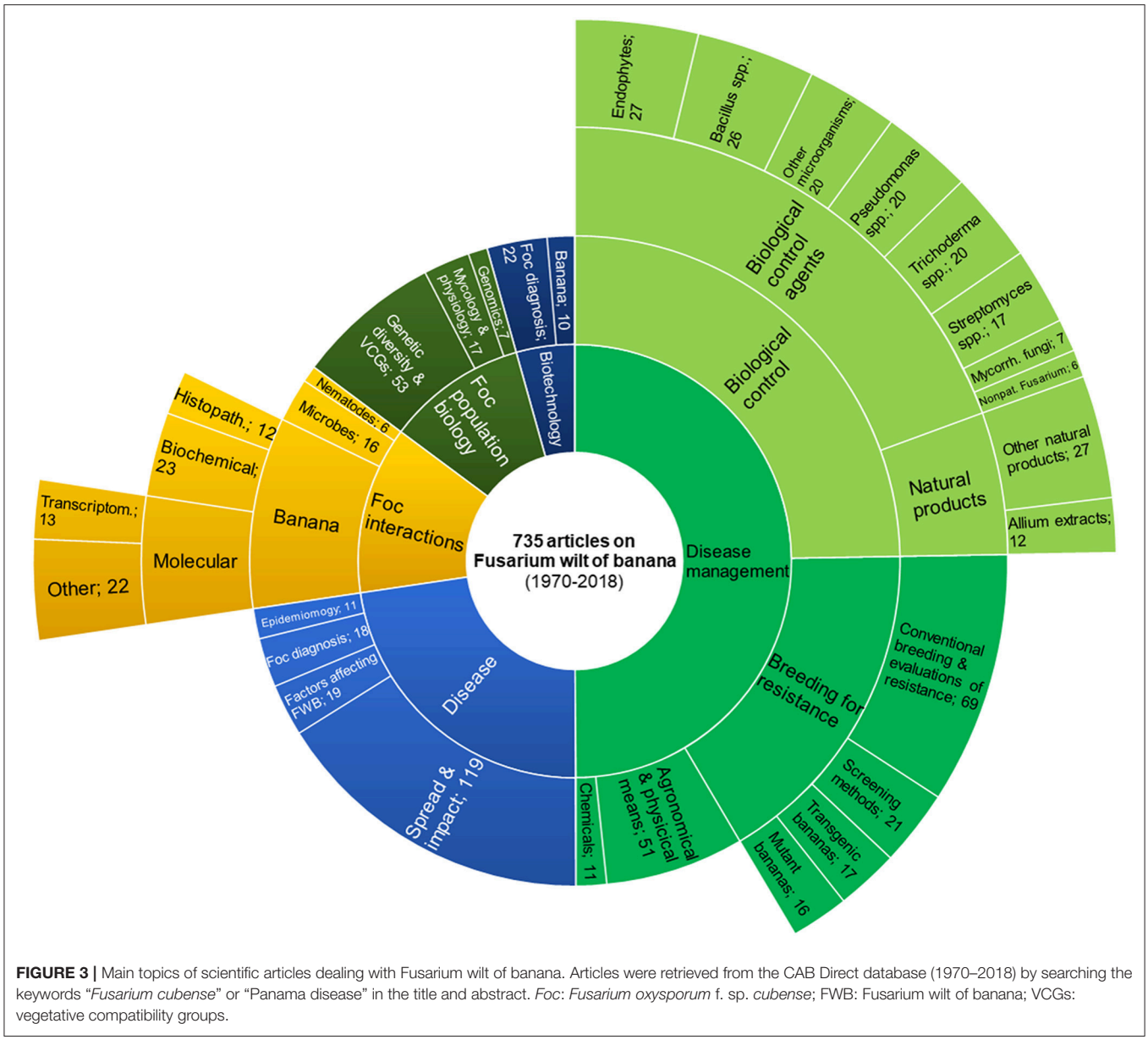

FIGURE 3 | Main topics of scientific articles dealing with Fusarium wilt of banana. Articles were retrieved from the CAB Direct database (1970–2018) by searching the keywords "*Fusarium cubense*" or "Panama disease" in the title and abstract. *Foc*: *Fusarium oxysporum* f. sp. *cubense*; FWB: Fusarium wilt of banana; VCGs: vegetative compatibility groups.

disulfide, and dipropyl trisulfide) were identified from the leaves and roots and found particularly active against the pathogen *in vitro* (Zhang et al., 2013). The pineapple-banana rotation has also given promising results. A 2-year crop rotation culminated with an 81% FWB reduction, and such control level was also attributed to changes in the bacterial and, more importantly, fungal communities in the soil (Wang B. et al., 2015). Lower disease control levels have been obtained by using rotations with maize, sugarcane, sunflower or eggplant (Hwang, 1985; Hermanto et al., 2012; Wang B. et al., 2015; Hong et al., 2017).

Biological (or reductive) soil disinfestation (BSD) has surfaced as another encouraging control means against FWB. Field experiments have shown that FWB can be reduced up to 82% in flooded soil incorporated with 0.5% rice straw (Huang et al., 2015c). The BSD simultaneously reduced the *Foc* inoculum in the soil and ameliorated the beneficial microbial communities, making the soil more suppressive to FWB (Huang et al., 2015a). Also, some microbes (e.g., *Clostridium* spp.) producing organic acids toxic for *Foc* (acetic, butyric, isovaleric and propionic acids) were found more abundant after BSD (Huang et al., 2015b).

BIOLOGICAL CONTROL AGENTS AND THEIR MODES OF ACTION: ACTUAL AND EFFECTIVE TOOLS TO CONFRONT FUSARIUM WILT OF BANANA

From a historical perspective, biocontrol of FWB has been studied for more than 70 years (Thaysen and Butlin, 1945).

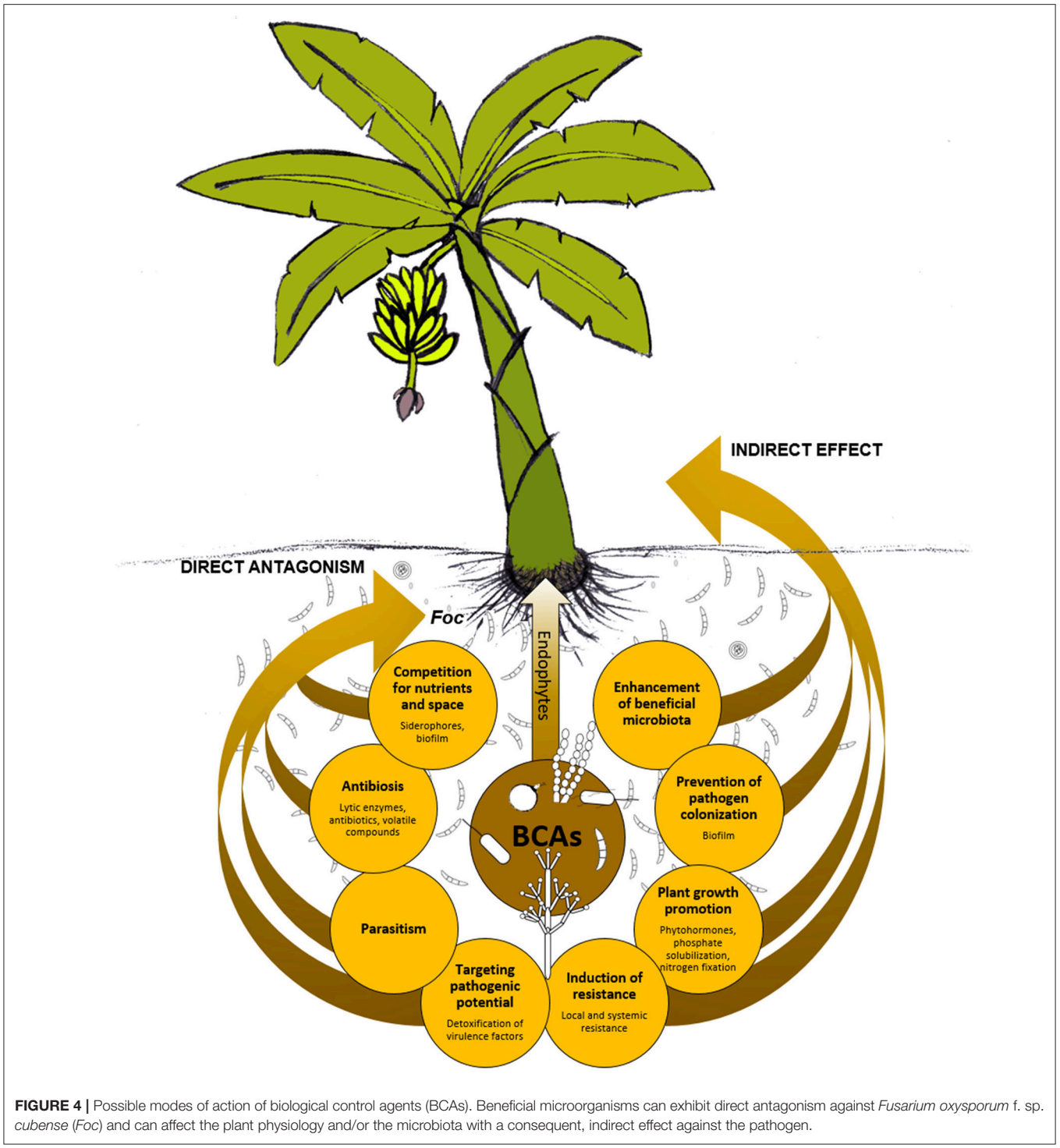

FIGURE 4 | Possible modes of action of biological control agents (BCAs). Beneficial microorganisms can exhibit direct antagonism against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and can affect the plant physiology and/or the microbiota with a consequent, indirect effect against the pathogen.

Studies have reported that disease suppressive sites showed microbial communities displaying higher richness and diversity (Shen et al., 2015b; Köberl et al., 2017), and possibly a higher number of antagonistic members, as observed for streptomycetes (Kinkel et al., 2012; Jauri et al., 2017). Moreover, differences in the composition of these communities correlated with the disease suppressiveness (abundance of *Acidobacteria*) or conduciveness (abundance of *Bacteroidetes*) of soil (Shen et al., 2015b). The manipulation of banana rhizosphere microbiota by the introduction of well-characterized antagonists

alone or in combination with organic amendments (bio-organic fertilizers) has already yielded very promising results against *Foc* TR4 in China (Shen Z. et al., 2013; Shen et al., 2015a; Xue et al., 2015). This strategy also leads to changes in the structure and composition of the microbial community that can be harnessed for more effective control of FWB (Shen et al., 2015a; Fu et al., 2016b).

The mechanisms underlying the BCAs' biocontrol activity are many and variegated (Narayanasamy, 2013; Singh, 2014). It is essential to know the BCAs' modes of action, including weakness and requirements, in order to exploit their potential for disease management in the most effective manner. Also, the combination of BCAs with different modes of action might result in a better biocontrol due to additive, or even synergistic, interactions between BCAs (Parnell et al., 2016; De Vrieze et al., 2018). **Figure 4** schematizes how BCAs can act directly or indirectly against *Foc*. Direct antagonism may be due to antibiosis (e.g., antibiotics, lytic enzymes, volatile organic compounds, etc.), parasitism, or competition (for space and/or nutrients). Induction of plant local/systemic resistance, plant growth promotion, or changes of soil/plant microbiota in favor of more beneficial microbial taxa are typical mechanisms that indirectly act against the pathogen, or at least contribute to reducing the infections or the disease. Antibiosis is one of the primary mechanisms possessed by BCAs. Indeed, initial *in vitro* selection of new BCAs often relies on the evaluation of the sole anti-microbial activity against the pathogen (**Figure 5A**), while other mechanisms are studied later on, possibly once the BCA effectiveness is demonstrated at least under controlled conditions (**Figure 5B**).

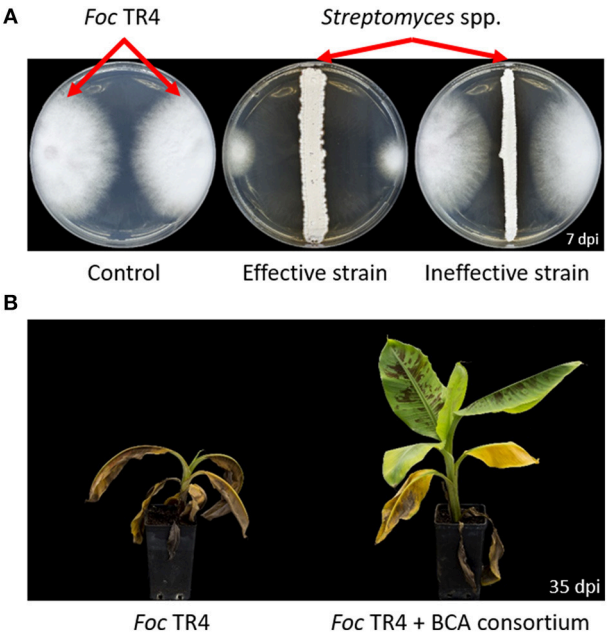

FIGURE 5 | Effectiveness of biological control agents against *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The dual culture method **(A)** allows testing the *in vitro* antifungal activity of metabolites produced by biological control agents (BCAs). Pot experiments **(B)** provide first evidence for the *in planta* efficacy of BCAs.

Literature Overview Shows That Biocontrol of Fusarium Wilt of Banana Is a Widely Studied Research Topic

In order to understand the relevance of the research on biological control of FWB, we searched the keywords "*Fusarium cubense*" or "Panama disease" in the title and abstract of articles indexed by the CAB Direct database (CAB Direct, 2018). The search yielded a comprehensive overview of the literature from 1970 to date. Amongst 735 retrieved articles, 367 were focused on disease management and, with 182 articles (ca. 25% of the total), biological control represents the largest research sub-topic on FWB (**Figure 3**). Records of FWB presence, distribution or impact on the crop productivity rank second for their abundance (119 articles), suggesting how much the disease is feared worldwide (**Figure 3**). With 123 retrieved articles, breeding for resistance is the third relevant FWB-related research topic (17% of the total articles), which in our article categorization included conventional breeding, screening for resistance, development, and evaluation of mutant and transgenic bananas. Therefore, biological control and host genetic resistance have been considered the most important strategies for the management of FWB. It should be noted that conventional breeding in banana is particularly laborious because cultivated varieties are polyploid; hence, diploid parents must be used (or generated) for crosses, and then promising progeny must be polyploidized. In the late 70's, the research on breeding was much more intense than that on biological control, which increased only since 2000s (**Supplemental Figure 1**). At least 143 articles deal with BCAs. Endophytes and strains of *Trichoderma* spp., *Pseudomonas* spp., and *Bacillus* spp. have been the most studied BCAs, followed by arbuscular mycorrhizal fungi. *Streptomyces* spp. have been investigated mainly *in vitro*, with very few *in planta* experiments, and none conducted in the field (**Figures 3** and **Supplemental Figure 2**). In 39 papers, the effects of natural products against *Foc* is showed and, interestingly, 12 articles published since 2011 report studies on the efficacy of *A. tuberosum* extracts (**Supplemental Figure 1**). The remaining articles are focused on the interactions of *Foc* with other microorganisms and its population biology, where the "-omics" approach has been used since the 2010s. Experiments conducted under field, pot and *in vitro* conditions are listed in **Tables 1**, **2** and **Supplemental Table 1**, respectively, and their by-taxon abundance is summarized in **Supplemental Figure 2**.

Data mining from the literature allowed us to infer on the potential and actual efficacy of several microbial genera in the control of FWB. In particular, from each retrieved article we mined the best FWB control value obtained, but not all the data. Although the experiments are not so numerous, plotting these data provides some evidence for microbial genera more prone to control FWB than others (**Figure 6A**). Interestingly, we realized that several biocontrol trials reached the field stage, and results even showed high effectiveness. In the field, FWB was controlled up to 77% (median of 5 articles) by *Pseudomonas* spp.

strains, and up to 71% by several endophytic strains (8 articles) and other *Trichoderma* spp. strains (4 articles) (**Figure 6A**). Lower biocontrol was obtained with *Bacillus* spp. (69% as the median of 5 articles), arbuscular mycorrhizal fungi (55%, 3 articles), and non-pathogenic *Fusarium* strains (42%, 2 articles), whereas most studies on *Streptomyces* spp. have been limited to *in vitro* conditions. Biocontrol under field conditions has been often reported to be less effective than under (semi)controlled, pot experimental conditions. Surprisingly, a different scenario appears for FWB biocontrol. In fact, pot- (median = 65) and field-experiments (median = 70) resulted substantially similar in terms of disease control efficacy. Moreover, articles dealing with *Pseudomonas* spp. reported on average a higher efficacy in the field (median = 77%) than in pot-experiments (median = 50%), as also observed for endophytes, *viz.* 71% in the field and 65% in pots. Non-pathogenic *Fusarium* strains, however, resulted more effective in pot-experiments (median = 87%) than in the field (median = 42%). Finally, *in planta* effectiveness of BCAs was not dependent on the target *Foc* race (R1 and R4), meaning that *Foc* R1 and R4 showed overall comparable sensitivity to BCAs (median disease control efficacy of 62%; **Figure 6B**).

Endophytes: The Help From Inside

All plants harbor a huge diversity of beneficial or neutral microorganisms living inside their tissues without causing any deleterious effect in the host (reviewed by Hardoim et al., 2015). Since beneficial endophytes can promote plant fitness and growth through a range of different mechanisms (i.e., phytohormones synthesis, nitrogen fixation, phosphate solubilization, induction of defense responses, alleviation of abiotic stress by reducing ethylene level, etc.) (Compant et al., 2016), they have a considerable agro-biotechnological potential yet to be fully exploited (Mercado-Blanco and Lugtenberg, 2014, and references therein). Endophytes have found evolutionary solutions to live within the plant interior (i.e., nutrient availability, evading/modulating host defense responses, etc.), where they can also deploy biocontrol activity against pathogens. Thus, an increasing number of studies are available on the isolation, characterization, and assessment as BCAs of specific culturable members of indigenous endophytic communities. Endophytic bacteria and fungi from banana plants have received early attention as BCAs against *Foc* and other biotic constraints (Ortiz and Pocasangre, 2012; Niere et al., 2014). Although many of them belong to genera that will be described in more detail in the following sections, we would like to summarize some reports focused on endophytes to highlight the importance of this special group of the plant-associated microbes.

It is interesting that many experiments with endophytes against FWB have been conducted in the field during the last years. One of the early research showed how *P. aeruginosa* FJAT-346-PA reduced FWB by 82–84% in semi-field and field conditions. The strain was proved to colonize the roots and stems of banana and to promote the plant growth (Yu C. et al., 2010). Cao et al. (2004) reported on the actinomycete communities found in the interior of leaves and roots of both healthy and diseased banana plants. Most of the isolates were *Streptomyces griseorubiginosus*-like strains. Isolates displaying antagonistic activity against *Foc* originated from roots of healthy plants, whereas no difference in this phenotype was reported between antagonists isolated from healthy and wilted leaves. *In vitro*, the antagonism of *S. griseorubiginosus* S96 was lost when $FeCl_3$ was introduced in the Petri plates, suggesting that it relies on siderophore production. *In vivo*, FWB severity (R4) was reduced by 47%, and plant fresh weight increased in plantlets treated with strain S96 compared to the control (Cao et al., 2005). Similarly, three endophytic *Bacillus* spp. isolated from different *Musa* cultivars in Brazil showed antagonistic activity against *Foc* and *Colletotrichum guaranicola* (Souza et al., 2014). More recently, endophytic bacteria from the classes Actinobacteria (genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*, *Kytococcus*, *Micrococcus*, *Naumanella*, *Rothia,* and *Tessaracoccus*), α- and γ-Proteobacteria (*Brevundimonas*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia,* and *Sphingomonas*) and Firmicutes (*Bacillus* and *Staphylococcus*) were isolated from the shoot-tips of banana cv. Grand Naine (Sekhar and Pious, 2015). From the collection generated in this study, strains of *Pseudomonas aeruginosa*, *Klebsiella variicola*, and *Enterobacter cloacae* showed variable antagonistic activity against *Foc*, but their potential as effective BCAs remained to be proved. Other studies reported on fungal and bacterial endophytes originating from plants other than banana such as weeds and medicinal plants (Ting et al., 2009b) or *Capsicum frutescens* (He H. et al., 2002), making them interesting reservoirs of *Foc* antagonists worth to be explored. Finally, some studies go a step forward, exploring the potential mechanism involved in the antagonistic effect. For instance, Ting et al. (2010) investigated the role of volatiles produced by several fungal endophytes in the growth inhibition of *Foc* R4. Even if with inconsistent disease control, the same authors also suggested the induction of host resistance as a mechanism involved in FWB control mediated by *Penicillium citrinum* BTF08 isolated from banana internal stem tissues (Ting et al., 2012).

Interestingly, some of the reports were focused on the inoculation of BCAs at the *in vitro* propagation stage of banana plants. Banana tissue culturing is widely used as a source of pathogen-free planting material. Since banana plantlets produced by these micropropagation schemes are more susceptible to diseases due to the absence of the natural-associated microbiota, the reintroduction of endophytic and rhizospheric microorganisms to protect the plant against subsequent challenges by *Foc* is an approach that yielded promising results (Kavino et al., 2014, 2016; Kavino and Manoranjitham, 2018). The *in vitro* co-culturing of banana plants with *Pseudomonas fluorescens* Pf1, *Bacillus subtilis* EPB 10 and EPB 56 provided a successful control of FWB in the field, combined with increased leaf nutrient status, vegetative growth, bunch yield and fruit quality (Kavino et al., 2016). In two other field trials, the endophyte and rhizobacteria strain led to a FWB control of 78% and a significant higher bunch weight (Kavino and Manoranjitham, 2018). Furthermore, this strategy was also proved to be successful when inoculating banana tissue culture plantlets with a mixture of naturally-occurring uncultivated endophytes from healthy banana plants

TABLE 1 | List of biocontrol field trials against Fusarium wilt of banana.

Biocontrol agents	Mode of application	*Foc* race	*Foc* inoculum	Best disease control obtained (%)	Highest yield increase obtained (%)	Relevant remarks	References
Endophytes							
Pseudomonas aeruginosa FJAT-346-PA				82		Colonization study with antibiotic-marked strains	Yu C. et al., 2010
Acremonium sp. Q34	Root dripping into the fermentation broth	4	10^5 conidia mL^{-1}	71		Strain isolated from disease-free *Kandelia candel*	Liu and Lu, 2013
Burkholderia cenocepacia 869T2	Root dipping (OD_{600} = 0.6–0.7)	TR4	Natural infestation	86	11	Growth-promoting effects	Ho et al., 2015
Pseudomonas putida C4r4, *Achromobactrum* sp. Gcr1, *Rhizobium* sp. Lpr2, *Bacillus flexus* Tvpr1	Talc powder formulation of bacterial consortia (10^8 cells g^{-1})	1	Natural infestation	42.2	214	Combined effect with rhizospheric isolates (*B. cereus* Jrb1, *P. putida* Jrb2, *Bacillus* sp. Jrb6 and Jrb7)	Thangavelu and Gopi, 2015b
Serratia marcescens ITBB B5-1	Pre-planting soil drenching	4	Pre-planting soil drenching (10^6 conidia mL^{-1})	70		Isolated from the rubber tree	Tan et al., 2015
Pseudomonas fluorescens Pf1, *Bacillus subtilis* EPB 10 and EPB 56	*In vitro* co-culturing of plants with bacteria		*In vitro* co-culturing of plants with *Foc* after bacteria inoculation			Increased leaf nutrient status and enhanced growth, bunch yield and fruit quality	Kavino et al., 2016
Pseudomonas fluorescens Pf1, *Bacillus subtilis* EPB 10 and EPB 56	Root dipping ($3{\cdot}10^{10}$CFU mL^{-1})	1	Injection into the corm (10^6 conidia mL^{-1})	78	119		Kavino and Manoranjitham, 2018
Fusarium oxysporum CAV553 and CAV255, *Pseudomonas fluorescens* WCS417	Root dipping and soil drenching (10^6 conidia mL^{-1})	STR4	Natural infestation	0		Endophytes from healthy micropropagated Cavendish banana roots (South Africa)	Belgrove et al., 2011
Trichoderma asperellum Prr2 (endophyte), *Trichoderma* sp. NRCB3 (rhizospheric)	Colonized rice chaffy grains	1	Natural infestation	47	45	Growth-promoting effects	Thangavelu and Gopi, 2015a
***Trichoderma* spp.**							
T. viride	Root dipping (10^6 conidia mL^{-1}), followed by application of colonized wheat bran:saw dust mixture		Natural infestation	75	60		Raguchander et al., 1997
T. viride NRCB1	Colonized rice chaffy grains ($1{\cdot}10^{31}$ CFU g^{-1}) + 5% jaggery solution		Colonized sand:maize mixture	80		Induction of peroxidase, phenylalanine ammonia lyase, and total phenolic content	Thangavelu and Mustaffa, 2010
T. harzianum TH UH and TH13		TR4		0			Wibowo et al., 2013
T. harzianum (ECO-T®)	Soil inoculation in the nursery			68		*Foc* reduction: 68% in Humic Nitisol, 6% in Rhodic Ferralsol	Mukhongo et al., 2015
***Pseudomonas* spp.**							
P. fluorescens	Root dipping (10^6 conidia mL^{-1}), followed by wheat bran application		Natural infestation	77	68		Raguchander et al., 1997

(Continued)

TABLE 1 | Continued

Biocontrol agents	Mode of application	*Foc* race	*Foc* inoculum	Best disease control obtained (%)	Highest yield increase obtained (%)	Relevant remarks	References
P. fluorescens Pf1	Different combinations of paring and pralinage with a *P. fluorescens* formulation ($2.5{\cdot}10^8$ CFU g^{-1}), soil application, and capsule application		Natural infestation	80.6		Two field trials. Pairing and pralinage with *P. fluorescens* formulation + capsule application at 3 and 5 months after planting gave the best results	Raguchander et al., 2000
P. fluorescens Pf1	Soil inoculation at transplanting and post-planting with a talc-based powder formulation			100	68		Rajappan et al., 2002
P. fluorescens Pf1	Soil application combined with *Bacillus subtilis* TRC 54 and a plant extract-based fungicide		Natural infestation	75		Also, 64% FWB reduction in greenhouse	Akila et al., 2011
P. fluorescens Pf1	4 L ha^{-1} of liquid formulation ($9{\cdot}10^8$ CFU mL^{-1})			60	47	Three field trials. Also, 41.3–89% reduction of *Helicotylenchus multicinctus*	Selvaraj et al., 2014
***Bacillus* spp.**							
B. subtilis TR21	Root dipping			73.9			Yu G. et al., 2010
B. subtilis TRC 54	Soil application combined with *Pseudomonas fluorescens* Pf1 and a plant extract-based fungicide		Natural infestation	75		Also, 64% FWB reduction in greenhouse	Akila et al., 2011
Bacillus spp. (PHC Biopak®)	Soil inoculation in the nursery			50		*Foc* reduction: 50% in Vertisol, 47% in Humic Nitisol	Mukhongo et al., 2015
B. amyloliquefaciens NJN-6	Colonized bio-organic fertilizer			68.5	100	Isolated from suppressive soil	Xue et al., 2015
B. amyloliquefaciens W19	Colonized bio-organic fertilizer (10^9 CFU g^{-1})		Natural infestation	44.4	34.5	Banana root exudates Enhanced colonization	Wang et al., 2016
Non-pathogenic *Fusarium oxysporum*							
F. oxysporum Ra-1, Ro-3	Colonized sand:maize mixture	1	Colonized sand:maize mixture	84			Thangavelu and Jayanthi, 2009
F. oxysporum UPM31P1	Colonized peat:perlite:oats (2:1:2 vol.) (10^6 UFC g^{-1})	TR4		0			Ting et al., 2009c
Arbuscular mycorrhizal fungi							
Glomus mosseae, *Trichoderma harzianum*	Inoculation at transplanting	1	Colonized sorghum grains ($1.5{\cdot}10^6$ CFU g^{-1})	68 (measured by ELISA)	75	Growth promotion	Mohandas et al., 2010
Glomus clarum	Soil inoculation in the nursery			23		Ineffective when *G. clarum* was combined with *Pseudomonas putida* and *Trichoderma asperellum*	Lin et al., 2012
Rhizatech®	Soil inoculation in the nursery			55		*Foc* reduction in Humic Nitisol on cv. Gros Michel	Mukhongo et al., 2015
Other microorganisms							
Serratia marcescens	Bentonite and kaolin formulations	TR4				Bentonite performed better. In bentonite formulation, PABA should be omitted, while NFSM, and sucrose levels should be optimized	Ting A.S.Y. et al., 2011

TABLE 2 | List of biocontrol pot-experiments against Fusarium wilt of banana.

Biocontrol agents	Mode of application	*Foc* race	*Foc* inoculum	Best disease or *Foc* UFC reduction (%)	Relevant remarks	References
Endophytes						
Streptomyces griseorubiginosus S96	Root dipping (10^6 spores mL^{-1})	4	10^4 conidia mL^{-1}	47	Siderophore-producing strain, selected from 131 banana roots-endophytic actinomycetes	Cao et al., 2005
F. oxysporum BRIP 29089, 29093 and 45952	Colonized ground millet	1, STR4	Colonized ground millet	75 (R1) 67 (R4) (vascular discoloration)	Obtained from banana roots in suppressive soil	Forsyth et al., 2006
Burkholderia spp. AB202 and AB213, *Herbaspirillum* spp. BA227 and BA234	Root dipping ($5{\cdot}10^7$ CFU mL^{-1})			97 (CFU)	Isolated from roots and stem of pineapple and banana	Weber et al., 2007
Endophytic bacteria (mainly γ-Proteobacteria)	Crude endophytes inoculum (7.1 log CFU g^{-1})	4	10^5 conidia mL^{-1}	67	Growth-promoting effects	Lian et al., 2009
Erwinia chrysanthemi E353		4		60.67	Endophytic strain from a healthy banana plant in a *Foc*-infested field	Yin et al., 2009
Penicillium citrinum	Soil drenching (10^6 conidia mL^{-1})	4	10^6 UFC mL^{-1}	2	Host defense response	Ting et al., 2012
Pseudomonas fluorescens Pf1, *Bacillus subtilis* EPB 10, and EPB 56	*In vitro* co-culturing of plants with bacteria		*In vitro* co-culturing of plants with *Foc* after bacteria inoculation			Kavino et al., 2014
Trichoderma sp. TJ5	Root dipping (10^6 CFU mL^{-1})	1	Soil inoculation (10^6 CFU mL^{-1})	62.5	Plant growth promotion	Caballero Hernández et al., 2013
T. asperellum		1				Chaves et al., 2016
***Trichoderma* spp.**						
T. viride	Soil inoculation					Shamarao et al., 2001
T. harzianum	Soil inoculation	4		0	*T. harzianum* + $Ca(NO_3)_2$	Ting et al., 2003
T. viride	Colonized organic amendments				Neem cake, groundnut cake, *Pongamia* cake	Satheesh and Venu, 2004
T. viride	Colonized corn grits		Colonized corn-meal:sand	81.76	Abaca (*Musa textilis*)	Bastasa and Baliad, 2005
T. harzianum A34	Soil inoculation ($8{\cdot}10^9$ UFC g^{-1})	4	Naturally infested soil	95	Plantains	Pérez Vicente et al., 2009
Trichoderma sp. TR76	Soil drenching (10^6 UFC ml^{-1})			41		Hima and Beena, 2016
Trichoderma spp. T22 and T5	Soil drench (10^7 spores mL^{-1})	4	Colonized millet seeds	62	Isolated from suppressive soil	Nel et al., 2006
T. viridae	Root dipping and soil drenching					Pushpavathi et al., 2017

(Continued)

TABLE 2 | Continued

Biocontrol agents	Mode of application	*Foc* race	*Foc* inoculum	Best disease or *Foc* UFC reduction (%)	Relevant remarks	References
T. asperellum PZ6	Root-injury irrigating method	4	Root-injury irrigating method	48	Plant growth promotion	Qin et al., 2017
***Pseudomonas* spp.**						
P. fluorescens Pfcp	Root dipping (10^8 CFU mL^{-1})	1, 4	10^8 conidia mL^{-1}	80	Less severe wilting and internal discoloration. Improved root growth and enhanced plant height in *M. balbisiana*	Sivamani and Gnanamanickam, 1988
P. fluorescens	Root dipping (10^8 UFC mL^{-1})					Shamarao et al., 2001
P. fluorescens Pf10	Soil inoculation			50	Detoxification of fusaric acid	Thangavelu et al., 2001
P. fluorescens Pf10	Soil drenching (10^9 CFU mL^{-1})	1	Colonized sand:maize mixture		Induction of defense enzyme and phenolics	Thangavelu et al., 2003
P. fluorescens Pfm	Talc powder formulation (10^8 CFU g^{-1})	1	10^6 conidia mL^{-1}	50 (vascular discoloration)	Enzymatic activity assay	Saravanan et al., 2004a
P. fluorescens Pfm. Pf1, Pf2, and Pf3	Talc powder formulation (10^8 CFU g^{-1})	1		7.4 (spore germination)	Rifampicin resistant strain of *P. fluorescens*	Saravanan et al., 2004b
P. fluorescens	Colonized charcoal (10^8 CFU mL^{-1})		Colonized sorghum seeds ($9.2{\cdot}10^4$ CFU mL^{-1})	72	Immunolocalization of both organisms in banana roots	Mohandas et al., 2004
P. aeruginosa FP10	Inoculation of *in vitro* plants				Plant growth promotion	Ayyadurai et al., 2006
P. fluorescens	Root dipping and soil drenching					Pushpavathi et al., 2017
***Bacillus* spp.**						
B. subtilis						du Plessis, 1994
B. thuringiensis	Root dipping (10^6 CFU mL^{-1})					Shamarao et al., 2001
B. licheniformis C-4	Root dipping					Sun and Wang, 2009
B. subtilis KY-21	Soil drenching ($5{\cdot}10^5$ CFU mL^{-1})	4	Soil drenching ($5{\cdot}10^5$ CFU mL^{-1})	33	Induction of defense-related enzymes	Sun et al., 2011
B. subtilis N11	Colonized bio-organic fertilizer			82	Biofilms formation and enhancement of root elongation and differentiation zones	Zhang et al., 2011
Bacillus spp. RZ-1, 3, 10, 34, 35, 60, 69, and 76	Root dipping ($OD_{540} = 0.5$)		Soil drenching (10^5 CFU mL^{-1})	16	Also, a dual effect on mortality and motility of *Meloidogyne javanica* second stage juvenile	Ribeiro et al., 2012
B. amyloliquefaciens W19	Colonized bio-organic fertilizer (10^9 CFU g^{-1})		Naturally infested field soil ($1.5{\cdot}10^4$ CFU g^{-1})	77	Antifungal lipopeptides	Wang et al., 2013

(Continued)

TABLE 2 | Continued

Biocontrol agents	Mode of application	*Foc* race	*Foc* inoculum	Best disease or *Foc* UFC reduction (%)	Relevant remarks	References
B. amyloliquefaciens WJ22	Colonized bio-organic fertilizer ($3{\cdot}10^8$ CFU g^{-1})		Naturally infested field soil ($1{\cdot}10^3$ CFU g^{-1})	75.7	Antifungal lipopeptides	Wang J. et al., 2015
Non-pathogenic *Fusarium oxysporum*						
F. oxysporum CAV 255 and CAV 241	Soil drench (10^7 spores mL^{-1})	TR4	Colonized millet seeds	87.4	Obtained from suppressive soil	Nel et al., 2006
***Streptomyces* spp.**						
Streptomyces sp. g10	Soil drenching (10^8 CFU mL^{-1})	4	10^4 or 10^6 conidia mL^{-1}	47	Effective against *Foc* at 10^4 conidia mL^{-1} but not at 10^6 conidia mL^{-1}	Getha et al., 2005
S. lunalinharesii B-03	Fermentation broth	4	10^6 CFU mL^{-1}	73	Effective *in vitro* against nine pathogens	Zhou et al., 2017
8 actinomycetes	Fermentation broth	4	$1.85{\cdot}10^6$ conidia mL^{-1}	87	Selected from 139 isolates. Effective *in vitro* against several *F. oxysporum* ff. spp.	Qin et al., 2010
Arbuscular mycorrhizal fungi						
Glomus intraradices, *Glomus* spp.	Soil inoculation in the nursery				Plant growth promotion	Jaizme-Vega et al., 1998
Glomus fasciculatum	Soil culture (500 chlamydospores)			43 (CFU)	Increased cell size and number. More total insoluble polysaccharides, total proteins, and total nucleic acids	Habeeba et al., 2003
Gigaspora margarita	Soil inoculation in the nursery				FWB reduction dependent on AMF and *Foc* inoculum concentrations	Borges et al., 2007
Native arbuscular mycorrhizal fungi	$3.5{\cdot}10^3$ or $7{\cdot}10^3$ kg^{-1}		10^6 conidia mL^{-1}		More mycorrhiza in plants treated with a biofertilizer rather than three concentrations of Hoagland solution	Sampaio et al., 2012
Other microorganisms						
Rhizospheric strains FB5, FB2, T2WF, T2WC, and W10	Root dipping	4		81		Yang et al., 2006
Bacteria 0202 and 1112		4				Wang et al., 2011
Paenibacillus spp. RZ-17, and RZ-24	Root dipping ($OD_{540} = 0.5$)		Soil drenching (10^5 CFU mL^{-1})	16	Dual effect on mortality and motility of *Meloidogyne javanica* second stage juveniles	Ribeiro et al., 2012
Marine rhizobacteria YS4B1, YS1A3, YS2A5					Isolated from mangrove rhizosphere. Effective also against *Ralstonia solanacearum* and *Mycosphaerella fijiensis*	Bonsubre et al., 2016
F. oxysporum f. sp. *cubense* (dead)			10^4 CFU mL^{-1}	100		Chand et al., 2016

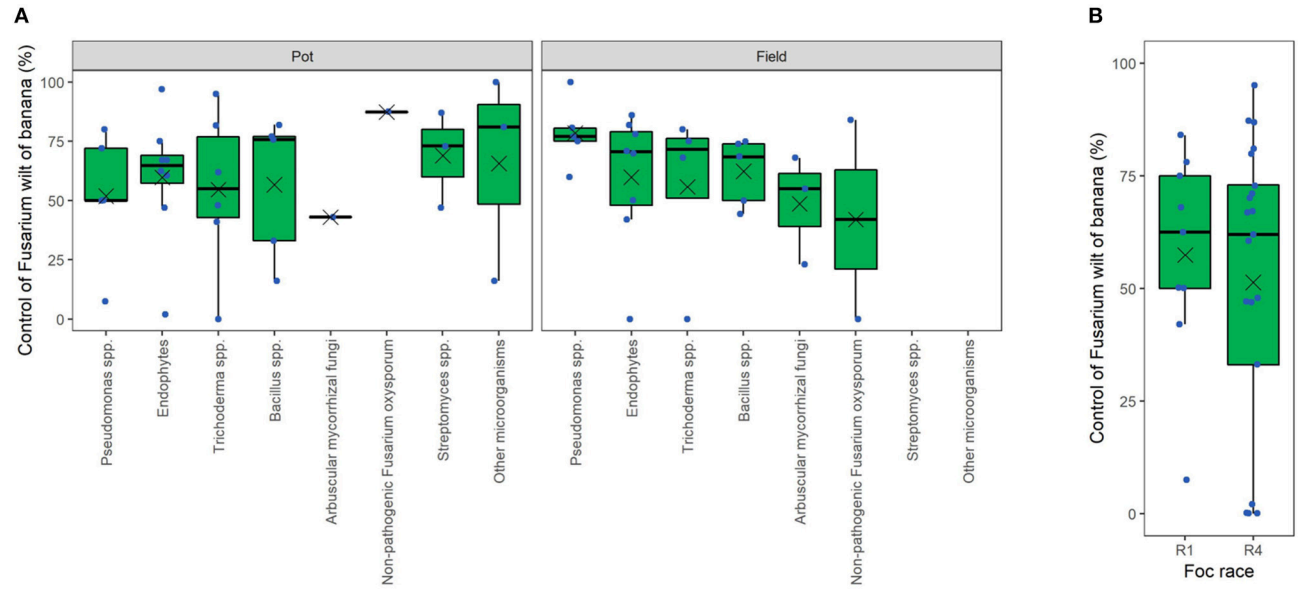

FIGURE 6 | Overview of the efficacy of biological control agents against Fusarium wilt of banana. Data of pot and field trials separated by microbial groups **(A)** or by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) races **(B)**. Dots correspond to the best control levels (*viz.* reduction of disease incidence or severity) obtained in each scientific article in the literature. In the boxes, the mean is reported as a cross and the median as a horizontal line.

of a commercial plantation (Lian et al., 2009). In this latter study, the re-introduction of endophytes to the banana tissue culture led to a significant reduction of wilt disease (67%) caused by *Foc* R4 (artificial inoculation) and growth promotion under greenhouse conditions. Ten non-pathogenic *F. oxysporum* isolates obtained from healthy micro-propagated "Cavendish" banana roots were able to significantly reduce FWB under greenhouse conditions, but none of them nor *P. fluorescens* WCS417 gave protection from the disease (STR4) in the field (Belgrove et al., 2011). Some researchers tried to have more chances to obtain effective BCAs by isolating from FWB-suppressive soil, e.g., non-pathogenic *F. oxysporum* strains (Forsyth et al., 2006), or even from healthy banana plants located in *Foc*-infested soil, e.g., *Erwinia chrysanthemi* E353 (Yin et al., 2009).

Furthermore, endophytes effective against FWB did not originate only from banana plants. For instance, Ho et al. (2015) isolated *Burkholderia cenocepacia* 869T2 from surface-sterilized vetiver grass (*Chrysopogon zizanioides*) roots. Banana tissue culture plantlets inoculated with 869T2 showed a lower disease incidence caused by *Foc* TR4 (86% incidence reduction) as well as significant plant growth promotion under field conditions. The endophytic strain *Serratia marcescens* ITBB B5-1 was isolated from the rubber tree (*Hevea brasiliensis*) (Tan et al., 2015). A sharp reduction in disease severity caused by *Foc* R4 was scored under both greenhouse (79%) and field conditions (70%) upon inoculation of banana plants with this strain. Moreover, chitinase and glucanase activities were suggested to be involved in its antifungal activity (Tan et al., 2015). Endophytic diazotrophic bacteria (strains of *Burkholderia* sp. and *Herbaspirillum* sp.) isolated from roots and stems of pineapple (Weber et al., 2007), and an *Acremonium* sp. strain isolated from *Kandelia candel* (Liu and Lu, 2013) are additional examples of promising biocontrol and biofertilizer candidates for the banana crop that originate from a different host. Finally, effective control of FWB and production increase (number of banana hands and bunch weight) was reported under field conditions when different combinations of endophytic (*P. putida* C4r4, *Achromobactrum* sp. Gcr1, *Rhizobium* sp. Lpr2, and *B. flexus* Tvpr1) and rhizospheric bacteria (*B. cereus* Jrb1, *P. putida* Jrb2, *Bacillus* sp. Jrb6, and Jrb7), in this case isolated from different banana accessions, were applied to a naturally infested soil (Thangavelu and Gopi, 2015b). In another field trial, combined applications of the endophytic *T. asperellum* prr2 together with the rhizospheric *Trichoderma* sp. NRCB3 resulted in a 47% reduction of FWB incidence and a 45% increase of the bunch weight (Thangavelu and Gopi, 2015a).

Bacillus spp.: The Endospore-Forming Bacteria

The use and number of *Bacillus* spp. strains displaying suppressive effect against plant diseases caused by soil-borne phytopathogens has been increased rapidly, and a large body of knowledge on the biocontrol mechanisms involved as well as on their application and effectiveness under diverse conditions is available for the interested reader (Fira et al., 2018; Aloo et al., 2019). The spore-forming ability of *Bacillus* species confers them an important advantage over other beneficial microorganisms in the field of biological control. On the one hand, this capability enables these bacteria to endure adverse environmental conditions. On the other hand, and from the agro-biotechnological point of view, it favors the development and manufacturing of commercial formulations more stable over time. In addition, many *Bacillus* species show rapid growth

rates and the ability to synthesize a large number of secondary metabolites which play a key role in the antibiosis against many deleterious microorganisms (Radhakrishnan et al., 2017; Fira et al., 2018). Some species, such as *B. subtilis*, are also able to produce volatile organic compounds (VOCs), which are important sometimes for plant growth promotion and the activation of plant defense mechanisms by triggering induced systemic resistance (Raaijmakers et al., 2010; Cawoy et al., 2014). *Bacillus*-mediated plant growth promotion can also be due to the capacity to promote phytohormone (i.e., gibberellic acid and indole-3-acetic acid) biosynthesis, thereby enhancing nutrient uptake ability in the host and stimulating plant defense responses against biotic and abiotic stresses (Chen et al., 2007; Harman, 2011). Besides the production of antibiotics and the elicitation of systemic resistance in plants against pathogens, *Bacillus* species are also able to produce lytic enzymes like chitinase and β-1,3-glucanase, involved in the degradation of the fungal cell wall (Kumar et al., 2012). Considering their versatility, the combination of different *Bacillus* spp. strains (or with other BCAs) displaying different biocontrol mechanisms appears as an interesting approach to improve biocontrol effectiveness under different cropping scenarios and environmental conditions.

Bacillus spp. are commonly found in the banana rhizosphere (Xue et al., 2015), and many members of this genus have already been investigated as BCAs of diverse *Fusarium*-induced plant diseases (Khan et al., 2017). Among them, representatives of *B. subtilis, B. amyloliquefaciens*, *B. pumilus* and *B. thuringiensis* are found in the literature. *Bacillus subtilis* is well known for its antagonistic activity against several fungal and bacterial plant diseases. Its biocontrol activity is mainly attributed to antibiotic production (Cawoy et al., 2015), and its enzymatic products are highly active against many fungal pathogens. The biocontrol effect of the plant endophytic *B. subtilis* strain TR21 against FWB was investigated in Brazilian fields, and promising results (74% effectiveness) were reported (Yu G. et al., 2010). Likewise, *B. subtilis* strain N11 isolated from the rhizosphere of a healthy banana plant showed biocontrol activity in pot experiments under greenhouse conditions (Zhang et al., 2011). Addition of 10% (v/v) of culture filtrate of the endophytic *B. subtilis* strain EBT1 to the plant growth medium increased bud multiplication, plantlet weight, pseudo-stem height, and conferred resistance to plantlets against *Foc* conidia and toxin (Yang et al., 2010). *Bacillus subtilis* strain B25, isolated from banana rhizosphere soil in Hainan, is another example of an effective antagonist, not only against *Foc*, but also against other plant pathogenic fungi including *Corynespora cassiicola*, *Alternaria solani*, *Botrytis cinerea,* and *Colletotrichum gloeosporioides* (Tan et al., 2013; Yu et al., 2016). Results on its capability to control FWB under greenhouse and field conditions can be found in the literature, although they are not easily accessible (Liu, 2011). The antifungal protein of B25, identified as a disease-resistance protein, produced mycelium and spore tumescence and abnormal growth of the pathogen (Tan et al., 2013). The chitinolytic and heat tolerant strain *B. subtilis* TSA3 showed *in vitro* inhibition of *Foc* growth although effectiveness *in planta* and under field conditions has not been demonstrated yet (Nawangsih and Purba, 2013). Similarly to previous examples, *B. subtilis* strain S-1 not only inhibited *in vitro Foc* growth but also antagonized fungal pathogens such as the *formae speciales lycopersici*, *vasinfectum* and *niveum* of *F. oxysporum*, as well as *Curvularia lunata*, *C. gloeosporioides*, *Verticillium dahliae,* and *Gibberella zeae* (Sun et al., 2008).

The *B. amyloliquefaciens* strain NJN-6 was isolated from the rhizosphere of a healthy banana plant in a FWB-suppressive soil. Field plots with plants pre-treated (in nursery pots) with a bio-organic fertilizer colonized by NJN-6 showed a decreased disease incidence by 68.5%, resulting in doubled yield (Xue et al., 2015). The mode of action of this strain relies on several metabolites. The lipopeptide iturin A, a powerful antifungal surfactant, is produced by several *Bacillus* strains including NJN-6 (Yuan et al., 2011). Two homologs of bacillomycin D and three homologs of members of the macrolactin family were identified in NJN-6 using HPLC/electrospray ionization mass spectrometry. Bacillomycin D and macrolactin exhibited significant antagonistic effects against *Foc* and *R. solanacearum*, respectively (Yuan et al., 2012a). Finally, among 36 VOCs detected in NJN-6, 11 compounds completely inhibited the *Foc* growth (Yuan et al., 2012b). Wang et al. (2013) isolated 57 bacterial strains from the rhizosphere of healthy banana plants grown in a field severely affected by the disease, all showing antagonism against *Foc*. Six strains (W2, W10, W14, W15, W17, and W19) displaying the best survival abilities in the rhizosphere soil were tested in greenhouse experiments, *B. amyloliquefaciens* W19 being the most effective against FWB. Moreover, and even more suggestive, biocontrol effectiveness of a bio-organic fertilizer colonized by W19 was then proved in a naturally infested field, where it reduced FWB by 44% and increased yield by 35% (Wang et al., 2016). Similarly to *B. amyloliquefaciens* NJN-6, the strain W19 produces several antifungal metabolites, including lipopeptides (e.g., iturin, bacillomycin D, and surfactin), 18 VOCs (Wang et al., 2013), and indole-3-acetic acid (Wang et al., 2016). Interestingly, banana root exudates seemed to enhance the ability of this strain to colonize roots by augmenting bacteria biofilm formation, due to surfactin production (Wang et al., 2016).

Munimbazi and Bullerman (1998) reported on extracellular antifungal metabolites produced by *B. pumilus* which inhibited the mycelial growth of different strains of *Aspergillus* sp., *Paenibacillus* sp., and *Fusarium* sp. The chitinolytic *B. pumilus* strain CH4 caused inhibition of *Foc* mycelial growth under *in vitro* conditions, but its effectiveness *in planta* has not been tested yet (Nawangsih and Purba, 2013).

The interest of *B. thuringiensis* as a BCA has been mainly focused on the Cry protein and its effect as bio-insecticide, a topic outside the scope of this review (Bravo et al., 2013, 2017). Nevertheless, it has also potential in biocontrol of phytopathogenic fungi due to the chitinase production. In fact, two chitinolytic strains of *B. thuringiensis* (50E and 48F) caused complete growth inhibition of *Foc* R4 *in vitro*. Indeed, uneven thickening and swelling of hyphae tips were observed at the sites where interaction with the bacterial crude chitinase took place (Usharani and Gowda, 2011).

Pseudomonas spp.: The Metabolically-Versatile Biological Control Agents

Pseudomonas is a genus that comprises more than a hundred species (Mulet et al., 2010; Loper et al., 2012; Hesse et al., 2018). Many *Pseudomonas* spp. strains are indigenous inhabitants of the plant endosphere, rhizosphere, and/or phyllosphere, mostly established in these niches as commensals. Some of them are able to suppress the deleterious effects caused by different phytopathogens thereby promoting plant growth and health, and have thus been successfully used as plant inoculants (Arshad and Frankenberger, 1997; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009; Pliego et al., 2011; Schreiter et al., 2018). These bacteria displayed characteristics such as: (i) high colonization competence for plant surface, internal plant tissues (endophytism), and/or phytopathogen structures; (ii) versatility in the production of antibiotics suppressing diverse phytopathogens; (iii) ability to use specific nutrients in the target niche like plant exudates, that enable them to outcompete many components of the plant-associated microbiota; and (iv) capability to trigger (systemic) defense responses in the host plants (Mercado-Blanco, 2015, and references therein).

A relatively large number of *Pseudomonas* spp. strains have been studied as antagonists of *Foc*, the majority of which has been focused on species of the *fluorescens* group. A screening for the antagonistic activity of fluorescent pseudomonads isolated from the rhizoplane of several crops led to the identification of *P. fluorescens* Pf1 (Vidhyasekaran and Muthamilan, 1995). Since then, this strain has been studied, and proved effective, on several plant diseases, such as Fusarium wilt of chickpea (*F. oxysporum* f. sp. *ciceris*) (Vidhyasekaran and Muthamilan, 1995), rice blast (*Pyricularia oryzae*) (Vidhyasekaran et al., 1997), and rice sheath blight (*Rhizoctonia solani*) (Vidhyasekaran and Muthamilan, 1999; Nandakumar et al., 2001). *Pseudomonas fluorescens* Pf1 produces siderophores, hydrogen cyanide, the antibiotics 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin, and induces resistance-associated enzymes (e.g., PO and PPO) in banana roots (Akila et al., 2011; Selvaraj et al., 2014). Repeated field trials have also demonstrated the effectiveness of *P. fluorescens* Pf1 against FWB, using diverse application protocols and formulations. Carriers such as wheat bran-saw dust (Raguchander et al., 1997), paring and pralinage, capsule (Raguchander et al., 2000; Rajappan et al., 2002), and talc (Rajappan et al., 2002; Saravanan et al., 2004b) have been explored. However, it is known that liquid formulations of *P. fluorescens* offer numerous advantages over solid formulations, e.g., high cell count, zero contamination, longer shelf life, greater protection against environmental stresses, and increased field efficacy (Hegde, 2002). Different chemicals, such as trehalose, polyvinylpyrrolidone, and glycerol, were tested for the development of a liquid formulation, and glycerol supported the highest Pf1 survival until 6 months of storage (Manikandan et al., 2010). A liquid formulation of Pf1 provided good results against FWB in multiple trials and locations (Selvaraj et al., 2014).

Saravanan et al. (2004a) reported a significant *in vitro* inhibitory effect on *Foc* R1 when testing five different *P. fluorescens* strains (Pf1, Pf2, Pf3, Pf4, and Pfm) isolated from banana rhizosphere, with the strain Pfm showing the highest antagonist effect against the pathogen growth. When greenhouse experiments were conducted using a talc-based formulation of strain Pfm, a significant reduction in vascular discoloration of the banana rhizome was observed (Saravanan et al., 2004a). Additionally, *P. fluorescens* Pfm systemically induced the accumulation of three key defense enzymes (PAL, PO, and polyphenol oxidase or PPO) in roots that contributed to induce resistance against *Foc* (Saravanan et al., 2004b).

Sivamani and Gnanamanickam (1988) investigated the possibility of suppressing FWB by bacterization with different *P. fluorescens* strains originating from roots of rice (strain Pfrl3), peanut (Pfgn), banana (Pfb), black gram leaves (Pfbg), citrus (Pfcp), and cotton (Pfco). These strains were tested for their *in vitro* antagonism ability against *Foc* R1 and R4. Results showed that strain Pfcp showed maximum inhibition of *Foc* mycelial growth, and was chosen to bacterize seedlings of *Musa balbisiana.* Seedlings treated with Pfcp showed less severe wilting symptoms and internal discoloration under greenhouse conditions. In addition, they also showed enhanced root growth and overall plant height.

In another study, 11 strains of *P. fluorescens* isolated from the banana rhizosphere were tested for their *in vitro* antagonistic effect against *Foc*. Among the tested isolates, strain Pf10 was the most effective in inhibiting the pathogen mycelial growth (Thangavelu et al., 2001). Further studies were focused on the effect of strain Pf10 treatment and *Foc* inoculation on the induction of banana plant enzymes and compounds known to be related with defense responses (e.g., PAL, POX, chitinase, β-1,3 glucanase and phenolics) (Thangavelu et al., 2003).

In vitro growth inhibition of *Foc* was also observed with *P. fluorescens* strain IIHRPf12. In greenhouse experiments using banana cv. Neeypovan, this strain reduced *Foc* colonization and FWB severity symptoms. Interestingly, structural modifications in the cortical cells at the site of fungal entry were observed, indicating that bacterized root cells were somehow "alerted" to mobilize a number of defense structures aiming to hinder the pathogen progress (Mohandas et al., 2004).

Goswami et al. (2015) reported on the *P. aeruginosa* strain BG, isolated from marine water from the Gulf of Khambhat in Gujarat, and its ability to inhibit *Foc* growth under *in vitro* conditions. *Pseudomonas aeruginosa* BG displayed plant growth promotion, biocontrol abilities, secretion of enzymes such as catalase, urease, and phosphatase, as well as the synthesis of metabolites such indole-3-acetic acid, siderophores, ammonia, and hydrogen cyanide. Other *P. aeruginosa* were also tested against *Foc in vitro* (Ayyadurai et al., 2006; Sekhar and Pious, 2015) and *in planta* (Yu C. et al., 2010).

Trichoderma spp.: Antagonists and Plant Growth Promoters

Trichoderma is a genus of asexually-reproducing fungi widely distributed in nearly all temperate and tropical soils. The sexual

teleomorph (genus *Hypocrea*) can be found frequently, but many strains, including most biocontrol strains, have no known sexual stage. *Trichoderma* spp. show a wide genetic diversity, and are producers of several extracellular proteins, enzymes such as cellulase and chitinase, and more than 100 different metabolites with antibiotic activities. This genus can also parasitize a range of other fungi (e.g., *R. solani*). Besides antibiosis and mycoparasitism, *Trichoderma*-mediated biocontrol also relies on the induction of plant resistance (Harman et al., 2004). Therefore, due to their metabolite arsenal, rhizosphere-competence, and ability to stimulate plant growth, *Trichoderma* species have long been recognized as BCAs (Harman et al., 2004; Vinale et al., 2008; Woo et al., 2014), and they are widely studied against FWB (**Supplemental Figure 2**). These fungi are efficient colonizers of plant roots, where they establish intense interactions with plants (Vinale et al., 2008). They colonize root surfaces and invade the root epidermis, usually not beyond the first or second layer of cells (Yedidia et al., 2000), but some authors have claimed the endophytic nature of the strains tested in their research, even in banana (Caballero Hernández et al., 2013; Thangavelu and Gopi, 2015a; Chaves et al., 2016).

A rhizospheric strain, namely *T. viride* NRCB1, was identified *in vitro* among 37 isolates and tested in the pot- and field-experiments, where it reduced FWB by a maximum of 75–80% in terms of external symptoms and vascular browning (Thangavelu and Mustaffa, 2010). The authors proved that a bioformulation based on the rice chaffy grains conferred a higher efficacy over the talc cum powder formulation. Finally, the strain was able to induce the peroxidase (PO), phenylalanine ammonia lyase (PAL), and total phenolic content in treated plants (Thangavelu and Mustaffa, 2010). Later, the same authors identified a new rhizospheric strain (*Trichoderma* sp. NRCB3) which was combined with the endophyte *Trichoderma asperellum* Prr2, and successfully tested against FWB in the field (Thangavelu and Gopi, 2015a). Significant disease protection was obtained in the field using a *T. viride* strain applied by root dipping at transplanting and by a colonized wheat bran:saw dust mixture 3 months later. The treatment reduced FWB incidence by 75% and increased the yield by 60% (Raguchander et al., 1997). Interesting results were also obtained using a commercial product namely ECO-T® (containing *T. harzianum*; Plant Health Products, South Africa), though they were context-specific. In fact, the product reduced by 68% the *Foc* inoculum in Humic Nitisol, but only by 6% in Rhodic Ferralsol (Mukhongo et al., 2015). With the assumption that potential microbial antagonists are more abundant in soil with a history of low disease incidence, Nel et al. (2006) selected two *Trichoderma* spp. isolates, T22 and T5, from FWB-suppressive soil in South Africa, and obtained with them a 62% FWB control in a glasshouse (*Foc* R4). These strains were among the most effective strains tested in the experiment (33 strains) and were superior to a *Trichoderma*-based commercial product. Interesting results (efficacy higher than 80%) were also obtained on abaca (*Musa textilis*) and plantain (Bastasa and Baliad, 2005; Pérez Vicente et al., 2009). The literature, however, also reports examples of failure in the control of *Foc* (R4) using *Trichoderma* spp. strains, albeit resulting effective *in vitro* or able to delay the disease onset (Ting et al., 2003; Wibowo et al., 2013).

Arbuscular Mycorrhizal Fungi: Not Only Nutrient Providers for the Plants

The root cortical tissues enable the host plant to live in a symbiotic association (intercellularly or intracellularly) with fungi of the phylum Glomeromycota, which develop morphological structures called arbuscules and transform the infected root into the so-called "mycorrhiza." These arbuscular mycorrhizal fungi (AMF) gain some nutrients from the plant and return others to it, thus enhancing the plant growth. Additionally, AMF protect plants against phytopathogens and abiotic stresses (Parniske, 2008; Bonfante and Genre, 2010; Lenoir et al., 2016). The AMF's beneficial effects have been also demonstrated in banana, albeit sometimes with inconstant results. While AMF are generally considered as plant growth promoters rather than pathogen antagonists, it is fascinating to see how AMF inoculated in the banana nursery could provide, in some cases, protection from FWB in the field during several subsequent weeks. Indeed, an early study reported that either *Glomus intraradices* or *Glomus* spp. stimulated the growth of banana plants cv. Grande Naine and reduced both rhizome necrosis and external FWB symptoms (Jaizme-Vega et al., 1998). Inoculation with *Gigaspora margarita* was found to reduce FWB in pot-grown plants cv. Maçã, especially under low concentration of *Foc* inoculum (Borges et al., 2007). Also, the pathogen population in roots of banana plants at 7 months after treatment with a combination of *G. mosseae* and *T. harzianum* was significantly reduced, as measured by ELISA (Mohandas et al., 2010). In the field, banana plants pre-treated in the nursery with *G. clarum* had higher biomass than untreated plants, and showed lower FWB incidence (67%), compared to the untreated control (88%). But plants pre-treated with *G. clarum* and then inoculated at transplanting with a commercial product based on *P. putida* and *T. asperellum* did not differ from the untreated control in FWB incidence and severity (Lin et al., 2012). The lack of knowledge about the interactions among those BCAs might have led to unexpected results.

It is known that plant colonization by AMF is stimulated by the soil organic matter and hindered by mineral fertilization. In banana cv. Maçã, the application of a bio-fertilizer promoted abundant mycorrhizal colonization and was associated to lower expression of FWB symptoms, compared to applications of the Hoagland solution at three concentrations (non-fertilized control was not established) (Sampaio et al., 2012). However, inconsistent results obtained with AMF have been a common experience. In a factorial experiment, the effectiveness of a commercial AMF-based product and two other commercial BCAs was context-specific. Three products were inoculated in the nursery and their efficacy against FWB evaluated on "Gros Michel" banana grown in fields with three different soil types. Rhizatech® (Dudutech, Kenya) reduced *Foc* inoculum by 55% in Humic Nitisol. In this soil type, PHC Biopak® (*Bacillus* spp.; Plant Health Care Inc., USA) reduced *Foc* by 47%, a level similarly reached in Vertisol (50%). ECO-T® (*T. harzianum*; Plant Health Products, South Africa), also showed the best efficacy in Humic Nitisol (68% disease control), while it was almost ineffective in Rhodic Ferralsol (6%) (Mukhongo et al., 2015).

Non-pathogenic *Fusarium oxysporum*: Beneficial Relatives

The species *F. oxysporum* includes pathogenic strains as well as plant beneficial endophytes and saprophytes living in soil and on organic debris (Di Pietro et al., 2003). Non-pathogenic *F. oxysporum* (np*Fo*) strains are primarily recognized upon their inability to infect plants. Since about 120 *formae speciales* are known for *F. oxysporum*, np*Fo* should be validated on as many species by pathogenicity tests. Nevertheless, it is generally accepted that a limited number of plant species is enough to declare an *F. oxysporum* as np*Fo* (Fravel et al., 2003). Determining the vegetative compatibility group (VCG) cannot be used as a universal tool to identify np*Fo* isolates. Nel et al. (2006) developed a PCR-based restriction fragment length polymorphism (RFLP) analysis of the rRNA intergenic spacer (IGS) region for discriminating np*Fo* strains from *Foc* among 100 isolates obtained from banana rhizosphere in South Africa. The mechanisms underlying the biocontrol exerted by np*Fo* strains are based on the competition for infection sites or nutrients, as well as the induction of systemic resistance (He C. Y. et al., 2002; Fravel et al., 2003; Olivain et al., 2006). The fact that np*Fo* share the same niche with the pathogen is advantageous from the biocontrol perspective (Larkin and Fravel, 2002).

Two np*Fo* namely Ro-3 and Ra-1 reduced FWB (*Foc* R1) by 80% on cv. "Rasthali" in a field trial. They were selected *in vitro*, proved to be effective in reducing the disease (up to 89% reduced severity) and promoting the plant growth when applied three times on both tissue-cultured and sucker-derived plants under greenhouse conditions (Thangavelu and Jayanthi, 2009). Another np*Fo* isolate, UPM31P1, alone or in combination with *S. marcescens* isolate UPM39B3, resulted effective *in vitro* against *Foc* TR4, and reduced FWB under greenhouse condition. In the field, this strain only delayed the onset of FWB (*Foc* TR4), as it reduced the percentage of diseased plants by 75% at 15 weeks post-transplanting, but no significant difference between treated and untreated plants was observed at 28 weeks post-transplanting (Ting et al., 2009c). Plants treated with two np*Fo* isolates obtained from disease suppressive soils in South Africa, CAV 255 and CAV 241, showed a FWB incidence reduced by 87.4 and 75.0%, respectively (Belgrove et al., 2011). In the same trial, the widely studied strain Fo47 did not suppress significantly the disease. Forsyth et al. (2006) pointed out that np*Fo* isolates may unexpectedly be synergistic with *Foc*, thus they are not necessarily antagonists. In fact, although more evidence would be needed, the strain BRIP 45952 increased Fusarium wilt disease severity on "Cavendish." Nevertheless, another isolate, BRIP 29089, reduced disease severity in artificially inoculated "Lady Finger" (*Foc* R1) and "Cavendish" (*Foc* STR4) plants. At least three more research works have dealt with np*Fo* isolated from FWB suppressive soil and/or banana plants, though their assays remained at the laboratory stage (Nita and Harsh, 2015) or were not related to biocontrol (Nel et al., 2006; Deltour et al., 2018).

Streptomyces spp. and Other Actinomycetes: Natural Antibiotics Factories

Streptomycetes are the most important antibiotic-producing microbes. They also produce a broad range of additional secondary metabolites and lytic enzymes. For this reason, they receive attention for biotechnological, pharmaceutical and agricultural purposes. Streptomycetes are widely distributed in the soil, where they are strong competitors and antagonists. The use of streptomycetes as BCAs is largely documented in the literature and, recently, their application against plant diseases incited by *Fusarium* species has been reviewed by Bubici (2018). In the literature, we found only four pot trials where streptomycetes were tested against FWB (**Table 1**). All four experiments were conducted against *Foc* R4 and showed that FWB could be reduced between 46 and 87%. Interestingly, the highest disease reductions were obtained using the streptomycete fermentation broth (Qin et al., 2010; Zhou et al., 2017), compared to the experiments where spore suspensions were applied by drenching or root dipping (Cao et al., 2005; Getha et al., 2005). Qin et al. (2010) selected 8 out of 139 isolates using *in vitro* assays against several *F. oxysporum formae speciales* and demonstrated that the application of their fermentation broth provided a FWB control ranging from 78 to 87% in pot experiments. In particular, using $1.85 \cdot 10^6$ conidia mL^{-1} of *Foc* R4, plants treated with the best streptomycete strain, ZJ-E1-2, showed FWB incidence of 12%, while it was 76% on untreated trees. With a similar disease pressure, *viz.* 78% incidence on the control plants upon inoculation with $1 \cdot 10^6$ CFU mL^{-1} of *Foc* R4, Zhou et al. (2017) observed a FWB reduction of 73% after treatment with *S. lunalinharesii* B-03. The application of fermentation broth introduces into the soil both the microbial cells and their metabolites and, hence, it has a stronger impact on soil *Foc* inoculum than the sole microbial cells. In fact, when introduced alone, the cells must first proliferate to produce antifungal metabolites enough for effective control of *Foc*. Trees treated before planting with 10^6 CFU mL^{-1} of *Streptomyces* sp. strain S96, and later inoculated with 10^4 conidia mL^{-1} of *Foc* R4, showed significant reductions in FWB incidence, severity, and vascular browning. *Streptomyces* sp. strain S96 was selected from 131 endophytic actinomycetes isolated from surface-sterilized banana roots (Cao et al., 2005). The soil application of a spore suspension (10^8 CFU mL^{-1}) of *Streptomyces* sp. strain g10 reduced FWB severity index by 47% and rhizome discoloration by 53% when banana plantlets were inoculated with 10^4 conidia mL^{-1} of *Foc* R4. The same treatment was ineffective under higher pathogen pressure, i.e., 10^6 conidia mL^{-1} (Getha et al., 2005). *Streptomyces* sp. strain g10 was effective *in vitro* against several phytopathogenic fungi, including different physiological races of *Foc* (Getha and Vikineswary, 2002). Nevertheless, *Foc* and *R. solani* were more resistant than other fungi (i.e., *P. oryzae* and *Phytophthora palmivora*) to the antagonistic streptomycete (Getha et al., 2004). Crude fractions containing antifungal metabolites excreted in liquid media by g10 produced swelling, distortion and excessive branching of *Foc* R4

hyphae, as well as inhibition of spore germination. Antibiosis-mediated *Foc* antagonism was also demonstrated in sterile soils for the strain g10 by using an indirect method, i.e., the paper disc method (Gunji et al., 1983).

Several articles reported on *in vitro* experiments with *Streptomyces* spp. strains, but assays under *in vivo* conditions to fully demonstrate their biocontrol effectiveness have not made yet (**Table 2**). In these studies new antifungal metabolites were discovered, such as (6S,8aS,9S,11S,12aR)-6-hydroxy-9,10-dimethyldecahydrobenzo[d]azecine-2,4,12(3H)-trione (termed as 210-A) (Wu et al., 2009), and fungichromin (Wei et al., 2011). Other three compounds were isolated from *S. albospinus* 15-4-2: 2-methyl-2,5,6-bornantriol, 4,4′-(3-hydroxypropane-1,1-diyl)diphenol, and 7-(4-methoxybenzyl)-4,5,6,7-tetrahydro-1,3-oxazepine-5,6-diol. These compounds did not show an inhibitory effect against *Foc* R4, though the streptomycete was effective against the same pathogen (Yu et al., 2011). Two other studies demonstrated the efficacy of crude culture filtrate or methanol extracts of streptomycetes, but the effective metabolites were not identified (Shih et al., 2013; Wang L. et al., 2015). Soil inoculation with *S. griseus* St 4 viable cells was more effective in suppressing *Foc* TR4 (6 $\log_{10}$ CFU g^{-1} soil of *Foc*) than cell-free crude extracts (7 $\log_{10}$ CFU g^{-1} soil) at 20 days after inoculation (Zacky and Ting, 2013). The formulation of *S. griseus* St 4 with kaolin clay, sodium alginate, or a kaolin-alginate combination increased the effectiveness of the streptomycete, compared to non-formulated cells. The kaolin clay formulation reduced *Foc* TR4 soil inoculum from 6 to 5.4 $\log_{10}$ CFU g^{-1} soil (Zacky and Ting, 2015).

Other Genera or Unidentified Species of Biological Control Agents

A *Serratia marcescens* strain, isolated from roots of wild bananas, has shown plant growth promoting effect both in glasshouse and field, and suppressed FWB, though only in the glasshouse. The loss of control efficacy stimulated the evaluation of diverse formulations in an attempt to improve its viability and efficacy in field applications. Results showed that bentonite performed better and that further advantage could come from optimization of non-fat skimmed milk and sucrose levels, whereas para-aminobenzoic acid should be omitted from bentonite formulations (Ting et al., 2009a; Ting A.S.Y. et al., 2011). Other studies in greenhouse evaluating several rhizospheric bacterial strains (FB5, FB2, T2WF, T2WC, and W10) of unidentified species culminated in the successful control of FWB (81% reduction) (Yang et al., 2006). Moderate control (16%) was obtained using *Paenibacillus* spp. strains RZ-17 and RZ-24 which also had additional effects on mortality and motility of *Meloidogyne javanica* second stage juveniles (Ribeiro et al., 2012). Multiple beneficial effects have been also reported for strains of marine rhizobacteria isolated from mangrove (YS4B1, YS1A3, and YS2A5), which were also effective against *R. solanacearum* and *Mycosphaerella fijiensis* (Bonsubre et al., 2016). Finally, a number of studies identified *Foc* antagonists among strains of *Talaromyces* spp., *Eutypella* sp., *Paenibacillus polymyxa*, *Herbaspirillum* spp., *Tsukamurella paurometabola*, *Brevibacillus brevis*, and *Streptoverticillium lavenduligriseum* (Sun et al., 2010; Manoch and Dethoup, 2011; Ting S. et al., 2011; Marín et al., 2013; Shen L. et al., 2013; Sun and Hsieh, 2015; Qi et al., 2017). A spectacular control of FWB was claimed by Chand et al. (2016), who applied a dead *Foc* to plant roots before inoculation with live *Foc*. Although no data were showed, the authors stated that inoculated plants, grown in a sick plot, did not show disease symptoms even 2 months after inoculation, while they occurred on control plants 1 week after inoculation. This approach appears as interesting as it is unusual, and no other researchers have replicated the technique against *Foc* until now.

FUSARIUM WILT OF BANANA AND THE BANANA-ASSOCIATED MICROBIOMES

The development of next-generation sequencing (NGS) technologies, along with advanced bioinformatics tools, is rapidly increasing our knowledge on many biological processes. Diverse "-omics" techniques such as (meta)genomics, (meta)transcriptomics, proteomics, metabolomics, microbiomics, etc., are currently available to better understand plant-microbe(s) interactions from a holistic perspective (Massart et al., 2015). However, the implementation of "-omics" in the study of BCAs effective against FWB as well as their interaction with the banana-*Foc* pathosystem is still very scant.

The NGS-based approaches are very useful for the in-depth study of the structure, composition, and diversity of plant-associated microbiomes. Yet, microbiomics is waiting to be used in a more frequent way in the research field of banana, FWB, and biocontrol. Recent studies (*16S rRNA* and *ITS* amplicon sequencing profiling) have been focused on endophytic bacterial communities present in different parts and micro-environments of banana plants (Köberl et al., 2015, 2017; Zhai et al., 2016; Suhaimi et al., 2017), as well as in the microbiota of banana rhizosphere (Fu et al., 2016a) and soil (Xue et al., 2015; Rames et al., 2018).

Healthy plants and healthy soils have higher microbial diversity and more abundant beneficial microbes, which can improve nutrients uptake, promote plant growth, and control soil-borne diseases (Bulluck and Ristaino, 2002; Bailey and Lazarovits, 2003; Raaijmakers et al., 2009; Luan et al., 2015). Köberl et al. (2015) studied the impact of biogeography and agroforestry on the banana-associated microbiome, mostly γ-proteobacteria. Banana plants grown under agroforestry systems showed a higher abundance of potentially beneficial plant-associated bacteria and lower presence of phytopathogenic bacteria. Thus, γ-proteobacteria diversity and community members were identified as potential health indicators. Healthy plants revealed an increase in potentially beneficial microbes like *Pseudomonas* and *Stenotrophomonas*, while diseased plants showed a preferential occurrence of *Enterobacteriaceae* (Köberl et al., 2017). Another study correlated FWB positively with the abundance of *Proteobacteria*, *Ascomycota*, *Fusarium*, *Cylindrocarpon*, *Gymnascella*, *Monographella*, *Pochonia*, and *Sakaguchia*, but negatively with *Acidobacteria*, *Firmicutes*, *Leptosphaeria*, and *Phaeosphaeriopsis* (Shen et al., 2015a). In such

pot-experiment, 2 years of biofertilizer application manipulated the composition of the rhizosphere microbial community and induced the FWB suppression. The relationship among suppression of *Foc* under field conditions, the use of ground cover management, and changes in the soil microbiome was also investigated in "Ducasse" banana (synonym "Pisang Awak," ABB) (Pattison et al., 2014; Rames et al., 2018). Results showed that suppression of FWB tended to increase over time when the banana was cultivated with ground covers compared to bare soil conditions. Statistically significant changes over time in the structure of soil microbial communities in the vegetated treatment were observed, and potential biomarkers related to disease suppression were identified. In addition, fungal amplicon sequencing demonstrated that reduction of *Foc* in the vegetated treatment was associated with disease suppression.

Similarly, analyzing the banana/*R. solanacearum* pathosystem (bacterial wilt of banana or Moko), five major microbial genera were found in both symptomatic and non-symptomatic plant samples: *Sphingomonas*, *Methylobacterium*, *Flavobacterium*, *Pseudomonas*, and *Ralstonia*, the latter being more abundant in symptomatic (59% out of the entire genera) than in non-symptomatic plants (only 36%). In addition, several genera were only assigned to non-symptomatic plants (Suhaimi et al., 2017). Another experiment showed that the soil cultivated with tobacco and infested by *R. solanacearum* had lower microbial diversity than the soil free from the pathogen, which harbored more abundant beneficial microbes such as *Bacillus*, *Agromyces*, *Micromonospora*, *Pseudonocardia*, *Acremonium*, *Lysobacter*, *Mesorhizobium*, *Microvirga*, *Bradyrhizobium*, *Acremonium*, and *Chaetomium*. Also, the activities of catalase, invertase, and urease, as well as soil pH, available phosphorus and potassium content were lower in the infested soils (Wang et al., 2017).

Soil microbial community varies because of many factors. The main drivers of the rhizosphere microbiome are soil type and plant genotype (Berg and Smalla, 2009), but fertilizers (Ikeda et al., 2011), crop rotation (Hilton et al., 2013), and pesticides (Jacobsen and Hjelmso, 2014) may also play a significant role. Soil microbiota also fluctuates with the plant growth stages, mostly due to changes in the root exudates (Yang and Crowley, 2000; Okubo et al., 2016; Wang et al., 2017). Finally, the soil microbial assembly may be influenced by BCAs artificially introduced in the system (Xue et al., 2015). However, several studies have revealed that edaphic and anthropic factors had a deeper and more durable effect on the rhizosphere microbiota than a BCA application. For example, compared to untreated plants, *R. solani* had a much higher impact on lettuce rhizosphere bacterial communities than the applications of diverse BCAs such as *Trichoderma* sp. (Grosch et al., 2006), *P. jessenii* RU47 (Adesina et al., 2009), or *B. amyloliquefaciens* FZB42 (Chowdhury et al., 2013; Erlacher et al., 2014). On the other hand, co-inoculation of different BCAs may cause a more pronounced impact on the microbial community structure compared to the single strain application, as demonstrated in the lettuce rhizosphere (Grosch et al., 2012). Nonetheless, some systems may be more reluctant to changes, sometimes even contrasting the applied BCAs (Garbelotto et al., 2019).

The "-omics" technologies do not only provide a global overview of the banana-associated microbiota but may also yield useful information to develop more effective biological control strategies. For instance, dominant bacterial groups can be identified in FWB suppressive soils, thereby leading to the development of strains or consortia of strains serving as new biocontrol tools. For example, the above mentioned *B. amyloliquefaciens* strain NJN-6 was isolated from a FWB-suppressive soil after the NGS analysis had evidenced *Bacillus* as the dominant taxon (Xue et al., 2015). A metagenomic study has been targeted to microbes that harbor the non-ribosomal peptide-synthetase (NRPS) gene, which encodes for one of the largest groups of natural microbial secondary metabolites, such as the antibiotics vancomycin and gramicidin, as well as the lipopeptides surfactin, iturin A, and bacillomycin. The research evidenced that these microbes were more abundant in FWB-suppressive soil than in FWB-conducive soil. The main microbial taxa harboring the NRPS gene and related to FWB suppression were *Pseudomonas* spp. and *Streptomyces* spp. (Zhao et al., 2018). As potential probiotic candidates, plant vertically transmitted actinobacteria are beneficial to the growth and health of host plants (Du et al., 2018). The majority of bacteria from healthy banana shoot tips were affiliated with actinobacteria, being *Mycobacterium* and *Nocardia* the dominant taxa. The streptomycetes were isolated from shoot tips and proved to enhance the growth and resistance to *Foc* of pot-grown banana plants. The research elegantly presented how microbiomics can foster the selection for probiotic agents (Du et al., 2018). Another study has shown that the endophytic root microbiome of healthy banana plants was dominated by Nocardioidaceae (56.37%), Pseudonocardiaceae (14.36%) and Nocardiaceae (9.77%) (Zhai et al., 2016).

But metagenomics and microbiomics are not the sole "-omics" technologies that can help the research on biocontrol of FWB. Nevertheless, the implementation of "-omics" techniques other than microbiomics for studying biocontrol mechanisms of *Foc* is still absent or very limited. The study of a tripartite interaction among banana, *Foc*, and *T. asperellum* strain Prr2 is one of the few examples available, however with a non-NGS approach, *viz.* the suppression subtractive hybridization or SSH (Thangavelu et al., 2016).

GAPS IN THE KNOWLEDGE AND CONCLUDING REMARKS

Banana production systems lack phytosanitary certification schemes. In Australia, where *Foc* TR4 is considered a quarantine pest, the organization that governs standardization and certification of agricultural practices, GlobalG.A.P., launched in 2017 an "add-on" in its standards which encourages farmers to take preventive biosecurity measures following a strict protocol (GLOBALG.A.P, 2019). However, it is only a preventive awareness measure rather than a true certification. Phytosanitary certification would be necessary either for tissue culture- or sucker-derived plants, because *Foc*-contamination may occur at different stages of plant production. In fact, undesired infected

plants can derive not only from infected asymptomatic suckers but also from pathogen-free tissue culture plantlets, which can be later contaminated by the pathogen during the acclimation period (e.g., by contaminated irrigation water or substrate).

It is worth noting that, despite its dangerousness, *Foc* is not yet a quarantine pathogen in several countries, and where it is considered as such, the regulation is limited to TR4 only. For example, in countries joining to the European and Mediterranean Plant Protection Organization (EPPO), *Foc* is not yet reported in the A1 or A2 lists, which include pests and pathogens absent or present, respectively, in the EPPO region and recommended for quarantine regulations (EPPO, 2019). Among the *Fusarium* species, only *F. circinatum*, *F. euwallaceae*, *F. foetens* and *F. oxysporum* f. sp. *albedinis* are included in the A2 list, while *F. oxysporum* f. sp. *lactucae* is provisionally placed in an alert list because it is pending for a risk assessment that will designate or not it as a quarantine pathogen. Therefore, in the EPPO region, no evaluation for the inclusion of *Foc* among the quarantine pathogens seems to have initiated yet. Nevertheless, awareness campaigns, pest risk assessments, and research works have largely emphasized the importance of quarantine measures against *Foc* TR4 (Baker et al., 2008; Pocasangre et al., 2011; Blomme et al., 2013; Sánchez, 2013; Anses, 2018). Both quarantine measures and phytosanitary certification schemes require huge efforts by the governments in terms of legal framework, personnel training, and protocols for inspection, sampling, diagnosis, etc.

Following the pathogen exclusion and quarantine measures, which are critical to hinder the current *Foc* TR4 expansion, the use of pathogen-free certified plating material is certainly one of the first key preventive steps toward successful management of FWB. It has been implementing well in countries of Latin America, where almost 100% of plants derived from tissue culture, but only partially in Africa, where suckers are still largely used to establish new (especially small) plantations, very likely for economic and cultural reasons. On the other hand, the introduction of beneficial and well-characterized microorganisms during banana propagation protocols can be an excellent strategy to "prepare" (or "pre-condition," or "prime") plantlets to cope with *Foc* inoculum in the field.

A large number of studies have contributed to select BCAs with variable effectiveness against *Foc*, and many of them reached the field-testing stage (**Table 1** and **Supplemental Figure 2**). Interestingly, the disease control degree obtained experimentally has been surprisingly high, even considering that *Foc* is a vascular pathogen enduring in the soil for a long time and, thus, it is generally considered difficult to control using BCAs. Bio-formulation, which is one of the key factors affecting the BCA efficacy, has been tested in several cases, but new aspects, such as nanotechnology and formulation of microbial consortia and/or their metabolites (Keswani et al., 2016), merit to be studied. Moreover, the effectiveness of these formulations could be improved by the amendment with specific nutrients/factors aiming to enhance the biosynthesis of these metabolites as well as the survival of the BCAs in the banana rhizosphere. Microbial metabolites have proved their value in nutrition, agriculture, and healthcare, but poorly evaluated so far against FWB. In contrast, plant extracts like *A. tuberosum* (**Figure 3**) have been repeatedly tested, especially during the last years (Singh et al., 2017). Bioformulations combining different strains, nutrients, metabolites and/or other natural products must take into account the compatibility (i.e., the absence of antagonism) of the different components (Sarma et al., 2015; Gómez-Lama Cabanás et al., 2018). Blends of microorganisms beyond their mere mixture, but based on tailor-made combinations of strains with complementary and/or synergistic modes of action are encouraged (Lutz et al., 2004). Solidly supported by the powerful currently-available "-omics" methodologies, biocontrol strategies can now aim to provide novel tools based on *ad hoc* tailored consortia of BCAs originating from the indigenous microbiota associated with the target plant (Gopal et al., 2013; Berg et al., 2017; Mercado-Blanco et al., 2018), thereby overcoming problems and inconsistencies frequently observed when biopesticides are based on formulations of a single microorganism or combinations of few of them. Furthermore, successful colonization and endurance in the target niche must be guaranteed as the first requisite for successful biocontrol. Therefore, a comprehensive understanding of the mode of action, ecology, and trophic interactions established upon the application of BCAs is instrumental for their success (Saraf et al., 2014; Eljounaidi et al., 2016; Shafi et al., 2017). Monitoring the microbial strains in the field after their application should be addressed in order to understand their fate in the environment and optimize their application protocol. For these aspects, "-omics"-based approaches are of great help.

The availability of the *M. acuminata* draft genome marked a milestone in the genetic research of banana (D'Hont et al., 2012), and the genomic sequence of several *Foc* races is also available. Furthermore, transcriptomics has been implemented in studies on *Musa* spp. (e.g., Backiyarani et al., 2015) enhancing our knowledge on physiological processes such as the fruit ripening (Asif et al., 2014) and the responses to abiotic stresses such as low-temperature (Yang Q. S. et al., 2015). This "-omics" has been largely used to study the interaction between BCAs, plant and/or microbiome, but no studies are available on the banana/*Foc* pathosystem. Moreover, integrating genomics, metagenomics and (meta)transcriptomics would allow understanding the microbiota structure and the roles and functions of its members, as well as the intricate interactions between BCAs, microbiota, plant and the environment. This approach has not been applied to plant microbiomes yet, but it is providing new insight in other fields like the human microbiome (Massart et al., 2015). Proteomics and metabolomics analyses have been also conducted in banana, but they have been focused mainly on the plant-pathogen interaction while BCAs have not been involved (Li et al., 2013, 2017; Lu et al., 2013; Sun et al., 2014; Ramu et al., 2016; Gopalakrishnan, 2017; Yuan et al., 2017). Therefore, at least to the best of our knowledge, there is still an important gap in our knowledge of biocontrol against *Foc*, including insight on the mechanisms involved in the antagonism, plant colonization, plant growth promotion, etc.

Biocontrol should not be considered as an independent tool, but adequately implemented in an integrated management framework. Actually, besides the combination with organic fertilizers, very little has been investigated on the integration

of BCAs with other control means. For instance, the effect of an organic amendment against FWB was enhanced by the combination with a BCA, *B. amyloliquefaciens* strain NJN-6, namely biofertilizer (Shen et al., 2015a). Furthermore, the biocontrol efficacy of such biofertilizer resulted even higher when it was applied after ammonia fumigation (Shen et al., 2019). Other studies have shown that combinations of biocontrol organisms with silicon and mulching, or with neem cake can be advantageous compared to the individual applications, and therefore can provide a better control option for banana growers who have to deal with FWB in their plantations (Saravanan et al., 2003; Kidane and Laing, 2010). Diverse combinations of treatments with silicon, *T. harzianum*, compost, various sources of nitrogen, phosphorus and potassium, and the cover crop *Crotalaria juncea* were applied in the field to banana varieties differing in the susceptibility level to *Foc*. The results highlighted the advantages of integrated disease management, especially the combination of different control means with the host genetic resistance. In fact, the treatment including all the control means was more effective than those including only some of them. Also, while in the highly susceptible cultivar Silk (AAB) the treatment was not effective in reducing FWB during the first crop cycle, in the moderately susceptible variety Prata Anã (AAB) it reduced the disease by 58%, with a yield increment of 157.3% (Haddad et al., 2018).

We showed that the literature offers numerous examples of encouraging results, suggesting that biocontrol can greatly contribute to limit the damage caused by FWB. More efforts should be done to further validate the currently available outcomes, to deepen the knowledge on the most valuable BCAs, and to improve their efficacy by setting up effective formulations, application protocols, and integrated strategies.

AUTHOR CONTRIBUTIONS

All the authors wrote sections of the manuscript, contributing equally to its first draft. GB coordinated and merged the individual contributions from the authors. All the authors read, revised, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00616/full#supplementary-material

Supplemental Figure 1 | Publication timeline of scientific articles dealing with Fusarium wilt of banana. Articles were retrieved from the CAB Direct database (1970–2018) by searching the keywords "*Fusarium cubense*" or "Panama disease" in the title and abstract (735 articles). *Foc*: *Fusarium oxysporum* f. sp. *cubense*; FWB: Fusarium wilt of banana.

Supplemental Figure 2 | Biological control agents studied for the control of *Fusarium oxysporum* f. sp. *cubense*. The numbers indicate the scientific articles retrieved from CAB Direct database (1970–2018) by searching the keywords "*Fusarium cubense*" or "Panama disease" in the title and abstract.

Supplemental Table 1 | List of *in vitro* experiments conducted against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) using beneficial microorganisms.

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Corrigendum: Biological Control Agents Against Fusarium Wilt of Banana

Giovanni Bubici[1]*, *Manoj Kaushal*[2], *Maria Isabella Prigigallo*[1], *Carmen Gómez-Lama Cabanás*[3] and *Jesús Mercado-Blanco*[3]

[1] Consiglio Nazionale delle Ricerche (CNR), Istituto per la Protezione Sostenibile delle Piante (IPSP), Bari, Italy, [2] International Institute of Tropical Agriculture (IITA), Dar es Salaam, Tanzania, [3] Department of Crop Protection, Institute for Sustainable Agriculture (CSIC), Córdoba, Spain

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***Correspondence:**
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giovanninicola.bubici@cnr.it

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It should be "Kalaiponmani, K., Thangavelu R., and Varun, G. (2017). Optimization of protein isolation and preliminary comparative proteomics of pathogenic *Fusarium oxysporum* f. sp. *cubense* (p-Foc) and non-pathogenic *Fusarium oxysporum* (np-Fo). *J. Plant Pathol.* 99, 361–369. doi: 10.4454/jpp.v99i2.3883"

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Assessing Variations in Host Resistance to *Fusarium oxysporum* f sp. *cubense* Race 4 in *Musa* Species, With a Focus on the Subtropical Race 4

Andrew Chen[1], Jiaman Sun[2], Andrea Matthews[1], Liz Armas-Egas[1], Ning Chen[1], Sharon Hamill[3], Sharl Mintoff[4], Lucy T. T. Tran-Nguyen[4], Jaqueline Batley[1,5] and Elizabeth A. B. Aitken[1]*

[1] School of Agriculture and Food Science, The University of Queensland, Brisbane, QLD, Australia, [2] Guangxi Crop Genetic Improvement and Biotechnology Key Lab, Guangxi Academy of Agricultural Sciences, Nanning, China, [3] Department of Agriculture and Fisheries, Maroochy Research Facility, Nambour, QLD, Australia, [4] Department of Primary Industry and Resources, Northern Territory Government, Darwin, NT, Australia, [5] School of Biological Sciences, The University of Western Australia, Perth, WA, Australia

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***Correspondence:**
Andrew Chen
a.chen2@uq.edu.au

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Fusarium oxysporum f. sp. *cubense* (*Foc*) has severely curtailed banana production in the tropical regions of the world. The tropical race 4 (TR4) of *Foc* was detected in Australia in the 1990s and it is virulent to all Cavendish type banana cultivars, which represents the majority of banana production in Australia. Genetic resistance to *Foc* race 4 is urgently needed. To characterize sources of resistance, we have assessed the *Foc* resistance response of 34 *Musa* cultivars with plants grown under controlled settings. Amongst diploid banana cultivars carrying the AA genome, resistance is found in *Musa acuminata* sub-species including *malaccensis* 'Pahang' and *burmannica* 'Calcutta4.' In the polyploid group, the hybrids such as 'FHIA-18' and 'FHIA-25' are highly resistant against both *Foc*-TR4 and subtropical race 4 (*Foc*-STR4). Interestingly, 'FHIA-2' and 'CAM020' appear to be resistant to *Foc*-TR4 but susceptible to *Foc*-STR4, suggesting potential differences in the resistance mechanisms against the different race 4 strains. Using a GFP tagged *Foc*-STR4 strain challenged onto both resistant and susceptible *M. a. malaccensis* lines, a high inoculum dosage rapidly induced vascular wilt in the susceptible *M. a. malaccensis* lines at 2.5 weeks. This was associated with an accumulation of micro-conidia in the rhizome and the movement of the fungus through the xylem vessels. In contrast, the fungal movement was restrained in the rhizome of the resistant *M. a. malaccensis* lines and no sporulation was observed. Overall, this research suggests that the resistance response is dependent to an extent on inoculum dosage and that the plant host's response, in the rhizome, plays an important role in inhibiting the fungus from spreading to the rest of the plant. Identifying race 4 resistant accessions can help to understand mechanisms of resistance and provide banana breeders with the genetic resources to integrate resistance genes into commercial varieties.

Keywords: *Fusarium* wilt, banana, *Musa acuminata* ssp. *malaccensis*, green fluorescent protein, *Fusarium oxysporum* f. sp. *cubense*

INTRODUCTION

Banana and plantain (*Musa* spp.) serve as important sources of staple food and fruit around the world and collectively are considered the world's leading fruit crop, with a production value reaching over 100 million tons per annum (FAO, 2019). As a staple food, banana is an important export commodity in Africa and Asia, ensuring food security for millions of people (Aurore et al., 2009). One of the major constraints in the global production of banana is the disease, *Fusarium* wilt. It is also known as Panama disease, which is caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Ploetz, 2006).

Foc gains entry into the plant host via roots. Once inside, it colonizes the rhizome and travels up the pseudostem, where it blocks the water-conducting xylem vessels and thus prevents the transport of water and nutrients to the aerial parts of the plant. External symptoms of *Fusarium* wilt start with the yellowing and wilting of the older leaves and progress to the younger leaves until the plant dies. Internally, the plants show brown discoloration and necrosis of xylem vessels in the rhizomes and stems. The disease incidence varies depending on the cultivar, the environment and the level of inoculum, but can extend to total crop loss in heavily infested fields (Moore et al., 2001).

Foc is a soil-borne pathogen that produces chlamydospores enabling its survival in the soil in the absence of the host. It is also known to survive on weed hosts in a non-pathogenic manner (Hennessy et al., 2005). Once the soil is infested with *Foc*, it generally becomes unsuitable for replanting for many years thereafter (Stover, 1990). Furthermore, unlike the black Sigatoka leaf spot caused by *Pseudocercospora fijiensis*, the other major fungal pathogen affecting the banana industry worldwide, *Fusarium* wilt cannot be controlled by fungicides (Ploetz, 2006).

Outbreaks of *Fusarium* wilt decimated a banana industry primarily based on the cultivar 'Gros Michel' (AAA) in Central America in the 1950s. The pathogen was subsequently named *Foc* race 1, and the outbreak forced the industry to shift production to the *Foc* race 1 resistant cultivars of the Cavendish (AAA) subgroup (Stover, 1990). Cavendish now accounts for >40% of world banana production, with export markets amounting to 15% of the total production (FAO, 2019).

Within the *forma specialis* (f. sp.) *cubense*, which include all isolates pathogenic to *Musa* spp. (Snyder and Hansen, 1940), there is significant pathogenic variation; members of this f. sp. are divided into four races based on their host range (Stover, 1990). Race 1 is pathogenic to 'Gros Michel' (AAA) and a range of other cultivars carrying the AAB genome. Race 2 targets those race 1-susceptible cultivars, as well as the hybrid triploid 'Bluggoe' (AAB). Race 3 affects *Heliconia* species and is no longer considered to be part of the *cubense* race structure. Race 4 is pathogenic to all race 1 and race 2 susceptible cultivars plus the Cavendish subgroup (AAA) (Su et al., 1986). Race 4 is further divided into two groups: tropical (TR4) and subtropical (STR4) race 4. *Foc*-STR4 isolates cause disease in Cavendish in the subtropics, whereas *Foc*-TR4 isolates are pathogenic to Cavendish under both tropical and subtropical conditions (Buddenhagen, 2009).

Foc isolates can also be grouped according to vegetative compatibility, which is the ability of an isolate to anastomose and form a stable heterokaryon (Moore et al., 1993). Isolates that are vegetatively compatible with one another form a VCG and typically share common biological, physiological and pathological traits (Caten and Jinks, 1966). At least 21 different VCGs of *Foc* have been characterized, with the majority of the groups present in Asia, where the pathogen is thought to have evolved (Fourie et al., 2009). *Foc*-TR4 isolates are designated as VCG 01213 (or VCG 01216, which is a different designation for the same VCG) (Bentley et al., 1998). *Foc*-STR4 isolates are designated as VCGs 0120, 0121, 0122, 0129, and 01211 (Buddenhagen, 2009). Since its appearance in Southeast Asia in the 1990s, *Foc*-TR4 (VCGs 01213 or 01216) has caused severe damage to Cavendish plantations in Malaysia, Indonesia, China, the Philippines and the Northern Territory and Queensland in Australia as well as Mozambique (Ploetz, 2006, 2015; Buddenhagen, 2009). The virulence and epidemic nature of *Foc*-TR4 is due to its potent pathogenicity and wide host range within the genus *Musa*.

Genetic resistance can offer a long-term means of control of *Fusarium* wilt, but no truly *Foc*-TR4 resistant commercially viable cultivar is available. Efforts have been made to identify *Foc*-TR4 resistant cultivars (Zhang et al., 2018; Zuo et al., 2018) but transferring these favorable alleles into a commercially viable cultivar with good agronomic traits has been a challenge (Dita et al., 2018). Somaclonal variants of Cavendish known as the 'giant Cavendish tissue culture variants' or 'GCTCVs' have been generated in Taiwan through tissue culturing and field trials and have shown that they possess some improved level of tolerance to *Foc*-TR4 (Hwang and Ko, 2004). However, these lines vary in their levels of tolerance and are considered by some to contain undesirable agronomic traits (Ploetz, 2015). Therefore, genotypic screening of cultivated and wild germplasm is extremely important in characterizing existing or novel sources of *Foc* race 4 resistance, which could assist phenotypic selection in breeding programs or facilitate the isolation of gene(s) underlying resistance for the purpose of engineering resistance in commercial cultivars via gene technologies.

In cultivated banana, four genome types have been identified and they include *M. acuminata* (A), *M. balbisiana* (B), *M. schizocarpa* (S) and those of the *Australimusa* section (T) (D'Hont et al., 2000). So far, most of the cultivated banana plants are diploid or triploid, originating from intra- or inter-specific hybridizations between the diploid A and the B genomes (Perrier et al., 2011). Most of the commercial cultivars are seedless and produce fruit by parthenocarpy, resulting in limited selection process and mono-culture productions. Most of these cultivars are therefore susceptible to biotic and abiotic stress, indicating a limited gene pool (D'Hont et al., 2012). Wild relatives of commercial varieties and other cultivated diploids such as 'Pisang Jari Buaya,' produce seeds and are considered good sources of genetic diversity worthy of exploration for improving resistance to *Fusarium* wilt. Indeed, *resistance gene analog* 2 (*RGA*2) was

Abbreviations: dpi, days post-inoculation; *Foc, Fusarium oxysporum* f sp. *cubense*; FHIA, Fundacion Hondurena de Investigación Agrícola; GFP, green fluorescent protein; Ma, *Musa accuminata* ssp. *malaccensis*; SH, synthetic hybrid; STR4, subtropical race 4; TR4, tropical race 4; VCG, vegetative compatibility group.

isolated from *Musa acuminata* ssp. *malaccensis* and has recently been shown to confer resistance to TR4 in field trials conducted in Australia (Peraza-Echeverria et al., 2008; Dale et al., 2017).

In this study, we assessed the resistance level of 34 banana cultivars against *Foc*-TR4 and *Foc*-STR4. These include diploids (AA and BB groups, and wild relatives) and intra- or inter-specific hybrids such as Cavendish banana 'GCTCVs' and plantains from the FHIA. We used a pot based bio-assay method to assess the level of resistance in each accession grown under glasshouse conditions. We characterized several *Foc* race 4 resistant diploids as a source of resistance; these sources may have already been incorporated into the resistant hybrids through conventional breeding. We also show that resistance levels vary amongst the genotypes and resistance is likely of quantitative nature across a spectrum. The level of observed resistance under controlled environmental conditions can be determined by factors such as inoculum dosage, maturity of plants and inoculation technique. Using a GFP tagged *Foc*-STR4 strain, we show that the rhizome plays an important role as a barrier to the pathogen preventing it from migrating toward the rest of the plant. We identified a diploid wild relative that exhibits a strong resistance response to *Foc* manifested by reduced fungal penetration in root cells and containment of the fungi in the rhizome. This is therefore, a potential novel source of resistance to *Foc* race 4 types.

MATERIALS AND METHODS

Fusarium Strains

The monoconidial *Foc*-TR4 strain (NTPDc 35673) was originally collected from the Coastal Plains Research Farm at in the Northern Territory, Australia in the early 2000s. In 2016, VCG testing performed on this strain by Department of Agriculture and Fisheries (DAF, Nambour, QLD, Australia) confirmed that it is VCG 01213/16.

Three monoconidial isolates of *Foc* VCG0120 were obtained from the Queensland Plant Pathology Herbarium (BRIP; 63488, 43781, and 42331) at the Queensland Department of Agriculture and Fisheries. *Foc* BRIP 23598 from VCG 0120 was previously transformed with GFP (Henceforth, GFP-*Foc*-STR4) and stored at −80°C (Forsyth, 2006).

All *Foc* isolates were cultured on half-strength potato dextrose agar (PDA) Difco (Becton, Dickson and Co., Sparks, MD, United States) for 7 days at 25°C. The GFP-*Foc*-STR4 isolate was regenerated on the same media but with the addition of 50 mg L^{-1} of hygromycin B.

Preparation of Millet Inoculum

To prepare millet grain for *Foc*-STR4 inoculum, millet (*Pennisetum glaucum*) seed was washed in tap water, covered with distilled water, and then soaked overnight in a suitable container. Excess water was drained from the seed using a sieve, then the grain was rinsed a second time in distilled water to remove leached carbohydrate. The grain was then placed into Erlenmeyer flasks or other suitable containers and autoclaved twice for 20 min at 120°C on consecutive days. Once cooled, the grain was inoculated with approximately five 1 cm squared mycelial plugs cut from *Foc* cultures grown on half strength PDA. Flasks were shaken daily to distribute *Foc* evenly. When the millet was fully colonized by the *Fusarium*, it was used as plant inoculum.

The *Foc*-TR4 inoculum was prepared with the following modifications to allow an up-scale. Batches of 1.5 kg of millet were placed in small autoclave bags. After the addition of 500 mL of RO water, the bags were sealed and autoclaved twice over consecutive days. Half of a PDA plate containing *Foc*-TR4 was added to each bag of autoclaved millet. The bags were shaken every second day.

For GFP studies, four to five mycelial agar plugs of GFP-*Foc*-STR4 were added to half strength potato dextrose broth (PDB, Difco) containing 50 mg L^{-1} hygromycin B and shaken gently at 28°C for 5 days. The culture was processed using a previously described method (Li et al., 2011) to extract viable spores and a final concentration of 2×10^6 conidia mL^{-1} was prepared for root dipping. All work involving GFP transformed strains of *Foc*, was conducted under conditions of an NLRD (notifiable low risk dealing) permit according to the Office and Gene Technology Regulator, Australia.

Plant Materials and Growth

Wild relatives of the cultivated banana are known to harbor resistance against *Foc*. So far, field studies have identified resistant genotypes against *Foc* race 4 and those include diploids (AA genome) from *Musa accuminata* ssp. *malaccensis*, *Musa accuminata* ssp. *Banksii,* and *Musa accuminata* ssp. *burmannica* (**Table 1**). In this study, we selected 34 accessions of various ploidy levels to assess their resistance response to *Foc*-STR4 and *Foc*-TR4 in pot trials (**Table 1**). The diploids used include *M. a. malaccensis* from Sumatra, Indonesia ('Ma846,' 'Ma848,' 'Ma850,' 'Ma851,' 'Ma852') and from Malaysia, 'Pahang' and *M. a. malaccensis* of ITC0250. We also examined 'Calcutta4' (*M. a. burmannica*) and 'Pisang Jari Buaya' (AA) that have been shown to carry race 4 resistance in field trials (**Table 1**). Other diploid cultivars tested include 'SH3217,' 'SH3362,' and 'SH3142' that have been used extensively in breeding programs such as FHIA. The hybrids with polyploid genomes tested include 'FHIA-1,' 'FHIA-2,' 'FHIA-3,' 'FHIA-18,' 'FHIA-23,' 'FHIA-25,' 'FHIA-26.' We also looked at the two 'GCTCVs' lines (119 and 218) of Taiwan origin that exhibited improved tolerance against *Foc*-TR4 in the field (Hwang and Ko, 2004). Lines from breeding programs were also examined. These include 'M61 Guadeloupe,' an elite bred diploid from the Jamaican breeding program and 'CAM020' which is an F_1 individual from a cross between 'Calcutta 4' and 'Banksii Madang.' It is part of the 'AFCAM20' population developed by INIBAP (International Network for the Improvement of Banana and Plantain).

Banana germplasm was kindly supplied by the Maroochy Research Facility at the Queensland Department of Agriculture and Fisheries. Germplasm is listed in **Table 1** and those with known corresponding ITC numbers are indicated. Six to eight clones of each accession from **Table 1** were micro-propagated and maintained *in vitro* as per previous study (Smith and Hamill, 1993). Seedlings approximately 10 cm in

TABLE 1 | List of genotypes presented in this study, and the available corresponding field studies that determined their resistance responses against *Foc* race 4 types.

Genotype name	Genome	Origin	ITC No	Field resistance	Present study
Ma851	AA	*malaccensis*	–	R^T(a)	R^S
Ma852	AA	*malaccensis*	–	R^T(a)	R^S
Calcutta4-IV9	AA	*burmannica*	–	R^T(b)	R^T, R^S
Pahang	AA	*malaccensis*	ITC0609	R^T(b)	R^T, R^S
SH-3217	AA	Hybrid	–	–	R^S
SH-3362	AA	Hybrid	–	–	R^T, R^S
SH-3142	AA	Hybrid	ITC0425	SS^T(b)	R^T, R^S
Madang Gaudelope	*M. acuminata*	*banksii*	–	–	R^T, R^S
FHIA-1	AAAB	Hybrid	ITC0504	SS^T(a), S^T(c), R^S(d)	R^T, R^S
FHIA-25	AAB	Hybrid	ITC1418	R^T(a), R^T(b)	R^T, R^S
GCTCV-119	AAA	Cavendish	ITC1282	SS^T(a), R^T(e)	R^T, R^S
Ma850	AA	*malaccensis*	–	R^T(a)	R^T, R^S
Pisang Jari Buaya	AA	c.v.	–	SS^T(a), R^T(b)	R^T, R^S
Calcutta-4	AA	*burmannica*	ITC0249	R^T(b)	R^T, R^S
FHIA-18	AAAB	Hybrid	–	SS^T(a), R^S(d)	R^T, R^S
Ma250	AA	*balaccensis*	ITC0250	–	R^S
Pisang bangkahulu	AA	c.v.	ITC0689	–	R^S
M61 Gaudelope	–	–	–	–	R^T, SS^S
GCTCV-218	AAA	Cavendish	ITC1597	S^T(a), R^T(e)	SS^S
Williams	AAA	Cavendish	–	HS^T(a), S^T(b), MS^S(d)	S^T, SS^S,
Khae (Phrae)	*M. siamea*	*siamea*	ITC0660	–	SS^S
Pisang raja	AAB	c.v.	ITC0243	R^T(b)	MS^S
Pisang madu	AA	c.v.	ITC0258	SS^T(b)	MS^S
FHIA-23	AAAA	Hybrid	ITC1265	HS^T(a), S^T(b)	SS^T, S^S
Ma846	AA	*malaccensis*	-	S^T(a)	S^S
FHIA-2	AAAA	Hybrid	ITC0505	HS^S(d)	R^T, S^S
Balbisiana	BB	*balbisiana*	-	MS^T(c)	S^S
Musa zebrina	AA	*zebrina*	ITC1177	SS^T(b)	S^S
FHIA-3	AABB	Hybrid	ITC0506	S^T(b)	SS^T, S^S
CAM-020	AAA	Cavendish	–	–	R^T, S^S
Ma848	AA	*malaccensis*	–	S^T(a)	S^T, S^S
FHIA-26	AABB	Hybrid	1422	–	S^T
Lady Finger	AAB	c.v.	–	HS^T(a), HS^S(d)	S^T
Pisang Gajih Merah	ABB	Hybrid	Indonesia	–	SS^T

R, SS, MS, S, and HS abbreviate resistant, slightly susceptible, moderately susceptible, susceptible, and highly susceptible over-all wilting symptoms from the field studies, respectively. Symbols ^{S} and ^{T} indicates Foc sub-tropical race 4 and Foc tropical race 4, respectively. All except the study performed by Walduck and Daly (2007), used naturally infested soil types. The latter study used additional susceptible plants as inoculum between the trial plants. References are listed in brackets and are abbreviated using the following letters, a = Walduck and Daly, 2007; b = Zuo et al., 2018; c = Li et al., 2015; d = Smith et al., 2014; e = Hwang and Ko, 2004. In the present study, resistance responses are categorized according to the mean scores of the rhizome discoloration per cultivar; resistant (R) = 1 to 3; slightly susceptible (SS) ≥ 3 and ≤ 4; moderately susceptible (MS) ≥ 4 to ≤ 5; susceptible (S) ≥ 5.

height with three to four true leaves were de-flasked into 30 well seedling trays to harden off in the laboratory under constant LED lights. The seedlings were transferred into 200 mm diameter pots in designated spaces for disease screening. *Foc*-TR4 is strictly quarantined in Australia and pathogen screening was instead carried out in a screen house at the Coastal Plains Research Station (Department of Primary Industry and Resources, Northern Territory) in Darwin, Australia. The average daily temperature in Darwin ranged from 30 to 35°C during the August to November months when the pot trials were conducted. *Foc*-STR4 screening was performed in the University of Queensland (UQ) glasshouse facility with temperature control set at 25 to 28°C. The potting mix contained 70% composted pink bark and 30% coco peat at a pH range of 5.5 to 6.5.

Plant Inoculation

Once the pseudostem of most plants reached 30 cm in height and each plant carried five to six true leaves, the plants were inoculated with either *Foc*-STR4 or *Foc*-TR4 infested millet or spores of GFP-*Foc*-STR4 at pre-determined concentrations. A total of 40 g of *Foc*-STR4 or *Foc*-TR4 infested millet was used as inoculum per 200 mm diameter pot. For inoculation, plants were briefly removed from the pots and 20 g of millet was added to the bottom of the pots before plants were re-potted. Another 20 g of millet was then spread

evenly on the surface soil layer and finally covered with additional soil.

Assessment of Symptoms

Plants inoculated with *Foc*-STR4 or *Foc*-TR4 infested millet were scored for internal symptoms 10 to 12 weeks post-inoculation. The rhizome was vertically cut in halves to score the extent of discoloration. The rating scale for rhizome discoloration ranges from 1 to 8, with 1 being no discoloration and 8 indicating the entire rhizome is discolored and the plant dead (Mak et al., 2004). The mean score from three to eight plants was used to quantify the plant response.

Confocal Microscopy

Confocal microscopy was used to visualize GFP-*Foc*-STR4 in two separate experiments. Firstly, 'Ma851' self-derived progeny 'p168' (resistant) and 'p248' (susceptible) were examined for the presence of GFP-*Foc*-STR4 in the rhizome. Plants were inoculated with GFP-*Foc*-STR4 infested millet. Assessment was performed at 3 months post-inoculation. In the second experiment, the parent 'Ma851' (resistant) and 'Ma848' (susceptible) were used. Plant roots were dipped in a GFP-*Foc*-STR4 spore suspension containing 2×10^{-6} spores per mL for 2 h. Inoculated plants were then re-potted using soil containing 50,000 GFP-*Foc*-STR4 conidia per g of soil which is 5–10-fold of what is typically used in this type of assay (Li et al., 2012). The pathogen infection processes in these plants were observed at 1, 2, 3, 4, 7, and 14 dpi.

Transverse and longitudinal sections were hand-prepared to visualize pathogen development on the root surface and in the vascular bundles and the corms. Sliced sections were counter-stained with propidium iodide (PI, Sigma Aldrich) for 5 min at a concentration of 10 $\mu g\ mL^{-1}$. A Zeiss 700 laser scanning microscope was used to visualize and acquire the confocal images. The GFP and the PI were detected using the 488 and 555 nm lasers, respectively. Z-stack acquisition mode was used to obtain 3D images consisting of 10–20 optical slices taken at intervals of 1–5 μm. T-PMT (transmission detector setting) was used to obtain the light images of the sectioned tissue.

Koch's Postulates

Primary isolations were performed on three resistant (104A, 3A, 18A) and one susceptible (96B) self-derived progeny of 'Ma851' to investigate the presence or absence of GFP-*Foc*-STR4. Fungal strains were isolated 3 months post-inoculation with GFP-*Foc*-STR4 infected millet. Five regions were isolated per plant, which included the upper stem just below the first leaf petiole, mid-point of the stem, stem just above the rhizome, the central cylinder of the rhizome and the outer layer of the rhizome connected to the cortex. Four pieces of tissues were isolated in each region. These sectioned pieces were surface sterilized for 1 min in 1% hypochlorite solution, then rinsed twice in distilled water for 30 s each time. Each piece was air-dried and plated onto water agar plates containing 100 mg L^{-1} streptomycin for 5 days at 24°C. Segments showing *Fusarium*-like growth under a dissecting microscope were further isolated and then hyphal tipped to generate monoconidial isolates on half strength potato dextrose agar (PDA) plates containing 50 mg per L hygromycin B. Each isolate was then transferred to 20 mL of potato dextrose broth containing 50 mg L^{-1} hygromycin B (PDB) and incubated at 26°C on a platform rotating at 160 rpm for 4 days. GFP fluorescence of the isolates was visualized under Zeiss 700 confocal microscope to confirm the presence of the GFP-*Foc*-STR4 strain.

RESULTS

Foc-STR4 Screening Trial

Thirty-four genotypes were tested for their response to *Foc*-STR4 under glasshouse conditions (**Table 1**). Following inoculation, the rhizome discoloration was scored using a predetermined scale (Mak et al., 2004) which is presented in **Table 2**. A rhizome score of 1 or 2 indicates no discoloration in the rhizome 3 months after inoculation and is associated with the AA diploids 'Ma851,' 'Ma852,' 'Calcutta4-IV9,' 'SH-3217,' 'SH-3362,' 'SH-3142,' as well as the hybrids 'FHIA-1' (Gold Finger), 'FHIA-25' and 'GCTCV-119' (**Figure 1**). Accessions that had a clear rhizome but had little or some discoloration around the junctions of roots joining the rhizome are categorized by a score of 2 to 3 ($\leq$5% discoloration) and include the diploids 'Ma850,' 'Pisang Jari Buaya,' 'Calcutta-4,' 'FHIA-18,' 'Ma250,' and 'Pisang bangkahulu.' In this group, some of these lines, namely 'Ma850,' 'Pisang Jari Buaya,' 'FHIA-18,' and 'Ma250' had large phenotypic variation in the degree of rhizome discoloration amongst individual clones, possibly due to somaclonal variations of genetic or epigenetic origin (**Figure 1** and **Supplementary Figure 1**). These lines also exhibited a good level of resistance against *Foc*-TR4 in previous field studies (**Table 1**). Accessions that display up to 20% rhizome discoloration fall into the slightly susceptible (SS) group. These include' 'M61 Gaudelope,' 'GCTCV218,' 'Williams,' 'Khae' (**Figure 1**). 'Pisang raja' and 'Pisang madu' showed discoloration in the 21 to 50% range and are hence considered moderately susceptible (**Figure 1**). The accessions that showed more than 50% discoloration are considered susceptible (S) to *Foc*-STR4. These include 'FHIA-2,' 'FHIA-23,' 'Ma846,' *Musa balbisiana*, *Musa accuminata* ssp. *zebrina*, 'FHIA-3,' 'CAM020,'

TABLE 2 | Rhizome discoloration index as per previously described (Mak et al., 2004).

Discoloration index	Description
1	No discoloration of the tissue in the stelar region of the rhizome and the surrounding region.
2	No discoloration of the stellar region of the rhizome. Discoloration at the junctions of root and rhizome.
3	Trace up to 5% of the stellar region discolored
4	6 to 20% of the stellar region discolored.
5	21 to 50% of the stellar region discolored.
6	More than 50% of the stellar region discolored.
7	The entire rhizome stele is discolored.
8	The plant is completely dead.

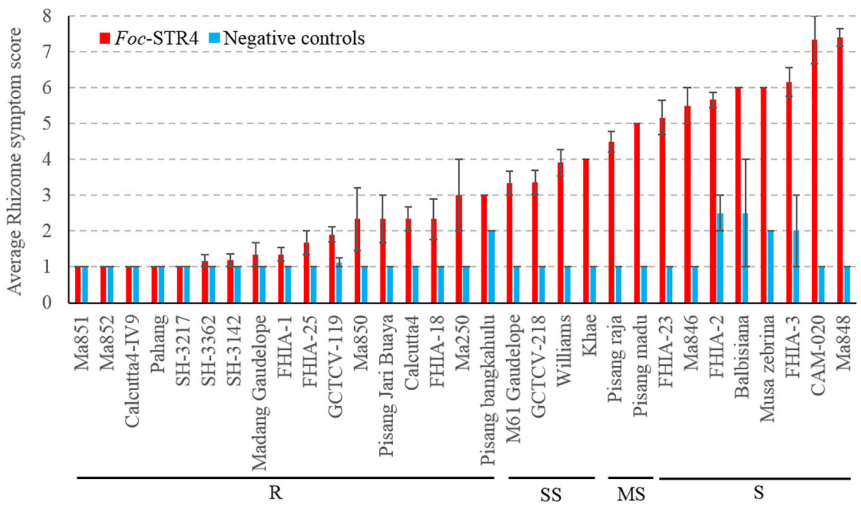

FIGURE 1 | Sensitivity of different banana genotypes to *Foc*-STR4 in glasshouse pot trials. Internal symptoms were assessed by using a scoring system based on the percentage of rhizome discoloration (Mak et al., 2004). Millet was inoculated separately using three *Foc*-STR4 isolates BRIP63488, BRIP43781, and BRIP42331 of VCG 0120. Fully colonized millet from each strain was mixed in equal amounts and 40 g of inoculum was applied to each 200 mm diameter pot. Clean autoclaved millet was used as negative controls. Error bars indicate standard deviations of the mean derived from three to eight individual plants. Genotypes that have rhizome symptom scores between 1 and 3 (≤5% rhizome discoloration) are categorized in the resistant group (R); those with a score between 3 and 4 (>5 to ≤20% discoloration) are categorized in the slightly susceptible group (SS); those that score between 4 and 5 (>21 to ≤50%) are categorized as susceptibles (MS); those that score greater than 5 (>50%) is categorized as susceptible (S).

and 'Ma848' (**Figure 1**). 'CAM-020' and 'Ma-848' were extremely susceptible to *Foc*-STR4; half of the clonal plants were dead at the time of assessment.

Foc-TR4 Screening Trial

Due to logistical constraints, only a subset of accessions was selected for testing against *Foc*-TR4 in the Northern Territory, Australia under shade house conditions. At the time of the assessment, the susceptible plants did not show noticeable external symptoms, however, internal symptoms were present. Internally, the diploids *M. a. malaccensis* (AA) 'Pahang' and 'Ma850' displayed clean rhizomes that suggests a high level of resistance against *Foc*-TR4 (**Figure 2**). Other accessions that produced resistant (R) rhizome phenotypes include 'FHIA-1,' 'FHIA-2,' 'FHIA-18,' 'FHIA-25,' 'Calcutta4,' 'Calcutta4-IV9,' 'SH-3142,' 'SH-3362,' 'Pisang Jari Buaya,' 'Madang

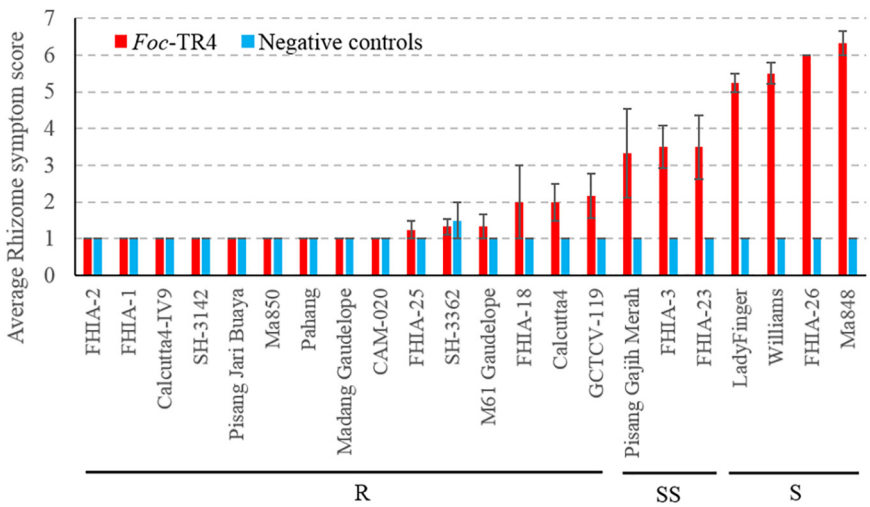

FIGURE 2 | Sensitivity of different banana genotypes to *Foc*-TR4 in the shade house pot trial. Internal symptoms were assessed by using a scoring system based on the percentage of rhizome discoloration (Mak et al., 2004). Millet infested with *Foc*-TR4 strains carrying VCG 01213/16 was used as the inoculum. For each 200 mm diameter pot, 40 g of the inoculum was applied. Clean autoclaved millet grains were used in uninoculated control treatments. Error bars indicate standard deviations of the mean derived from three to eight individual plants. Abbreviations and categories of resistance are the same as per **Figure 1**.

Guadelope,' 'M61 Guadelope,' 'CAM-020,' and 'GCTCV-119' (**Figure 2**). The SS group includes 'Pisang Gajih Merah,' 'FHIA-3,' and 'FHIA-23.' All three cultivars displayed a high level of phenotypic variation in clonal plants, possibly owing to somaclonal variations (**Figure 2** and **Supplementary Figure 2**). 'Lady Finger,' 'Williams,' 'FHIA-26,' and 'Ma848' all showed severe discoloration in their rhizomes and hence are considered as susceptible (S) to *Foc*-TR4 (**Figure 2**).

Comparison Between *Foc*-STR4 and *Foc*-TR4 Induced Responses

Most accessions showed consistent responses between the two race 4 types (**Table 1**). 'Williams,' which belongs to the Cavendish subgroup, is the dominant variety used in commercial production in Australia. In our study, it was highly susceptible to *Foc*-TR4 but was only partially susceptible against *Foc*-STR4 when tested under glasshouse conditions (**Figures 1**, **2**). Our results are consistent with the resistance response of 'Williams' to race 4 types detected in the field (**Table 1**). In these studies, Cavendish types (AAA) have generally shown high susceptibility against *Foc* race 4 but improved resistance has been detected in Cavendish variants in the field (Bhagwat and Duncan, 1998; Hwang and Ko, 2004). The giant Cavendish type somatic mutant 'GCTVC119' showed resistance against both *Foc*-STR4 and *Foc*-TR4 in our study (**Figures 1**, **2**). However, the variant 'GCTCV-218' showed slight susceptibility against *Foc*-STR4. *Foc*-TR4 resistance levels of 'GCTCV-218' does not appear to be consistent as shown by field results (**Table 1**), possibly suggesting the presence of genotype-environment interactions at the different trial locations. 'M61 Gaudelope,' 'FHIA-3,' 'FHIA-23,' 'Pisang raja' all showed a relatively higher level of susceptibility to *Foc*-STR4 than *Foc*-TR4 (**Table 1**). Interestingly, 'FHIA-2' and 'CAM-020' were both resistant to *Foc*-TR4 but showed highly susceptible rhizome phenotypes to *Foc*-STR4 (**Figures 1**, **2**).

FIGURE 3 | Self-derived F_2 progeny of the resistant parental line 'Ma851' showing contrasting resistance response against GFP-*Foc*-STR4. Plants were inoculated with GFP-*Foc*-STR4 infested millet and photographs were taken 3 months after inoculation. **(A)** The resistant progeny plant 'p168' showing no leaf wilting symptoms and was relatively healthy. **(B)** The base of the pseudostem just above the soil showing no signs of stem splitting, which is a manifestation of the wilting disease. **(C)** The rhizome of 'p168,' cut in half longitudinally, showed no traces of brown discoloration in the lower and center regions. **(D)** The susceptible progeny plant 'p248' derived from the same F_2 cross displayed wilting of the old leaves. **(E)** Stem splitting at the base of 'p248' was clearly visible just above the soil level. **(F)** The rhizome of 'p248' rhizome developed extensive brown discoloration which is typically associated with high *Foc* susceptibility. Arrows indicate the sites of external and internal symptoms at the time of assessment. w = wilting of old leaves, ps = pseudostem splitting, rd = rhizome discoloration, pd = pseudostem discoloration.

Pathogen Infection Process

Fluorescence produced by GFP was visualized to assess the extent of the spread of the GFP-*Foc*-STR4 inside the rhizome of plants that had been inoculated with millet infested with the fungus. Resistant 'p168' and susceptible 'p248' progeny of 'Ma851' were compared. 'P168' and 'p248' were the self-pollinated progeny between the clonal plants of the parent 'Ma851.' Previously, segregation analysis using the self-derived progeny of 'Ma851' showed that they segregated for both *Foc* race 4 type resistance at a 3 : 1 (resistance : susceptibility) ratio indicating the respective presence of a single dominant resistance gene in 'Ma851' (Fraser-Smith et al., 2016). Three months after the initial inoculation, 'p168' showed minimal leaf symptoms, no stem splitting and a healthy rhizome (**Figures 3a–c**). In contrast, 'p248' showed necrotic lesions and the wilting of old leaves, a split stem and moderately discolored rhizome (**Figures 3d–f**). Confocal imagery of the inoculated resistant 'p168' line showed that the GFP was associated with the vascular bundles in the central cylinder toward the lower part of the rhizome (**Figures 4A,B**). GFP fluorescing hyphae was present in the cortex region near the xylem perforation plates (**Figure 4B**). Furthermore, patches of mycelial networks were detected at a low rate in the cortex region of 'p168' rhizome (**Figure 4C**). In contrast, mycelia associated with a strong GFP signal was detected in the mid and lower regions of the 'p248' rhizome where black discoloration was observed (**Figures 4D,E**).

The GFP signal of GFP-*Foc*-STR4 is strongly associated with the xylem perforation plates. Hyphal structures containing GFP were clearly observed in the cortex cells surrounding the vascular bundles (**Figures 4D,E**). The GFP-*Foc*-STR4 was strictly localized to the vascular bundles of the xylem and in the surrounding region (**Figure 4F**) suggesting that the fungus moves via the xylem. The cortex region of the uninoculated control showed little to no GFP fluorescence (**Figure 4G**). The xylem vessels of the uninoculated control were observed under the transmitted light setting (**Figure 4H**) and when stained with propidium iodide, they were associated with red-fluorescence at 555 nm (**Figure 4I**).

Although no internal symptoms were detected in the resistant genotype 'p168,' the presence of GFP-*Foc*-STR4 in its rhizome suggests that resistance does not inhibit the pathogen from gaining entry into its roots. To further evaluate the resistance response, we used two of the *Musa acuminata* ssp. *malaccensis*

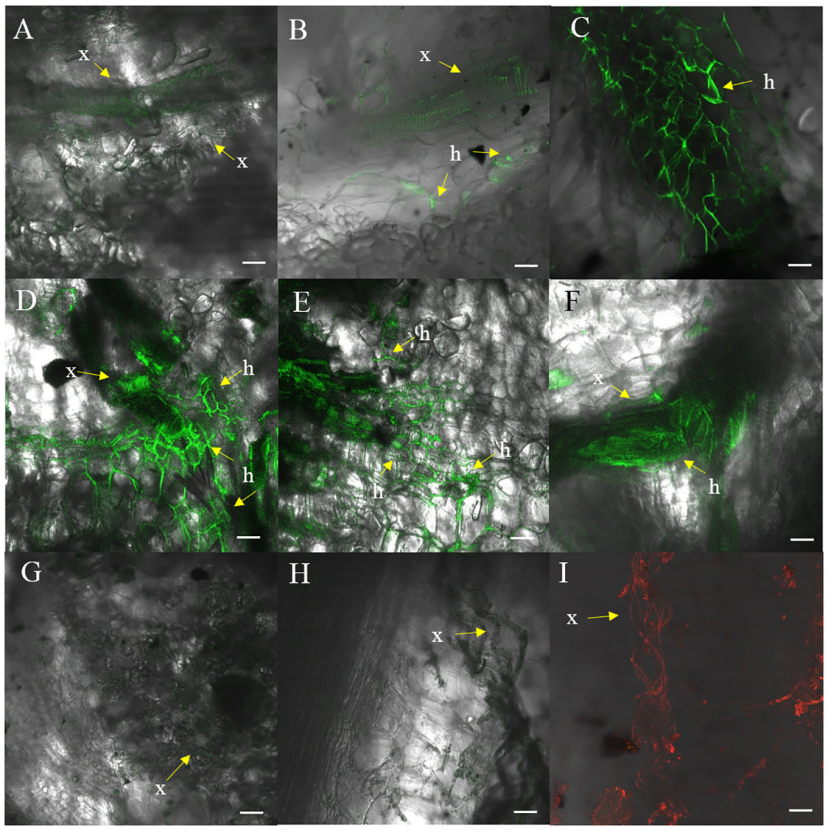

FIGURE 4 | Confocal images of 'p168' (resistant) and 'p248' (susceptible) at 3 months post-inoculation performed using millet and GFP-*Foc*-STR4. **(A)** Xylem perforation plates observed containing GFP observed in the lower region of the 'p168' rhizome. **(B)** GFP linked to the perforation plates and the hyphae structure in the lower region of the rhizome bordering the cortex cells in 'p168.' **(C)** Presence of GFP tagged mycelial networks in the lower region of the rhizome in 'p168.' **(D)** A cross section of the lower region of the 'p248' rhizome showing xylem perforation plates and the expansion of mycelial networks associated with GFP. **(E)** A longitudinal section of the lower region of the 'p248' rhizome showing GFP associated with perforation plates and the expanded mycelial networks. **(F)** Mycelial structures establishing near the boundary between the central region of the rhizome and the cortex area in 'p248.' **(G)** Confocal showing the absence of GFP in the lower rhizome of the un-inoculated control 'p4-19' derived from 'Ma851.' **(H)** Xylem perforation plates with no associated fluorescence in the non-inoculated controls of 'p4-19.' **(I)** Xylem perforation plates visualized under a 555 nm laser using non-inoculated 'p4-19' rhizome stained with propidium iodide. GFP was visualized using the 488 nm laser. Light images are created using the T-PMT (transmitted light detector) setting. Scaled bars represent a 50 μm unit. Arrows indicate the presence of xylem vessels (x) and hyphae (h).

parental lines, one that has been shown to be resistant, 'Ma851,' and one susceptible, 'Ma848,' against both *Foc*-STR4 and *Foc*-TR4 (Fraser-Smith et al., 2016). Progeny of 'Ma851' segregates for *Foc* resistance (**Figure 3**) whereas 'Ma848' showed an extremely susceptible phenotype indicative of the absence of any resistance genes (**Figures 1**, **2**). In this assay, a *Foc* spore suspension and a root dipping inoculation method was used which has been reported previously to study the onset of the infection process (Li et al., 2011; Zhang et al., 2018). The inoculation rapidly induced wilting in the susceptible 'Ma848' lines with the first signs of wilting observed at 2–3 weeks post-inoculation (**Supplementary Figure 3**). 'Ma848' plants were observed to be dead at 4 weeks (**Figure 5A**). Inoculated 'Ma851' and un-inoculated controls of both lines did not show any external symptoms at 4 weeks (**Figures 5A,B**). When cut in halves, the rhizomes of 'Ma848' showed extensive discoloration as typically associated with necrotic lesions of vascular vessels (**Figure 5C**). The symptoms also include the discoloration of the pseudostem and a root structure of reduced size (**Figures 5D,E**). While 'Ma851' showed some noticeable discoloration in the lower part of the rhizome, discoloration in the pseudostem was not observed (**Figures 5C,D**).

To study the infection process, the presence of GFP-*Foc*-STR4 in 'Ma848' and 'Ma851' was visualized with confocal microscopy. The GFP tagged *Foc*-STR4 spores were confirmed to be constitutively fluorescing prior to the start of the experiment (**Figure 5F**). Adhesion of GFP-*Foc*-STR4 spores to the lateral roots of 'Ma848' was observed at 1 day post-inoculation (dpi, **Figure 5G**). The presence of hyphae on the epidermis of lateral roots of 'Ma848' was first observed at 2 dpi (**Figure 5H**). A longitudinal section of a lateral root shows the elongation of hyphae and the establishment of a mycelial network on the epidermis of lateral roots of 'Ma848' at 4 dpi (**Figure 5I**). Complete colonization of a lateral root tip by GFP-*Foc*-STR4 was observed in 'Ma848' at 7 dpi (**Figure 5J**). The presence of GFP-*Foc*-STR4 was not detected on the main roots of 'Ma848'

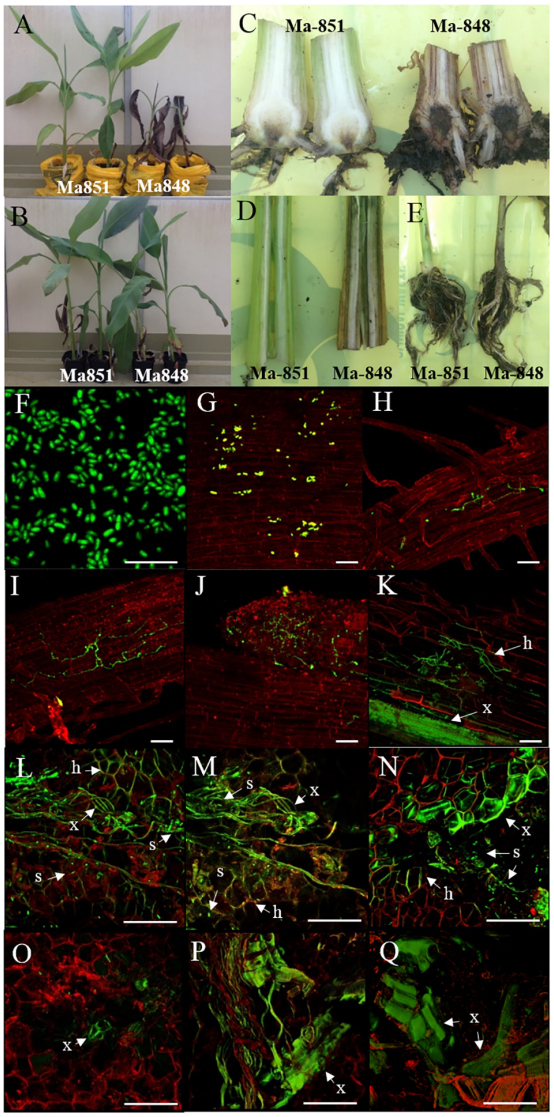

FIGURE 5 | Characterization of *Foc*-race 4 resistance from *Musa acuminata* subsp *malaccensis* using a GFP tagged *Foc*-STR4 isolate (BRIP 23598, VCG 0120). **(A)** Plant phenotypes of the resistant 'Ma851' and susceptible 'Ma848' *M. a malaccensis* plants at 28 days post-inoculation (dpi). **(B)** Non-inoculated controls of 'Ma851' plants (left) and 'Ma848' plants (right). Longitudinal sections of **(C)** rhizomes, **(D)** pseudostem and **(E)** entire roots of 'Ma851' and 'Ma848' at 28 days dpi, respectively. **(F)** Spores collected from suspension culture of BRIP23598 after 5 days of growth in half strength PDB containing 50 mg per L of hygromycin B. **(G–K)** Visualization of the GFP protein on 'Ma848' under a confocal microscope. **(G)** Attachment of spores to the lateral root surface at 1 dpi. **(H)** Movement of hyphae in the epidermis layer of a lateral root at 2 dpi. **(I)** A mycelial network established on the epidermis at 4 dpi. **(J)** The apical meristem region of a root tip completely colonized by GFP-*Foc*-STR4 at 7 dpi. **(K)** Mycelial networks are established in the cortex and vascular bundles of the xylem at 14 dpi. The **(L)** upper, **(M)** mid and **(N)** lower sections of the rhizome from a 'Ma848' plant at 14 days dpi. The **(O)** upper, mid **(P)** and lower **(Q)** sections of the rhizome from a 'Ma851' plant at 14 days dpi. White bars indicate a 50 μm scale. GFP fluorescence was detected at 488 nm wavelength using a Zeiss 700 laser scanning microscope. The tissues were stained with propidium iodide to produce a red fluorescence which was detected at 555 nm wavelength. x = xylem vessels, s = individual or clumps of spores, h = hyphae.

during the first 7 dpi. A single hypha was observed attempting to penetrate the epidermis cell layer of the lateral roots in 'Ma848' at 7 dpi (**Supplementary Figure 4A**). Presence of hyphae was also detected on the epidermal cell layer of the fine roots in 'Ma848' (**Supplementary Figure 4B**). The presence of GFP-*Foc*-STR4 was not detected in the roots of the resistant line 'Ma851' (**Supplementary Figures 4C–E**). At 14 dpi, GFP tagged mycelium was observed in the root cortex region of 'Ma848' (**Figure 5K**). The xylem vessels also showed a strong GFP signal indicating the presence of the fungus within the xylem. The rhizome was sectioned into three parts, which include the upper region closer to the pseudostem, the middle region which is the central cylinder and the lower region that connects to the cortex and the roots. At 14 dpi, all three regions of the 'Ma848' rhizome showed the abundant presence of spores near the xylem perforation plates which were also infected with GFP-*Foc*-STR4 (**Figures 5L–N**). GFP-*Foc*-STR4 can be seen colonizing the cortex cells around the xylem vessels in 'Ma848.' In contrast, no spores were observed in the rhizome of the inoculated resistant 'Ma851' plants (**Figures 5O–Q**). Movement of hypha was also not observed. However, strong GFP fluorescence was observed in the xylem vessels, indicating the likely presence of GFP-*Foc*-STR4 in these vessels (**Figures 5O–Q**). These data suggest that the frequency of fungal penetration via the roots was greatly induced in 'Ma851.'

Isolation of GFP-*Foc*-STR4 Using Koch's Postulates

Susceptible 'p96' and resistant 'p3,' 'p18,' 'p104' self-derived progeny of 'Ma851' were inoculated with GFP-*Foc*-STR4 and grown in a glasshouse for 3 months. At harvest, internal and external symptoms of *Fusarium* wilt were visible on the 'p96' plants (**Supplementary Figures 5**, **6**). Extensive leaf wilting, corm rot and stem splitting were present in the susceptible '96' plant but not in the resistant plants. Some slight discoloration of the corm was evident in the resistant plants (**Supplementary Figure 5**).

The presence of GFP-*Foc*-STR4 was detected in most of the regions isolated from the susceptible 'p96' plant, which include the throat and the mid-stem region of stem, and the mid-corm and cortex region of the corm (**Supplementary Figures 7**, **8** and **Table 1**). GFP-*Foc*-STR4 was not detected at all in two resistant 'p3' and 'p18' plants. However, it was isolated with the presence of the GFP confirmed in the regions of the throat in the third resistant 'p104' plant (**Supplementary Figures 7**, **8** and **Table 1**).

DISCUSSION

Fusarium wilt is one of the major threats confronting the banana industry today. An effective long-term solution to this problem would be to identify genetic resistance from the untapped gene pools in the wild relatives of the cultivated banana and introduce the identified sources of resistance loci back into commercially viable varieties. Resistance screens are traditionally performed in the field, which is labor-intensive owing to the long growth cycle and the large size of banana plants. In this study, a

glasshouse screen method was adopted to assess resistance level in relatively young plants grown in pots. This type of assay can have a short turn-over time due to the up-scaling of the number of plants that can be tested at a given time under controlled conditions with minimized cross infection by other micro-organisms (Smith et al., 2008; Li et al., 2015; Zuo et al., 2018). In addition, high inoculum dosage can be applied to identify highly resistant germplasm.

Resistance against *F. oxysporum* f. sp. *cubense* was assessed in a collection of 34 genotypes of diploid and polyploid banana plants in the glasshouse (**Table 1**) and their resistance levels were ranked using the internal discoloration of the rhizome. A wide range of disease responses from completely resistant, to partially resistant and highly susceptible, was revealed in the plant rhizomes against *Foc* race 4 types (**Figures 1**, **2**). Resistance levels of the genotypes tested were mostly consistent with previously published field data (**Table 1**). This response shows that resistance to *Foc* race 4 is mainly of a quantitative nature. Quantitative resistance has been detected in other pathosystems including *Pisum sativum* and *Medicago truncatula* against *Fusarium oxysporum* f. sp. *pisi* and *Fusarium oxysporum* f. sp. *medicaginis*, respectively (Bani et al., 2012; Rispail and Rubiales, 2014). In this study, wilt resistance can be detected in each of the diploid, triploid and tetraploid genome groups and in different *Musa accuminata* ssp. *malaccensis*, *Musa accuminata* ssp. *burmannica*, *Musa accuminata* ssp. *banksia*.

The *M. acuminata* ssp. *malaccenis* line 'Ma851' appears to carry strong wilting resistance to *Foc*. Wild *M. acuminata* diploids are highly diversified and have been heavily integrated in the breeding of edible banana cultivars (Perrier, 2009; Perrier et al., 2011; Li et al., 2013; Christelova et al., 2017). Geographically *M. acuminata* sub-species shows a wide-spread pattern of distribution in East Asia with each sub-species localized in distinct regions (Perrier et al., 2011). This suggests that some of these diploids may have evolved different mechanisms of resistance independently of one another. Similar findings were obtained in other studies, particularly in *Medicago truncatula* and *Pisum sativum*, which showed that quantitative resistance in their respective collections corresponds to accessions originating from multiple locations (Grünwald et al., 2003; Rispail and Rubiales, 2014). Future work will aim to identify QTLs controlling resistance in *M. a. malaccensis* lines.

The screen for *Foc* race 4 resistance revealed that the collection of *Musa* spp. contained sufficient genetic variations for the resistance responses to be detected. Cultivars shown to be highly susceptible to *Foc* race 4 in the field showed strong necrotic lesions in the rhizomes under our conditions (**Table 1**). In our study, the extent of the discoloration also depends on the inoculation technique used as it may influence the inoculum dosage and thereby the rate of the infection process. This observation is supported by studies that show a positive correlation between the amount of *F. oxysporum* detected within plant tissue and the resistance level in several species, including pea, tomato, watermelon, and chickpea (Gao et al., 1995; Zhou and Everts, 2004; Jimenez-Fernandez et al., 2011; Rispail and Rubiales, 2014).

In banana, contrasting resistance responses against *Foc* race 4 types have not been previously reported. In the present study, two cultivars, namely 'FHIA-2' and 'CAM020' showed excellent resistance to *Foc*-TR4 but were highly susceptible to *Foc*-STR4 (**Figures 1**, **2**). This indicates that resistance to the two race 4 type VCGs might be differentially regulated. One possible explanation is that both cultivars lack a gene(s) or a component of the PAMPs (pathogen-associated molecular patterns) triggered immunity (PTI) or the effector-triggered immunity (ETI) that is specifically required for *Foc*-STR4 mediated resistance in these lines. Mechanisms of PTI and ETI are discussed in detail in recent reviews (Jie and Zhou, 2010; Petit-Houdenot and Fudal, 2017). Furthermore, both *Foc* race 4 type resistance can potentially be controlled by a single gene. For example, it has been shown in tomato that the immune receptor Ve1 mediates resistance against multiple pathogens by recognizing not only the *Verticillium* effector *Ave1*, but also related homologs of *Ave1*, from *Fusarium oxysporum* f. sp. *lycopersici* and *Cercospora beticola* (de Jonge et al., 2012).

In soil borne plant diseases, such as those caused by *Foc*, where the pathogen gains entry via plant roots, the state of the rhizome is a good indicator of the severity of infection. Necrotic lesions in the rhizome are the consequences of *Foc* colonizing the vascular tissues to cause senescence in a localized manner. This was evident in the rhizomes of the susceptible banana plants used in this study (**Figures 3**, **4**). As shown using GFP tagged *Foc*-STR4, mycelial networks typically migrated along the xylem vessels and expanded outwards from the pits in susceptible plants. Our results are consistent with the behavior and the strong virulence of *Foc* race 4 observed in susceptible banana cultivars (Li et al., 2011, 2017; Zhang et al., 2018). A high level of sporulation further supports this observation in the present study (**Figure 4**).

In our study, the resistant plants typically showed no discoloration when millet was used as the inoculum (**Figures 1**–**3**). Furthermore, fungal isolation performed on rhizome and stems tissues of the resistant plants 3 months post-millet inoculation also failed to recover the GFP-STR4 strain (**Supplementary Table 1**). This suggests that the frequency of fungal colonization was too low to be detected in these lines at the respective early and late stages. This finding is consistent with a previous study which showed that *Foc* was not detectable at an early stage of infection in resistant banana plants (Li et al., 2011). A possibility could be that the growth of *Foc* was inhibited. Root exudates can potentially inhibit spore germination and fungal growth. In pea, phytoalexin pisatin, one of the metabolites detected in the root exudate extracts of pea, negatively correlated with the extent of *Fusarium oxysporum* f. sp. *pisi* germination (Bani et al., 2018a). It is of note that the millet inoculation technique minimized root wounding, whereas artificially induced wounding can enhance the infection rate in the roots and cause GFP tagged *Foc* to be detected in the rhizome of resistant banana cultivar Pahang (Zhang et al., 2018). However, in our study, *Foc* was never-the-less inhibited from traveling further up the pseudo-stem of the plant.

When a relatively more invasive method, root dipping, and concentrated micro-conidia were applied, GFP signals were not detected in the roots of resistant 'Ma851' plants at 7 dpi (**Supplementary Figure 4**). However, necrotic lesions were observed in the rhizome of the resistant plants at 18 dpi (**Supplementary Figure 3**). At this time, GFP fluorescence was associated with the xylem perforation plates. However, no

sporulation or mycelial networks were observed (**Figure 4**). This suggests that the colonization by the fungus was mainly contained in the rhizome of 'Ma851.' Furthermore, no GFP-*Foc*-STR4 was detected in the stems of 'Ma851' suggesting that the xylem was likely uninfected (**Supplementary Table 1**). A similar pattern of restricted colonization in *Dianthus caryophyllus* by *Fusarium oxysporum* f. sp. *dianthi* has been reported and further characterization revealed that the infected regions of the xylem became compartmentalized by cell wall thickening and hyperplasia of parenchyma cells (Ouellette et al., 1999).

This type of resistance mechanisms against vascular pathogens have been characterized in several plant species (Beckman et al., 1982; Bishop and Cooper, 1983a,b; Baayen et al., 1989; Tessier et al., 1990; Pereira et al., 2013; Bani et al., 2018b). Plant hosts develop physical and chemical barriers to block pathogen progressions at different stages during the infection process. These include cell wall strengthening by lignification and suberization, formation of papillae at penetration sites, the accumulation of tyloses inside cells and production of antifungal compounds (Beckman et al., 1982; Bishop and Cooper, 1983a; Baayen and Elgersma, 1985; Charchar and Kraft, 1989; Ouellette et al., 1999; Grayer and Kokubun, 2001; Yadeta and Thomma, 2013; Pouralibaba et al., 2017; Bani et al., 2018b). Beckman et al. (1982) showed that *F. oxysporum* triggered callose deposition in the parenchyma cells of tomato plants and that the rate of deposition was faster in the resistant than the susceptible plants. In a separate experiment, Robb et al. (1991) showed that *Verticillium* infected tomato petioles induced suberization in the membranes of the pits and the intercellular spaces around the vascular vessels. Furthermore, the level of vascular coating positively correlated with resistance and negatively correlated with the frequencies of pathogen penetration of pit membranes in alfalfa (Newcombe and Robb, 1988). One or any of these mechanisms could potentially explain the resistance mechanism observed in this study.

In the present study, we have assessed a collection of 34 banana cultivars for resistance against *F. oxysporum* f. sp. *cubense* race 4 types and identified a range of resistance responses in the rhizomes. The rhizome appears to be a key factor in preventing the fungus from further spreading to other parts of the plant. We characterized diploid wild relative *M. a. malaccensis* lines that exhibit strong wilt resistance to *Foc* by inhibiting fungal growth in its rhizome. They are potential sources of 'complete' resistance to *Foc* race 4. Furthermore, contrasting resistance responses to different *Foc* race 4 types were observed. Phenotypic methods used in this study can help accelerate the efforts in breeding programs. Overall, this study paves the way for further characterizations in the defense mechanisms of *Foc* resistance at the cellular and molecular level in this important plant species.

AUTHOR'S NOTE

Plants used in this study were generated from the Australian in vitro banana cultivar collection that is maintained in the Quality Banana Approved Nursery (QBAN) scheme accredited Plant Biotechnology Laboratory located at the Maroochy Research Facility, Department of Agriculture and Fisheries, Nambour, Queensland, Australia. The cultivars were directly sourced from the owners or institutions under verbal or written agreement and where plants were directly collected, such as *Musa acuminata* ssp. *malaccensis* lines, they were sourced prior to the development of International treaties for germplasm. All cultivars were sourced under agreements allowing that they are able to be used for research purposes. No ownership is claimed for those cultivars sourced from owners or other institutions. The plants were destructively sampled for the purposes of this experiment and will not be further propagated.

AUTHOR CONTRIBUTIONS

AC, SH, JB, and EA proposed, organized, and planned the experiments. AC, JS, AM, LA-E, NC, SM, and LT-N carried out and performed the experiments. AC wrote the manuscript draft. All authors commented and contributed to the preparation of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01062/full#supplementary-material

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Molecular Diagnostics of Banana Fusarium Wilt Targeting *Secreted-in-Xylem* Genes

Lilia C. Carvalhais[1], Juliane Henderson[1], Vivian A. Rincon-Florez[1], Cecilia O'Dwyer[1], Elizabeth Czislowski[2], Elizabeth A. B. Aitken[2] and André Drenth[1]*

[1] Queensland Alliance for Agriculture and Food Innovation, Centre for Horticultural Science, Ecosciences Precinct, The University of Queensland, Brisbane, QLD, Australia, [2] School of Agriculture and Food Sciences, The University of Queensland, Saint Lucia, QLD, Australia

Fusarium wilt is currently spreading in banana growing regions around the world leading to substantial losses. The disease is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*), which is further classified into distinct races according to the banana varieties that they infect. Cavendish banana is resistant to *Foc* race 1, to which the popular Gros Michel subgroup succumbed last century. Cavendish effectively saved the banana industry, and became the most cultivated commercial subgroup worldwide. However, *Foc* tropical race 4 (TR4) subsequently emerged in Southeast Asia, causing significant yield losses due to its high level of aggressiveness to cultivars of Cavendish, and other commonly grown cultivars. Preventing further spread is crucially important in the absence of effective control methods or resistant market-acceptable banana cultivars. Implementation of quarantine and containment measures depends on early detection of the pathogen through reliable diagnostics. In this study, we tested the hypothesis that *secreted in xylem* (*SIX*) genes, which currently comprise the only known family of effectors in *F. oxysporum*, contain polymorphisms to allow the design of molecular diagnostic assays that distinguish races and relevant VCGs of *Foc*. We present specific and reproducible diagnostic assays based on conventional PCR targeting *SIX* genes, using as templates DNA extracted from pure *Foc* cultures. Sets of primers specifically amplify regions of: *SIX6* in *Foc* race 1, *SIX1* gene in TR4, *SIX8* in subtropical race 4, *SIX9*/*SIX10* in *Foc* VCG 0121, and *SIX13* in *Foc* VCG 0122. These assays include simplex and duplex PCRs, with additional restriction digestion steps applied to amplification products of genes *SIX1* and *SIX13*. Assay validations were conducted to a high international standard including the use of 250 *Fusarium* spp. isolates representing 16 distinct *Fusarium* species, 59 isolates of *F. oxysporum*, and 21 different vegetative compatibility groups (VCGs). Tested parameters included inter and intraspecific analytical specificity, sensitivity, robustness, repeatability, and reproducibility. The resulting suite of assays is able to reliably and accurately detect R1, STR4, and TR4 as well as two VCGs (0121 and 0122) causing Fusarium wilt in bananas.

Keywords: tropical race 4, molecular diagnostics, Panama disease, *SIX* genes, plant pathogens, *Fusarium oxysporum*

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****Correspondence:***
Lilia C. Carvalhais
l.carvalhais@uq.edu.au

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INTRODUCTION

Commercial banana production is under serious threat worldwide. A destructive disease caused by the fungus *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) is spreading rapidly throughout the banana growing regions around the globe (Ordonez et al., 2015; Zheng et al., 2018). The soilborne nature of this pathogen is one of the main reasons why eradication and containment of TR4 are very challenging. In the absence of effective disease resistance in market-accepted banana varieties, one of the remaining measures to avoid further losses is to prevent spread through early detection with reliable diagnostics, and subsequent containment of new incursions.

Tropical race 4 was first identified in samples obtained from Sumatra, Indonesia, in 1992 (Ploetz, 1994; Ploetz, 2004). However, reports of infected Cavendish banana (*Musa* spp. AAA genome group) date from nearly 30 years before in Taiwan (Su et al., 1977; Ploetz, 1994). Since its emergence, TR4 has not only decimated the Cavendish banana industry in Taiwan, but has been making its way through Southeast Asia in China, Indonesia, Malaysia, Philippines, Laos, Myanmar, Vietnam, and Pakistan (Ordonez et al., 2015, 2016; Chittarath et al., 2018; Hung et al., 2018; Zheng et al., 2018). Intercontinental spread has also occurred with reports of the pathogen in Australia, Jordan, Lebanon, Oman, and Mozambique (Butler, 2013; García-Bastidas et al., 2014; Ploetz, 2015b; Dita et al., 2018).

The present situation of Fusarium wilt is familiar: another race of the causal agent (race 1 or R1) triggered a devastating epidemic in the banana growing regions of Central America in the first half of last century (Stover, 1990; Ploetz, 2005). It was "widely regarded as one of the most destructive plant diseases in recorded history" (Moore et al., 1995). A major commercial subgroup at the time, "Gros Michel" (*Musa acuminata* AAA genome group), was highly susceptible to R1 (Stover, 1962). The banana industry could only recover because cultivars belonging to the Cavendish subgroup were found to be resistant to R1 (Ploetz, 1994).

Resistance to Fusarium wilt R1, high productivity, the use of temperature-controlled transportation in boxes, and consumer acceptance turned Cavendish into the main subgroup used worldwide for export (Ploetz, 2005). Other varieties of banana are fundamental to the subsistence of households of many developing countries with low incomes, either as a staple or as a cash crop (FAOSTAT, 2018). With predictions of many of these countries being the main contributors to population growth (Gerland et al., 2014), the protection of banana production is essential to safeguard not only one of the most popularly consumed and exported fruits globally, but also livelihoods of millions of people (FAOSTAT, 2018).

To facilitate reference to groups that infect specific plant species, *F. oxysporum* has been sub-divided into *formae speciales* (Armstrong, 1981). However, given that not all varieties of the same plant species are necessarily susceptible to a particular *forma specialis*, these are further classified into races, e.g., *F. oxysporum* f. sp. *lycopersici* race 1 infects a specific group of tomato varieties (Alexander, 1945). The term *forma specialis cubense* is utilized to delineate those populations that can cause disease on banana. Nevertheless, the race classification can be inconsistent as surprisingly little work focused on identifying the host specificity of this pathogen (Ploetz, 2015a). In general, the cultivars within the subgroup Gros Michel (AAA), Pisang Awak (ABB) and others that belong to the AAB genomic group (e.g., Maqueno, Silk, and Pome) are susceptible to R1 (Stover and Buddenhagen, 1986; Stover and Simmonds, 1987; Stover, 1990). The susceptibility of banana varieties to races 2 and 4 is still unclear as insufficient variety trials have been systematically conducted for these two races. Race 4 was further divided into two groups, TR4 and subtropical race 4 (STR4). Lower temperatures are typically associated with STR4 infection and disease progression in cultivars of the Cavendish subgroup in subtropical regions, while TR4 is virulent to these varieties in all environments (Ploetz, 2005). Reclassification of TR4 into the new species *Fusarium odoratissimum* was proposed based on the genetic diversity of *Foc* isolates in the Indonesian centre of origin (Maryani et al., 2019).

An additional classification commonly used for the fungi causing Fusarium wilt is based on vegetative compatibility. Strains are classified into the same vegetative compatibility group (VCG) when they are able to anastomose and form a stable heterokaryon with each other (Leslie, 1993). This system reflects well the similarity of strains based on phenotypical traits (Caten and Jinks, 1966); however, genetic relatedness between different groupings cannot be inferred as mutations in the *vic* loci can lead to vegetative incompatibility even in closely related isolates (Bentley et al., 1998). Although different races can be associated with most VCGs, there are several limitations in the use of VCGs to support race classifications. Various VCGs can be associated with the same race, and evolutionary relationships cannot be taken into consideration (Ordonez et al., 2015). For example, VCG 0126 exhibits a closer phylogenetic affiliation to race 4 and, similarly to isolates classified into this race, have the ability to produce odorous aldehydes on media (Moore et al., 1991; Czislowski et al., 2017). Nonetheless, they are considered race 1 due to the host range on which it is able to cause disease (Pegg et al., 1994).

Breeding of new banana cultivars has been attempted, but until now has only had very limited success. Progress in banana breeding has been hampered by many factors, which include lack of germplasm evaluation, lack of fundamental genetic studies of diploid plants to identify characteristics, as well as lack of a long-term commitment to fund breeding. Besides, the occurrence of varying ploidy levels in cultivated varieties (which are polyploid) and the need for diploids for crosses, parthenocarpy, sterility, poor seed set, germination, and survival have hampered progress in banana breeding programs (Pillay and Tenkouano, 2011). Nonetheless, the growing impact of TR4 and other diseases such as black leaf streak (black Sigatoka, caused by the fungus *Pseudocercospora fijiensis*) and several improvements in breeding processes have yielded a renewed interest in this area (Pillay and Tenkouano, 2011; Li et al., 2015), while transgenic approaches to obtain TR4-resistant Cavendish are undergoing field-testing (Dale et al., 2017).

Once banana plants are infected, there is no effective treatment for Fusarium wilt. Fungicides or soil fumigation are ineffective to control or eradicate this disease (Ploetz, 2015b). Thus, in the

absence of effective resistance or control methods, one of the remaining ways to manage this disease is preventing further spread and ensuring rapid containment of new infected plants. For this, early identification of infected plants combined with sensitive, accurate and robust diagnostic methods to detect incursions at an early stage are paramount. Diagnosing the specific VCGs and races is required as similar early symptoms caused by distinct strains are observed in various locally grown non-Cavendish and commercialized banana varieties (Karangwa et al., 2016). Diagnostics based solely on morphology is not reliable to differentiate distinct *Foc* and non-pathogenic strains of *F. oxysporum* (Pérez Vicente et al., 2014). Several molecular diagnostics methods have been developed, which mostly rely on core genomic regions and hence are unlikely to be closely linked or associated with pathogenic characteristics of different races. A molecular diagnostic method based on the intergenic spacer region (IGS) of the nuclear ribosomal gene cluster of *Foc* is widely used and is efficient for detecting TR4 (Dita et al., 2010). However, this assay was not designed to detect other strains that are closely related to TR4 and are also able to infect Cavendish, such as those affiliated to VCG 0121 and VCG 0122 (Ordonez et al., 2015; Czislowski et al., 2017; Mostert et al., 2017). Another study conducted by Lin et al. (2009) reported primers that are able to detect all race 4 strains, and hence can detect strains assigned to VCG 0121 and VCG 0122. However, these primers are unable to distinguish TR4 and STR4. Despite being closely related to TR4 VCGs 01213/16 and being able to attack Cavendish varieties in the tropics, the classification of R4 VCGs 0121 and 0122 as TR4 or STR4 is still a matter of debate (Moore et al., 1993; Bentley et al., 1998; Buddenhagen, 2009). Some of the isolates belonging to VCGs 0121 and 0122 have been reported to infect Cavendish causing symptoms that were less severe than those caused by TR4 isolates (Buddenhagen, 2009), which suggests that predisposing conditions may need to be in place for symptoms to be expressed. Therefore, we opted for classifying here VCGs 0121 and 0122 as R4, without further sub-classifications as tropical or subtropical. *Foc* strains affiliated to these VCGs can cause disease in Cavendish (Mostert et al., 2017), thus having molecular tools available to detect VCG 0121 and VCG 0122 is especially beneficial for countries reliant on banana cultivation under subtropical conditions.

A common target for diagnostics is genes that encode proteins that are strongly correlated with virulence (de Sain and Rep, 2015). In *F. oxysporum*-infected tomato, some of these proteins have been detected in the xylem sap and named Secreted-in-Xylem (SIX) (Rep et al., 2004; Houterman et al., 2007; Gawehns et al., 2014). When these proteins or other molecules (e.g., secondary metabolites and small RNAs) are associated with processes in the host in favor of colonization by the pathogen and disease progression, they are known as effectors (Weiberg et al., 2013; de Sain and Rep, 2015). Homologs of the *SIX* genes are found across different *formae speciales* and their profile can be used to distinguish distinct *formae speciales*, races and isolates (Lievens et al., 2009; Chakrabarti et al., 2011; van Dam et al., 2016; Czislowski et al., 2017). *SIX* gene profiling has been successfully used to distinguish the three races in *F. oxysporum* f. sp. *lycopersici* (Lievens et al., 2009). The mode of action of these proteins in the pathogenicity process remains to be elucidated, but some studies have suggested an effect on plant defense signaling (Thatcher et al., 2012; Kazan and Lyons, 2014).

Diagnostic tools to identify races associated with banana Fusarium wilt are needed to allow early detection of new incursions and distinguish isolates infecting varieties that are generally more susceptible to a wider range of *Fusarium* (e.g., Lady Finger). However, poor assay validation often leads to a huge gap between development and implementation of diagnostic methods (Chilvers, 2012). Evidence for the specificity and reliability of diagnostic assays is required and therefore validation needs to be conducted according to rigorous standards.

The gene *SIX8* has been previously shown to contain enough variation to differentiate *Foc* race 4 from the races 1 and 2 as well as STR4 from TR4 through a conventional PCR (Fraser-Smith et al., 2014). However, only three non-pathogenic isolates of *F. oxysporum* were included in the screening, and the detection of TR4 was based on the absence of an amplification product. Diagnosing a disease based on the absence of an amplification product is unreliable because the absence of a pathogen would provide the same outcome. In addition, basic validation parameters such as sensitivity and robustness cannot be assessed if there is no amplicon for the assessment of positive diagnostics. Therefore, the overall objective of our study was to assess whether sequence variation in *SIX* genes of *Fusarium* races could be used to develop a series of assays that can detect relevant VCGs and races of Fusarium wilt of banana. We specifically sought to address whether: (1) *SIX* genes exhibit specific sequences that are conserved within each race; (2) there are sufficient single nucleotide polymorphisms (SNPs) across *SIX* gene homologs to enable the design of primers which target unique sequences within the above races; (3) the primers targeting specific *SIX* genes homologs can be used in PCR assays to reach a level of specificity, sensitivity, robustness and repeatability that meets international standards for diagnostic assays; and (4) our proposed assays can detect all races and VCGs mentioned above and outperform popularly used molecular diagnostic tests previously designed to detect R4 (STR4 and TR4).

Reliable detection of different races and VCGs of *Fusarium* will enable decision-making by the banana industry stakeholders.

MATERIALS AND METHODS

Regions in the *SIX* genes conserved within races of *Foc* were identified by analyzing sequences generated by Czislowski et al. (2017), which are available in the National Center for Biotechnology Information database[1] (NCBI Resource Coordinators, 2017), under accessions KX434886-KX435052. These sequences had been generated by whole-genome sequencing (WGS) analysis followed by *Foc-SIX* specific PCR to characterize the diversity and evolution of the *SIX* genes in a collection of 89 isolates representing 23 genetic lineages of *Foc* (Czislowski et al., 2017). Based on the data of

[1]https://www.ncbi.nlm.nih.gov/nuccore/

Czislowski et al. (2017), it was possible to identify in this current study an exclusive *SIX* gene homolog for each race of *Foc*. Each of these exclusive homologs was present in a different *SIX* gene. Consensus sequences of these *SIX* gene homologs were aligned for the identification of conserved regions within races of *Foc*. Alignments were performed either in Geneious (v10.0.9) (Kearse et al., 2012) or Clustal Omega[2] (Sievers and Higgins, 2014). SNP-rich regions within the distinct *SIX* genes were then targeted for race-specific primer design, as described in the following sections.

Primer Design

Sets of primers were designed either to anneal to SNP-rich regions within the targeted *SIX* gene or to anneal to regions flanking a specific SNP that contained a sequence which included a restriction enzyme site. Details of races, VCGs, target *SIX* gene homologs, primer sequences, and annealing temperatures are listed in **Table 1**. The strategy for identifying TR4 isolates was based on the exclusive presence of the homolog "a" of *SIX1* in TR4 (Czislowski et al., 2017). For the design of specific primers to detect TR4 isolates, full-length sequences from all *SIX1* gene homologs ("a" to "i") were aligned using the software Clustal Omega[2]. The primer set SIX1_266 (**Table 1**) was designed to anneal to regions which were rich in SNPs that were exclusive to the *SIX1* gene homologs "a," "b," or "c." These gene homologs are unique to TR4, R4 VCG 0121 and R4 VCG 0122, respectively (Czislowski et al., 2017). The 266 bp-product amplified by these primers contains a unique recognition site for the restriction enzyme HpyAV (New England BioLabs), which is present in *SIX1* homolog "a" and absent in homologs "b" and "c." After restriction digestion, two DNA fragments are predicted to be generated, one with 124 bp and the other with 142 bp.

A different approach was adopted to detect R4 VCG 0121 and R4 VCG 0122. To specifically detect R4 VCG 0121, primers were designed to target the *SIX10* gene which is present exclusively in this VCG (Czislowski et al., 2017) using Primer-BLAST[3]. A 309 bp-product is predicted to be amplified with this primer set. However, we obtained the same amplicon for several *F. oxysporum* strains isolated from crops other than banana (data not shown). We thus opted for the use of a duplex PCR combining the primer set that targets *SIX10* in combination with the *SIX9* gene that is conserved in all isolates of *Foc* (Czislowski et al., 2017). The primer set SIX9_Foc was designed to amplify a 260-bp product targeting the homolog "a" of *SIX9* using the software Primer3 (Untergasser et al., 2012).

To detect VCG 0122, primers were designed to amplify a 343-bp region of the gene *SIX13* that contains a restriction site exclusive to this VCG recognized by the restriction enzyme EagI (New England Biolabs). A restriction digestion step after amplification was necessary as there was insufficient sequence variation to design primers that would amplify only the homolog "c" of *SIX13* (Czislowski et al., 2017). Two fragments of this PCR product were predicted to be generated after the restriction digestion, one with 102 bp and the other with 241.

To identify STR4 isolates, primers were designed to anneal to SNP-rich regions of the *SIX8* gene which are unique to the homolog "b," only present in STR4 (Czislowski et al., 2017). Likewise, to detect R1 isolates, primers were designed to anneal to the polymorphic regions of the homolog "b" of the *SIX6* gene, found to be present only in R1 by Czislowski et al. (2017).

PCR and Restriction Digestion Conditions

DNA material extracted from isolates of *Fusarium* was used as templates for the amplification tests in this study. DNA was extracted from 5 to 7-day-old monoconidial cultures, grown on half-strength potato dextrose agar (PDA, Difco Laboratories) at 25°C, using the DNeasy Plant Mini Kt (Qiagen) on the QIACube (Qiagen) following the manufacturer's recommendations. Approximately 50–100 mg of mycelium were used as initial material in the extractions (amount recovered by scraping the medium surface of a 100-mm diameter standard Petri dish fully covered with mycelium).

For all amplifications, MyTaq^{TM} Red DNA Polymerase (Bioline) was used in 20 uL reactions according to the manufacturer's recommendations. The primer and DNA template concentration in the final PCR reaction were 0.4 μM and 5 pg. uL^{-1}, respectively. The following cycling conditions were used for all PCR: 95°C for 3 min, 30 cycles of 95°C for 15 s, and annealing temperature were primer-set dependant (see **Table 1**) for 15 s and 72°C for 10 s. In the cases of follow-up restriction digestions (for *SIX1* and *SIX13* products), no purification step of the PCR products is needed before enzymatic digestions. Restriction digestions for the enzymes HpyAV and EagI (New England Biolabs) were performed using 4 μL of the amplification product and 0.4 units (U) of enzyme in a total volume of 10 μL. The enzyme EagI is also known as Eco521. Star activity is a property exhibited by some restriction enzymes in which they show relaxed or inaccurate sequence recognition (Wei et al., 2008). HpyAV and EagI-HF have not been predicted to have star activity in the buffers recommended by the manufacturer[4]. If the recommended conditions are used as reported here, no restriction enzyme star activity is expected to occur. The restriction enzymes digestions were conducted at 37°C for 1 h and subsequently inactivated for 20 min at 65°C. A volume of 5 μL of amplification products and 10 μL restriction digestions were run on a 1.5% agarose gel and post-stained with ethidium bromide (1 μg mL^{-1}).

The primer set that partially amplifies the translation elongation factor 1α gene (*TEF-1α*) was used in an additional PCR for all samples to confirm that each fungal DNA sample was amenable to amplification and control for false negatives (O'Donnell et al., 1998).

Method Validation

To validate the diagnostic assays, the international standards as proposed in the "guidelines for the validation and verification of quantitative and qualitative test methods" were followed (National Association of Testing Authorities, 2018). These guidelines delineate particular requirements that a proposed

[2]https://www.ebi.ac.uk/Tools/msa/clustalo/

[3]https://www.ncbi.nlm.nih.gov/tools/primer-blast

TABLE 1 | Primer sequences designed in this study based on *SIX* gene sequences generated in Czislowski et al. (2017).

Race	VCG	Targeted *SIX* gene	Primer name	Sequence (5′ -> 3′)	Primer annealing position within gene	Product length (bp)	Anneal. temperature (°C)	Restriction digestion	Restriction enzyme recognition site
All *Foc*	All	*SIX9*a	SIX9_Foc_F SIX9_Foc_R	ATCGCTGAAGCCCAGAACAA TTCTGTCCGTCGATCGTTCC	46–65291–305	260	58	No	N/A
R1	0123, 01210, 01217, 01218, 0124, 0124/5, 0124/22, 0125, 0128, 01220	*SIX6*b	SIX6b_210_F SIX6b_210_R	ACGCTTCCC**A**ATACCG**T**CTGT AAG**TT**G**T**G**AGT**A**TC**AA**T**GC	181–201371–390	210	55	No	N/A
TR4	01213/16	*SIX1*a	SIX1a_266_F SIX1a_266_2_R	GTGACCAGAACTTGCCCACA CTTTGATAAGCACCATC**AA**	442–461689–707	266	55	HpyAV, 124 bp/142 bp	**CCTTC**$(N)_6$
STR4	0120, 0120/15, 0129, 01211, 01215 0126*	*SIX8*b	SIX8b_206_F SIX8b_206_R	GCCTGCATAACAGGTGC**C**GG**T** **T**TC**C**TCACCTCACCCGGCA**G**GAT**TC**	267–287448–472	206	62	No	N/A
R4[#]	0121	*SIX10*a	SIX10a_309_F SIX10a_309_R, to be used as duplex in combination with SIX9_Foc	**CCACTGGCACCAAAGACTTG** **CGATGCGGAGTACTGGTTGA**	62–81351–370	309	58	No	N/A
R4[#]	0122	*SIX13*c	SIX13c_343_F SIX13c_343_R	CAGCCTCCTAGCGTCGAAAA CCGTGATGGGGT**A**CGTTGTA	91–110414–433	343	57	EagI102 bp/241 bp	**C**GGC**C**G

*Nucleobases in the primer sequences represented **in bold** correspond to polymorphic sites in the target gene homolog. 'N/A' stands for not applicable.*

**The race of VCG 0126 is arguable as isolates are phenotypically and genetically similar to race 4 (e.g., ability to synthesize odorous aldehydes on media) (Moore et al., 1991; Dale et al., 2017). However, there is limited evidence to suggest that VCG 0126 is capable of infecting Cavendish and is therefore often considered race 1 based on its host range (Pérez Vicente et al., 2014).*

[#]The classification of VCGs 0121 and 0122 as tropical or subtropical race 4 is arguable. Isolates of these VCGs are not as aggressive as those of VCG 01213/16 on Cavendish banana, which suggests that predisposing conditions may need to be in place for symptoms to be expressed. We thus opted for classifying these VCGs as R4, abstain from giving a further sub-classification and point out here that this is a matter of debate which needs further clarification.

method should meet to fulfill the purpose for its intended use (i.e., "fit-for-purpose"). As validation parameters, we included inter and intraspecific analytical specificity, sensitivity, robustness, repeatability, and reproducibility.

A total of 250 isolates of *Fusarium* spp. were screened in the validation tests. These included 16 different plant-associated *Fusarium* species in addition to 21 VCGs commonly associated with *Foc* (VCGs 120 to 01223).

For validation of assay specificity, we tested each primer set with 16 *Fusarium* species (**Table 2**). These *Fusarium* isolates were obtained from infected plant material in Australia. We also included in the validations nine different *formae speciales* of *F. oxysporum* and 21 different VCGs of *Foc*. Sensitivity was tested in a 10-fold serially diluted positive control within the range of 10 ng to 0.1 fg.μL^{-1} (**Supplementary Table S1**). Before the preparation of dilutions, DNA concentrations were measured in a Qubit fluorometer with the Quant-iT High-Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific).

To test for assay robustness, two different *Taq* polymerases were used for the amplification of eight positive controls, in duplicate, in two different thermal cyclers. The DNA polymerases used were MyTaq^{TM} HS DNA polymerase (Bioline, MyTaq reaction buffer with 1 mM dNTPs, 3 mM $MgCl_2$) and ThermoFisher Scientific *Taq* Polymerase [*Taq* Buffer with $(NH_4)_2SO_4$], using reagent concentrations and cycling conditions as previously described. The robustness of the assays was tested by changing the manufacturer of the DNA polymerases. Different manufacturers of restriction enzymes were not used for the assays that included an additional restriction digestion step (*SIX1* and *SIX13*). The reason for that is based on reportedly different outcomes observed according to the type of polymerase (Schierwater and Ender, 1993), while the results obtained with restriction enzymes from different manufacturers are more consistent due to the intrinsic specificity of these enzymes.

For repeatability, six to eight positive controls from a variety of VCGs in six separate occasions were tested by the same operator for each primer set. For all assays, two negative controls were also included: one that contained a DNA sample that was not targeted by the primers and another that lacked DNA template. Reproducibility was tested by conducting separate assays with two different operators, on three different occasions, with the same set of samples. All tests included two technical replicates. Two previously reported conventional PCR methods were also compared with our assays: one utilized primers which amplify a region of the intergenic spacer (IGS) region of the nuclear ribosomal operon to detect TR4 (Dita et al., 2010); the other utilized primers developed from a Random Amplified Polymorphic DNA marker specific to *Foc* Race 4 (Lin et al., 2009).

TABLE 2 | Strains used for inter-specific validation of the diagnostic assays using the primer sets SIX1a_266 (TR4), SIX9_Foc/SIX10a_309 duplex (R4 VCG 0121), SIX13c_343 (R4 VCG 0121), SIX8b_206 (STR4) and SIX6b_210 (R1) and DNA extracted from *Fusarium* isolates obtained from infected plant material in Australia.

Code	Identification	Host
BRIP 61484a	*Fusarium acutatum*	*Solanum lycopersicum*
BRIP 53804a	*Fusarium ananatum*	*Ananas comosus*
BRIP 47260a	*Fusarium equiseti*	*Fragaria ananassa*
BRIP 26010	*Fusarium equiseti*	*Citrullus lanatus*
UQ6530	*Fusarium fujikuroi*	*Musa* sp. (Lady Finger)
UQ6540	*Fusarium fujikuroi*	*Musa* sp. (Lady Finger)
BRIP 61879a	*Fusarium meridionale*	Musa sp.
UQ6547	*Fusarium proliferatum*	*Musa* sp. (Lady Finger)
BRIP 63776	*Fusarium pseudocircinatum*	*Gossypium arboreum*
BRIP 61022a	*Fusarium pseudocircinatum*	*Mangifera indica*
BRIP 16558a	*Fusarium roseum* 'Gibbosum'	*Passiflora edulis*
BRIP 53693a	*Fusarium roseum* 'Gibbosum'	*Gossypium hirsutum*
BRIP 53695a	*Fusarium roseum* 'Gibbosum'	*Rosa* sp.
UQ6659	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
148	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
212	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6561	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6562	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6564	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6567	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6569	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6576	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6586	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6588	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6589	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6590	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
UQ6675	*Fusarium sacchari*	*Musa* sp. (Lady Finger)
BRIP 53261b	*Fusarium semitectum*	*Sorghum bicolor*
UQ6527	*Fusarium solani*	Musa sp. (Lady Finger)
UQ6609	*Fusarium solani*	*Musa* sp. (Lady Finger)
UQ6528	*Fusarium solani*	*Musa* sp. (Lady Finger)
UQ6610	*Fusarium solani*	*Musa* sp. (Lady Finger)
UQ6673	*Fusarium solani*	*Musa* sp. (Lady Finger)
UQ6548	*Fusarium solani/falciform*	*Musa* sp. (Lady Finger)
UQ6524	*Fusarium solani/falciform*	*Musa* sp. (Lady Finger)
BRIP 61517e	*Fusarium* sp.	*Persea americana*
BRIP 59719a	*Fusarium sterilihyphosum*	*Mangifera indica*
BRIP 52901a	*Fusarium tricinctum*	*Vitis vinifera*

No amplification product was generated for any of the Fusarium isolates tested above.

RESULTS

Sequences from previously reported homologs of each *SIX* gene (Czislowski et al., 2017) were aligned and conserved regions within each race were identified as potential primer annealing sites. The alignment of different homologs of the genes *SIX1*, *SIX13*, *SIX8*, *SIX6* allowed the identification of exclusive sequences for TR4, R4 (VCG 0122), STR4 and R1, respectively. As *SIX10* was reported to be only present in R4 VCG 0121, and only one homolog has been reported (Czislowski et al., 2017), no alignment was needed prior to primer design targeting this gene.

Details on the presence of sufficient sequence polymorphisms that enabled race-specific primer design are described in the subsections dedicated to each *Foc* race below. All diagnostic assays were validated using DNA extracted from 38 isolates

of *Fusarium* affiliated to 16 different species and 59 isolates of *F. oxysporum* obtained from healthy plant tissues of a range of plant species including, amongst others, *Asparagus officinalis*, *Citrullus lanatus*, *Cucumis melo*, *Euphorbia dallachyna*, *Musa* spp., *Phoenix* sp., *Solanum lycopersicum*, *Solanum tuberosum*, *Trianthema portulacastrum*, and *Zingiber officinale*. This included endophytes that were asymptomatic on their hosts and *F. oxysporum* classified into different *formae speciales*. A total of 150 *Foc* isolates affiliated to different races were used in our tests, including 32 TR4 isolates obtained in various countries (**Tables 2–4**).

Diagnostic Assay to Detect TR4 Using PCR and Restriction Digestion

Sequences of the *SIX1* homologs "a" to "i" were aligned and the primer set SIX1a_266 was designed to anneal to regions that were conserved within homologs "a," "b," and "c," and were exclusive to TR4, R4-VCG 0121, and R4-VCG 0122, respectively (**Table 1**). These primers flanked a recognition site of the enzyme HpyAV, which is only present in the *SIX1* gene homolog "a," which is unique to TR4 (Czislowski et al., 2017). **Figure 1** shows amplification products for TR4 isolates and HpyAV-digested fragments. The amplified product of 266 bp was digested into two fragments with the enzyme HpyAV, one with 124 bp and the other with 142 bp (**Figure 1**). The 266-bp amplification product is also obtained for R4-VCG 0121 and R4-VCG 0122; however, this product is not digested by HpyAV in strains associated with these VCGs (**Figure 1**). No false positives or false negatives were obtained for other *Fusarium* species, *F. oxysporum* strains, *formae speciales* of other *F. oxysporum*, or races tested (**Tables 2–4**). This assay was validated and proven to be repeatable, robust, and specific. The limit of detection is 1 pg.μL^{-1} (**Supplementary Table S1**).

Diagnostic Assay to Detect R4-VCG 0121 Using a Duplex PCR

The primer set which targets the gene *SIX10* in VCG 0121 (SIX10a_309) generated the expected amplification product in nine isolates of *F. oxysporum* that were asymptomatic endophytes in plant genera distinct to *Musa* (data not shown). For this reason, we developed a duplex PCR as the diagnostic assay for the detection of VCG 0121, which then included primers that target the homolog "a" of the gene *SIX9*. According to currently available data, this gene homolog seems to be present in *Foc* but absent in other *formae speciales* of *F. oxysporum* (Czislowski et al., 2017; **Tables 3**, **4**). Two amplicons with the expected lengths were obtained using the primer sets SIX9_Foc and SIX10a_309, with 260 and 309 bp, respectively (**Figure 2**). The resultant duplex PCR has a limit of detection of 0.1 ng.μL^{-1} and met most validation criteria (**Tables 2–4** and **Supplementary Table S1**). Four of 32 TR4 isolates from Indonesia tested positive for this duplex-PCR assay (**Table 4**), inferring the presence of *SIX10* in these isolates. The limit of detection was 0.01 ng.μL^{-1} (**Supplementary Table S1**).

TABLE 3 | Intra-specific validation of the diagnostic assays using the primer sets SIX1a_266 (TR4), SIX9_Foc/SIX10a_309 duplex (R4 VCG 0121), SIX13c_343 (R4 VCG 0121), SIX8b_206 (STR4) and SIX6b_210 (R1) and DNA extracted from *Fusarium* isolates obtained from infected plant material in Australia.

Code	Identification	Host
1756	*Fusarium oxysporum*	*Phoenix* sp.
1755	*Fusarium oxysporum*	*Phoenix* sp.
WEED6	*Fusarium oxysporum*	*Trianthema portulacastrum*
WEED5	*Fusarium oxysporum*	*Euphorbia dallachyna*
WEED10	*Fusarium oxysporum*	*Rhynchosia minima*
BRIP 13106	*Fusarium oxysporum*	*Cucumis melo*
23732	*Fusarium oxysporum*	*Musa* sp.
23733-P	*Fusarium oxysporum*	*Musa* sp.
23733-W	*Fusarium oxysporum*	*Musa* sp.
BRIP 14928	*Fusarium oxysporum*	*Pisum sativum*
BRIP 16617	*Fusarium oxysporum*	*Trifolium repens*
BRIP 62618	*Fusarium oxysporum*	*Musa* sp. (DPM25)
BRIP 62577a	*Fusarium oxysporum*	*Musa* spp. (Cavendish)
GRS1058	*Fusarium oxysporum*	*Asparagus officinalis*
GRS585	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS1054	*Fusarium oxysporum*	*Citrullus lanatus*
GRS1049	*Fusarium oxysporum*	*Solanum tuberosum*
GRS1034	*Fusarium oxysporum*	*Solanum tuberosum*
GRS1021	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS1017	*Fusarium oxysporum*	*Asparagus officinalis*
GRS1008	*Fusarium oxysporum*	*Petroselinum crispum*
GRS1007	*Fusarium oxysporum*	*Citrullus lanatus*
GRS1002	*Fusarium oxysporum*	*Solanum lycopersicum*
FCC0778	*Fusarium oxysporum*	*Pinus patula*
FCC0776	*Fusarium oxysporum*	*Pinus patula*
CMH6015B	*Fusarium oxysporum*	*Citrullus lanatus*
CMH6008	*Fusarium oxysporum*	*Citrullus lanatus*
CMH6007	*Fusarium oxysporum*	*Citrullus lanatus*
CMH6002	*Fusarium oxysporum*	*Citrullus lanatus*
CMH6001	*Fusarium oxysporum*	*Citrullus lanatus*
CMH5727A	*Fusarium oxysporum*	*Petroselinum crispum*
GRS942(3)	*Fusarium oxysporum*	*Citrullus lanatus*
GRS920	*Fusarium oxysporum*	*Ocimum basilicum*
GRS932	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS897	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS895	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS894	*Fusarium oxysporum*	*Solanum lycopersicum*
GRS652	*Fusarium oxysporum*	*Solanum lycopersicum*
BRIP 63620	*Fusarium oxysporum* f. sp. *basilici*	*Ocimum basilicum*
BRIP 63545	*Fusarium oxysporum* f. sp. *basilici*	*Ocimum basilicum*
BRIP 63616	*Fusarium oxysporum* f. sp. *basilici*	*Ocimum basilicum*
BRIP 63616	*Fusarium oxysporum* f. sp. *basilici*	*Ocimum basilicum*
BRIP 62106	*Fusarium oxysporum* f. sp. *fragariae*	*Fragaria ananassa*
BRIP 13039	*Fusarium oxysporum* f. sp. *lycopersici*	*Solanum lycopersicum*
BRIP 53843	*Fusarium oxysporum* f. sp. *lycopersici*	*Solanum lycopersicum*

(Continued)

TABLE 3 | Continued

Code	Identification	Host
BRIP 5181	*Fusarium oxysporum* f. sp. *niveum*	*Citrullus lanatus*
BRIP 5177	*Fusarium oxysporum* f. sp. *niveum*	*Citrullus lanatus*
BRIP 5178	*Fusarium oxysporum* f. sp. *niveum*	*Citrullus lanatus*
BRIP 28044	*Fusarium oxysporum* f. sp. *passiflorae*	*Passiflora edulis*
BRIP 57641	*Fusarium oxysporum* f. sp. *tracheiphilum*	*Vigna unguiculata* subsp. *sesquipedalis*
BRIP 43336	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 43344	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 43351	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 43356	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 43365	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 63607	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 25374	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 43339	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*
BRIP 44986	*Fusarium oxysporum* f. sp. *zingiberi*	*Zingiber officinale*

No amplification product was generated for any of the F. oxysporum isolates tested above.

Diagnostic Assay to Detect R4-VCG 0122 Using PCR and Restriction Digestion

This assay was designed to target the gene *SIX13* and flanked a R4-VCG 0122-specific recognition site for the restriction enzyme EagI (**Table 1**). The expected 343 bp product was amplified and, after digestion with the enzyme EagI, expected fragments of 102 and 241 bp were obtained (**Figure 2**). The assay using the primer set SIX13c_343 followed by an EagI-restriction digestion was specific, with no false negatives or false positives detected (**Tables 3**–**5**). The limit of detection of this assay was 0.01 g.μL^{-1} (**Supplementary Table S1**). An example of a gel showing amplification products of *SIX13* obtained with a range of DNA template concentrations is shown is **Supplementary Figure S1**. All validation criteria of repeatability and robustness were met.

Diagnostic Assay to Detect STR4 Using PCR

The primer set SIX8b_206 was designed to amplify exclusively STR4 isolates (**Table 1**). A 206 bp product was amplified for all tested STR4 isolates (**Figure 2**) and no false positives were obtained (**Tables 2**–**4**). Amplification products with the expected sizes were also obtained for isolates belonging to the VCGs 01219 (whose race is still undetermined) and VCG 0126 (classified as R1) (**Table 4**). This assay was proven to be repeatable, reproducible, robust, sensitive and specific. The limit of detection was 0.01 ng.μL^{-1} (**Supplementary Table S1**).

Diagnostic Assay to Detect R1 Using PCR

The primer set SIX6b_210 was designed to detect exclusively R1 isolates (**Table 1**). The primers generated the expected 210 bp amplicon for most R1-associated VCGs (60 isolates out of 65, **Figure 1** and **Table 4**). Three strains classified as R1 did not generate the expected PCR product, with one of them belonging to the VCG 0123 (BRIP 62890), three to the VCG 0124 (BRIPs 42125, 42190, and 59087), and the other to the VCG 0124/5 (BRIP 58774) (**Table 4**). The test was shown to be repeatable, robust, reproducible, and the limit of detection was 0.1 ng. uL^{-1} (**Supplementary Table S1**).

Although all primer sets generated amplicons using DNA templates at a concentration of 0.5 ng. μL^{-1} in the PCR reaction for most isolates, some false negatives were observed at this particular concentration. Thus, we recommend using DNA concentrations between 50 pg. μL^{-1} and 5 pg. μL^{-1} in the PCR reaction as these concentrations worked consistently well in the assays conducted to determine the limit of detection (**Supplementary Table S1**).

Comparison With Previously Reported Molecular Diagnostics Methods to Detect R4

We confirmed unpublished results obtained by the Department of Agriculture and Fisheries (Henderson J., unpublished), which revealed that the primers FocTR4, previously reported to detect TR4 (Dita et al., 2010), tested positive for one endophytic strain isolated from an asymptomatic banana plant grown in Northern Queensland, Australia (BRIP62577, **Table 5**). Furthermore, the same primer set and the Foc1/2 primer set reported by Lin et al., 2009 tested positive for one STR4 and three *F. oxysporum* f. sp. *vasinfectum* isolates (BRIP 59052, 43336, 43344, and 43365, **Table 5**). The Foc1/2 primer set also produced amplification products for two endophytic strains of *F. oxysporum* colonizing watermelon plants (*Citrullus lanatus*) (GRS1054 and CMH6002, **Table 5**). As the Foc1/2 primer set cannot distinguish between TR4 and STR4, it is expected that amplicons for STR4 strains are produced, such as for BRIP 59052 (**Table 5**).

DISCUSSION

This study supports the utilization of *SIX* genes as targets for molecular diagnostics for races of *Foc*. The results demonstrate that for each race, specific conserved *SIX* gene sequences can be identified especially for TR4, R4-VCG 0122, and STR4. Sufficient SNPs across *SIX* gene homologs enabled the differentiation of TR4, R4-VCG 0121, R4-VCG 0122, STR4, and R1 strains through conventional PCR using primers targeting polymorphic regions of the genes *SIX1*, *SIX9*/*SIX10*, *SIX13*, *SIX8*, and *SIX6,* respectively. However, only a small number of SNPs was present to distinguish TR4 and R4-VCG 0122 from other races and

TABLE 4 | Validation of the diagnostic assays using the primer sets SIX1a_266 (TR4), SIX9_Foc/SIX10a_309 duplex (R4 VCG 0121), SIX13c_343 (R4 VCG 0121), SIX8b_206 (STR4) and SIX6b_210 (R1) and DNA extracted from isolates from the *Fusarium oxysporum* f. sp. *cubense* (*Foc*) species complex infecting *Musa* spp.

Code	Race*	VCG	Country of origin	TR4-specific *SIX1*a_266	R4 VCG 0121-specific duplex SIX9_Foc and *SIX10a*_309	R4 VCG0122-specific SIX13c_343	STR4-specific *SIX8*b_206	R1-specific *SIX*6b_210
58625	TR4	01213/16	Indonesia	+	–	–	–	–
58671	TR4	01213/16	Indonesia	+	–	–	–	–
58686	TR4	01213/16	Malaysia	+	–	–	–	–
58688	TR4	01213/16	Malaysia	+	–	–	–	–
58712	TR4	01213/16	Malaysia	+	–	–	–	–
58715	TR4	01213/16	Malaysia	+	–	–	–	–
58732	TR4	01213/16	Malaysia	+	–	–	–	–
58734	TR4	01213/16	Malaysia	+	–	–	–	–
58750	TR4	01213/16	Malaysia	+	–	–	–	–
58754	TR4	01213/16	Malaysia	+	–	–	–	–
58760	TR4	01213/16	Malaysia	+	–	–	–	–
59047	TR4	01213/16	Indonesia	+	–	–	–	–
59049	TR4	01213/16	Indonesia	+	–	–	–	–
59072	TR4	01213/16	Indonesia	+	–	–	–	–
59094	TR4	01213/16	Indonesia	+	–	–	–	–
59127	TR4	01213/16	Indonesia	+	–	–	–	–
59132	TR4	01213/16	Indonesia	+	–	–	–	–
59136	TR4	01213/16	Indonesia	+	–	–	–	–
59150	TR4	01213/16	Malaysia	+	–	–	–	–
62765	TR4	01213/16	Indonesia	+	–	–	–	–
62922	TR4	01213/16	India	+	–	–	–	–
62963	TR4	01213/16	Taiwan	+	–	–	–	–
63144	TR4	01213/16	Indonesia	+	–	–	–	–
63160	TR4	01213/16	Indonesia	+	–	–	–	–
63181	TR4	01213/16	Indonesia	+	+	–	–	–
63184	TR4	01213/16	Indonesia	+	–	–	–	–
63188	TR4	01213/16	Indonesia	+	+	–	–	–
63199	TR4	01213/16	Indonesia	+	–	–	–	–
63203	TR4	01213/16	Indonesia	+	–	–	–	–
63211	TR4	01213/16	Indonesia	+	–	–	–	–
63213	TR4	01213/16	Indonesia	+	–	–	–	–
63246	TR4	01213/16	Indonesia	+	+	–	–	–
58666	R4	0121	Indonesia	–	+	–	–	–
58738	R4	0121	Malaysia	–	+	–	–	–
58741	R4	0121	Malaysia	–	+	–	–	–
59084	R4	0121	Indonesia	–	+	–	–	–
59104	R4	0121	Indonesia	–	+	–	–	–
59106	R4	0121	Indonesia	–	+	–	–	–
59165	R4	0121	Taiwan	–	+	–	–	–
62962	R4	0121	Taiwan	–	+	–	–	–
63220	R4	0121	Indonesia	–	+	–	–	–
59154	R4	0122	Philippines	–	–	+	–	–
62808	R4	0122	Philippines	–	–	+	–	–
62892	R4	0122	Philippines	–	–	+	–	–
62894	R4	0122	Philippines	–	–	+	–	–
62901	R4	0122	Philippines	–	–	+	–	–
39259	STR4	0129/11	Australia	–	–	–	+	–
40309	STR4	0129	Australia	–	–	–	+	–
40334	STR4	0129	Australia	–	–	–	+	–

(Continued)

TABLE 4 | Continued

Code	Race*	VCG	Country of origin	TR4-specific *SIX1*a_266	R4 VCG 0121-specific duplex SIX9_Foc and *SIX10a*_309	R4 VCG0122-specific SIX13c_343	STR4-specific *SIX8*b_206	R1-specific *SIX*6b_210
42113	STR4	0129	Australia	–	–	–	+	–
42130	STR4	0120	Australia	–	–	–	+	–
42131	STR4	0129	Australia	–	–	–	+	–
42134	STR4	0129	Australia	–	–	–	+	–
42135	STR4	0129	Australia	–	–	–	+	–
42186	STR4	0129	Australia	–	–	–	+	–
44012	STR4	0120	Australia	–	–	–	+	–
44027	STR4	0120	Australia	–	–	–	+	–
44073	STR4	01211	Australia	–	–	–	+	–
58610	STR4	0120/15	Canary Islands	–	–	–	+	–
58614	STR4	0120	Canary Islands	–	–	–	+	–
58620	STR4	0120	Indonesia	–	–	–	+	–
59052	STR4	0120/15	Indonesia	–	–	–	+	–
59093	STR4	0120/15	Indonesia	–	–	–	+	–
59162	STR4	0120	South Africa	–	–	–	+	–
59163	STR4	0120	South Africa	–	–	–	+	–
59787	STR4	0120/15	South Africa	–	–	–	+	–
59791	STR4	0120	South Africa	–	–	–	+	–
62581	STR4	0129	Australia	–	–	–	+	–
63532	STR4	0120	Australia	–	–	–	+	–
63615	STR4	0129	Australia	–	–	–	+	–
58637	R1**	0126	Indonesia	–	–	–	+	–
58657	R1**	0126	Indonesia	–	–	–	+	–
59044	R1**	0126	Indonesia	–	–	–	+	–
59062	R1**	0126	Indonesia	–	–	–	+	–
59152	R1**	0126	Philippines	–	–	–	+	–
59153	R1**	0126	Philippines	–	–	–	+	–
59161	R1**	0126	Papua New Guinea	–	–	–	+	–
63200	R1**	0126	Indonesia	–	–	–	+	–
42102	R1	01220	Australia	–	–	–	–	+
42174	R1	01220	Australia	–	–	–	–	+
42177	R1	01220	Australia	–	–	–	–	+
58617	R1	01218	Indonesia	–	–	–	–	+
58627	R1	01218	Indonesia	–	–	–	–	+
58680	R1	01217	Malaysia	–	–	–	–	+
58681	R1	01217	Malaysia	–	–	–	–	+
58683	R1	01217	Malaysia	–	–	–	–	+
58691	R1	01217	Malaysia	–	–	–	–	+
58698	R1	01217	Malaysia	–	–	–	–	+
58700	R1	01218	Malaysia	–	–	–	–	+
58710	R1	01217	Malaysia	–	–	–	–	+
58722	R1	0123	Malaysia	–	–	–	–	+
58723	R1	01217	Malaysia	–	–	–	–	+
58737	R1	0123	Malaysia	–	–	–	–	+
58742	R1	01217	Malaysia	–	–	–	–	+
58778	R1	0123	Philippines	–	–	–	–	+
58811	R1	0123	Thailand	–	–	–	–	+
59051	R1	0123	Indonesia	–	–	–	–	+
59071	R1	01218	Indonesia	–	–	–	–	+
59109	R1	01218	Indonesia	–	–	–	–	+

(Continued)

TABLE 4 | Continued

Code	Race*	VCG	Country of origin	TR4-specific *SIX1*a_266	R4 VCG 0121-specific duplex SIX9_Foc and *SIX10a*_309	R4 VCG0122-specific SIX13c_343	STR4-specific *SIX8*b_206	R1-specific *SIX*6b_210
59145	R1	01217	Malaysia	–	–	–	–	+
59147	R1	01218	Malaysia	–	–	–	–	+
62542	R1	0123	Indonesia	–	–	–	–	+
62890	R1	0123	Philippines	–	–	–	–	–
63162	R1	0123	Indonesia	–	–	–	–	+
63175	R1	01218	Indonesia	–	–	–	–	+
63236	R1	01218	Indonesia	–	–	–	–	+
63581	R1	01220	Australia	–	–	–	–	+
63582	R1	01220	Australia	–	–	–	–	+
63583	R1	01220	Australia	–	–	–	–	+
63584	R1	01220	Australia	–	–	–	–	+
63585	R1	01220	Australia	–	–	–	–	+
63586	R1	01220	Australia	–	–	–	–	+
40176	R1	0125	Australia	–	–	–	–	+
40188	R1#	0125	Australia	–	–	–	–	+
42125	R1#	0124	Australia	–	–	–	–	–
42190	R1#	0124	Australia	–	–	–	–	–
44010	R1#	0125	Australia	–	–	–	–	+
44013	R1#	0128	Australia	–	–	–	–	+
44014	R1#	0125	Australia	–	–	–	–	+
44015	R1#	0128	Australia	–	–	–	–	+
44479	R1#	0128	Australia	–	–	–	–	+
44480	R1#	0128	Australia	–	–	–	–	+
44614	R1#	0128	Australia	–	–	–	–	+
58692	R1#	0125	Malaysia	–	–	–	–	+
58693	R1#	0124/5	Malaysia	–	–	–	–	+
58774	R1#	0124/5	Mexico	–	–	–	–	–
58788	R1#	0125	Thailand	–	–	–	–	+
58790	R1#	0124	Thailand	–	–	–	–	+
58813	R1#	0124/22	Uganda	–	–	–	–	+
59023	R1#	0124	Brazil	–	–	–	–	+
59033	R1#	0124	India	–	–	–	–	+
59036	R1#	0125	India	–	–	–	–	+
59087	R1#	0124	Indonesia	–	–	–	–	–
59788	R1#	0125	India	–	–	–	–	+
62794	R1#	0124/5	Philippines	–	–	–	–	+
62924	R1#	0125	India	–	–	–	–	+
62947	R1#	0124	Uganda	–	–	–	–	+
62950	R1#	0124/5	Brazil	–	–	–	–	+
62952	R1#	0125	Brazil	–	–	–	–	+
62961	R1#	0124	India	–	–	–	–	+
63531	R1#	0124	Australia	–	–	–	–	+
63537	R1#	0124	Australia	–	–	–	–	+
63600	R1#	0124/5	Australia	–	–	–	–	+
58624	Unknown	01219	Indonesia	–	–	–	+	–
58634	Unknown	01219	Indonesia	–	–	–	+	–
58635	Unknown	01219	Indonesia	–	–	–	+	–
58636	Unknown	01219	Indonesia	–	–	–	+	–
59037	Unknown	01212	India	–	–	–	–	+
59115	Unknown	01219	Indonesia	–	–	–	+	–

(Continued)

TABLE 4 | Continued

Code	Race*	VCG	Country of origin	TR4-specific *SIX1*a_266	R4 VCG 0121-specific duplex SIX9_Foc and *SIX10a*_309	R4 VCG0122-specific SIX13c_343	STR4-specific *SIX8*b_206	R1-specific *SIX*6b_210
59170	Unknown	01222	Uganda	–	–	–	–	–
62955	Unknown	01212	India	–	–	–	–	+
63186	Unknown	01219	Indonesia	–	–	–	+	–
63187	Unknown	01219	Indonesia	–	–	–	+	–

The symbols '+' and '−' represent positive and negative results, respectively.

**The race structure used here was based in the literature (Rep, 2005; Moore et al., 1993; Ploetz, 2004; Bentley et al., 1995, 1998; Katan and Di Primo, 1999; Gerlach et al., 2000; Jones, 2000; Groenewald et al., 2006).*

***The race of VCG 0126 is considered equivocal as isolates are phenotypically and genetically similar to race 4 (e.g., ability to synthesize odorous aldehydes on media) (Moore et al., 1991; Dale et al., 2017). Since the ability of VCG 0126 to infect Cavendish has not been supported by solid evidence, we considered here as race 1 based its currently known host range (Pérez Vicente et al., 2014).*

#These VCGs have been sometimes classified as R1/R2. However, limited evidence suggests its ability to cause disease in the Bluggoe subgroup.

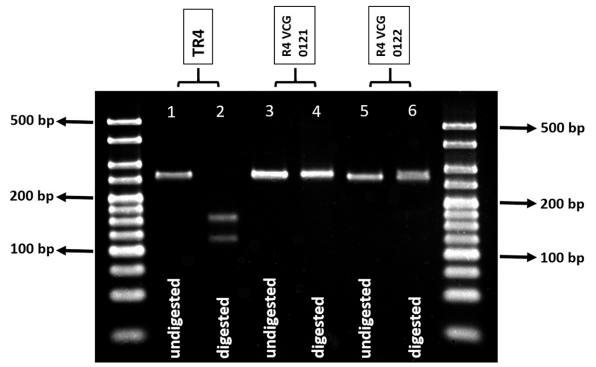

FIGURE 1 | Agarose gel (3%) showing undigested and digested 266-bp amplification products obtained with the primer set (SIX1a_266) designed to target the *SIX* gene homolog present exclusively in TR4 (*SIX1*a). Ladder used: 25 bp Bioline Hyperladder. Lane 1, undigested amplicon from TR4 isolate (5 μL); lane 2, HpyAV-treated amplicon from TR4 isolate (10 μL); lane 3, undigested amplicon from R4 VCG 0121 isolate (5 μL); lane 4, HpyAV-treated amplicon from R4 VCG 0121 isolate (10 μL); lane 5, undigested amplicon from R4 VCG 0122 isolate (5 μL); and lane 6, HpyAV-treated amplicon from R4 VCG 0122 isolate (10 μL).

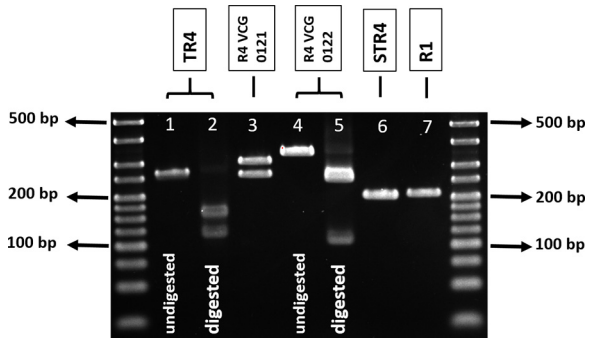

FIGURE 2 | Agarose gel (3%) showing amplification products obtained with primers designed to target different races and VCGs of *Fusarium oxysporum* f. sp. *cubense*. Ladder used: 25 bp Bioline Hyperladder. Lane 1, undigested 266 bp *SIX1* gene amplicon (5 μL); lane 2, TR4-specific HpyAV-treated 266 bp *SIX1* gene amplicon (10 μL), showing 124 and 142 bp digests; lane 3, R4 VCG 0121-specific 309 and 260 bp amplicons from duplex PCR of the genes *SIX10* and *SIX9* (5 μL), respectively; lane 4, undigested 343 bp *SIX13* gene amplicon (5 μL); lane 5, R4 VCG 0122-specific EagI-treated 343 bp *SIX13* gene amplicon, showing 102 and 241 digests (10 μL); lane 6, STR4-specific 206 bp *SIX8* gene amplicon (5 μL); and lane 7, R1-specific *SIX6* 210 bp gene amplicon (5 μL).

VCGs. Therefore, following the PCRs targeting *SIX1* and *SIX13*, a restriction digestion step with the enzymes HpyAV and EagI was required to detect the specific homologs present in TR4 and R4 VCG 0122, respectively. Overall, our validation experiments revealed that our assays are specific, sensitive, robust and repeatable.

Strains with VCG 0122 have been recently reported to infect the Cavendish cultivar Grande Naine (AAA) in the Davao del Norte area in the Philippines between 2006 and 2007 (Mostert et al., 2017), but Maryani et al. (2019) reported that these strains were not able to infect Cavendish in glasshouse trials. These contradictory results may stem from the low aggressiveness of these strains, which may not infect plants under artificial conditions. Some factors influencing the success of the infections in the field may not have been known and taken into account. The primer set SIX1a_266 generates amplicons for not only TR4, but also the R4 VCGs 0121 and 0122. Therefore, the primer sets SIX10a_309 and SIX13c_343 were additionally designed to detect exclusively R4 VCGs 0121 and 0122, respectively. Other PCR primers that can detect VCG 0121 and TR4 have been reported by Lin et al. (2009), but they cannot discriminate between TR4 and STR4 isolates. In the study by Czislowski et al. (2017) within *Foc*, only one homolog of the gene *SIX10* was identified and it was present exclusively in VCG 0121; consequently *SIX10* appeared to be a suitable specific target for VCG 0121. The *SIX10* gene is however, present in other *F. oxysporum* strains, as we observed from amplification products using isolates other than *Foc*. This led to the use of the additional primer set SIX9_Foc, which targets a gene homolog of *SIX9* previously shown to be present in all *Foc* (Czislowski et al., 2017). Despite the reported monophyletic nature of TR4 isolates (Ordonez et al., 2015;

TABLE 5 | List of strains tested with primer sets reported in previous studies, which target TR4 and/or R4 strains and produced false positive results.

Code	Identification	Host	Country	TR4 primer set FocTR4[a]	R4 primer set Foc1/2[b]
BRIP 62577a	*Fusarium oxysporum*	*Musa* sp.	Australia	+	–
GRS1054	*Fusarium oxysporum*	*Citrullus lanatus*	Australia	–	+
CMH6002	*Fusarium oxysporum*	*Citrullus lanatus*	Australia	–	+
BRIP 59052	*Foc* STR4 (VCG 0120/15)	*Musa* sp.	Indonesia	+	+
BRIP 43336	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*	Australia	+	+
BRIP 43344	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*	Australia	+	+
BRIP 43365	*Fusarium oxysporum* f. sp. *vasinfectum*	*Gossypium hirsutum*	Australia	+	+

The symbols '+' and '–' represent positive and negative results, respectively.
[a]*FAOSTAT (2018)* and [b]*Lin et al. (2009).*

Maryani et al., 2019), our results indicate that there is some level of variability associated with the *SIX* gene profiles of these isolates given that amplicons of *SIX10* in the duplex PCR were observed in 12.5% of the TR4 isolates tested (4 out of 32). However, this finding does not affect the use of our diagnostic toolkit. For example, if Fusarium wilt symptoms are reported in a Cavendish plantation in a region where there is no record of TR4, the first molecular diagnostic method that should be conducted is a PCR using the SIX1a_266 primer set, followed by restriction digestion with the HpyAV enzyme. This is important given the increased risks associated with a TR4 incursion compared to the other races. If the expected 124 and 142 bp digestion products are obtained after the treatment with HpyAV, there is strong evidence to suggest that the isolate is TR4. An amplification reaction with primers targeting the gene *SIX9* should be run simultaneously with the primer set SIX1a_266 to verify whether the isolate is *Foc*. If this is confirmed, the isolate may be considered TR4. DNA from isolates with VCGs 0121 and 0122 will also generate an amplification product with this primer set but, because they lack the HpyAV restriction site, the 266-bp product will not be digested into two fragments. If this is the case, then the next step would be either to use the duplex PCR to verify whether it detects R4 VCG 0121, or the SIX13c_343 primer set to identify R4 VCG 0122. We advise that a PCR targeting the *SIX9* gene should be conducted in parallel with the race-specific PCR assay to confirm that the tested isolate is a *Foc* and not an endophytic *Fusarium* strain that shares the same *SIX* gene homolog with a pathogenic strain. In **Figure 3** we suggest a workflow for the use of the different primer sets designed in this study.

The primer set SIX8b_206 was designed to amplify exclusively STR4 isolates. Amongst a wide range of strains tested, 24 STR4 isolates from the eight different VCGs considered to be STR4 were included in the assays (**Table 4**). A 206 bp product was amplified for all tested STR4 isolates and no false positives were obtained (**Table 2**). Amplification of products with the expected size were also obtained for isolates belonging to the VCGs 01219 and 0126, whose races are either not determined or still controversial. The race associated with the VCG 01219 has not been determined yet. However, our findings corroborate those of other studies that documented strains which belong

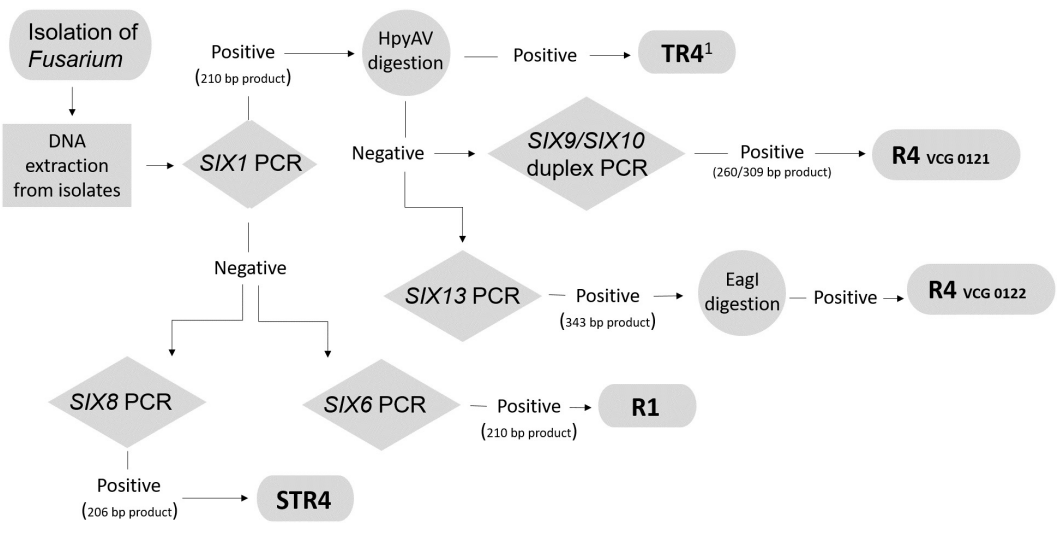

FIGURE 3 | Proposed molecular diagnostic workflow to distinguish races and vegetative compatibility groups (VCGs) of *F. oxysporum* f. sp. *cubense*. based on a conventional PCR targeting the *SIX* genes. TR4[1] has been proposed to be reclassified as *Fusarium odoratissimum* (Maryani et al., 2019).

to the VCG 01219 have the same *SIX* gene profile as all the other VCGs commonly associated with STR4 (Czislowski et al., 2017). In addition, despite VCG 0126 having been associated with R1 given its host range, our results are in agreement with reports suggesting closer genetic relatedness of VGC 0126 to isolates belonging to VCGs associated with STR4 than those associated with R1 (Bentley et al., 1998; Groenewald et al., 2006; Czislowski et al., 2017).

Although the expected results were obtained for most strains tested with the *SIX6* primers designed to amplify R1, five R1 isolates did not show the expected amplification product. Three of them belong to VCG 0124 and one to VCG 0123. A possible reason is the polyphyletic nature of R1 strains (Ordonez et al., 2016), which complicates the design of specific primers for this race. A recent phylogenetic analysis of *Foc* using strains isolated in the Indonesian center of origin revealed that diversity among genetic lineages of *Foc* is higher than previously anticipated (Maryani et al., 2019). Identification and phylogeny of these isolates were based on the genes encoding the translation elongation factor-1alpha (*tef1*), the RNA polymerase II largest submunit (*rpb1*), and the RNA polymerase II second largest subunit (*rpb2*), leading to the formal description of nine independent lineages (Maryani et al., 2019). TR4 isolates belong to the same lineage and were proposed to be reclassified as *F. odoratissimum* (Maryani et al., 2019). A previous attempt to design a real-time PCR specific for *Foc* R1 isolates has been reported but only four isolates belonging to this race were included and no VCG information was given (Yang et al., 2015). The distribution of putative effectors in natural and agro-ecosystems suggests that *SIX6*, which was chosen in this study to detect R1, may have been horizontally transferred across *F. oxysporum* strains (Rocha et al., 2016).

Pathogenicity genes have been common targets for molecular diagnostic assays of plant diseases (Loreti and Gallelli, 2002; Zaccardelli et al., 2007; Serdani et al., 2013). In fungi, the most typical avirulence factors are secreted small proteins (less than 200 amino acids) (Rep, 2005). Disease resistance is often triggered when the host's innate immune system identifies these proteins (Kim et al., 2016). However, it is not unusual that fungi adopting endophytic lifestyles also harbor genes that encode these proteins (Kim et al., 2016). The occurrence of putative effector genes has been investigated in strains of the *F. oxysporum* species complex isolated from asymptomatic plants occurring in natural environments (Rocha et al., 2016; Jelinski et al., 2017). The genes *PDA1*, *PELD*, *SGE1*, and *SIX* were evaluated because of their involvement in pathogenicity and functional diversity (Rocha et al., 2016). From these putative effector genes, *SIX* genes were reported to be generally less prevalent, which suggests that they may not have a crucial function in natural populations of *F. oxysporum* (Rocha et al., 2016). *SIX* genes have already been used as targets for molecular diagnostics, as in the case of a loop-mediated isothermal amplification assay targeting the *SIX3* gene of *F. oxysporum* f. sp. *lycopersici* (Ayukawa et al., 2017). The proposed method claims to detect point mutations and distinguish race 3 strains from other races of *F. oxysporum* f. sp. *lycopersici* (Ayukawa et al., 2017). Furthermore, a previous study detected enough sequence variation in the *SIX8* gene to design a primer set which distinguished R4 strains from races 1 and 2 and another set which differentiates STR4 from TR4 by the presence of an amplification product only in STR4 (Fraser-Smith et al., 2014). The main issue with this approach is its reliance on the absence of a band to test positive for TR4, which is not acceptable as a robust diagnostic test.

A range of molecular diagnostics methods has been previously proposed to distinguish R4 from other races of *Foc*. Lin et al. (2009) proposed primers to detect R4 isolates based on a random amplification of polymorphic DNA (RAPD) product that was unique to R4 strains. However, their validation tests included only seven reference *Foc* isolates from other races (two R1 isolates, two R2, and three STR4 isolates). This primer set was further tested in at least two other studies. Dita et al., 2010 found that these primers amplified nine different *Foc* VCGs, including several that included strains which are not classified as R4, such as the ones belonging to the VCG 01210 that typically includes R1 isolates (Dita et al., 2010). Another study reported that the primers developed by Lin et al. (2009) tested positive for endophytic strains isolated from healthy banana plants and R1 isolates (Magdama, 2017). This primer set is also unable to distinguish STR4 from TR4 isolates, which is a major drawback for regions where STR4 is endemic and that still have no record of TR4. Alternative primers targeting a ribosomal IGS have been developed and found to be quite reliable (Dita et al., 2010). This region was chosen as it was deemed more polymorphic than others and more suitable as a sensitive diagnostic test due to the multi-copy nature of this region (Fourie et al., 2009; Dita et al., 2010), which was confirmed by our tests (**Supplementary Table S1**). Nonetheless, the primers FocTR4 which were proposed by Dita et al., 2010 were tested in another study which included endophytic strains isolated from healthy banana plants (Magdama, 2017). An endophytic *F. oxysporum* strain which was obtained from Gros Michel roots tested positive although it was isolated from an asymptomatic plant grown in a region where TR4 is absent (Magdama, 2017). In our study, we also obtained false positives for one endophytic strain isolated from an asymptomatic banana plant, one STR4 and three *F. oxysporum* f. sp. *vasinfectum* isolates with primers FocTR4 (Dita et al., 2010; **Table 5**). It is possible that there is a higher risk of obtaining false positives when targeting core genomic regions, as genetically related strains may contain the same sequence and still differ in pathogenicity. Primers designed to amplify genes or sequences associated with pathogenicity may be more specific in a diagnostic assay; however, their sensitivity may be lower compared to other regions that are often present as multiple copies in the genome, such as ribosomal regions (Black et al., 2013).

It is important to point out that our molecular diagnostics toolkit was designed and validated to be used only with DNA extracted from pure cultures of *F. oxysporum* as templates. Thus, we strongly discourage the use of our assays beyond the parameters validated here. Further tests with the use of DNA extracted from infected plant material are needed and require extensive validation. The ubiquitous nature of *F. oxysporum* existing in plant tissue as saprophytic and/or endophytic strains with apparent diverse and fickle genotypes

renders the development of molecular based diagnostic assays direct from plant tissue, challenging. This would be the main reason why we would be hesitant to convert our conventional PCR assays into loop-mediated isothermal amplification (LAMP) assays, which are mainly used either in crude samples extracts or DNA extracted directly from tissues rather than cultures (Zhang et al., 2014). LAMP is a technique that is used for amplifying a specific segment of DNA under isothermal conditions through the strand-displacing *Bst* DNA polymerase (Zhang et al., 2014). This tool is commonly advocated as being sensitive, low cost and mobile; however, the combined price of the needed equipment, kits and reagents is considerable. In addition, some expertise is needed for the interpretation of the results in the field, suitable controls are also necessary, and results need to be confirmed in a centralized laboratory through an alternative effective diagnostic assay. Our results also suggest that there is not enough variability in the *SIX* gene sequences across *Foc* races to allow the design of four primers that recognize six distinct regions. For example, our assay for TR4 relies on the presence of two SNPs in the *SIX1* homolog "a," which is exclusive to TR4 strains and is part of the recognition site of the HpyAV enzyme.

The availability of specific, sensitive, and robust diagnostic assays to identify plant pathogens is vital for the early detection and further containment or eradication of plant diseases. Accurate identification of the race or VCG of *Foc* using a sensitive, robust, user-friendly, and accessible assay by laboratories in any part of the world can provide reliable diagnostics to growers. This would also assist local governments to take suitable control measures to prevent threats to food security or economic losses to the banana industry.

AUTHOR CONTRIBUTIONS

JH, EA, EC, and AD conceived the study. JH, AD, and LC designed the experiments. EA and EC provided the sequences for primer design. CO, VR-F, and LC carried out the experiments. LC and AD drafted the manuscript. All authors contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00547/full#supplementary-material

FIGURE S1 | Example of agarose gel showing the limit of detection of amplification products obtained with the primer set SIX13c_343 within a range of DNA template concentrations.

TABLE S1 | Limit of detection of primer sets SIX1a_266, SIX6b_210, SIX8b_206, SIX9_Foc, SIX10a_309, SIX13c_343, and the previously published primers Foc-TR4 (Dita et al., 2010), and EF (O'Donnell et al., 1998) within the DNA template concentration range of 10 ng·μL^{-1} to 0.1 fg·μL^{-1}.

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METHODS
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An Improved Phenotyping Protocol for Panama Disease in Banana

Fernando A. García-Bastidas[1], Alexander J. T. van der Veen[1], Giuliana Nakasato-Tagami[1], Harold J. G. Meijer[2], Rafael E. Arango-Isaza[3] and Gert H. J. Kema[2]**

[1] *Laboratory of Plant Breeding, Wageningen University and Research, Wageningen, Netherlands,* [2] *Laboratory of Phytopathology, Wageningen University and Research, Wageningen, Netherlands,* [3] *Escuela de Biociencias, Universidad Nacional de Colombia, Medellín, Colombia*

Fusarium oxysporum (*Fo*) belongs to a group of soil-borne hyphomycetes that are taxonomically collated in the *Fusarium oxysporum* Species Complex (FOSC). Hitherto, those infecting bananas were placed in the forma specialis *cubense* (*Foc*). Recently, however, these genetically different *Foc* lineages were recognized as new *Fusarium* spp. placed in the Fusarium of Banana Complex (FOBC). A member of this complex *F. odoratissimum* II-5 that uniquely comprises the so-called Tropical Race 4 (TR4), is a major problem sweeping through production zones of Cavendish banana in several regions of the world. Because of this, there is an urgent need for a phenotyping method that allows the screening for resistance to TR4 of large numbers of banana genotypes. Most Fusarium 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, depending on inoculum concentration. Pouring inoculum directly onto 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. Moreover, this method allows the inoculation of 250 plants per hour by one individual thereby facilitating the phenotyping of large mutant and breeding populations.

Keywords: Panama disease, TR4, *Fusarium oxysporum* ff. spp. *cubense*, mung bean, microconidia, phenotyping, spore production

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****Correspondence:***
Fernando A. García-Bastidas
fernando.garcia-bastidas@keygene.com
Gert H. J. Kema
gert.kema@wur.nl

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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, 1983; 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 names of strains infecting banana and causing Fusarium wilt, or Panama disease (*Musa* spp.). 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 (Ordonez et al., 2015b). Therefore, Maryani et al. (Maryani et al., 2019) 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). Race 1 strains 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 these 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* II-5, 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; Ordonez 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.

Therefore, efficient inoculum production is the first critical factor in optimizing phenotyping protocols. For *Fusarium* spp. several protocols were developed (Sun and Su, 1984; Adesemoye and Adedire, 2005; Amorim et al., 2009; Dita et al., 2011; García-Bastidas et al., 2014; Li et al., 2014; Ordonez 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., 2014; Ordonez et al., 2015a). However, these methods cannot be up scaled 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 medium or costly infrastructure. Moreover, the procedures are very labor intensive. Hence, these practical constraints have 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 hour^{-1} person^{-1} (Dita et al., 2010; Dita et al., 2011; Ordonez 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 suffices for screening up to 250 banana plants per hour per person. Moreover, its wide applicability was shown for other *Fusarium* spp. affecting different crops.

MATERIALS AND METHODS

Fusarium spp. and Growth Conditions

In total, eleven *Fusarium* spp. genotypes 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,

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

Fusarium spp.[1]	Code	Host	VCG[2]	Origin	Provider
FOSC clade 4	*Foc* R1	Banana	Unknown	Cruz das Almas, Bahia (Brazil)	M. Dita, Netherlands
F. tardichlamydosporum	*Foc* R2	Banana	0124	United States	K. O'Donnell, United States
F. odoratissimum II-5	TR4	Banana	01213	Indonesia	R. Ploetz, United States
F. phialophorum	*Foc* ST4	Banana	0120	Canary Islands (Spain)	J. Hernandez, Spain
F. oxysporum f. sp. *melonginae*	*Fom*	Eggplant	n.a.	Israel	Unpublished
F. oxysporum f. sp. *lycopersici*	*Fol*	Tomato	n.a.	Netherlands	Unpublished
F. oxysporum f. sp. *cepae*	*Foce*	Onion	n.a.	Australia	Unpublished
F. oxysporum f. sp. *gladiola*	*Fog*	Gladiola	n.a.	–	Unpublished
F. oxysporum f. sp. *albedinis*	*Foca*	Date palm	n.a.	Canary Islands (Spain)	Unpublished
FOSC Clade 3	*Fo*47	Biocontrol	n.a.	France	Lemanceau et al. (1991)
–	*Fo*618-12	Biocontrol	n.a.	Netherlands	Postma and Luttikholt (1996)

[1]*Names adapted based on latest classification on Maryani et al. (2019).* [2]*VCG, vegetative compatibility group; Fo, Fusarium oxysporum.*

United States) for 5 days in the dark and plugs were then taken from the edge of the colony to inoculate liquid media.

Plant Material

In vitro plants were obtained from various sources (**Supplementary Table 1**), transferred to individual 1L pots containing a standard soil from the WUR Radix, 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, 16 h light, and ∼85% relativity humidity). Plants were acclimatized under plastic for 2 weeks to maintain high humidity conditions and thereafter grown 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 *F. odoratissimum* II-5 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 (SMB20; Bai and Shaner, 1996), or 20 gr (SMF20), 5 gr (SMF5), 2 gr (SMF2), 1 gr (SMF1), or 0.5 gr (SMF05) 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 8 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, United States), 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. After evaluations of the different media, SMF2 was used for spore production of all other *Fusarium* spp. infecting banana (**Table 1**) with quantification of spore concentration 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 the infected substrate in relation 1:12, then flasks with the mixture were incubated for an additional 6 weeks. Infected maize kernels were produced by inoculating 100 g of sterilized grains in Erlenmeyer flasks with five TR4 plugs derived from a fresh growing colony on PDA plate. Flasks were incubated at 25°C in the dark for 10 days.

Inoculation and Plant Maintenance

Five phenotyping methods were compared: (i) dipping banana plants with trimmed roots in spore suspension and transplanting them into either non-sterilized soil (*dipping soil*) or (ii) sterilized sand (*dipping sand*), (iii) uprooting banana plants and transplanting them into soil infested with chlamydospores (*chlamydospore method*), (iv) adding TR4 colonized maize kernels to soil of potted banana plants (*kernel method*), and (v) pouring inoculum directly on the soil of potted banana plants (*pouring method*). For the dipping methods with transplanting either to 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 min in inoculum of various concentrations (10^3, 10^4, 10^5, 10^6 spore ml^{-1}). For the chlamydospore 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 spore ml^{-1}) were directly added onto the pots. For all experiments, we selected five plants with 6/7 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 yellowing/wilting leaves – were scored following a 1–4 class scale in which $1 = 0 > x \leq 25\%$, $2 = 25 < x \leq 50\%$, $3 = 50 < x \leq 75\%$, and $4 = 75 < x \leq 100\%$. In addition, morphological changes of leaves and pseudostem splitting were recorded. For internal evaluation, plants were uprooted, cleaned and cut longitudinally at the rhizome (corm) of each plant. Disease severity was visually assessed following a 1–6 scale where 1 = No discoloration in the corm, 2 = isolated points, $x < 5\%$, $3 = 5 < x \leq 30\%$, $4 = 30 < x \leq 50\%$, $5 = 50 < x \leq 90\%$, and 6 = plant totally decayed $x < 90\%$. To guarantee an accurate quantification of the affected and discolored tissues we conducted image analyses (ImageJ 1.49)[1] 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 48 h (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 min). Hundred mg tissue was processed for DNA extraction using a Kingfisher robot (Thermo Labsystems, Finland) and the AGOWA Sbeadex® Maxi plant DNA isolation kit (LGC Genomics, Germany). Samples were mixed with 600 µl lysis buffer, bead beaded (Bertin technologies, Ampere montigny-le-Bretonneux, France) for 40 s and then incubated at 65°C for 15 min, followed by centrifugation for 20 min at maximum speed in an Eppendorf centrifuge (Eppendorf 5415D, 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 1× PicoGreen and 99 µl TE after which the samples were measured with Tecan Infinite M200Pro using Icontrol 107 software (Morrisville, NC, United States). Verifications 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, Netherlands) in a real time PCR machine (ABI 7500, Thermo Fisher Scientific, United States). 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 assay experiment was set up in 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 analyzed 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 *F. odoratissimum* II-5 (TR4) commenced within 24 h after inoculation on the media. All SM, except SMF20 (20 g), showed a significant increase of conidia compared to the control SMB20 (original protocol) (**Figure 1A**). After 24 h, a steep increase in conidia production was observed in SMF5 (5gr), SMF2 (2gr), and SMF1 (1gr), while a moderate increase was observed in SMF05 (0,5 gr). In contrast, no conidia were observed for SMB20 and SMF20 at that time. During final quantification at 8 dai, the SMB20 culture contained 4.5×10^6 conidia ml^{-1} while the highest concentrations were obtained in SMF5 and SMF2, with over 1.1×10^8 conidia ml^{-1} (**Figure 1A**). Albeit that SMF5 and SMF2 excelled in spore production at 8 dai, the former was eventually chosen as the final filtering and dilution of conidia was much easier due to the higher content of mycelia in SMF5.

The New Mung Bean Protocol Is Applicable for Other *Fusarium* spp.

To test whether the SMF2 protocol is applicable for other *Fusarium* spp. and two *Fo* biocontrol strains (**Table 1**) we compared spore production with SMB20 (**Figure 1B**). All strains produced significantly more conidia in SMF2 than in SMB20 media. Despite the fact that *Foce* was the least productive (1.6×10^7 spores ml^{-1}), SMF2 produced 10-fold the quantity over SMB20. The highest concentration of conidia was obtained for *Fom, Fo*47 and ST4 reaching $> 6.0 \times 10^7$ spores ml^{-1} (**Figure 1B**), but TR4 produced more than 5.0×10^7 spores ml^{-1}

[1] http://rsb.info.nih.gov/ij/

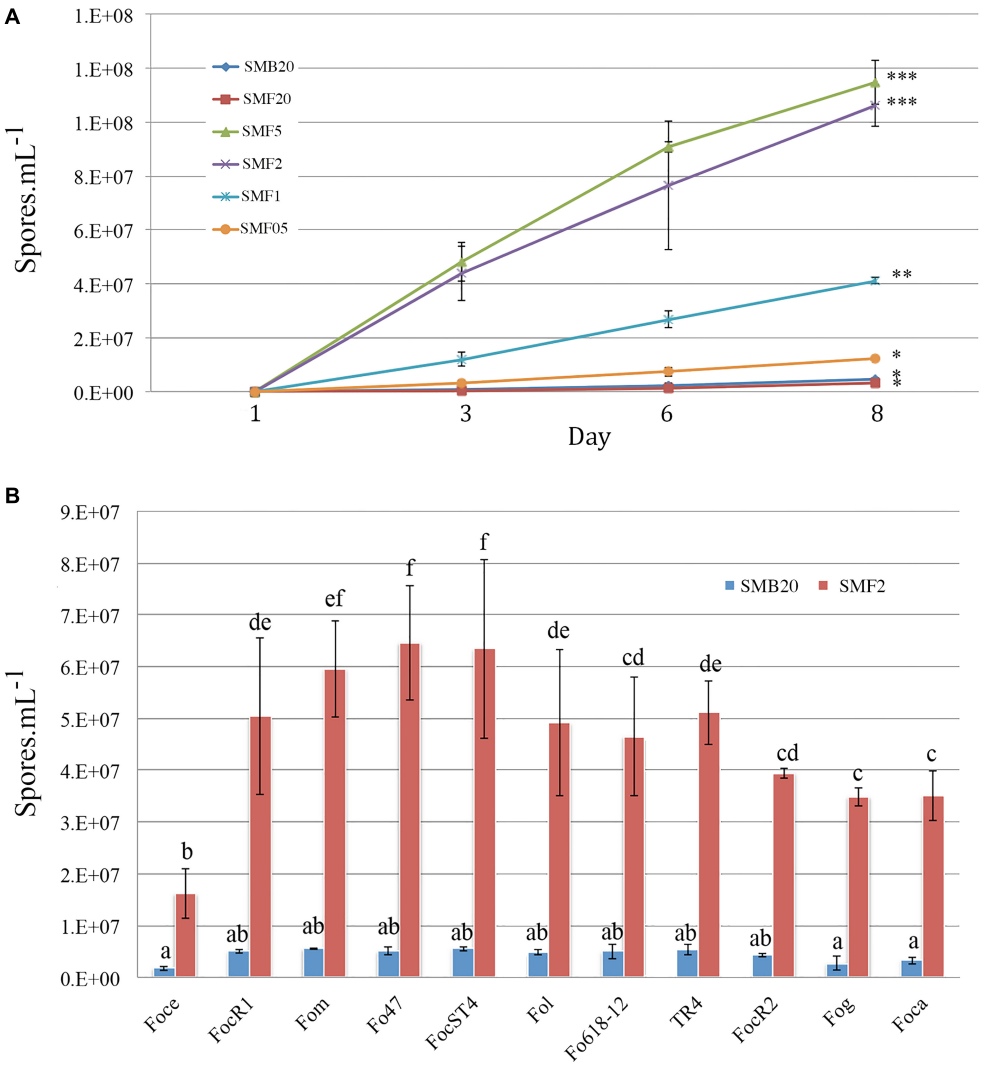

FIGURE 1 | Conidia production of *Fusarium* spp. in sporulation media (SM). **(A)** Spore production over time for Tropical Race 4 (*F. odoratissimum* II-5) in different media. **(B)** Spore production in SMB20 and SMF2 for 11 *Fusarium oxysporum* ff. spp. Spores were quantified after 6 days ($n = 3$) and the experiment was repeated at least twice ($P > 0.05$, treatments with the same letter/symbol are not significantly different).

(**Figure 1B**), which was sufficient to inoculate 250 plants per hour by one person (see infographic **Figure 2**).

Comparison of Inoculation Assays and Inoculum Concentrations

To determine the most optimal inoculation method we compared five commonly used protocols (**Figure 3**). Initial chlorosis/wilting was observed for the dipping and chlamydospores inoculation methods, as well as their un-inoculated controls but these recovered after 2 weeks suggesting that these effects resulted from root trimming. Depending on the method used, the latency period was between 2 and 4 weeks after inoculation (wai), but was shortest in plants 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 (**Supplementary Table 2**). 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 (sand) and pouring methods, as well as after using the maize kernels assay. The dipping (sand) and pouring methods with 1×10^4 spores ml^{-1} consistently scored 3, whereas all other treatments resulted in score 4 for foliage discoloration.

All inoculation assays resulted in internal corm discoloration, but affected areas differed significantly between the applied methods and inoculum concentrations (**Figures 4**, **5** and

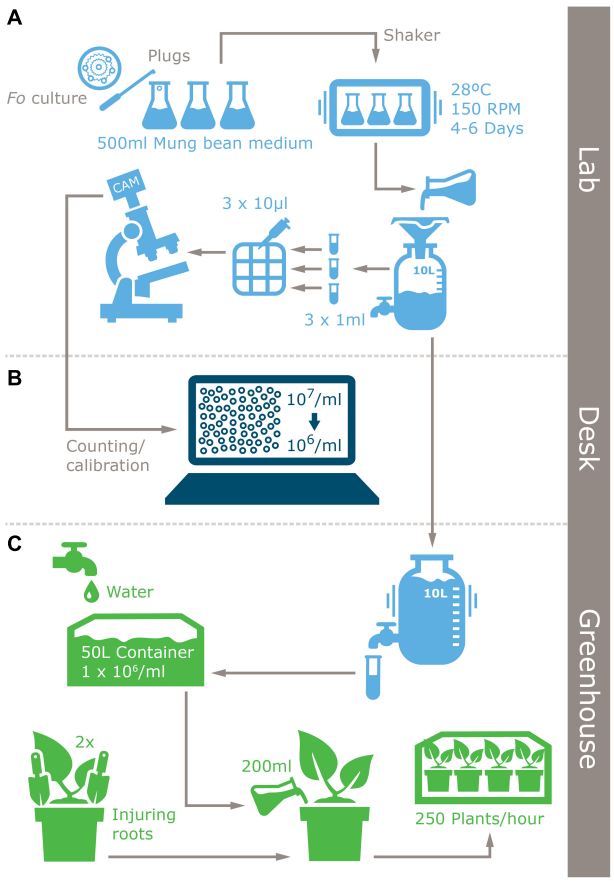

FIGURE 2 | Infographic of the improved inoculum production protocol for *Fusarium* spp. and plant inoculation method. **(A)** Laboratory inoculum production, **(B)** inoculum quantification, and **(C)** inoculation.

Supplementary Table 2). 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 (I-III) based on their severity (**Figure 4** and **Supplementary Table 2**): 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 and 50; pouring (10^5 spores ml^{-1}) and chlamydospore (2,5g) treatments exhibited moderate severities with DIs between 50 and 80; all remaining treatments developed high severities with DIs between 80 and 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 4C**). This enabled the ranking of these accessions by their DIs from immune to highly susceptible (**Figure 4**). Across all experiments, "Grand Naine" plants inoculated with race 1 (**Figure 5F**) as well as "Pahang" and "cv Rose" inoculated with TR4 did not develop any external and internal symptoms, independent of the used inoculum concentrations (**Figures 5K,L**). All water controls remained healthy (**Figures 5G–J**).

Additional Corm Analyses

Corm dry-weights correlated with DIs, except for the colonized maize kernels assay (**Supplementary Table 1**). 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. 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 (**Supplementary Table 2**), but the correlation with DI was rather low ($R^2 = 0.697$, **Figure 4D**).

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 and first published in 1978 (Sun, 1978), the occurrence in Jordan and other countries outside South East Asia drew eventually global attention (Butler, 2013; García-Bastidas et al., 2014; Ordonez 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 2 grams of Mung bean seeds to produce between 1-7.5 × 10^7 spores ml^{-1} in 6 days, irrespective of the *Fusarium* species and more than 1 × 10^8 at day 8 with TR4. Comparable results were also obtained for other *Fo* species, including the biocontrol strains *Fo*47 and *Fo*618-12. The production of 5 × 10^4 to 3 × 10^6 spores ml^{-1} was reported for *Rhyzopus oligosporus* using 100 gr L^{-1} of Mung bean sprouts (Nout et al., 1987) 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

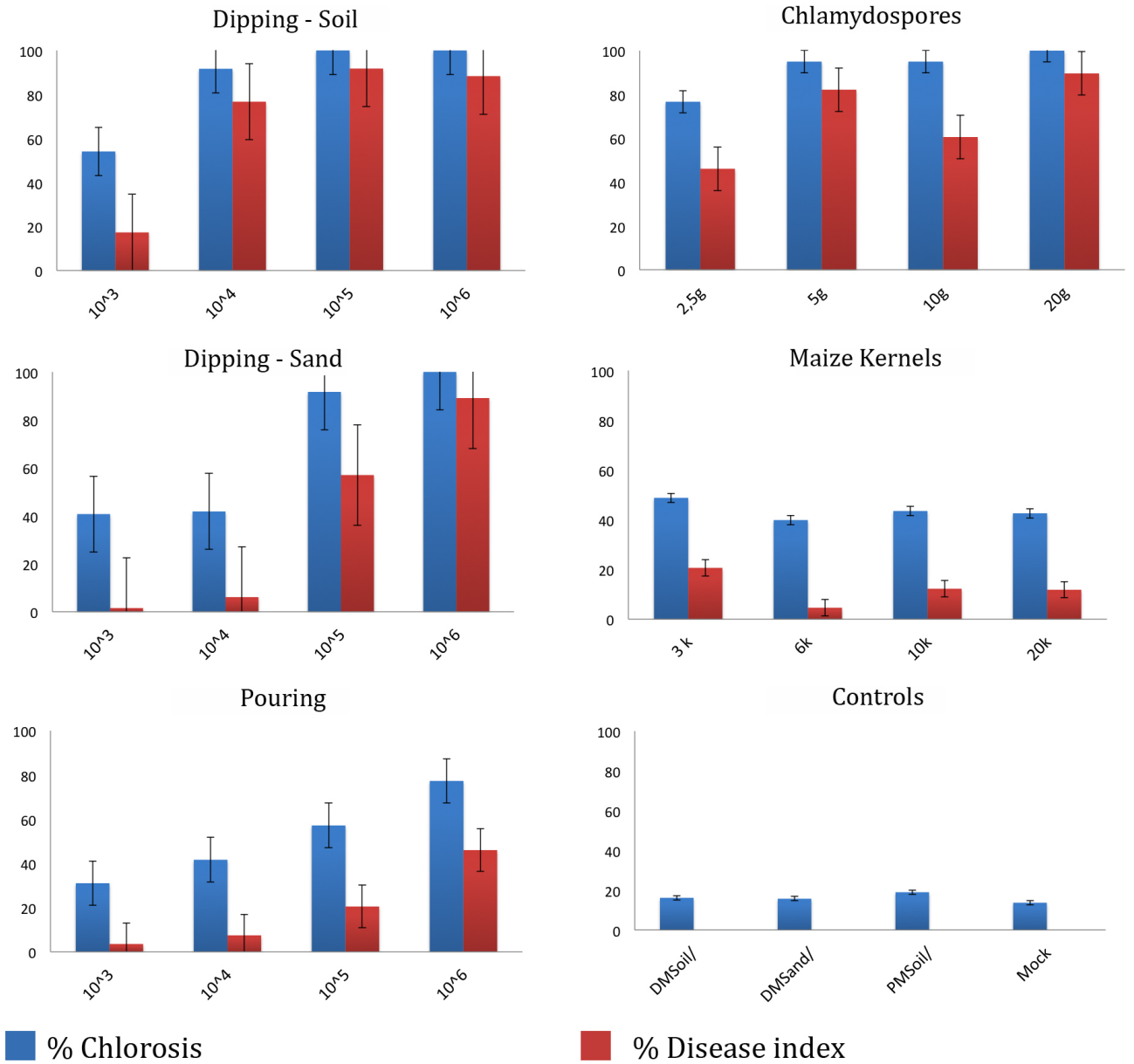

FIGURE 3 | Dose-response disease indexes of "Grand Naine" plants at final score (5–10 weeks after inoculation) with *Fusarium odoratissimum* II-5 (Tropical Race 4) using five inoculation methods. For each treatment the average result for four inoculum doses are expressed as percentage of foliar chlorosis and as the disease index at the time of scoring.

7 dai, 4.6×10^5 at 21 dai and only 6.2×10^5 spores ml^{-1} at 4 wai. The latter was confirmed as the production of SMB20 resulted in 8.3×10^5 spores ml^{-1}. Thus, our improved protocol allowed us to produce on average 100 times 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 6 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

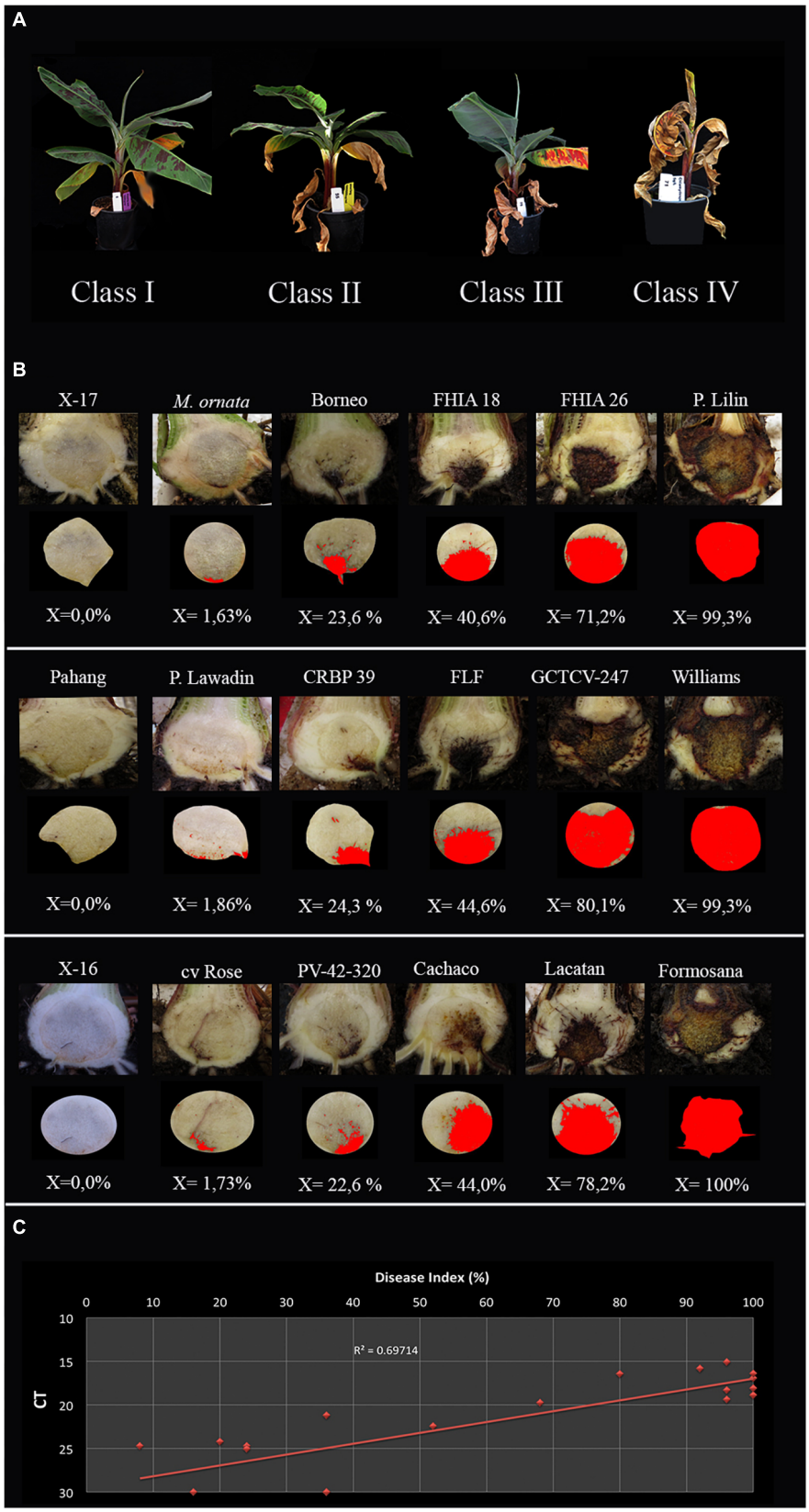

FIGURE 4 | Continued

FIGURE 4 | Panama disease progress incited by Tropical Race 4 (*Fusarium odoratissimum* II-5). **(A)** Four class rating scale of leaf chlorosis: I = ($0 > x \leq 25\%$), II = ($25 < x \leq 50\%$), III = ($50 < x \leq 75\%$), and IV ($75 < x \leq 100\%$); **(B)** Internal severity levels of 18 banana accessions (**Supplementary Table 2**) and the accompanying percentages of affected tissue as calculated by ImageJ; **(C)** The correlation between qPCR quantification and disease index per corm with trendline and *R* value.

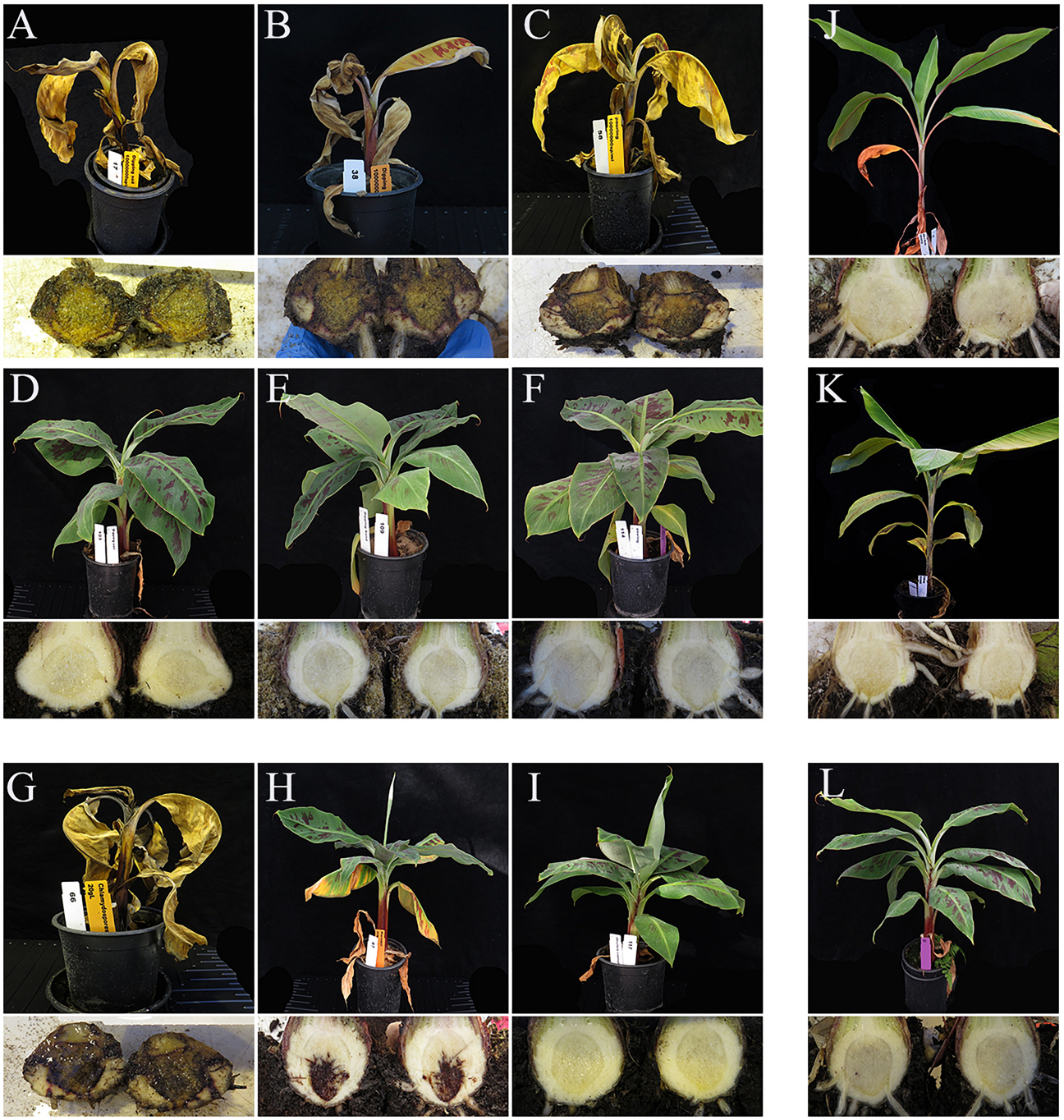

FIGURE 5 | External and internal symptoms of 'Grand Naine' at six weeks after inoculation with *Fusarium odoratissimum* II-5 (Tropical Race 4). The panels **(A–F)** show the results of different inoculation methods (top panels); **(A)** DM soil, **(B)** DM Sand, **(C)** pouring method and their respective controls in panels **(D–F)**. Panels **J,K** are the associated negative controls using resistant accessions cv. Rose **(J)** and 'Pahang' **(K)**. Plants inoculated with chlamydospores **(G)** or Maize kernels **(H)**, also developed similar symptoms but the latency period differs from that of conidial inoculations. Plants shown were challenged with the highest inoculum doses described for each method. The negative controls were 'Grand Naine' inoculated with race 1 using the dipping method **(I)** and the mock **(L)**.

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., 2010; Dita et al., 2011; García-Bastidas et al., 2014; Ordonez 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., 2014). 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). 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 (Smith et al., 2008; Amorim et al., 2009), 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 80–100). 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 $\times$ 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 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 min per plant. Subsequently, plants must be immersed 30 min 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 3 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 9 months before disease expression, due to the mostly unknown distribution of inoculum in the soil (Sun and Su, 1984). Current phenotyping assays often rely on visual (external) scoring, which is straightforward but has severe limitations. Here, we complement 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.

Taken together we described an improved method for spore production for *Fusarium* spp. and compared five banana inoculation methods concluding that – contrary to all other methods – the pouring method enables the inoculation of a large number of plants, can be done by one person and yields a final disease index that is proportionate with the applied inoculum concentration.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

FG-B and GK contributed to the conception and design of the study. FG-B performed the experiments, and organized and analyzed the database. AV contributed with the evaluation of inoculation methods, and organized and analyzed the data. GN-T contributed with the conidia production experiments, and organized and analyzed the data. FG-B, GK, RA-I, and HM wrote

the paper. All authors contributed to the manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01006/full#supplementary-material

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Coordination as Management Response to the Spread of a Global Plant Disease: A Case Study in a Major Philippine Banana Production Area

Marilou O. Montiflor[1,2], Sietze Vellema[1] and Larry N. Digal[2]*

[1] Knowledge, Technology and Innovation Group, Wageningen University & Research, Wagenignen, Netherlands [2] School of Management, University of the Philippines Mindanao, Davao, Philippines

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****Correspondence:***
Marilou Montiflor
momontiflor@up.edu.ph

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An integrative management approach to the spread and emergence of global plant diseases, such as the soil-borne fungus Fusarium oxysporum f. sp. cubense tropical race 4 (Foc TR4), entails a combination of technical measures and the responsiveness and awareness of area-specific constellations supporting conditions conducive to interactions and coordination among organizations and actors with different resources and diverse interests. Responses to banana diseases are mostly studied through technical and epidemiological lenses and reflect a bias to the export industry. Some authors, however, indicate that cross-sector collaboration is crucial in responding to a disease outbreak. Earlier studies on the outbreak of diseases and natural disasters suggest that shared cognition and effective partnerships increased the success rate of response. Hence, it is important not to focus exclusively on the impacts of a pathogen at farm or field level and to shift attention to how tasks and knowledge are coordinated and shared. This paper aims to detect whether and how the emergence of Foc TR4 is a driver of coordination. The case study focuses on the interactions between a variety of banana producers and among a range of public and private actors in southern Philippines. The analysis identifies distinct forms of coordination emerging in the context of three organizational fields responding to Foc TR4, which underlie shared capacity to handle and understand the spread of a global plant disease. The research is based on qualitative key informant interviews and document analysis and on observations of instructive events in 2014–2017. Analysis of the composition and actions developed in three organizational fields leads to distinguishing three theory-driven forms of coordination: rule-based, cognition-based, and skill-based. The combination of these three forms constitutes the possibility of a collaborative community, which conditions the implementation of an integrative management approach to mitigate Foc TR4.

Keywords: fusarium wilt, Southeast Asia, cross-sector partnerships, plant disease management, Foc TR4

INTRODUCTION

The global emergence and spread of Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), threaten both local food securities related to the cultivation and availability of a wide range of banana varieties and the mainly large-scale production of bananas for international trade (Ordoñez et al., 2015; Ploetz et al., 2015; Mostert et al., 2017). An earlier spread of the Foc devastated commercial production of the "Gros Michel" variety in Central and Latin America in the 20th century. The Cavendish sub-group was resistant to this earlier strain of the Foc TR4. However, the current strain of the pathogen that emerged in Southeast Asia in the 1990s also endangers the Cavendish subgroup, and thus the commercial interests of major banana companies (Ploetz, 2015). Foc TR4 infects a range of banana varieties grown under both tropical and subtropical conditions (Buddenhagen, 2009). The pathogen has spread in different banana production systems in Asia, Africa, and the Middle East (Ploetz, 1994; Jeger et al., 1996; Magnaye, 2001; Singh, 2008; Buddenhagen, 2009; García-Bastidas et al., 2013; Ordoñez et al., 2015; Ploetz, 2015). The TR4 is a soil-borne pathogen, microscopic, lacks visible symptoms on suckers and fruits (Brunschot, 2006), displays observable symptoms in the plant during its advanced stage (Ploetz, 1994; Buddenhagen, 2009), and cannot be controlled by a fungicide (Dita et al., 2010). Methods of contamination are not exhaustive but some observations indicated that it may be transferred through infected suckers, soil, surface water, and farm implements (Ploetz, 2015). Zheng et al. (2018) attribute this to the biological complexity of the soil-borne disease, and Dita et al. (2018) emphasize the complex epidemiological dynamics of the fungus that can survive in the soil for a long time (Li et al., 2013). Quarantine and good hygiene practices are means of control currently implemented in affected areas, which is according to Warman and Aitken (2018) especially important in the context of the absence of commercially viable cultivars resistant to Foc TR4 and lack of effective fungicide. Numerous efforts by private and public actors in the commercial banana export sector to contain the spread of the global disease had only a limited effect (Zheng et al., 2018). The responses of other, small-scale banana growers are largely unknown: they can leave infected plants in the field or convert their farms to other crops, and consequently, the pathogen remains in the soil. Hence, from a technical perspective, management of the disease has proven to be difficult, and no cures are readily available.

This limited capacity to control the disease and to contain the spread of Foc TR4 importantly conditions the implementation of an integrative disease management approach (Dita et al., 2018). Research and management approaches addressing the spread of TR4 have adopted a strong focus on plant and field levels. However, some authors in the plant sciences point out that there is a need to go beyond merely technical measures at field level. Ordoñez et al. (2015) and Pocasangre et al. (2011) mention action plans such as awareness campaigns, quarantine measures, the mobilization of joint investments in research, and knowledge exchange in workshops. Accordingly, the feasibility and effectiveness of an integrative management approach also depend on the responsiveness and awareness of area-specific constellations of diverse actors, such as small-scale and large banana producers, industry associations, local government bodies and plant protection organizations, researchers, and extension workers. Therefore, our research complements technical management responses with strategies that emphasize procedures for decision-making and sharing responsibilities and for improving preparedness, horizon scanning, and contingency plans (Ward, 2016). Ward (2016) argues that it is important not to focus exclusively on the impacts of a pathogen at farm or field level and to shift attention to how tasks and knowledge are coordinated and shared at area levels. Consequently, plant disease management strategies are affected by contextual epidemiological dynamics and imply coordinated efforts between different actors embedded in spatially bounded areas with diverse production systems.

The spread of Foc TR4 into areas with multiple banana production systems, e.g., including backyard farming, and production for local markets and for export complicates finding a coordinated response to its spread as well as searching possible control measures. Moreover, in the case of Foc TR4, uncertainties regarding containment of the spread or management of the disease hamper coordinated and integrative plant disease management. The epidemiology and management of other plant diseases, in particular the Banana Xanthomonas Wilt (BXW) that has spread throughout most of East Africa, are equally challenging, and research confirms the importance of understanding emerging forms of coordination for area-based responses to plant diseases (Shimwela et al., 2016; Shimwela et al., 2017). Similarly, the analysis by McAllister et al. (2015) of the Australian response to the outbreak of the fungal pathogen black sigatoka (*Mycosphaerella fijiensis*) demonstrates that success of response was credited to effective partnerships, which mobilized local action. This is in line with the findings of Macleod et al. (2016), who report on consultations among plant protection authorities and identified cooperation among stakeholders and along supply chains as a key mechanism to strengthen weak links, develop spatially explicit integrated decision-making, and reduce plant health risks, especially those moved in trade and in a highly connected world. This is particularly relevant because Foc TR4 ripples through the system and crosses spatial, organizational, and administrative boundaries, which complicates coordinated endeavors and the emergence of novel trajectories supporting resilience to global plant diseases in production areas or communities.

Our research builds on the work of De Vos et al. (2016) and aims to detect whether and how the emergence of Foc TR4 is a driver of new forms of coordination. The Foc TR4 not only connects the pathogen and their hosts (bananas), but also affects other components of the system and their mutual relationships. This mix of complexities requires a typical multidisciplinary approach that this paper endeavors to elaborate. Understanding the coupling of human actions and the biology and epidemiology of pathogens (Berbés-Blázquez et al., 2014) benefits from adopting a landscape or area perspective (Meentemeyer et al., 2012). Accordingly, we adopt an area lens to assess whether the recurrence of Foc TR4 reshapes the interactions between public and

private stakeholders and may eventually generate conditions conducive for coordination and collaboration.

This paper presents a qualitative case study, with well-defined spatial and temporal boundaries, of emerging forms of coordination in distinct organizational fields in a major banana production area in southern Philippines severely affected by TR4 (Bureau of Plant Industry, 2012; Molina et al., 2016). The paper qualitatively maps responses to and interactions around pathogen incursions shaped in both formal organizational structures and informal collaborations (McAllister et al., 2015). The case study concentrates on the period in which the manifestation and dramatic effects of TR4 were widely discussed by various stakeholders in the industry: 2014–2017. It traces emerging forms of coordination in different organizational fields observed in the banana sector in the southern part of Mindanao, the most southern island in the Philippines. For this paper, we define coordination as the self-organization and mobilization of responses aligned with those of other actors (Comfort, 2007). The notion of organizational field origins in the work of DiMaggio and Powell (1983) and refers to sets of organizations that, in the aggregate, constitute a recognized area of institutional life that gives direction to problem-solving strategies and sense-making of unanticipated risks and uncertainties. The unknowns related to Foc TR4 as well as the limitations of past experiences to respond to the disease are addressed in the evolving interactions in an organizational field. For this study, we drew empirical boundaries around inductively identified organizational fields anchored in (i) industry alliances and formalized partnerships, (ii) *ad hoc* organizational fields facilitating knowledge exchange and shaping public discourse, and (iii) less salient professional and cross-sector collaborations in which experts share coping strategies for navigating risks and uncertainties related to Foc TR4. We describe the actors and interactions in each organizational field to detect specific emerging forms of coordinated efforts responding to the global pant disease threatening a range of banana producers assembled in a single geographical area.

The paper continues as follows. The next section presents the research area and describes the qualitative and case study methods used for data collection and analysis. The section *Results* identifies and describes three organizational fields that were selected after preliminary research as empirical domains for tracing emerging processes of coordination. In each field, different stakeholders co-create responses to the spread and emergence of Foc TR4. The descriptions of these fields identify the involved actor groups and their connections, the sequential actions and events, and the form of coordination visible. The discussion section 4 matches the empirically identified forms of coordination with theoretically relevant building blocks. To qualify each emerging forms of coordination, we made the connection to theoretical accounts from management, organization, and public management science. The conclusion it explores whether these forms of coordination may configure a collaborative community, which potentially makes an area capable of developing an integrative management approach to Foc TR4 specifically, and other plant diseases more generally.

MATERIALS AND METHODS

Research Area

The case study focused on interactions between a variety of banana producers and among a range of public and private actors in the leading production area of export banana in the Philippines: southern Mindanao. Since the 1960s, the island of Mindanao in southern Philippines emerged as the country's main producer of bananas, contributing to 82.8% of the country's total volume in 2015. Mindanao produced almost all of the country's Cavendish-AAA (*Musa acuminata*), 84.8% of the Lakatan-AA (*Musa acuminata*), and 55.6% of the Saba-ABB (*Musa acuminata* x *Musa balbisiana*) (Philippine Statistics Authority, 2017). The Davao Region produced half of the Cavendish variety in 2015 (Philippine Statistics Authority, 2017).

Plant diseases have been a major problem for both the export industry and producers serving domestic markets. Besides the recurrence of Foc TR4, the following diseases were reported: Moko, black sigatoka, (Stover, 1986), nematodes (Davide, 1988; Herradura et al., 2012; Aggangan et al., 2013), and bunchy top (Kohnen et al., 2013; Molina et al., 2009). The Foc TR4 had been reported in Mindanao since 2009 and affected a range of banana production systems located in the area. The production systems were connected as adjacent farms or *via* the movement of people, banana fruits or leaves, vehicles, and farm tools and machinery (Ploetz, 2006). In 2015, Foc TR4 affected an area of around 15,500 hectares of banana production in Davao Region, which accounted for 32% of the total Cavendish production area (**Table 1**).

This multi-actor landscape made the area interesting for a study of coordination: a variety of stakeholders tried to find

TABLE 1 | Estimated areas in Davao Region affected by TR4 in 2015 (in hectares).

	Banana (all varieties)	Cavendish	Area affected with TR4	Percentage of Cavendish area affected with TR4
Philippines	443,369.91	85,808.90	Not available	Not available
Davao Region	88,274.80	48,050.00	15,507.53	32.27
Davao del Norte	036,368.00	28,972.00	13,743.00	47.44
Davao del Sur	15,413.00	3,642.00	436.00	11.97
Davao Oriental	10,528.80	156.00	36.00	23.08
Compostela Valley	19,131.00	11,934.00	1,082.78	9.07
Davao City	6,834.00	3,346.00	209.75	6.27

Sources: Department of Agriculture; Philippine Statistics Authority.

ways to respond to the spread of Foc TR4 (Pocasangre et al., 2011) and developed distinct strategies to control plant diseases (de la Cruz and Jansen, 2018). Disease management in banana plantations has been one of the controversial issues, specifically concentrating on the unintended effects of aerial spraying on the health of workers and communities (Panganiban et al., 2004). Large banana plantations have their own research departments while smallholder farmers receive information from government line agencies through the local government. Aside from farmers and corporations, other actors also influenced and co-created trajectories for managing plant diseases (de los Reyes and Pelupessy, 2009; Dalayon, 2013). These included commercial input and service providers and strategic alliances of academe, government, and financial institutions. In addition, the presence of major city in the banana production area connected consumers directly to disease management practices in banana farms, which paved the way for strict regulation of pesticide use and promoted organic production (Davao City ordinance No. 0384-10, 2010).

In addition to distinct perspectives on disease management, the area featured a diverse landscape of banana producers with different landholdings and sometimes opposed interests and perspectives. (De Leon and Escobido, 2004; Borras and Franco, 2005; Digal, 2007; Lockie et al., 2015). The export banana production on large and medium plantations expanded since the 1960s. The industry became influential toward policy and developed infrastructure for international trade, such as roads and ports. Large plantations were either family-owned or managed by multi-national corporations. These corporations were mostly members of the Pilipino Banana Growers and Exporters Association (PBGEA). The export-oriented industry produced bananas on managed plantations, used contractual arrangements with cooperatives and individual growers, or sourced directly from smallholder farmers (Digal, 2007; de la Cruz and Jansen, 2018). This was partly a response of the industry to land reform program implemented by the Philippine government. Coordination and collaboration between the export industry and agricultural producers supplying other markets were limited.

Case Study of Emerging Forms of Coordination

For investigating the possible emergence of coordination, the study adopts an area-perspective approach. The case study method was appropriate for documenting specific events and tracing unfolding coordination processes situated in a spatially bounded landscape affected by the spread of a global plant disease, Foc TR4. The case study (Yin, 1994) advances a contextual understanding of the degree to which actors with different interests, worldviews, and management styles agreed to coordinate their responses to Foc TR4. The case study involved identifying organizational fields in which distinct groups of actors interact and exchange information and knowledge. In addition, it entailed using process tracing as a method to document procedures that plausibly generate different forms of coordination in real-life situations marked by multiple interaction effects, and where it was difficult to explain coordination as the outcome of a limited number of independent variables (George and Bennett, 2005). The case study revealed temporarily and spatially unique coordination processes, which, as a first step, were inductively identified and subsequently elaborated by matching empirical findings with theory (Flyvbjerg, 2006). Process tracing generated a range of observations and identified the sequences of events, which exposed processes leading to the outcome of our interest: coordination.

Data Collection

Data collection took place in the years 2014–2017 and involved (i) the study of formalized collaboration agreements, (ii) the observation of *ad hoc* interactions during public events, (iii) the documentation of career histories and professional affiliations of key players, and (iv) document analysis of information shared in media and trainings.

The first step was to collect data on formalized arrangements assembling key private sector actors associated with the export banana industry, in particular the Pilipino Banana Growers and Exporters Association (PBGEA, representing larger corporations) and Mindanao Banana Farmers & Exporters Association (MBFEA; a platform assembling small and medium Cavendish farmers and exporters). This was based on one structured interview, with a representative of the association, and semi-structured and unstructured interviews with members during public events.

Second step was to collect data during public events, which were considered to be *ad hoc* and temporary organizational fields bringing together multiple stakeholders from private and public sectors, non-governmental organizations (NGOs) working with smallholder farmers, and representatives of organizations and cooperatives formed by agrarian reform beneficiaries. From 2014 to 2016, there were four banana events observed which focused on Foc TR4 (**Table 2**). These events provided venues for the examinations of spontaneous (unrehearsed) interactions among the actors. Participation in these events allowed observation of the ways in which stakeholders discussed coping strategies and a menu of solutions to address the spread of Foc TR4. Thirty six unstructured interviews consisted of conversations with industry actors during field visits, workshops, symposia, and conferences. Unstructured interviews with randomly chosen industry actors lasted between 15 and 30 min. Their responses became entries to conversational journals, which contained notes and narratives to provide context

The fourth step consisted of interviews with a purposefully selected sample of key actors from the private and public sector active in 21 in-depth key-informant interviews provided insights into their educational and career histories, their linkages and interactions with professionals in public and private sectors, and their approaches to the spread and management of Foc TR4. Key informant and unstructured interviews were recorded and transcribed with consent from the respondents. Answers of those who refused to be recorded were noted and encoded. The languages used in the interviews were English, Cebuano, Filipino, and Ilonggo/Hiligaynon. The quotes were translated in English in this paper. To protect the anonymity of the research participants, their names were withheld, identified only by their sector, location, and date of interview.

The final step involved reviewing 128 media reports and training material, which served as a source for documenting how

TABLE 2 | Summary of methods of data collection.

Methods	#	Source	Focus
Key informant interviews	21	-3 university employees/researchers -10 local/national government employees -3 NGO employees -2 private sector representatives -3 farmers	-Involvement with the banana industry -Activities related to banana and Foc TR4 -Perceptions/opinions about banana-related issues
Ad hoc unstructured interviews	36	-14 local/national government employees -15 farmers -5 private sector representatives -1 NGO employee -1 university employee	-Familiarity with the Foc TR4 problem -Interactions with other industry players -Perceptions/opinions about banana-related issues
Observations	4	-International Stakeholder Workshop (February 2014, Davao City, Philippines) -International Banana Symposium (November 2014, Davao City; estimated 568 participants) -International Banana Congress (April 2016, Miami, Florida, United States of America; estimated 600 participants) -8th International Conference on Agribusiness Economics and Management (October 2016, Davao City, Philippines; 218 participants)	-Interactions with other industry players -Studies about Foc TR4 -Solutions and mitigating actions -Perceptions/opinions about banana-related issues
Documentation of media coverage	128	-Online newspapers -Websites	-Solutions and mitigating actions -Identification of actors (specific names, sector, or organizations) -Observation of signals of coordination and blaming

Foc TR4 was discussed in the media and whose perspectives and views were represented.

Data Analysis

The interviews, observations, and document analysis resulted in indications of how actors with different interests approached coordination and tensions around TR4 spread. Data analysis involved three steps (**Table 3**): (i) qualitative mapping of the actors and their interactions, (ii) constructing time paths of events and activities, and (iii) identifying the variation of responses to Foc TR4. Firstly, we determined what types of actors were connected in the three identified organizational fields. The key informant and informal interviews were transcribed, then manually scanned to find themes, compared it with the responses of other respondents on the same topic, and analyzed the answers. There were observed recurring names mentioned during the interviews, media releases, and public events. Based on media reports, structured and unstructured key informant interviews, the career histories, and visualization of the professional networks, the recurring names of actors were listed, and mutual linkages were detected.

TABLE 3 | Qualitative data analysis.

Step	Focus of the analysis
Step 1. Examine who are the actors in the organizational field	To discover what groups or individuals are in the organizational field
Step 2. Observe and examine the activities of the organizational field	To map the actions developed in the organizational fields and establish the sequence of events
Step 3. Derive patterns of coordinated activities and typify the response	To distinguish emerging forms of coordination

It was observed that these individuals moved in the same circles because they belong to the same professional group, shared the same alumni network, worked together in previous projects or company, or served as keynote speakers in a conference. Secondly, chronological and thematic clustering of events was mentioned in the interviews, outlining the activities and discovering indications of coordination in these documented and observed processes. In addition, during this second step, the observed dialogues at public activities and in media releases were analyzed to determine the approaches of banana companies, government line agencies, and local government units (LGUs) in detecting and mitigating Foc TR4. Finally, a selection of instructive events were investigated in more detail to discover the specific types of responses to Foc TR4 emerging in each of the organizational fields and to establish who formulated these responses.

Next, the qualitative case study analysis followed the process proposed by Ruona (2005, p. 236): (1) sensing themes, (2) constant comparison, (3) recursiveness, (4) inductive and deductive thinking, and (5) theory-informed interpretation to generate meaning and typify emerging forms of coordination. The properties of the empirically detected forms of coordination were elaborated by making a connection to a selection of theoretical explanations of why and how different actors agreed to act in a coordinated manner. Building on Ruona, (2005) process of analyzing qualitative data, we identified three empirical manifestations of organizational fields: the industry-based and formalized partnerships, temporary and *ad hoc* multi-stakeholder settings, and professional associations and networks. The study first focused on formal industry associations with an established history of coordination and advocacy in the banana industry, mainly supported by resourceful and powerful firms

working in the export sector. The emergence of Foc TR4, however, induced a series of public events accommodating interactions between stakeholders that did not connect before and now used multi-stakeholder platforms to express their concerns and to agree or disagree on the diagnosis of the origins, implications, and possible management of Foc TR4. The documentation of career histories of key informants in the private and public sectors indicated less obvious interactions taking place, which seemed to be related to a distinct organizational field. These three organizational fields were used to structure the result section and to derive patterns of coordinated activities.

RESULTS

This section distinguishes three organizational fields in which multiple actors interact to come to grips with the unknowns and uncertainties related to Foc TR4 and to explore and evaluate open-ended solutions for an unprecedented plant disease problem. The three organizational fields are instructive for detecting emerging forms of coordination depicted in each section. The descriptive account of who interacts, how and in what ways, set the stage for matching observed processes and sequences of events with theoretical accounts of coordination in the next section.

Organizational Field 1: Industry-Based and Formalized Partnerships

Two prominent industry-based and formalized partnerships in the Philippine banana industry were the Pilipino Banana Growers and Exporters Association (PBGEA) and Mindanao Banana Farmers and Exporters Association (MBFEA) Inc. The associations coordinated activities among its corporate members. PBGEA was composed of around 31 large multinational and family-owned corporations (Tourism Promotions Board Philippines, 2017) while MBFEA was composed of around 23 small and medium banana growers, exporters, and federations of cooperatives (Mindanao Banana Farmers & Exporters Association Inc, 2017). PBGEA and MBFEA represented companies predominantly producing and trading Cavendish bananas for export. Both associations have Technical Committees, which focus on Research and Development and on regulatory aspects of managing diseases in the banana industry.

Prior to the emergence of Foc TR4, the associations' coordinated efforts existed for the management of other diseases such as Black Sigatoka which can be controlled, by fungicide treatment (while TR4 cannot). Additionally, the PBGEA and MBFEA successfully lobbied and advocated for issues beneficial for their corporate members, such as repealing restrictions on the limit of banana hectares, banning aerial spray, and installing stricter policies on agricultural venture agreements. A clear example of joint advocacy is how the PBGEA asserted the illegality of the Davao City Ordinance No. 0309-07: *Banning Aerial Spraying as an Agricultural Practice in All Agricultural Activities by All Agricultural Entities*. This resulted in litigations and dialogues with the private, government, and non-government organizations (NGOs). The local NGOs were supportive of the ordinance and managed a campaign to implement the ordinance and ban aerial spraying. PBGEA, on the other hand, appeared in legislative hearings to push their own agenda. Eventually, in 2016, the Supreme Court ruled Davao City Ordinance No. 0309-07 as unconstitutional (Perez, 2016), and banana companies in Davao City were allowed to engage in aerial spraying. Decisions concerning an "industry-sanctioned" solution needed the agreement of not only the technical officers but all members of PBGEA had to consent. Decision-making within the associations was bounded by rules and based on reaching consensus. When a collective approval was not possible, interested individual companies were approached for collaboration. Since the members were business competitors, some were more cautious when it comes to working with non-members on banana-related issues. One member of PBGEA explained that collaborative projects were not easily supported by PBGEA. This member said to be after the collective interest and look for what is good for the group, but other members may be less open to joint endeavors (Interview with Private Sector, Davao City, 04 November 2016). Any decision regarding collaborative efforts had to pass the bureaucratic procedures installed in the associations.

Foc TR4 has been on the agenda of the industry-based associations, but concerted action was not automatically the outcome of their deliberations. One member of PBGEA explained.

> *We nonchalantly talked about Foc TR4. In fact, as early as 2005, someone was already proposing some sort of a Task Force to be organized by the government to monitor the disease and the extent of the infection. That time, he was also proposing that we sort of come up with a war chest to be used to arrest or address this Panama disease. But considering that the industry was facing many problems that time, it did not push through.*
> *(Interview with private sector, Davao City, 18 November 2014)*

The technical committees of the associations played an influential role in the decision-making about how to respond to Foc TR4 and with what other actors to align. Members of the associations tried to implement their individual disease management strategies and exchanged experiences during meetings of the association. Existing interdependencies of large plantations and other farms supplying the banana companies also shaped interactions around disease management. For example, both managed/leased farms and those under contract growing utilized aerial spraying as a disease-control method. These managed farms, with an average of more than 300 hectares, tried to control Foc TR4 through quarantine measures, i.e., foot baths at every gate or entrance to the farm, which was also common practice in the larger plantations. Larger companies expressed concerns about adjacent farms, since these have limited technical and financial capacities to address outbreak of Foc TR4, and the pathogen might spread from these farms. Even more so, farms supplying spot markets were considered a threat: these are relatively dispersed compared to farmers under lease and contract-growing arrangements. For area-based management strategies, however, representatives of banana companies recognized that collaboration was crucial for controlling a large track of contiguous farms. This, however, contrasted with the

history of working with relatively isolated plantations. During observed meeting of PBGEA, this issue was raised, but no specific actions were prioritized to stimulate area-based coordination.

The industry-based partnership aligned with government line agencies, such as the Department of Agriculture (DA). Politically, the DA had close connections with the banana industry through a series of secretaries coming from the industry, but cross-sector collaborations in the production areas were rare. However, Foc TR4 induced more interactions, in particular around quarantine. The alignment of the industry-based associations and government agencies resulted in a Task Force *Fusarium* initiated by the DA. This task force was a response to the lobbying of the associated Cavendish growers. The Task Force (TF) was composed of government line agencies, banana companies, LGUs, PBGEA, MBFEA, and other stakeholders. One of its functions was to identify stakeholders to collaborate with disease management. The TF made plans for the training of LGUs on the recognition, containment, and handling of Foc TR 4. Furthermore, bureaus and divisions of the DA were mandated to work on specific issues, such as Foc TR4. The regional DA offices coordinated with LGUs, which in turn identified the target beneficiaries of government programs and projects. After the passage of the Local Government Code of 1991 (Republic Act 7160), the agricultural extension was devolved to the LGU's Provincial/Municipal/City Agriculturist's Office. The technical committees of the industry-based associations oversaw collaborative activities with external stakeholders in banana plantations, such as universities (Interview with university employee, Davao City, 22 October 2014). In addition, coordinated activities were initiated in villages where plantations were located. There were multi-partite monitoring teams (MMT) involving village leaders, non-government organizations, Fertilizer and Pesticide Authority of the Department of Agriculture, Department of Environment and Natural Resources-Environment Management Board representatives, and heads of banana companies operating in the area. The MMTs were organized to encourage stakeholder vigilance and monitoring of Foc TR4 in the villages concerned. It was also intended to monitor compliance of companies to Philippine environmental laws. Interviews and observations disclose that both the decision-making procedures with the industry-based associations and the alignment of the private sector and public sector suggest a form of coordination building both on bureaucratic procedures to agree on resolutions and joint actions and regulatory interventions meant to monitor Foc TR4 and explore conditions for installing quarantine measures.

Organizational Field 2: Temporary and *Ad Hoc* Multi-Stakeholder Settings

The Foc TR4 discourses became prominent in the Philippine media and was reported on *ad hoc* knowledge exchanges and interactions such as several banana congresses, workshops, and symposiums where multiple stakeholders discussed the implication of and possible solutions to Foc TR4 (**Table 4**). Some of these exchanges were public while others were by invitation only. We observed interactions in three multi-stakeholder settings in Davao City: International Workshop on Panama Disease in February 2014, International Banana Symposium in November 2014, and the 8th International Conference on Agribusiness Economics and Management (ICAEM) in October 2016. Aside from banana producers, other participants came from government agencies, LGUs, non-government organizations, funding agencies, academe, and input suppliers.

While the main purpose of these multi-stakeholder gatherings was to share information and make connections, the public events were also used to make sense of the risk and its effects on a variety of banana producers. Perhaps, more importantly, participants hoped to find solutions to the disease problems destroying their farms, which hampered investigating the many uncertainties and unknowns also mentioned during the presentations and discussions. Stakeholder discussed a variety of mitigating strategies in the *ad hoc* gatherings, such as quarantine measures including setting up of footbaths in designated farm entrance (for humans) and tire baths in roads leading to the plantation (for vehicles). The chemicals vary but many farms used formaldehyde. Other strategies focused on information dissemination, directed mainly to smallholder banana farmers. Although some companies implemented the quarantine measures and the LGU disseminated information, participants in the meetings also recognized that these strategies were short-lived due to budgetary and jurisdiction issues.

The divergent views on how to manage and mitigate Foc TR4 were also a prominent feature of the research inputs to multi-stakeholder meetings. In particular, the use of somaclones, which were claimed to survive Foc TR4 for a certain period, led to debate and controversies, although some major banana companies decided to try this as a solution. When scientists with different perspectives on short- and long-term solutions shared a stage during the 6th International Banana Conference in Miami, Florida, organized by Costa Rica's National Banana Corporation (CORBANA), and the Association for Research and Integrated Management of Banana and Plantain (ACORBAT), the rebuttals on methods, results, and scientific viability and importance of each other's contribution were highlighted. Similar tensions between research-based preferences for disease management strategies were observed during the multi-stakeholder meetings in Davao City.

Despite the differences in opinions, the interactions in multi-stakeholder for a created a common concern in Foc TR4 and even stimulated the implementation of joint projects. For example, a project funded by the Department of Science and Technology was about "S&T (Science and Technology) Management Approaches against *Fusarium (Fusarium oxysporum* f. sp. *cubense (Foc*) on Cavendish in Mindanao." It had a budget of ₱34.05 million, with the Department of Agriculture-Bureau of Plant Industry (BPI), University of Southeastern Philippines, and Southern Philippines Agri-Business and Marine and Aquatic School of Technology (SPAMAST) as partners. The program aimed to reduce disease infestation with a focus on adaptability trials of resistant varieties, biological control strategies, and assessment of the distribution of the Foc TR4 in Mindanao. It supported institutions through human resource development, laboratory facilities upgrading, establishment of molecular laboratory, green houses, and other needed facilities and equipment (Valencia, 2015). Another project, funded by the Department of Agriculture, had a budget of ₱102 million and set out to

TABLE 4 | Examples of *ad hoc* encounters and interactions.

Type of action	Description	Participants	Focus of actions
Research collaborations	Collaborative and interdisciplinary research between private and public sectors	Universities, Inter-government (i.e., Department of Agriculture, Department of Science and Technology), plantations, individual growers, smallholder farmers, research institutes, local government units	Coming up with technical solutions, new varieties, quarantine measures, and experiments
International Banana Symposium/ congress	Sharing of banana related issues, ranging from pests, plant, people, and the environment	For example, in November 2014: 25 International and local speakers with 568 delegates	Public debate of veracity of the problem, which solutions to use and who is to blame
Workshops and training	Usually among partners, long-time or new collaborators	Selected people/partners, depending on the type of workshop	Knowledge sharing, information dissemination and identification of possible partners
Meetings	Broader attendance and participation of stakeholders especially if it is industry wide	Selected people/partners, depending on the type of the meeting	More thorough discussion of the issue from different perspectives, not only on the technical side but also on organizations and people involved

Source: Fieldwork, news articles, websites.

stop the spread and occurrence of Panama disease in Region XI (Valencia, 2015). Since the *ad hoc* forms of collaboration were composed of newly assembled members, team building activities were also included in the project as in the case of the Southern Mindanao Agriculture and Resources Research and Development Consortium (SMARRDEC)'s project on "Science and Technology Management Approaches against Fusarium Wilt *(F. oxysporum* f. sp. *cubense (Foc)* on Cavendish in Mindanao."

Besides projects, trainings also allowed different stakeholder to interact on an *ad hoc* basis. For example, one training focused on a participatory approach to Foc TR4 management. Participants included farmers, researchers, and extension agents. This training workshop aimed to enhance the capabilities of local researchers, extension agents, and local farmers in providing effective disease management approaches. Other elements of the training were the proper detection of Foc TR4 in the field. Another example was a training on diagnosis and characterization provided to banana companies, universities, research organizations, and government agencies. Resource persons were from Bioversity International, University of the Philippines Los Baños, and Stellenbosch University (South Africa). There were also trainings for diagnosis and knowledge sharing by the Department of Agriculture. The DA shared four interventions to assist banana farmers to mitigate *Fusarium*: cash for work program for eradication, planting of GCTCV 219, crop conversion program, and *Trichoderma* distribution (Maestre, 2015).

These temporary and often *ad hoc* gathering resulted in the exchange of information, but observations also signaled that participants were quite desperate to find solutions. Tensions arose when participants proposed a solution, while others were doubtful about the effectiveness or demanded careful evaluation first. Moreover, the multi-stakeholder meetings were also used to discuss where the disease came from, and consequently who is to blame for the destruction of many banana fields. The multi-stakeholder meetings became avenues for public debate, where turfing, positioning, information-sharing, and blaming mingled. It was noted during the February 2014 workshop that farmers blamed the large companies for denying the spread of Foc TR4 and non-disclosure of the disease's presence in their plantations. The farmers felt the public information about the disease was late because many bananas were already infected. Moreover, people's clothes and footwear and farm tools (such as bolos and scythe), which were believed to be carriers of the disease, had been exposed to TR4, and farmers unknowingly contaminated other areas/ plants. Meanwhile, an employee during the same workshop blamed the small farmers, working in villages adjacent to their plantation, for the spread and contamination. The employee shared the company's frustrations that despite adequate efforts to disseminate information (in collaboration with government agencies), farmers and residents were uncooperative. An MBFEA representative, who stated that DA was not supportive to all of banana farmers, which prompted a meeting with some DA Region 11 personnel, other government representatives, and selected banana farmers to clarify the issue. This meeting (details were confidential), which was attended by one of the authors, concluded on the agreement that one farmer leader's opinion did not represent the majority.

Observations of various temporary and *ad hoc* occurrences of multi-stakeholder encounters not only exposed tensions and different interest and views, but also signaled emerging space for collective sensemaking of the risk for the range of banana producers located in a single area. Despite controversies and tensions, the meetings and related media attention also raised awareness of the unknowns and uncertainties related to management of the disease. The observed interactions also induced recognition of new type of interdependencies underlying any form of collective mitigation of a disease crossing organizational boundaries and affecting large and small banana producers alike.

Organizational Field 3. Professional Associations and Networks

The interviews and documented career histories of key informants from the private and public sectors indicate that long standing relations were rooted and reproduced within alumni associations

and professional networks. These communities were composed of alumni networks from the University of the Philippines (particularly the Los Baños campus), University of Southeastern Philippines, and University of Southern Mindanao—all agricultural universities in the Philippines. The connections among professionals were reproduced by membership of scientific societies such as the Philippine Association of Agriculturists, Crop Science Society of the Philippines, and Philippine Phytopathological Association. Six key informants attended the annual meetings of these associations, which included presentations of new findings and researches. Moreover, the meetings became a venue for sharing ideas, some of which translated into actual projects and collaborations.

The professional associations and networks enabled interactions based on personal ties or social connections. A key informant from the private sector related how he tapped a friend from an international research institute and a local politician to discuss urgent issues and future plans for the banana industry:

> *I organized a summit, in which we talked about Fusarium. I tapped my friend who is from the Taiwan Banana Research Institute and I also invited a congressman to talk about the National Banana Research Center. We also talked about the bills regarding pro and anti-aerial spray. (Interview with Private Sector, Davao City, 04 November 2016)*

Another key informant from government indicated that cross-sector interactions in informal professional networks were easier if you were trained at the same university or belonged to the same scientific society:

> *I am a member of UP Phytopathological. Of course, if you are from UP (University of the Philippines), even if it's not plant path, for as long as you are from UP, you will be introduced and access is easier. Especially if you belong to the Philippine Phytopathological Society body. (Interview with National government employee, Davao City, 7 February 2017)*

A university employee explained that mobilizing friendships with other professionals helped to move things forward when formal partnerships were not able to agree on a collective resolution:

> *If we want to do things in a corporate farm, they (the corporate farm) cannot decide. They have to go to PBGEA because PBGEA has a governing technical committee. One company allowed us to do research because we have a friend who was the Director of Research and who can allocate a field for trials. In the end, PBGEA only allowed this company to be involved.*
> *(Interview with university employee, 22 October 2014, Davao City)*

The interviews revealed that most of the people working in the banana industry, particularly those who were involved in actions addressing Foc TR4, were familiar with each other, both personally and professionally. It was observed that the same people attended similar *ad hoc* actions, research projects, workshops, meetings, and task forces. There were also events where they met as university alumni or as professional group members. The acquaintance made cross-sector collaboration easier; a university employee explained that personal relations with a local government unit enabled access to production areas and regularly organize a forum with farmers and link with partner agencies. Moving through the personal networks was essential for this (Interview with university employee, Davao City, 22 October 2014)

Media reports in newspapers and websites (Philippine News Agency, 2011; Madrazo-Sta. Romana, 2012; Valencia, 2015) expose indications of interactions among alumni, colleagues, and peers within and between organizations opened space to explore contextual and experimental pathways addressing Foc TR4. Industry actors from different companies and government agencies recognized that Foc TR4 did not have a single solution and looked for ways to temper the effect or pragmatically continue banana production (Cayon, 2011; Peña, 2012). This type of response was inclined to look for a combination of disease management practices. Responding to the disease was too complicated, and it was unlikely that a single solution would be available. In unstructured interviews, employees of large-scale banana plantations indicated to adopt quarantine measures at the boundaries of the plantation, work with skilled foremen for detecting diseased plants and eradicate them, support trials with somaclones, recognize that soil management matters (as was suggested by one of the banana cooperatives for agrarian reform beneficiaries), and engage in long-term research projects looking for resistant varieties (Lumawag, 2015). Multiple key informants confirmed that they exchange insights from trial and error procedures with peers working in different sectors. Private sector employees borrowed hands-on measures experimented by peers and were open to evaluate the results together in informal settings.

The interviews indicate how professionals crossed organizational boundaries, both between the public and private sectors, and between large companies and smallholder farmers. In the history of the banana industry in Mindanao, these boundaries used to be less permeable, but the management approaches to Foc TR4 adopted by expert professionals indicate a certain willingness to move outside established silos. Private sector employees reached out to government officials whom they shared a professional affiliation with, in particular plant pathology. In addition, some companies also realized that reaching out to neighboring smallholder banana growers could be part of the response to TR4, which was further complicated by farmers who decided to convert their land to other crops without treating the Foc TR4, and therefore allow the pathogen to stay in the soil.

DISCUSSION

The previous section identified three organizational fields in which different stakeholders interacted and co-created responses to the spread and emergence of Foc TR4. This section matches the above descriptive accounts with theoretical insights in coordination to further typify the observed emerging forms of

coordination. The emerging forms of coordination are labeled as: rule-based, cognition-based, and skill-based.

Rule-Based Coordination

The organizational field including industry alliances and formal partnerships is rooted in the dominant export sector producing and trading Cavendish. This exclusive organizational field has strong ties to the globalized economy, in which people and products move, through air, water and land. These movements are a factor in spreading plant diseases. These partnerships build on coordinated actions prior to the emergence of Foc TR4, which included lobbying for legislation to lift the ban on aerial spraying. These same adversaries could become allies in other issues, such as searching for mitigating factors and solutions for emerging plant disease threats. However, the issue of aerial spraying mainly relates to the management of Black Sigatoka in large-scale plantations and is different compared to Foc TR4 issue in terms of the coordination requirements. Foc TR4 has the risk of spreading the disease when efforts of players in addressing the issue vary. Resultantly, large firms have good reasons to share their knowledge to those who supply to them under contract as well as to those who supply other buyers (e.g., spot market) as they pose a risk. The presence and the nature of Foc TR4 encouraged the rather cautious and exclusive export-oriented industry players to be open for more opportunities to collaborate.

As leading industry players, this organizational field tried to coordinate among themselves and with regulatory government bodies in order to agree on rules, particularly on quarantine, and to prescribe preferred disease management practices. Regular organizational members/representatives met for meetings or projects, with some information privately and exclusively available to them. Representatives of individual companies behaved and reacted based on their organizations' directives. The formal partnerships feature bureaucratic interactions and are inclined to set of rules and standards to govern its operations. Formal arrangements such as memoranda of agreement or membership regulations enable coordination (Quero, 2012). The private sector alliance coordinates with the public sector in formulating guidelines and laws and implementing rules and regulation (Leone and Gaillard, 1999). This kind of rule-based coordinated action is structured, procedural, and formal.

Cognition-Based Coordination

In the *ad hoc* organizational fields of stakeholder meeting during workshop, training, and conference interactions and membership are flexible and cover a wider scope of issues, and the orientation leans toward exchange of information. Since it is an *ad hoc* interaction, its mandates depend on what is collectively agreed by the interim members. This means that protocols, rules, and process are constantly changing, including the functions of participants (individuals or representing their organizations). The more the actors talk and interact with each other, the more effective is their governance (Andersson, 2004). This form of interaction in the form of public meetings and exchanges, also including media coverage, features a degree of inclusiveness. Information, attendance, and memberships are often open and accessible to the public. The *ad hoc* organizational field accommodates interactions that connect actors recognizing the treats affecting many in an area.

This organizational field provides a platform for different stakeholders trying to make sense of the risks and exploring possible solution pathways, and therefore plays a cognitive role in the landscape of actors affected by Foc TR4. Comfort (2007) identifies cognition as a vital ingredient of any concerted action in responding to natural hazards or biological risks affecting a variety of actors in a single area. This is the case with TR4, which has uncertainties and has no universally accepted chemical, biological and cultural cures. Comfort (2007) argues that capacities to communicate, control, and coordinate remain disconnected if cognition is absent. Comfort derives this insight from several studies mostly in the United States of America analyzing responses to natural disasters, such as typhoons or floods, in spatially bounded areas (Comfort et al., 2001; Comfort et al., 2004; Comfort, 2005; Comfort and Kapucu, 2006; Comfort and Haase, 2006). Cognition may start with technical experts working in laboratories of multinational companies or local governments, but normally these experts elevate concerns to their organizations and other stakeholders. This type of expert knowledge has become part of deliberations and interactions taking place in *ad hoc* organizational fields that contribute to the recognition of threats and construct shared or contrasting cognitive understanding of Foc TR4.

Skill-Based Coordination

This study highlights the invisible and informal form of skill-based coordination as an important resource for navigating the tensions and conflicts around the formal and public responses to the spread of the disease. It identifies professionals that belong to a specific technical profession, i.e., plant pathology, and most often do not hold management positions as key players in this form of skill-based coordination. Bardon et al. (2015) emphasize the relevance of professional association for coordination because peers incorporate shared elements in approaching management problems and interacting with other professionals. This connectivity allows actors to tweak organizational protocols while navigating risks and uncertainties. This form of skill-based coordination entails interactions among alumni, colleagues, and peers within and between organizations (Grindle, 1977; Lawrence, 2004; Mudambi and Swift, 2009; Hatmaker et al., 2011; Noordegraaf, 2011; Levine and Prietula, 2013; Cohen and Malloy, 2014; Bardon et al., 2015). It has the capacity to find and recognize contextual pathways addressing a virulent plant disease, to avoid prescribing single solutions, and to contain the tendency to blame others (Vellema and Jansen, 2018). We observed trust in the form of pursuing the solutions and mitigating actions recommended by colleagues and getting access to people and information, which formed a significant ingredient in informal coordination (Zanini and Migueles, 2013). Cross-sector coordination is importantly realized by a variety of peer groups that are able to cross-organizational boundaries and

share informal, symbolic, or task-oriented views associated with a profession.

Following Adler et al. (2008), our findings emphasize this peer community principle as a mechanism underlying hidden forms of coordination that entail careful selection of trusted collaborators. In this way, individuals associated with principles and management views of a specific profession (Mudambi and Swift, 2009; Noordegraaf, 2011) and employed by different organizations mobilize their networks to solve problems and develop collaborative capacity (Kaplan, 2000). These professional associations generate a certain degree of common understanding (Yanow, 2004) and share practices and routines to manage problems (Lawrence, 2004). The skill-based coordinated action is dynamic. It enables actors to cross both organizational boundaries and borders between private and public sectors. This hidden/invisible interaction also flourished because the professional organizations follow what Noble and Pym (1970, p. 438) termed a "collegial pattern of authority" and stimulated an openness for trial and error. This could explain why some interactions within the industry remain hidden because there is careful selection of trusted collaborators who share a set of skills associated with a specific professional association.

Recognizing and Configuring Emerging Forms of Coordination As Response to Global Plant Diseases

The case study of emerging forms of coordination in distinct organizational fields, i.e., industry-based and formalized partnerships, temporary and *ad hoc* multi-stakeholder settings, and professional associations and networks. These organizational fields were shaped by both formal organizational structures and informal preferences identified distinct forms of coordination. Tracing a variety of interaction processes and encounters in these organizational fields and matching these with theoretical accounts enabled us to typify emerging forms of coordination. Based on this typology, we propose a set of qualitative indicators to recognize these three forms, acknowledging that coordination is difficult to quantify (**Table 5**). These indicators based on our theory-informed typology may inform stakeholders involved in management other plant diseases in banana or other crops to recognize and assess the plausible emergence of forms of coordination. The three typical forms serve as a sufficiently exhaustive representation of coordination to guide an integrative plant disease management approach, while leaving space for additional forms of coordination.

This paper considers the emergence and precise forms of coordination as highly contingent on both the characteristics of the disease itself, i.e., the long-term persistence in the soil and the uncertainties related to spread and control in the case of TR4, and the area-specific history of actor constellations, i.e., in the case of the area in Mindanao the entangled presence of export-oriented multinational companies, processes of agrarian reform, and decentralized government responsibilities. Our area-based perspective used for the case study indicates that these contextual conditions importantly determine whether and how distinct forms of coordination both emerge and combine in an area affected by Foc TR4. The configuration of distinct forms of coordination is an important determinant for the possible emergence of a so-called collaborative community. According to Adler et al. (2008, p. 366), participants in a collaborative community "coordinate their activities through a shared commitment to a set of ultimate goals." These authors show that the structure of a collaborative community is characterized by the combination of more global and open ties and stronger local ties. Its values are based on trust, with emphasis on contribution, concern, honesty, collegiality, and value-rationality. However, according to Kolbjørnsrud (2016), collaborative communities have challenges relating to governance such as mutual monitoring, membership restrictions, values and rules, and incentives. Our analysis indicates that the emergence of Foc TR4 influenced organizational dynamics in the Philippine banana sector. This may encourage the multinational banana companies to become part of a locally embedded collaborative community, as is suggested by Faulconbridge (2010). However, it is still difficult to assess whether this increases the likelihood of the formation of such a collaborative community, in which different actors converge to address a common problem.

TABLE 5 | Indicators to recognize emerging forms of coordination.

Descriptions	Forms of coordinated action		
	Rule-based	**Cognition-based**	**Skill-based**
Action and events	Conduct mandated activities, formulate, and follow rules	Organize activities that generate or share information	Accomplish research, development, and extension activities with long standing connections/partners
Composition	Requires regular organizational membership	Coordinate interim/temporary, multi-disciplinary members	Established by specialists or professional groups
Organization set-up	Formal and structured	*Ad hoc* and flexible	Dynamic and cross organizational boundaries
Objectives	Advocacy, formulation, and imposition of policies	Knowledge generation and finding solutions/answers	Implementation and application of solutions
Orientation	Bureaucratic	Exchange of information	Problem solving
Specific type of response	Recommending policies, implementation of rules	Creating Task Forces and activities that require interactions and dialogues	Formulating and evaluating specific technical solutions

CONCLUSION

This paper expounded that coordination is one of the responses of a sector confronted with an emerging global plant disease affecting an important export variety embedded in a landscape of diverse production systems of the same crop. The paper exposed that the spread and emergence of Foc TR4 either changed or induced coordination among stakeholders with different interests, e.g., large-scale export or diverse food provision, or from different domains, e.g., private and public. Coordinated action prior to the emergence of TR4 was modified or intensified. Based on qualitative analysis and theory matching, the paper distinguished three forms of coordinated action: rule-based, cognition-based, and skill-based. The qualitative analysis indicates that formalized partnership importantly continued its routinized forms of coordination but also opened space for new collaborations. Multi-stakeholder setting offered opportunities for sense-making jointly with surfacing tensions and different perspectives on how to handle the unknowns and uncertainties attached to Foc TR4. Finally, it highlights the informal form of skill-based coordination as an important resource for navigating the uncertainties and tensions and engage with trusted collaborators in trial and error based experimentation and trials, which entail an unprecedented crossing of organizational boundaries. Our analysis suggests that integrative capacity to manage a global plant disease and its spread entails a combination of technical measures and conditions conducive to interactions and coordination among organizations and actors with different resources and diverse interests. We consider it important to recognize these different forms of coordination and understand how they combine in the specific regional circumstances of doing business and interactions between private and public sectors. A careful suggestion from this study is that, rather than in the form of formalized partnerships, a collaborative community may emerge from the invisible layer of professionals working in different organizations and crossing sectoral boundaries.

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MM and SV contributed to research design, conceptualization, analysis and writing. LD contributed to analysis, writing and subsequent discussions.

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Genetic Diversity in *FUB* Genes of *Fusarium oxysporum* f. sp. *cubense* Suggests Horizontal Gene Transfer

Siwen Liu[1†], Bo Wu[2†], Shuxia Lv[3†], Zongzhuan Shen[4], Rong Li[4], Ganjun Yi[2], Chunyu Li[2] and Xiuwu Guo[1*]*

[1] College of Horticulture, Shenyang Agricultural University, Shenyang, China, [2] Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization, Ministry of Agriculture, Key Laboratory of Tropical and Subtropical Fruit Tree Research of Guangdong Province, Institution of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, China, [3] College of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang, China, [4] Jiangsu Key Lab for Solid Organic Waste Utilization, Key Laboratory of Plant Nutrition and Fertilization in Low-Middle Reaches of the Yangtze River, Ministry of Agriculture, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China

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***Correspondence:**
Chunyu Li
lichunyu881@163.com
Xiuwu Guo
guoxw1959@163.com

[†]These authors have contributed equally to this work

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Fusaric acid (FA) is an important secondary metabolite of many Fusarium species and involved in the wilt symptoms caused in banana by *Fusarium oxysporum* f. sp. *cubense (Foc)*. To investigate the evolution characteristics of the 12 *Foc* FA biosynthetic genes (*FUB*), coding sequences of the 12 *FUB* genes and three housekeeping genes, *EF-1α/RPB1/RPB2* (translation elongation factor-1α/RNA polymerase II subunit I/RNA polymerase II subunit II), were subjected to genetic diversity analysis, phylogenetic analysis, recombination detection, and selective pressure analysis. The results of selective pressure analysis showed that the 15 genes were mainly subjected to negative selection. However, a significantly higher number of silent mutations, which could not be simply explained by selective pressure difference, were observed in the 12 *FUB* genes in *Foc* than in the three housekeeping genes. Infraspecies phylogeny and recombination detection analysis showed that significantly more horizontal gene transfer (HGT) events (normalized) had occurred in the *FUB* genes than in the three housekeeping genes. In addition, many of these events involved outgroup isolates and significantly increased the genetic diversity of *FUB* genes in *Foc*. The infraspecies phylogenetic analysis suggested that the polyphyletic phylogeny proposed for Foc requires further discussion, and the divergence of race 1, race 4, and the common ancestor of several *F. oxysporum* (*Fo*) isolates pathogenic to nonbanana plants should have diverged over a short period. Finally, our results suggest that the *FUB* genes in *Fo* should have benefited from HGT to gain a relatively high genetic diversity to respond to different host plants and environments despite mainly being subject to negative selection.

Keywords: Panama disease, *Fusarium oxysporum* f. sp. *cubense*, fusaric acid, horizontal gene transfer, phylogeny

INTRODUCTION

Fusarium oxysporum (*Fo*) f. sp. *cubense* (*Foc*), the causal agent of Fusarium wilt of banana (*Musa* spp.), which is also known as Panama disease, is the most important soil-borne pathogen limiting banana production in the world (Ploetz, 2015). Like other *Fusarium* f. sp., *Foc* produces many toxic secondary metabolites; among these, fusaric acid (FA) is one of the most studied mycotoxins (Desjardins and Proctor, 2007; Bohni et al., 2016; Singh et al., 2017; López-Díaz et al., 2018) and a host-nonspecific toxin. FA is involved in the toxicity of *Fusarium* spp. towards plants, animals, and human beings (Ghazi et al., 2017; Singh et al., 2017; López-Díaz et al., 2018) and in interactions with environmental microorganisms (Brown et al., 2015; Bohni et al., 2016; Simonetti et al., 2018). FA has been reported to be phytotoxic to different plants involving multiple biological processes. For instance, it has been reported to chelate metal ions inside tomato (López-Díaz et al., 2018), disturb the water balance in cucumber (Wang et al., 2015), and cause programmed cell death in tobacco suspension cells (Jiao et al., 2013). In Panama disease, FA production by *Foc* is involved in disturbing the water balance and causing the wilt symptom (Dong et al., 2012; Li et al., 2013; Dong et al., 2014) and has been shown to be essential for its virulence on banana plantlets (Ding et al., 2018).

Due to the important function of FA in many *Fusarium* spp., much attention has been paid to the genes involved in its biosynthetic and related regulatory processes (Brown et al., 2012; Niehaus et al., 2014; Brown et al., 2015; Studt et al., 2016; Ding et al., 2018). Like many other secondary metabolite biosynthetic genes, which are generally located in gene clusters (Hoogendoorn et al., 2018), a *FUB* gene cluster consisting of 12 genes (*FUB1* to *FUB12*) was identified in different *Fusarium* species (Brown et al., 2015; Studt et al., 2016). The functions of the *FUB* genes in *Fusarium* were predicted through the functions of their annotated homologs, including a polyketide synthase (PKS, *FUB1*), an unknown protein (*FUB2*), an aspartate kinase (*FUB3*), a serine hydrolase (*FUB4*), a homoserine *O*-acetyltransferase (*FUB5*), an NAD(P)-dependent dehydrogenase (*FUB6*), an *O*-acetylhomoserine (thiol-) lyase (*FUB7*), a nonribosomal peptide synthetase (NPRS)-like enzyme (*FUB8*), an FMN-dependent dehydrogenase (*FUB9*), two C6 transcription factors (*FUB10* and *FUB12*), and a major facilitator superfamily transporter (*FUB11*) (Studt et al., 2016). FA production is differentially altered in deletion mutants of each *FUB* gene (Brown et al., 2015; Studt et al., 2016), suggesting that the importance of the 12 genes is different. *FUB1* is the key gene in the *FUB* gene cluster, and FA production was abolished or reduced by 95% in the *Fo FUB1* deletion mutant (Brown et al., 2015; López-Díaz et al., 2018). FA production was abolished in deletion mutants of *FUB10* in *Fusarium verticillioides* (*Fv*), *Fusarium fujikuroi* (*Ff*), and *Fo*, and *FUB10* has been proven to be a transcription factor positively regulating the transcription of other *FUB* genes (Brown et al., 2015; Studt et al., 2016). Deletion mutants of the other transcription factor-encoding gene, *FUB12*, exhibited incomplete loss of FA production in *Fv* and *Ff* (Brown et al., 2015; Studt et al., 2016). *Fo* deletion mutants of *FUB3*, *FUB6*, or *FUB8* also abolished FA production, but in corresponding *Fv* mutants, FA production was reduced to only a certain extent (Brown et al., 2015). A *FUB4* deletion mutant of a *Foc* race 4 strain lost its capacity for FA production and was significantly weakened in its virulence towards banana (Ding et al., 2018).

The infraspecies phylogeny of *Foc* has been suggested to be complex. Three races of *Foc* that are pathogenic to different host banana types have been reported: race 1, affecting Gros Michel (AAA) and some AAB or ABB bananas; race 2, pathogenic to ABB cooking bananas; race 4, affecting Cavendish bananas; and race 1 and race 2 suspects (Mostert et al., 2017). Race 4 isolates were further divided into two groups according to their different requirements of disease-predisposing conditions: tropical race 4 (TR4) and subtropical race 4 (STR4) (Ploetz, 2015; Ploetz et al., 2015). According to vegetative compatibility, *Foc* isolates were assigned to 24 vegetative compatibility groups (VCGs) (Fourie et al., 2009). *Foc* was suggested to have multiple evolutionary origins based on different nuclear and mitochondrial genes (O'Donnell et al., 1998; Fourie et al., 2009; Czislowski et al., 2018). In the study of Fourie et al. (2009), polyphyletic phylogeny was supported for not only *Foc* but also race 1 and race 4, and the same sets of VCGs were generally clustered together. An infraspecies phylogeny analysis based on three housekeeping genes (*EF-1α*/*RPB1*/*RPB2*) supported monophyletic phylogeny of race 1 VCGs and race 4 VCGs (Czislowski et al., 2018). However, significant discordance was observed between some of the phylogenetic trees based on the *SIX* (Secreted In Xylem) genes and the infraspecies tree, suggesting horizontal gene transfer (HGT) events in the *SIX* genes in *Foc* (Czislowski et al., 2018). The study of Maryani et al. (2019) based on *EF-1α*/*RPB1*/*RPB2* also suggested that the *Foc* TR4 group should be monophyletic, but *Foc* race 1 isolates were clustered into multiple lineages. Horizontal gene transfer rather than convergent evolution was suggested to explain the polyphyletic phylogeny of race 1 and race 4 observed (Czislowski et al., 2018), and it was also suggested to have contributed to the high diversity level observed in race 1 compared with TR4 (Maryani et al., 2019). According to the infraspecies analysis in the study of Czislowski et al. (2018), some *Fo* f. sp. isolates and nonpathogenic *Fo* isolates should have originated from *Foc* race 1. *Foc* has no known sexual cycle and is supposed to undergo sexual production only rarely, if at all (Kerényi et al., 2004; Ploetz, 2015), and the mechanism of HGT in *Foc* remains unknown.

As stated above, FA production plays an important role in pathogen plant/environmental microorganism interactions in many *Fusarium* (sub)species including *Foc*. Considering its specific function, we hypothesize that the conservation of protein function (negative selection) should be the main selective force on *Foc FUB* genes when the host and environment are relatively stable, but selection of new advantageous mutations (positive selection) should also be possible if the fungus is adapting to a new host or environment. Based on coding sequences (CDSs) of the 12 *FUB* genes and three housekeeping genes from 33 *Foc* isolates, this study aimed to reveal the genetic diversity and evolutionary characteristics including nonsynonymous/synonymous substitution ratio and HGT in *FUB* genes which are largely determined by the selective forces. And in the meantime, an effort was also made to infer the infraspecies phylogeny of *Foc*.

MATERIALS AND METHODS

Isolates and Group Assignments

Whole-genome sequences of 33 *Foc* isolates (**Table 1**) collected from multiple countries in South Asia, South Africa, and America were obtained from the *Foc* genome sequencing project (data unpublished). Published genomes of five *Fo* isolates, II5 (NRRL#54006, GCA_000260195.2, belonging to *Foc* race 4), Fo25433 (NRRL#54006, GCA_000260175.2), Fo47 (NRRL#54002, GCA_000271705.2), Fo4287 (NRRL#34936, GCA_000149955.2), and Fo26406 (NRRL#26406, GCA_002318975.1), and an outgroup *Fv* isolate, Fv7600 (GCA_000149555.1), were downloaded from the National Center for Biotechnology Information (NCBI) assembly database. The Northern Regional Research Laboratory (NRRL) codes were assigned by the Agricultural Research Service Culture Collection, US Department of Agriculture, and the IDs following the NRRL codes in the brackets are GenBank accession numbers. A total of 16 race 1 isolates from the *Foc* genome sequencing project were assigned to R1, including two nonpathogenic *Fo* isolates JB255 and JB553, which should belong to R1 according to phylogenetic analysis; and 18 race 4 isolates, including 17 from the *Foc* genome sequencing project and II5, were assigned to R4. Fo4287, Fo47, and Fo26406, which formed a monophyletic group in the phylogenetic analysis in this study (**Figures 2A**, **B**), were assigned to the 3Fo group. Fo25433 was not assigned to the R1 group considering its special identity, although it should have originated from R1 according to phylogeny analysis.

Sequence Alignment and Genetic Diversity Analysis

CDSs of *EF-1α*, *RPB1*, *RPB2*, and 12 *FUB* (*FUB1–12*) genes were obtained from the reference genome sequence of *Foc* race 4 isolate II5 (GenBank accession no. from MH972571 to 973155) (Guo et al., 2014). Then, the CDSs of the 15 genes were pulled out from all the genomes according to the results of local BLASTN alignment using II5 CDSs as queries, and no highly similar (>95% nucleotide similarity) paralog was identified for any gene. Multiple sequence alignment for each gene and concatenated sequences of *EF-1α/RPB1/RPB2*, the *FUB* gene cluster and the 15 genes was carried out using Clustal X version 2 (Larkin et al., 2007). Some of the CDSs were slightly different (probably due to different gene models applied in genome annotations or mutations causing transcript alteration that could not be distinguished and should

TABLE 1 | Information of sequenced *Fo* isolates.

Group	Strain	VCG*	Host banana cultivar	Sampling location
R1	Race1-CAV2013	VCG0128	Chuoi tay cao	Van Giang, Vietnam
R1	Race1-22994	VCG0128	Bluggoe	South Johnstone, Australia
R1	Race1-188	VCG01212	Ney Poovan	Tenguero Station, Tanzania
R1	Race1-623	VCG01220	Williams	Carnarvon, Australia
R1	Race1-871	VCG01217	Pisang Rastali	Malaysia
R1	Race1-939	VCG0123	Kluai Namwa	Thailand
R1	Race1-967	VCG0124/5	Latundan	Philippines
R1	Race1-MW2	VCG01214	Harare	Misuku, Malawi
R1	Race1-Mal6	VCG01217	Pisang Rastali	Kg. Taboh, Malaysia
R1	Race1-1983	VCG0123	Pisang Awak	Taiwan
R1	Race1-24223	VCG1220	Williams	Carnarvon, West Australia
R1	Race1-GD01	VCG01220	Pisang Awak	Guangdong, China
R1	Race1-HN05	VCG0123	Gros Michel	Hainan
R1	Race1-MW40	VCG01214	Harare	Misuku, Malawi
R1	JB255	–#	Non-pathogenic, soil	Kiepersol, South Africa
R1	JB553	–	Non-pathogenic, soil	Kiepersol, South Africa
R4	TR4-CAV2318	VCG0121	Namwa	Kuosin E, Taiwan
R4	TR4-CAV300	VCG01213	Valery	Southeast Sumatra, Indonesia
R4	TR4-STSUM5	VCG01213	Pisang Batan	Sumatra, Indonesia
R4	TR4-HN17	VCG01216	Cavendish	Hainan, China
R4	TR4-Mal2a	VCG01216	Pisang Raja	MARDI, Selangor, Malaysia
R4	STR4-105	VCG0120	Cavendish	Kiepersol, South Africa
R4	STR4-612	VCG01215	Gros Michel	Costa Rica
R4	STR4-SH3142	VCG01211	SH3142	Queensland, Australia
R4	STR4-980	VCG0120/15	–	Canary Island
R4	STR4-Pacovan	VCG0120	Pacvan	Bahia, Brazil
R4	STR4-187	VCG01210	Apple	Florida, USA
R4	STR4-195	VCG01219	Pisang Ambon	Indonesia
R4	STR4-618	VCG0122	Cavendish	Philippines
R4	STR4-M3	VCG01210	Gros Michel	FHIA Villa Clara, Cuba
R4	STR4-II12	VCG01219	Pisang raja garing	Cibinong Collection, Indonesia
R4	STR4-GD26	VCG0126	Pisang Awak	Guangdong, China
R4	STR4-1089	VCG0129	Lady finger	Cooloolabin, QLD, Australia

**VCG, abbreviation of vegetative compatibility group. #The short dash line indicates the information is unknown or un-recorded.*

not be a problem in this study) and were adjusted using their genome sequences to accommodate the CDS of the *Foc* II5 isolate.

Parameters, including *Pi*, the number of intragroup/intergroup variations, the number of synonymous/nonsynonymous mutations, the number of haplotypes, Dxy (the average number of nucleotide substitutions per site between populations), and the haplotype diversity of R1, R4 and *Foc* were calculated for each gene using DnaSP v6.12.01 (Rozas et al., 2017). Some of the mutations occurred on the same codons and could be assigned to be either non-synonymous or synonymous, depending on their occurrence orders. A total 9 such mutations were not assigned to either class. Silent loci in CDSs are generally subjected to neutral evolution in the genome, and the synonymous substitution rates among different genes have been shown to match a uniform point mutation rate across genes in *Escherichia coli* (Maddamsetti et al., 2015). A high level of synonymous mutations could be utilized as a potential signal of HGT (Proctor et al., 2013; Chen et al., 2018). Because the three housekeeping genes were mainly subjected to negative selection, which would not induce loss of *PIsi*, the *PIsi* level in these three genes was utilized as the control level to detect regions with significantly increased *PIsi*. *PIsi* was calculated in 100 silent site windows with a step size of 25 across the concatenated sequences of the 15 genes in R1, R4 and *Foc* using DnaSP v6.12.01. The first 97 windows were located within the three housekeeping genes and used as controls. The 99% cumulative probability threshold *PIsi* value was calculated for R1, R4 and *Foc* based on the normal distribution parameters calculated from the 97 windows in EXCEL, and *PIsi* values higher than this threshold are supposed to be significantly ($p < 0.01$) higher than those for the three housekeeping genes.

Infraspecies Phylogenetic Analysis

With the aligned sequences, jModelTest-2.1.10 (Darriba et al., 2012) was applied to choose the best models for phylogenetic tree construction based on ML and BI. Then, PhyML 3.1 (Guindon et al., 2010) was applied to produce ML trees using the best-fit model for concatenated sequences of *EF-1α/RPB1/RPB2* and the *FUB* gene cluster and individual genes with 1,000 bootstrap replicates, and MrBayes v3.2 was used for tree construction by BI (Ronquist et al., 2012). Partitioned analysis unlinking models and parameters among different gene loci was applied on concatenated sequences by MrBayes v3.2. The SH test (Shimodaira and Hasegawa, 2001) was implemented in RAxML v8.2.12 (Stamatakis, 2014) to determine whether there was a significant difference in the tree topologies supported by different genes.

When concatenated gene sequences were used in phylogenetic inference, large regions of weak phylogenetic signals (produced when three or more species diverged in a short time) that support the true species tree topology could be obscured by short regions of strong phylogenetic signals caused by recent HGT events, and in this case, the standard nonparametric bootstrap could have lost its power (Heled and Drummond, 2010). In theory, the introgressed genetic material should account for a smaller proportion of the genome than the original species/infraspecies. Accordingly, the BSRRSS method was carried out by our Python script by calculating the branch support rate of branches in the best trees (obtained from 500 independent ML tree searches using RAxML v8.2.12) constructed for 1,000 randomly sampled 1 or 2 kbp gene segments from the concatenated sequence. A majority rule extended consensus tree was obtained from the 1000 best trees using RAxML v8.2.12, which should be a more reliable species tree. The low support rate of inner-group branches could have derived from either HGT or low sequence difference; thus, our main attention would be focused on the splitting of *Fo* groups. In theory, it would be more reasonable to sample an equal number of gene segments from each 'unlinked' locus (at most four in this study, *EF-1α, RPB1, RPB2* and the *FUB* gene cluster), but considering that the gene sequences of the three housekeeping genes were short, it was not applied in this study.

Recombination Detection

Recombination detection was carried out using 7 different algorithms implemented in RDP v.4.95 (Martin et al., 2015), including RDP, Chimaera (Posada and Crandall, 2001), BootScan (Salminen et al., 1995), 3Seq (Boni et al., 2007), GENECONV (Padidam et al., 1999), MaxChi (Smith, 1992) and SiScan (Gibbs et al., 2000). The detected recombination events were required to have a Bonferroni corrected P-value < 0.05 and to be supported by topological evidence and at least two different methods. Two recombination events detected only by MaxChi that could explain the significant tree differences between *EF-1α*, *RPB2,* and *RPB1* were also accepted because they were well supported by the infraspecies phylogenetic analysis. Manual inspection and correction were carried out by checking whether the recombined region supported a tree topology (using the ML and BI methods implemented in the software) different from that based on the ML/BI tree based on the three housekeeping genes. To analyze if there were significant differences in the number of recombination events in the three housekeeping genes and the *FUB* gene cluster, a chi-square test was carried out against the null hypothesis that the number of recombination events should be proportional to the length of the analyzed regions.

Ka/*Ks* and Selective Pressure Analysis

Ka and *Ks* were calculated between each pair of isolates from *Foc* and 3Fo using DNAsp v6.12.01, and the means of the *Ka*/*Ks* values of all of the genes were compared by one-way ANOVA implemented in SPSS v22 (IBM, New York).

Selection analysis of branches and amino acid sites was carried out using algorithms implemented in the web server Datamonkey 2.0 (Weaver et al., 2018). Isolates with introgressed outgroup genetic material were excluded from the analysis of the corresponding recombinant genes. Branch-site model-based methods aBSREL (Smith et al., 2015) and BUSTED (Murrell et al., 2015) were carried out to test whether the branches leading to R1, R4 and 3Fo had been subjected to positive selection. ML-based methods FEL, SLAC (Kosakovsky Pond and Frost, 2005) and MEME (Murrell et al., 2012) were performed for selective pressure analysis of individual sites.

RESULTS

Genetic Diversity of *Foc* Based on 12 FUB Genes and Three Housekeeping Genes

CDSs of three housekeeping genes (*EF-1*α, *RPB1*, and *RPB2*) and 12 *FUB* genes with a total length of 33,168 bp were subtracted

from the assembled genome sequences of 16 race 1 group (R1) isolates and 18 race 4 group (R4) isolates (**Table 1**). A total of 1,505 single-nucleotide variations were detected in the CDSs (**Table S1**), including 1,464 biallelic and 41 triallelic loci. Of the 1,505 variations (1,546 mutations), 1,228 mutations were synonymous, 309 were nonsynonymous, and the remaining nine mutations were not assigned (explained in the *Materials and Methods* section). No highly deleterious mutation that caused an open reading frame shift or protein truncation was detected in any of the 15 genes in any isolate.

A higher level of genetic diversity was identified in R1 than in R4. Based on the 15 genes, 12 different genotypes were identified from the 16 R1 isolates, and nine different genotypes were identified from the 18 R4 isolates (**Figure S1**). More group-specific intragroup variations were detected in R1 than in R4 on all genes except *FUB5* (**Figure 1A** and **Table S1**). A total of

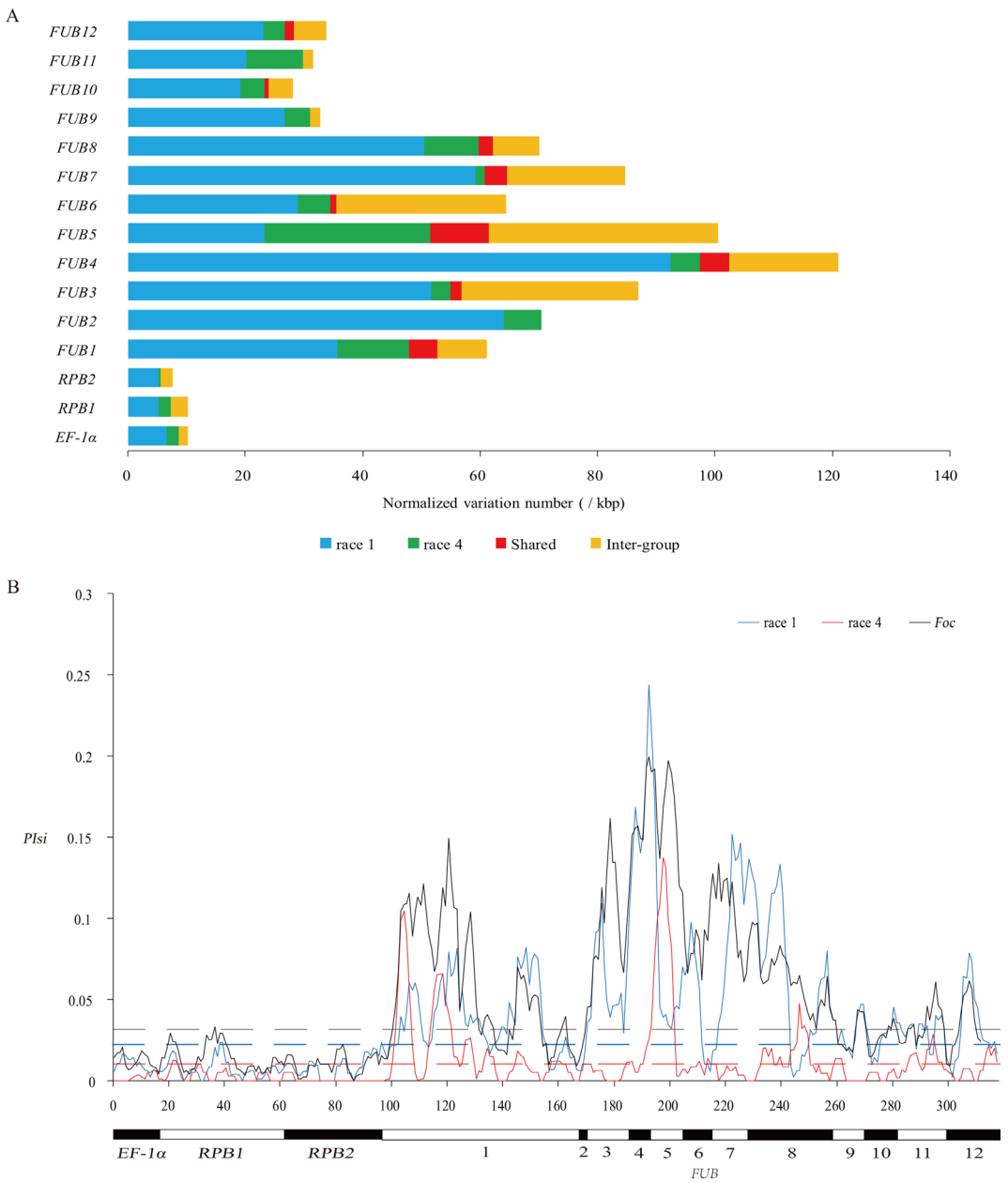

FIGURE 1 | Genetic diversity of 34 *Foc* isolates based on 15 genes. **(A)** Distribution of intragroup and intergroup variations in *Foc* isolates based on 15 genes. Blue and green bars indicate the number of intragroup variations specific in R1 and R4, respectively. The red bar indicates the amount of intragroup variation shared by R1 and R4 isolates. Yellow bar indicates the number of intergroup variations bet ween R1 and R4. **(B)** Distribution of *PIsi* on windows of 100 silent sites across the 15 genes. The horizontal dashed lines indicate the threshold *PIsi* value, which was significantly ($p < 0.01$) higher than the *PIsi* level of the three housekeeping genes in R1, R4, and *Foc*.

71 intragroup variations in *FUB* genes were shared between the two groups, which is most likely a sign of horizontal transfer between the two groups rather than convergent evolution. No shared intragroup variation was discovered in the three housekeeping genes. In total, 298 variations (51 nonsynonymous and 247 synonymous) were identified as intergroup variations in the 15 genes. The nucleotide diversity (*Pi*) of the R1 was generally higher (>2-fold and on average 6.43-fold) than that of the R4 in all genes except *FUB5*, on which R1 had lower (0.82-fold) *Pi* than R4. The *Pi* in the *FUB5* gene of R4 was significantly higher (on average 5.35-fold, $p < 0.001$) than the *Pi* in any other gene of the same group. The haplotype diversity of R1 is higher than that of R4 for all genes, including *FUB5*.

As shown in **Table S1**, the *Pi* in the *FUB* genes of both R1 and R4 is significantly (on average 7.5- and 11.1-fold, respectively, $p < 0.01$) higher than that in the three housekeeping genes. Differences in *Pi* could be the result of the combined effect of different selective pressures and different amounts of HGT, but differences in the number of synonymous variations were supposed to be affected by only HGT. According to the results (**Table S1**), the number of synonymous substitutions per synonymous site (*Ks*) between R1 and R4 was significantly ($p < 0.01$) higher for *FUB* genes than for the three housekeeping genes. As shown in **Figure 1B**, significantly higher nucleotide diversity of silent variations (*PIsi*) ($p < 0.01$) was observed in 77.3%, 45.7%, and 76.0% of the regions of the *FUB* gene cluster than in the three housekeeping genes in R1, R4, and *Foc*, respectively. The observed high *Ks* and *PIsi* in *FUB* genes should indicate introgression of outgroup genetic material into the *FUB* genes in either or both group(s).

Infraspecies Phylogeny Analysis

Molecular data on the 15 genes were used to infer the infraspecific phylogeny of *Fo*. To reveal the phylogenetic relationships of the sequenced *Fo* isolates and four published *Fo* isolates, Fo4287, Fo26406, Fo47, and Fo25433, maximum likelihood (ML) and Bayesian inference (BI)-based phylogenetic methods were applied on concatenated nucleotide sequences of *EF-1α*/*RPB1*/*RPB2* and 12 *FUB* genes with Fv7600 as the outgroup. As shown in **Figure 2A** and **B**, phylogenetic trees obtained by different phylogenetic methods on the same gene set were highly consistent, and in phylogenetic trees based on both concatenated sequences, isolates of the same VCGs were clustered into the same clades. However, the phylogenetic trees based on the *EF-1α*/*RPB1*/*RPB2* gene set were poorly supported [significantly worse by the Shimodaira–Hasegawa (SH) test at the 1% level] by the 12-*FUB*-gene set and *vice versa*. In the trees based on the three housekeeping genes, four isolates (JB255, JB553, Race1-MW2, and Race1-MW40) were clustered in the R1, but in the 12-*FUB*-gene cluster, they clustered out of R1 and formed a monophyletic group with Fo4287, Fo26406, and Fo47, which are three nonbanana *Fo* isolates (referred to as the 3Fo group). The phylogenetic relationship of R1 isolates and R4 isolates based on *EF-1α*/*RPB1*/*RPB2* should be robust, since any recombination signal was seldom detected in R1 and R4 isolates on these three genes (see the following section).

To check the difference in the phylogeny of the 15 genes, ML and BI-based methods were applied to each of the 15 genes for all of the above accessions. In trees based on both concatenated sequences and most genes, the nonbanana isolate Fo25433 was highly supported as belonging to R1. As shown in **Figure S2**, contradictory branches were widely observed in the 15 gene trees. The results of SH tests showed that the molecular trees based on most genes were significantly different ($p < 0.05$, **Figure 2C**), and only *EF-1*α and *RPB2* did not reject each other ($p > 0.05$ by SH test), but both rejected the tree based on *RPB1*, showing that at least one HGT event occurred in the three genes. For the trees based on *EF-1*α and *RPB2*, the tree topology supports the idea that the 3FO diverged from the common ancestor of R1 and R4, while the tree based on *RPB1* supported that 3Fo and R1 shared a more recent common ancestor than with R4, which is also supported by phylogenetic trees based on both concatenated gene sets (**Figures 2A, B**).

Considering the widely present topological differences among gene trees, a method named branch support rate in randomly sampled segments (BSRRSS) was used to infer a more reliable infraspecies tree and assess the support rate of controversial branches. Randomly sampled 1- and 2-kbp segments from the CDSs of the 15 genes were both applied in this study and produced similar results. As shown in **Figure 2D**, in the branches best supported by randomly sampled 1-kbp (2-kbp) gene segments, the monophyly of R1 and the monophyly of R4 were supported by 12% (15%) and 86% (91%) of sampled segments, respectively. The monophyletic clade compromising R1 and 3Fo was supported by 31% (36%) of sampled 1-kbp (2-kbp) segments, and the ratios of sampled 1-kbp (2-kbp) segments supporting the monophyly of R1 and R4 and the monophyly of 3Fo and R4 were 15% (15%) and 4% (2%), respectively. When most recombinant R1 and R4 isolates identified in the following section were excluded from the analysis, the support for the monophyly of R1 and 3Fo was only slightly increased to 35% (38%), while the support for monophyly of R1 increased to 77% (81%). Considering that at most four loci with different lengths were sampled, the support for the monophyletic clade including R1 and 3Fo is not robust based on the 15 genes according to the BSRRSS test method. In the BSRRSS analysis, most 1- and 2-kbp gene segments did not contain phylogenetic signals supporting either monophyletic phylogeny or polyphyletic phylogeny of *Foc*, indicating that the differentiation of R1, R4, and 3Fo should have occurred in a short period, leaving only a weak phylogenetic signal in the genes.

Enrichment of HGT Events for *FUB* Genes

According to the above results, HGT should have played a role in producing some of the *Foc* genotypes observed in this study, such as the four isolates JB255, JB553, Race1-MW2, and Race1-MW40. To reveal the distribution of recombination events that led to the 15 analyzed genes in the *Fo* isolates, seven different methods implemented in RDP4 were applied, and the topology in **Figure 2D** was assumed to be the true infraspecies tree in the process. Three recombination events were detected to have occurred in the three housekeeping genes. Two of the

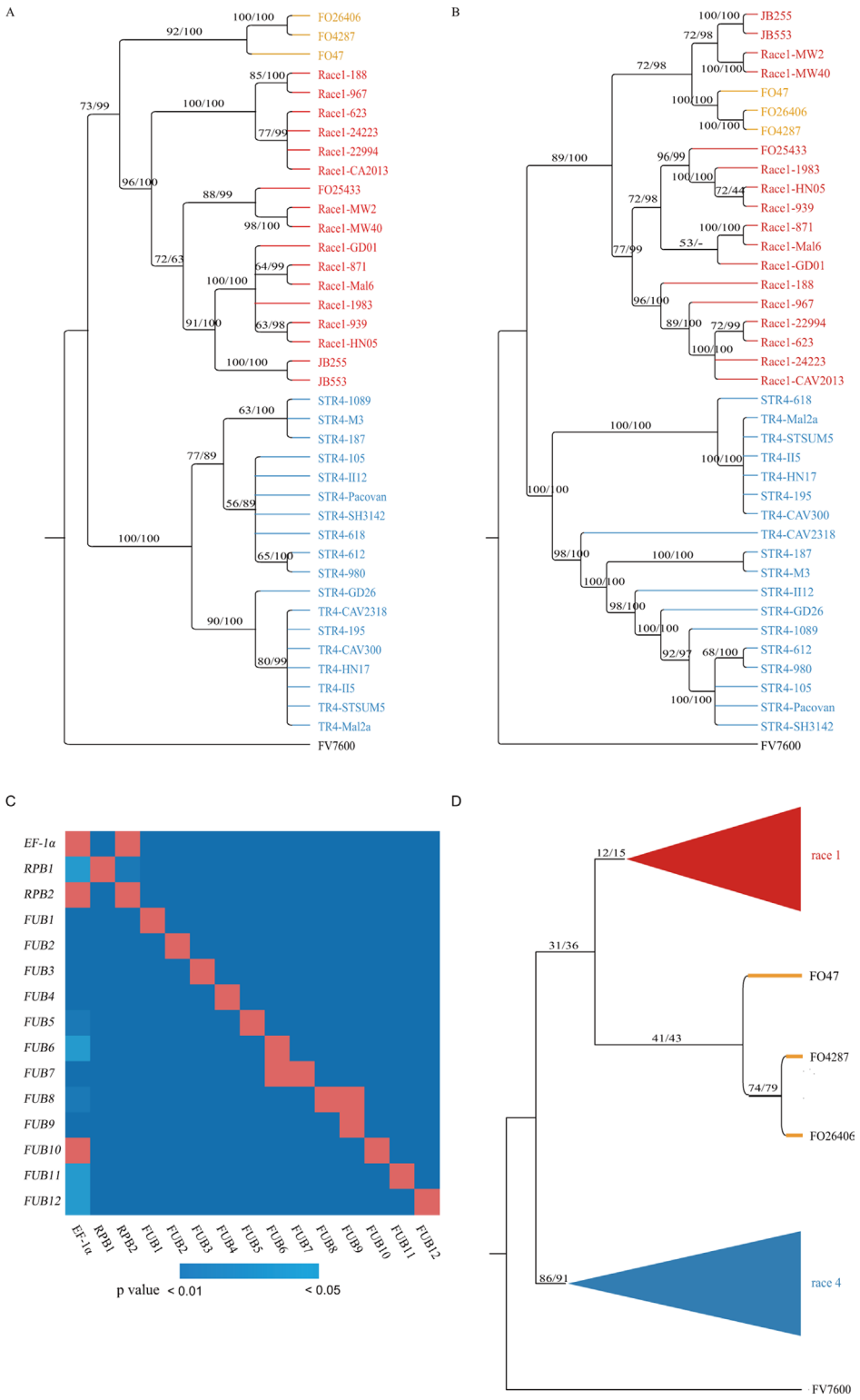

FIGURE 2 | *Foc* infraspecies phylogeny inference and discordance between phylogenetic trees based on different genes. **(A** and **B)** Phylogenetic trees constructed based on concatenated sequences of *EF-1α/RPB1/RPB2* and the 12 *FUB* genes, respectively. The support values on the branches are ML bootstrap proportions/BI posterior probabilities. R1, R4 and 3FO isolates were denoted by red, blue, and orange colors respectively. **(C)** SH test of the tree concordance between different genes. The best ML tree based on genes listed on the vertical axis was tested using the best tree and sequence of the gene listed on the horizontal axis. The color of the squares was determined by the p value of the SH test: blue indicated significance, and red indicated nonsignificance ($p > 0.05$). **(D)** Infraspecies tree inferred using the BSRRSS method. The numbers on branches are the support rates of the branch in 1,000 randomly sampled 1-kbp/2-kbp gene segments.

events were supported by only one method and were HGTs of the outgroup (which diverged earlier than the divergence of R1 and R4) *EF-1*α and *RPB2* genes into the 3Fo isolates, which could explain the conflict between the phylogenetic trees based on *EF-1*α, *RPB2*, and *RPB1*. The third recombination was identified within the R4 with support from two methods and involved HGT of partial *RPB1* of the TR4-STSUM5-like isolate into STR4-GD26. As shown in **Figure 3**, a significantly greater number of well-supported (detected by at least two methods and passing manual inspection) recombination events (14.7-fold, $p < 0.001$) were detected in the *FUB* gene cluster (1.41 recombination/kbp) than in the three housekeeping genes (0.096 recombination/kbp) in the *Fo* isolates. The recombination detection methods were supposed to detect only relatively large recombinant regions (Martin et al., 2015), and there should be an undetected recombination because the *PIsi* values were significantly higher in the *FUB* genes than in the three housekeeping genes in more regions (**Figure 1B**).

As shown in **Figure 3**, bottom panel, many more outgroup genetic materials were transferred into R1 than into R4, which partially explained why R1 had a much higher genetic diversity than R4. Large regions of horizontal gene-transferred outgroup genetic materials were observed in the *FUB* genes in Race1-MW2, Race1-MW40, JB553, and JB255. A recombination region that involved the horizontal transfer of ancient outgroup genetic material in *FUB5* was detected in II5 and five other R4 isolates, which should explain why the R4 had higher genetic diversity in only *FUB5*. In two R1 isolates, JB255 and JB553, which have lost their pathogenicity towards banana, *FUB1* was identified to be recombined with an outgroup isolate.

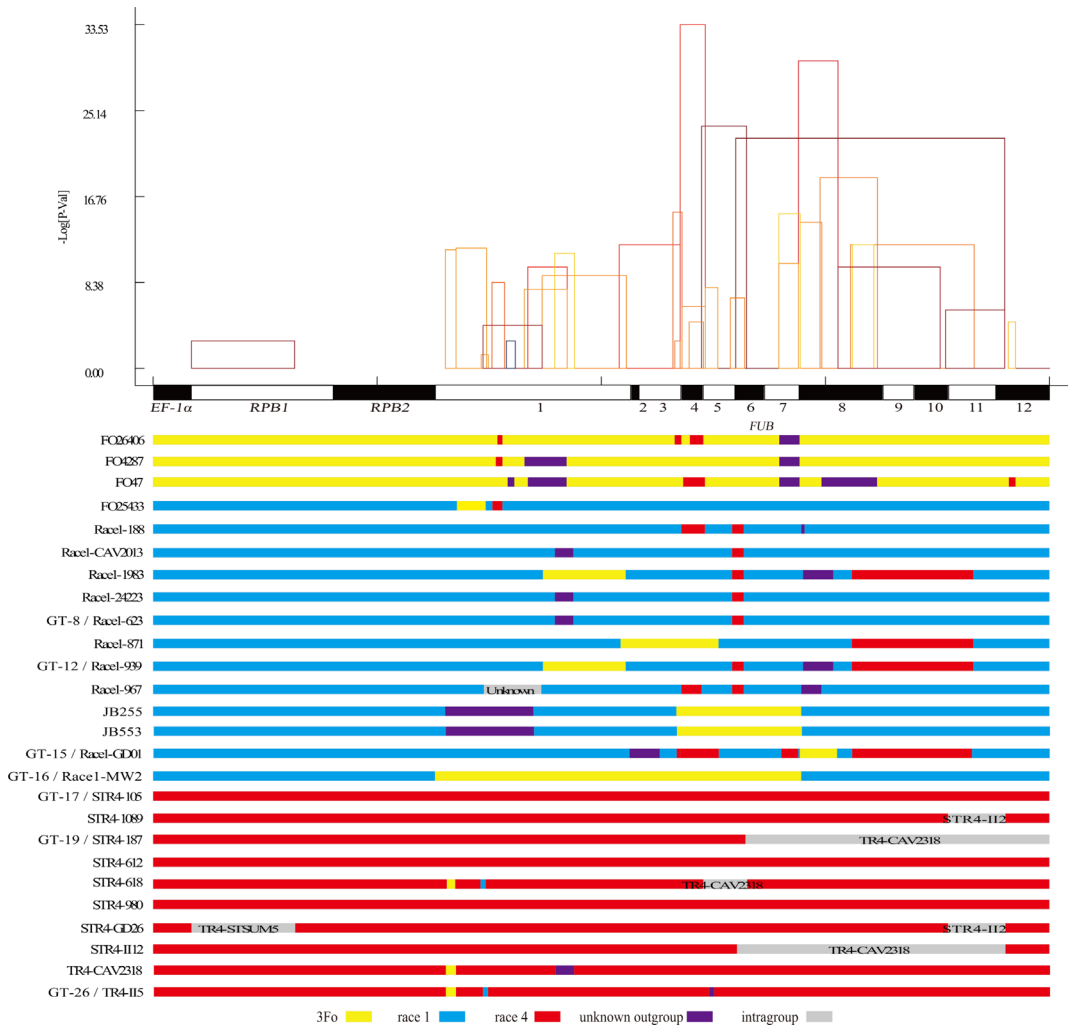

FIGURE 3 | Distribution of inferred recombination events in the 15 genes. Top panel: each rectangle represents an inferred recombination event, and the horizontal coordinates of the two vertical edges of the rectangles indicate the inferred most likely recombination points of the recombination event by RDP4. The height (vertical axis) of each rectangle is the lg (*p* value) of the recombination event inferred by the algorithm implemented in RDP4. Bottom panel: introgressed gene segments identified in the 26 different *Fo* genotypes. Gray bands indicate intragroup recombination events in R1, R4, or 3Fo, and the name of putative source isolate (most likely the isolate which is the most similar to the source isolate) was shown on the graph.

Selective Pressure on *Fo* Genes

The average *Ka*/*Ks* values for the 15 genes over different *Fo* isolates suggested that most coding loci on the 15 genes should have been subjected to negative selection (**Table S1**), because all of these values were much smaller than 1. The *FUB* genes other than *FUB11* had a significantly higher mean *Ka*/*Ks* values than each of the three housekeeping genes (**Figure 4**). The largest average *Ka*/*Ks* value was observed for *FUB2* (0.166 ± 0.122), and the *Ka*/*Ks* values between some isolates from 3Fo and R4 were as high as 0.84, suggesting that many loci of this gene could have been subjected to neutral evolution in some branches.

Branch-site models were applied to test for positive selection on the branches leading to R1, R4, and 3Fo groups. Positive selection was not identified in any of the tested branches for the three housekeeping genes and 12 *FUB* genes. Site analysis was carried out to test whether there were any codons subjected to positive or negative selection on the *FUB* genes. As shown in **Table S2**, 173 negatively selected amino acid sites but not a single positively selected site was detected ($p < 0.05$ by at least two methods) on the 12 *FUB* genes, showing that the translations of analyzed genes were mainly subjected to negative selection or neutral selection.

DISCUSSION

FA is an important virulence factor in *Fo* and other *Fusarium* species that are associated with the wilt symptom in banana and other plants (Li et al., 2013; Dong et al., 2014; Singh et al., 2017; Ding et al., 2018). Considering the function of FA involved in interactions with the host and environmental microorganisms (Brown et al., 2015), revealing the evolution characteristics of the *FUB* genes could help us understand how *Fo* isolates have evolved to accommodate different host types and environments. Indeed, in this study, we demonstrated that the genetic diversity and phylogeny of *Foc* isolates did have some specific characteristics in the 12 *FUB* genes compared with the housekeeping genes.

Our results showed that all 12 *FUB* genes in *Foc* were mainly subjected to negative selection according to selection pressure analysis (**Figure 4**) and that not a single highly deleterious mutation was identified on any of the 12 *FUB* genes in any isolate. The conservation of the *FUB* genes observed in this study suggested most of the FUB genes were functionally important, consistent with the report that FA is essential for the virulence of *Foc* against banana (Ding et al., 2018). Consistent with the report that the functions and importance of the 12 *FUB* genes are different (Brown et al., 2015), significant differences in selective pressure were found among the 12 *FUB* genes according to *Ka*/*Ks* analysis. The function of *FUB2* is unknown and seemed to be unimportant in deletion analysis (Brown et al., 2015); however, the *Ka*/*Ks* analysis suggested that *FUB2* was conserved in some phylogenetic branches, though it has the highest average *Ka*/*Ks* value and had probably been subjected to neutral evolution during the divergence of 3Fo and R4. Additionally, no single fixed variation was observed in *FUB2* between R1 and R4 (**Table S1**), which suggested that it could also have a function in some unknown circumstances. *FUB11* (FA transporter) and *FUB7* [*O*-acetylhomoserine (thiol-)lyase] are important in transporting FA from the intracellular space to the extracellular space and in FA production, respectively (Brown et al., 2015), and in this study, *FUB11* and *FUB7* were shown to be the most conserved of the 12 *FUB* genes by the *Ka*/*Ks* analysis. *FUB1* has been known as the key gene in the gene cluster and encodes a PKS, and a recombinant *FUB1* gene including a partial gene from an unknown outgroup isolate was observed in both JB255 and JB553, which have lost their pathogenicity towards banana. The correlation between the two facts deserves further experimental verification.

Significantly higher genetic diversity was observed in the *FUB* genes than in the three housekeeping genes, which is not explained by the difference in selective pressure. According to our analysis, the high genetic diversity of the *FUB* genes was mainly derived from significantly enriched introgression of outgroup genetic materials into the *Foc* groups, as shown in **Figures 1B** and **3**. In other words, recombination at *FUB* genes

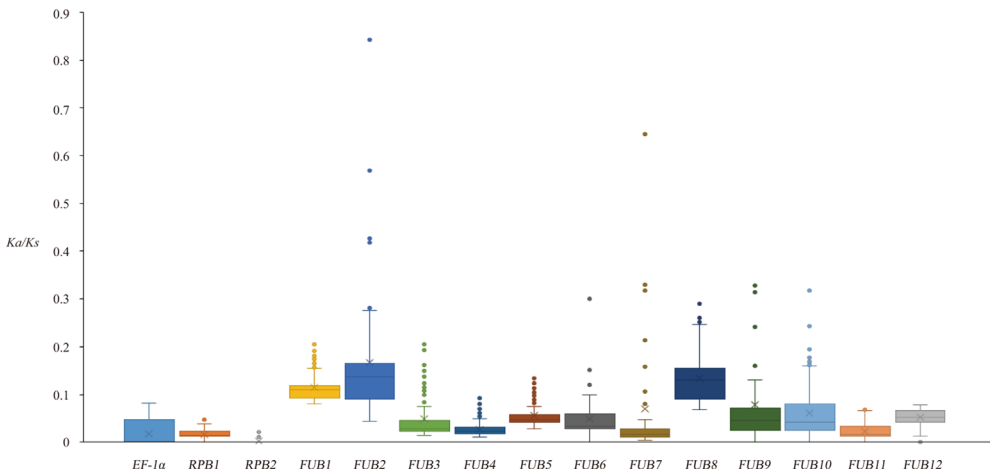

FIGURE 4 | Boxplot of *Ka/Ks* distribution between *Fo* isolates based on the 15 genes. The mean *Ka/Ks* value is indicated by the small crosses on the graph.

could have been positively selected, significantly enlarging the gene pool in *Foc* and other *formae speciales* of *Fo*. Because mutations in *FUB* genes have been mainly reported to affect the regulation and efficiency of FA production in *Fusarium* isolates (Brown et al., 2015), some recombination events should have the ability to change the pattern of FA production in *Fo* isolates. Considering that FA is involved in the interaction of *Fusarium* isolates with both plant hosts and environmental microorganisms (Brown et al., 2015; Bohni et al., 2016; López-Díaz et al., 2018), when the plant host and living environment are relatively stable, the *Foc FUB* genes should be mainly subjected to negative selection. However, when *Foc* isolates were brought into a new environment, recombination of the *FUB* genes with local *Fo* lineages or even other *Fusarium* species (ancient outgroup genetic materials were identified in some *Foc* isolates) could have a positive effect on their adaption, and this process is supposed to save much more time than developing new beneficial mutations. Several recombination events were also supposed to have occurred at the *SIX* genes, which encode the only identified family of effectors in *Fo*, as reported by Czislowski et al. (2018). These results suggested that recombination enrichment might be a common phenomenon for genes involved in pathogen–host interactions or interactions with environmental microorganisms in *Fo*; however, this hypothesis still needs further verification.

The previously inferred polyphyletic phylogeny (Fourie et al., 2009; Czislowski et al., 2018) of race 1, race 4, and *Foc* is not sufficiently robust according to our analysis. Polyphyletic phylogeny of *Foc* was inferred based on the concatenated sequence of *EF-1α*/*RPB1*/*RPB2* in the study of Czislowski et al. (2018), and the inferred infraspecies tree also supported that race 1 and race 4 should be two monophyletic groups. However, as shown in our results based on individual genes, only the gene tree based on *RPB1* supported the polyphyletic phylogeny of analyzed *Foc* isolates, while *EF-1α* and *RPB2* supported the opposite case, which indicated that the phylogenetic inference based on the concatenated sequence could have resulted in a well-supported (by bootstrap or posterior probability) but incorrect tree (Heled and Drummond, 2010). Moreover, recombination analysis suggested that a recombination event had occurred at RPB1 or that two separate recombination events had occurred at *EF-1α* and *RPB2*, respectively, in the 3Fo group, depending on which infraspecies tree is accurate. In addition, in the study of Czislowski et al. (2018) and our study, introgression of outgroup genetic materials was observed at both the *SIX* genes and the *FUB* genes in both R1 and R4 isolates, which could easily render gene trees supporting polyphyletic phylogeny of R1 and R4 similar to that discovered in a previous study (Fourie et al., 2009), although the difference in the results could also be caused by the difference in *Foc* isolates used. The BSRRSS analysis in this study showed that the polyphyletic phylogeny of *Foc* was supported by <40% of the regions of the 15 analyzed genes and that R1, R4, and 3Fo should have diverged within a short time period, which made the phylogeny signal representing the true intraspecific phylogeny relatively weak and easily covered by HGT events. This study also supported that Fo25433, which is a pathogen detected on cotton, should have originated from *Foc* race 1, and the infraspecies tree in the study of Czislowski et al. (2018) also suggested that many *formae speciales* of *Fo* should have originated from the *Foc* race 1. Considering the limited number of sequenced genes and the HGT commonly observed in all the studies, to achieve a highly reliable infraspecific phylogeny tree for *Fo*, it is necessary to sequence more genome regions of more isolates and resolve the complex recombination events in sequenced isolates first.

In conclusion, this study showed that negative selection on a majority of amino acids and enriched HGT events are the main evolutionary characteristics of the 12 *FUB* genes. Though no positive selection signal on any gene or amino acid has been detected in this study, the genetic diversity on the 12 *FUB* genes was greatly increased due to significantly enriched HGT events, suggesting some of the natural mutations could have an adaptive effect and positively selected. But such mutations were not detected in this study, either because they are too limited in number compared with synonymous mutations or because they do not exist in the coding regions. Infraspecies phylogeny analysis in this study suggested that *Foc* race 1, *Foc* race 4, and some other *Fo* f. sp. should have diverged in a short time, and the previously suggested polyphyly of *Foc* still needs more evidence.

AUTHOR CONTRIBUTIONS

SL, BW, and SXL analyzed the data and wrote the manuscript; ZS, RL, GY, CL and XG conceived and designed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01069/full#supplementary-material

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REVIEW
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The Epidemiology of Fusarium Wilt of Banana

Kenneth G. Pegg[1], Lindel M. Coates[1], Wayne T. O'Neill[1] and David W. Turner[2]*

[1] Ecosciences Precinct, Department of Agriculture and Fisheries, Brisbane, QLD, Australia, [2] School of Agriculture and Environment, Faculty of Science, The University of Western Australia, Perth, WA, Australia

Fusarium wilt of banana (also known as Panama disease) has been a problem in Australia since 1874. Race 1 of the pathogen (*Fusarium oxysporum* f. sp. *cubense*) is responsible for damage to 'Lady Finger' (AAB, Pome subgroup) and other less widely grown cultivars such as 'Ducasse' (Pisang Awak, ABB). Subtropical Race 4 (STR4) also affects these cultivars as well as Cavendish cultivars (AAA) in southern Queensland and northern New South Wales where cold temperature predisposition is involved. Tropical Race 4 (TR4) has led to the demise of the Cavendish industry in the Northern Territory, and its presence was confirmed in a North Queensland plantation in 2015, which warranted destruction of all banana plants on the property; as of this writing (April 2019), TR4 has spread to two adjacent properties. This review, which was commissioned by Biosecurity Queensland in response to the 2015 TR4 outbreak, considers the key epidemiological factors associated with the onset of a Fusarium wilt epidemic. Resistance to TR4, which is mediated by events following entry by the pathogen into the xylem, is not present in any commercially acceptable banana cultivar. Also, there is no effective chemical agent that can be used to manage the disease. Besides prevention, very early recognition and rapid containment of a disease outbreak are necessary to prevent epidemic development. A good understanding of the key factors responsible for disease development is required when devising practical protocols for the destruction of infected plants, treatment of surrounding infested soil, and reduction of inoculum in plant residues and soil.

Keywords: Fusarium wilt, *Fusarium oxysporum* f. sp. *cubense*, *Musa* spp., disease containment, infection process

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****Correspondence:***
Kenneth G. Pegg
Ken.Pegg@daf.qld.gov.au

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INTRODUCTION

"*Panama disease can transform a living plantation to a dead loss in a few months*" (Carefoot and Sprott, 1969).

This review of the epidemiology of Fusarium wilt of banana (Panama disease) is in response to an outbreak of the disease that was detected in a Cavendish (AAA) banana plantation at Tully (17.9329°S, 145.9236°E) in North Queensland, Australia in March 2015. Molecular and vegetative compatibility group (VCG) analyses showed that the pathogen [*Fusarium oxysporum* Schlecht. f. sp. *cubense* (E.F. Smith) Snyder and Hansen] (Foc) belonged in clonal population VCG 01213/16, colloquially known as tropical race 4 (TR4). This population, which is thought to have evolved with its banana host in the Indo-Malayan region (Ploetz and Pegg, 1999), was first detected in Australia in a Cavendish plantation near Darwin in the Northern Territory in 1997.

Foc is genetically heterogeneous and possibly has had several origins (polyphyletic). Vegetative compatibility has been used to characterize the pathogen with more than 20 VCGs being reported (Ploetz and Pegg, 1997; Ploetz, 2019). TR4 (VCG 01213/16) is just one of several distinct populations of Foc that can attack Cavendish (Pegg et al., 1993; Bentley et al., 1998; Ploetz and Pegg, 1999; Moore et al., 2001; Buddenhagen, 2009). It is much more aggressive than the so-called subtropical race 4 (STR4) strains that have been reported from Australia, Canary Islands and South Africa. STR4 strains attack Cavendish plants that have been predisposed by cold winter temperatures, soil water saturation, or drought, but are not known to attack Cavendish in the tropics. TR4 does not require predisposing factors to affect Cavendish, and susceptibility to this strain does not change with the physiological status of the host.

TR4 is a soil-borne fungus with strictly asexual reproduction, producing microconidia, macroconidia and chlamydospores as survival structures. It is extremely difficult to manage because the pathogen persists in soil or colonized host tissue by producing chlamydospores with a long survival potential, by growing as hyphae in organic residues, and by invading and surviving as asymptomatic endophytes in a range of non-host plants (Pittaway et al., 1999; Hennessy et al., 2005). In addition, the pathogen can cause disease at low inoculum levels, can reside at some depth in the soil, and the disease can have a long incubation period. Presently, there is no practical and effective way of detecting an infected plant until external symptoms are expressed.

Symptomatic plants are the basic parameter for studying the progress of the epidemic, and also for indicating the distribution of potential new inoculum that may be released into the soil. With all plant diseases there is an incubation period during which no symptoms are expressed in the host. The time between root infection and the development of Fusarium wilt symptoms can be between two to six months (Rishbeth, 1957). This long incubation period is influenced by the initial inoculum level, the susceptibility or resistance of the host plant, and prevailing environmental conditions. When symptoms are not evident it is difficult to know where and whether the pathogen is in a plantation. However, even in scarcely infested fields, infection eventually occurs as banana roots ramify though the soil, contact the fungus, and become infected. This process may take five or more years (Rishbeth and Naylor, 1957).

The ultimate solution to TR4, an acceptable resistant cultivar, is so far unavailable. Thus the application of exclusion and early quarantine measures is the only effective way of managing the disease. Once the pathogen is found in a new area, exclusion from non-infested plantations is difficult, especially if factors that influence the epidemiology and pathology of the disease are not understood. Once exclusion has failed, it is also extremely difficult to predict the extent and duration of quarantine measures that will be needed on an infested property as it will depend on the rate of disease development, the location of new disease foci, and whether banana production is continued on the site (Stover, 1962). Most of our current knowledge is derived from studies reviewed by Stover (1962) during the 'Gros Michel' era. The export trade (mainly in Central America) was based on this cultivar until the 1950s when it collapsed due to devastation caused by race 1. The amount of research on the epidemiology of Fusarium wilt declined significantly following the substitution of resistant Cavendish clones for susceptible 'Gros Michel.' The appearance of the less aggressive STR4 strains in the 1980s and 1990s did stimulate a lot of valuable research on pathogen diversity but little on the disease itself.

Little is known about the disease cycle and host pathogen interactions of TR4 (Buddenhagen, 2009); much more research is required as there are still many difficulties associated with the development and application of containment measures. A more effective method of detecting and destroying the host plant and treating the surrounding soil to disrupt the production and dispersal of initial propagules of the pathogen is a priority (Ordonez et al., 2015). In combination with established clean production methods to minimize pathogen dispersal (see the section *TR4 Control and Containment in Queensland, Australia*), this will slow down, but may not stop the spread of this threatening disease.

HISTORY OF THE DISEASE IN AUSTRALIA

The first report and description of Fusarium wilt of banana in the world was from Australia. In 1874, Dr Joseph Bancroft (**Figure 1**)

FIGURE 1 | Dr Joseph Bancroft (John Oxley Library).

found banana plants ('Sugar,' AAB, Silk subgroup) with a fungal wilt disease at Eagle Farm (27°S 431°E) near Brisbane (Bancroft, 1876). Bancroft's description of the symptoms leaves no doubt that he was dealing with a Fusarium wilt disease (Pegg et al., 1996). He recognized the ease by which the pathogen could be spread by vegetative propagation and advocated careful selection of disease free planting material. It is of interest to note that this was also the first recorded plant pathological investigation in Queensland. The disease was again recognized by Tryon (1912), who noted the susceptibility of 'Sugar' and 'Gros Michel' and the resistance of Cavendish.

In 1947, Magee and Simmonds independently reported the susceptibility of 'Lady Finger' (AAB, Pome subgroup) and recognized that this cultivar was not as susceptible as 'Sugar' or 'Gros Michel' (Magee, 1947; Simmonds, 1947). The 'Lady Finger' cultivar became widely grown, but by the 1960s Fusarium wilt was also recognized as a serious problem for this cultivar. The disease currently causes significant damage in 'Lady Finger' plantations in subtropical, eastern Australia.

Cavendish was regarded as being highly resistant to the disease, but in 1953 a small number of plants of the Cavendish cultivar 'Williams' were found affected by Fusarium wilt in southern Queensland (Purss, 1953). An isolate from 'Williams' was pathogenic to both Cavendish and 'Lady Finger,' but an isolate from 'Lady Finger' did not affect Cavendish. This was possibly the first occurrence of Fusarium wilt caused by STR4 in Australia.

The disease did not appear again in Cavendish plantations in southern Queensland until 1976 (RA Peterson, personal communication, 1976), but since only an occasional plant was affected, it was not recognized as being important. It was considered to be a break down in resistance in Cavendish due to poor soil conditions, as affected plants were growing in shallow clay soils subject to waterlogging. However, by the early 1980s many Cavendish plantations were being seriously affected in southern Queensland (Mayers, 1983) and northern New South Wales (Pegg and Langdon, 1987). Most outbreaks occurred in plantations where wilt susceptible 'Lady Finger' had been replaced with resistant Cavendish cultivars. These outbreaks were shown to be caused by a new strain of the fungus (Pegg and Langdon, 1987) which is now referred to as subtropical race 4 (STR4) (Ploetz et al., 1990; Ploetz, 2015). This strain was also found to be present, often with race 1 strains, in 'Lady Finger' plantations.

In 1992 Fusarium wilt was detected in 10 out of 153 Cavendish plantations at Carnarvon in Western Australia. A race 1 strain of the pathogen was involved and adverse environmental conditions (flooding and drought) were implicated in the disease outbreak. This strain of Foc was thought to have been introduced with windbreak banana plants (an edible diploid of *Musa acuminata*), which were imported directly from Java and Singapore before 1930 (Pegg et al., 1995; Shivas et al., 1995).

In 1997, an outbreak of Fusarium wilt occurred on Cavendish in the Northern Territory. This was the first detection of TR4 in Australia (Conde and Pitkethley, 2001). It has caused significant damage and a dramatic decline in commercial production of bananas in the Northern Territory. In March 2015, TR4 was detected in Australia's major banana production region near Tully in North Queensland (O'Neill et al., 2016) where it has created a landscape of fear.

INFECTION AND DISEASE PROGRESSION

Fusarium wilt of banana is a classical vascular wilt disease. The pathogen is considered to be a hemibiotroph, since the initial infection establishes a biotrophic relationship with the host but eventually transitions to a necrotroph where host tissue is killed (Dita et al., 2018; Ploetz, 2019). Multiple cycles of infection occur in a banana plantation that is infested with Foc (Ploetz, 2015). With the exception of sucker rhizomes being infected directly *via* vascular connection with diseased parent plants, all infections originate from secondary and tertiary roots (Wardlaw, 1961). The larger roots are rarely infected directly, and while the plant can occasionally be infected through the rhizome or pseudostem, such infections usually remain localized. When surface wounds or weevil borer (*Cosmopolites sordidus*) tunnels are invaded by the pathogen, there is some hyphal invasion of surrounding cells (Ploetz and Pegg, 1999). However, deeper invasion of rhizome tissue or leaf bases is prevented by the rapid formation of suberized protective barriers by the host plant. When a sucker is severed from the parent plant, there are numerous exposed injured vessels. These vessels rarely become infected, and if they do become infected, the pathogen rarely penetrates more than one centimeter before a protective barrier is produced by the host plant (Wardlaw, 1961).

Chlamydospores in the soil are stimulated to germinate by nutrients in the exudates from banana roots and non-hosts, or contact with pieces of non-colonized plant residues (Ploetz and Pegg, 1999; Dita et al., 2018). Those infecting the tips of secondary and tertiary roots of banana penetrate the root cap and zone of elongation and establish an intercellular parasitic relationship in the root cortex, before entering xylem vascular elements (**Figure 2**). The pathogen gains its nutrients from cell exudates rather than from the cell wall or the cytoplasm. The pathogen may also infect through natural wound sites along the roots. Living xylem is present in the banana root close to the apex, and as xylem becomes mature the vessels become vacuolated and the protoplasm and nucleus disappears from such elements. The living xylem is a barrier against the advance of the pathogen and the probability that the infection may reach the rhizome is low, estimated to be about one root in 20 by Rishbeth (1957). However the pathogen can move passively in the empty lumen of mature vessels (Trujillo, 1963). Initial movement through the roots can be slow, requiring some four weeks to advance 75 cm, but in mature xylem vessels, the pathogen can advance in surges of 30 cm in two to three days, with every new generation of spores. Once reaching the rhizome the pathogen can become distributed within the pseudostem in less than two weeks (MacHardy and Beckman, 1981) (**Figure 3**). Studies by Beckman et al. (1961) and Trujillo (1963) suggested that this colonization is facilitated by the extensive formation of conidia within the xylem elements, and that these spores move freely in the transpiration stream until they are temporarily blocked by the perforation plates at the end of the xylem vessels.

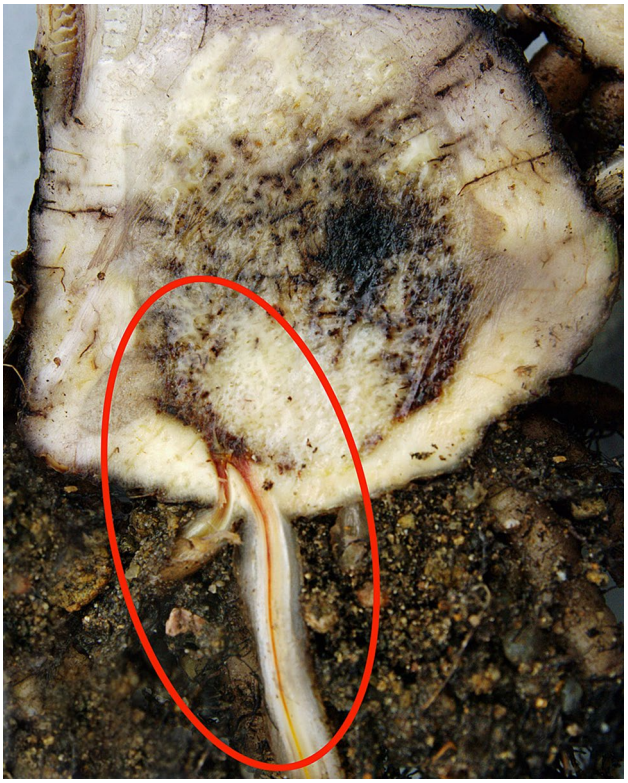

FIGURE 2 | Dark brown discoloration of vascular tissue in a banana root caused by Foc. (W. O'Neill).

FIGURE 3 | Vascular discoloration in xylem vessels (W. O'Neill).

Once impeded by the perforation plate, the microconidia reportedly germinate and germ tubes grow through the plates and again sporulate to produce more conidia. The sequence of trapping, germination, penetration and sporulation would allow the host to be colonized quite quickly. Wardlaw (1961) however reported slender, unbranched, hyphae growing in the vascular elements of 'Gros Michel.' Trujillo (1963) suggested that movement of the pathogen in the plant was too rapid to be explained by growth of mycelium, noting that Foc migrated from the rhizome to the top of a 12 m-tall plant in less than two weeks. However, Cook (1981) suggested that, although less efficient, banana plants may also be systemically infected *via* hyphal extension. In more recent studies of young glasshouse plants inoculated with GFP (green fluorescent protein)-transformed STR4, Warman and Aitken (2018) sometimes observed microconidia prior to any signs of hyphae, whereas at other times observed single or multiple strands of hyphae without the presence of any microconidia in the vascular elements of the pseudostem. Based on the studies conducted to date, it would appear that both microconidia and hyphae are present in the xylem of infected plants, but the relative importance of each in the infection process is yet to be precisely clarified. Although less efficient, spread within the xylem *via* hyphal growth should not be dismissed. The extent to which this occurs, and whether this method of host colonization depends on the banana cultivar and the identity of the pathogen should be investigated.

Infections trigger a host defense response involving gels, tyloses and lignification that leads to vascular occlusion. This response is present even in susceptible cultivars (Ploetz and Pegg, 1999). In addition to trapping by the perforation plates, the pathogen can be immobilized by gels forming on the upper side of these plates. These gels will not persist unless there are other host reactions in the xylem elements, and therefore only impede colonization for a short time. If the gel persists long enough to allow tyloses to form, and then become impregnated by phenolic materials, the pathogen can be successfully contained. In resistant bananas infection is checked by the rapid deployment of these host defenses in their rootlets, roots or at the root base (MacHardy and Beckman, 1981). In susceptible banana plants, after the pathogen has systemically invaded the xylem vessel elements with appreciable invasion of the rhizome (Simmonds, 1966), a severe water shortage develops due to vascular plugging. This impaired water movement leads to reduced transpiration and the expression of external symptoms. A common initial symptom is the appearance of a faint pale yellow streak at the base of the petiole of the oldest leaf. This is followed by leaf chlorosis which progresses from lower to upper leaves, wilting of leaves and longitudinal splitting of their bases. Splitting is more

common in young, rapidly growing plants (Stover, 1962) (**Figure 4**). Systemic invasion of the xylem vessels in the pseudostem does not necessarily need to occur for wilt symptoms to become apparent (Moore, 1995). The involvement of toxic metabolites in pathogenesis has been proposed. Fusaric acid which is produced by Foc and other *Fusarium* species is believed to contribute to symptom expression (Dong et al., 2014). The banana plant may be slow to show external symptoms because it has a vascular capacity two to three times its need for growth and reproduction (Beckman, 1990).

MacHardy and Beckman (1981) comment on the weak ability of Foc to invade living plant tissues. They describe how the fungus traverses the energy-rich cells of the root cortex to colonize the nutrient-poor, rather inhospitable xylem vessels encased in lignified walls. The xylem fluid contains only water which is low in carbohydrates, amino acids and minerals, has a low oxygen concentration and a fluctuating pH. Perhaps through its prolonged coevolution with its banana host, it has developed the ability to adapt to the isolated xylem environment where there is no microbial competition and where it can move freely with help from the transpiration stream to complete its life cycle. The transpiration stream will also deliver nutrients to the hyphae. Late in pathogenesis, when the vascular tissue has been fully colonized, the pathogen escapes from the xylem into the adjacent parenchyma and cortex to invade plant tissue weakened by water deficit. Chlamydospores and conidia are then produced in the degraded host tissue, and are released into the environment when these tissues collapse.

FIGURE 4 | External symptoms of Fusarium wilt in 'Lady Finger' banana: pseudostem splitting of leaf bases (W. O'Neill).

A number of recent studies have used GFP-transformed isolates of Foc to visualize the movement of the pathogen through the banana plant. Despite variation in the Foc strains, banana cultivars, plant age at inoculation and inoculum levels used in the individual studies, all demonstrated a similar pattern of root infection whereby the fungus directly penetrates the epidermal cells of the root tip followed by intercellular growth along the elongation zone (Xiao et al., 2013; Li et al., 2017; Warman and Aitken, 2018). By 10 days after inoculation with GFP-transformed Foc, the pathogen was found inside the xylem tissue of roots in Cavendish cv. B.F. plants (Xiao et al., 2013) and Cavendish cv. Williams (Warman and Aitken, 2018). Colonization of the roots, rhizome and lower pseudostem tissue of 'Lady Finger' plants by STR4 was markedly slower than in Cavendish plants (Warman and Aitken, 2018). Hyphae were confined to the xylem vessels of both Cavendish and 'Lady Finger' plants while the leaf sheaths were healthy and intact, but were observed in the gas spaces of leaf sheaths once the outer leaf sheaths and leaves began to senesce. At a more advanced stage of disease development, hyphae and sporodochia were observed protruding from stomata in the leaf sheaths. Production of chlamydospores also occurred, both internally within the gas chambers and externally on the outer surface of leaf sheaths. This suggests that senescent leaf sheaths are a significant source of Foc inoculum, and that cultural practices such as de-leafing may increase the risk of returning chlamydospores to the soil.

THE INFLUENCE OF CLIMATIC AND SOIL FACTORS ON DISEASE DEVELOPMENT

Many factors affect Fusarium wilt of banana under field conditions, most importantly the susceptibility of the banana cultivar. Plant development stage is also significant, as mature plants are more resistant than younger plants (Brake, 1990). However, weather events (prolonged wet or dry conditions, extremes in temperatures, storm damage) and soil conditions (poor soil drainage and aeration, unfavorable chemical or physical conditions) also have a major influence on the disease (Brake et al., 1995). Many of these factors directly impact the host plant and its response to the pathogen, whereas others have a direct effect on the pathogen.

Weather Events

Weather affects the incidence and severity of Fusarium wilt during infection, systemic infection of the xylem, and the development of wilt symptoms (Cook, 1981). Growth and survival of the pathogen in the root zone are favored by dry soil conditions under which the fungus is still able to extract sufficient water for its own growth and reproduction but is less likely to be antagonized by or compete with other microorganisms. After infection, disease development will depend on sufficient water being available for pathogen growth and dispersion in the xylem fluid. An internal

water deficit caused by dry conditions or waterlogging promotes symptom expression (Peng et al., 1999; Pattison et al., 2014).

Rainfall

In Puerto Rico, Fawcett (1913) noted that shortly after the wet season began the incidence of symptomatic plants increased. Rishbeth (1957) found that wilt incidence was greatest when conditions were most favorable for plant growth. He observed that there was a slow but steady appearance of diseased plants during dry conditions, but that the incidence of wilted plants increased four-fold following two months of heavy rainfall. Stover (1962), reported that the disease developed more slowly on 'Gros Michel' in Honduras in the dry season, and that wilt incidence was highest when rainfall and temperatures favored maximum plant growth. Simmonds (1966) suggested that drought depressed, and heavy rain favored the development of, the disease. He also indicated that an actively growing plant favored disease development.

Epp (1987) studied the epidemiology of Fusarium wilt in Cavendish type banana 'Umalag' for 4 years in the Philippines. He showed that symptoms developed after rain events, and that there was a correlation between heavy rains and increased disease incidence (**Figure 5**). He also noted that 95% of the symptomatic plants had bunched or were about to bunch; the disease was rarely found on Cavendish plants less than six months old, but even young plants of 'Gros Michel' succumbed, presumably due to their greater susceptibility. It should be noted that VCG 0122 (a far less aggressive population of the pathogen), rather than VCG 01213/16 (TR4), was probably involved in these studies, as VCG 01213/16 was not confirmed in Mindanao for another decade. Epp reports that he was able to control the disease by the early and accurate identification of infected plants, creation of adequate buffer zones around infected mats, good weed control and optimal nutrition. The successful management of Fusarium wilt using such interventions would be highly unlikely to have been possible in the presence of TR4 as it is much more aggressive than other known populations (Ploetz, 2019).

Temperature

Temperature is a critical factor in wilt development (Rishbeth, 1957). Peng et al. (1999) noted that the growth of Fusarium wilt pathogens is usually greatest at 28°C, and inhibited above 33°C and below 17°C. Stover (1962) also noted that plant growth was reduced and disease development was slow during winter, possibly due to reduced transpirational stress on the plant. It has also been proposed that cold winter temperatures in the subtropics predispose Cavendish to systemic infection by STR4 (Moore, 1995). The optimum temperature for growth of Cavendish is between 22 and 31°C (Turner and Lahav, 1983; Robinson 1990). The assimilation of carbon dioxide and rate of leaf emergence decrease when the temperature falls below 22°C, with growth ceasing at 14°C. At low temperatures, light bleaches chlorophyll, primary root growth stops, and tertiary roots die without the formation of new ones; thus, the uptake of water and nutrients is severely affected.

Moore (1995) studied the effect of winter temperatures on susceptible ('Williams') and resistant ('Dwarf Parfitt' Cavendish plants. In August, 'Dwarf Parfitt' had a higher F_v/F_m ratio (chlorophyll fluorescence induction) than 'Williams,' indicating that it sustained less damage due to winter chilling (**Table 1**). 'Dwarf Parfitt' maintained leaf area during winter, while that of 'Williams' declined. Even though 'Dwarf Parfitt' had a lower CO_2 assimilation efficiency per kg dry weight of total biomass than 'Williams' plants at the end of autumn, it maintained a higher efficiency than 'Williams' during cold weather.

At both 20 and 28°C, Brake et al. (1995) reported severe symptom development in plants inoculated with race 1 or STR4. In a glasshouse experiment, Peng et al. (1999) found that disease severity increased as temperature increased from 24 to 34°C. At 14°C (a temperature unfavorable for the growth of banana) symptoms did not develop but the pathogen could be isolated from the plants.

Cyclonic Weather

Rishbeth (1957) found that disease increased markedly following cyclonic weather. He attributed this to the production of many highly susceptible new roots by storm damaged plants. He had previously demonstrated that young roots of 'Gros Michel' were more susceptible to infection than older roots. Any catastrophic event that causes mechanical breakage of the root system will rapidly increase disease incidence and severity, especially if flooding or waterlogging occurs following the storm event.

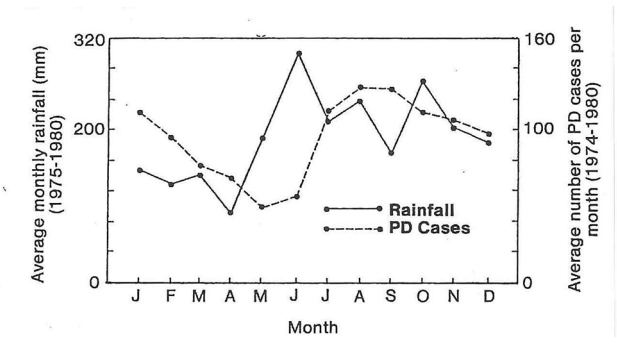

FIGURE 5 | The average monthly rainfall and number of Fusarium wilt cases per month show increased disease incidence peaking at three months after high rainfall (from Epp, 1987).

Soil Factors

Influence of Soil Type

Stover (1956) suggested that some soil factors weaken plant resistance and play a vital role in disease development. He

TABLE 1 | Chlorophyll fluorescence induction F_v/F_m ratio of Cavendish in winter and spring at Wamuran, Queensland (27.0359° S, 152.8627° E). Data are mean values of single leaves of three plants of each cultivar ± standard errors. From Moore (1995).

Chlorophyll Fluorescence Induction (F_v/F_m ratio)		
Cultivar/genotype	**F_v/F_m ratio**	
	Winter (August)	**Spring (October)**
Dwarf Parfitt	0.520 ± 0.038	0.722 ± 0.013
Williams	0.354 ± 0.061	0.653 ± 0.018

indicated that the disease is more serious in light sandy soils than in heavy clay soils. This is probably due to the effect of the sandy soil on the water relations of the host plant, but also *Fusarium* species are strongly aerobic and are favored by soil water contents of less than field capacity. Presumably there would also be less competition or antagonism from other soil microorganisms in the sandy soil.

Suppressive Soils

There are some banana soils with chemical, physical and microbiological properties that suppress disease development. These are referred to as suppressive soils, as opposed to conducive soils in which the disease progresses unrestrained on susceptible banana clones. Suppressive banana soils have been reported in Central and South America, Australia, Canary Islands and South Africa for races other than TR4. Even with TR4, one would expect some variation in the incidence and severity of the disease over time in different production areas.

Stover (1962) summarized studies on the impact of soil type on the development of Fusarium wilt on 'Gros Michel,' where different soils in Central America were initially categorized as 'non-resistant,' 'semi-resistant,' and 'resistant,' based on the rate of disease spread; they were later referred to as 'short-life' or 'long-life' soils. Reinking and Manns (1933) also described suppressive soils in Central America. Stotzky and Martin (1963) correlated suppressiveness with the presence of montmorillonoid clay in soils in Central America and Ecuador; they suggested that suppressiveness was associated with greater biological activity in these soils.

Although suppressive soils often have similar abiotic properties (higher pH, higher organic carbon content, clay texture), it is generally accepted that suppression is mainly through the impact of these properties on the saprophytic soil microflora. Soil microorganisms play a key role in suppressing soil-borne diseases, mainly *via* antagonism or competition (Cook and Baker, 1983; Alabouvette, 1986).

Control of Fusarium wilt in susceptible cultivars has not been achieved using practical biological or cultural methods. However, the existence of soils where *Fusarium* is naturally suppressed does indicate that such control may be possible with a better understanding of the physical and biological environment, pathogen activity and host susceptibility. Peng et al. (1999) observed differences in disease expression in a Cavendish planting at Carnarvon (25°S, 114°E) in Western Australia and found differences in chlamydospore germination and disease severity in laboratory and glasshouse tests using soil from areas where there were no wilted plants and soil nearby where disease was clearly evident. The suppressive soil had a higher population of actinomycetes and bacteria than the conducive soil, and the potassium concentration in the suppressive soil was 3.5 times higher.

In a study of 'Pisang Awak' and race 1, Pattison et al. (2014) demonstrated that symptom incidence and severity of Fusarium wilt was reduced with a ground cover of Pinto peanut (*Arachis pintoi*) that reduced water stress and enhanced soil microbial activity and diversity. Disease reduction was significant, but not great (20% compared to the non-ground cover treatment). Although it was not tested against TR4, the authors suggested that ground cover could reduce its impact in Cavendish somaclones which are not completely resistant to Fusarium wilt, but currently no such information is available.

Soil Water Content and Aeration

Both Rishbeth (1957) and Stover (1962) reported a higher incidence of wilt disease in poorly drained soil, when there was temporary flooding of the root zone. Stover (1962) found reduced activity of *Fusarium* in wet soils and attributed this to elevated levels of carbon dioxide which favor chlamydospore germination and hyphal development, but inhibit the formation of new chlamydospores, thus decreasing the population of the pathogen. However, he also suggested that water-saturated, oxygen-deficient conditions predispose the host to infection. Turner et al. (2007) noted that banana growth and productivity are negatively impacted by poor drainage due to low oxygen content. The first part of the banana root to die as oxygen is reduced in soil is the root tip. Roots begin to die if soil is waterlogged for more than six hours. Aguilar et al. (2000) found that soil flooding and the resultant hypoxia or anoxia greatly restrict oxygen to banana roots, which made them more susceptible to infection.

The Influence of Nematodes on Fusarium Wilt

In some plants, nematodes predispose the host to infection by Fusarium wilt pathogens. For example, the interaction between the root-knot nematode *Meloidogyne incognita* and strains of *Fusarium oxysporum* f. sp. *vasinfectum* causing Fusarium wilt of cotton in the USA is probably the most widely recognized disease complex in the world (Smith et al., 1981; Colyer, 2007).

Fusarium oxysporum was a common inhabitant of root lesions caused by the burrowing nematode (*Radopholus similis*), although isolates recovered by Blake (1961) were unable to invade the host vascular system. On Cavendish in the Philippines, Epp (1987) found no association between nematode infestations (*Radopholus*, *Meloidogyne*) and the incidence or severity of Fusarium wilt. However, with 'Gros Michel,' Loos (1959) reported that *R. similis* aggravated disease expression and reduced the time between inoculation with Foc and symptom expression, even though its presence was not necessary for symptoms to develop.

In 2010–2011, Cavendish plants growing in subtropical New South Wales succumbed to a race 1 clonal population (VCG 0124) (**Figure 6**). The plants were heavily infested with both *R. similis* and *Helicotylenchus multicinctus*, but Fusarium wilt symptoms did not develop in Cavendish in a glasshouse pathogenicity test with the isolate, alone or in combination with two different inoculation rates of *R. similis*.

Chemical Factors

Reports on the role of chemical factors on disease development, especially fertilizer applications and soil pH, are often contradictory. This can be attributed in part to the difficulty in separating their effects on the host from those on the pathogen.

Fusarium wilt diseases in other crops are usually most severe in sandy soils with a low pH. Thus, the adjustment of pH to near neutrality by liming is used to inhibit wilt development in a range

FIGURE 6 | Cavendish plant affected by Race 1 Foc (D. Peasley).

of crops. Disease suppressive banana soils in Jamaica and Central America had a neutral or slightly alkaline pH (Stover, 1962). Reducing the impact of Fusarium wilt diseases by liming soils may in part be due to limiting the availability of micronutrients to the fungus, as well as enhancing the activity of a competing microbial flora (Jones and Woltz, 1970). It is interesting to note that Peng et al. (1999), in pot experiments with conducive and suppressive soils from Carnarvon, Western Australia, found the reverse effect of pH. Disease developed more slowly in acid soils than in alkaline soils (pH 8) and they attributed this to the influence of pH on chlamydospore germination, as high pH favored chlamydospore germination. A survey by Turner et al. (1989) included one plantation at Carnarvon that had soil with a pH of 8.7 and a measured annual yield of 38 t/ha. This indicated that under the growing conditions and soils at Carnarvon, WA, bananas can be productive at a high pH, so it was important for Peng et al. (1999) to investigate the behavior of Foc with treatments in the high pH range.

Mineral nutrients that influence wilt development include nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), zinc (Zn), iron (Fe), and manganese (Mn) (Datnoff et al., 2007). Although none of these alone would be useful in reducing disease incidence or severity, maintaining a correct nutritional balance is important for plant health and prolonging the life of the plantation (Rishbeth, 1957).

Although N affects many plant diseases, its form rather than its total amount is often most important in these interactions (Huber and Watson, 1974). Regarding Fusarium wilts, nitrate (NO_3^-) reduces, whereas ammonium (NH_4^+) increases, their severity. For example, Simmonds (1966) noted that applying excessive ammonium sulfate was the most effective way of encouraging Fusarium wilt. Epp (1987) found that NO_3^- increased the time between the initial expression of symptoms and death of the plant compared to NH_4^+; regardless, all plants died regardless of the nitrogen source.

Brown et al. (1997) stated that ammonium nitrogen lowers the pH in the rhizosphere, as hydrogen ions are released into the soil as a result of nitrification. High rates of urea are used to treat infested soil during destruction and containment procedures. Ammonia (NH_3), produced by enzymic hydrolysis of urea, and nitrite (NO_2^-/HNO_2), produced during subsequent nitrification, are directly toxic to *Fusarium* propagules (Sequeira, 1963). Ammonia accumulation also occurs when high amounts of some forms of organic matter (e.g. chicken manure) are incorporated into soils, and this can lead to disease suppression through toxicity to the pathogen and by increasing the population of microbial antagonists.

"Other than nitrogen, calcium nutrition is perhaps the most important nutritional factor for management of diseases" (Rahman and Punja, 2007). Nevertheless, there are few examples that document the impact of calcium on a Fusarium wilt. Peng et al. (1999) added calcium carbonate, calcium hydroxide, calcium sulfate and iron chelates to soils in pot experiments and reduced disease severity and chlamydospore germination of Foc by one-third to one-half. Although it was unclear how/whether the calcium materials affected the development of Fusarium wilt, it was suggested that the iron chelate acted by reducing the availability of iron that is necessary for chlamydospore germination. There is a need to take such studies to the field to see what happens in the natural world.

Although the effect of K on disease development cannot be generalized, it can reduce the severity of plant diseases (Datnoff et al., 2007). Rishbeth (1957) indicated that soil potassium levels and Fusarium wilt incidence were inversely correlated, and that the highest correlations were where potassium levels were 3.3 times higher than surrounding areas. Similarly, a putatively suppressive soil at Carnarvon in Western Australia had 3.5 times more K than conducive soil (Peng et al., 1999).

Zinc is the most important minor element in bananas. Fernandez-Falcon et al. (2004) reported that wilt was more severe in two-month-old 'Dwarf Cavendish' plantlets with zinc deficiency. They indicated that there was relationship between zinc concentration and IAA levels, and the timely formation of tyloses. Tylose formation in the xylem is stimulated by IAA.

Stover (1990) reviewed studies in the Canary Islands where 'healthy' and 'diseased' soils were identified using chemical and physical parameters. The 'healthy' soils had good structure, permeability and drainage, high levels of organic matter, zinc, calcium and magnesium. Suppressive soils in Jamaica and Central America had very high phosphate and potassium levels (Simmonds, 1966). It is evident that highly fertile soils with good physical structure, and balanced fertilization will prolong the life of an infested plantation.

Although the potential for manipulating chemical factors to manage Fusarium wilt in banana has not been fully exploited, it is improbable that this disease could be fully controlled by altering soil pH or adding a specific nutrient. The complex soil environment makes this a most difficult goal, as it complicates the host × pathogen interaction and impacts how it and soil chemistry ultimately interact.

PATHOGEN SPREAD

Pathogen spread is either passive or active. Active spread (spread of an existing infection) depends on banana roots growing to the inoculum, whereas with passive spread (spread to new areas) the inoculum is carried to the roots.

The only means of active spread of the pathogen through the soil is from plant to plant by root proximity. The fungus is present in some of the major roots of an infected plant, and as the plant dies, these roots decay and spores are released into the soil. The short-lived secondary and tertiary roots are more likely to be infected than the major roots, and they will release a small but constant supply of inoculum into the soil while the plant is still growing (Stover, 1962). Roots of adjacent healthy banana plants will grow into the root zone of the diseased plants thus accounting for mat-to-mat spread.

Banana roots arise in groups of two to four from primordia at the inner edge of the cortex of the rhizome. These primary roots (main, cord) give rise to secondary and tertiary roots. Primary roots may be several meters long, secondary roots less than 1 m, and tertiary roots, which only remain functional for about three weeks, are several centimeters long. The primary roots produce the framework for the root system and spread well out from the plant. The secondary and tertiary roots explore the volume of soil near the primary, supporting root (Turner et al., 2007), and a small undetectable inoculum level in this soil can lead to serious disease. A single propagule entering the vascular system can multiply greatly inside the plant (Nash-Smith, 1970).

Active spread can be limited by quarantine of the affected plants and their neighbors. However, once passive spread is involved the situation changes dramatically and managing spread becomes much more difficult.

Passive spread may occur in the following ways:

- Movement of the pathogen in water can be significant. Outbreaks of the disease commonly occur in association with irrigation and/or flooding (Stover, 1962; Su et al., 1986) (**Figure 7**). When plantations have been irrigated from contaminated dams or rivers, and when flood waters from infested land have inundated alluvial river valleys, propagules of the pathogen and infested organic residues can move considerable distances. The chlamydospores will survive better in a running river than in dam water due to superior aeration. Stover (1962) found that a large percentage of chlamydospores die after 40 days of submersion in still water, such as in dams. They survive for much longer in oxygenated water, such as in rivers. He also noted that Foc can tolerate lack of oxygen in soil for a long period, but suggested that it was lack of free oxygen plus submerged soil conditions that are the destructive factors in dams. Stover found that submerged fresh pseudostems were destroyed in 2–3 months as a result of anaerobic decay.
- If a dam is located immediately below an infested plantation, inflowing water becomes a constant threat as a source of inoculum in the dam water. As most spores sink in a day or two, it is best to take irrigation water from the surface layers of the dam and not irrigate until at least 2 days after surface run-off (from a rain event) into the dam (Deacon, 1984). Rattink (1990) also showed that microconidia of the cyclamen vascular pathogen *Fusarium oxysporum* f. sp. *cyclaminis* settled to the bottom of water containers within 24 h. This further indicates that water should not be pumped from the bottom of the dam. An original Queensland Banana Industry Protection Board recommendation for dealing with Fusarium wilt states that "Irrigation water should be taken from areas which are not contaminated by surface run-off from diseased plantations. A flotation inlet for the irrigation system should be used because fungal spores are heavy and most will sink after a day or two" (BIPB, 1989). Regardless of what precautions may be employed, irrigating from a potentially contaminated water supply is always a very risky practice.
- The pathogen can be moved in infested soil by both humans (farm machinery, vehicles, shoes and clothing of farm staff) and animals (movement greater on sticky clay soils).
- Asymptomatic planting material, either infected with or contaminated by the pathogen, is probably the most effective means of local, national and international dispersal (Stover, 1962). Su et al. (1986) found that 30–40% of suckers taken from a diseased plantation in Taiwan were infected even though they were free of symptoms, and symptoms may develop up to two years after planting such material (Rishbeth, 1957; Deacon, 1984). Tissue cultured plantlets grown under aseptic conditions in accredited nurseries with high standards of hygiene are the most reliable source of disease-free planting material.
- Infested dust and infected dry leaf debris can disperse the pathogen, as chlamydospores survive for months under dry conditions.
- The pathogen infects banana leaves, which are often used to transport fruit, as well as banana bunch stalks (**Figure 8**), which are often put back into the plantation (Deacon, 1984). Infected leaves and stalks can both initiate new disease foci.
- In infected banana fibers and other pieces of banana tissue that cling to the blade of the cane knife (machete) (Rishbeth, 1955). Rishbeth (1955) found that a machete used to cut the pseudostem of an infected plant carried 3,000 viable conidia,

FIGURE 7 | Aerial view of a cotton field affected by Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*) showing spread along rows with flood irrigation water (J. Kochman)

FIGURE 8 | Vascular discoloration in Foc infected bunch stalk (W. O'Neill).

FIGURE 9 | Collection of laticifer fluid and xylem sap from an infected plant for epidemiology studies (W. O'Neill).

and that a single drop of sap contained the same number of microconidia. The sap can dribble from the machete blade and contaminate the soil. He also suggested that the spores in the sap on the blade will remain viable for several days and be a potent source of inoculum for other plantings. Wardlaw (1961), quoting Stover (1954, 1956), suggested that green diseased pseudostems only show sparse growth of hyphae in discolored vascular strands, and sporulation is sparse or absent. It was also suggested that the abundant sap exuding from the cut end of such pseudostems is free from conidia but contains fragments of hyphae. There is the need to re-assess the risk sap poses during the detection and destruction of an infected plant. Nevertheless, cane knives should be cleaned and sterilized between plants. In their sap studies, Rishbeth (1955) and Stover (1954, 1956) did not differentiate between the laticifer, phloem and xylem fluids. The initial sap that exudes from a cut pseudostem comes from laticifers. The osmotic potential of the contents of the laticifer is lower (more negative) than the surrounding tissues, so they have a positive turgor. The contents of the laticifer exude when cut because the cutting sets the turgor pressure of the laticifer to zero, and then the osmotic potential gradient between the latex and surrounding cells causes water to flow into the laticifer along its length. This causes the sap to flow out of the cut surface of the pseudostem (**Figure 9**). Sap stops flowing when the osmotic gradient becomes zero. Once the laticifers empty, the xylem will start to exude fluid because of root pressure. Laticifer sap is quite milky whereas xylem fluid is quite clear. Foc does not grow in a healthy laticifer. If it was infected it would lose turgor. It is the xylem vessels that harbor the pathogen.

– There is considerable potential for insects to spread conidia from plant to plant. Meldrum et al. (2013) found the pathogen on exoskeletons of the banana weevil borer, *C. sordidus*, and suggested that it may be a vector.
– There is no evidence of dispersal in fruit, even when the bunch stalk is infected. Fruit from seriously infected plants rarely produce a marketable bunch. However, the Australian Government considered that the pathogen could move as symptomless infections of the vasculature of fruit crowns (DAFF, 2004).
– Sporodochia (macroconidia bearing hyphal masses) could provide local dispersal of TR4 directly by rain splash, or by being washed into the soil. Although these structures have been observed on the stems of infected banana plants in a glasshouse, their role in the field needs to be examined (Ploetz, 2015).

DISEASE SPREAD

This section is concerned with the spread of the disease as distinct from the dispersal of the pathogen. It involves the banana plant and its reaction to the pathogen and the environment.

An epidemic of the disease usually begins with one or a few affected plants. The spread from one plant to another takes place essentially by overlapping roots of interconnected mats (active spread), whereas new centers of infection result from the passive dispersal of inoculum. Stover and Waite (1954) suggested that high inoculum levels were necessary for rapid disease development as this increased the number of roots that were infected, and the possibility that the pathogen would enter the rhizome. Intensive cultivation, vehicle movement, failure by farm staff to apply basic farm hygiene procedures and (perhaps most importantly) frequent irrigation from a contaminated water source, will greatly facilitate the development of new foci and accelerate the epidemic.

Stover (1962) noted that the disease spread more slowly on small, compared to large, plantations of 'Gros Michel.' He attributed this to small farms being separated by banana-free blocks of land that slowed disease spread. Also, small farms may rely on rainfall and not have irrigation water which can spread the pathogen.

When populations of the pathogen are low in soil, new foci usually appear as single or paired plants, often some distance from previous centers of disease (Stover, 1962; Deacon, 1984). There may also be a long incubation period before symptoms appear. However, as the epidemic progresses and inoculum increases, symptoms develop more rapidly and clumps of six to

twenty or more plants are affected in a random pattern (Stover, 1962). Fawcett (1913) also noted that when inoculum levels were low, only isolated cases of the disease were found and external symptoms in such plants were not expressed until after bunch initiation. He also reported that there was 'dwarfing or stunting' of plants in fields where the disease had been present for some time and inoculum levels had increased.

The fungus can cause disease at very low initial inoculum levels (Nash-Smith, 1970). Since the banana plant remains in the soil for many years (Stover and Waite, 1954) and the roots search through a large volume of soil, the probability that these roots will contact inoculum present in the soil is high. Thus, limiting inoculum production is an important management goal.

SURVIVAL

TR4 is a soil-borne fungus that is well adapted to long-term survival in soil. It readily forms chlamydospores, which remain dormant in the remnants of decayed host tissue until stimulated to germinate by root exudates from banana, alternative hosts or pieces of fresh plant remains (Stover, 1962). It is virtually impossible to eliminate from infested soil by crop rotation and even by bare fallowing. Stover and Waite (1954) mention a survival figure of 40 years for Foc in abandoned fields but there are many suggestions that it may be much longer.

Chlamydospores produced by Foc in dead and dying banana plants are released into the soil when the plant material decays (**Figure 10**). They are undoubtedly the most important survival propagule of the pathogen, but it is difficult to determine how long they survive naturally in the soil. Chlamydospores can persist for an extended period in plant debris in soil in the absence of a suitable host plant.

When examining infested banana soil, Trujillo and Snyder (1963) found chlamydospores associated with the cortical tissue of decayed banana roots, but could not find them in the decayed tissue of other plants. Their presence in soil without plant tissue was difficult to ascertain. They found them to be very sparse and unevenly distributed in the soil and suggested that chlamydospores in plant tissue survive longer than those unprotected in the soil. Chlamydospores of the Fusarium wilt pathogen of date palm (*Fusarium oxysporum* f. sp. *albedinis* Gordon) are known to persist in soil at a depth of 1 m (Louvet and Toutain, 1981). It is thought that chlamydospores of Foc are probably found at a similar depth in the soil to which the primary roots can penetrate.

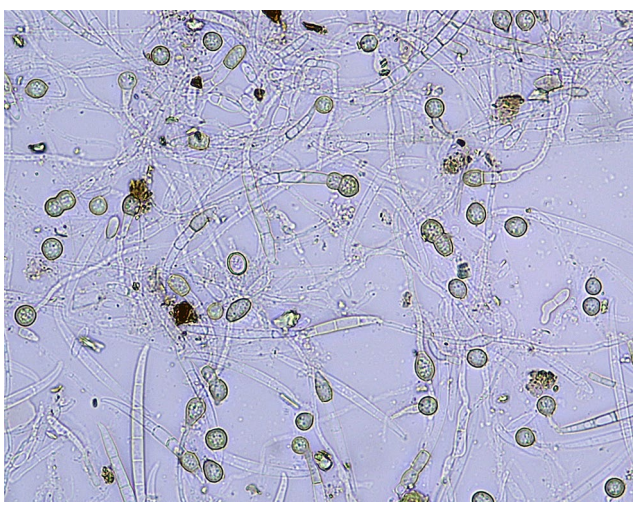

FIGURE 10 | Chlamydospore production in a Foc culture (W. O'Neill).

Stover (1962) suggested the long survival in soil is indicative of multiplication on wild hosts or to its saprophytic ability, and suggested it was a soil saprophyte with pathogenic potential. However it was not a frequent saprophytic colonizer of dead banana tissue in soil. This suggests that chlamydospores in plant tissue in the soil originated from a living plant. Early studies do not indicate how these propagules survived; whether in a dormant state or by germination and formation of new chlamydospores when activated by nutrients. Macro- and microconidia are found on the surface of dead plants, but are very fragile and die quickly when exposed to sunlight (Manicom, 1989).

Better understandings are needed for the formation, germination and survival of chlamydospores, as it could inform the containment of TR4. Nonetheless, the ability of the pathogen to colonize non-susceptible weeds and grasses complicates containment (see the section *Alternative Hosts* below). It can be seen from this review that the majority of studies on Foc survival in soil are more than 50 years old. This brings into sharp relief the critical need for more research on survival of the pathogen and inoculum management, especially given the continuing spread of TR4 epidemics.

To summarize, these studies indicate that the pathogen in soil originates in tissues of diseased banana plants which were colonized while the plant was still alive. The highest populations of the fungus occurred around a diseased plant, and they declined significantly once affected plants were removed (Stover, 1962). This illustrates the importance of the current destruction protocol for TR4, which is designed to reduce the amount of inoculum reaching the soil. Early detection is essential; once the vascular pathogen reaches the leaf lamina, the fungus is no longer confined to the xylem; it will move into the phloem and parenchyma and produce chlamydospores and microconidia (Wardlaw, 1961).

Flood fallowing was used extensively at the end of the 'Gros Michel' era where production occurred in alluvial flood plains (where flooding was possible). In general, pathogen survival was minimized in saturated soils after 6 weeks (Stover, 1954). However, since flooding reduced the populations of most soil organisms, recontamination of the resulting biological vacuum by the pathogen was a routine occurrence (Stover, 1962). Crop rotation with paddy rice was considered in Taiwan. Su et al. (1986) found that in submerged soils, the population of Foc dropped to a non-detectable level within 4 months. Field tests showed that rotation with paddy rice for 1 and 3 years reduced the disease incidence from 40 to 12.7% after 1 year and 3.6% after 3 years. Disease incidence was not decreased when infested banana fields were rotated with alternate crops such as sugarcane or sunflowers

for 3 years. Crop rotation with a perennial pineapple crop in TR4 infested soil in Northern Territory, Australia, allows the land to be re-planted with banana to produce two cycles (plant crop and first ratoon). Readers who are interested in crop rotation should refer to Dita et al. (2018).

ALTERNATIVE HOSTS

At an international symposium on soil-borne plant pathogens in 1963, S. D. Garret (1970) stated that despite the great volume of work done on the non-host phase of Foc, "it is still uncertain which is the most important among several modes of survival in the non-host phase." This statement is still applicable.

Chlamydospores of Foc in dead plant material are generally accepted as the major means of its survival, but its persistence may be primarily due to its capacity to colonize the outer root cells of the epidermis and cortex of grasses and weeds as asymptomatic endophytes (Ploetz, 2015). Armstrong and Armstrong (1948) first showed that vascular wilt Fusaria were able to parasitize the roots of plants without pathogenesis.

Grass and weed populations may serve as a reservoir of inoculum that influences the occurrence of disease in banana plantations. They are also possibly involved in the contamination of irrigation sources and rivers with the pathogen. However, alternative hosts are usually regarded as a mechanism of long-term survival and not for the build-up of the fungus in the soil. A better understanding is needed of the importance of alternative hosts when managing TR4.

Waite and Dunlap (1953) isolated Foc (presumably race 1) from alternative grass and weed hosts. They collected grass and weed species from a field in Honduras with a high incidence of Fusarium wilt, planted them in a sterile sandy loam and inoculated them with Foc grown in corn meal sand. Of many plants tested, three species of grass [*Paspalum fasciculatum* Willd., *Panicum purpurascens* Raddi, *Ixophorus unisetus* (Presl.) Schlecht.], and one herb (*Commelina diffusa*) were infected. They also recovered Foc from the roots of these plants in the field.

In Taiwan, Su et al. (1986) examined 25 species of weed and eight rotation crops growing in a race 4-infested field. Three sedges (*Cyperus iria* L., *Cyperus rotundus* L., *Fimbristylis koidzumiana* Ohwi) and one herb (*Gnaphalium purpureum* L.) were infected, and they confirmed the pathogenicity of these isolates on Cavendish.

In Australia, Pittaway et al. (1999) reported that STR4 (VCG 0120) was isolated from surface sterilized roots of a grass (*Paspalum* sp.) and a weed (*Amaranthus* sp.) in soil adjacent to Cavendish plants affected by STR4 in southern Queensland. In 2005, 18 species were collected from within a 1-m radius of Cavendish bananas affected by TR4 in the Northern Territory (Hennessy et al., 2005). TR4 (VCG 01213) was isolated from four species: *Chloris inflata* (a common grass), *Euphorbia heterophylla* (Euphorbiaceae), and *Cyanthilium cinereum* and *Tridax procumbens* (Asteraceae).

The presence of Foc as asymptomatic endophytes in weeds and grasses calls into question the value of current containment protocols for Fusarium wilt where the focus is on killing infected banana plants and asymptomatic hosts are largely ignored. Their importance as reservoirs for the pathogen and as potential vehicles of spread should be examined under natural conditions. Likewise, whether their removal would be helpful also requires study.

Since most alternative host studies have been conducted either in glasshouse trials or in heavily infested field soils, these results may well overstate the capacity of the plant species to be an alternative host under natural field conditions. Based on the old literature, it is clear that Foc is capable of surviving at very low levels which, nonetheless, provide sufficient inoculum to start epidemic disease development. With modern techniques it should be possible to follow the survival of TR4 in soil in the absence of a banana host and understand the importance of asymptomatic weed infection and/or chlamydospore survival in perpetuating this pathogen.

UREA TO REDUCE POPULATIONS OF *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* IN SOILS

Urea, when incorporated in soil, reduces the populations of certain soil-borne fungi by the production of volatile ammonia upon hydrolysis (Tsao and Oster, 1981; Chang and Chang, 1999). In the containment of TR4 in North Queensland, urea is utilized to reduce the soil population of the fungus. Regular surveillance results in the early detection of wilt symptoms. Once the pathogen is confirmed, affected plants are injected with herbicide and a high rate of urea (1 kg per square meter) is applied to the soil surface surrounding the diseased plants to reduce propagules of the fungus in the soil. The area is then covered with plastic sheeting to produce a lethal concentration of ammonia. There is currently no systemic fungicide available for killing the pathogen in an affected pseudostem. As the pseudostem and leaves are a potent source of inoculum, they are chopped up and placed in large plastic bags and 1 kg of urea is added to each bag (Persley et al., 1989). When the urea treatment was used previously to contain an outbreak of STR4 in South-east Queensland and northern New South Wales, symptomatic plants were not injected with herbicide prior to bagging. This was because killing by herbicide is thought to predispose plants to rapid fungal colonization with a greater production of inoculum in the affected plant. For this reason, the use of herbicide in the current TR4 destruction protocol is being reviewed.

Urease, which plays a vital role in overall nitrogen metabolism and catalyzes the hydrolysis of urea to give volatile ammonia, occurs in many bacteria, yeasts and higher plants. Production in fungi is variable (Veverka et al., 2007). Foc contains urease (Sequeira, 1963) and it is also present in the growing point and floral shoot apex of the banana plant (Freiberg and Payne, 1957) and these sources likely catalyze the urea to produce ammonia when the infected plant material is bagged.

Although volatile ammonia produced from the hydrolysis of urea causes a rapid decline in the population of *Fusarium*, nitrite accumulation is considered to provide an additional longer term source of fungicidal action where ammonia levels have been

sufficiently high to inhibit the activity of *Nitrobacter*, which converts nitrite to nitrate (Sequeira, 1963; Smiley et al., 1970; Loffler et al., 1986).

The urea treatment would be expected to kill Foc present in infected banana roots. Chang and Chang (1999) found that 3,000 mg kg^{-1} of urea incorporated in soil to give 400 mg kg^{-1} ammonia completely killed *Phellinus noxius*, which causes brown root disease in numerous orchard and forest tree species, in wood pieces less than 3 cm in diameter.

Ammonia is reported to be more effective in soils with relatively low soil moisture (–0.75 to –0.15 MPa) than in soils with high soil moisture (–0.025 MPa) (Chang and Chang, 1999). In wet soils the ammonia dissolves in water to form the ammonium ion and would not be present as a fumigant. Ammonium/ammonia and nitrite/nitrous acid each exist in equilibrium in aqueous solution. The non-ionized form of each compound penetrates cell membranes more easily and is thus more toxic to Foc than the ionized form (Tsao and Oster, 1981). The ammonium ion generated in moist soils may eventually stimulate the activity of an antagonistic microflora which further reduces the survival of Foc.

Many nitrogenous amendments are phytotoxic when used in high concentrations. When urea is applied at a high rate, ammonium toxicity and alkalinity, along with nitrite accumulation, will damage plants. Crop safety is not an issue when using urea to treat soil in the vicinity of an infected banana plant. It may even have a positive effect in that soil toxicity will be detrimental to the health of alternative weed hosts.

FUNGICIDES TO REDUCE INOCULUM PRODUCTION IN PLANTS DURING THE DESTRUCTION PROCESS

While fungicides have not been effective for managing Fusarium wilt in the field, they could reduce inoculum when infected plants are destroyed in an eradication or containment program. Injection of pseudostems with herbicides is a common strategy for destroying infected and associated plants; however, dead and dying plants could continue to be a potent source of inoculum. During the death of an infected plant, there is a high likelihood that the fungus will move from the colonized xylem vessels into the surrounding parenchyma and cortical tissues, where it would undoubtedly produce many microconidia and chlamydospores unless the infected plant material is treated in some way. In Cavendish and 'Lady Finger' plants inoculated with GFP-labelled STR4, Warman and Aitken (2018) demonstrated the production of sporodochia, hyphae and chlamydospores on the outer surface of senescing leaf sheaths, as well as chlamydospores in the gas spaces of senescing leaf sheaths. This result suggests that the use of herbicides is likely to encourage rapid colonization of senescing plant material, thus increasing inoculum potential.

Injection of infected pseudostems with an appropriate fungicide at the time of herbicide injection could provide some reduction in fungal sporulation, although which fungicides would do so is unclear. In India, injection of rhizomes with carbendazim (3 ml of a 2% suspension) was reported to protect 'Rasthali' (AAB, 'Silk' subgroup) bananas against the disease (Lakshmanan et al., 1987), but this was done within the context of disease management rather than plant destruction/inoculum reduction. While rhizome injection with carbendazim in association with an herbicide would likely reduce inoculum production in dead and dying banana plant tissue, its use, or the use of other fungicides, may raise health and safety concerns for those applying the treatments. Other fungicides may be appropriate for this purpose but require further evaluation. Nel et al. (2007) reported that prochloraz and propiconazole significantly inhibited *in vitro* mycelial growth of STR4, with prochloraz giving complete inhibition at all concentrations tested (1–100 μg ml^{-1}). Prochloraz and propiconazole were also highly effective when applied as a root dip to 'Chinese Cavendish' plants inoculated with STR4, reducing disease severity by 80% and 75% respectively compared with control plants. From a health and safety perspective, these fungicides may be more appropriate alternatives to the benzimidazole fungicides such as carbendazim and benomyl, although studies are required to establish their ability to reduce sporulation of Foc in infected banana tissue treated with herbicides, as well as their mobility in infected plant tissue following injection.

TR4 CONTROL AND CONTAINMENT IN QUEENSLAND, AUSTRALIA

Following the confirmation of a TR4 incursion in Queensland in 2015, a cooperative effort between the State Government and the banana industry resulted in the rapid containment of affected sites, implementation of best-bet control strategies and accelerated research on management options. This program has resulted in minimal spread of the disease, with only three properties affected more than four years later. The key aspects of this program have been:

- An intensive surveillance and diagnostics program for early detection of diseased plants.
- Destruction of symptomatic and surrounding healthy plants at disease foci (including the use of urea, as detailed in the section Urea to Reduce Populations of *Fusarium oxysporum* f. sp. *cubense* in Soils), decontamination of all equipment and personnel involved in the destruction process, and restriction of further entry into affected areas.
- Implementation of on-farm biosecurity practices and hygiene, including farm zoning to restrict movements onto (and within) banana farms, and the use of disinfectants in footbaths and for vehicle and machinery decontamination.
- Use of clean (tissue cultured) planting material.

Nguyen et al. (2019) found that quaternary ammonium compounds containing a minimum of 10% active ingredient were the most effective against race 1 and TR4. When used at a 1:100 dilution, the survival of all Foc propagules was completely inhibited, regardless of the absence or presence of soil (at 0.05 g ml^{-1}). Further resources are available online *via* the Australian Banana Growers Council and Biosecurity Queensland websites[1].

[1]https://abgc.org.au/panama-tropical-race-4/ https://publications.qld.gov.au/dataset/panama-disease-tropical-race-4-grower-kit

THE EPIDEMIOLOGY APPROACH TO CONTAINMENT

Epidemiology (derived from the Greek epi = on, demos = population) is concerned with disease on populations of plants; it recognizes the influence of the environment, and the importance of time in disease development (MacKenzie et al., 1983; Brown et al., 1997). Quantitative epidemiology is all about numbers, and for Fusarium wilt might consider the number of pathogen propagules that reside in soil or infected plants, the number of successful infections that enter the rhizome, the ratio of diseased to healthy plants, the number of days for disease latency (inoculum generation period) and incubation (infection to symptom expression period), and the number of days that an epidemic lasts.

The epidemiological implications of the source of the initial inoculum, the means of pathogen dispersal, sanitation strategies, host range of the pathogen, environmental conditions for disease development, latency, and time required to manage the epidemic, are all important when managing containment.

As bananas are a perennial crop and Foc inoculum carries over from the previous season and increases during the current season, Fusarium wilt is regarded as a polycyclic or 'compound interest' disease. The pattern of the epidemic, in terms of the number of symptomatic plants, is given by a curve called the disease-progress curve, which shows the progress of the epidemic over time. A sigmoid curve (S-shaped) is characteristic for polycyclic diseases (**Figure 11**). The curve has three phases:

a. initial lag phase where inoculum levels are low.
b. exponential or logarithmic phase where inoculum levels and plants available for infection are not limiting.
c. decline (plateau) phase where very few plants are available for infection.

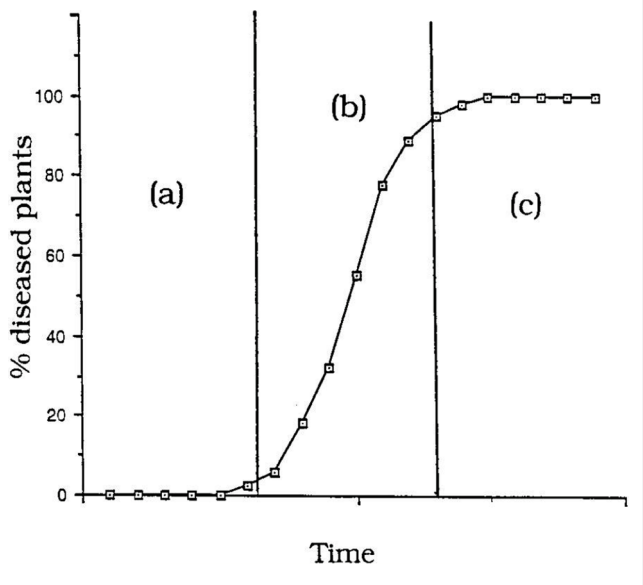

FIGURE 11 | 'Compound interest' disease epidemic showing a sigmoidal (S-shaped) epidemic progress curve: (a) initial lag phase, (b) exponential phase, (c) decline phase (from Brown et al., 1997).

With compound interest diseases new inoculum produced (= interest) is continuously added to the previous amount of inoculum (= interest bearing capital). The epidemic will start slowly (lag phase) but accelerate rapidly in the exponential phase, often with catastrophic losses.

A major focus for the TR4 epidemic in Queensland is to keep the amount of inoculum available for infection as low as possible to reduce the rate of disease spread, i.e. keep the disease in the lag phase. Once a diseased plant is detected, it and surrounding plants are killed with herbicide before inoculum is released into the soil. This will eliminate the disease but inoculum of the pathogen in the plant is not killed. Therefore plants are cut down, sealed in bags and surrounding soil treated with urea before covering with plastic. Plants are not dug out in the current strategy as this causes soil disturbance and possible spread of infested soil during flooding rains. Rhizomes are gouged out and urea applied to the cavity to hasten decomposition and reduce inoculum levels. At the time of writing, the most effective way of treating infected plants is being investigated (see the section Urea to Reduce Populations of *Fusarium oxysporum* f. sp. *cubense* in Soils).

CASE STUDY: THE DEVELOPMENT OF THE TR4 EPIDEMIC IN TAIWAN

The progress of the TR4 epidemic in Taiwan was first documented by Su et al. (1986). Commercial banana production in Taiwan involves many small farms (0.5–1.0 ha) rather than the extensive plantations managed by multi-national companies in the more tropical regions of the world. Taiwan grew Cavendish for export to Japan.

Since TR4 is virtually impossible to eradicate, their attempt to contain the disease was originally aimed at reducing the soil population of the fungus. Their disease management strategy was to dig out diseased plants, cut them into pieces, treat the pieces with lime and bury them 60 cm or deeper in the soil. The surrounding eight 'healthy' plants were destroyed in the same way. However this strategy proved difficult and failed to provide long term control. As the epidemic progressed, growers became careless, did not bury infected material, but left it lying on the ground or threw infected pseudostems into irrigation channels.

The first diseased Cavendish plant in Taiwan was detected in 1967. By 1976 there were half a million infected plants spread over 1,200 ha (Su et al., 1986). In 1983, 1,500 ha were infested (Hwang and Ko, 2004) and in 1999, 3,000 ha were infested (Hwang, 2001).

The following graph illustrates the progress of the TR4 epidemic in Taiwan (1967–1999) (**Figure 12**).

Over more than 30 years the total area infested has increased, with some important changes in the rate of spread of the disease. Initially, at least on an absolute basis, the spread appeared slow, but increased very rapidly in the mid-1970s (**Figure 12**). After this there seems to have been a steady increase in the area infested. Rigid control measures introduced in 1970 were abandoned in 1973 because they were unpopular, difficult to apply and had not prevented the spread of the disease.

When data for the spread of the disease is expressed as the number of hectares newly infested each year (ha/y), a rapid expansion of the disease in Taiwan in the mid-1970s is clearly

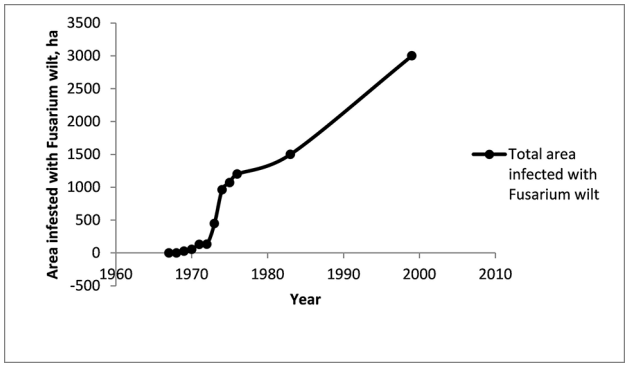

FIGURE 12 | The area infested with Fusarium wilt in the Cavendish banana industry in Taiwan. Data from Su et al. (1986), Hwang (2001), Hwang and Ko (2004). The industry covered 50,000 ha in the mid-1960s and was 6,000 ha in 1999.

evident. This illustrates the need for early intervention if the disease is to be contained.

Calculations from the data from Taiwan show the rapid increase in the rate of spread of the disease in the early years of the epidemic (**Figure 13**). This demonstrates the need for urgent action when an outbreak is first detected.

The origin of inoculum for the initial TR4 outbreak in North Queensland is unknown. Based on the number of plants infected, and the long incubation period of the disease, it is possible that the disease was present for some time before it was detected. However, there was very limited secondary spread on the infested property from the initial incursion prior to the banana farm being taken out of production. TR4 has subsequently been found on another two properties.

CONCLUSIONS

Despite the long history of Fusarium wilt in Australia (145 years), the only effective way of managing the disease is still by exclusion or early containment. To safeguard existing Cavendish plantations until acceptable resistant cultivars become available, a better understanding of the epidemiology and pathology of TR4 is required. The greatest challenge will be to confine the fungus to an existing area and to prevent the dispersal of its propagules. This will depend on focused research to provide information on how to improve early detection and destruction of infected plants and intensive treatment of soil in their vicinity. Research into better methods for detecting the presence of the pathogen in soil and water is also required.

The only reliable way of measuring the progress of the epidemic is the symptomatic plant. With any plant disease there is an incubation period during which symptoms are not evident. The susceptibility or resistance of the host plant, as well as environmental factors, influence incubation period. The shorter the incubation period, the more rapidly pathogen populations increase. Soil populations of the pathogen are extremely important. A high population will increase disease incidence and severity as well as the potential for disease spread. It will result in an earlier development of wilt symptoms and plants will die more quickly. Low soil populations will give systemic invasion, but plants will generally take longer to produce recognizable symptoms. Disease development is also strikingly influenced by both environmental and host factors, and disease incidence and severity may fluctuate independently of the population level. For example a saturated soil, which reduces the oxygen concentration in the roots, will make the plant more susceptible to infection. Once symptoms are detected, early intervention is possible. However, the absence of symptoms does not necessarily indicate that the pathogen is not present. It is possible for a very low population of the fungus to be present in a banana field where plants do not show any wilt symptoms. It may take as long as five years for wilt symptoms to appear in a field with a very low population (Rishbeth and Naylor, 1957). Thus once the pathogen enters a growing area, long-term surveillance will be required.

It is important to clarify the role of sap from laticifers and xylem fluid in the dispersal of the pathogen. It is generally accepted

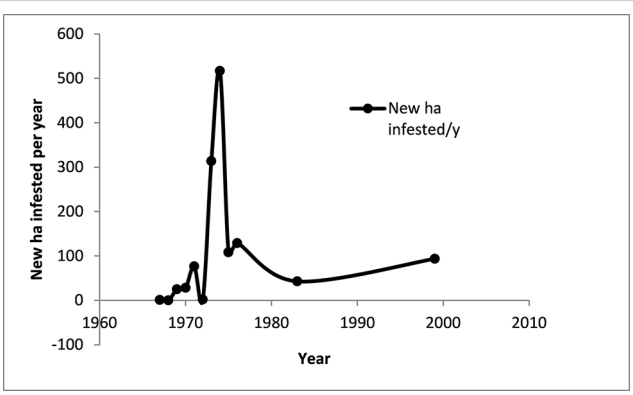

FIGURE 13 | The annual rate of spread of Fusarium wilt in the Cavendish banana industry in Taiwan. Data derived from Su et al. (1986), Hwang (2001), Hwang and Ko (2004).

FIGURE 14 | Cavendish banana plants affected by Fusarium wilt tropical race 4, showing yellowing and collapse of leaves at the petiole base (John E. Thomas).

that a large number of microconidia are extruded in xylem fluid and thus it is a potent source of inoculum for pathogen spread. Testing using traditional and molecular (fluorescence microscopy and qPCR) methods is required to track the movement of the pathogen in the vascular system of banana. As the disease can have a long incubation period, the rate of colonization may not preclude spread solely by hyphal growth.

Another important factor to consider is growing banana somaclones which are not completely resistant to TR4 when containment is being attempted, and the disease has not become widespread. There are Cavendish somaclones available from Taiwan, China, Australia and Indonesia which have a measure of resistance to Foc. However, resistance is not complete and growing such plants becomes an epidemiological risk, especially in regions where the pathogen has a limited distribution, as they may mask its presence and spread. It may be prudent to delay the deployment of such cultivars until containment strategies have failed and the disease is widespread. The current approach is to grow these somaclones using an integrated approach to managing Fusarium wilt. The underlying theme of this program is the promotion of practices to enhance 'soil health' as well as the management of plant stress. Such an approach needs to consider temporal variations in host susceptibility and pathogen activity. There are a number of environmental factors which will affect both; especially physical environmental factors in the soil (temperature, moisture, aeration). Many banana growing areas are subject to periodic cyclones and tropical storms where the best drained soils become temporarily saturated and overland flow of water occurs. A water-saturated, oxygen deficient soil will increase plant susceptibility, and partially resistant plants will succumb. Thus, extreme environmental events may quickly override any gains made in disease suppression through integrated management.

Despite current containment and surveillance protocols, confinement is likely to fail where there has been a long time period between the introduction of the pathogen and its detection, along with increased mechanization in the modern industry, high annual rainfall and flooding in tropical storms, and failure to apply simple farm hygiene procedures. One also must be realistic and recognize that the inoculum level in a TR4-infested soil will never be zero, and that these soils will remain a source of inoculum for decades and not sustain economic banana production as we know it today unless an acceptable resistant cultivar becomes available.

Although the entire banana industry is at risk from TR4, even if the pathogen is not contained, the disease is unlikely to spread as rapidly as it has in the export plantations in Asia and Mozambique. During the 'Gros Michel' epidemic the disease spread more slowly on small farms than in large plantations. Banana farms in Queensland are quite dispersed, growers are very progressive, and most have implemented on-farm biosecurity measures.

AUTHOR'S NOTE

The aspects of Fusarium wilt of banana discussed in this review are generally related to race 1 and subtropical race 4 of *Fusarium oxysporum* f. sp. *cubense*, but are considered by the authors to be important to understanding the epidemiology and pathology of tropical race 4 (**Figure 14**). The basic biology of the fungus should be considered when evaluating surveillance and containment options and quarantine protocols for the disease.

AUTHOR CONTRIBUTIONS

KP and LC reviewed the literature and wrote the main body of the article. WO'N had primary input into the sections on the influence of nematodes, alternative hosts, urea soil applications and TR4 containment, and supplied many images. DT prepared the section on the Taiwan TR4 case study and had significant input into the sections on influence of climatic factors and pathogen spread. All authors contributed to manuscript revision and editing.

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Ex Ante Assessment of Returns on Research Investments to Address the Impact of Fusarium Wilt Tropical Race 4 on Global Banana Production

Charles Staver[1], Diemuth E. Pemsl[1], Lars Scheerer[1], Luis Perez Vicente[2] and Miguel Dita[3]*

[1] Bioversity International, Montpellier, France, [2] Instituto de Investigaciones de Sanidad Vegetal, Habana, Cuba, [3] Bioversity International, Santiago de Cali, Colombia

The spread of Fusarium *oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), causal agent of Fusarium wilt of banana (FWB), has been projected to reach 17% of the global banana-growing area by 2040 equaling 36 million tons of production worth over US$10 billion. This potential loss has fueled (inter)national discussions about the best responses to protect production and small-scale growers' livelihoods. As part of a multi-crop ex ante assessment of returns on research investments conducted by the CGIAR Research Program on Roots, Tubers, and Bananas (RTB) from 2012 to 2016, four FWB research options were assessed: (i) improved exclusion, surveillance, eradication, and containment (ESEC) measures to reduce Foc TR4 spread, (ii) integrated crop and disease management (ICDM) to facilitate production of partially FWB resistant cultivars on Foc-infested soils, (iii) conventional breeding of FWB-resistant cultivars (CBRC), and (iv) genetically modified (GM) FWB-resistant cultivars (GMRC). Building on a risk index (Foc scale) predicting the initial occurrence and internal spread of Foc TR4 in 29 countries, an economic surplus (ES) model, cost-benefit analysis, and poverty impact simulations were used to assess impact under two adoption scenarios. All options yield positive net present values (NPVs) and internal rates of return (IRRs) above the standard 10% rate. For the conservative scenario with 50% reduced adoption, IRRs were still 30% for ICDM, 20% for CBRC, and 28% for GMRC. ESEC has IRRs between 11 and 14%, due to higher costs of capacity strengthening, on-going surveillance, farmer awareness campaigns, and implementation of farm biosecurity practices, which could be effective for other diseases and benefit multiple crops. The research investments would reach between 2.7 million (GMRC) and 14 million (ESEC) small-scale beneficiaries across Asia/Pacific, Sub-Saharan Africa, and Latin America/Caribbean. The options varied in their potential to reduce poverty, with the largest poverty reduction resulting from CBRC with 850,000 and ESEC with 807,000 persons lifted out of poverty (higher adoption scenario). In the discussion, we address the data needs for more fine-grained calculations to better guide research investment decisions. Our results show the potential of public investments in concerted research addressing the spread of Foc TR4 to yield high returns and substantially slow down disease spread.

Keywords: Fusarium wilt, banana, research priorities, ex ante, impact assessment

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****Correspondence:***
Charles Staver
c.staver@gmail.com

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INTRODUCTION

The threat of the tropical race 4 of Fusarium *oxysporum* f. sp. *cubense* (Foc TR4), causal agent of Fusarium wilt of banana (FWB), to world banana supplies has been raised frequently in the popular press in the past several years[1]. These articles highlight Cavendish, the dominant banana cultivar group, which accounts for around 90% of current export production and is highly susceptible to Foc TR4 (Ploetz, 2005). However, other cultivars consumed and traded locally are also susceptible, although characterization is still ongoing (Hermanto et al., 2011; Zuo et al., 2018; Chen et al., 2019). Currently, Foc TR4 is present in 27 countries where thousands of hectares have been affected. A recent projection concluded that 17% of today's banana growing area with an annual production of 36 million tons worth approximately US$10 billion at current prices could be lost over the next 20 years (Scheerer et al., 2018a). When the race 1 of this pathogen (Foc R1) threatened the export banana business during the period 1900–1950 (Stover, 1990), commercial producers successfully switched from the highly susceptible cultivar Gros Michel to the resistant Cavendish. Today, Cavendish constitutes about 50% of global banana production (FRuiTRoP, 2016) and the boxed banana postharvest supply chain is based completely on Cavendish requirements. While FWB ceased to be a concern for export banana growers, Foc R1 and R2 strains continue to spread and threaten banana-based livelihoods especially of smallholder farmers growing diverse susceptible cultivars. In response, contract growers of the highly FWB-susceptible Maça (Silk, AAB) cultivar in Brazil move production to clean soils every one to two crop cycles. Due to FWB in East Africa, small farmers have replaced Pisang Awak (ABB) used for banana juice with other cultivars. Gros Michel is still a preferred national market cultivar in Central America as an intercrop in shaded coffee, but an increasing number of producers have lost this income option because their fields are highly infected with the FWB pathogen and no efficient management options are available (Siles et al., 2013).

Frequent calls have been made for increased global investment to reduce the impact of FWB (Kema and Weise, 2013) and the Food and Agricultural Organization of the United Nations recently launched a global initiative which is seeking donors to invest US$98 million for a concerted response (FAO, 2017). However, while there is certain consensus that the threat of Foc TR4 is real and needs to be addressed, agreeing on a global investment strategy is challenging. Often the collapse of banana production is presented as imminent through words like banana extinction, apocalypse, and the end of banana (see news headlines in Footnote 1). However, the example of Foc R1 suggests that decades may pass until disease spread impacts large numbers of smallholder producers thereby critically affecting supplies. Cook et al. (2015) project high losses, even with a slow rate of spread, for bananas in Australia due to Foc TR4, where production is primarily based on Cavendish. Clearly, the threat is huge to the Cavendish-based production sector both for export and large internal markets like China, India, Indonesia, Brazil, and Mexico, because Cavendish has been shown to be highly susceptible to Foc TR4 and production is often concentrated in large monocultures in coastal lowlands subject to flooding, an accelerator of the pathogen spread (Dita et al., 2018).

The spread of the disease across China with the loss of over 100,000 hectares of production area and into four other countries of the Mekong sub-region over two to three decades as contract Cavendish growers have sought out clean lands for production illustrates a worst case scenario in the absence of action (Zheng et al., 2018). However, the extent of the threat to smallholder systems with a wide diversity of cultivars and cropping systems is uncertain. Is this sector protected by its diversity? Is the pathogen spread slower than in highly intensive monocrops, but inexorable, even for cultivars which show partial tolerance?

Breeding has been proposed as the most viable response through genetic modification (Loeillet, 2019) and with even greater promise in gene editing (Dale et al., 2017). Somaclonal variants tolerant to Foc TR4, a strategy pioneered in Taiwan (Hwang and Ko, 2004) and field tested in more tropical regions (Molina et al., 2016), have been taken up commercially and several national programs have established on-going selection. However, the touted success of these somaclonal Cavendish in infested areas (Molina, 2016) and the promise of short-term success of gene-edited cultivars cited above may put at risk the investment on exclusion and containment of FWB. Other authors (e.g., Dita et al., 2018) emphasize the importance of research on surveillance, exclusion, and containment to slow down and limit the spread as well as research on cropping systems management to both facilitate the production of susceptible cultivars and increase the durability of new resistant clones in infested lands.

Globally, the public research budget to address opportunities and threats to agricultural production has increased faster in larger countries, while smaller countries have faced many competing expenses (Beintema and Elliott, 2011). At the same time, the agricultural research agenda now addresses an expanded list of topics beyond increasing or maintaining productivity, such as climate change, environmental conservation, and poverty reduction (Place et al., 2013). In addition to conducting ex post impact assessment studies to demonstrate to donors and the global public that invested funds have generated (large) positive returns (e.g., Renkow and Byerlee, 2010), ex ante assessments (with different levels of rigor and formality) that determine *a priori* expected returns on investment have been widely applied. These studies are generally used to support and justify strategic research portfolio decisions in order to maximize the benefit of limited resources (see, e.g., case studies in Raitzer and Norton, 2009) and respond to increased up-front accountability demands from donors (Pardey et al., 2016). Quantitative ex ante studies have been conducted for a range of different agricultural technologies and locations (recent examples are Ainembabazi et al., 2015; Komarek et al., 2019).

As a global research partnership, CGIAR is the world's largest international agricultural research network, implementing 15 thematic Consortium Research Programs (CRPs) in collaboration

[1]e.g., D. Koeppel (2011) in The Scientist: The beginning of the end for Bananas; Popular Science (2014): Has the end of the banana arrived?; P. Tullis in The Washington Post (2017): Bananapocalypse; F. Kentish (2015) in The Telegraph: Is this the end of the banana?; N. Fleming (2018) in The Guardian: Science's search for a super banana (see reference list for full citations).

with 1000+ partners worldwide[2]. The Research Program focusing on Roots, Tubers, and Bananas (RTB) comprises five research centers (Bioversity International, the International Center for Tropical Agriculture (CIAT), the International Institute of Tropical Agriculture (IITA), and the French Agricultural Research Center for International Development (CIRAD) who work with more than 360 other partners. RTB, as a new program in 2012, was requested to conduct a priority assessment and implemented a rigorous, harmonized quantitative ex ante study for its five major crops (banana, cassava, potato, sweet potato, and yam).

The RTB centers and institutes working on banana (Bioversity, IITA, and CIRAD) together with national banana programs articulated through four regional banana networks carried out a banana priority assessment as part of the multi-crop cross-Center effort. The assessment started with a participatory elicitation of major constraints and opportunities to (small-scale) banana production, processing, and marketing. The results of this global expert consultation with responses from 523 banana experts are summarized in an RTB publication (Pemsl et al., 2014). Globally, respondents ranked FWB fourth in this survey behind pest and disease-infected planting material, black leaf streak, and water deficits. In a subsequent workshop held in April 2013, 34 banana scientists, representing different geographic regions and areas of expertise, proposed initial research lines to address these major yield constraints. Ex ante analysis for eight of these research lines, five for banana breeding and three for crop management, was completed as part of the RTB priority assessment (Pemsl and Staver, 2014). Subsequently, four research options addressing the threat of FWB were assessed. Since the time of the assessment, Foc TR4 has spread to more countries in South and South-East Asia and Africa as well as Latin America[3], increasing the urgency to invest in FWB research guided by a systematic and quantitative priority assessment.

We have three objectives in this paper:

1) Describe the methodological approach, data compilation, and results of the ex ante assessment of FWB research lines (incl. returns on investment and poverty impacts);
2) Assess the validity of the results with reference to their use by policy makers and funders and identify priority areas for future data collection and curation and complementary research for follow-up studies;
3) Discuss steps to improve the use of priority assessment studies to guide research funding decisions on FWB.

MATERIALS AND METHODS

The analytical framework used for the quantitative ex ante assessment follows the methodology used in the wider RTB priority assessment study across crops and is described in Alene et al. (2018). For the assessment of the Foc research options, these steps comprised the selection and detailed description of research options, compilation of data and parameter estimation, the quantification of potential impacts using a partial equilibrium economic surplus (ES) model and subsequent cost-benefit analysis, sensitivity analysis, and an online stakeholder feedback survey to validate parameter assumptions.

Selection and Description of Research Options

In order to narrow down and describe the specific research options to be assessed, we clustered research interventions addressing Fusarium wilt around three general themes: (i) preventing the spread of the disease (especially Foc TR4) to currently unaffected regions/countries through research on (and implementation of) improved exclusion, surveillance, eradication, and containment (ESEC) measures; (ii) research on integrated crop and disease management (ICDM) to recover banana yields in areas affected by (all strains of) Fusarium; and (iii) research focused on developing banana cultivars resistant to Fusarium wilt. There are two fundamentally different approaches to developing resistant varieties: conventional breeding using the genetic diversity of banana or genetic modification of susceptible cultivars of economic importance, with the latter likely being applicable only for a smaller area due to country biosafety regulations.

Based on these considerations, four distinct potential research options to address FWB were selected and quantitatively assessed for this study:

- Improved ESEC measures to avoid Foc TR4 spread (ESEC);
- Integrated crop and disease management to reduce impact of Foc TR4 (ICDM);
- Conventional breeding for FWB-resistant banana cultivars (CBRC);
- Genetically modified (GM) FWB-resistant banana cultivars (GMRC).

The adoptable, public good innovations resulting from the research in the form of knowledge, practices, and technologies were formulated for each research option and the specific research agenda was detailed (**Table 1**). This provided a scope of work for each research option, required to budget expected research costs for the cost-benefit analysis. Even though some topics, e.g., epidemiology, pathogenicity, diagnostic protocols, clean seed, and mapping relate to more than one research option (see ESEC and ICDM overlap in **Table 1**), we costed each option separately so they can later be compared. If investment in both options occurred, there would be substantial synergies that would result in lower research costs.

Each research option has a distinct target domain, since each option focuses on or is applicable to certain cultivar groups and is thus (more) relevant in certain countries. We considered major banana producing countries in Asia and the Pacific, Africa, and Latin America and the Caribbean (LAC). Our focus was on countries with predominantly small-holder producers and a substantial dependency on bananas for livelihoods.

The research on ESEC is applicable to all six cultivar groups in 29 major banana producing countries threatened by Foc TR4 (**Table 2**). The agenda will contribute to the

[2]www.cgiar.org

[3]http://www.promusa.org/Tropical+race+4+-+TR4

TABLE 1 | Description of the four assessed research options to address Fusarium wilt of bananas.

Research option	Improved exclusion, surveillance, eradication and containment (ESEC)	Integrated crop and disease management (ICDM)	Conventional breeding of Fusarium resistant banana cultivars (CBRC)	Genetically modified Fusarium resistant banana cultivars (GMRC)
Adoptable innovation	(Improved) exclusion, surveillance, containment, and early eradication measures on farm, community, national, and international level	Crop and disease management package	High yielding and market accepted Fusarium resistant varieties	High yielding and market-accepted genetically modified (GM) Fusarium resistant varieties
Research agenda	• Strengthen science-based risk analysis protocol for Foc movement for local, national, regional, and intercontinental use • Develop/improve protocol to produce Foc-free planting material from tissue culture (TC), suckers, and macro propagation • Develop model for Foc epidemiology and pathogenicity and more efficient tools for epidemiological studies • Determine pathogen population structure, cultivar-specific disease intensity, and current distribution of Foc populations in key banana producing countries • Develop and optimize diagnostic protocols for TR4 and other relevant Foc strains Evaluate susceptibility/resistance of major cultivars to Foc TR 4 and other races		• Prospection for new sources of resistance to Foc in germplasm collection, including breeding lines • Identify and characterize resistance genes (and molecular markers) to support breeding processes including Marker Assisted Selection • Generate diploid pre-breeding lines with Foc resistance (emphasis on TR4) for major cultivar groups • Develop efficient protocols for phenotyping of breeding lines	• Identify pathogenicity factorsand defense/resistance genes and develop cisgenic and/or trans-genic constructs to generate Foc resistant bananas cultivars • Develop GM banana cultivars with Foc resistance • Phenotype GM bananas lines for Foc resistance at greenhouse level • Evaluate and select commercial GM lines resistant to Foc on multi-site field experiments
	• Validate efficient surveillance protocols to detect, delimitate, and monitor Foc spreading • Understand risk and pathways of Foc dissemination in soil, suckers, humans, other banana parts, diverse agricultural and non-agricultural practices within country, across borders, and between continents • Determine effectiveness of different eradication and isolation procedures for first detected Foc affected banana plants in Foc-free areas	• Identify and evaluate cover crops, intercrops, and other agronomic and soil management practices that suppress or accelerate Foc in banana and clarify mechanisms involved • Understand functional diversity of suppressive vs. conducive soils in banana production contrasting biological, physical, and chemical properties • Screen and characterize root-associated microorganisms w/Foc suppressive and growth promotion capacity • Prototype integrated Foc management strategies based on biological inputs (incl. microorganisms), crop (incl. resistant genotypes, chemical fertilizers fine tuning), and cropping systems	• Employ conventionally breeding methods to develop bananas with Foc resistance • Strengthen protocols and develop somaclonal and clonal selection for Foc resistance in susceptible (and partially resistant) cultivars • Identify possible Foc resistant substitutes for the major susceptible market and food security cultivars and select for clones with superior traits • Evaluate and select resistant genotypes on multi-site field experiments • Evaluate and develop post-harvest and market oriented strategies	• Evaluate and develop post-harvest and market oriented strategies

effectiveness of national plant protection offices, starting with a better understanding and assessment of risks of Foc TR4 introduction and spread (Dita et al., 2013; Biosecurity of Queensland, 2016). Field studies on movement of planting material, banana products, and soil and other practical experience (Pegg et al., 2019) will contribute to ESEC strategies. Basic information about the disease in the plant is central not only to ESEC, but to ICDM (Warman and Aitken, 2018) and should be expanded. Such basic knowledge is also applicable to a spatial model for the analysis of scenarios for different locations of first outbreak and the speed and likelihood of spread depending on the actions taken (see Flores et al., 2017, for an example on citrus greening disease). Such models will also contribute to more effective surveillance routines and routes and to more strategic actions for eradication and containment. Research also includes development of more effective measures of biosecurity both in large plantations and in zones of diversified small farms with bananas (Pérez Vicente, 2015; Kukulies and Veivers, 2017).

The research on ICDM is primarily applicable to commercial production for national and export markets (**Table 2**). The deployment of cover crops, microbial organisms, systematic crop rotation, and careful rogueing was considered unlikely among small growers who grow bananas for home consumption and local sale. Delivery systems for inputs and seed are also often deficient where small growers predominate. Research to develop such ICDM approaches centers on healthy soils with capacity to suppress disease build-up and crop management to

TABLE 2 | Target domains for the assessed research options to address Fusarium wilt of bananas.

Research option	Improved exclusion, surveillance, eradication, and containment (ESEC)	Integrated crop and disease management (ICDM)	Conventional breeding of Fusarium resistant banana cultivars (CBRC)	Genetically modified Fusarium resistant banana cultivars (GMRC)
Target domain	Production areas of all six cultivar groups in countries in Africa, LAC, and Asia/Pacific where Fusarium Foc TR4 is either already present or will very likely spread in the near future	Production areas of all six cultivar groups in countries in Africa, LAC, and Asia/Pacific where soils are infested with Fusarium R1 and/or TR4	Production area of all six cultivar groups in Africa, LAC, and Asia/Pacific	Production area of "Cavendish AAA" in countries where local markets are important (export-oriented countries are less likely to adopt GM varieties due to political and consumer concerns in importing countries)
Applicable cultivars	Cavendish AAA; other AAA + Gros Michel + AA; East African Highland AAA; AAB Plantain; other AAB; ABB	***Foc TR4:*** •Cavendish AAA; other AAA + Gros Michel + AA; East African Highland AAA; AAB Plantain; other AAB; ABB (in Asia/Pacific and LAC) Cavendish AAA (in Africa) ***Foc R1:*** Other AAA and "other AAB" (in LAC and Asia/Pacific)	• Cavendish AAA; other AAA + Gros Michel + AA; East African Highland AAA; AAB Plantain; other AAB; ABB	Cavendish AAA
Countries included in assessment	***Africa***: Burundi, Cameroon, Congo, D.R., Côte d'Ivoire, Ghana, Kenya, Mozambique, Nigeria, Rwanda, Tanzania, Uganda ***Asia/Pacific:*** China, India, Indonesia, Malaysia, Myanmar, Pakistan, Papua New Guinea, Philippines, Thailand, Vietnam ***LAC***: Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Nicaragua, Peru	***Africa***: Cameroon, Côte d'Ivoire, Ghana ***Asia/Pacific:*** China, India, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Thailand, Vietnam ***LAC:*** Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Nicaragua, Peru	***Africa:*** Burundi, Cameroon, Congo, D.R., Côte d'Ivoire, Ghana, Kenya, Mozambique, Nigeria, Rwanda, Tanzania, Uganda ***Asia/Pacific:*** China, India, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Thailand, Vietnam ***LAC:*** Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Mexico, Nicaragua, Peru	***Africa:*** Burundi, Congo, D.R., Kenya, Mozambique, Nigeria, Rwanda, Tanzania, Uganda ***Asia/Pacific:*** China, India, Indonesia, Malaysia, Myanmar, Pakistan, Thailand, Vietnam ***LAC:*** Brazil, Mexico, Peru

reduce crop susceptibility and to limit inoculum accumulation. Research results on specific bacterial antagonists of the pathogen (Pérez Vicente et al., 2009; Shen et al., 2015; Bubici et al., 2019), crop suppressive effects (Huang et al., 2012), and integrated systems approaches (Haddad et al., 2018; Huang et al., 2019) already provide evidence for the potential of this strategy. Basic knowledge on Foc populations, cultivar susceptibility, and more advanced quantitative diagnostic tools should underlie the applied management approaches. While the ICDM approaches are applicable for Foc races 1 and SR4, the calculation of returns only takes into account the recovery of losses due to Foc TR4 projected based on the risk index or FOC scale.

The development of CBRC is proposed to have the widest applicability after ESEC with relatively easy uptake for home consumption, local and national markets and export, as long as eating and cooking quality and handling traits are acceptable (**Table 2**). A broad range of varieties for different cultivar groups is proposed. Different improvement approaches are considered (**Table 1**) based on improved varieties already available through conventional breeding by Embrapa, the Brazilian Agricultural Research Corporation (Silva et al., 2013), clonal selection in Cavendish by the Taiwan Banana Research Institute already cited, and mutation breeding in Australia (Smith et al., 2006). Screening for resistance among existing lines continues to cover more and more *Musa* diversity (Zuo et al., 2018; Chen et al., 2019; García-Bastidas et al., 2019). Important areas of work include screening for sources of resistance, more effective phenotyping tools, development of pre-breeding lines, marker-assisted crossing, and multi-site evaluation trials.

The development of GMRC is projected only for Cavendish with application to countries with limited export, but relatively large national markets. Current status of biosafety regulations and laws on GM crops were not taken into account but could reduce the number of countries included. The use of resistance genes for cis and trans modification and the identification of genes linked to resistance to guide gene editing have already advanced beyond proof of concept (Dale et al., 2017; Maxmen, 2019). Resulting materials are screened for resistance to all Foc races in greenhouse and then field trials with evaluation of postharvest handling and taste qualities are conducted.

Data Compilation and Parameter Estimation

Data required for our ex ante assessment were collected from three general sources: statistical databases, other published sources, and expert estimates. For consistency, we followed the same procedure for compiling and cleaning/adjusting

TABLE 3 | Estimated banana production area lost due to Fusarium wilt over time (by country and region).

Country	Banana production area*['000 ha]	Banana production area lost due to Fusarium wilt [% of total]over time assuming 50% internal spread rate once in country (loss with 25% spread rate in parentheses if applicable)					Banana production area lost ['000 ha] in 25 years due to FW with 50% internal spread (25% spread)
		2019 (year 5)	2024 (year 10)	2029 (year 15)	2034 (year 20)	2039 (year 25)	
Africa							
Burundi	371.05	0	3	7 (6)	12 (10)	20 (15)	75.1 (45.5)
Cameroon	184.41	0	3	7 (6)	12 (10)	20 (15)	37.6 (27.3)
Congo, D.R.	391.62	0	0	4	11 (9)	19 (15)	74.4 (60.5)
Côte d'Ivoire	411.19	0	2	5 (5)	10 (8)	17 (12)	68.0 (49.1)
Ghana	191.75	0	0	4	9 (8)	16 (13)	30.2 (24.5)
Kenya	80.49	0	1	3 (3)	7 (5)	11 (8)	8.8 (6.3)
Mozambique	27.86	6	14 (12)	25 (20)	39 (29)	55 (38)	15.3 (10.7)
Nigeria	455.55	0	0	1	1 (1)	3 (2)	12.6 (10.1)
Rwanda	343.64	0	0	1	3 (3)	6 (5)	19.7 (15.8)
Tanzania	537.68	0	4	10 (9)	18 (15)	29 (21)	156.7 (115.6)
Uganda	1866.25	0	0	1	2 (1)	3 (2)	55.1 (44.2)
Subtotal	**4861.49**						**553.5 (418.7)**
Asia/Pacific							
China	398.19	8	19 (17)	34 (28)	52 (39)	71 (51)	283.4 (202.3)
India	1858.28	0	0	2	5 (4)	9 (7)	163.3 (131.8)
Indonesia	320.03	4	10 (9)	18 (14)	29 (21)	43 (29)	137.6 (91.6)
Malaysia	56.82	2	5 (4)	9 (7)	15 (11)	23 (15)	13.2 (8.5)
Myanmar	65.43	0	8	18 (17)	33 (27)	50 (38)	32.8 (24.7)
Pakistan	31.98	8	19 (17)	33 (27)	51 (39)	71 (50)	22.6 (16.1)
Papua New Guinea	45.18	0	4	10 (9)	18 (14)	29 (21)	13.1 (9.6)
Philippines	391.88	8	19 (17)	34 (28)	52 (39)	71 (51)	278.9 (199.1)
Thailand	132.08	0	8	19 (17)	33 (27)	50 (38)	66.6 (50.2)
Vietnam	102.17	8	19 (17)	34 (28)	52 (39)	71 (51)	72.7 (51.9)
Subtotal	**3402.04**						**1084.1 (785.7)**
Latin America and Caribbean (LAC)							
Brazil	498.45	0	0	0	1	2 (2)	12.0 (10.8)
Colombia	461.43	0	0	1	2 (1)	3 (2)	13.8 (11.1)
Costa Rica	61.22	0	0	1	2 (2)	4 (3)	2.7 (2.1)
Ecuador	266.88	0	0	1	2 (2)	4 (3)	10.2 (8.2)
Guatemala	50.55	0	0	0	2	8 (4)	4.1 (2.0)
Mexico	86.31	0	0	0	1	2 (2)	2.0 (1.7)
Nicaragua	14.46	0	0	0	0	1	0.1 (0.1)
Peru	120.83	0	0	0	1	2 (2)	2.5 (2.1)
Subtotal	**1560.13**						**47.3 (38.1)**
ALL	**9823.66**	0.9	2.8 (2.6)	6.3 (5.5)	11.1 (8.8)	17.1 (12.6)	**1684 (1242)**

*Source: *Source for production area FAO STA (averages of 2010–2012 values) and FRuiTRoP (2012) Results of the "Foc Scale" as part of the Strategic Assessment of Banana Research Priorities (Scheerer et al., 2018a,b).*

data that was used in the previous assessment of RTB banana research options that is described in detail in Pemsl and Staver (2014). To facilitate the disaggregation of production data by cultivar group, we used information from FRuiTRoP (2012) in addition to the FAO[4] statistics. To avoid bias due to annual abnormalities, we computed a 3-year average for banana yield and price for each country included (using 2010–2012 data for consistency with the previous assessments, older data if necessary). Since FAO does not separate production from large scale, commercial plantations from (semi−) subsistence production under smallholder conditions, some yield figures for cultivar groups other than Cavendish for countries with sizable banana export industry were capped using expert judgment to reflect smallholder conditions. Yield and production data were then used to compute the banana production area for each included country (see **Table 3**).

To populate the poverty impact model, we relied on data included in the World Development Indicators[5], namely, the most recent information for each included

[4]FAOSTAT http://faostat.fao.org/

[5]World Bank, http://data.worldbank.org/indicator

country on poverty prevalence (total population and poverty rate) and (agricultural) gross domestic product (GDP) by country.

Predicting Pathogen Spread

The benefit of the FWB research interventions is the yield loss avoided[6] by either preventing or delaying the spread of the disease (ESEC) to an area, (partially) recovering yields in areas with infested soils through crop or disease management (ICDM), or replacing susceptible with resistant banana cultivars that are not affected by the disease (CBRC and GMRC). The magnitude of the benefits from each of these options largely depends on the scale and pace of disease spread, i.e., the size of the affected area that constitutes the target domain for the intervention.

In the absence of an established epidemiological model to predict the future spread of Foc TR4, we relied on the risk-index model developed by Scheerer et al. (2018a) to project the future disease spread and thus determine the expected yield losses that could be avoided by investing in the four research options. The risk-index model consists of two steps where for each country a score is assigned based on (i) the likelihood of initial outbreak of Foc TR4 (time lag in years) and (ii) the internal spread rate of the disease (disaggregated per cultivar type) once present in the area. Factors considered when scoring for the time lag for Foc TR4 to reach a country include the importance of mono-cropped Cavendish, global banana traffic to and from a country, quality of borders and internal plant quarantine measures, and land and other links to countries where Foc TR4 is already present. The higher the aggregated score for a country, the shorter the time lag for Foc TR4 to be introduced and established in the country. The internal spread rate is scored based on three factors: the quality of internal quarantine measures, the importance of Cavendish, and the importance of banana for research investment and public policy. The higher the aggregated score for a country, the faster the spread and thus the higher the expected loss of banana production due to Foc TR4 with differentiated losses depending on cultivar types. Loss of production was proposed between 1 and 8% of area planted during the first 5 years after detection depending on cultivar group and the country score for internal spread risk. Assuming an accelerating expansion of the disease-affected area, especially in the early years, in each successive 5-year period after first detection, the spread rate was calculated to increase by 50%. In a second, more conservative scenario, the spread rate only increased by 25% for each successive 5-year interval.

In **Table 3**, we show the projected banana area lost to FWB using the risk index for each country over time (2014–2039, in 5-year intervals) in percent of the national banana production area lost. The last column gives the absolute area rendered unsuitable for production due to FWB in the absence of interventions thus constituting the target domain for our research interventions. The projected national losses are organized by region to illustrate the projected impact of the disease in different parts of the world over the next 25 years. The large effect in Asia/Pacific is due to the fact that Foc TR4 has already been detected in several countries in this region and internal spread has progressed. Though the current spread of the disease is more limited in Africa, the results indicate the potential for very high negative impact especially on smallholders. In the model results, spread onset was most delayed for LAC. However, the recent detection of Foc TR4 in Colombia is expected to accelerate the spread in LAC and shows how difficult the prediction of initial outbreak is.

The accelerating nature of the spread over time as well as the slowing effect of a more conservative internal spread assumption is visualized in **Figure 1**.

Quantifying the Potential Impact of Research Options

Following the methodology used in the multi-crop RTB priority assessment, we simulated adoption of the innovations resulting from ICDM, CBRC, and GMRC over time based on the target domain and estimated parameters on adoption lag (time until first adoption of innovation), the adoption pace, and ceiling. The adoption pace is indicated by the time until full adoption. The adoption ceiling is defined as the maximum share of the total production area affected by the disease on which the innovation(s) will be adopted. The definition of adoption is different for the ESEC research option, since the decision to implement is taken on the national (or even regional) level instead of the individual producer level and thus either none or all of the (affected) production area in the country benefits. **Table 4** gives an overview of the parameter assumptions used in the assessment.

The largest adoption area reached over the assessment period represents the reach of the intervention and is reported as one impact indicator in the results section. This largest adoption area figure is also the basis for the computation of the number of beneficiaries, an additional impact indicator. The conversion is conducted by division with country specific estimates of the average banana area per household and multiplication with the country specific average household size (RTB, 2011).

To quantify the benefits from adopting the innovations developed under each of the research options at the national level, we used a partial equilibrium ES model estimated over a 25-year period (2014–2039). This quantitative approach of computing the ES resulting from a research-induced supply shift is a standard procedure in the agricultural economics and impact assessment field (see, e.g., Alston et al., 1995) and has been used in many previous studies (e.g., Alene et al., 2009, 2018; Fuglie and Thiele, 2009). We assumed elasticities of supply and demand to be 1 and 0.5, respectively, across all technologies and countries due to lack of other information.

In the subsequent cost-benefit analysis, we discount the benefits computed in the ES model (i.e., apply an interest rate to account for the difference in time when benefits occur) and compare them with the discounted costs associated with

[6]In line with previous studies on (potential) plant/animal disease outbreaks applying a cost-benefit analysis framework (e.g., Cembali et al., 2003; Wittwer et al., 2005; Breukers et al., 2008; Cook et al., 2013, 2015) the benefits of delaying or preventing FWB occurrence/spread in our assessment are the avoided losses/costs that would have occurred without the (research) intervention within the same timeframe.

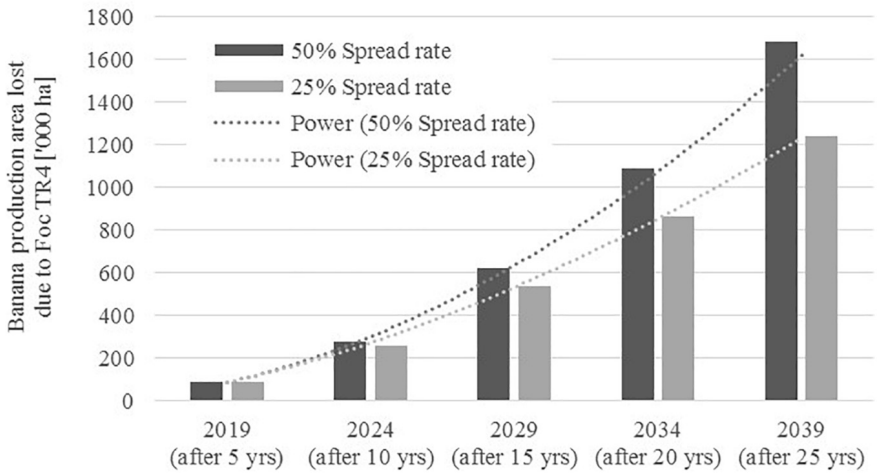

FIGURE 1 | Projected loss of global banana production area due to FW over time (two scenarios). Source: Adapted from Scheerer et al. (2018a).

TABLE 4 | Summary of parameter estimates and assumptions.

Parameter	Improved exclusion, surveillance, eradication, and containment (ESEC)	Integrated crop and disease management (ICDM)	Conventional breeding of FWB resistant banana cultivars (CBRC)	Genetically modified FWB resistant banana cultivars (GMRC)
Change in yield (%) = avoided yield loss	100	80	100	100
Production cost change (%)	1	20	NA	NA
R&D costs (US$ million)[1]	16.2	30.5	47.7	8.5
Dissemination costs (US$ha of new adoption)	50	80	50	50
Additional costs	***Establishing quarantine system***[2] : US$50/ha ***Maintaining quarantine system***: US$5/ha/year prior to Foc arrival and US$10/ha/year with Foc	NA	NA	NA
Adoption ceiling (% of target domain)	100[3]	30–50	80	40
Adoption ceiling (% of total national production area)	2–51	0.3–25	0.8–41	0.1–18
Research lag (years)	8	10	17	12
Time between first adoption and adoption ceiling (years)	10	15	15	15
Chance of research success (%)	80	90	60	40
Chance of national uptake[4] (%)	80	25, 50, or 75[5]	90	70

[1]Matched 1:1 with additional costs expected to occur at the national level for the cost-benefit analysis. [2]In year 5 for countries with high importance of banana and in year 10 for countries with low importance of banana. [3]Given that quarantine and surveillance measures are executed at the national level, we assumed that all farmers "adopt" or benefit from the technology once the country implements the quarantine scheme, i.e., "adoption" is 100% of the target domain. [4]Probability of successful up-take of the technology by national level (extension) agencies to enable adoption. [5]Depending on the importance of banana to national public policy and national research and extension investments.

each research options. The estimated research and development (R&D) costs for each research option (**Table 4**) were doubled for the cost-benefit analysis to account for (in-kind) contributions of national partners. Additionally, dissemination costs based on the marginal annual adoption area were included. Dissemination costs were set at US$50 per hectare for improved varieties and US$80 per hectare for knowledge intensive innovations such as crop or pest management practices. For ESEC, additional costs were included for (i) establishing the quarantine system in the amount of US$50 per hectare reflecting the initial capacity strengthening effort and (ii) maintaining the quarantine and surveillance system in the amount of US$5 per hectare and year prior to Foc TR4 arrival and US$10 per hectare and year once Foc TR4 is present.

The key indicators resulting from the cost-benefit analysis are the net present value (NPV) and the internal rate of return (IRR) on the investment.

In addition to computing the standard economic indicators NPV and IRR, we also assessed the potential impact of each of the research options to reduce poverty. To do so, we estimated the marginal impact of an increase in the value of agricultural production on poverty reduction using elasticities of agricultural productivity growth. We applied the regional elasticities proposed by Thirtle et al. (2003) who found that a

1% growth in agricultural productivity reduces the number of rural poor by 0.72% in Africa, 0.48% in Asia, and 0.15% in LAC. Following Alene et al. (2009), we calculated the reduction in the number of poor by considering the estimated economic benefit of the respective research option as an increase in the agricultural production value.

Sensitivity Analysis of Results

Ex ante assessments by their nature predict uncertain future outcomes of potential investments and results are based on (expert) estimates of the costs and effects. Results have notoriously been too optimistic with regard to the benefits while underestimating (unknown) costs and problems. Alston et al. (1995) proposed sensitivity analysis to remedy this situation. In order to probe the robustness of results and take into account the tendency of technical experts to be overly optimistic with regard to the performance of and demand for their technologies, we embedded different adoption scenarios in the assessment. We also conducted sensitivity testing for additional key variables, focusing on parameters elicited from experts rather than model inherent parameters (such as elasticities and discount rate) or parameters populated based on statistics (e.g., banana production area, yield, or farm-gate prices). In order to keep the number of scenarios manageable, we focused on the three most crucial parameters which at the same time seem most prone to overly optimistic assumptions: (1) adoption area of the new technology, (2) time when adoption starts, and (3) magnitude of the farm-level benefit realized when adopting the technology.

For ICDM, CBRC, and GMRC, we assessed two different adoption scenarios: the first uses the adoption ceiling estimated by experts, while the second one is more conservative and uses a 50% reduced adoption assumption. Subsequently, we tested additional scenarios with delayed adoption and reduced effects for these three research options.

Since adoption is either 0 or 100% at the national level for ESEC, we constructed three increasingly cautious scenarios in order to test sensitivity of the results to changes in key parameters (see Scheerer et al., 2018b). For Scenario 1, we assume that ESEC will lead to a doubling of the arrival time (e.g., the disease will first occur in a country after 10 instead of 5 years) and a 50% reduced increase in spread rate once Foc reaches the country (i.e., 12.5 instead of 25% increase of spread rate every 5 years). Scenario 2 has a less delayed first arrival [Scenario 1 minus 5 years, i.e., 5 years earlier than under Scenario 1 in our example after (5 years $\times$ 2) – 5 = 5 years] and the same reduced increase in spread rate of 12.5%. Scenario 3 has the same less delayed arrival from the second scenario and a small reduction in the increase in spread rate (18.75 instead of 25%) once the disease breaks out in a country.

RESULTS

Economic Impact

The assessment results show that all research options for FWB generate positive NPVs under all adoption scenarios (**Table 5**), indicating that investment in all research options is profitable. The magnitude of NPVs, however, varies considerably across options ranging from US$35 million for the most conservative scenario of improved quarantine and surveillance measures to reduce Foc TR4 spread (ESEC) to slightly over US$1 billion for ICDM to facilitate commercial production of partially Foc-resistant cultivars on Foc-infested soils (expert adoption assumption scenario). Since R&D costs, or the level of investment required, vary substantially across research options (US$8.5—47.7 million), the NPVs cannot be used to rank the research options. Instead, the IRRs (the interest rate realized on the invested amount) are a preferred measure for ranking alternative technologies.

All assessed FWB research options yield positive IRRs that are above the standard 10% interest rate (**Table 5**). Even under the lower adoption scenario the IRRs are positive and mostly well above 10%. Again, there is considerable variation in the return on investment among research options and adoption scenarios. ESEC Scenario 3 yields an estimated 11% return on the investment while the higher adoption scenario for ICDM reaches an estimated 36% IRR. The three ESEC scenarios show the lowest returns on investment, just slightly above the 10% threshold. These lower returns result from additional cost variables we included compared to the other research options. In addition to the R&D costs (matched 1:1 with national partner costs for the assessment) and the dissemination costs included for all research options, we added the costs of establishing quarantine systems reflecting the initial capacity strengthening effort and the costs of maintaining the quarantine and surveillance system. The resulting costs during the first ten years are exceptionally high, thereby lowering the IRR. At the same time, we did not include any additional benefits resulting from reduced or prevented losses due to pests and diseases other than Foc, that would very likely result from strengthening national level of surveillance and quarantine systems. We consider our results for ESEC to be very conservative.

Table 5 displays the estimated area on which the new technologies will be adopted under each of the adoption scenarios. In the case of ESEC, since the adoption decision takes place on the national level, the adoption area represents the area after 25 years for which losses could be avoided (in this case all area affected) due to the execution of the quarantine and surveillance measures at a national level. In comparison, the adoption area for the other three research options is the area on which farmers apply the new technologies and thus individually avoid or reduce losses. The estimated adoption area translates into the number of people that benefit from the new technologies which is highlighted in the second last column of **Table 5**. These figures are based on the largest adoption area reached over the assessment period.

The estimates show that conventional breeding of FWB-resistant cultivars (CBRC) reaches the largest number of beneficiaries across all research options under both the higher (14 million beneficiaries) and lower adoption scenario (7 million beneficiaries) because of the largest estimated adoption area. The investment in breeding GMRC reaches the lowest number of beneficiaries (2.7 million and 1.4 million beneficiaries for the

TABLE 5 | Results of ex ante assessment: adoption area, NPV and IRR, beneficiaries, and poverty reduction.

Research option w/adoption scenario	Adoption area after 25 years ('000 ha)	All benefits		Number of beneficiaries ('000 persons)	Poverty reduction ('000 persons)
		NPV (US$ million)	IRR (%)		
ESEC—Improved exclusion, surveillance, eradication, and containment					
*Scenario 1**	404	260.84	14	9107	807
*Scenario 2***	363	156.69	13	8237	714
*Scenario 3****	300	35.10	11	6654	615
ICDM—Integrated crop and disease management					
Expert estimated adoption scenario	344	1040.29	36	7875	157
50% reduced adoption scenario	170	501.08	30	3926	79
CBRC—Conventional breeding of FWB resistant banana cultivars					
Expert estimated adoption scenario	593	418.54	25	14,040	850
50% reduced adoption scenario	297	183.36	20	7020	422
GMRC—Genetically modified FWB resistant banana cultivars					
Expert estimated adoption scenario	127	286.03	34	2743	89
50% reduced adoption scenario	63	137.02	28	1371	44

*NPV calculated using a real interest rate of 10%. The adoption area of ESEC represents the area after 25 years where losses could be avoided due to the execution of the quarantine and surveillance measures at a national level. *Doubled arrival time and 50% reduced increase of spread rate (12.5%) once Foc reaches the country compared to counterfactual (no intervention); **Arrival time as in Scenario 1 minus 5 years; 50% reduced increase of spread rate (12.50%) once Foc reaches the country; ***Arrival time as in Scenario 1 minus 5 years; 25% reduced increase of spread rate (18.75%) once Foc reaches the country.*

two adoption scenarios, respectively) due to the assumption that countries with export-oriented banana production would not adopt GM varieties due to political and consumer concerns in importing countries. Similar to the NPV and IRR results, these numbers should be interpreted in combination with the size of the investments required for each research option.

Impact on Poverty

The last column of **Table 5** shows the poverty reduction impact of the different research option, measured as the estimated number of persons lifted out of poverty. This indicator is largely driven by the total economic benefits, national poverty rates, and region-specific elasticities of poverty reduction with respect to agricultural productivity growth. With Africa having the highest poverty rates and largest poverty elasticity, the poverty reduction measure thus favors research options that generate a larger part of the benefits in Africa compared to the other regions. This partly explains why the estimated impacts on poverty reduction are highest for ESEC (615,000–807,000 people lifted out of poverty based on the scenario) and CBRC (850,000 and 422,000 for the two adoption scenarios, respectively), whereas the investment in GMRC has the lowest poverty reduction effect. The lower importance of Cavendish cultivars in Africa also contributes to these lower adoption figures. Research options that generate comparable global economic benefits may have different poverty reduction impacts depending on share of benefits generated in countries in Africa.

Impact by Region

We estimated the regional distribution of the adoption area (**Table 6**) and find that most adoption occurs in Asia/Pacific (45–92%) followed by Africa (2–44%). The regional benefits are determined by the extent of spread over the period of the calculation with only small areas affected in Latin America[7]. The benefits are mainly determined by the adoption area, but also other parameters used in the model, such as cost effects, yield levels (which are currently much higher in LAC compared to most countries in Africa), crop prices, and likely success rate.

Sensitivity Analysis

In addition to the adoption scenarios included in results (**Tables 5**, **6**), we conducted sensitivity analysis for the pace of adoption and size of the effects. We also computed impacts for an even more conservative adoption scenario (25% of expert estimated adoption). The results of these additional scenarios are displayed in **Table 5** for ESEC and **Table 7** for the other options.

Even under these increasingly, extremely, and conservative scenarios, all but one assessed research options yield positive NPVs and IRRs well above the 10% benchmark level (**Tables 5**, **7**). These findings confirm our conclusion that investments in the assessed FWB research options generate positive economic and poverty reduction effects.

DISCUSSION AND CONCLUSION

All four assessed research lines yield positive IRRs, ranging from 11 to 36%, suggesting that investment in research to reduce the impact of Foc TR4 is worthwhile. Even under scenarios of delayed uptake or lower adoption ceiling, benefits were robustly above the 10% benchmark. The ESEC and CBRC lines have the largest

[7]In the time span between conducting our analysis and writing this paper, Foc TR4 has been detected in Colombia, with subsequent efforts and investments to confine and manage the disease. Once present in the LAC region, it is very possible that the disease will further spread and this threat will likely result in increased adoption of Foc-related technologies and management approaches since many of the key banana exporters are located in this region and will have a keen interest to protect/recover their production.

potential for poverty reduction since they cover more cultivars and more countries, particularly in Africa.

While these positive results are useful evidence for donors and development institutes and national research programs to include research on Foc TR4 among their priorities, the approximate nature of the research costs and the uptake costs mean that the results cannot be used for fine grain decision making about research priorities. Decision makers may be reluctant to base their decisions to fund one research approach rather than the other three based on these calculations. To make the results more useful for decision-making about research investments, the analysis could be improved by converting from global or aggregate country level to more targeted, location or context specific assessments.

The usefulness of disaggregation to refine results was demonstrated in our division of banana production by cultivar groups. This allowed us to differentiate rate of loss to Foc TR4 and to target different countries based on our prior knowledge of the production systems thereby differentiating uptake among the different research lines by both of these variables. In our initial research design, we planned to employ spatial data on banana production at the sub-country scale, but such data were not available at the time of the study. To improve granularity, the priority assessment cost-benefit analysis requires geographic distribution of banana production within country by cultivar group, production system, market destination, and potentially even degree of organization among growers. This last dimension has been highlighted by Montiflor et al. (2019) in their analysis of the institutional dimension of management of response to the outbreak and spread of Foc TR4 in the Philippines.

The availability of more detailed spatial data of banana production by cultivar, production system, and market is also a necessary resource to improve the projection of losses beyond our simple risk index model. The degree of dispersion of banana production areas and their location in river plains or on uplands have large potential effects on the rate of

TABLE 6 | Results—regional breakdown of adoption area.

Technology	Adoption area after 25 years						
	Africa		LAC		Asia/Pacific		ALL
	('000 ha)	Share (%)	('000 ha)	Share (%)	('000 ha)	Share (%)	('000 ha)
ESEC—Improved exclusion, surveillance, eradication, and containment							
*Scenario 1**	174	43	35	9	194	48	404
*Scenario 2***	157	43	30	8	175	48	363
*Scenario 3****	133	44	32	11	135	45	300
ICDM—Integrated crop and disease management							
Expert est. adoption scenario	6	2	21	6	317	92	344
50% reduced adoption scenario	3	2	8	5	158	93	170
CBRC—Conventional breeding of FWB resistant banana cultivars							
Expert est. adoption scenario	201	34	18	3	373	63	593
50% reduced adoption scenario	101	34	9	3	187	63	297
GMRC—Genetically modified FWB resistant banana cultivars							
Expert est. adoption scenario	18	14	3	2	106	83	127
50% reduced adoption scenario	9	14	2	2	53	83	63

*The adoption area of ESEC represents the area after 25 years where losses could be avoided due to the execution of the quarantine and surveillance measures at a national level *Doubled arrival time and 50% reduced increase of spread rate (12.50%) once Foc reaches the country as compared to a scenario without intervention; **Arrival time as in Scenario 1 minus 5 years; 50% reduced increase of spread rate (12.50%) once Foc reaches the country; ***Arrival time as in Scenario 1 minus 5 years; 25% reduced increase of spread rate (18.75%) once Foc reaches the country.*

TABLE 7 | Sensitivity analysis—benefits under different adoption scenarios.

Technology	Benefits of lower adoption (25% of estimate)		All benefits (based on50% lower adoption scenario)							
			Scenario I: *Adoption delay of 2 years*		Scenario IIa: *25% reduced effect*		Scenario IIb: *50% reduced effect*		Scenario III: (I+IIb) *Adoption delay + 50% reduced effect*	
	NPV (US$'000)	IRR (%)	NPV (US$'000)	IRR (%)	NPV (US$'000)	IRR (%)	NPV (US$'000)	IRR (%)	NPV (US$'000)	IRR (%)
ICDM	230,709	24	329,066	26	332,224	27	160,871	22	97,208	18
CBRV	66,937	15	19,155	12	124,657	18	66,148	15	-15,103	8
GMRV	63,055	23	80,352	24	99,872	26	62,812	23	34,606	19

Note: *NPV calculated using a real interest rate of 10%.*

disease spread. Other production system characteristics like the quality of seed, the use of inputs which accelerate Foc inoculum build-up such as ammonium-based fertilizers and herbicides (Dita et al., 2018), and the nature of post-harvest packing and transport can also be incorporated into spatial models to generate more realistic scenarios of losses. Such a framework could also generate insights into the risks and mechanisms for dispersion of Foc TR4 from Cavendish monocrop production systems where most of the losses have occurred to smallholder diversified production areas whether contiguous or distant. Field studies are needed to build scenarios through modeling to indicate whether Foc TR4 is a Cavendish problem or can be projected to cause progressive losses in more diversified smallholder banana production as we have suggested in the loss model.

The information base to not only improve loss projections, but also segment growers of bananas and plantains based on more detailed characterization should facilitate the contrast among investments in the research lines by user groups. Research costs can be estimated with greater specificity than our general costs based on CG center working budgets. This segmentation would also provide the opportunity to make more detailed calculations of the dissemination costs. In our calculations, we have used a single estimate for all countries (following the standard value used across the different root and tuber research investments included in the wider RTB priority assessment study). In addition to the one-time dissemination costs, we have proposed annually recurring maintenance costs to surveillance operations. However, the investments required to upgrade and maintain national plant protection operations are certain to vary from country to country. Countries like Costa Rica and Mexico already have strong tradition and capacity, while other countries are much more incipient. Farm scale biosecurity measures (Kukulies and Veivers, 2017) are an additional dimension of costs to be included, although very little documentation exists of costs. A large transnational company recently reported their initial investment in biosecurity measures to prevent Foc TR4 at US$800 per hectare with additional recurring costs to maintain foot baths and vehicle wash-down facilities in operation. At the same time, supermarkets concerned about future banana supplies have also been incorporating farm level biosecurity for FWB into their certification programs[8]. However, addressing farm scale biosecurity among small scale growers for national markets in diversified farms remains to be developed which will provide a better basis for costing.

Increased production costs were added in the case of ICDM for purchased inputs. These costs may vary depending on the degree and nature of resistance to FWB of the cultivar. The GMRC presents a different challenge to countries open to this technology. We have included Peru and Mexico in target countries, but in practice, they have generally strong regulation against any type of GM crops. An additional complexity is the possible differentiation between genetic modification and gene editing. Procedures and documentation on biosafety for cultivar registration are potential costs which may be needed for more targeted priority assessment calculations (see example in Ainembabazi et al., 2015).

To move beyond the contrast of investments in different research options, optimization analysis could be considered. The spatial models linked with economic analysis could be used to explore mixed investments of ESEC, ICDM, and the breeding options as the disease spreads. Such models may generate different strategies for Africa than for Latin America, both continents at early stages in Foc TR4 spread, but with quite different cultivar preferences and grower groups. Such an optimization exercise for Asia with already advanced spread may need a greater emphasis on the use of already infested soils than on ESEC, although internal exclusion remains important.

In conclusion, the priority assessment exercise provides evidence that investments in all assessed research lines to address the threat and projected losses from Foc TR4 will provide positive returns and contribute to poverty reduction. A more fine-grained estimate of costs and benefits to assess alternative research lines would require more complete characterization and spatial distribution of banana production systems, improved projection of losses, more site-specific costs of research, and more detailed calculations of costs of uptake and impacts on production costs and viability. Such exercises, applied in different regions and countries from Asia to Latin America, should serve not only to improve research efficiency, but also provide science-based documentation for the debates about the severity of the Foc TR4 threat and alternative ways to address it.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MD, CS, and LP identified and defined the Fusarium research options. DP and LS conducted the economic analysis and all other data tasks. CS and DP did the final manuscript writing based on the existing RTB working manuscript. All authors contributed to the article and approved the submitted version.

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[8]See, e.g., https://www.globalgap.org/uk_en/media-events/news/articles/Tesco-Takes-Crucial-Step-to-Protect-Global-Banana-Production/

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