THE DEVELOPMENT OF NOVEL MOLECULAR DIAGNOSTIC ASSAYS FOR *FUSARIUM OXYSPORUM* F. SP. *CUBENSE*

by

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SUMMARY

Banana (*Musa* sp.) is an important crop for food security and income generation in developed and developing nations. Most bananas are grown for local consumption, with approximately only 14% exported to Europe, the US, Japan and Russia. The export industry is almost exclusively reliant on Cavendish bananas. Cavendish bananas also constitute approximately 47% of bananas grown globally.

A major constraint to sustainable banana production is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (Foc). Race 1 and 2 Foc isolates cause disease of Gros Michel, other dessert varieties and some cooking banana like Bluggoe, but not Cavendish bananas. Subtropical (STR) and tropical (TR4) race 4 Foc isolates affect Cavendish and most banana cultivars susceptible to Foc races 1 and 2 in the subtropics and tropics, respectively. Fusarium wilt is difficult to manage as Foc chlamydospores can survive in the soil for decades. Phytosanitary regulation and clean planting material to exclude Foc, and the planting of resistant banana cultivars, are therefore required to manage the disease. Early detection and the geographic mapping of Foc can inform farmers and governing bodies about the distribution of the fungus and aid in containment strategies. DNA-based detection with PCR is favoured over phenotypic identification due to its speed and accuracy. PCR detection, however, is qualitative and lacks sensitivity when DNA is isolated from environmental samples.

Quantitative (q)PCR has been developed to directly detect plant pathogens in environmental samples. Molecular markers are available to quantitatively detect Foc races 4 and TR4, but the Foc TR4 markers lack specificity. Molecular markers for Foc Lineage VI (race 1 and 2) and STR4 are also required. In this study, DNA markers and qPCR assays were developed to detect Foc Lineage VI, TR4 and STR4 in plant, water and soil samples. Markers were designed from the RNA polymerase III subunit, a hypothetical protein and the Foc supercontig 1.57 gene regions. Marker suitability and specificity was evaluated, and standard curves produced for Foc detection based on specificity, repeatability, reproducibility, limit of quantification (LOQ) and limit of detection (LOD). The Foc TR4 and Lineage VI markers were specific for qPCR detection, but the Foc STR4 markers amplified two non-target *Fusarium* members and two non-pathogenic *F. oxysporum* isolates.

Quantitative PCR can detect Foc collected in the environment. DNA from non-viable cells, however, is then also amplified, which can lead to an overestimation of inoculum levels. In this study, propidium monoazide (PMA) and qPCR were combined to quantify Foc Lineage VI and TR4 spores that survive following sanitiser treatments. PMA applied at 20 µM, incubated in the dark for 1 min and activated with light for 5 min effectively separate viable from non-viable Foc spores. The PMA-qPCR results also correlate well with colony forming unit counts.

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OPSOMMING

Piesangs (*Musa* sp.) is ‘n belangrike gewas wat voedselsekuriteit en inkomste bied aan beide ontwikkelde en ontwikkelende lande. Meeste piesangs word verbou vir plaaslike verbruik, met net ongeveer 14 % wat uitgevoer word na Europa, die VSA, Japan en Rusland. Die uitvoer industrie maak eksklusief staat op Cavendish piesangs. Omtrent 47% van piesangs wat wereldwyd verbou word bestaan ook uit Cavendish piesangs.

Volhoubare piesang produksie word bedreig deur Fusarium verwelking, veroorsaak deur *Fusarium oxysporum* f. sp. *cubense* (Foc). Foc ras 1 en 2 veroorsaak siekte van Gros Michel, ander nagereg tipes en kook tipes soos Bluggoe, maar nie Cavendish tipes nie. Foc subtropiese (STR4) affekteer Cavendish en meeste varieteite vatbaar vir ras 1 en 2, in subtropiese gebiede, terwyl Foc tropiese ras 4 (TR4) siekte veroorsaak op dieselfde variete in tropiese gebiede. Fusarium verwelking is moeilik op te bestuur, want Foc chlamydospore kan in die grond oorleef vir dekades. Fitosanitêre regulasies en skoon plant material, sowel as die gebruik van weerstandbiedende variteite, word vereis vir die bestuur van die siekte. Vroeë deteksie en opstel van kaarte van die geografiese verspreiding van Foc, kan boere en regerings inlig en te hulp staan. DNS-gebaseerde deteksie met PKR word verkies oor fenotipiese identifikasie, omdat dit meer spoedige en meer akkurate resultate lewer. PKR identifikasie is kwalitatief en vir DNS monsters wat isoleer word uit die omgewing, moet sensitiwiteit verbeter word.

Kwantitatiewe (k)PKR is ontwikkel om plant patogene direk uit omgewingsmonsters te identifiseer. Molekulêre merkers is reeds beskikbaar vir kwantitatiewe deteksie van Foc ras 4 en TR4, maar Foc TR4 merkers se spesifisiteit moet verbeter word. In hierdie studie is DNS merkers en kPKR toetse ontwikkel vir die deteksie van Foc Linie VI, TR4 en STR4 in plant, water en grond monsters. Merkers is ontwikkel in die RNS polymerase III subeenheid, ‘n hipotetiese protein en die Foc nukleotiedversameling 1.57 geen areas. Merker geskiktheid en spesifisiteit is evalueer en standaard kurwes produseer vir Foc deteksie gebaseer op spesifisiteit, herhaalbaarheid, reproduseerbaarheid, die limiet van kwantifisering (LOK) en die limiet van deteksie (LOD). Die Foc TR4 en Linie VI merkers was spesifiek vir kPKR deteksie, maar die Foc STR4 merkers het twee ander *Fusarium* spesies en twee nie-patogene *F. oxysporum* montsters, wat nie teikens van die merkers was nie, amplifiseer.

Kwantitatiewe PKR kan gebruik word om Foc uit omgewingsmonsters te identifiseer. DNS van dooie selle kan egter ook amplifiseer word, wat kan lei tot oorskattings van inokulum vlakke. In die studie is propidium monoazied (PMA) en kPKR kombineer om Foc Linie VI en TR4 spore te kwantifiseer na behandeling met ontsmettingsmiddels. PMA aangewend by 20 µM, inkubeer vir 1 minuut in die donker, en lig-geaktiveer vir 5 minute, kon lewende en dooie selle van Foc effektiek onderskei. Die PMA-kPKR resultate het goed gekorreleer met bepaling van kolonie-vormende eenhede.

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CHAPTER 1

Diagnostic assays for *Fusarium oxysporum* f. sp. *cubense*: a review

INTRODUCTION

Bananas (*Musa* spp.) are ranked among the world's leading agricultural crops (Potts *et al*., 2014). Cultivated bananas include cooking bananas, which are important staple crops in developing countries and important for food security, and dessert bananas, which are eaten as fresh fruit and serve as important cash crops for export (Stover, 1986; Price, 1995). The export banana industry relies mainly on one type of banana, the Cavendish variety, which is planted mostly in large-scale monoculture production systems, leaving it vulnerable to biological constraints (Ploetz, 2000).

Fusarium wilt (also called Panama disease), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) (Stover, 1962), is one of the major constraints to sustainable banana production. In the early 1900s Fusarium wilt, caused by Foc race 1, decimated export banana plantations in Central and South America, the Caribbean and West Africa (Stover, 1962; Ploetz, 2005). The epidemic was one of the most devastating plant diseases in agricultural history. The only successful management strategy was the replacement of the highly susceptible Gros Michel bananas with Cavendish bananas (Stover, 1986; Ploetz and Correll, 1988). Outbreaks of Fusarium wilt in Cavendish bananas in tropical Asia in the past two decades are indicative of a renewed Fusarium wilt epidemic (Ploetz, 2005). The pathogen responsible for the new epidemic, Foc tropical race 4 (TR4), has destroyed Cavendish plantations throughout Southeast Asia (Ploetz, 2015). More recently it has also been introduced into the Middle East (Ploetz, 2015) and Africa (northern Mozambique) (IITA, 2013). In subtropical countries, such as South Africa, a strain of Foc called 'subtropical' race 4 (STR4) has reduced banana production by 70% in some areas (Viljoen, 2002).

Fusarium wilt management is challenging, as Foc is a fungus that can persist in soil for decades (Ploetz, 1994; Buddenhagen, 2007). The only means to manage Fusarium wilt effectively is by exclusion of the pathogen (phytosanitary regulation and clean planting material) and the replacement of susceptible banana cultivars with resistant ones. There are, however, no suitable varieties available to replace Cavendish bananas globally (Ploetz, 2015; Dale *et al*., 2017). Accurate diagnosis of Foc, thus, plays a vital role in the early detection and geographic mapping of Foc spread, which can, in turn, be used to inform farmers and guide containment strategies (Dita *et al*., 2010; Nakato *et al*., 2016).

DNA-based diagnostics using PCR are favoured today because of their accuracy and efficiency compared to other methods of pathogen identification. A drawback of DNA-based

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diagnostics, however, is that DNA from viable cells cannot be distinguished from those from non-viable cells (Nogva *et al*., 2003; Mackay, 2004). This can lead to an overestimation of inoculum levels if pathogen numbers are quantified by quantitative (q)PCR. This manuscript will review the current Foc classification system, its epidemiology and management, with a special focus on PCR-based diagnostic techniques. Since no DNA-based diagnostic assay exists to distinguish viable from non-viable Foc cells, methods to differentiate between viable and non-viable cells of other fungal species will also be discussed (Vesper *et al*., 2008; Andorrà *et al*., 2010; Crespo-Sempere *et al*., 2013; Vilanova *et al*., 2017).

THE BANANA FUSARIUM WILT PATHOGEN

Morphology and pathogenicity

Foc is a member of the *Fusarium oxysporum* (Snyder and Hansen, 1940) species complex (FOSC). Members of the FOSC are anamorphic filamentous fungi that are ubiquitous in soil. They produce three distinctive spore types; microconidia, macroconidia and chlamydospores (Wardlaw 1961; Burgess, 1981; Leslie and Summerell, 2006). Microconidia are usually abundant, formed in false heads on short monophialides and are oval, elliptical or reniform in shape. Macroconidia are falcate with three to four septa and have short apical cells and notched or foot-shaped basal cells. They are formed on short monophialides of hyphae or on branched conidiophores in sporodochia (Snyder and Hansen, 1940; Ohara *et al*., 2004; Leslie and Summerell, 2006). Chlamydospores are formed singly or in pairs with double-layered walls in hyphae and macroconidia (Nelson *et al*., 1981; Ploetz, 2006).

The FOSC includes both pathogenic and non-pathogenic strains. The pathogenic strains can cause vascular wilt diseases and root or bulb rots (Jarvis and Shoemaker, 1978; Nelson, 1981; Baayen *et al*., 2000), while the non-pathogens live as endophytes or saprophytes (Steinberg *et al*., 2016). Pathogenic and non-pathogenic *F. oxysporum* strains are generally considered indistinguishable based on morphological characteristics (Guadet *et al*., 1989). FOSC isolates can be separated from each other based on their pathogenicity to crops they affect. Strains of *F. oxysporum* that affect the same crop are known as a *forma specialis* (f. sp.), with the strains causing disease to banana known as *F. oxysporum* f. sp. *cubense* (Stover, 1962).

Three different pathogenic races of Foc are known, based on the ability of strains to infect a cultivar or group of cultivars. Foc race 1 strains are pathogenic to Gros Michel, Silk, Apple, Lady Finger and Latundan cultivars and Foc race 2 strains are pathogens of certain cooking banana varieties like Bluggoe (Stover, 1962; Ploetz, 1994; Ploetz, 2005). Foc race 4 infects Cavendish varieties and most varieties affected by Foc races 1 and 2 (Waite and Stover, 1960; Su *et al*., 1986; Moore *et al*., 1993). Foc race 4 is further divided into two groups.

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Foc STR4 causes disease to Cavendish bananas under subtropical conditions, and Foc TR4 causes disease to Cavendish bananas both in the tropics and subtropics (Viljoen, 2002; Ploetz, 2006). The race concept in Foc is cumbersome, as it relies on pathogenicity tests on multiple banana cultivars (Stover and Buddenhagen, 1986; Ploetz and Correll, 1988).

Pathogen diversity

To compensate for the limitations associated with the Foc race concept, vegetative compatibility groups (VCG) have been used to study the diversity in Foc. Twenty-four different VCGs have been described for Foc (Ploetz and Correll 1988; Moore *et al*. 1993; Bentley *et al*. 1995; Katan 1999; Katan and Di Primo 1999). Vegetative compatibility is the ability of individual isolates to recognise each other by the fusion of their hyphae to form a heterokaryon (Leslie and Summerell, 2006). For this to occur, the loci governing vegetative compatibility, called the *vic* or *het* loci, must be identical (Correll *et al*., 1987). In an anamorphic population, such as Foc, differences at the *vic* loci prevent heterokaryon formation between incompatible isolates (Ploetz and Correll, 1988; Elias and Schneider, 1991; Leslie, 1993). Isolates within the same VCG often share pathological and physiological traits (Kistler, 1997). For Foc, however, VCGs and race do not always correlate as multiple VCGs may occur in the same race, and a single VCG may contain strains from different Foc races (Ploetz, 1990). VCGs can give an indication of genetic diversity but cannot indicate genetic distance between groups (Bentley *et al*., 1998; Fourie *et al*. 2009).

DNA-based techniques, such as amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), DNA amplification fingerprints (DAFs), multi-gene sequencing and diversity array technology (DArT) have been used to indicate genetic distance among Foc VCGs (Bentley *et al*., 1995; Bentley *et al*., 1998; O'Donnell *et al*., 1998; Groenewald *et al*., 2006; Fourie *et al*. 2009; Ordonez *et al*., 2015). These techniques showed that Foc is divided into two polyphyletic clades, indicating that pathogenicity to banana evolved at least twice (Ploetz and Pegg, 1997). Within the two clades, five to nine clonal lineages are found that consist of closely-related VCGs (Bentley *et al*., 1998; Groenewald *et al*., 2006; Fourie *et al*., 2009). Foc Clade A contains Lineages I-V, and Clade B contains Lineages VI-VIII (Fourie *et al*., 2009).

Life cycle of Foc

Foc persists in soil as dormant chlamydospores for decades and can also survive as an endophyte of certain weeds (Stover, 1962; Hennessy *et al*. 2005) (Fig. 1). The fungus infects susceptible banana plants after exudates released by their roots induce chlamydospore germination. The germination tubes enter the plant by direct penetration of the root epidermis or via pre-existing wounds (Stover 1962; Beckman and Roberts 1995). The fungus then

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progresses through the cortex and endoderm into the vascular tissue. Host cell walls are penetrated when the fungus releases enzymes such as pectin methylesterases (PMEs), polygalacturonases (PGs) and polymethylgalacturonases (PMGs) (Brandes 1919; Gothoskar *et al*., 1953; Vorwerk *et al*., 2004; Cantu *et al*., 2008). Once inside the vascular tissue, Foc proliferates and produces secondary inoculum that progresses to the pseudostem where it disrupts water translocation causing wilt symptoms. Wilting results as the host secretes gums and tyloses that occlude the infected xylem vessels to isolate Foc at the cost of the vascular function (Wardlaw, 1930; Beckman and Halmos, 1962; Pegg *et al*., 1996). External symptoms that develop include chlorosis and wilting of foliage from old to young leaves, necrosis and longitudinal splitting of the pseudostem (Stover, 1962; Beckman 1990). Internal symptoms include discolouration of the vascular tissue due to necrosis. As the banana host dies and nutrients become limited, Foc produces chlamydospores that go back into the soil to serve as primary inoculum for subsequent infections (Nash *et al*. 1961; Rowe *et al*., 1977).

Dispersal

Foc is dispersed with infected planting materials and with soil attached to shoes, machinery and plantation tools. Symptomless infected suckers, as well as non-host plants, may also carry the pathogen (Stover, 1962; Hennessy *et al*., 2005). The spores of Foc can also be spread in irrigation and run-off water from infested fields (Stover, 1962; Su *et al*., 1986; Ploetz, 1994). Once introduced into new fields, Foc is often not detected until symptoms begin to develop on infected plants. This may take several months, allowing the fungus to spread to new areas while undetected.

FUSARIUM WILT MANAGEMENT

Managing Fusarium wilt is complex due to the diversity of the pathogen, the resilience of the pathogen in soil and the way in which banana is cultivated. Bananas are grown as a perennial crop, which enables pathogen inoculum to build up over time (Ploetz, 2000). Fusarium wilt requires an integrated disease management strategy (Viljoen, 2002), which can be divided into three steps: prevention, containment and disease management.

Prevention

Prevention is the primary strategy when Foc has not been detected in an area. It includes increasing awareness about Fusarium wilt and the training of farmers in biosecurity (Pérez Vicente *et al*., 2014). Good biosecurity practices include the use of disease-free tissue culture plantlets to establish new fields and proper sanitation routines (Dita *et al*., 2018). Limiting the

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movement of soil and water between plantations, and the cleaning and disinfection of farm equipment, prevents the introduction of Foc to uninfested areas (Nel *et al*., 2007). Sanitisers that have been shown to inhibit Foc growth include quaternary ammonia compounds such as SporeKill (Nel *et al*., 2007; Meldrum *et al* 2013; Nguyen *et al*., 2019).

Early detection and containment

When Fusarium wilt has been detected early in a plantation, containment measures become necessary. This include rapid and accurate diagnostics, surveillance, and providing farmers with information on disease dispersal and prevalence (Nakato *et al*., 2016). Fencing of diseased areas can be done to limit the movement of people and animals, which in turn can prevent the spread of Foc (Hennessy *et al*., 2005). Trenches need to be dug around contaminated areas to limit runoff water spreading the pathogen (Nel, 2004). Proper destruction of affected plants is also vital to limit inoculum build-up (Eagling, 2009). This can be done by glyphosate injection of affected and surrounding plants (Meldrum *et al* 2013; Nguyen *et al*., 2019). Chemical disinfectants, such as quaternary ammonium or demethylation-inhibiting compounds can be used to clean farm equipment, thereby preventing the spread of Foc to non-infested areas (Nel *et al*., 2007).

Disease management

Once Fusarium wilt becomes widespread in an area, the primary concern is to keep producing bananas economically. This can be achieved by growing resistant varieties in the area and by managing the pathogen's inoculum levels in the soil.

Several banana improvement programs have sought to provide industry with a suitable Foc-resistant banana variety. These include conventional banana breeding programmes such as Fundación Hondureña de Investigación Agrícola (FHIA), the International Institute of Tropical Agriculture (IITA), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), the African Research Centre on Banana and Plantain (CARBAP) and the National Research Centre for Banana (NRCB). Conventional breeding of banana is time-consuming and costly, and commercial cultivars are mainly infertile (Rowe, 1981; Stover and Buddenhagen, 1986). Their fruit is also often not acceptable to the market.

Cavendish banana has been genetically modified with *Ced9* (a nematode derived gene) and *RGA2* (a gene from diploid bananas with Foc TR4 resistance), which resulted in plants resistant to Foc TR4 that retain the Cavendish taste (Dale *et al*., 2017). Still, public perception of genetically modified food prevents large-scale production of genetically modified bananas (Panis, 2014; Stergiopoulos *et al*., 2014). Mutation breeding, an unconventional plant improvement technique achieved by *in vitro* mutagenesis, has produced Cavendish somaclones tolerant to Foc TR4 (Molina, 2011; Molina *et al*., 2016). Due to their longer

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production cycle, somaclonal variants are planted mainly in areas where Foc TR4 makes Cavendish banana production uneconomical (Molina, 2011).

Conventional plant protection practices to mitigate Fusarium wilt, such as chemical and biological control, are not very successful (Ploetz, 2015). Such practices are primarily aimed at promoting disease suppression. Methyl bromide fumigation has been used to suppress Fusarium wilt in soil environments (Herbert and Marx, 1990). Synthetic chemicals such as soil fumigants have not been favoured in recent years most likely due to the associated negative effects on the environment and human health (Gamliel *et al*., 2000; Woo *et al*., 2014; Ploetz, 2015). As an alternative to synthetic chemicals biofumigants (biologically active plant substances used to control disease) produced by Chinese chives have been used to inhibit the growth of Foc (Zhang *et al*., 2013). The introduction of beneficial microbes in soil has also contributed to the suppression of banana Fusarium wilt (Ho *et al*. 2015; Shen *et al*. 2015; Xue *et al*. 2015; Fu *et al*. 2017; Köberl *et al*. 2017), but it does not control the disease. Soil microbes that suppress Foc in banana fields include non-pathogenic *Fusarium* spp., *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* spp. (Raza *et al*. 2017). Crop rotations and inter-cropping can be used to lower soil inoculum levels (Su *et al*. 1986; Zhang *et al*., 2013). An increase in plant diversity leads to increased microbial diversity that may decrease pathogen inoculum levels in the soil over time (Curl, 1963; Hwang *et al*, 1985; Zhang *et al*., 2013).

DIAGNOSTIC TECHNIQUES FOR FUSARIUM OXYSPORUM, WITH SPECIAL REFERENCE TO FOC

Fusarium wilt diagnosis is based on host symptoms and pathogen identification. For Fusarium wilt diseases, field symptoms most often include the progressive yellowing and wilting of host foliage and the internal browning of roots and stems (Beckman 1964). In banana, a longitudinal splitting of the pseudostem can also be seen, with a yellow to brownish-red discoloration of the inner rhizome and pseudostem (Stover 1962; Ploetz, 2000). Traditionally, the responsible pathogen will be isolated from infected host tissue and identified based on their morphological characteristics. Because of the similarity of FOSC members' morphology, phenotypic and DNA-based identification techniques are being used to identify FOSC pathogens such as Foc.

Phenotypic identification

Selective media

Artificial media can be used for isolating and enumerating *Fusarium* species. These media include Nash-Snyder medium, Komada's (K2) medium, malachite green agar and selective *Fusarium* agar (Leslie and Summerell, 2006). When grown on modified K2 media, Foc race 4 Foc isolates from Australia, Taiwan and mainland China have formed lacinated colonies, which

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distinguished them from Foc races 1 and 2 isolates (Su *et al*., 1986; Qi *et al*., 2008). Ploetz (1990), however, found this technique inconsistent as some Foc race 4 isolates were hard to distinguish from Foc races 1 and 2 isolates.

Volatiles

Foc isolates grown on starch media can be differentiated based on the volatile organic compounds (VOCs) produced (Pegg *et al*., 1994). Foc isolates from Panama, Costa Rica and Jamaica have been distinguished from Cuban isolates based on the production of aromatic aldehydes (Brandes, 1919). The VOCs of 57 Australian Foc isolates representing Foc races 1, 2 and 4 were compared with gas chromatography. Foc race 1 and 2 isolates produced no chromatogram peaks ('Inodoratum'), but Foc race 4 isolates had characteristic gas chromatogram profiles ('Odoratum') (Moore *et al*., 1991). To differentiate Foc isolates based on VOCs the sensitivity of the technique must be improved as currently Foc race 4 can only be distinguished from other races.

Pathogenicity testing

Pathogenicity testing is used to identify *formae speciales* and races of *F. oxysporum* (Waite and Stover, 1960; Correll, 1991). This can be done either in a greenhouse or in the field. For Foc, pathogenicity tests can reliably be used to confirm a pathogen's ability to cause disease to banana, but many factors may influence the race-typing of the banana Fusarium wilt fungus. These include the inoculation method, host age and environmental conditions. The requirement of large sets of banana cultivars and the lack of a standardised inoculation technique makes comparing pathogenicity tests difficult (Stover and Buddenhagen, 1986; Ploetz and Correll, 1988; Fourie *et al*., 2011).

VCG testing

VCG testing involves the generation of mutants, incapable of utilising nitrates, on chlorate ($KCLO_3$) media (Leslie and Summerell, 2006). When grown on a minimal media that includes nitrate as nitrogen source, the mutations can be classified as *nit* 1(structural mutation in nitrate reductase), *nit* 3 (regulatory gene for nitrate reduction) and NitM (mutation in at least one of five *nit* genes involved in molybdenum cofactor construction) (Klittich and Leslie, 1988). To identify unknown VCGs, *nit* 1 and 3 mutants of the unidentified isolate is paired with a NitM testers isolate of a known VCG on minimal media (Leslie and Summerell, 2006). VCG testing is a relatively tedious exercise that can take up to 2 months to complete.

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DNA-based identification

PCR is a widely used diagnostic technology that has improved the speed and accuracy with which plant pathogens are identified (Henson and French 1993). PCR involves the enzymatic amplification of target DNA with DNA polymerase and thermal cycling (Mullis *et al*., 1986). PCR primers can be designed to detect specific target organisms based on tandem repeats or variations at single or multiple loci (Wright and Wynford-Thomas, 1990; Dieffenbach *et al*., 1993; Agarwal *et al*., 2008). Multi-locus variations include RFLPS, AFLPS or RAPD markers that can be amplified to produce sequence characterised amplified region (SCAR) markers to increase reproducibility (Cheng *et al*., 2015). Locus-specific variations include simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) (Wilkinson, 2000). Common target genes used for marker design of FOSC species include the β-tubulin, calmodulin, translation elongation factor-1 alpha (TEF), mitochondrial small subunit and ribosomal DNAs (rDNAs) (includes the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions) genes (Chandra *et al*., 2011). These genes are favoured due to their high copy numbers, intra-species conservation and inter-species variation. Variations in effector genes associated with pathogenicity, such as Foc-*SIX*, sucrose non-fermenting, cytochrome P450 and F-box protein in Foc, are also being investigated (Meldrum *et al*., 2012; Myburg *et al*., 2013; Czislowski *et al*., 2017). Primers based on the Foc-*SIX8* gene were used by Fraser-Smith *et al.* (2014) to differentiate Foc race 4 from Foc races 1 and 2, and Foc STR4 from Foc TR4. Pathogenicity genes may thus correlate pathogen identity with host specificity (Czislowski *et al*., 2017).

PCR markers used for Foc identification include those based on sequence variation within the IGS region (Dita *et al*., 2010; Li *et al*., 2011) and a putative virulence gene (Li *et al*., 2013a) for Foc TR4, as well as a SCAR marker for Foc race 4 (Lin *et al*., 2009). The SCAR markers developed by Lin *et al*. (2009) were used for PCR and insulated isothermal PCR (iiPCR) in a POCKIT device (Fig. 2) (Lin *et al*., 2016). To our knowledge PCR markers for Foc race 1 and 2 are not currently available. Limitations of traditional PCR include the generation of qualitative results and a lack of sensitivity when DNA is extracted from environmental samples.

Detection with quantitative PCR technologies

Quantitative PCR technologies have been designed to address the limitations of PCR but are more expensive and require specialised expertise (Espy *et al*., 2006). Quantitative PCR technologies use fluorescent detection to track the amplification of DNA in real time (Heid *et al*., 1996; Wittwer *et al*., 1997). The ability to track DNA amplification in real time allows detection at very low concentration and provides quantitative data (Higuchi *et al*., 1993; Mackay, 2004). The increased sensitivity of quantitative PCR technologies relative to traditional PCR makes detection of pathogens from environmental samples more efficient

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(Schaad and Frederick, 2002; Yadav and Singh, 2017). Examples of quantitative PCR technologies include loop-mediated isothermal PCR (LAMP) and quantitative PCR (qPCR).

Loop-mediated isothermal PCR

LAMP-PCR is designed for in-field diagnostics by using a single tube-based reaction, a visualisation system and a portable heat source to amplify and detect target DNA (Khiyami *et al*., 2014; Abdullahi *et al*., 2015). Target DNA amplification is quantified using colour-changing dyes like SYBR green or from increases in the turbidity of magnesium pyrophosphate (Parida *et al*., 2008). The portable heat source keeps the LAMP-PCR reaction isothermal at 60-65°C. At this temperature, the *Bst* DNA polymerase large fragment and LAMP primers within the reaction tube amplify target DNA in a loop pattern. The main drawback of LAMP is the complexity of LAMP primer design (Kaneko *et al*., 2007; Gill and Ghaemi, 2008). In the field, portable qPCR technologies are also exposed to different environmental conditions that may influence specificity, and cross-sample contamination is thus more likely than in a laboratory setting.

LAMP assays were developed for the detection of Foc race 4, which includes both TR4 and STR4 (Li *et al*., 2013b; Zhang *et al*., 2013; Peng *et al*., 2014). Primers where designed based on a SCAR marker (Li *et al*., 2013b), the IGS region (Zhang *et al*., 2013) and a RAPD marker (Peng *et al*., 2014). These studies used a limited number of Foc samples for specificity testing. Given the diversity amongst Foc isolates, the specificity of available LAMP assays needs to be re-evaluated against representatives of all Foc VCGs.

Quantitative PCR

qPCR is the preferred method for pathogen detection from environmental samples due to its sensitivity, precision and reproducibility (Klein, 2002; Bustin *et al*., 2009; Capote *et al*., 2012). In a qPCR assay, DNA is amplified via PCR and quantified using non-specific dsDNA intercalating dyes or sequence-specific DNA probes (Navarro *et al*., 2015). As fluorescence is detected, an exponential curve of DNA amplification is created from which quantitative analysis can be performed (Higuchi *et al*., 1993). The ability to quantify DNA allows analysis of environmental samples at very low quantities of pathogen DNA (Yadav and Singh, 2017).

In contrast to LAMP, qPCR is not portable and requires comparatively bulky equipment for quantitative detection. Molecular markers have been developed for qPCR detection of race 4 and TR4 (Lin *et al*., 2013; Aguayo *et al*., 2017). Markers developed by Lin *et al*. (2013) cannot differentiate Foc TR4 from Foc STR4, and markers developed by Aquayo *et al*. (2017) target VCG 0121 and VCG 01213/16 simultaneously (Foc TR4). The quantitative assays developed by Lin *et al*. (2013) and Aguayo *et al*. (2017) can detect Foc *in planta*, and LAMP based assays

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developed by Zhang *et al*. (2013) and Peng *et al*. (2014) can detect Foc in soil. No assays are available for detecting Foc in water.

Limitations of PCR-based techniques

Some of the limitations of PCR based techniques such as qPCR include the reliance on molecular markers that require knowledge of the host genome, the necessity of good quality DNA, and the inability to differentiate DNA from viable or non-viable cells (Mackay, 2004; Kralik and Ricchi, 2017). Adequate sequencing data is not always available for developing primers that are suitable and specific for qPCR detection. Environmental samples may also contain inhibitors like humic substances or plant metabolites that interfere with DNA extraction and amplification (Porteous and Armstrong, 1991; Wilson, 1997; Khanuja *et al*., 1999). The amplification of DNA from both viable and non-viable cells, can lead to overestimation of pathogen quantities. Relative to PCR, qPCR is more expensive and requires more specialised expertise (Espy *et al*., 2006).

Quantitative detection of viable Foc

Viability based on RNA markers

RNA has a short half-life outside viable cells. Reverse transcription qPCR (RT-qPCR), can therefore be used to detect RNA markers, providing quantitative detection of viable pathogens (Keer and Birch, 2003; Martínez-Blanch *et al*., 2011). RNA markers include regulatory RNAs associated with the target organism or transcripts that may be under or over-expressed in response to environmental conditions (Ludwig and Weinstein, 2005). RT-qPCR has been used for the detection of viable viruses (Monaco *et al*., 2011; Loconsole *et al*., 2012) pathogenic bacteria (Pichon and Felden, 2005; Chinni *et al*., 2010), algae (Bai *et al*., 2015), archaea (Babski *et al*., 2014) and fungi (Hierro *et al*., 2006; Morton *et al*., 2012; Ogata *et al*., 2015). RNA markers have not been developed for the quantitative detection of Foc. RNA marker development is expensive compared to other viability tests and requires specialist skills.

PMA, PCR and qPCR

Dyes such as ethidium bromide monoazide (EMA) and propidium mono-azide (PMA) can be used in conjunction with PCR/qPCR to preferentially detect DNA from viable cells (Nocker et al., 2006). PMA is less toxic than EMA and the preferred dye for the quantitative detection of viable cells (Nocker and Camper, 2009). It is a membrane impermeable, propidium iodide analogue with a photo reactive azide group. The azide group is capable of covalently binding of nearby organic moieties such as nucleic acids when exposed to light (Nocker *et al*., 2007; Tamburini *et al*., 2013). Unbound PMA molecules can be removed during DNA extraction (Taylor *et al*., 2014). DNA within membrane-compromised cells is bound by PMA and PCR

inhibited. The DNA within viable cells is not bound by PMA and can be PCR-amplified because the dye is membrane impermeable (Hellein *et al*., 2012).

PMA has been applied to distinguish viable from non-viable bacterial pathogens to monitor food safety procedures. A study by Nocker *et al*. (2007) compared the effect of inhibitory treatments on bacteria using a PMA-qPCR method. Treatments included the hypochlorite disinfection of *Salmonella enterica* serovar *Typhimurium*, benzalkonium disinfection of *Listeria monocytogenes*, and heat disinfection of *Mycobacterium avium*. PMA has been applied to viability testing of fungal plant pathogens, including *Alternaria* spp. (Crespo-Sempere *et al*., 2013) and *Monilinia* spp. (Vilanova *et al*., 2017). PMA has never been used to test the viability of any *Fusarium* spp.

Optimising a propidium monoazide quantitative PCR (PMA-qPCR)

PMA treatment conditions must be optimised in order to combine it with qPCR. Firstly, PMA treatment optimisation requires a membrane-compromised or non-viable target organism. Heat treatment is the most popular method for preparing a non-viable control because it is effective, easy to do and is unlikely to interfere with DNA extraction (Nocker *et al*., 2006; Richter *et al*., 2010; Àlvarez, *et al*., 2013). The efficacy of membrane disruption can be tested by plating the non-viable control out on nutrient agar (Yáñez *et al*., 2011). If no growth occurs the treatment was effective. The effect of PMA treatment conditions such as PMA concentration, dark incubation time and light activation time can be compared by the differences in Ct values between viable samples and the non-viable control (Crespo-Sempere *et al*., 2013; Vilanova *et al*., 2017). A large difference between viable and non-viable samples indicates the PMA dye is working optimally (Banihashemi *et al*., 2012). The cytotoxicity of PMA concentrations should be evaluated with colony forming unit (CFU) counts to ensure the dye does not kill viable cells that reduces the difference between viable and non-viable cells (Yáñez *et al*., 2011; Fittipaldi *et al*., 2012).

The limitations of propidium monoazide

A limitation of using PMA dye is the reliance on membrane integrity as an indication of viability. Only treatments that compromise the membrane integrity of the target organism can be compared (Girones *et al*., 2010). PMA requires direct contact with the target cell membranes in order to enter the cell and bind DNA, which proves challenging in complex environmental samples. PMA-PCR has nevertheless been applied to environmental water and effluent samples (Bae and Wuertz, 2009; Varma *et al*., 2009), biofilms (Àlvarez, *et al*., 2013; Tavernier and Coenye, 2015) and several plant matrices, including stone fruit (Vilanova *et al*., 2017), berries (Verhaelen *et al*., 2012), tomato (Crespo-Sempere *et al*., 2013) and lettuce (Liang *et*

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al., 2011). The use of PMA on plant tissue appears limited to softer tissue in which homogenisation can be performed without exposing pathogen DNA prior to PMA application.

CONCLUSIONS

Fusarium wilt of banana is one of the most devastating constraints to sustainable banana production. Management of the disease is challenging due to the resilience of the pathogen in soil and the perennial nature of banana production. Early detection of Foc can alert farmers and governing bodies and can increase the success of containment strategies (Hennessy *et al*., 2005; Nakato *et al*., 2016). Diagnostic techniques of Foc include phenotypic and DNA-based molecular identification. PCR has improved the speed and accuracy with which plant pathogens are identified (Henson and French 1993). LAMP and qPCR have been developed for the quantitative detection of plant pathogens such as Foc in environmental samples. Of the two, qPCR is the more trusted technique. Molecular markers available for Foc TR4 qPCR assays do not target VCG 01213/16 exclusively (Aguayo *et al*., 2017). They have also only been optimised for detection from plant samples. Assays for the quantitative detection of Foc TR4 from plants, soil and water are therefore urgently required. Foc Lineage VI and STR4 are also important pathogens to bananas in non-Cavendish bananas grown in the tropics and Cavendish bananas grown in subtropical countries, respectively. qPCR assays for the detection of Foc Lineage VI, TR4 and STR4 in plant, water and soil samples will therefore be developed and evaluated in **Chapter 2**.

A major drawback of qPCR is that DNA is amplified from both non-viable and viable cells, making the DNA-based quantification of disease management outcomes difficult (Mackay, 2004; Kralik and Ricchi, 2017). Viability tests, such as CFU counts, are often not suitable for Foc because FOSC strains are morphologically indistinguishable from one another. When used in conjunction with qPCR, the viability dye PMA has the potential to differentiate viable from non-viable Foc cells, thereby giving an indication of viability (Nocker and Camper, 2009). The potential use of a PMA-qPCR assay will be investigated in **Chapter 3.** The technique will also be used to test the efficacy of sanitiser treatments of Foc.

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Figure 1. Life cycle of *Fusarium oxysporum* f. sp. *cubense* within the banana host (Dita *et al*., 2018). (A) Foc spores are present in the soil or associated with alternative hosts. (B) Host root exudates stimulate Foc spore germination and the germ-tubes penetrate banana root tissue (C) Foc progresses through the root cortex to the epidermis into vascular tissue. (D) Inside vascular tissue, Foc spores are produced rapidly and spread throughout the host. (E) As the fungus spreads, the host responds by secreting gums or tyloses to isolate the pathogen. The disruption of water translocation results in wilting symptoms. (F) As the infected plant dies, Foc can spread to follower plants or the soil when the dead host eventually falls down.

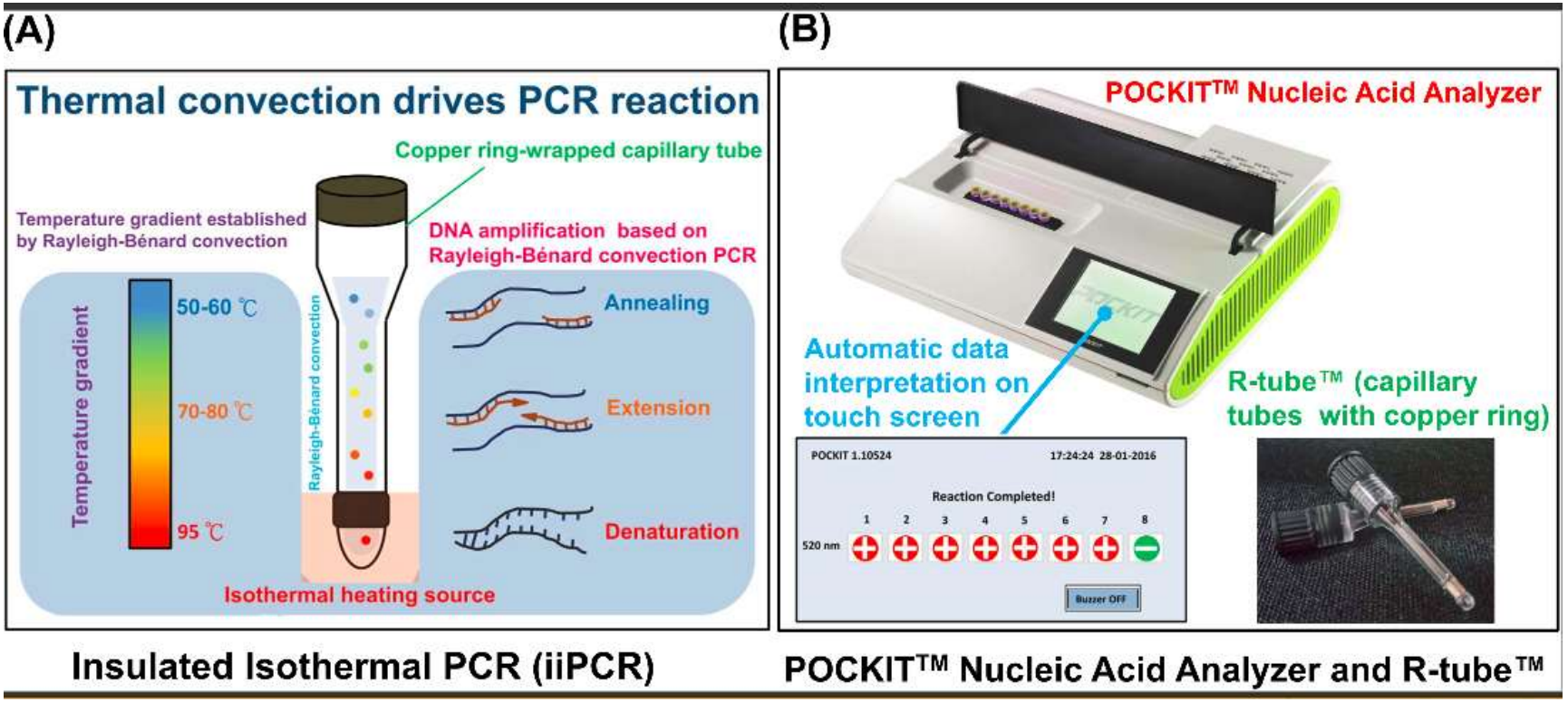

Figure 2. The insulated isothermal PCR (iiPCR) system (A) used in the POCKET nucleic acid analyzer (B) (Lin *et al*., 2016

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CHAPTER 2

Developing assays for the quantitative detection of *Fusarium oxysporum* f. sp. *cubense* Lineage VI, tropical and sub-tropical race 4 in Africa

ABSTRACT

Banana is an important food crop and source of income in Africa. Cooking bananas are often consumed as staples, while exotic varieties such as Gros Michel, Cavendish and Silk are used as dessert bananas and for export. Diseases like Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), limit banana production in Africa. Foc race 1 infects Silk, Pisang Awak, and Gros Michel bananas and Foc race 2 infects cooking bananas like Bluggoe. Foc subtropical race (STR) 4 and Foc tropical race (TR) 4 cause disease to Cavendish bananas and bananas susceptible to races 1 and 2. Foc can be disseminated from infested to disease-free fields with infected plant material, water and soil. Once introduced into a new plantation, the fungus cannot be eradicated, and resistant varieties must be planted to continue production. The early detection of the Fusarium wilt fungus in Africa is important to improve the success of management strategies and follow the spread of the pathogen. The aim of this study was to develop quantitative PCR (qPCR) assays for the detection of Foc Lineage VI that contains Foc race 1 and 2 strains from Africa, Foc TR4 and Foc STR4 in plant, water and soil samples. Markers were designed based on single nucleotide polymorphisms (SNPs) in gene regions for RNA polymerase III subunit beta (*RPC2*), a hypothetical protein and a region within Foc supercontig 1.57. The markers were then tested using a collection of *Fusarium* isolates representing different *Fusarium* species, pathogenic and non-pathogenic *F. oxysporum* strains, and Foc isolates from all 24 vegetative compatibility groups (VCGs). Specific markers were used to produce standard curves for absolute quantification. Each qPCR was evaluated on the quality of the standard curve, repeatability, reproducibility, limit of quantification (LOQ) and limit of detection (LOD). Markers for Foc TR4 and Lineage VI were specific in qPCR, but Foc STR4 markers amplified non-target *Fusarium* members and two non-pathogenic *F. oxysporum* isolates. The qPCR assays for Foc Lineage VI and TR4 and STR4 were repeatable and reproducible, with LOQ values of 10^{-2}-10^{-4} ng/µL and a LOD

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of 10^{-3}-10^{-5} ng/µL. The Lineage VI and TR4 qPCR assays proved specific when tested against Foc-infected plant, water and soil samples.

INTRODUCTION

Millions of Africans rely on banana (*Musa* spp.) as a staple food and a source of income (Karamura *et al*., 1998; Arias *et al*., 2003). Bananas such as the East African Highland bananas (EAHB) in Eastern and Central Africa (ECA) and the plantains in Central and West Africa are cooked or roasted (Wilson, 1987; Pillay *et al*., 2001) whereas dessert varieties such as Pisang Awak, Silk, Cavendish and Gros Michel are consumed as snacks, brewed or exported (Karamura *et al*., 1998; Rutherford, 2001; Blomme *et al*., 2013). In Africa bananas are grown by small-scale subsistence farming, in gardens and in large plantations (Karamura *et al*., 1999). Factors that limit banana production include diseases such as the banana bunchy top disease (BBTD), banana Xanthomonas wilt (BXW), black Sigatoka and Fusarium wilt (Stover, 2000; Viljoen, 2002). Of these, Fusarium wilt is considered the most devastating.

The causal agent of banana Fusarium wilt, *Fusarium oxysporum* f. sp *cubense* (Foc), is a diverse anamorphic soil-borne fungus (Stover, 1962). Foc is classified into races, vegetative compatibility groups (VCGs), clades and clonal lineages. The Foc races are assigned based on pathogenicity to specific banana cultivars (Waite and Stover, 1960). Foc race 1 strains affect Gros Michel (AAA), Pisang Awak (ABB) and Silk (AAB) bananas, Foc race 2 strains affect Bluggoe (ABB) and other cooking bananas (Waite and Stover, 1960; Stover, 1962; Su *et al*., 1977; Ploetz and Correll, 1988), and Foc race 4 affects Cavendish (AAA) and banana varieties susceptible to Foc races 1 and 2. Foc race 4 can be further divided into a subtropical race 4 (STR4) and tropical race 4 (TR4) (Ploetz, 2006a; Viljoen, 2002). Foc STR4 predominantly causes Fusarium wilt in Cavendish bananas subjected to environmental stresses like cold stress during winter in the subtropics (Ploetz and Pegg, 2000). Foc TR4 infects Cavendish under sub-tropical and tropical conditions. Foc isolates are divided into two clades. Isolates that affect Cavendish bananas (Foc TR4 and STR4) group in Clade A, and those affecting non-Cavendish bananas (Foc races 1 and 2) in Clade B. Foc is also divided into 24 vegetative compatibility groups (VCGs) based on the ability of individuals to form heterokaryons between compatible strains (Leslie and Summerell, 2006). The VCGs are divided into eight to nine clonal lineages (Bentley *et al*., 1998; O'Donnell *et al*., 1998; Fourie *et al*., 2009). Foc TR4

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and STR4 isolates are in Lineage V and IV respectively. Foc race 1 and 2 isolates in ECA are clustered in Foc Lineage VI, which include VCGs 0124, 0125, 0124/5, 0128, 01212, 01220 and 01222 (Karangwa *et al*., 2018).

It is believed that Foc was introduced into Africa with contaminated plants from Asia (Blomme *et al*. 2013). Today, all races of Foc are present on the continent. Foc Lineage VI isolates are widespread and affect Gros Michel, Pisang Awak, Silk, Mchare (AA) and Bluggoe bananas. However, the EAHB and plantain grown in ECA and in West Africa, respectively, are not susceptible to Foc races 1, 2 and STR4. A pilot study on a small selection of EAHB and plantain cultivars indicated that they are resistant (Molina *et al*., 2016) to Foc TR4, but response to a larger collection of cultivars still needs to be resolved. Cavendish bananas, which are resistant to Foc races 1 and 2, are affected by Foc TR4 (VCG 01213/16) in Mozambique (IITA, 2013) and Foc STR4 (VCG 0120) in South Africa (Visser *et al*., 2010). With the expansion of dessert bananas in Africa for beer and wine production, fresh consumption and export, there is a significant risk that Foc TR4 may cause significant damage to small-grower and commercial production in future (Wilson, 1987; Karamura *et al*., 1998; Beed and Markham, 2008).

Foc is a member of the *F. oxysporum* species complex (Snyder and Hansen, 1940). Traditional diagnostics of *F. oxysporum* requires lengthy culturing, microscopy and pathogenicity tests. *Fusarium oxysporum* can be distinguished from other *Fusarium* species based on morphology, but pathogenic and non-pathogenic *F. oxysporum* strains (*formae specialis*) are morphologically indistinguishable (Booth, 1971; Windels, 1991; O'Donnell *et al*., 1998 and Meldrum *et al*., 2012). Pathogenicity tests can be used to differentiate Foc from other *formae specialis* of *F. oxysporum* and to differentiate Foc races. Yet, they are time consuming and variable climatic conditions may yield inconsistent results (Stover and Buddenhagen, 1986; Persley and De Langhe, 1987; Ploetz and Correll, 1988).

DNA-based diagnostics with PCR has improved the speed and accuracy with which plant pathogens are identified (Henson and French 1993). Conventional PCR, however, still may require culturing and only provides qualitative results (Schaad and Frederick, 2002; Niu *et al*., 2009). Quantitative (q)PCR has been developed to address the inadequacies of traditional PCR and can be used for the absolute quantification of pathogen DNA at low concentrations directly from environmental samples (Valasek and Repa, 2005; Bustin *et al*., 2009; Pabinger *et al*., 2014).

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Quantitative diagnostic assays have been developed for Foc race 4 (Lin *et al*., 2009; Li *et al*., 2012, Peng *et al*., 2014; Yang *et al*., 2015) and TR4 (Zhang *et al*., 2013; Aguayo *et al*., 2017) in plant or soil samples, but detection in environmental water should still be optimised. The specificity of markers developed by Zhang *et al*. (2013) was only determined against four Foc isolates and markers developed by Aguayo *et al*. (2017) amplify DNA from closely related Foc TR4 VCGs 0121 and 01213/16.

Early detection of the pathogen plays a vital role in the prevention and early management of disease outbreaks. The quantitative detection of Foc strains in Africa could substantially reduce the time and improve the accuracy for identifying the Fusarium wilt pathogen on the continent. It could potentially also be used by quarantine officials, in resistance breeding projects, and to determine Foc inoculum load in water and soil. The objective of this study, therefore, was to develop qPCR assays for the detection of Lineage VI, VCG 0120/15 and VCG 01213/16 from banana plant, water and soil samples. For ease, VCG 0120/15 will be referred to as Foc STR4 and VCG 01213/16 as Foc TR4 in this Chapter.

MATERIALS AND METHODS

Fungal isolates

A total of 127 isolates were included in this study (Table 1). These included *Fusarium* species such as *F. verticillioides*, *F. thapsinum*, *F. sporotrichioides*, *F. solani*, *F. semitectum*, *F. sacchari*, *F. proliferatum, F. konzum*, *F. graminaerum*, *F. fujikuroi*, *F. equiseti*, *F. chlamydosporum*, *F. avenaceum*, *F. anthophilum, F. redolens* and *F. oxysporum*, *F. oxysporum* isolates affecting different crops, Foc and non-pathogenic *F. oxysporum* isolates. The Foc isolates included VCGs representing all Foc lineages and races (Bentley *et al*., 1998; Fourie *et al*., 2009). The non-pathogenic Foc isolates originated from *Musa* root samples collected in Kiepersol, South Africa. All isolates are deposited at the *Fusarium* collection at the Department of Plant Pathology, Stellenbosch University in South Africa.

Primer design

A database with the full genome sequences of all known lineages and VCGs of Foc was used for primer design (Mostert, 2014). Sequences were aligned with MEGA 7.0.18. software (https://www.megasoftware.net/), and single nucleotide polymorphisms (SNPs)

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identified that were specific for Foc TR4 (VCG 01213/16), STR4 (VCG 0120/15) and Lineage VI (VCGs 0124/5, 0128, 01212, 01220 and 01222) (Fig. 1). The primers were designed to have lengths of 20-25 bp, produce product sizes of 100-200 bp, have less than a 2°C difference in annealing temperature between forward and reverse primers, to produce no primer dimers, and with the SNPs present on the 3' end.

Primer specificity in PCR

To test primer suitability, the designed primers were first evaluated using conventional PCR. DNA that was extracted from Foc isolates which represent the eight Foc lineages described by Fourie *et al*. (2009) were used for the initial screening. Each 25 µL PCR reaction contained 12.5 µL of KAPA ready mix with dye (Roche, Basel, Switzerland), 9.5 µL autoclaved dH_2O, 0.5 µL forward primer (0.2 µM), 0.5 µL reverse primer (0.2 µM) and 2 µL of DNA. PCR cycles included a denaturation step at 95°C for 5 min, 30 cycles of 95°C for 45 s, annealing temperature (56-66°C was used to optimise primer performance) for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. Amplification products were separated via electrophoresis in a 1% agarose gel containing 0.5 µL SYBR safe immersed in 1× TAE buffer, and visualised under UV light with Gel Doc™ XR+ Imager and Image Lab software 5.2.1 (Biorad, Herculese, California, USA) after electrophoresis.

Primer specificity in qPCR

Primer sets that were suitable and specific in conventional PCR were tested for use in qPCR. Conditions were optimised for each primer set using annealing temperatures of 60-70°C, different chemistries (SYBR no-Rox Sensimix or SensiFAST, Bioline, Fremont, California, USA) and primer concentrations of 0.3 µM or 0.15 µM in a three-step Rotor-Gene™-6000 (Bio-Rad, Hercules, California, USA) machine. Conditions that resulted in the highest fluorescence of target DNA were selected for subsequent qPCRs. Once qPCR conditions were optimised for the detection of Foc Lineage VI, TR4 and STR4 isolates, the specificity of assays were assessed.

DNA was extracted using Nucleospin® Plant II miniprep extraction kits (Machel-Nagel, Düren, Germany), according to manufacturer guidelines, from representative isolates of *Fusarium* species, non-pathogenic *F. oxysporum* and Foc were used to assess marker specificity in qPCR (Table 1). For each qPCR sample, 2 µL DNA, 10 µL SYBR no-Rox mix (Bioline), 6.8 µL dH_2O, and 0.6 µL of the forward and reverse primers were used. SYBR no-Rox mix Sensimix conditions included an initial denaturing step of 10 min,

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followed by 40 amplification cycles of 10 s at 95°C, 15 s at the annealing temperature, and 20 s at 72°C. SYBR no-rox mix SensFAST conditions included an initial denaturing step of 3 min followed by 40 amplification cycles of 5 s at 95°C, 10 s at the annealing temperature, and 20 s at 72°C. Corbett-Type Strip tubes (0.1 mL) and caps (SSIBio, Lodi, California, USA) were used in all qPCR reactions. For each qPCR reaction, specificity in qPCR was assessed via melt curve analysis (temperature gradients from 72-95°C, with a hold for 90 s on the first step and 5 s on the next steps.

Preparation of Foc-infected samples

Preparation of Foc*-infected plant material*

Foc inoculum was prepared by growing the Foc isolates representing all VCGs (Table 1.) on 20 g/L potato dextrose agar (PDA) for 10 days. Each culture received 5 mL of sterile distilled water, a sterile scalpel was used to loosen conidia from the mycelia and a pipette (Labnet International, Inc., Edison, New Jersey, USA) was used to transfer 5 mL of spore suspension to a 15 mL falcon tube (NEST®, New District, Wuxi, Jiangsu, China). Collected spore suspensions were filtered with cheesecloth and adjusted to 10^5 spores/mL based on haemocytometer counts.

The pathogenicity tests were performed at the National Quarantine Station of the Department of Agriculture, Forestry and Fisheries in Stellenbosch, South Africa. Tissue cultured-derived 10 cm Cavendish and Gros Michel plantlets were inoculated with each of the Foc isolates using the method described by Viljoen *et al.* (2017). Foc VCG 0120 isolate CAV 179 was used as a pathogenic positive control, and water was used as a negative control. After 6 weeks at 25°C disease development was determined based on the external yellowing of leaves and the discolouration of the inner rhizome. The plants that were inoculated with water were also used as a source of background DNA for qPCR standard curves.

Preparation of Foc-infected water

Mycelia from Foc isolates representing all VCGs were used to inoculate 100 mL Armstrong media (Booth, 1971) in CELLSTAR® culture flasks (Greiner bio-one Gmbh, Frickenhausen, Germany) to mass produce spores. The isolates were then gently rotated at 100 x g in a Labcon incubation shaker (Labcon®, Petaluma, California, USA) at room temperature for 2 weeks. Each spore suspension was thereafter filtered through cheesecloth and washed with autoclaved dH_2O (20mL) before being centrifuged at 4 000

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x g for 2 min in an Eppendorf® 5810 R Centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and the pellet dissolved in water collected from the Coetzenberg dam in Stellenbosch, South Africa. Dam water was selected to simulate an environmental sample. A haemocytometer was then used for counting spores, and the spore concentrations were adjusted to ~10^5 spores/mL. Uninoculated water samples were used as negative controls and as a source of background DNA for qPCR standard curves.

Preparation of Foc-infected soil

Soil samples were prepared by autoclaving 3 kg soil from a Kiepersol banana plantation in South Africa. After autoclaving, 1 g of soil was plated onto PDA+ (PDA with 40 mg/L of streptomycin sulphate) in triplicate to ensure no viable *F. oxysporum* was present. Autoclaved soil (100 g) was transferred into Magenta™ boxes (Magenta LLC, Lockport, Ilinois, USA) and inoculated with isolates representing all Foc VCGs that were grown on 5 g of millet seed (Strauss and Labuschagne, 1995). The inoculated soil was incubated for 6 weeks at room temperature before use. Uninoculated soil samples were used as negative controls and as a source of background DNA for qPCR standard curves.

DNA extractions for qPCR

DNA extraction for plant qPCRs

Total genomic DNA was extracted from pure fungal isolates and from the rhizomes of infected banana plantlets for qPCR analysis using an optimised protocol for Nucleospin® Plant II miniprep extraction kit. The rhizomes were first lyophilised with liquid nitrogen, and ~100 mg of the lyophilised material was mixed with 350 µL lysis buffer (Nucleospin® PL2) and 30 glass beads in a 1.5 mL Eppendorf™ tube (Eppendorf, Hamburg, Germany). The tube was then placed in a tissue lyser for 5 min at a frequency of 30/s. After lysis the DNA extraction was performed as per manufacturer guidelines up to the elution step. During elution, 30 µL buffer (Nucleospin® PE) was added instead of 50 µL.

DNA extraction for water qPCRs

Total genomic DNA was extracted from pure fungal isolates and from 1 mL samples of Foc-infected water samples for qPCR analysis using AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) and a DynaMag™-96 side magnetic plate (Thermo Fischer, Waltham, Massachusetts, USA). The 1 mL samples were centrifuged at 13 200 x g for 2 min in 1.5 mL Eppendorf™ tubes and the supernatants discarded. Glass beads

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(~10) and lysis buffer (Nucleospin® SL2) were added to each tube. Samples were then placed in a tissue lyser for 1 min at a frequency of 30/s and incubated in a water bath at 65°C for 30 min. Samples were centrifuged at 13 200 x g for 2 min, and 35 µL supernatant transferred to a single PCR tube (Nolato Treff AG, Degersheim, Switzerland). DNA was then extracted according to the AMPure XP manufacture guidelines.

DNA extraction for soil qPCRs

Total genomic DNA was extracted from pure fungal isolates and soil samples for qPCR analysis using an optimised protocol for Nucleospin® Soil extraction (Machel-Nagel, Düren, Germany) miniprep kit. Soil samples were lyophilised with liquid nitrogen, and 1 g per sample was them transferred to 15 mL Falcon tubes (NEST®, New District, Wuxi, Jiangsu, China). Glass beads and 2 mL of extraction buffer (10 mL of 0.5 EDTA pH 8, 10 mL of 1 M Tris pH 8, 16.6 mL of 3 M NaCl, 0.7 mL of beta mercaptoethanol, 1.25 mL of 20 % SDS made up to 100 mL with autoclaved dH_2O) were added to each 15 mL tube Falcon tube. The tubes were then vortexed for 1 min and incubated at 65°C for 1 hr with 1 min of vortexing at 15 min intervals. Each 15 mL tube was centrifuged at 4 000 x g for 1 min in an Eppendorf centrifuge (Hamburg, Germany), and 450 µL clear supernatant transferred to 2 mL Eppendorf™ tubes with ceramic beads. DNA was then extracted according to manufacturer guidelines until the elution step that used 30 µL of elution buffer instead of 50 µL. The quality and quantity of DNA samples used to produce qPCR standard curves was evaluated with a NanoDrop™ spectrophotometer (Thermo Fischer, Waltham, Massachusetts, United States).

Optimisation and sensitivity of quantitative PCR

Tests for linearity and the presence of inhibitors

Standard curves were constructed using four-fold dilutions of Foc Lineage VI, TR4 or STR4 DNA in a fixed background of DNA extracted from Foc-free plant, water and soil samples. Dilution points were used in triplicate qPCR reactions in a Rotor-Gene™-6000 (Bio-Rad, Hercules, California, USA) with optimised qPCR conditions. The Rotor-Gene™ Q-series software was used to plot the cycle threshold (Ct) values of dilution points against a logarithm of the initial DNA concentration (measured with NanoDrop™ Spectrophotometer). Standard curves were acceptable if the efficiency was close to 1.00 and the correlation coefficient (R^2) was above 0.99 (Bustin *et al.*, 2009).

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The DNA concentration estimated with qPCR of target Foc DNA extracted from plant, water and soil samples was compared before and after inhibitor removal. NucleoSpin® Gel and PCR Clean-up kits (Machel-Nagel, Düren, Germany) were used for inhibitor removal. Inhibition is less prominent with dilutions and can be indicated by low amplification efficiencies along a DNA dilution series (like a standard curve) (Bustin *et al*., 2009). For plant, soil and water assays, standard curve efficiencies were assessed to ensure inhibitors in the environmental samples did not affect DNA amplification.

Reproducibility and repeatability

DNA extracted from Foc-infected plant, water and soil samples were used to test the reproducibility and repeatability of the DNA extraction methods and qPCR assays. To evaluate DNA extraction repeatability and reproducibility, DNA was extracted from four different Foc samples infected with Lineage VI in triplicate and from one sample six times (within sample), on two separate days. The extracted DNA was used in the appropriate Lineage VI qPCR assay and standard deviations recorded between average DNA concentrations across days and within sub-samples. SAS® version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used for Leven's test for homogeneity, Shapiro Wilk's test for normality and ANOVA to check if significant differences in average DNA concentration occurred between days as there is no threshold for DNA extraction standard deviations.

To test the reproducibility (inter-assay variation) and repeatability (intra-assay variation) of each qPCR assay, DNA from one sample was used in three or six technical repeats in two different qPCR runs. Standard deviations between average Ct values across qPCR runs and between technical repeats with the highest and lowest mean Ct values were recorded. Standard deviations between replicate Ct values below 0.35 were considered acceptable (Hellemans and Vandesompele, 2011).

Limit of quantification and limit of detection

The dilution point below which positive samples can not be reliably quantified is called the limit of quantification (LOQ). The dilution point below which positive samples can not be detected with certainty is called the limit of detection (LOD) (Bustin *et al*., 2009; Ramírez *et al*., 2015). For each qPCR assay, a ten-times dilution series was made with DNA from three different positive samples in DNA from the appropriate background. The DNA from positive samples was 20 ng/µL for plant and soil and 5 ng/µL for water. Background DNA

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consisted of ~20 ng/µL of DNA extracted from a Gros Michel rhizome, ~1.5 ng/uL of DNA extracted from banana plantation soil or ~1 ng/uL of DNA extracted from dam water. DNA at each dilution point was extracted and used in triplicate in an appropriate qPCR assay. Standard deviations between the Ct values of technical replicates were recorded at each respective LOQ and LOD.

Quantitative PCR within infected environmental samples

The specificity of the qPCR assays in environmental samples was evaluated using DNA extracted from Foc-infected plant, water or soil samples (Table 1) and environmental samples void of Foc. This was done to ensure inhibitors within the environmental samples did not affect specificity.

RESULTS

Primer design

The number of primer sets designed and tested for Foc Lineage VI, TR4 and STR4 detection was 3, 11 and 15, respectively. The top performing primer sets for Foc Lineage VI, TR4 and STR4 were RTLinVI_F3 and FocLinVI-R, RT_13.16_F2.5 and RT_13.16_R2.5 and 0120_15_F4 and 0120_15_R4 respectively. Primer pair RTLinVI_F3 (5'-GACATTTGACGACTTTCTGA-3') and FocLinVI-R (5'-GTGTCACTTGGTCCTCGTAT-3') was designed to amplify a 98-bp product within the DNA-directed RNA polymerase III subunit beta (*RPC2*) (Fig. 1A). RT_13.16_F2.5 (5'-GAATATAAAGAGGAAGTAGCCG-3') and RT_13.16_R2.5 (5'- CCTCGCTGAATTATATCTAAACC-3') were designed to amplify a 157-bp DNA fragment in position 25794-25951 of supercontig 1.57 of the Foc reference isolate's (II5) genome (Fig. 1B). Primers 0120_15_F4 (5' TCTGGTCTCTCAACGTCCACC-3') and 0120_15_R4 (5'- ACCGTGTTATCGAGGAGGGA-3') were designed to amplify a 235-bp DNA fragment within the gene area for a hypothetical protein (NCBI reference: XM_018378748.1) (Fig. 1C).

Primer specificity in PCR

When primer specificity was evaluated in PCR, primer sets; RTLinVI_F3 and FocLinVI-R, RT_13.16_F2.5 and RT_13.16_R2.5 and 0120_15_F4 and 0120_15_R4 only amplified DNA from the appropriate lineage. The optimal annealing temperatures were 62°C, 56°C

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and 63°C for RTLinVI_F3 and FocLinVI-R, RT_13.16_F2.5 and RT_13.16_R2.5 and 0120_15_F4 and 0120_15_R4 respectively.

Primer specificity in qPCR

The Foc Lineage VI primers RTLinVI_F3 and FocLinVI-R performed best at a 66°C annealing temperature, with SYBR Sensimix chemistry and were specific for the detection of all the Foc Lineage VI isolates. The Foc TR4 primers RT_13.16_F2.5 and RT_13.16_R2.5 performed best at a 62°C annealing temperature, with SYBR SensiFAST chemistry and were also specific in detecting Foc TR4 isolates. The Foc STR4 primers 0120_15_F4 and 0120_15_R4 performed best at a 68°C annealing temperature, with SYBR Sensimix chemistry. The Foc STR4 primers, however, amplified some non-target isolates including a *F. proliferatum* (CAV 386), *F. sacchari*, (CAV 388) and two non-pathogenic *F. oxysporum* isolates (CAV 528 and CAV 531). All primer sets worked best with 0.3 µM primer concentrations. The melting points of target amplicons were 80.2-80.5°C (Fig. 2A), 77.0-77.8°C (Fig. 2B) and 84.2-84.5°C (Fig. 2C) for RTLinVI_F3 and FocLinVI-R, RT_13.16_F2.5 and RT_13.16_R2.5 and 0120_15_F4 and 0120_15_R4 respectively.

Optimisation and sensitivity of quantitative PCR

Tests for linearity and the presence of inhibitors

Inhibitor removal kits did not improve DNA quality and DNA amplification efficiencies were close to 100% indicating there was no significant inhibition of DNA amplification within all three matrix matched backgrounds. The standard curves of all the qPCR assays had acceptable efficiencies and R^2-values close to 1.00, indicating they were suitable for quantitative detection (Table 2). Foc Lineage VI calibration curves efficiencies and R^2 values were 0.98 and 0.99584 (plant background), 0.99 and 0.99546 (water background) and 1.00 and 0.99030 (soil background), respectively (Fig. 3A-C). The Foc TR4 calibration curves efficiencies and R^2 values were 1.00 and 0.99672 (plant background), 1.03 and 0.99024 (water background) and 0.98 and 0.99660 (soil background), respectively (Fig. 3D-F). The Foc STR4 calibration curves had efficiencies and R^2 values were 1.00 and 0.99902 (plant background), 1.01 and 0.99729 (water background) and 0.99 and 0.99952 (soil background), respectively (Fig. 3G-I).

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Reproducibility and repeatability

There is no threshold for DNA extraction repeatability and reproducibility, but standard deviations were reported for comparative purposes (Table 3 and 4). Levene's test for homogeneity and Shapiro Wilk's test showed the data to be homogenous and normally distributed (data not presented). No significant differences ($P<0.5$) were found between the DNA extraction methods used on two different days, indicating that DNA extractions from plant, soil and water was reproducible. Significant differences were found among Foc Lineage VI isolates when DNA was extracted from plant and soil samples at a 95% significance level (Table 5).

Standard deviations between target DNA quantified in separate qPCR assays (reproducibility) and within the same qPCR assay (repeatability) did not exceed 0.35 (Table 6 and 7). This indicated that all the qPCR assays were reproducible and repeatable.

Limit of quantification and limit of detection

The LOQ and LOD in plant material was ~10^{-4} and ~10^{-5} ng/µL for both Foc TR4 and Lineage VI, and ~10^{-3} and ~10^{-4} ng/µL for Foc STR4 (Table 8). In water the LOQ and LOD was ~10^{-2} and ~10^{-4} ng/µL for Foc Lineage VI, ~10^{-2} and ~10^{-3} ng/µL for Foc TR4, and 10^{-3} and 10^{-4} ng/µL for Foc STR4. The LOQ and LOD in soil was ~10^{-4} and ~10^{-5} ng/µL for Foc Lineage VI and TR4, and 10^{-3} and 10^{-4} ng/µL for STR4. In general, higher LOQs and LODs were determined for qPCR assays from water samples. Foc STR4 quantified in soil and plant qPCR assays thus had a higher LOQ and LOD than Foc Lineage VI and TR4.

Quantitative PCR within complex environmental DNA

Foc Lineage VI and TR4 qPCR assays were specific when tested with DNA from Foc-infected plant, water and soil samples. Plant samples infected with TR4 isolates (CAV 300, CAV 789, CAV 3326 and CAV 3049) tested positive with the Foc TR4 plant qPCR assay (Table 1). Water and soil samples infected with Foc TR4 isolates (CAV 789, CAV 3326 and CAV 3049) tested positive with the respective TR4 water and soil qPCR assays (Table 1). Plant samples infected with Lineage VI isolates (CAV 184, CAV 188, CAV 606, CAV 620, CAV 629, CAV 853, CAV 893, CAV 1004, CAV 1024, CAV 2400 and NRRL 36117) tested positive with the Lineage VI plant assay. Water and soil samples infected with Lineage VI isolates (CAV 184, CAV 188, CAV 606, CAV 620, CAV 893, CAV 1004, CAV 2400 and NRRL 36117) tested positive with the respective Lineage VI water and soil qPCR assays.

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DISCUSSION

Quantitative PCR assays with newly designed primer sets were developed in this study to identify and quantify Foc Lineage VI, TR4 and STR4 strains in soil, water and banana plants. To our knowledge, this is the first study where qPCR markers were designed for Foc Lineage VI and STR4. The Foc TR4 markers amplified only VCG 01213/16 and not the closely related VCG 0121. The Foc TR4 markers designed from a putative virulence gene by Aguayo *et al*. (2017) for qPCR detection of tropical strains of Foc race did not distinguish between these VCGs.

LOQs reported for Foc in host material or soil were 10^{-3}-10^{-4} ng/µL (Li *et al*., 2013; Aquayo *et al*., 2017) and 10^{-5} (Zhang *et al*., 2013), respectively. LODs reported for quantitative detection of Foc were 10^{-5} in host material and 10^{-6} ng/µL (Zhang *et al*., 2013) in soil, respectively. In this study, similar LOQ and LOD were observed. The LOQ and LODs were higher in water than in plant material and soil. Therefore, if the Foc markers developed in this study is to be used to quantify Foc levels in environmental water, the spores may have to be concentrated with ultrafiltration or baiting. Extracting DNA from water is a challenge due to low DNA concentrations and large sample volumes (Aw and Rose, 2012; Winton and Hansen, 2001).

Markers for Foc Lineage VI and TR4 were robust and specific for quantification with qPCR, but the Foc STR4 markers amplified some non-target isolates. Foc STR4 specificity therefore need to be improved, as *F proliferatum* and *F. sacchari* are common endophytes of banana (Li *et al*., 2013), while non-pathogenic *F. oxysporum* are ubiquitous in soil (Ploetz, 2006b). The STR4 markers could be used after initial diagnostics with available qPCR markers for *F. oxysporum* (Jiménez-Fernández *et al*., 2010: Haegi *et al*. 2013) and race 4 (Lin *et al*., 2013). Alternatively, more robust Foc STR4 markers can be developed, or the current markers improved, with a fluorescent probe, as such probes are generally considered more specific than intercalating dyes such as SYBR (Arya *et al*., 2005).

Quantitative PCR assays can detect very small quantities of pathogen DNA in an environmental sample. It has potential for use as a tool to screen plant material at border post quarantine stations. Seemingly healthy plants may harbour Foc and spread banana Fusarium wilt to new areas (Ploetz, 2005). With the recent incursion of Foc TR4 in northern Mozambique, there is considerable concern that the pathogen may spread to

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neighbouring countries as borders are porous and people often move planting material and animals across. The screening of banana plants moved across country borders, however, depends on the practicality of a diagnostic test, the availability of the required equipment such as PCR and real-time PCR machines, and the expertise of quarantine officials. For Africa, these do not readily exist, but such services can still be obtained when unauthorised planting material is intercepted by quarantine officials in developed countries.

The quantitative detection of pathogens in their plant hosts can be used for resistance screening. In studies by Vandemark and Barker (2003) and Markakis *et al*. (2009) significantly more pathogen DNA was detected in susceptible hosts than resistant hosts. Pathogen DNA quantity was therefore correlated with host susceptibility. If threshold inoculum levels of a pathogen are ascertained, it can be used to identify high-risk areas and guide disease management (Cullen *et al*., 2001). In a study by Ophel-Keller *et al* (2008) a routine DNA-based testing service was developed to assist farmers in predicting the extent of losses due to soil-borne diseases prior to planting. The service included sampling from farm soil, DNA extraction and qPCR detection of several soilborne pathogens. If detected pathogens exceeded pre-determined thresholds disease, incidence was expected. The quantitative detection of pathogens like Foc Lineage VI and TR4 in soil could be used to evaluate soil inoculum levels necessary for disease and develop a similar service to that of Ophel-Keller *et al*. (2008).

Amplification of DNA from both viable and non-viable cells (Mackay, 2004; Kralik and Ricchi, 2017) is a limitation of qPCR and can lead to overestimation of pathogen inoculum quantities. Reverse transcription and certain nucleic acid binding dyes such as propidium monoazide (PMA) can be considered to improve qPCR assays designed in this study, to detect DNA from viable cells selectively. Reverse transcription is used to convert RNA to complementary DNA (cDNA). The cDNA is then detected with qPCR as an indication of viable inoculum as RNA has a short half-life outside living cells (Keer and Birch, 2003; Martínez-Blanch *et al*., 2011). In order to use the developed assays for RT-qPCR the expression of RNA, complementary to target gene regions, will have to be confirmed. PMA is membrane impermeable but can bind DNA within membrane compromised (non-viable) cells, PMA bound DNA is excluded from DNA amplification (Nocker and Camper, 2009; Tamburini *et al*., 2013). PMA-qPCR has been used to detect DNA from viable fungal spores (Vesper *et al*., 2008; Andorrà *et al*., 2010; Crespo-Sempere

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et al., 2013; Vilanova *et al*., 2017) but not *Fusarium* species. In order to use PMA-qPCR the treatment of Foc spores with PMA will have to be optimised.

In conclusion, the designed Foc Lineage VI and TR4 markers were used to develop qPCR assays that are specific for quantifying the target pathogens in plant, water and soil samples. The Foc STR4 markers, however, still need to be improved. Foc Lineage VI and TR4 qPCR assays can be applied for early detection of target pathogens in areas bordering where the disease is present, like northern Mozambique, to monitor whether or not disease is spreading to bordering farms or to neighbouring countries. It can also be applied for host resistance screening and for quantifying Foc DNA in soil.

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Table 1. *Fusarium* isolates used to develop quantitative PCR assays for *Fusarium oxysporum* f. sp. *cubense* Lineage VI, Tropical race 4 (TR4) and Subtropical Race 4 (STR4).

CAV number[a]	Alternative culture no.	Species	Foc Lineage/Pathogenicity	VCG	Location
92[c]	-	Foc	Lineage IV	0120	South Africa
180[b c]	Taiwan 14	Foc	Lineage V	0121	Taiwan
181[b c]	Phil 36	Foc	Lineage II/IV	0122	Philippines
184[b c]	-	Foc	Lineage VI	0125	Australia
185[c]	Phil 6	Foc	Lineage I/II	0126	Philippines
186[c]	-	Foc	Lineage II/III	0129	Australia
188[b c]	STNPZ (RP59)	Foc	Lineage VII/VIII	01212	Tanzania
189[b c]	RPMW 40	Foc	Lineage VII/VIII	01214	Malawi
191[b c]	Indo 160	Foc	Lineage IV	0120/15	Indonesia
193[b c]	Mal 6	Foc	Lineage VII	01217	Malaysia
194[b c]	Indo 5	Foc	Lineage VII	01218	Indonesia
195[b]	Indo 25	Foc	Lineage I/II	01219	Indonesia
202	-	*F. oxysporum*	Non-pathogen	-	South Africa
291	C1	Foc	Lineage IV	0120	Canary Islands
300[b]	CV-1	Foc	Lineage V	01213	Indonesia
344	CBS 489.97	*F. redolens*	-	-	-
346	CBS 680.89	*F. oxysporum*	-	-	-
349	MRC 3236	*F. anthophilum*	-	-	-
352	MRC 8381	*F. avenaceum*	-	-	-
354	MRC 8391	*F. chlamydosporum*	-	-	-
367	MRC 1813	*F. equiseti*	-	-	-
369	MRC 8532	*F. fujikuroi*	-	-	-
372	MRC 4927	*F. graminearum*	-	-	-
374	MRC 8544	*F. konzum*	-	-	-
386	MRC 8549	*F. proliferatum*	-	-	-
388	MRC 8551	*F. sacchari*	-	-	-
396	MRC 6715	*F. semitectum*	-	-	-

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399	MRC 8454	*F. solani*	-	-	-
400	MRC 43	*F. sporotrichioides*	-	-	-
404	MRC 8554	*F. subglutinans*	-	-	-
406	MRC 8558	*F. thapsinum*	-	-	-
411	MRC 8560	*F. verticillioides*	-	-	-
528	-	*F. oxysporum*	Non-pathogen	-	South Africa
529	-	*F. oxysporum*	Non-pathogen	-	South Africa
531	-	*F. oxysporum*	Non-pathogen	-	South Africa
531	-	*F. oxysporum*	Non-pathogen	-	South Africa
532	-	*F. oxysporum*	Non-pathogen	-	South Africa
533	-	*F. oxysporum*	Non-pathogen	-	South Africa
534	-	*F. oxysporum*	Non-pathogen	-	South Africa
535	-	*F. oxysporum*	Non-pathogen	-	South Africa
545	-	*F. oxysporum*	Non-pathogen	-	South Africa
548	-	*F. oxysporum*	Non-pathogen	-	South Africa
551	-	*F. oxysporum*	Non-pathogen	-	South Africa
554	-	*F. oxysporum*	Non-pathogen	-	South Africa
557	-	*F. oxysporum*	Non-pathogen	-	South Africa
561	-	*F. oxysporum*	Non-pathogen	-	South Africa
564	-	*F. oxysporum*	Non-pathogen	-	South Africa
565	-	*F. oxysporum*	Non-pathogen	-	South Africa
566	-	*F. oxysporum*	Non-pathogen	-	South Africa
600 [c]	Indo 16	Foc	Lineage IV	Gen 2*	Indonesia
606 [b c]	Thai 13	Foc	Lineage VI	0124/5	Thailand
612 [c]	RPCR1-1	Foc	Lineage IV	01215	Costa Rica
615 [c]	PHIL18	Foc	Lineage III	Gen 8*	Phillipines
616 [c]	PHIL1	Foc	Lineage V	Gen 7*	Phillipines
617 [c]	23707	Foc	Lineage III	0129/11	Phillipines
620 [b c]	PHIL24	Foc	Lineage VI	Gen 9*	Phillipines
624 [c]	PHIL26	Foc	Lineage VI	Gen 10*	Phillipines
629 [b]	22468	Foc	Lineage VI	0125	Australia
632 [b c]	RP53	Foc	Lineage I/II	01210	USA
785 [b c]	RP JAK 4	Foc	Lineage I/II	0126	USA

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789 [b c]	25111	Foc	Lineage V	01213/16	Australia
818 [b c]	Indo 54	Foc	Lineage IV	Gen 3*	Indonesia
849	Indo 57	Foc	Lineage I/II	01219	Indonesia
853 [b]	Mal 4	Foc	Lineage VI	Gen 4*	Malaysia
893 [b c]	23997	Foc	Lineage VI	0128	Australia
898 [b c]	Phil 4	Foc	Lineage VI	Gen 11*	Philippines
909 [b c]	Mal 65	Foc	-	Gen 5*	Malaysia
997 [c]	Tanz 9	Foc	Lineage VI	0124/5/8	Tanzania
1003 [b c]	Viet 1	Foc	-	Gen 13*	Vietnam
1004 [b c]	Viet 3	Foc	Lineage VI	Gene 14*	Vietnam
1011 [b c]	Viet 10	Foc	-	Gen 12*	Vietnam
1024 [b]	Mex 2	Foc	Lineage VI	Gen 6*	Mexico
1036 [b c]	RP 2	Foc	Lineage VII	0123	Philippines
1089 [b]	N5447	Foc	Lineage III	0129	Australia
1563	-	Foc	Lineage V	01213/16	Oman
1683	-	Foc	Lineage V	01213/16	Philippines
1703	-	*Fusarium spp.*	-	-	Philippines
1824	-	*F. oxysporum*	Non-pathogen	-	Uganda
1830	-	*Fusarium* spp.	-	-	Philippines
1833	-	Foc	-	-	Philippines
1842	-	Foc	Lineage I/II	0126	Philippines
1897	-	Foc	Lineage VI	0125	India
1905	-	*Fusarium* spp.	-	-	India
1907	-	*Fusarium* spp.	-	-	India
1910	-	*Foc*	-	-	India
1912	-	Foc	Lineage VI	0125	India
1927	-	Foc	Lineage VI	-	India
1933	-	Foc	Lineage VI	-	India
1960	-	Foc	Lineage VI	-	Sri Lanka
1970	-	Foc	Lineage VI	-	Sri Lanka
1974	-	Foc	Lineage VI	-	Sri Lanka
2030	-	*Fusarium* spp.	-	-	Bangladesh
2031	-	*Fusarium* spp.	-	-	Bangladesh

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2107	-	*Fusarium* spp.	-	-	Cambodia
2151	-	*Fusarium* spp.	-	-	Cambodia
2154	-	*Fusarium* spp.	-	-	Cambodia
2251	-	Foc	Lineage VI	0124	Vietnam
2266	-	Foc	-	-	Sri Lanka
2267	-	Foc	-	-	Sri Lanka
2270	--	*Fusarium* spp.	-	-	Malaysia
2271	-	Foc	-	-	Malaysia
2281	-	Foc	-	-	Malaysia
2287	-	Foc	-	-	Malaysia
2351	-	Foc	-	-	Taiwan
2354	-	*Fusarium spp.*	-	-	Taiwan
2400 [b c]	-	Foc	Lineage VI	01220	Bangladesh
2407	-	Foc	Lineage VI	-	Bangladesh
2413	-	Foc	Lineage VI	-	Bangladesh
2443	-	*Fusarium spp.*	-	-	Malaysia
2611	-	*F. oxysporum*	Non-pathogen	-	Tanzania
2633	-	*F. oxysporum*	Non-pathogen	-	Tanzania
3049 [b c]	-	Foc	Lineage V	01213/16	Mozambique
3127	-	Foc	-	-	Phillipines
3128	-	Foc	Lineage VI	-	Phillipines
3130	-	Foc	Lineage VI	-	Phillipines
3142	-	Foc	Lineage VI	-	Philippines
3143	-	Foc	Lineage VI	-	Philippines
3326 [b c]	-	Foc	Lineage V	01213/16	Jacaranda
3351	-	Foc	-	-	Philippines
3371	-	Foc	-	-	Philippines
3372	-	Foc	Lineage V	01213/16	Philippines
3475	-	Foc	Lineage VI	-	Philippines
3478	-	Foc	Lineage VI	-	Philippines
3481	-	Foc	Lineage VI	-	Mauritius
3484	-	Foc	Lineage VI	-	India
3534	-	Foc	-	-	Indonesia

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3540	-	Foc	-	-	Australia
-	NRRL 36115 [b c]	Foc	Lineage V	01224	Malaysia
-	NRRL 36116 [b c]	Foc	Lineage V	01223	Malaysia
-	NRRL 36117 [b c]	Foc	Lineage VI	01222	Malaysia
-	NRRL 36118 [b c]	Foc	Lineage VII	01221	Thailand

Genotypes which are not compatible to known *Fusarium oxysporum* f. sp. *cubense* (Foc) VCGs as described by Bentley *et al*. (1998) are indicated with; *' unavailable data is indicated with "-".

[a] Culture collection Altus Viljoen (CAV) housed at Stellenbosch University Plant Pathology department

[b] Isolates which were used to infect plant samples

[c] Isolates which were used to infect water and soil samples

[bc] Isolates which were used to infect plant, water and soil samples

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Table 2. Standard curve parameters for *Fusarium oxysporum* f. sp. *cubense* Lineage VI, TR4 and STR4 quantitative detection in plant, water and soil samples.

Standard Curve	R^{2a}	Efficiency[b]	[DNA] range[c]	Ct range[d]	Slope[e]
Lineage VI Plant	0.99584	0.98	4.62-0.0002 ng/µL	19.22-33.11	3.380
TR4 Plant	0.99672	1.00	4.80-0.0002 ng/µL	18.59-32.42	3.333
STR4 Plant	0.99902	1.00	22.52-0.0015 ng/µL	13.26-27.12	3.315
Lineage VI Water	0.99546	0.99	4.46-0.0170 ng/µL	17.64-25.72	3.343
TR4 Water	0.99204	1.03	2.60-0.0100 ng/µL	20.17-27.95	3.252
STR4 Water	0.99729	1.01	5.00-0.0048 ng/µL	20.40-30.43	3.290
Lineage VI Soil	0.99030	1.00	5.81-0.0003 ng/µL	19.71-32.95	3.312
TR4 Soil	0.99660	0.98	1.26-0.0003 ng/µL	18.23-30.18	3.382
STR4 Soil	0.99952	0.99	22.05-0.0013 ng/µL	11.73-25.64	3.341

[a] The correlation coefficient (R^2) of the standard curve representing linearity.

[b] The efficiency ($E = (10^{(-1/slope)}) - 1$) of the standard curve.

[c] The range of DNA concentrations within the standard dilution series.

[d] The range of cycling thresholds (Ct) within the standard dilution series.

[e] The slope of the standard curve (M-value).

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Table 3. The reproducibility of DNA quantification, based on quantitative PCR, from plant water and soil samples inoculated with different *Fusarium oxysporum* f. sp. *cubense* (Foc) isolates.

	Day 1[a]	Day 2	
	Target DNA concentration (ng/µL)[c]	Target DNA concentration (ng/µL)	SD_{Days}[d]
Plant samples[b]			
CAV 188	0.003	0.005	0.001
CAV 184	0.003	0.008	0.004
CAV 2400	0.025	0.011*	0.010
NRRL 36117	0.039	0.054	0.011
Water samples[b]			
CAV 188	0.066	0.035	0.022
CAV 184	0.088	0.056*	0.023
CAV 2400	0.266	0.352	0.061
NRRL 36117	0.354	0.345	0.006
Soil samples[b]			
CAV 188	0.038	0.060	0.016
CAV 184	0.060*	0.046	0.010
CAV 2400	0.087	0.041	0.033
NRRL 36117	0.020	0.014	0.004

SD – Standard deviation

[a] The day on which the target DNA was extracted.

[b] Environmental sample type (plant/water/soil) infected with Foc Lineage VI isolates (CAV 188, CAV 184, CAV 2400 or NRRL 36117).

[c] The average DNA concentration of three or six (*) sub-samples measured with qPCR.

[d] The standard deviation (SD) between the average DNA concentrations of Day 1 and Day 2.

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Table 4. The repeatability of DNA quantification, based on quantitative PCR, from plant water and soil samples inoculated with different *Fusarium oxysporum* f. sp. cubense isolates (Foc).

	Day 1[b]		Day 2	
	Target DNA concentration (ng/µL)[c]	SD of target DNA[e]	Target DNA concentration (ng/µL)	SD of target DNA
Plant samples[a]				
CAV 188	0.003	0.002	0.005	0.002
CAV 184	0.003	0.001	0.008	0.004
CAV 2400	0.025	0.006	0.011*	0.004*
NRRL 36117	0.039	0.027	0.054	0.014
Water samples[a]				
CAV 188	0.066	0.021	0.035	0.010
CAV 184	0.088	0.009	0.056*	0.021*
CAV 2400	0.266	0.086	0.352	0.036
NRRL 36117	0.354	0.008	0.345	0.028
Soil samples[a]				
CAV 188	0.038	0.016	0.060	0.039
CAV 184	0.060*	0.022*	0.046	0.022
CAV 2400	0.087	0.014	0.041	0.018
NRRL 36117	0.020	0.002	0.014	0.002

SD – Standard deviation[a] Environmental sample type (plant/water/soil) infected with Foc Lineage VI isolates (CAV 188, 184, 2400 or NRRL 36117).

[b] The day on which the target DNA was extracted.

[c] The average DNA concentration of three or six (*) sub-samples measured with qPCR.

[e] The standard deviation between target DNA concentrations from three or six (*) sub-samples.

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Table 5. Analysis of variances of the different extraction methods to isolate *Fusarium oxysporum* f. sp. *cubense* DNA from environmental samples

DNA extraction method:	Nucleospin® Plant II minipreps		AMpure water extraction		Nucleospin® Soil extraction	
Sources of variation	DF[a]	Pr > F[b]	DF	Pr > F	DF	Pr > F
Day of the DNA extraction [c]	1	0.8903	1	0.1370	1	0.0790
Lineage VI isolate[d]	3	<.0001	3	0.0057	3	<.0001
Day x Lineage VI isolate[e]	3	0.0907	3	0.0782	3	0.0207

[a] Degrees of freedom (DF).

[b] The significance probability (P) value associated with the F-Value.

[c] The day of extraction (1 or 2).

[d] Lineage VI isolate the environmental sample (plant/water soil) was infected with (CAV 184/CAV 188/CAV 2400/ NRRL 36117).

[e] The interaction between the day of the DNA extraction and the Lineage VI isolate which infected the sample.

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Table 6. The reproducibility of the qPCR assays quantifying *Fusarium oxysporum* f. sp. cubense in plant, water and soil samples.

	Lineage VI Ct values			TR4 Ct values			STR4 Ct values		
Plant[a]	A1[b]	A2	SD[c]	A1	A2	SD	A1	A2	SD
Isolate 1	31.04[d]	31.39	0.295	16.99	16.87	0.087	16.76	16.98*	0.149
Isolate 2	31.47	31.98*	0.342	21.36	21.57	0.153	21.11	21.33	0.155
Isolate 3	27.85	27.57	0.301	31.29	31.56*	0.194	21.02	21.37	0.273
Water									
Isolate 1	24.62	24.47	0.106	18.30	18.39	0.066	21.84	21.92	0.059
Isolate 2	23.59	24.03	0.336	17.77	17.75*	0.018	21.22	21.21	0.009
Isolate 3	21.39	21.42*	0.021	24.94	24.76	0.123	22.49	22.47*	0.015
Soil									
Isolate 1	29.51	29.24	0.323	15.84	15.71	0.117	14.68	15.01	0.233
Isolate 2	28.10*	27.73	0.239	14.34	14.32	0.014	18.46	18.31	0.304
Isolate 3	32.42	32.25	0.281	24.32*	24.73	0.223	25.19	25.45*	0.242

Ct values – Cycle threshold value according to qPCR analyses.SD – Standard deviation

[a] The environmental sample type (plant, water or soil) infected with three different positive isolates; CAV 184, CAV 188 and CAV 2400 for Lineage VI, CAV 789, CAV 3326 and CAV 3049 for TR4 and CAV 92, CAV 191 and CAV 612 for STR4.

[b] The qPCR assay in which DNA, extracted from the target isolates, was analysed in triplicate.

[c] The standard deviation between average Ct values between duplicate qPCR assay

[d] The average Ct of three or six (*) technical replicates.

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Table 7. The repeatability of qPCR assays quantifying *Fusarium oxysporum* f. sp. cubense in plant, water and soil samples.

	Cycle threshold values			
Plant[a]	High[b]	Low[c]	SD_H[d]	SD_L[e]
Lineage VI	31.98*	27.57	0.136	0.228
TR4	31.56*	16.87	0.248	0.021
STR4	21.37	16.76	0.302	0.111
Water				
Lineage VI	24.62	21.39	0.146	0.125
TR4	25.00	17.75*	0.268	0.083
STR4	22.49	21.21	0.035	0.036
Soil				
Lineage VI	32.42	27.73	0.279	0.284
TR4	24.73*	15.71	0.091	0.070
STR4	25.45*	14.68	0.343	0.117

SD – Standard deviation

[a] The environmental sample type (plant, water or soil)

[b] The highest mean Ct value of target (Lineage VI/TR4/STR4) DNA in an environment with three or six replicates (*)

[c] The lowest mean Ct value of target (Lineage VI/TR4/STR4) DNA in an environment with three or six replicates (*)

[d] The standard deviation between Ct values from the sample with the highest mean CT

[e] The standard deviation between Ct values from the sample with the lowest mean CT

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Table 8. Limit of detection and limit of quantification of *Fusarium oxysporum* f. sp. *cubense* in plant, water and soil samples.

	Ave [DNA] (ng/µL) [a]		Ave Ct[c]		SD_{Ct}[d]	
Plant[b]	LOQ	LOD	LOQ	LOD	LOQ	LOD
Lineage VI	2.29×10^{-4}	8.00×10^{-5}	33.84	35.42	0.159	0.466
TR4	5.99×10^{-4}	5.98×10^{-5}	31.82	35.24	0.194	0.683
STR4	1.87×10^{-3}	3.17×10^{-4}	26.79	29.36	0.087	0.530
Water[b]						
Lineage VI	2.22×10^{-2}	7.74×10^{-4}	25.72	33.49	0.121	3.77
TR4	1.64×10^{-2}	1.02×10^{-3}	27.95	33.33	0.267	2.18
STR4	1.84×10^{-3}	7.22×10^{-4}	29.27	31.31	0.116	0.408
Soil[b]						
Lineage VI	4.86×10^{-4}	9.43×10^{-5}	33.32	36.02	0.327	0.762
TR4	1.64×10^{-4}	2.98×10^{-5}	30.98	33.00	0.153	1.879
STR4	1.13×10^{-3}	1.28×10^{-4}	26.38	29.16	0.233	0.573

Ct values – Cycle threshold values according to qPCR analyses.

LOD - Limit of detection.LOQ - Limit of quantification.

[a]The average DNA concentration at the LOQ or LOD.

[b]The environmental sample type (plant/water/soil) that was infected with the target Foc isolates (Lineage VI/TR4/STR4).

[c]Average Ct (cycle threshold) values obtained from three technical replicates of the LOQ/LOD.

[d]The standard deviation between Ct values at the LOD and LOQ.

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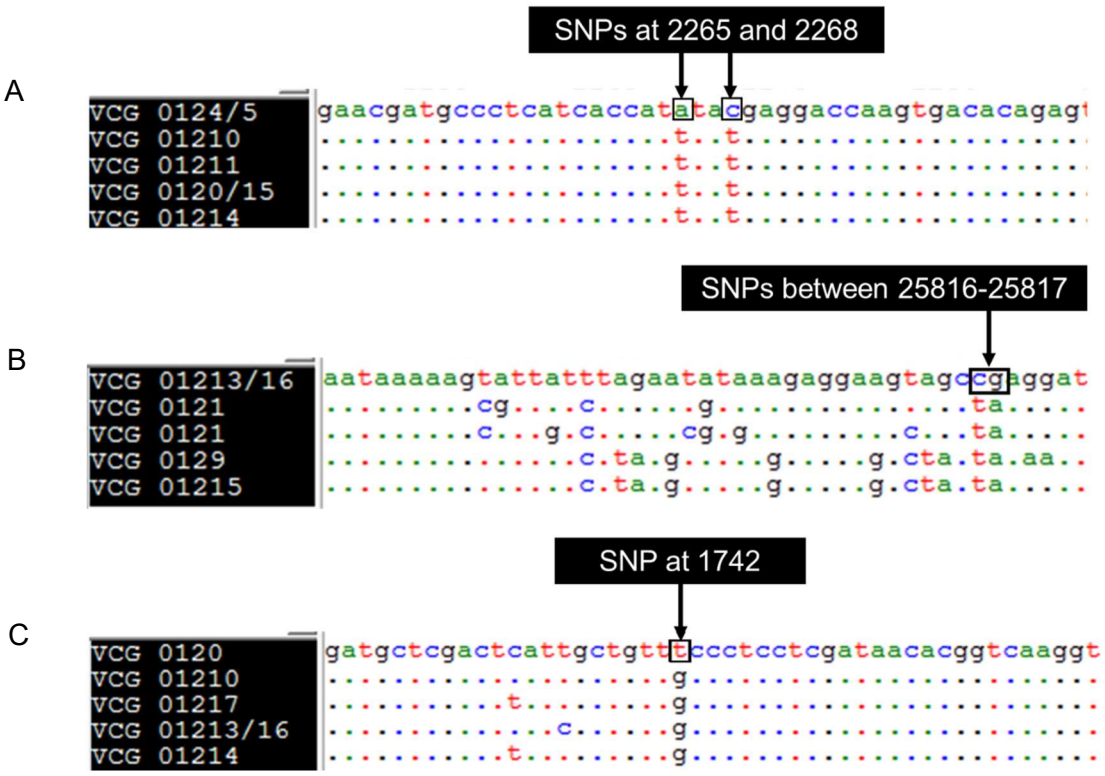

Figure 1. Sequence alignment and SNP identification for *Fusarium oxysporum* f. sp. *cubense* Lineage VI (A), TR4 (B) and STR4 (C) from gene regions RNA polymerase III subunit beta (*RPC2)*, a region within Foc supercontig 1.57 and a hypothetical protein, respectively. The position of each SNP/SNPs is indicated with arrows.

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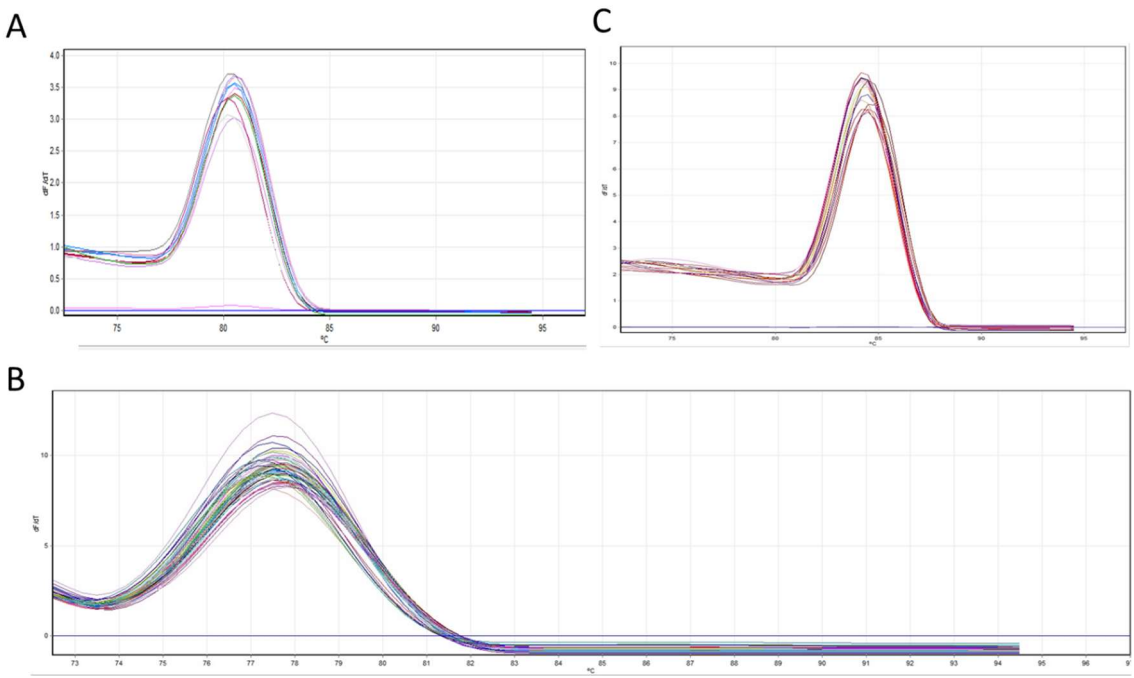

Figure 2. A: Melt curve analysis of seven *Fusarium oxysporum* f. sp. *cubense* Lineage VI target DNA samples with melting points of 80.2-80.5°C. B: Melt curve analysis of 50 TR4 target DNA samples with melting points of 77.2-77.8°C. C: Melt curve analysis of a 10 STR4 target DNA samples with melting points of 84.2-84.5°C.

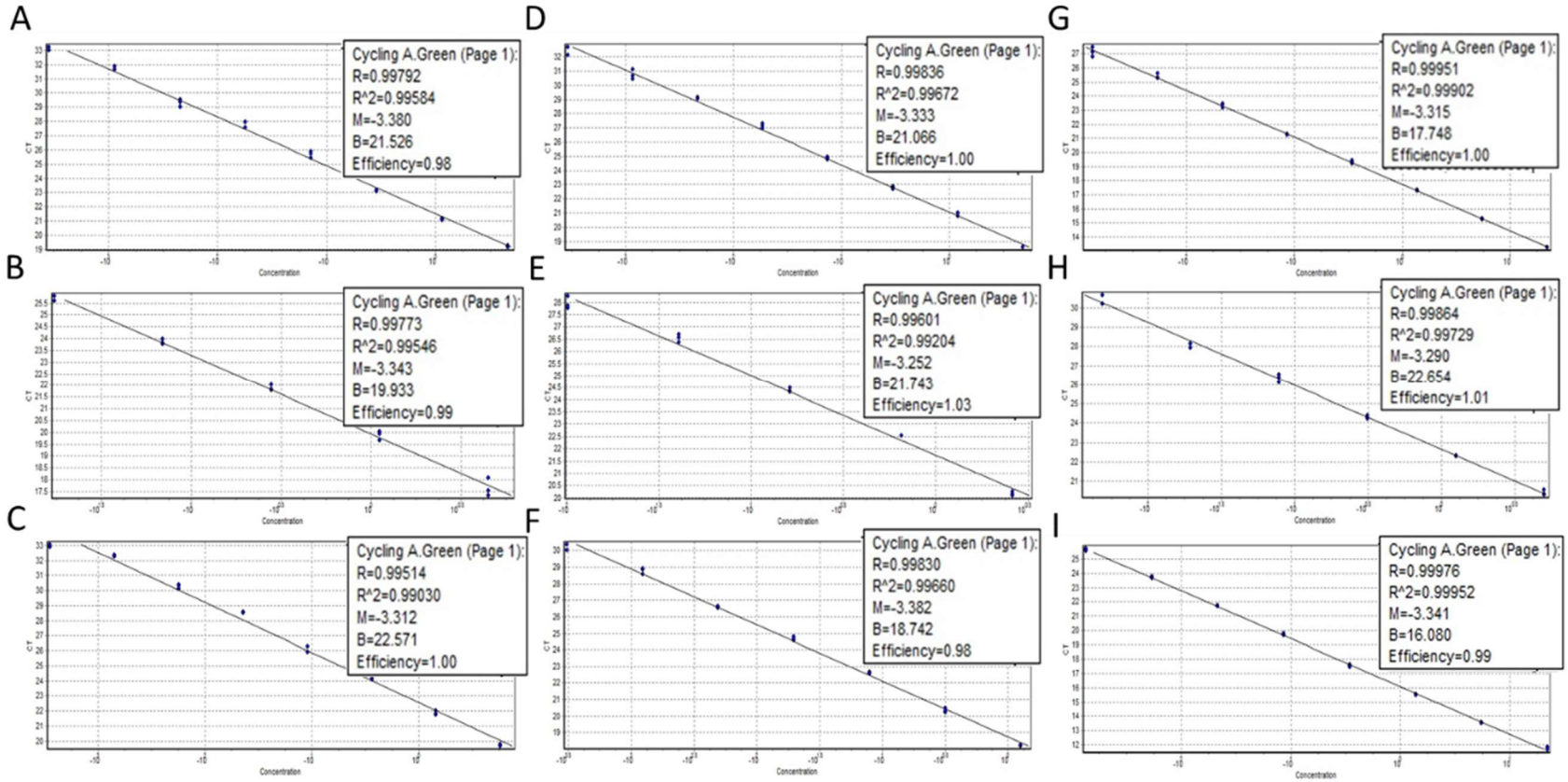

Figure 3. Standard curves of DNA from *Fusarium oxysporum* f. sp. *cubense* (Foc) Lineage VI in plant (A), water (B) and soil backgrounds (C). Standard curves of DNA from Foc TR4 in plant (D), water (E) and soil (F) backgrounds. Standard curves of DNA from Foc STR4 in plant (G), water (H) and soil (I) backgrounds.

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CHAPTER 3

Investigating the potential of propidium monoazide to distinguish between viable and non-viable *Fusarium oxysporum* f. sp. *cubense* spores using quantitative PCR

ABSTRACT

Fusarium oxysporum f. sp. *cubense* (Foc) causes Fusarium wilt of banana, considered to be one of the most devastating constraints to banana production. Foc produces highly resistant chlamydospores that spread with infected plant, water, soil and field equipment. The detection of Foc spores in infected plants, water and soil, therefore, is an important activity in managing banana Fusarium wilt. Quantitative PCR (qPCR) is the preferred method of pathogen detection in environmental samples. A major drawback of DNA-based diagnostic techniques, however, is the inability to distinguish between viable and non-viable cells. The dye propidium monoazide (PMA) can be used with qPCR to preferentially detect DNA from viable target cells. PMA-qPCR has been applied to detect bacteria and a few fungi in water and plant samples. PMA-qPCR has not been optimised for any *Fusarium* species. The objective of this study was to evaluate the potential of PMA-qPCR as a specific and quantitative test of Foc viability. Microscopy and qPCR were used to detect differences between viable and non-viable Foc samples as a measure of PMA dye performance. The PMA dye performed best with 20 µM PMA concentration, 1 min of agitation in the dark and 5 min light activation. The optimised PMA-qPCR assays were then used to compare the efficacy of sanitisers (chlorine, Farmcleanse and Sporekill) on Foc TR4 and Lineage VI spores at different exposure times. Based on PMA-PCR, PMA-qPCR and colony forming unit (CFU) counts, Sporekill was the most effective sanitiser. Viable spores/mL measured with qPCR correlated well to CFU counts for both Foc Lineage VI (R = 0.754) and TR4 (R = 0.702). This indicated that PMA-qPCR was useful in distinguishing between viable and non-viable Foc spores after treatment with sanitisers. Future studies could optimise the PMA-qPCRs for the detection in living Foc spores in water and soil.

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INTRODUCTION

Fusarium wilt is an economically important disease of banana that causes significant losses to bananas produced by commercial and small-scale subsistence farmers. The causal agent is *Fusarium oxysporum* f. sp. *cubense* (Foc), a diverse soil-borne pathogen (Stover, 1962). Foc is a member of the *Fusarium oxysporum* species complex (FOSC), and produces three characteristic spore types: microconidia, macroconidia and chlamydospores. FOSC members can be classified into *formae speciales* based on their pathogenicity to different agricultural crops, and races based on pathogenicity to different host cultivars. For instance, Foc race 1 strains are pathogenic to Gros Michel, Silk, Apple, Lady Finger and Latundan cultivars, Foc race 2 Foc to certain cooking banana varieties like Bluggoe (Stover, 1962; Ploetz, 1994; Ploetz, 2005) and Foc race 4 Foc to Cavendish bananas and many cultivars susceptibility to Foc races 1 and 2. Foc race 4 is divided into tropical Race 4 (TR4) and subtropical Race 4 (STR4), of which Foc TR4 affect Cavendish bananas in all environments, and Foc STR4 in the subtropics only. Foc isolates can also be divided into 24 vegetative compatibility groups (VCGs) and eight to nine clonal lineages based on DNA analysis (Bentley *et al*., 1998; Fourie *et al*., 2009). In Africa, Foc Lineage VI comprises of VCGs present in Foc races 1 and 2 (Karangwa *et al*., 2018). Foc TR4 is limited to northern Mozambique, and Foc STR4 occur in South Africa only (Viljoen *et al*., 2002; Blomme *et al*., 2013; Butler, 2013).

Fusarium wilt management is challenging because the soil-borne pathogen forms highly resistant chlamydospores which can survive in soil for decades. Symptomless plants, as well as infested soil and water can unintentionally move the pathogen to disease-free areas (Stover, 1962; Su *et al*., 1986; Ploetz, 1994). To prevent the spread of Foc, disease-free tissue culture plantlets should be used to establish new fields and proper sanitation measures implement. Chemical agents such as quaternary ammonium or demethylation-inhibiting compounds can be used to clean farm equipment, thereby limiting the spread of Foc (Nel *et al*., 2007; Nguyen *et al*., 2019). Once introduced into a banana field, the planting of resistant varieties is the only means to continue banana production. No resistant replacement bananas, however, are currently suitable to replace the Cavendish as the main export banana (Ploetz, 2015; Dale *et al*., 2017).

The early detection of Foc plays a vital role in banana Fusarium wilt management. Infested plantations can be isolated, plants destroyed, and water treated to prevent further pathogen dispersal (Dita *et al*., 2010). Traditional methods of identifying and quantifying

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fungal pathogens in water and soil relied on culturing methods. Culturing is, however, time consuming and not suitable for distinguishing closely-related members of the FOSC, as these are culturally and morphologically similar (Leslie and Summerell, 2006). DNA-based detection with PCR or quantitative (qPCR) has, therefore, been used increasingly for detecting pathogens (Mackay *et al*., 2004). Relative to qPCR, PCR is less sensitive and generates semi-quantitative results. Due to its robustness, sensitivity and ability to differentiate closely related pathogens quantitatively, qPCR is preferred for the detection of pathogens in environmental samples (Klein, 2002; Bustin *et al*., 2009; Capote *et al*., 2012.

The quantitative detection of Foc race 4 and TR4 in plant and soil samples has been accomplished (Lin *et al*., 2009; Zhang *et al*., 2013; Peng *et al*., 2014; Aguayo *et al*., 2017). In chapter 2 of this study assays were developed for detecting Foc Lineage VI and TR4 in plant, soil and water samples. A limitation of qPCR is that all DNA within a sample is amplified, even from non-viable cells. This can lead to an overestimations of pathogen inoculum in samples (Fittipaldi *et al*., 2012; Kralik and Ricchi, 2017).

Propidium monoazide (PMA) is a dye that can be used in conjunction with qPCR to allow the specific detection of viable pathogens. The technique was developed on the principle that the photo-reactive azide group of PMA is converted into a highly reactive nitrene intermediate upon exposure to UV light (photo activation). When cells are membrane-compromised (non-viable), PMA can enter the cell and bind the hydrocarbon molecules of DNA in the nucleus covalently (Nocker *et al*., 2006). PMA cannot enter cells with intact cell membranes to bind the DNA. PMA-bound DNA is not available for amplification by DNA polymerase, and therefore only DNA from viable cells is quantified by qPCR (Nocker *et al*., 2007; Tamburini *et al*., 2013). PMA-qPCR has mainly been applied to quantitatively detect viable bacteria in food (Josefsen *et al*., 2010; Elizaquível *et al*., 2012; Yang *et al* 2013), human waste (Bae and Wuertz, 2009; Fujimoto *et al*., 2011; Taskin *et al*., 2011) and environmental samples (Nocker *et al*., 2010; Hu *et al*., 2014). PMA-qPCR has also been used to compare the efficacy of inhibitory treatments, such as disinfectants and antibiotics, on bacteria (Nocker *et al*., 2007; Kobayashi *et al*., 2012). PMA-qPCR assays applied to fungi have focused on the detection of *Alternaria* in a tomato matrix (Crespo-Sempere *et al*., 2013), *Aspergillus*, *Mucor racemosus*, *Rhizopus, Paecilomyces variotii* in air and water samples (Vesper *et al*., 2008), *Monilinia* in a flower matrix (Vilanova *et al*., 2017) and several yeasts in wine (Andorrà *et al*., 2010).

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PMA has not been used to determine the viability of *Fusarium* species before. The objective of this chapter was, therefore, to evaluate the ability of PMA and qPCR to differentiate viable and non-viable Foc spores. This was accomplished by optimizing a PMA-qPCR for Foc spores and then applying the assay to compare the efficacy of three different sanitisers.

MATERIALS AND METHODS

Fungal inoculum

The soil suspension was prepared by adding 62.5 g potting soil to 250 mL of autoclaved distilled water in 250 mL flasks (Schott AG, Mainz, Germany). The flasks were agitated for 2 days at 120 x g on a digital orbital shaker (SCILOGEX, Rocky Hill, Connecticut, USA). The soil suspensions were then filtered through a 2.0-mm sieve and eight layers of sterile cheesecloth and poured into a 2 L flask (Schott AG, Mainz, Germany). Glucose (0.025 g/100 mL) was added to the filtrate, the pH adjusted to 7, and the solution autoclaved twice for 1 hr. Streptomycin (0.004 g/100 mL) was added and 100 mL of the solution transferred to 250-mL CELLSTAR culture flasks (Greiner bio-one Gmbh, Frickenhausen, Germany).

Foc TR4 (CAV 3049) and Foc Lineage VI (CAV 2260) were selected from the *Fusarium* collection deposited at the Department of Plant Pathology, University of Stellenbosch, South Africa. The isolates were previously characterised as members of VCG 01213/16 and VCG 0124, respectively. Both isolates were grown on PDA^{+} (PDA with 40 mg/L of streptomycin sulphate), transferred to carnation leaf agar and grown for 2 weeks at room temperature. Sterile distilled water (2 mL) was added to each plate, the spores were loosened with a sterile scalpel, and 500 µL of the spore suspension transferred to a 100 mL soil suspension in a CELLSTAR culture flasks (Greiner bio-one Gmbh, Frickenhausen, Germany). Soil suspensions were used to induce chlamydospore production. The inoculated soil suspensions (100 mL) were incubated in a digital orbital shaker (Rocky Hill, Connecticut, USA) at 150 x g at 22°C for 3 weeks. The suspensions were filtered through a double-layer of sterile cheesecloth, washed twice with autoclaved water (40 mL), and centrifuged at 4000 x g for 2 min using an Eppendorf 5810 R Centrifuge. Spore suspensions contained microconidia, macroconidia and chlamydospores. A haemocytometer was used to measure the concentration of the spore suspensions and the concentration was adjusted to 10^6 spores/mL.

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PMA treatment optimisation

Preparation of non-viable control

Foc TR4 spore suspensions (10^6 spores/mL) were treated with Sporekill to obtain a non-viable spore suspension (see method below). After treatment the spores were vortexed, incubated for 15 min at room temperature, and washed twice with dH_2O. The washed spores were centrifuged at 13 200 x g between each wash. The final spore suspension was plated on PDA^+ and incubated at room temperature for 2 days to validate their viability.

Evaluation of PMA toxicity

To assess the potential toxicity of PMA to Foc, PMA stock (Biotium, Fremont, California, USA) at a concentration of 20 mM was diluted in dH_2O to prepare 10, 20, 40 and 60 µM mixtures. Its toxicity was then assessed against 10^5 spores/mL, as this was the lowest spore concentration used in this study. From each PMA concentration, 500 µL was added to the Foc TR4 spore suspensions at 10^5 spores/mL in 1.5 mL Eppendorf tubes in triplicate. PMA and the spore suspensions were vortexed at 150 x g to ensure continued homogenisation of Foc spores and PMA dye, and incubated for 20 min in the dark. The Foc spores were then photo-activated with LED lights (460 nm) for 10 min in a PD80 photolysis device (Green Africa Renewable Energy (Pty) Ltd, Bloubergstrand, Cape Town). The dark incubation and light activation conditions were selected based on PMA treatment of *Alternaria* (Crespo-Sempere *et al*., 2013). The spores were washed twice and centrifuged at 13 200 x g to get rid of the extra dye molecules that might have had a cytotoxic affect. Spore suspensions were diluted and plated onto PDA+. Colony forming units (CFU) were counted after 72 hrs and the experiment repeated twice.

Optimising PMA treatment conditions

To optimise PMA treatment conditions, 500 µL of the viable and non-viable spore suspensions (10^6 spores/mL) were transferred into 1.5 mL Eppendorf tubes. Spores were treated with combinations of PMA at concentrations of 0, 10, 20, 40 and 60 µM, photo activation of 5, 10 or 15 min, and incubation in the dark for 1, 10, 20 min. PMA treated spore suspensions were vortexed prior to dark incubation on a benchtop orbital shaker to ensure continued homogenisation of spores and PMA. A PD80 photolysis device was used for photo activation. After incubation, the spores were washed twice to remove the excess PMA dye molecules. Each treatment was performed in triplicate, and the

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experiment was repeated before DNA was isolated from the different samples. DNA was extracted using AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) and a DynaMag-96 side magnetic plate as optimised in chapter 2. The Ct values were measured using the optimised Foc TR4 qPCR assay for water samples developed in Chapter 2. Treatment conditions that produced a large difference in Ct values between viable and non-viable spores were selected.

Confocal microscopy

PMA and Hoechst 3342- (Thermo Fisher Scientific, Waltham, Massachusetts, USA) stained viable and non-viable Foc TR4 spores were visualised using a LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany) and ZEN software (www.zeis.com). This was done to ensure that the PMA dye was binding DNA in non-viable cells and not in viable cells. The membrane permeable nucleic binding dye, Hoechst 33342, was used to visualise nuclei (Shahi *et al*., 2015), making it easier to evaluate if PMA dye was inside or outside the nuclei of Foc spores. After PMA treatment the spores were washed and centrifuged at 13 200 x g, and their volume adjusted to 199 µL for microscopy. From a 10 mg/mL stock of Hoechst 3342, 1 µL was added to 199 µL of the PMA-treated Foc spore suspension for 10 min to stain the nuclei. Excess Hoechst 3342 was washed off by centrifugation at 11 000 x g. The Hoechst 3342-treated spore suspension (200 µL) was added to the wells in a confocal microscope plate.

During confocal microscopy, a Plan-Apochromat 63x/1.4 Oil DIC M27 objective was used with a 90-µm pinhole. To visualise PMA (Abs/Em = 510/610 nm) alone, a 514 nm laser and 526-695 nm filter was used. To visualise PMA and Hoechst 33342 (Abs/Em 351/497) simultaneously, two lasers (561 and 405 nm) and two filters (622-718 nm and 410-471 nm) were used.

Application of PMA-qPCR to measure the efficacy of sanitation treatment

Optimising standard curves for sanitiser efficacy

To estimate sanitiser efficacy, additional standard curves were created for both Foc Lineage VI and TR4 in which a spore suspension (10^6 spores/mL) were subjected to a 4x serial dilution. DNA, isolated from each dilution, was used to generate a standard curve representing DNA from 10^{6-3} spores/mL. Standard curves for quantification were only used if amplification efficiency and the R^2 value were close to 1 (Bustin *et al*., 2009).

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To test the reproducibility (inter-assay variation) and repeatability (intra-assay variation) of the qPCR assays, DNA from three target samples of Foc Lineage VI (CAV 184, CAV 188 and CAV 2400) and Foc TR4 (CAV 789, CAV 3049 and CAV 3326) was used. Standard deviations between average Ct values across qPCR assays and between technical repeats were recorded. Standard deviations between replicate Ct values below 0.35 were considered acceptable (Hellemans and Vandesompele, 2011). To determine the limit of quantification (LOQ) and limit of detection (LOD) (Bustin *et al*., 2009; Ramírez *et al*., 2015), DNA was extracted from Foc Lineage VI and TR4 spore suspensions (10^{7-1} spores/mL) in triplicate and measured with the appropriate spore concentration standard.

Preparation of reagents

A neutralising agent was used to stop the effect of sanitisers on Foc spores. The neutraliser was made up of 0.5 g monopotassium phosphate (KH_2PO_4), 0.5 g sodium citrate ($Na_3C_6H_5O_7.3H_2O$), 8.0 g sodium taurocholate, 1.5 g sodium thiosulfate ($Na_2S_2O_3.5H_2O$), 8.0 g polyoxyethylene sorbitan mono-oleate and 1 L of distilled water, and autoclaved (Department of Trade and Industry, 1999). Chlorine, Farmcleanse and Sporekill sanitisers were prepared according to their recommended doses (Table 1). The pH of the chlorine stock was adjusted to 7.

Sanitiser treatments

Spore suspensions (500 µL) of Foc TR4 (CAV 3049) or Lineage VI (CAV 2260) at a concentration of 10^6 spores/mL were added to 4.5 mL of three sanitisers; chloride, Farmcleanse and Sporekill (Table 1); in 15 mL Falcon tubes (NEST, New District, Wuxi, Jiangsu, China). Sterile water was used for the control treatment. After 1, 30 and 120 s, 5 mL of the treated Foc spores were added to 5 mL of the neutralizing agent to neutralise the sanitiser's action.

To test the efficacy of the neutraliser, 5 mL of the neutraliser and 4.5 mL of each sanitiser were mixed in a 15 mL Falcon tube before Foc spores were added. The suspension was then plated out, and CFUs counted 72 hrs after incubation on PDA+. If the neutraliser was effective, no reduction in spore viability compared to the control was found. Both the sanitiser and neutraliser were washed off by centrifugation (13 200 x g for 2 min).

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Measuring sanitiser efficacy

The efficacy of the sanitisers on Foc was determined by counting CFUs, PMA-PCR and PMA-qPCR. For CFU counts, 500 µL of each sanitiser-treated sample was plated out onto PDA+ after dilution (10^3 and 10^2 spores/mL) and incubated for 2 days at room temperature. There were three technical replications for each treatment. For conventional PCR and qPCR analysis the remaining 500 µL of each sanitiser-treated spore suspension was subjected to the optimised PMA treatment conditions and transferred into 1.5-mL Eppendorf tubes. The PMA dye was washed off by centrifugation at 13 200 x g, and DNA extracted from the Foc spores as previously described.

DNA extracted from the three technical replications of each treatment was pooled. The primer sets RTLinVI_F3 (5'-GACATTTGACGACTTTCTGA-3') and FocLinV-R (5'-GTGTCACTTGGTCCTCGTAT-3') and RT_13.16_F2.5 (5'-GAATATAAAGAGGAAGTAGCCG-3') and RT_13.16_R2.5 (5'-CCTCGCTGAATTATATCTAAACC-3'), were used for the amplification of Foc Lineage VI and TR4 (Chapter 2). For conventional PCR analysis of Foc Lineage VI and TR4, optimised primers and conditions were used and 25 µL PCR reaction volumes were prepared that contained 12.5 µL of KAPA ready mix with dye (Roche, Basel, Switzerland), 9.5 µl autoclaved dH_2O, 0.5 µL forward and reverse primer (0.2 µM) and 2 µL of DNA. The PCR involved an initial denaturing step of 2 min at 95°C and then 30 cycles of 95°C for 3 min, 30 s at an annealing temperature of 62°C for Foc Lineage VI and 56°C for Foc TR4, followed by 30 s at 72°C. The final extension step continued for 5 min at 72°C. Amplification products were separated on a 1% agarose gel containing 0.5 µL SYBR safe and immersed in 1× TAE buffer, and visualised under UV lights with Gel DocTM XR+ Imager and Image Lab software 5.2.1 (Biorad, Herculese, California, USA).

DNA from sanitiser treatments and the control was used for qPCR in a three-step Rotor-Gene-6000 machine (Bio-Rad, 2000 Alfred Nobel Drive, Hercules, California 94547, USA) and analysed with Rotor-Gene Q-series software (version 2.3.1) (Qiagen, Venlo, The Netherlands). For each qPCR reaction, 10 µL of SYBR mix no-Rox mix (Meridian Bioscience, Inc, Cincinnati, Ohio, USA), 6.8 µL of dH_2O, 0.6 µL (0.3 µM) of forward primer and reverse primer and 2 µL of DNA were added to Corbett-Type Strip tubes (SSIBio, Lodi, California, USA). For Lineage VI SYBR mix no-Rox Sensimix was used with the following cycle conditions; 10 min for the hold step, followed by 40 cycles of 10 s at 95°C, 15 s at 66°C and 20 s at 72°C. For TR4 SYBR mix

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no-Rox SensiFAST was used with the following cycle conditions: a 3 min hold step followed by 40 cycles of 5 s at 95°C, 10 s at 62°C and 20 s at 72°C.

The highest Ct difference (9 cycles) between viable and non-viable DNA in the optimisation of PMA-qPCR was used as a cut-off point. Samples quantified after this point were excluded from the analyses.

Statistical analysis

SAS® version 9.4 software (SAS Institute Inc., Cary, North Carolina, USA) was used for Leven's test for homogeneity, Shapiro-Wilk's test for normality and analysis of variance (ANOVA). A 95% least significant difference was used to make pairwise comparisons using Fisher's (LSD) test. ANOVA was performed to determine what PMA concentrations significantly reduce Foc viability, and whether different factors (PMA concentration, light activation time and dark incubation time) significantly improved PMA performance. Weighted ANOVAs for CFU counts and PMA-qPCR were performed as variances were not equal. ANOVA was performed for CFUs and PMA-qPCR respectively to determine what sanitiser (chlorine, Farmcleanse, Sporekill) at which exposure time (1s, 30s, 120s) for which race (TR4 and Lineage VI) was most effective compared to an untreated control. Significant interactions were determined, and Pearson's correlation coefficient used to determine a correlation between the two datasets for both Foc Lineage VI and TR4.

RESULTS

PMA treatment optimisation

The disruption of Foc spore cell membranes with Sporekill was successful. No fungal growth was observed on the PDA+ plates after incubation. The treatment could, therefore, be used to produce the non-viable controls.

There were no significant differences in the viability of Foc Lineage VI spores treated with PMA at concentrations of 10 and 20 µM when compared to the non-treated control ($P < 0.05$). At 40 and 60 µM treatments, however, the PMA became toxic and significantly affected spore viability (Fig. 1).

The differences in Ct values between viable and non-viable Foc TR4 spores treated with 10 µM differed significantly from those treated with 20-60 µM PMA ($P < 0.05$) (Fig. 2). No significant differences were observed between PMA concentrations of 20, 40 and 60 µM, a PMA concentration of 20 uM was therefore selected for PMA-qPCR. This

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gave a minimum signal reduction of six cycles between the PMA-treated viable control and PMA-treated non-viable control (Fig. 2). Since no significant differences were recorded for different light activation times between the viable non-PMA-treated control and the non-viable PMA-treated control (Fig. 3), the shortest activation time of 5 min was selected. The dark incubation time of 1 min gave a significantly higher ($P< 0.05$) Ct difference (9.12 cycles) between viable and non-viable controls than dark incubation times of 10 min (6.6 cycles) and 20 min (6.8 cycles) (Fig. 4). The 1 min incubation time, thus, was selected for the PMA-qPCR assay. If a difference of nine Ct values between a sample and a viable control was observed, it was regarded as non-viable.

Confocal microscopy

The PMA dye was observed inside non-viable Foc spores treated with Sporekill (Fig. 5A), but not in the viable spores that were not treated with the disinfectant (Fig. 5B). This confirmed that PMA dye only enters membrane-compromised cells. When the PMA dye was combined with Hoechst 3342 the location of the PMA dye relative to the nucleus was more apparent. PMA was not present in viable spores (Fig. 6A and 6C) but present in non-viable spores (Fig. 6B and 6D).

Application of PMA-qPCR to assess efficacy of sanitiser treatments

Optimising standard curves for sanitiser efficacy:

The Foc Lineage VI standard curve was suitable for the absolute quantification of target spores, with an efficiency of 0.98, an R^2 value of 0.99, and a quantification range of ~10^6-10^3 spores/mL, with Ct values of between 16.91 and 25.31 (Fig. 7). The Foc TR4 standard curve had an efficiency of 1.00, a R^2 value of 0.99 and a quantification range of ~10^6-10^3 spores/mL, with Ct values of between 19.66 and 27.39, which also made it suitable for absolute quantification of Foc TR4 spores (Fig. 8). The LOQ and LOD was 10^3 and 10^2 spores/mL, respectively, for both Lineage VI and TR4. The qPCR assays were reproducible and repeatable with standard deviations between Ct values less than 0.35 (Tables 2 and 3).

Measuring sanitiser efficacy

Significant differences were observed between spores/mL obtained from Foc Lineage VI and TR4 isolates and between days for Lineage VI and TR4 spores/mL measured with CFUs or TR4 spores/mL measured with PMA-qPCR. ANOVA was therefore conducted

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separately for each isolate per day except for Lineage VI measured with PMA-qPCR. The treatment and exposure time had a significant effect on spores/mL measured in every ANOVA (Table 4.).

The spores/mL estimated from CFUs of Foc Lineage VI spores following treatment with chlorine, Farmcleanse and Sporekill at exposure times of 1, 30 and 120 s ranged from 4.90-5.32 x 10^5, 2.9-5.21 x 10^5 and 0 spores/mL, respectively (Table 5). The Foc TR4 spores treated with chlorine, Farmcleanse and Sporekill at exposure times of 1, 30 and 120 s produced 4.69-6.37 x 10^5, 3.52-6.12 x 10^5 and 0-1.48 x 10^4 spores/mL, respectively. Relative to the control, chlorine and Farmcleanse treatments did not significantly reduce spores/mL, except for three exceptions. These included Lineage VI spores treated with Farmcleanse for 30 s on Day 1 and Foc TR4 spores treated with Farmcleanse for 1 s and 120 s on Day 1. These treatments produced significantly more spores/mL than Sporekill, which was the most effective sanitiser. Sanitiser treatments did not differ significantly at different exposure times except for Lineage VI spores treated with Farmcleanse for 30 s on Day 1 and TR4 spores treated with Farmcleanse for 1 s on day 1. The neutraliser was effective with no reduction in Ct values compared to the non-treated control.

The DNA from Foc spores treated with chlorine and Farmcleanse produced amplification products of 98 bp for Foc Lineage VI and 157 bp for Foc TR4 when using conventional PCR, respectively, after exposure times of 1, 30 and 120 s (Fig. 9). This did not differ from the control treatments, which indicated that chlorine and Farmcleanse were ineffective at reducing Foc viability. The DNA of Foc spores treated with Sporekill was not amplified at any of the exposure times, which indicated that Sporekill was the most effective sanitiser evaluated.

The spores/mL estimated from PMA-qPCR of Foc Lineage VI spores treated with chlorine, Farmcleanse and Sporekill at exposure times of 1, 30 and 120 s ranged from 9.02-9.46 x 10^5 spores/mL, 3.74-6.26 x 10^5 spores/mL and 0 spores/mL, respectively (Table 6). The Foc TR4 spores ranged from 8.54 x 10^5 to 7.59 x 10^6 spores/mL, 9.17 x 10^5 to 5.38 x 10^6 spores/mL and 0-1.13 x 10^4 spores/mL, respectively. Relative to the control, chlorine and Farmcleanse treatments did not significantly reduce spores/mL. Sporekill, significantly redused spores/mL relative to the control and was the most effective sanitiser. Sanitiser treatments did not differ significantly at different exposure times except for TR4 spores treated with Farmcleanse on day 1.

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Pearson's correlation coefficient indicated that spore concentrations measured with PMA-qPCR correlated well with spore concentrations, with R values of 0.754 and 0.702 for Foc Lineage VI and TR4, respectively (Fig. 10).

DISCUSSION

Various studies in recently years have used qPCR assays for the detection of Foc from environmental samples (Lin *et al*., 2009; Li *et al*., 2012; Zhang *et al*., 2013; Peng *et al*., 2014; Yang *et al*., 2015; Aguayo *et al*., 2017). A limitation, however, was the inability of these molecular detection and quantification methods to distinguish between viable from non-viable or dead fungal propagules. In this study, PMA was used in combination with qPCR to quantify DNA isolated from viable Foc Lineage VI and TR4 spores. This is the first study where a preferential detection of viable over dead Foc cells was evaluated optimised for DNA based applications.

The PMA concentration had the greatest effect on Ct difference between viable and non-viable Foc spores. This agreed with observations made by Fittipaldi *et al*. (2012). Larger Ct differences between viable and non-viable controls indicate that PMA dye is working optimally and extends the range at which viable cells can be quantified. The maximum Ct difference between viable and non-viable Foc spores was 9 cycles, which was comparable to Ct differences of 7.13, 8.86 and 6-9 cycles reported for *Monolina fructicola*, *Alternaria* spp. and fungal strains of *Aspergillus* spp., *Rhizopus stolonifera*, *Mucor racemosus* and *Paecilomyces variotti*, respectively (Vesper *et al*., 2008; Crespo-Sempere *et al*., 2013; Vilanova *et al*., 2017).

PMA-qPCR assays developed in this study could in future be used to detect Foc from more complex environmental samples, such as from banana plant tissue, but require further optimization. Since PMA treatment requires direct contact with target cell membranes, it would be challenging to detect Foc spores in banana plant material. Non-target cells, as well as inorganic and organic compounds, may also interfere with the interaction between PMA and the target cells (Fittipaldi *et al*., 2012). The challenge with banana plant material will be to homogenise the dye in the sample without disrupting cell membranes. In a study by Crespo-Sempere *et al*. (2013) a Masticator stomacher was used to improved homogenisation of samples prior to PMA treatment. The preparation of a banana matrix for PMA-qPCR is harder than fruit or flower samples, and thus may

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require optimisation to achieve efficient sample homogenisation suitable for PMA treatment

PMA-qPCR can be used to determine the efficacy of sanitisers on Foc, as PMA-qPCR values correlated well with CFU counts in this study. Sporekill was more effective than chlorine and Farmcleanse as a disinfectant of Foc, as was previously reported by Nel *et al*. (2007), Meldrum *et al.* (2013) and Nguyen *et al*. (2019). Exposure times is important in the activity of disinfectants. The short exposure times and relatively high spore concentrations (10^5 spores/ mL) used in the current study may have contributed to the lack of efficacy of chlorine compared to Farmcleanse. Nel *et al*. (2007) reported that both Farmcleanse and chlorine resulted in significant reductions to Foc viability at 10^2 spores/ mL. Future studies, thus, could compare the performance of PMA-qPCR at lower spore concentrations and longer exposure times.

The major advantages of using PMA-qPCR to detect the viability of plant pathogens include a faster and more specific detection than culture plating. PMA-qPCRs can take 4-8 hrs to detect DNA of viable sample cells, depending on sample numbers. Foc CFUs require at least 48 hrs to grow into sufficiently large cultures to obtain reliable results. qPCR can also distinguish between Foc TR4 or Lineage VI isolates, which is not possible with CFUs. A limitation, however, include the high cost of qPCR relative to CFUs. PMA may also not bind to all DNA from non-viable cells and non-viable DNA might be amplified at later Ct's. Ideally, DNA from non-viable cells should not amplify, but this is hard to achieve due to the sensitivity of qPCR and the differences in cell membrane permeability. Factors that may improve the permeability of non-viable cells include the increase in temperature or reductions in pH or salt concentration (Fittipaldi *et al*., 2012).

In conclusion, a novel PMA-qPCR assay was developed for Foc Lineage VI and TR4 spores in this study. The PMA-qPCR assays correlated with CFU counts when sanitiser efficacy was evaluated at different exposure times. Future studies could optimise the PMA-qPCR assay for the detection of Foc in environmental plant and water samples.

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Table 1. Sanitisers, their active ingredients and concentrations used to treat *Fusarium oxysporum* f. sp. *cubense* Lineage VI and TR4 spores.

Sanitiser	Active ingredient	Concentration
Chlorine	Chlorine	0.005 g of calcium hypochlorite in 1000 mL
Farmcleanse	Alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide chlorine	10 mL/1000 mL
Sporekill	Didecyldimethylammonium chloride	10 mL/1000 mL

Table 2. Reproducibility and repeatability of the qPCR assay quanitifying *Fusarium oxysporum* f. sp *cubense* Lineage VI spores.

	Average Ct		Repeatability		Reproducibility
Isolate	Run 1[a]	Run 2	SD_{Run1}[b]	SD_{Run2}	SD_{Run}[c]
CAV 188	23.59	23.35*	0.191*	0.086	0.166
CAV 184	20.50	20.67	0.148	0.040	0.123
CAV 2400	20.81	20.68	0.085	0.129	0.087

[a]The qPCR reaction in which DNA was run in three or six (*) replicates.

[b]The standard deviation between Ct values from of three or six (*) technical replicates within the same qPCR run (repeatability).

[c]The standard deviation between average Ct values obtained from target DNA over two qPCR runs (reproducibility).

Table 3. Reproducibility and repeatability of the qPCR assay quanitifying *Fusarium oxysporum* f. sp *cubense* TR4 spores.

	Average Ct		Repeatability		Reproducibility
Isolate	Run 1[a]	Run 2	SD_{Run1}[b]	SD_{Run2}	SD_{Run}[c]
CAV 789	19.46*	19.52	0.086*	0.035	0.035
CAV 3326	22.78	22.56	0.167	0.127	0.127
CAV 3049	27.08	27.39	0.308	0.148	0.148

[a]The qPCR run in which DNA from the target isolates was run in three or six (*) replicates.

[b]The standard deviation between Ct values from three or six (*) technical replicates (repeatability).

[c]The standard deviation between average Ct values obtained from target DNA over two qPCR runs (reproducibility).

Table 4. Analysis of variance of *Fusarium oxysporum* f. sp *cubense* spores measured with colony forming units (CFUs) and quantitative PCR (qPCR).

Measurement[a] technique	Isolate[b]	Day[c]	Sources of Variation	DF[d]	Mean square[e]	Pr > F[f]
CFUs	Lineage VI	1	Treatment x Exposure[g]	9	1.50×10^{11}	<.0001
		2	Treatment x Exposure	9	1.65×10^{11}	<.0001
	TR4	1	Treatment x Exposure	9	1.51×10^{11}	<.0001
		2	Treatment x Exposure	9	1.99×10^{11}	<.0001
PMA-qPCR	Lineage VI	1+2	Day[h]	1	2.41×10^{10}	0.5961
			Treatment x Exposure	9	1.02×10^{12}	<.0001
			Day x Treatment x Exposure	9	2.53×10^{10}	0.9703
	TR4	1	Treatment x Exposure	9	1.51×10^{12}	0.0006
		2	Treatment x Exposure	9	1.15×10^{13}	0.0023

[a] The technique used to measure spores/mL after treatment.

[b]The isolate used (Foc Lineage VI or TR4).

[c]The day the sanitiser efficacy test was conducted.

[d]Degrees of freedom.

[e]The mean square spores/mL for each source of variation.

[f]The significance probability (P) value associated with the F Value.

[g]Treatment and exposure time combined as a factor.

[h]The interaction between treatment, exposure time and day.

Table 5. Colony forming units of *Fusarium oxysporum* f. sp *cubense* (Foc) Lineage VI and TR4 on days 1 and 2, respectively, as affected by sanitisers at different exposure times. Summary table of LSD test for CFUs

		Foc Lineage VI[a]		Foc TR4	
Treatment[b]	Exposure time[c]	Day 1[d]	Day 2	Day 1	Day 2
Control	0 s	4.71×10^{5}[e] a-c[f]	4.71×10^{5} a-b	4.97×10^{5} a	5.86×10^{5} a-c
Chlorine	1 s	4.90×10^{5} a-c	4.90×10^{5} a-b	4.69×10^{5} a-b	5.03×10^{5} c
	30 s	5.12×10^{5} a-b	5.32×10^{5} a	4.81×10^{5} a-b	6.37×10^{5} a
	120 s	5.00×10^{5} a	5.00×10^{5} a-b	4.91×10^{5} a-b	5.50×10^{5} b-c
Farmcleanse	1 s	4.57×10^{5} b-c	4.57×10^{5} a-b	3.52×10^{5} c	5.27×10^{5} c
	30 s	5.21×10^{5} d	2.9×10^{5} a	4.65×10^{5} a-b	5.68×10^{5} a-c
	120 s	4.28×10^{5} c	4.28×10^{5} b	4.59×10^{5} b	6.12×10^{5} ab
Sporekill	1 s	0 e	0 c	6.47×10^{3} d	1.48×10^{4} d
	30 s	0 e	0 c	0 d	0 d
	120 s	0 e	0 c	0 d	0 d

[a]The Foc isolate used (Lineage VI or TR4).

[b]The treatment used; control or one of three sanitisers (chlorine, Farmcleanse or Sporekill).

[c]The exposure times of the treatments (0 s, 1 s, 30 s or 120 s).

[e]The mean spores/mL within triplicate treatments.

[f]Letters from Fisher's least significant difference (LSD) test.

Table 6. Spore concentration, determined by quantitative PCR, of *Fusarium oxysporum* f. sp *cubense* Lineage VI and TR4 on day 1 and day 2, respectively, as affected by sanitisers at different exposure times. Summary table of LSD test for PMA-qPCR

		Lineage VI[a]	TR4	
Treatment[b]	Exposure time[c]	Days combined	Day 1	Day 2
Control	0 s	9.68×10^{5} c[e f]	1.17×10^{6} b	4.04×10^{6} b
	1 s	9.46×10^{5} a	1.49×10^{6} b	4.13×10^{6} a
Chlorine	30 s	9.18×10^{5} a-c	8.54×10^{5} b-d	4.91×10^{6} a-b
	120 s	9.02×10^{5} a	1.08×10^{6} b	7.59×10^{6} a
	1 s	6.26×10^{5} a-b	1.60×10^{6} b	3.42×10^{6} b
Farmcleanse	30 s	5.81×10^{5} a-b	2.58×10^{6} a	2.45×10^{6} b-c
	120 s	3.74×10^{5} b-c	9.17×10^{5} c-b	5.38×10^{6} a-b
	1 s	0 d	1.13×10^{4} d	7.37×10^{3} c
Sporekill	30 s	0 d	0 d	0 c
	120 s	0 d	0 d	0 c

[a]The *Fusarium oxysporum* f. sp. *cubense* isolate used (Lineage VI or TR4).

[b]The treatment used; control or one of three sanitisers (chlorine, Farmcleanse or Sporekill).

[c]The exposure times of the treatments (0 s, 1 s, 30 s or 120 s).

[e]The mean spores/mL within triplicate treatments.

[f]Letters from Fisher's least significant difference (LSD) test.

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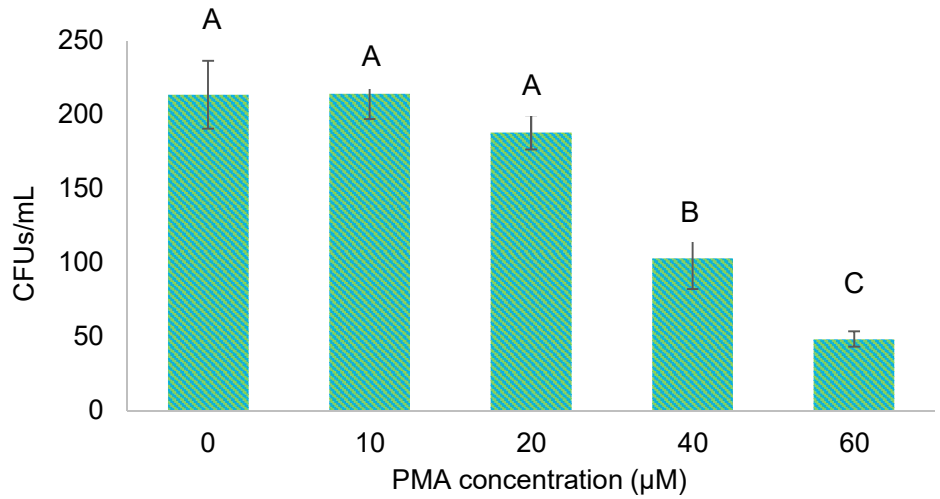

Figure 1. The toxicity of propidium monoazide (PMA) on *Fusarium oxysporum* f. sp *cubense* (Foc) TR4 applied at 10^5 spores/mL. Data is presented as the mean colony forming units (CFUs)/mL of Foc spores. The vertical bars indicate standard errors, and the different letters indicate significant differences according to Fisher's least significant difference (LSD) test.

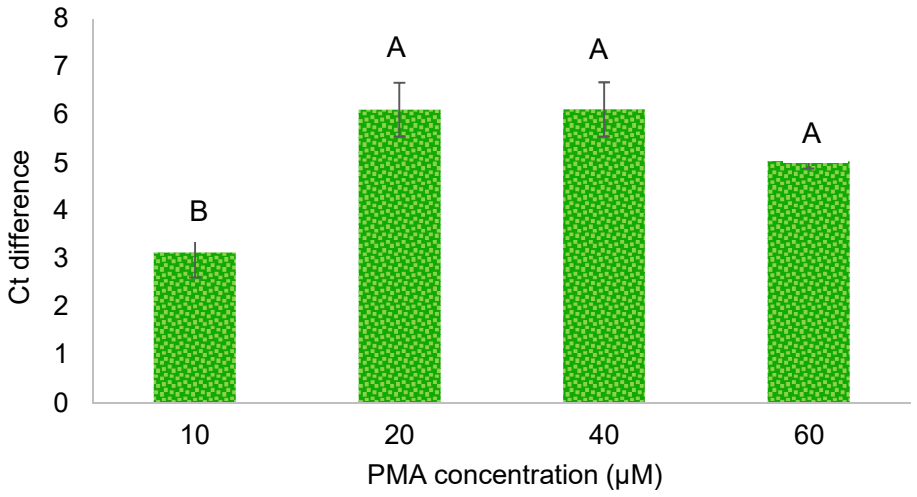

Figure 2. Mean Ct differences between viable and non-viable *Fusarium oxysporum* f. sp *cubense* (Foc) TR4 spores at four different propidium monoazide (PMA) concentrations. The vertical bars indicate standard errors, and the different letters indicate significant differences according to Fisher's least significant difference (LSD) test.

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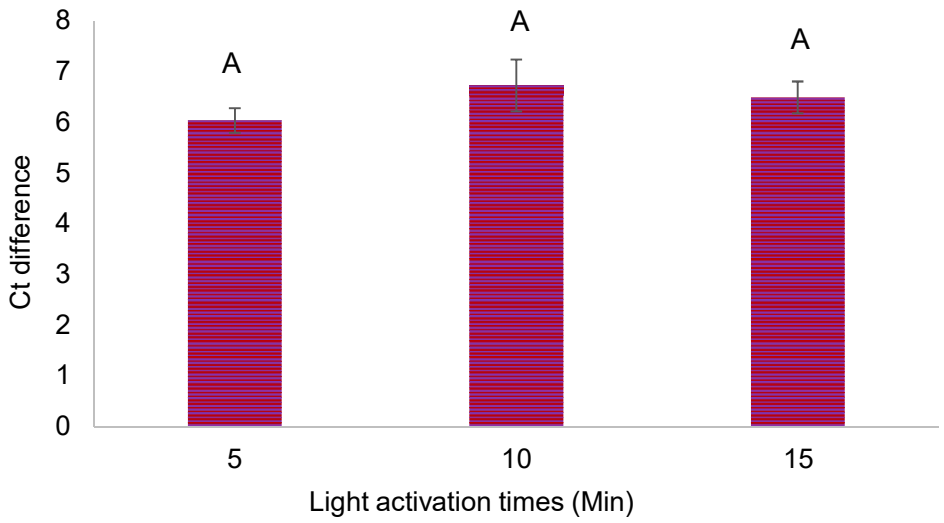

Figure 3. Mean Ct differences between viable and non-viable *Fusarium oxysporum* f. sp *cubense* (Foc) TR4 spores (CAV 3049) at three different light activation time periods. The vertical bars indicate standard errors, and the different letters indicate significant differences according to Fisher's least significant difference (LSD) test.

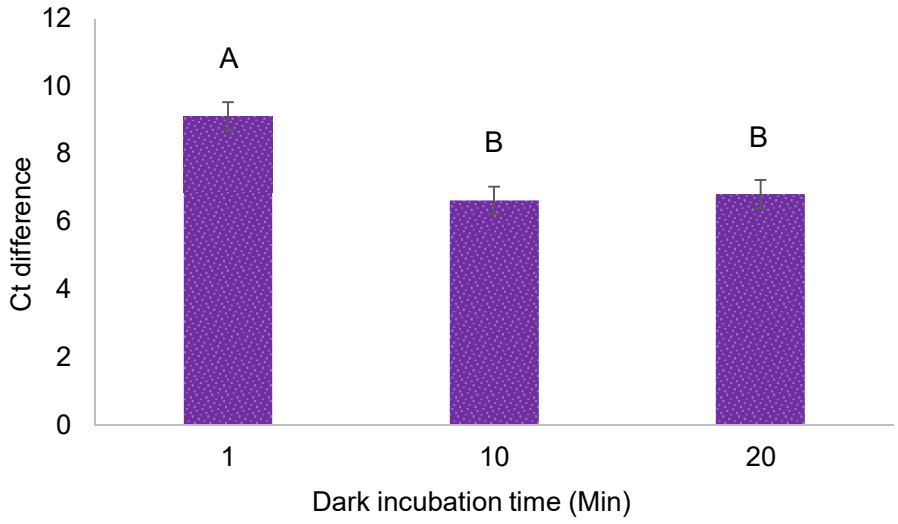

Figure 4. Mean Ct differences between viable and non-viable *Fusarium oxysporum* f. sp *cubense* (Foc) TR4 spores (CAV 3049) at three different dark incubation time periods. The vertical bars indicate standard errors, and the different letters indicate significant differences according to Fisher's least significant difference (LSD) test.

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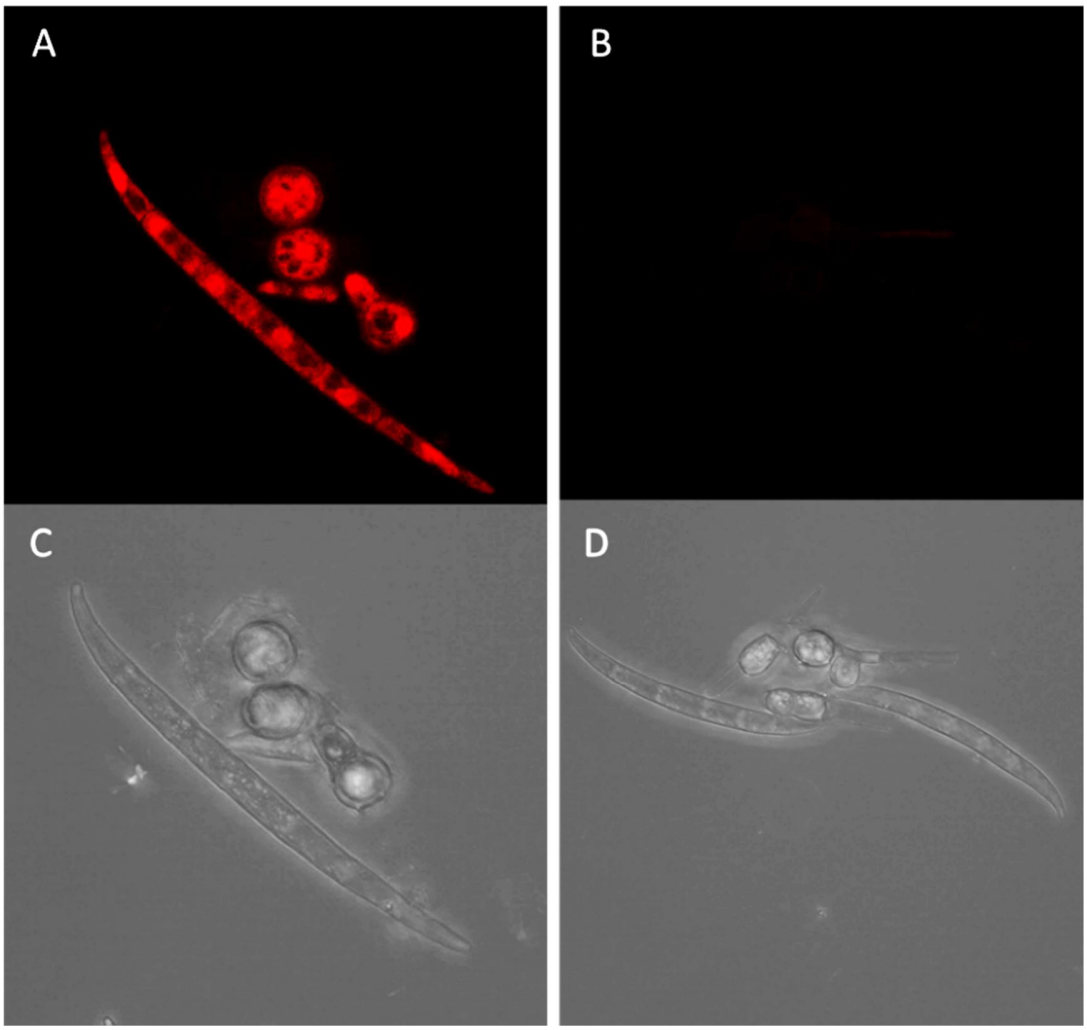

Figure 5. Confocal (A and B) and bright field (C and D) microscopy of *Fusarium oxysporum* f. sp *cubense* (Foc) spores. The propidium monoazide dye appears red under the Texas red filter used for confocal microscopy. Image A and C show spores treated with Sporekill prior to PMA treatment, and image B and D shows spores not treated with Sporekill.

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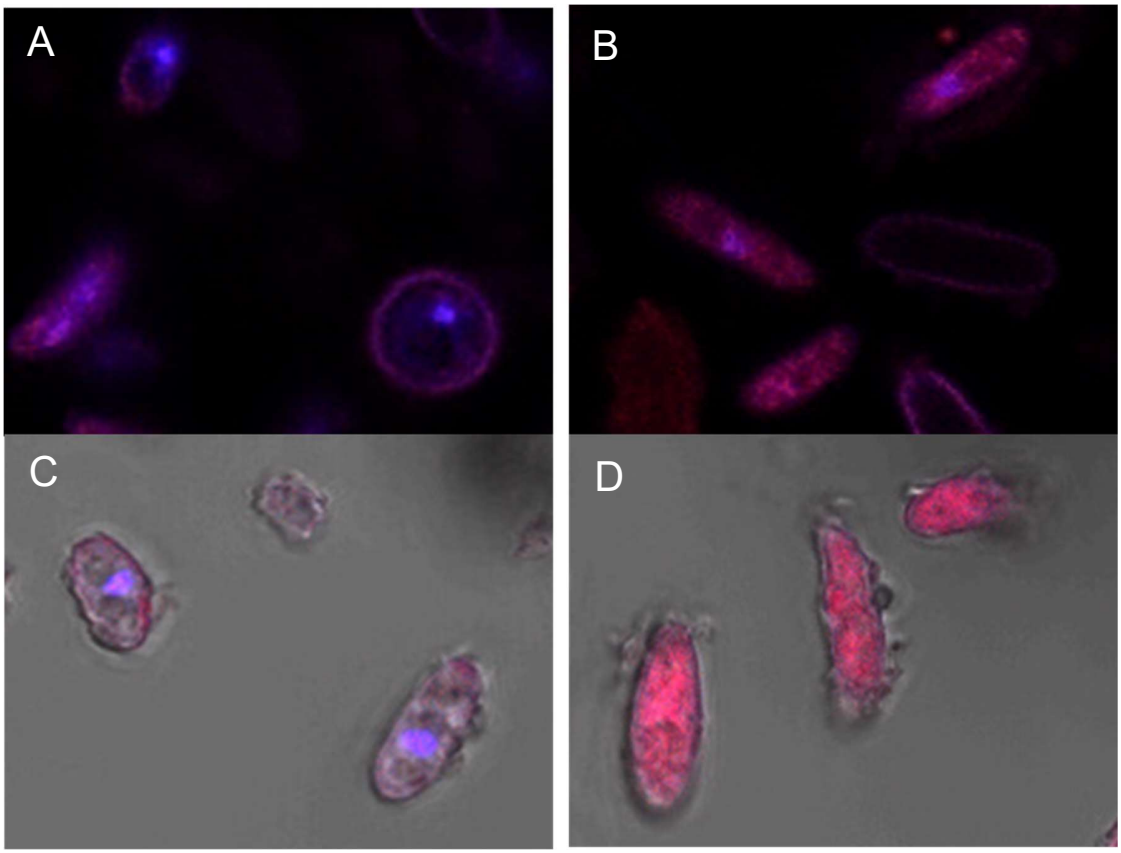

Figure 6. Image A and C show *Fusarium oxysporum* f. sp *cubense* (Foc) spores that have not been treated with Sporekill. Image B and D shows the Foc (CAV 3049) spores treated with Sporekill. Image A and B have black backgrounds to highlight fluorescence and images C and D have grey/bright field backgrounds to highlight the internal structures. The propidium monoazide dye is shown as red/pink while Hoechst 33342 is shown in blue and localised in the nucleus. The images were edited with ZEN software (www.zeis.com) after observation under a LSM 780 confocal microscope with the Plan-Apochromat 63x/1.4 Oil DIC M27 objective, a 90 µm pinhole, two lasers (405 nm and 561 nm) and two filters (622-718 nm 410-471 nm).

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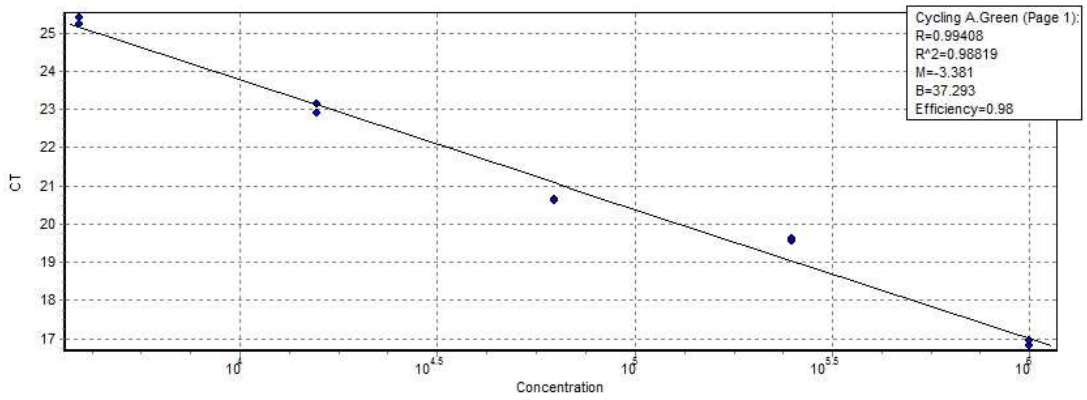

Figure 7. Standard curve relating Ct values to a 4x dilution of *Fusarium oxysporum* f. sp *cubense* Lineage VI (CAV 2260) spores (~10^6-10^3 spores/ mL).

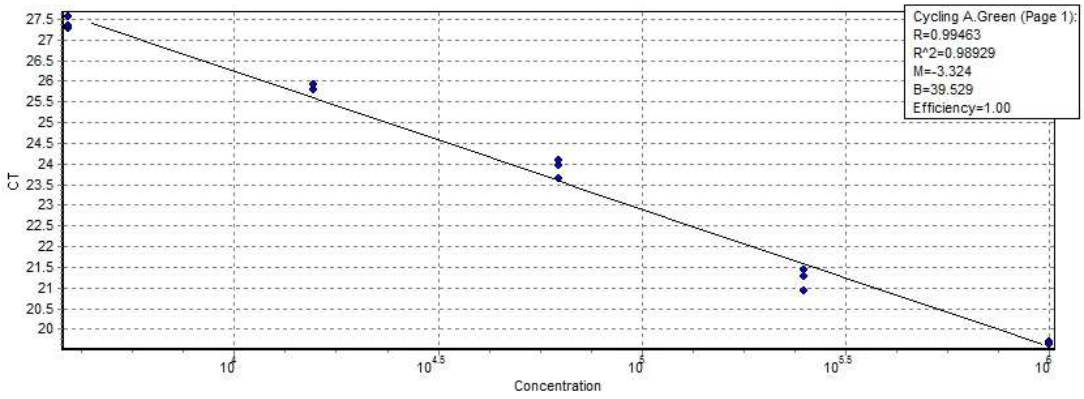

Figure 8. Standard curve relating Ct values to a 4x dilution of *Fusarium oxysporum* f. sp *cubense* TR4 (CAV 3049) spores (~10^6-10^3 spores/ mL).

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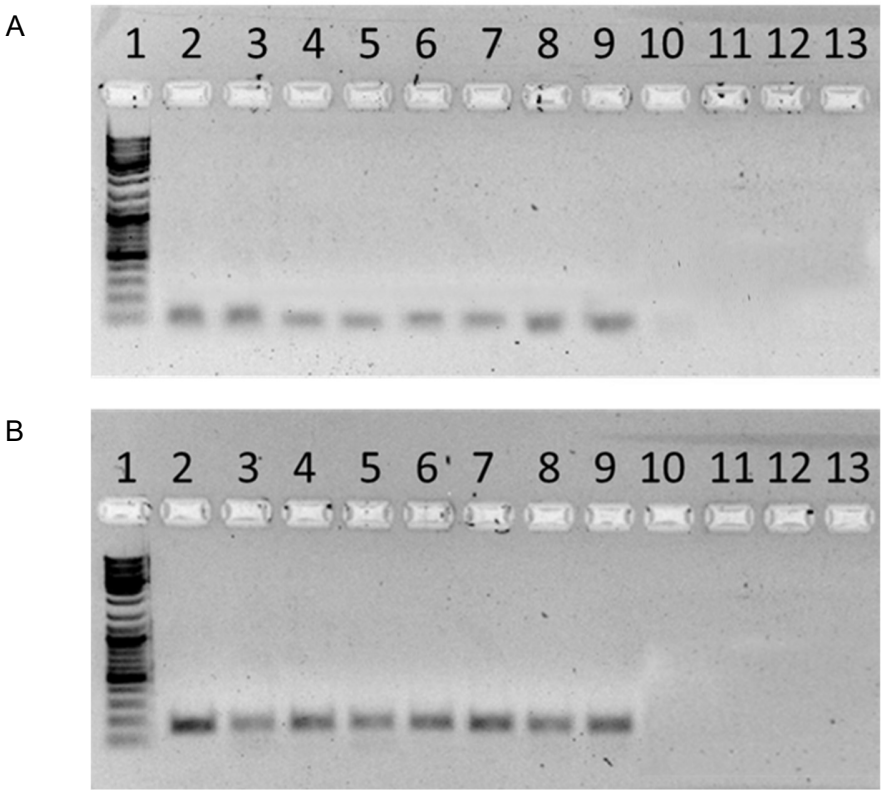

Figure 9. DNA of *Fusarium oxysporum* f. sp *cubense* (Foc) Lineage VI (A) and TR4 (B) spores as visualised on agarose gel. Lane: DNA ladder (Thermo Fischer, Waltham, Massachusetts, USA), lanes 2 and 3: amplified DNA from a positive control and propidium monoazide (PMA) treated viable spores, respectively, lanes 4-6: amplified DNA from PMA treated spores exposed to chlorine for 1 s, 30 s and 120 s respectively, lanes 7-9: amplified DNA from PMA treated spores exposed to Farmcleanse for 1 s, 30 s and 120 s respectively, lanes 10-12: amplified DNA from PMA treated spores exposed to SporeKill for 1 s, 30 s and 120 s respectively and lane 13: the NTC.

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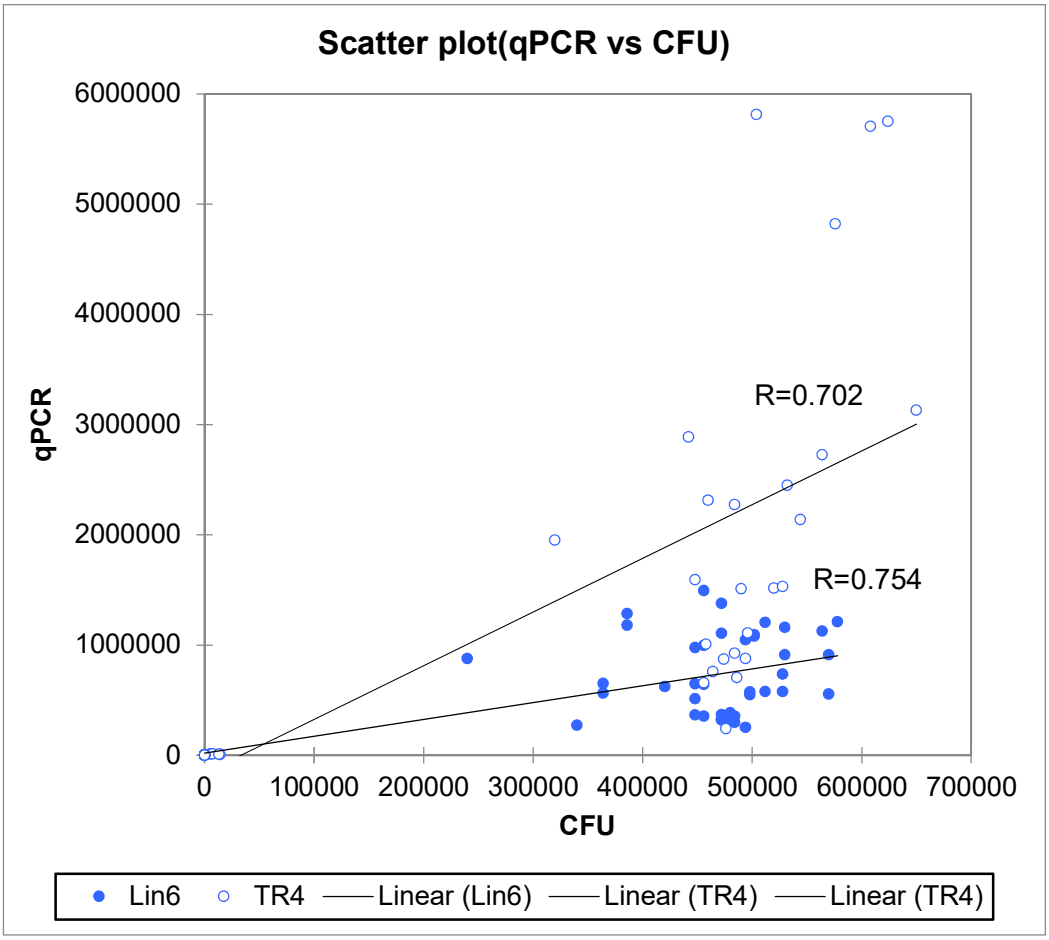

Figure 10. Pearson's correlation scatter plot to demonstrate the relationship between *Fusarium oxysporum* f. sp *cubense* Lineage VI and TR4 spores/mL measured by colony forming units (CFUs) and quantitative PCR (qPCR), respectively.