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Identification of New Hosts of *Pseudocercospora fijiensis* Suggests Innovative Pest Management Programs for Black Sigatoka Disease in Banana Plantations

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Abstract: Black Sigatoka is the main constraint to banana production worldwide, and epidemic outbreaks are continuously causing huge losses. Successful management of diseases requires a profound knowledge of the epidemiological factors that influence disease dynamics. Information regarding alternative hosts of *Pseudocercospora fijiensis*, the causal agent, is still very scarce. To date, only *Heliconia psittacorum* has been reported as an alternative plant host, and we hypothesized that other plants can house *P. fijiensis*. In the present report, ten plant species with suspicious leaf spots were collected inside and around commercial banana crops in Mexico. Diagnostic PCR gave positive amplification for six of these plant species, and DNA sequencing confirmed the presence of the pathogen in four. This is the first report of the presence of *P. fijiensis* in unrelated plants and it represents a breakthrough in the current knowledge of black Sigatoka. This finding is very important given the polycyclic nature of this disease whose successful management requires the control of initial inoculum to minimize epidemic outbreaks. The results presented herein can be used to introduce innovations in integrated black Sigatoka management programs to reduce initial inoculum, and help the international initiative to reduce the use of fungicides in banana production.

Keywords: *Pseudocercospora fijiensis*; *Musa* sp.; alternative reservoir

1. Introduction

The Sigatoka disease complex of banana involves three related ascomycetous fungi, *Pseudocercospora fijiensis* (causal agent of black leaf streak disease), *Pseudocercospora musicola* (causal agent of yellow Sigatoka disease), and *Pseudocercospora eumusae* (causal agent of eumusae leaf spot disease). The three pathogens were previously called *Mycosphaerella* [1]. *Pseudocercospora fijiensis* Morelet was first detected in the Sigatoka Valley on the island of Fiji in 1963 [2]. Currently, the pathogen is present in almost all banana producing regions worldwide [3,4]. In Mexico, the first report was published in 1980, and 13 years later, it had become endemic in the Mexican banana-producing regions [5]. The first symptoms appear as small specks, which grow progressively until they become black lesions with yellow halos, eventually resulting in extensive leaf necrosis. The damage caused by *P. fijiensis* is severe and costly; the drastic leaf death results in the loss of fruit quality and productivity, with a decrease of >50% in fruit yield [6].

Control of black Sigatoka is based mainly on weekly applications of fungicides; however, these are detrimental to the environment and people, and also increase the cost of production by 40% [4,6]. Black Sigatoka management also comprises cultural practices such as excising the speckled-zones from leaves (surgery) and the removal of severely damaged leaves [7]. Despite the intensive use of fungicides and surgery/defoliation practices, outbreaks of black Sigatoka occur periodically. Epidemiological factors that influence disease dynamics in agro-ecosystems include host genetics (i.e., resistant/susceptible), virulence of the pathogen, mode of pathogen dispersion and transmission, effect of environmental conditions, and alternative hosts and reservoirs [8]. Many of these aspects have been studied in black Sigatoka and taken into consideration in control programs of this disease [6,9], but there is still insufficient information regarding alternative hosts. Alternative hosts and reservoirs play key roles in agriculture because they provide a continuous source of inoculum and, in some cases, they are required to complete the pathogen's life cycle [8,10]. Gasparotto et al. [11] demonstrated that *Heliconia psittacorum* is an alternative host of *P. fijiensis* in Manaus, Brazil. To date, this is the only report of an alternative host for this pathogen. The aim of this work is to investigate if other plants, other than *Musa* spp. and *H. psittacorum*, might be alternative hosts for *P. fijiensis*. In banana plantations, vegetative cover on the soil is commonly used to prevent soil erosion and protect roots from heat stress. These plants might represent alternative hosts for *P. fijiensis* and, thus, they can negatively impact on crop protection practices. The survey was performed in banana growing regions in Mexico, screening first for visual symptoms and then by PCR and sequencing of the PCR product. The analysis revealed four different plant species were positive for *P. fijiensis* and, thus, they can be considered alternative hosts.

2. Materials and Methods

2.1. Plant Material

Leaves from plants with specks and similar symptoms to those induced by *P. fijiensis* in banana plants were collected inside and around commercial banana crops in Tabasco and Chiapas, Mexico: *Heliconia psittacorum*, *Heliconia wagneriana*, *Pontederia sagittata*, *Symgonium podophylum*, *Alpinia purpurata*, *Wedelia trilobata*, *Xantosoma robustum*, *Commelina elegans*, *Ipomoea* sp., and *Digitaria* sp. At least 10 individual samples were collected from each plant species analyzed. If any of those plants give consistently negative results, they will be the negative control in this work. All tools used to collect the samples were surface sterilized with absolute ethanol between samplings. After harvesting, plant leaf samples were placed inside a botanical press and transported to the laboratory in Merida, Yucatan. Alternative host plants were identified by the experienced taxonomist Paulino Simá Polanco (Botanical Garden of the Natural Resources Unit, Scientific Research Center of Yucatan). Before DNA extraction, leaves were washed with a solution of commercial bleach (10% *v*/*v*), then with sterile distilled water, and dried with sterile paper towels.

2.2. DNA Extraction and PCR

Genomic DNA (gDNA) was extracted using CTAB protocol [12] from collected plant material. The fungal DNA for positive control was isolated from *P. fijiensis* mycelium according to Conde-Ferráez et al. [13]. The DNA concentration and purity were determined by spectrophotometry from the 230/260 nm and 260/280 nm ratios, using a nanodrop 2000 c spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA integrity was examined on 1.0% agarose gel electrophoresis.

Molecular diagnosis of *P. fijiensis* was performed using the species-specific primer set MFactF: 5′-CTCATGAAGATCTTGGCTGAG-3 and ACTR 5′-GCAATGATCTTGACCTTCAT-3′ for actin fragment, according Arzanlou et al. [14]. Amplicons have an expected size of 500 bp. For PCR reactions the mixture contained 1× PCR buffer, 1 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 μM each primer, 1 ng of gDNA, and ultrapure water for a final volume of 15 μL. PCR amplification conditions were 5 min of denaturation at 95 °C, followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, and an extension step of 72 °C for 10 min; PCR reactions were carried out in a Thermocycler T-100 (BioRad, Hercules, CA, USA). The PCR products were analyzed by electrophoresis on 1.0% agarose gel containing ethidium bromide, and visualized and photographed using an UV transilluminator (Gel Doc system, BioRad).

PCR products were purified from gels (Qiaquick Gel Extraction Kit, Qiagen, Hilden, Germany), cloned into pGEM T-Easy vector (Promega), and used to transform *E. coli* Top 10 cells (ThermoFisher). White colonies were selected (up to 10 colonies from each plant) and the presence of the insert was confirmed by colony-PCR with the primers reported above. Colonies were cultured again and plasmids were isolated (Qiaprep Spin Miniprep Kit, Qiagen), and sent to Macrogen (Korea) for Sanger sequencing. The nucleotide sequences were used to query against GenBank database using BLASTn (website http://ncbi.nlm.nih.gov/BLAST) and against *Pseudocercospora* (*Mycosphaerella*) *fijiensis* v2.0 genome portal (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Mycfi2). The criterion for positive identification of *P. fijiensis* was an identity ≥98% [15].

Both DNA strands were sequenced in each clone. Sequences obtained with ACTR reverse primer were transformed in reverse complement (to convert them in plus strands) using the online tool, Bio-Web Resources for Molecular and Cell Biologists (http://www.cellbiol.com/cgi-bin/complement/rev_comp.cgi). The sequences of the PCR products, including primer regions, were aligned with the equivalent regions of reference sequences (XM_007925807.1 and MYCFIDRAFT_194801). Aligned was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) with default parameters. Primers were highlighted in the alignment.

When the query retrieved actin genes from other fungi (i.e., *Colletotrichum kahawae*, *Cladosporium herbarum*, *Aspergillus oryzae*), the primers were deleted from the query sequences to eliminate possible mismatches in these regions introduced artificially, since those sequences in PCR products are always the primer sequences, although in nature these regions in the hits were not 100% identical with the primers. Then, correct percentage of homology was recalculated in a second Blastn, performed with the shorter query. Homology was reported according to data from the second Blastn.

The primer set used here was reported as a diagnostic tool to identify *P. fijiensis* by PCR. To understand why these primers amplified false positives (fungi different from *P. fijiensis*), the first hit in the Blastn for each identification was downloaded from the GenBank, and aligned with the reference MYCFIDRAFT_194801 with Clustal Omega, as described above. The target regions for forward and reverse primers were localized, and matches and mismatches in the primer regions were highlighted in bold.

3. Results

3.1. PCR Diagnosis of Pseudocercospora Fijiensis in Plants with Leaf Spots, Collected Inside and Around Commercial Banana Plantations

There are several species of plants commonly coexisting with the crop in banana plantations. Ten species of plants showing similar symptoms to black Sigatoka were sampled in this survey, within and around banana plantations in Tabasco and Chiapas, Mexico. Most of the collected plants are used as vegetative mulch, but there are also ornamental plants (*Heliconia psittacorum*, *H. Wagneriana* and *Alpinia purpurata*) which are planted there by the banana growers.

Six of the 10 plant species studied gave positive amplification with the reported species-specific primer set MFactF and ACTR. Figure 1A shows the plants, which gave positive amplification in the diagnostic PCR reaction. Amplicons were consistent in size with the expected 500 bp diagnostic band (Figure 1B). Plants that gave negative or unexpected amplification were not included in further analyses, except *Commelina elegans*, which was used as negative control. *C. elegans* plants were collected in the plantations where the other plants were collected. None of these samples was positive in the PCR test. A representative result is shown in Figure S1, Supplementary materials.

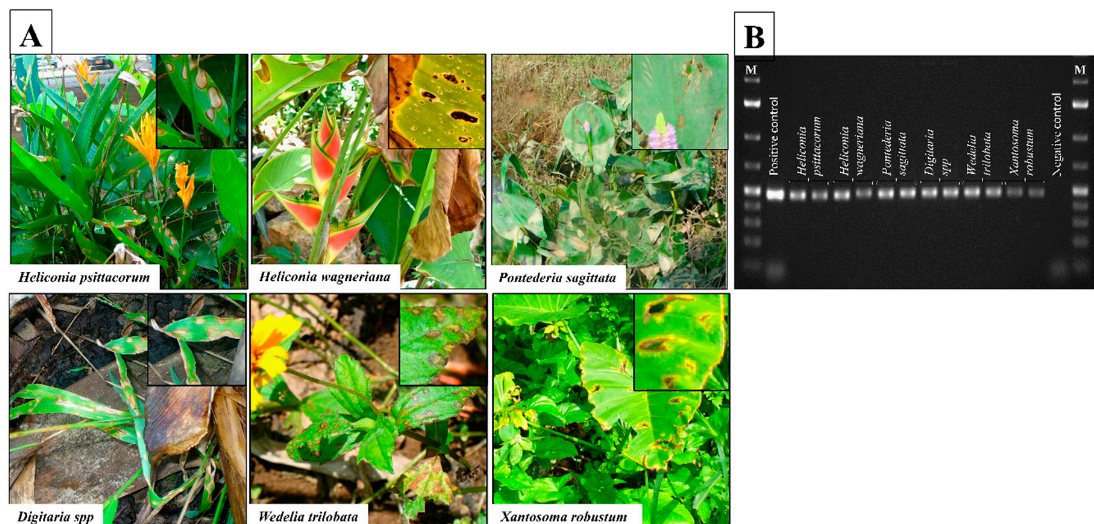

Figure 1. Potential alternative hosts of *P. fijiensis*. (**A**) Plants collected inside and around commercial banana crops, with symptoms similar to those induced by *P. fijiensis* in banana plants (leaf spots). Pictures in the boxes, close ups of symptoms. (**B**) PCR-based diagnostic of *P. fijiensis*. Those amplifying the expected diagnostic DNA product, a 500 bp fragment of actin gene, are presented. Positive control, gDNA of *P. fijiensis*; negative control, no DNA but water. M) Molecular marker (1 kb plus Invitrogen).

3.2. New Alternative Hosts of P. fijiensis: Confirmation by Specific PCR Amplification, DNA Sequencing and Bioinformatics

Positive diagnostic PCR products were cloned and sequenced to confirm the molecular identification of *P. fijiensis.* Each sequence was used as query to search in the Nucleotide Collection database from the National Center for Biotechnology (nr/nt), by Blastn tool.

For *Heliconia psittacorum*, *Wedelia trilobata*, *Xantosoma robustum* and *Digitaria* sp. first hits were gb|XM_007925807.1|, corresponding to "*Pseudocercospora fijiensis* CIRAD86 hypothetical protein partial mRNA", with 99% identity. Alignment of the sequences of PCR products with gb|XM_007925807.1| showed a 50 bp gap (not shown), because of an intron, since diagnostic PCR products were amplified on gDNA templates, and XM_007925807 sequence is mRNA. The full genomic sequence of this hit corresponds to Locus tag MYCFIDRAFT_194801 (1552 bp), and automatic annotation of molecular function for this gene in Pfam is PF00022.10: actin.

The sequences of PCR products amplified in samples of *H. psittacorum*, *W. trilobata*, *X. robustum* and *Digitaria* sp. were identical to each other; therefore, one of them was used as query for further analyses.

Alignment of partial sequence of MYCFIDRAFT_194801 (target regions for primers plus the flanking region), and one representative sequence of the diagnostic products from *H. psittacorum*, *W. trilobata*, *X. robustum* and *Digitaria* sp. show a single different nucleotide between the query and the reference sequences (Figure 2). Codon ATC in the actin gene of *P. fijiensis* strain CIRAD86 changed to ATT in the diagnostic PCR products from this study (highlighted in yellow in Figure 2). The "T" is in the reverse primer and therefore, all diagnostic products showed "T" instead of "C". Both codons codify for isoleucine. Thus, there is no actual change of amino acid in the protein. This difference is a silent single nucleotide polymorphism (SNP) in actin gene between strain CIRAD86 (available in the GenBank and the *Pseudocercospora* (*Mycosphaerella*) *fijiensis* genome portal) and the strain used by Arzanlou et al. [14] when they designed the diagnostic primers.

```
Diagnostic_PCR_product      CTCATGAAGATCTTGGCTGAGCGCGGTTACACTTTCTCCACCACCGCCGAGCGTGAAATT
MYCFIDRAFT_194801           CTCATGAAGATCTTGGCTGAGCGCGGTTACACTTTCTCCACCACCGCCGAGCGTGAAATT
                            ************************************************************
ACTF primer             5´- CTCATGAAGATCTTGGCTGAG-3´

Diagnostic_PCR_product      GTCCGCGACATCAAGGAGAAGCTCTGCTACGTTGCCCTTGACTTCGAGCAGGAGATCCAG
MYCFIDRAFT_194801           GTCCGCGACATCAAGGAGAAGCTCTGCTACGTTGCCCTTGACTTCGAGCAGGAGATCCAG
                            ************************************************************

Diagnostic_PCR_product      ACCGCCAGCCAGAGCTCCACCCTCGAGAAGTCCTACGAGCTTCCTGACGGCCAGGTCATC
MYCFIDRAFT_194801           ACCGCCAGCCAGAGCTCCACCCTCGAGAAGTCCTACGAGCTTCCTGACGGCCAGGTCATC
                            ************************************************************

Diagnostic_PCR_product      ACCATTGGCAACGAGCGTTTCCGTGCCCCAGAGGCCCTCTTCCAGCCATCCGTCCTTGGT
MYCFIDRAFT_194801           ACCATTGGCAACGAGCGTTTCCGTGCCCCAGAGGCCCTCTTCCAGCCATCCGTCCTTGGT
                            ************************************************************

Diagnostic_PCR_product      CTCGAATCTGGTGGTATTCACGTCACTACCTTCAACTCCATCATGAAGTGTGACGTCGAC
MYCFIDRAFT_194801           CTCGAATCTGGTGGTATTCACGTCACTACCTTCAACTCCATCATGAAGTGTGACGTCGAC
                            ************************************************************

Diagnostic_PCR_product      GTCCGTAAGGATCTCTACGGCAACATCGTCATGGTATGTATTGCGTATCCCACCACATGG
MYCFIDRAFT_194801           GTCCGTAAGGATCTCTACGGCAACATCGTCATGGTATGTATTGCGTATCCCACCACATGG
                            ************************************************************

Diagnostic_PCR_product      GTAATTAGCTGACACTTCTCCAGTCTGGTGGTACTACCATGTATCCAGGTATCTCCGACC
MYCFIDRAFT_194801           GTAATTAGCTGACACTTCTCCAGTCTGGTGGTACTACCATGTATCCAGGTATCTCCGACC
                            ************************************************************

Diagnostic_PCR_product      GTATGCAGAAGGAAATCACCGCGTTGGCCCCATCCAGCATGAAGGTCAAGATCATTGC
MYCFIDRAFT_194801           GTATGCAGAAGGAAATCACCGCGTTGGCCCCATCCAGCATGAAGGTCAAGATCATCGC
                            ******************************************************* **
ACTR primer                                                      3´-TGAAGGTCAAGATCATTGC-5
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Figure 2. Nucleotide alignment of partial sequences of the actin gene of *P. fijiensis*. Diagnostic PCR product is a representative sequence of PCR products amplified on DNA templates from leaf spot on *Heliconia psittacorum*, *Wedelia trilobata*, *Xantosoma robustum* and *Digitaria* sp. plants collected inside and around commercial banana plantations. MYCFIDRAFT_194801 corresponds to the genomic sequence of XM_007925807.1 (mRNA), the first hit in the GenBank retrieved with the query. One Single Nucleotide is observed (in bold red), which is present in the reverse primer. Highlighted in yellow: the different codons between our sequences and the reference sequence. Both codons, ATT and ATC, codify for isoleucine.

Why were our queries unable to retrieve "*Pseudocercospora (Mycosphaerella) fijiensis* actin gene" in the Blast, instead of "*Pseudocercospora fijiensis* CIRAD86 hypothetical protein" from the GenBank? A manual search for "*Pseudocercospora fijiensis* actin" in the nucleotide database retrieved seven hits for "*Pseudocercospora fijiensis* actin (act) gene, partial CDS", but they corresponded to 5′ end of the actin gene, and the diagnostic PCR products were amplified downstream on the gen. Therefore, the queries do not overlap those sequences, which explain why it was not possible to retrieve them.

In summary, taking all together, sequencing and bioinformatics confirm a positive diagnostic for *P. fijiensis* in *H. psittacorum*, *W. trilobata*, *X*, *robustum* and *Digitaria* sp. (Table 1).

Table 1. Identification of fungi in plant species with leaf spots similar to black Sigatoka symptoms. Plants were collected inside and around banana crops in Tabasco and Chiapas, México.

Plant Species	Family	Total Samples	Positive by PCR Test (%)	Pathogen Identification by Sequencing
Heliconia psittacorum	Heliconiaceae	21	47	*Pseudocercospora fijiensis*
Heliconia wagneriana	Heliconiaceae	10	50	*Colletotrichum kahawae*, *Cladosporium herbarum*, *Aspergillus oryzae*
Pontederia sagittata	Araceae	19	63	*Colletotrichum kahawae*
Digitaria spp	Poaceae	11	27	*Pseudocercospora fijiensis*
Wedelia trilobata	Asteracea	10	50	*Pseudocercospora fijiensis*
Xantosoma robustum	Araceae	19	63	*Pseudocercospora fijiensis*

3.3. Discovery of False Positives in the Diagnostic Polymerase Chain Reaction (PCR) of P. fijiensis

Sequencing did not confirm *P. fijiensis* for *Heliconia wagneriana and Pontederia sagittata* which previously gave positive in the diagnostic PCR test. We observed that regions where the primers target on the retrieved subject show differences with the queries. Since 5′ and 3′ ends of the PCR products always correspond to primer sequences, the ends of PCR products from non-*P. fijiensis* templates could contain incorrect nucleotides, introduced by the primers. Thus, the queries were edited by deleting sequences of primers, and those shorter queries were used again for Blastn. First hits in the second Blastn were consistent with the previous, but retrieved sequences matched 100% with their respective queries. *Cladosporium herbarum* (AJ300320.1), *Aspergillus oryzae* (AP007164.1) and *Colletotrichum kahawae* (KU579251.1) were molecularly identified (Table 1).

Finding false positives in the PCR with the primers used here was unexpected. MFactF and ACTR are species-specific primers for *P. fijiensis* and were designed after alignment of the actin gene of 17 *Mycosphaerella* spp. found in banana leaves, and these primers are able to distinguish *P. fijiensis* from closely related fungi such as *P.* (previously *Mycosphaerella*) *musicola* [14].

To understand why the diagnostic primers are amplifying the actin gene from those fungi, their sequences were downloaded from the GenBank. The target sequences of the primers were manually identified in each subject and the flanking region together with the primer targets were in silico extracted; they were aligned with the homologous reference sequence from *P. fijiensis* and the diagnostic primers (Figure 3). In the region corresponding to the forward primer, a few substitutions can be observed; each template has from one to three mismatches with the forward primer, but the 3′ end of this primer matches perfectly with the templates, enabling the primer to hybridize on them. The reverse primer has only one or no mismatch.

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Colletotrichum_kahawae                          CTCATGAAGATTCTGGCTGAGCGCGGTTACTCCTTCTCCACCACTGCCGAGCGTGAAATC
Aspergillus_oryzae                              CTCATGAAGATCCTGGCTGAGCGCGGTTACACTTTCTCCACCACCGCTGAGCGTGAAATT
Cladosporium_herbarum                           CTCATGAAGATCCTCGCCGAGCGCGGTTACACCTTCTCCACCACCGCCGAGCGTGAAATC
Mycosphaerella_fijiensis_reference_sequence     CTCATGAAGATCTTGGCTGAGCGCGGTTACACTTTCTCCACCACCGCCGAGCGTGAAATT
                                                ***********  * **  ********** * *********** ** ***********
ACTF_Primer                                  5´-CTCATGAAGATCTTGGCTGAG-3´

Colletotrichum_kahawae                          GTTCGTGACATCAAGGAGAAGCTCTGCTACGTCGCTCTCGACTTCGAGCAGGAGCTCCAG
Aspergillus_oryzae                              GTCCGTGACATCAAGGAGAAGCTTTGCTACGTCGCCCTCGACTTCGAGCAGGAGATTCAG
Cladosporium_herbarum                           GTTCGCGACATCAAGGAGAAGCTCTGCTACGTCGCCCTCGACTTCGAGCAGGAGATCCAG
Mycosphaerella_fijiensis_reference_sequence     GTCCGCGACATCAAGGAGAAGCTCTGCTACGTTGCCCTTGACTTCGAGCAGGAGATCCAG
                                                ** ** ***************** ******** ** ** *************** * ***

Colletotrichum_kahawae                          ACCGCGTCTCAGAGCTCCAGCTTGGAGAAGTCCTACGAGCTTCCCGACGGTCAGGTCATC
Aspergillus_oryzae                              ACCGCTTCTCAGAGCTCCAGCCTCGAGAAGTCCTATGAGCTTCCTGATGGCCAGGTCATC
Cladosporium_herbarum                           ACCGCCAGCCAGAGCTCTTCCCTCGAGAAGTCCTACGAGCTTCCCGACGGTCAGGTCATC
Mycosphaerella_fijiensis_reference_sequence     ACCGCCAGCCAGAGCTCCACCCTCGAGAAGTCCTACGAGCTTCCTGACGGCCAGGTCATC
                                                *****    ********    * * *********** ******** ** ** *********

Colletotrichum_kahawae                          ACCATCGGCAACGAGCGTTTCCGTGCTCCTGAGGCTCTGTTCGCTCCTTCCGTCCTTGGT
Aspergillus_oryzae                              ACCATCGGTAACGAGCGTTTCCGTGCTCCTGAGGCTCTCTTCCAGCCTAGCGTTCTGGGT
Cladosporium_herbarum                           ACCATCGGCAACGAGCGTTTCCGTGCCCCCGAGGCCCTCTTCCAGCCCTCCGTCCTTGGT
Mycosphaerella_fijiensis_reference_sequence     ACCATTGGCAACGAGCGTTTCCGTGCCCCAGAGGCCCTCTTCCAGCCATCCGTCCTTGGT
                                                ***** ** **************** ** ***** ** ***    **    *** ** ***

Colletotrichum_kahawae                          CTTGAGTCTGGTGGTATCCACGTCACCGTCTTCAACTCCATCATGAAGTCCGATGTCGAC
Aspergillus_oryzae                              CTGGAAAGCGGTGGTATCCACGTTACCACCTTCAACTCCATCATGAAGTGTGATGTTGAT
Cladosporium_herbarum                           CTCGAGTCCGGCGGAATCCACGTCACCACCTTCAACTCCATCATGAAGTGCGATGTCGAC
Mycosphaerella_fijiensis_reference_sequence     CTCGAATCTGGTGGTATTCACGTCACTACCTTCAACTCCATCATGAAGTGTGACGTCGAC
                                                ** **    ** ** ** ***** **   *******************  ** ** **

Colletotrichum_kahawae                          GTCCGTAAGGACCTGTACGGCAACATTGTCATGGTATGTTCCACGCAACACCAGTCCCC-
Aspergillus_oryzae                              GTCCGTAAGGATCTCTACGGTAACATCGTCATGGTATGTTCTCTATCTTGGGTTTTGGGT
Cladosporium_herbarum                           GTTCGCAAGGACCTCTACTCCAACATTGTCATGGTAAGATAATATTGTCTGACTTTGAAA
Mycosphaerella_fijiensis_reference_sequence     GTCCGTAAGGATCTCTACGGCAACATCGTCATGGTATGTATTGCGTATCCCACCACATGG
                                                ** ** ***** ** ***   ***** ********* *

Colletotrichum_kahawae                          ------------GAGGTCGCAAGCTAATGAAGTCTTAGTCTGGTGGTACCACCATGTACC
Aspergillus_oryzae                              CCATTCCAGAGGGTAACACATCACTAACGAATACTTAGTCTGGTGGTACTACCATGTACC
Cladosporium_herbarum                           ----------TCCCACACAAATAACTAACAAATCACAGTCTGGTGGTACCACCATGTACC
Mycosphaerella_fijiensis_reference_sequence     ----------G-----TAATTAGCTGACACTTCTCCAGTCTGGTGGTACTACCATGTATC
                                                                          *          ************* ******** *

Colletotrichum_kahawae                          CTGGTCTCTCCGACCGTATGCAGAAGGAGATCACTTCTCTTGCTCCTTCTTCCATGAAGG
Aspergillus_oryzae                              CTGGCATCTCCGATCGTATGCAGAAGGAAATCACCGCCCTTGCCCCCTCGTCCATGAAGG
Cladosporium_herbarum                           CCGGTATCAGCGACCGCATGCAGAAGGAGATCACCGCCCTGGCCCCCTCCAGCATGAAGG
Mycosphaerella_fijiensis_reference_sequence     CAGGTATCTCCGACCGTATGCAGAAGGAAATCACCGCGTTGGCCCCATCCAGCATGAAGG
                                                * **   **   *** ** *********** *****   *   * ** ** **   ********
ACTR_Primer                                                                                           3´-ATGAAGG

Colletotrichum_kahawae                          TCAAGATCATCGC
Aspergillus_oryzae                              TCAAGATCATTGC
Cladosporium_herbarum                           TCAAGATCATCGC
Mycosphaerella_fijiensis_reference_sequence     TCAAGATCATCGC
                                                ********** **
ACTR_Primer                                     TCAAGATCATTGC-5´
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Figure 3. Nucleotide alignment of actin gene (partial sequences) from different fungi, amplified with diagnostic primers for *Pseudocercospora fijiensis*, MFactF and ACTR. Identification was performed by Blastn in the Genbank and first hit was downloaded in each case: *Cladosporium herbarum* (AJ300320.1), *Aspergillus oryzae* (AP007164.1), and *Colletotrichum kahawae* (KU579251.1). In each case, the primer-free-query showed 100% identity with the first hit. In the alignment, target regions for the primers come from the Genbank sequences, and not from the PCR products, to compare with the true sequences. Sequence MYCFIDRAFT_194801 was used here as reference for *P. fijiensis*. Primer regions are highlighted in bold; mismatches in red.

4. Discussion

Black Sigatoka disease is the most important constraint to banana and plantain production worldwide. However, although its epidemiology has been studied extensively, knowledge of the alternative hosts for *P. fijiensis*, the causal agent, is still limited; only *Heliconia psittacorum* has been reported to harbor this pathogen in addition to the primary host. The current work aimed to use molecular diagnosis to investigate if other plant species could be alternative hosts for the pathogen.

To our knowledge, only *Heliconia psittacorum* has been previously reported as an alternative host for *P. fijiensis* [11] and, consistent with that report, *H. psittacorum* was found positive in the present study. The heliconias are plants from a tropical rainforest environment and they grow well in humid and warm conditions. In Mexico, heliconias are commonly found as ornamental plants inside

commercial banana plantations. The high abundance of these plants in banana growing areas emphasizes the need to consider heliconias in black Sigatoka control programs.

The Araceae plant *Xanthosoma robustum* also harbors *P. fijiensis*. This plant is considered ornamental and edible, but in agriculture may become a weed; it is not as abundant as heliconias in banana plantations. However, it could be also capable of spreading *P. fijiensis* and may represent a risk for banana plants.

We were not able to identify the species of one weed, which was positive for *P. fijiensis*, but it belongs to the genus *Digitaria*. Another weed housing *P. fijiensis* was *Wedelia trilobata*. Both weeds are commonly used in Latin America as ground cover plants in banana plantations, to protect the roots and keep them humid and fresh. However, uncontrolled weeds reduce banana production and complicate agronomical practices e.g., fertilization, desuckering and the control of pest and diseases, such as Moko disease [16]. Weeds also affect negatively the safety and comfort of the workers while performing their duties.

The findings reported here may be deemed somewhat surprising given that *P. fijiensis* is reported as specialist pathogen [17,18], as with many *Mycosphaerella* species, but some of these fungi are able to jump to different hosts, including those that are unrelated [19–21]. The genus *Musa* is the primary host of *P. fijiensis;* however, in this study, the presence of this pathogen was confirmed for the first time in four unrelated plants species: *Heliconia psittacorum* (*Heliconiaceae*), *Digitaria* spp (*Poaceae*), *Wedelia trilobata* (*Asteracea*), and *Xantosoma robustum* (*Araceae*) collected within or close to banana plantations in Tabasco and Chiapas, Mexico. None of the *C. elegans* plants was positive for black Sigatoka in PCR diagnosis, although they were collected below banana plants with black Sigatoka symptoms. Then, this result supports that the PCR amplification in positive plants is not just for deposition of spores of *P. fijiensis* on the surface of those plants. In addition, *P. fijiensis* was confirmed only in four of the suspicious plant species, reinforcing that positive diagnoses are not circumstantial by epiphyllic deposition of spores.

P. fijiensis is a hemibiotrophic fungus and both classes of spores, asexual spores (conidia) and sexual spores (ascospores) are produced on banana leaves during early and late necrotrophic stages, respectively, which means that the complete life cycle occurs in the same leaf of the primary plant host [6]. Therefore, there is no evidence that alternative hosts play a role in completing the pathogen's life cycle. Meanwhile the role of alternative hosts has been extensively studied in heteroecious fungi, i.e., which require at least two different hosts to complete their life cycles [8,22], little attention has been paid to alternative hosts for pathogens which can complete their life cycle on the primary host. However, the alternative hosts also play important roles in the ecology of these pathogens. Alternative hosts can be a shelter where the pathogen finds refuge against unfavorable conditions. For example, non-banana plants are not exposed to fungicides, or receive relatively little exposure to them, thus, they represent a shelter for *P. fijiensis* in banana plantations. In addition, alternative hosts can provide a large genetic diversity of hosts, which can result in a large genetic diversity of the pathogen [8]. *P. fijiensis* has high genetic diversity (large number of alleles), and produces large numbers of spores, which means population persistence and large mutational input [23]. This evolutionary dynamic increases the risk of emergence of new virulent strains, and can result in host range expansion and even host shift [10].

Finding *P. fijiensis* in other plants is of both scientific and practical interest. Alternative host plants are not expected to be uniquely responsible for starting epidemic outbreaks of black Sigatoka in rainy seasons given that this disease is present all the time in banana plantations. However, some alternative hosts are abundant in banana growing areas and can thus contribute to maintaining density of inoculum. The landscape structure in commercial banana plantations includes a single crop in a large area, high host density, and no crop rotation. These factors favor the rapid transmission of the propagule and promote black Sigatoka epidemics. Despite efforts to change these agricultural practices in some banana producing regions, it is more difficult to implement in countries in which banana exportation mean a significant economic income. However, other practices to dilute the inoculum can be compatible with the commercial production of banana, such as identification of alternative hosts and eradication from the vicinity of the banana crop, including corridors of

alternative hosts (narrow habitats that connect two otherwise non-contiguous habitat patches) to prevent spreading the pathogen between vicinal farms. In addition, some heliconias and weeds are reservoirs of other important banana diseases and should therefore be under phytosanitary monitoring in banana growing areas.

Black Sigatoka is a polycyclic disease, i.e., the disease initiates with primary inoculum (ascospores) and secondary inoculum (conidia) drives the epidemic, with several cycles of infection per rainy season. Density of inoculum is crucial in the epidemiology of a polycyclic disease: While the severity of a monocyclic disease is directly related to the amount of inoculum at the beginning of the season; for polycyclic diseases, intensity is exponentially related to the amount of the inoculum [24]. Control of polycyclic diseases requires control of both initial inoculum and inoculum multiplication [24], and the management of alternative hosts reduces the initial inoculum as well as the rate of inoculum production [25].

The findings described herein therefore, regarding the existence of alternative hosts of *P. fijiensis* is very important for black Sigatoka management programs. Currently, many of the programs for the control of inoculum multiplication involve intensive, weekly applications of fungicides, more than 50 times a year [4,26]; however, control of initial inoculum receives less attention (e.g., detached, infected banana leaves are left on the soil to decay inside the plantation). For sustainable management programs, it is important to achieve control of initial inoculum as well.

The management of alternative hosts to maintain them away from the primary hosts produces impressive results by drastically reducing diseases caused by heteroecious fungi [8,10,22]. The role of alternative hosts and the impact on disease control should be evaluated in detail for non-heteroecious pathogens as well, as in the case of *P. fijiensis*. In addition, the identification of alternative hosts for other important non-heteroecious fungal pathogens, previously described as specialist, was recently reported [27–29]. A change of paradigm in pest management programs of these fungi is necessary, taking in consideration the eradication of alternative host species from the vicinity of the primary hosts; or to include the alternative hosts in the fumigation programs.

Some alternative hosts are providing an environmental service in the banana farm, e.g., protection of the roots from heat and drying; these plants should be replaced by other non-host coverages (e.g., *C. elegans*), or otherwise be included in the fumigation programs. Over time this will reduce the inoculum and incidence of disease, leading to an effective reduction of fungicides.

Integration of biotechnology, epidemiology, and ecology is necessary to deal with crop diseases in modern agriculture and to improve productivity and sustainability with low environmental impact. In order to meet this challenge, it is important to go back to the basics and always take into consideration how dynamic the evolution of the pathosystems and the surrounding landscapes can be, in order to direct the biotechnology towards effective cultural practices in agriculture. The sensitive and accurate molecular diagnostic of *P. fijiensis* is a powerful tool to achieve these tasks efficiently. MFactF and ACTR primers were developed as a diagnostic tool for *P. fijiensis* [14], but they can work on *Colletotrichum kahawae*, *Cladosporium herbarum*, and *Aspergillus oryzae*, generating false positives. Differences throughout the actin fragments of these fungi and *P. fijiensis*, allow us to distinguish between them. Based on the results presented here, caution is recommended in the use of MFactF and ACTR primers. They are useful for rapid screening of *P. fijiensis*, but sequencing of the PCR products is mandatory, especially in samples collected directly from the field.

Banana is among the crops with the largest use of fungicides worldwide and, currently, international programs have been established with the aim of reducing these chemicals in banana production [4,30]. Our discovery of alternative hosts of *P. fijiensis* can be used to introduce innovations in black Sigatoka management and help these programs to succeed.

5. Conclusions

Identification of alternative hosts of pathogens is very important for the protection of plant industries. This is the first time that various unrelated plants have been reported as alternative hosts for *P. fijiensis* (*Heliconia psittacorum*, *Heliconiaceae*; *Wedelia trilobata*, *Asteracea*; *Xantosoma robustum Araceae*; and *Digitaria* sp., *Poaceae*). This finding has the potential to strongly improve the current black

Sigatoka management programs. Banana is the fourth most important crop after rice, wheat and maize, and black Sigatoka is one of the agricultural diseases which require the use of more fungicides in tropical and subtropical regions. Management of *P. fijiensis*´s alternative hosts and reservoirs through fumigation and cultural practices: weeding, elimination of infected plant materials and selection of *P. fijiensis*-free ground cover plants is crucial to decreasing local density of inoculum, which will allow farmers to reduce the use of fungicides, and achieve sustainable banana production.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Typical negative PCR detection of *P. fijiensis* in *Commelina elegans* (thirteen samples) collected in same plantations that those positive plants from.

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References

1. Chang, T.-C.; Salvucci, A.; Crous, P.W.; Stergiopoulos, I. Comparative Genomics of the Sigatoka Disease Complex on Banana Suggests a Link between Parallel Evolutionary Changes in Pseudocercospora fijiensis and Pseudocercospora eumusae and Increased Virulence on the Banana Host. *PLoS Genet.* **2016**, *12*, e1005904.
2. Rhodes, P. A new Banana disease in Fiji. *Commonw. Phytopathol. News* **1964**, *10*, 38–41.
3. Bennett, R.; Arneson, P. Black Sigatoka. *Plant Health Instr.* **2003**, *10*, doi:10.1094/PHI-I-2003-0905-01.
4. Friesen, T.L. Combating the Sigatoka Disease Complex on Banana. *PLoS Genet.* **2016**, *12*, e1006234.
5. Orozco-Santos, M.; Orozco-Romero, J. La sigatoka negra en bananos y plátanos: El caso de México. XVI Reunión Internacional ACORBAT. 2004; pp. 70–77. Available online: http://www.musalit.org/viewPdf.php?file=IN050657_spa.pdf&id=9611 (accessed on 28 June 2019).
6. Churchill, A.C.L. Mycosphaerella fijiensis, the black leaf streak pathogen of banana: Progress towards understanding pathogen biology and detection, disease development, and the challenges of control. *Mol. Plant Pathol.* **2010**, *12*, 307–328.
7. Etebu, E.; Young-Harry, W. Control of black Sigatoka disease: Challenges and prospects. Review. *Afr. J. Agric. Res.* **2011**, *6*, 508–514.
8. Plantegenest, M.; Le May, C.; Fabre, F. Landscape epidemiology of plant diseases. *J. R. Soc. Interface* **2007**, *4*, 963–972.
9. Henderson, J.; Pattemore, J.A.; Porchun, S.C.; Hayden, H.L.; Van Brunschot, S.; Grice, K.R.E.; Peterson, R.A.; Thomas-Hall, S.R.; Aitken, E. Black Sigatoka disease: New technologies to strengthen eradication strategies in Australia. *Australas. Plant Pathol.* **2006**, *35*, 181–193.
10. Morris, C.E.; Bardin, M.; Kinkel, L.L.; Moury, B.; Nicot, P.C.; Sands, D.C. Expanding the Paradigms of Plant Pathogen Life History and Evolution of Parasitic Fitness beyond Agricultural Boundaries. *PLoS Pathog.* **2009**, *5*. e1000693.
11. Gasparotto, L.; Pereira, J.C.R.; Urben, A.F.; Hanada, R.E.; Pereira, M. Heliconia psittacorum: Hospedeira de Mycosphaerella fijiensis, agente causal da sigatoka-negra da bananeira. *Fitopatol. Bras.* **2005**, *30*, 423–425.
12. Murray, M.; Thompson, W. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4326.
13. Conde-Ferráez, L.; Grijalva-Arango, R.; James, A. A rapid DNA extraction method from mycelium which is suitable for PCR. *Rev. Latinoam. Microbiol.* **2008**, *50*, 86–88.
14. Arzanlou, M.; Abeln, E.C.A.; Kema, G.H.J.; Waalwijk, C.; Carlier, J.; De Vries, I.; Guzmán, M.; Crous, P.W. Molecular Diagnostics for the Sigatoka Disease Complex of Banana. *Phytopathology* **2007**, *97*, 1112–1118.

15. Li, W.; Li, H.; Wu, H.; Chen, X.-Y. Use of chromosome walking in discovery of single-nucleotide polymorphism in noncoding regions of a candidateactin gene inPinus radiata. *J. Appl. Genet.* **2010**, *51*, 275–281.
16. Prieto-Romo, J.; Morales-Osorio, J.; Salazar-Yepes, M. Identification of new hosts for Ralstonia solanacearum (Smith) race 2 from Colombia. *Rev. Prot. Veg.* **2012**, *27*, 151–161.
17. Arzanlou, M.; Groenewald, J.; Fullerton, R.; Abeln, E.; Carlier, J.; Zapater, M.-F.; Buddenhagen, I.; Viljoen, A.; Crous, P. Multiple gene genealogies and phenotypic characters differentiate several novel species of Mycosphaerella and related anamorphs on banana. *Persoonia* **2008**, *20*, 19–37.
18. Crous, P. Taxonomy and phylogeny of the genus Mycosphaerella and its anamorphs. *Fungal Divers.* **2009**, *38*, 1–24.
19. Burgess, T.; Barber, P.; Sufaati, S.; Xu, D.; Hardy, G.; Dell, B. Mycosphaerella spp. on Eucalyptus in Asia; new species, new hosts and new records. *Fungal Divers.* **2007**, *24*, 135–157.
20. Crous, P.; Groenewald, J.; Pongpanich, K.; Himaman, W.; Arzanlou, M.; Wingfield, M. Cryptic speciation and host specificity among Mycosphaerella spp. occurring on Australian Acacia species grown as exotics in the tropics. *Stud. Mycol.* **2004**, *50*, 457–469.
21. Crous, P.; Summerell, B.; Mostert, L.; Groenewald, J. Host specificity and speciation of Mycosphaerella and Teratosphaeria species associated with leaf spots of Proteaceae. *Persoonia* **2008**, *20*, 59–86.
22. Peterson, P.D.; Leonard, K.J.; Miller, J.D.; Laudon, R.J.; Sutton, T.B. Prevalence and Distribution of Common Barberry, the Alternate Host of Puccinia graminis, in Minnesota. *Plant Dis.* **2005**, *89*, 159–163.
23. Giraud, T.; Gladieux, P.; Gavrilets, S. Linking the emergence of fungal plant diseases with ecological speciation. *Trends Ecol. Evol.* **2010**, *25*, 387–395.
24. Reece, R. Inoculum control. In *Introduction to Plant Pathology*; Strange, R.N., Ed.; John Wiley & Sons, Inc, 2003; pp. 107–144.
25. Arneson, P. *Plant Disease Epidemiology: Temporal Aspects*; The Plant Health Instructor; American Phytopathological Society (APS): Saint Paul, MN, USA, 2001.
26. Aguilar-Barragan, A.; García-Torres, A.; Odriozola-Casas, O.; Macedo-Raygoza, G.; Ogura, T.; Manzo-Sánchez, G.; James, A.; Islas-Flores, I.; Beltrán-García, M. Chemical management in fungicide sensivity of *Mycosphaerella fijiensis* collected from banana fields in México. *Braz. J. Microbiol.* **2014**, *45*, 359–364.
27. Fones, H.; Gurr, S. The impact of Septoria tritici Blotch disease on wheat: An EU perspective. *Fungal Genet. Biol.* **2015**, *79*, 3–7.
28. Oeser, B.; Kind, S.; Schurack, S.; Schmutzer, T.; Tudzynski, P.; Hinsch, J. Cross-talk of the biotrophic pathogen Claviceps purpurea and its host Secale cereale. *BMC Genom.* **2017**, *18*, 273.
29. Shaw, M.W.; Emmanuel, C.J.; Emilda, D.; Terhem, R.B.; Shafia, A.; Tsamaidi, D.; Emblow, M.; Van Kan, J.A.L. Analysis of Cryptic, Systemic Botrytis Infections in Symptomless Hosts. *Front. Plant Sci.* **2016**, *7*, 625.
30. Risède, J.; Lescot, T.; Cabrera-Cabrera, J.; Guillon, M.; Tomekpe, K.; Kema, G.; Côte, F. *Challenging Short and Mid-Term Strategies to Reduce Pesticides in Bananas*; From Science to Field Banana Case Study Guide; Endure. CIRAD, France, 2010; pp. 1–8.

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