Molecular Diagnostics in the Mycosphaerella Leaf Spot Disease Complex of Banana and for *Radopholus similis*

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Abstract

Mycosphaerella leaf spots and nematodes threaten banana cultivation worldwide. The Mycosphaerella disease complex involves three related ascomycetous fungi: *Mycosphaerella fijiensis*, *M. musicola* and *M. eumusae*. The exact distribution of these three species and their disease epidemiology remain unclear, since their symptoms and life cycles are rather similar. Diagnosing these diseases and the respective causal agents is based on the presence of host symptoms and fungal fruiting structures, but is time consuming and not conducive to preventive management. In the present study, we developed rapid and robust species-specific diagnostic tools to detect and quantify *M. fijiensis*, *M. musicola* and *M. eumusae*. Conventional species-specific PCR primers were developed based on the actin gene that detected as little as 100, 1 and 10 pg/µl DNA from, respectively, *M. fijiensis*, *M. musicola* and *M. eumusae*. Furthermore, TaqMan real-time quantitative PCR assays that were developed based on the β-tubulin gene detected quantities as low as 1 pg/µl DNA of each species from pure cultures and 1.6 pg/µl DNA/mg of *M. fijiensis* from dry leaf tissue. The efficacy of the tests was validated using naturally infected banana leaves. Similar technology has been used to develop a quantitative PCR assay for the banana burrowing nematode, *Radopholus similis*, which is currently being validated.

INTRODUCTION

Banana represents a very important staple food commodity in the tropics after wheat, rice and corn (Marin et al., 2003; Ploetz, 2000). Banana plants are susceptible to a variety of devastating diseases, including those in the Mycosphaerella leaf spot complex, caused by three related ascomycetous fungi. *Mycosphaerella fijiensis* (anamorph = *Pseudocercospora fijiensis*) causes black leaf streak or black Sigatoka disease, *M. musicola* (anamorph = *Pseudocercospora musae*) is responsible for Sigatoka disease (commonly known as Yellow Sigatoka) and *M. eumusae* (anamorph = *Pseudocercospora eumusae*) causes eumusae leaf spot disease. The diseases represent a serious threat to banana production worldwide. They cause necrotic leaf lesions, thus reducing the photosynthetic capacity of plants resulting in reduced crop yield and premature fruit ripening (Jones, 2002).

Sigatoka leaf spot was first reported from Java early last century, then spread to banana-producing areas in Asia, Africa and South America during the 1940s. In the early 1960s, *M. fijiensis* was identified in Fiji. It later spread to replace *M. musicola* as the most important constraint in many banana-producing areas. However, *Mycospaerella musicola*

still remains a serious pathogen of banana at higher altitudes, cooler temperatures and subtropical regions (Marin et al., 2003; Jones, 2002). In the mid-1990s, *M. eumusae* was recognised as the cause of a new Sigatoka-like disease of banana (Crous and Mourichon, 2002; Carlier et al., 2000). It is presently known from South-East Asia, the Indian subcontinent, Mauritius, Réunion and Nigeria (Jones, 2002).

South-East Asia is considered to be the centre of origin of the three species of *Mycosphaerella* causing diseases in the Mycosphaerella leaf spot complex (Rivas et al., 2004). This region is also the centre of origin and diversity of banana, and the place where earliest domestication of edible banana took place (Jones, 2000). The exact distribution of the three species still remains unclear, as their symptoms are rather similar and difficult to distinguish (Crous and Mourichon, 2002; Jones, 2002).

Infection of banana by nematodes causes yield losses of up to 60% in many countries. Nematodes damage the roots, which in turn leads to slower plant growth and ultimately reduced bunch weights. The burrowing nematode, *Radopholus similis*, is the most damaging species in most banana-producing countries. Araya et al. (2002) found that 97% of >60,000 root samples collected in Costa Rica were infected with *R. similis*. The pest appears to have been introduced to Uganda in the 1960s from where it spread to surrounding countries (Price, 2006). Some banana-growing areas have not yet been infested (Sharma et al., 2005).

The early detection of plant pathogens is essential for proper disease management. The species of *Mycosphaerella* in the Mycosphaerella leaf spot complex are distinguished on morphological characteristics of the conidia and conidiophores, but a trained eye is often needed to recognise slight differences (Crous et al., 2004). In recent years, conventional PCR-based techniques have emerged as robust tools for diagnosis and detection of phytopathogenic fungi and have contributed greatly to plant disease management (Lievens and Thomma, 2005). However, in conventional PCR, the amplification efficiency is not consistent during all PCR cycles and remains unreliable for quantitative analysis. Real-time PCR alleviates these difficulties, as it combines thermal cycling with online fluorescent detection of amplification. Unlike the end-point PCR, accumulation of amplicons is monitored during all PCR cycles. This technique enables DNA quantification at very low concentrations (pg/ml) and has been applied in plant-microbe interaction experiments (Valsesia et al., 2005) and monitoring studies (Waalwijk et al., 2004; Hietala et al., 2003).

The aim of the present study was to develop and optimise molecular-based detection and quantification tools for *M. fijiensis*, *M. musicola* and *M. eumusae* as well as a quantitative real-time detection for *R. similis*. These tools will facilitate ecological and epidemiological studies towards better understanding of these diseases and, after validation under field conditions, provide opportunities for optimised management strategies.

MATERIALS AND METHODS

The specificity of primers and probes was tested on a number of *Mycosphaerella* species and other fungal species found commonly on banana leaves. Genomic DNA was extracted as described by Arzanlou et al. (2007). DNA was retrieved from axenic cultures grown on agar plates using the PureGene kit (Gentra Systems Inc., Minneapolis, MN, U.S.A.), whereas the DNeasy Plant Mini Kit (Qiagen, Germany) was used to isolate DNA from noninfected banana leaves and banana leaves naturally infected with *M. fijiensis*, *M. musicola*, *M. eumusae* or a mixture of these species. *Radopholus similis*, maintained on carrot slices, was harvested and ground under liquid N_2. DNA was extracted using the Wizard Magnetic DNA purification System for Food (Promega). DNA from non-target nematode species, *Meloidogyne incognita*, *M. chitwoodi* and *Pratylenchus penetrans*, and DNA from both *R. similis*-infected roots as well as healthy roots were used as controls. Genomic DNA was used to test for possible cross-reactions and to verify the specificity and efficacy of the real-time PCR primers and probes.

Primers for conventional *Mycosphaerella* PCR were designed based on sequence

data from the actin gene for each of the target and other closely related species. Species-specific primer combinations were designed with expected amplicon sizes of 500 bp for *M. fijiensis* (MfACT-F/ACT-R, CTCATGAAGATCTTGGCTGAG/GCAATGATC TTGACCTTCAT), 200 bp for *M. musicola* (MmACT-F2/MmACT-Rb, ACGGCCAGGTCATCACT/GCGCATGGAAACATGA) and 630 bp for *M. eumusae* (ACT-F/MeACT-R, TCCAACCGTGAGAAGATGAC/GAGTGCGCATGCGAG). Specificity and sensitivity of conventional primers were verified using genomic DNA from pure cultures of each of the target species and naturally infected banana leaves with the respective target species, collected from various plantations in Costa Rica (Arzanlou et al., 2007).

TaqMan primer/probe sets were designed based on partial sequences of the β-tubulin gene. Primers TMG3 / TMG4 (5'-CTTTCTGGCAGACCATCTCC-3' and 5'-AAGAGCTGACCGAAAGGAACC-3') were used to amplify part of the β-tubulin gene, and the sequences were compared with those of other closely related *Mycosphaerella* species. One probe and primer combination was designed for *M. fijiensis*, and a general probe was designed for *M. musicola* and *M. eumusae* in combination with selective primers for these two species. *Potato leaf roll virus* (PLRV) sequences were used as a positive internal control to discriminate between noninfected samples and negative reactions caused by the presence of possible PCR inhibitors (Waalwijk et al., 2004). Banana leaves artificially inoculated with each target species or all three together were used to validate the TaqMan real-time assay (Abadie et al., 2005). Naturally infected banana leaves were used to verify the TaqMan reproducibility. Real-time PCR was performed using a MicroAmp Optical 96-well reaction plate and MicroAmp Optical Caps (Applied Biosystems, Foster City, USA). An ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used to perform the PCR and assess fluorescence. Each amplification reaction consisted of 1 ng of genomic DNA, 1× real-time PCR buffer, 5 mM $MgCl_2$, 83 nM of the FAM-labeled TaqMan probe, 83 nM of the VIC-labeled PLRV probe, 1.5 U of Hot Gold star DNA polymerase (Eurogentec, Belgium) and 333 nM of forward and reverse primer for each target DNA. The positive PLRV internal control was also included in each reaction volume of 30 µl. In each amplification assay, non-template DNA and noninfected banana DNA templates were run in parallel. The thermocycling profile for the PCR consisted of an initial incubation of 2 min at 50°C followed by incubation of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Standard curves were constructed using serial dilutions of pure genomic DNA of *M. fijiensis*, *M. musicola* and *M. eumusae* (10,000, 1,000, 100, 10 and 1 pg/µl). Dilution series were included in each run to calculate fungal biomass from infected plant materials.

Sequencing of ITS fragments of *R. similis* was undertaken using nematode generic primers, and LSU rDNA was amplified using one of the forward primers 28-61forward (5'-GTCGTGATTACCCGCTGAACTTA-3') or 28-81forward (5'-TTAAGCATATCA TTTAGCGGAGGAA-3') in combination with the reverse primers 28-1006reverse (5'-GTTCGATTAGTCTTTCGCCCCT-3') or 28-1032reverse (5'-TCGGAAGGAACCAGC TACTA-3'). TaqMan PCR for *R. similis* was based on the discriminative regions of the 28S rDNA. Alignment of *R. similis* data with partial LSU rDNA sequences from other members of the Tylenchomorpha resulted in the identification of a number of species-specific SNPs. In essence, quantitative detection assays were designed as described in Holterman et al. (2006).

RESULTS

The species-specific real-time PCR primers that were designed produced unique amplicons for *M. fijiensis*, *M. musicola*, and *M. eumusae*. The amplification profile of each primer-probe combination for target and non-target species showed that the combinations were specific for each of the target species. The fluorescent signal measured for non-target species was still at the base line even after 40 cycles of amplification. In contrast, fluorescence measured for target species became apparent after 22 cycles. TaqMan real-time PCR assay was sensitive enough to detect as little as 1 pg of DNA

from each of the target species. The standard curves for each of these *Mycosphaerella* species were generated by plotting Ct values obtained from software against the log of known amounts of serially diluted DNA. A linear relationship was observed with a R^2 value of 0.99.

The reproducibility of the real-time assay was confirmed by considering the three sources of variation that could affect the outcome. Both intra- and inter-assay variation had minimal effects on reproducibility. Inter-sample variation could affect reproducibility in real-time assays, which can be compensated, to some extent, by optimal sample selection.

Each primer-probe set successfully detected target species from artificially inoculated banana leaves. The species-specific conventional primer pairs amplified unique DNA fragments from each of the target species. No cross-reaction was observed with DNA from non-target species. Each PCR reaction was performed with a species-specific primer set in combination with a β-tubulin primer set, to check whether a fungus is responsible for leaf spot. The primer pairs for *M. fijiensis*, *M. musicola* and *M. eumusae* were also validated using naturally infected banana leaves with the target species showing bands of the expected size. Under these conditions, the sensitivity of the *M. fijiensis* primers was 100 pg of genomic DNA, whereas sensitivity levels for *M. musicola* and *M. eumusae* were 1 and 10 pg of genomic DNA, respectively.

DISCUSSION

The morphology and life cycle of *Mycosphaerella* species that constitute the Mycosphaerella leaf spot complex of banana are rather similar (Crous and Mourichon, 2002; Jones, 2000, 2002). Their distribution and the epidemiology of the diseases they cause are uncertain. In Southeast Asia, the centre of origin of the pathogens and hosts, the three species co-exist, while in many other banana-producing areas, *M. fijiensis* has replaced *M. musicola* and continues to colonise new ecological niches (Jones, 2002). In the Caribbean, *M. fijiensis* has been reported from Trinidad (Fortune et al., 2005), and its presence in Grenada has recently been confirmed (Carlier and Arzanlou, 2007, unpublished data). Knowledge of the identities of the species causing Mycosphaerella leaf spot diseases, especially where more than one may exist together, and their distribution in banana-producing areas on the one hand, and on host-pathogen interactions on the other hand, should enhance the ability of pathologists to understand the dynamics of these pathogens and enable the diseases they cause to be successfully managed. The quantitative and qualitative PCR methods we describe here could facilitate these objectives.

Several primer sets have been developed for ribosomal DNA of *R. similis*. Sequence divergence in the internal transcribed spacer regions led to the description of two new nematode species, *R. duriophilus* from durian (Nguyen et al., 2003) and *R. arabocoffeae* from coffee (Trinh et al., 2004). We used the sequences of the D2 and D3 regions in the 28S gene to identify diagnostic nucleotides to design primers and probes that distinguish *R. similis* from *M. incognita*, *M. chitwoodi* and *P. penetrans*. TaqMan PCR using DNA from healthy and infested roots collected in a banana plantation in Costa Rica, showed good fluorescence signals. The reproducibility of these analyses was good since replicated TaqMan PCR runs on three individual DNA extractions from each sample gave comparable results. However, the correlation between TaqMan results and conventional nematode counts from these samples was rather low. Further validation experiments are in progress to optimise these quantification methods.

The primary aim of the work was to develop a rapid and robust detection tool with a wide application. The primers for conventional PCR, which we developed and verified, differentiate the *Mycosphaerella* species constituting the Mycosphaerella leaf spot complex from each other and from other fungal species commonly occurring on banana. We consider these primer sets as valuable additions to the PCR-based detection tool developed by Johanson and Jeger (1993) that differentiated *M. musicola* from *M. fijiensis*.

TaqMan real-time PCR assays, as developed and validated in this study, facilitate

the quantification of fungal DNA even in very low amounts. Hence, TaqMan assays enable ecological and epidemiological studies, screening for fungicide resistance and efficacy of biological control agents (Hietala et al., 2003) and can be utilised to assess resistance levels in banana germplasm in support of banana resistance-breeding programs.

CONCLUSIONS

The molecular-based detection and quantification tools developed and optimised in this study are a starting point towards the better understanding of the Mycosphaerella leaf spot complex of banana. The probe and primer sets designed will facilitate ecological and epidemiological studies on *Mycosphaerella* pathogens of banana. For the burrowing nematode, *R. similis*, a TaqMan PCR was developed that discriminates between this and other nematodes frequently encountered in banana-growing regions.

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Figures

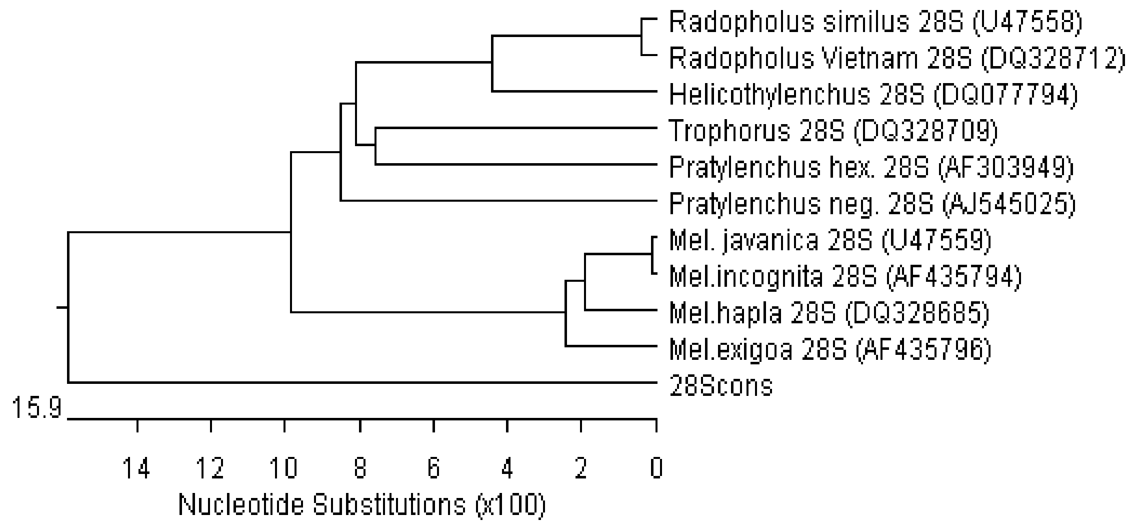

Fig. 1. Alignment of sequences of the D2 and D3 expansion regions of the ribosomal 28S gene of *R. similis* and several related nematode species (Subbotin et al., 2006) showing their dissimilarity. These results prompted us to design primers (and a probe) that proved to be *R. similis*-specific in later TaqMan PCR experiments.