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Research Article

Identification of Novel Potential Causal Agents of *Fusarium* Wilt of *Musa* sp. AAB in Southern Mexico

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ABSTRACT

A major threat for bananas and plantains production is the Panama Disease or *Fusarium* Wilt caused by *Fusarium oxysporum* f. sp. *cubense*. In order to characterize the causal agents of *Fusarium* wilt in Mexico, a sampling was performed in symptomatic plantains growing in fields of Oaxaca, a coastal southern state of Mexico. A phylogenetic analysis based on the sequences of *TEF 1-α* and *IGS* revealed that three isolates belonged to the *Fusarium oxysporum* species complex, while two other isolates were identified as members of the *Fusarium fujikuroi* species complex. Furthermore, isolates from the same complex shared the same *ITS2* sequence. Inoculation using spores of each isolate on the roots of *Musa* sp. AAB cv. Manzano produced wilting symptoms of varying severity, suggesting that the *Fusarium* wilt might not be caused only by *Fusarium oxysporum* f. sp. *cubense*. PCR-based detection of *Secreted in Xylem* (SIX) genes showed that each *Fusarium* isolate harbored a unique combination of genes typically found in banana pathogens, which might cause the disease.

Keywords: Breeding; *Fusarium oxysporum*; *Musa* sp.; Panama disease; genetic diversity; vascular pathogens; SIX genes

Abbreviations: *Foc*; *Fusarium oxysporum* f. sp. *cubense*; FOSC; *Fusarium oxysporum* Species Complex; FFSC; *Fusarium Fujikuroi* Species Complex; TEF 1-α; Translation Elongation Factor 1-α; IGS; Nuclear Ribosomal Intergenic Spacer Region; ITS; rDNA Internal Transcribed Spacer; SIX: Secreted in Xylem.

INTRODUCTION

Bananas and plantains (*Musa* spp.) are a main staple food worldwide. Their production represents an important income source in tropical countries where cultivation of those plants is favored [1]. The tropical and subtropical climate conditions also promotes the establishment of phytopathogens such as *Fusarium oxysporum* f. sp. *cubense* (*Foc*), which is the causal agent of *Fusarium* wilt or Panama disease [2]. The Panama disease caused by *Foc* is considered the utmost factor hampering the global banana production. *Foc* is an asexual ascomycete, belongs to the *Fusarium oxysporum* species complex (FOSC) that produces macroconidia, microconidia, and the quite resistant chlamydospores, that persist in the soil for years. When such asexual spores germinate, hyphae accede into the plant via the roots. Once inside, the fungus invades xylem vessels and gets dispersed throughout the whole plant, causing yellowing and wilting in old leaves, browning in the vascular tissue and splitting of the pseudostem [3]. Based on the affected *Musa* host plant, *Foc* is classified into race 1, 2 and 4 distributed in the eight clonal lineages [3]. *Foc* race 1 (*Foc*1) causes disease in the Gros Michel subgroup (genotype AAA), and the Silk subgroup (genotype AAB). *Foc1* was responsible of the Panama disease epidemic in the 19th century, that devastated the production of bananas, but it is avirulent to the resistant AAA Cavendish cultivar, whose introduction replenished banana production [3]. *Foc* race 2 (*Foc*2) is virulent in the Bluggoe subgroup that carries genotype ABB. *Foc* race 4 (*Foc*4) is virulent in Cavendish banana plants (genotype AAA) and cause an enhanced Panama disease in the hosts of *Foc*1. Because the disease can be present in subtropical or tropical conditions, *Foc*4 is divided into subtropical race 4 (STR4) and tropical race 4 (TR4) [4]. Since *Musa* spp. are asexually propagated clones, they have reduced genetic variability, which has promoted the emergence and rapid spreading of the TR4. Panama disease is currently a serious threat for worldwide banana production as TR4 has been detected in South East Asia, Australia, Africa, the Middle East, India, Pakistan, Lebanon [5], and recently in Israel [6]. These findings illustrate how TR4 has been dispersing, thus, identification of causal agents in banana plantations should include the monitoring of TR4 as

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a necessary early step to prevent economic losses or even export bans. Furthermore, little is known about the diversity of *Foc* in Latin America. *Foc*1 isolates have been detected in diverse banana and plantains cultivars in southern Brazil [7], Minas Gerais at the Southeastern Brazil [8], and recently in Puerto Rico [9]. It is worth to mention that a new lineage within *Foc* was also identified in Goiás, central Brazil, and it is highly virulent in Latundan bananas (AAA) [10]. To our knowledge, there are no reports of TR4 in Latin America, however, further screenings in diseased plants can broaden our knowledge about the fungal biodiversity causing the Panama disease.

States in southern and southeast Mexico with a coastline on the Pacific Ocean are the major producers of bananas and plantains. More than 2000 tons of bananas and plantains are produced per year [11]. Little is known about the incidence of the causal agents of Fusarium wilt and its corresponding genetic diversity in Mexico. In this work, we present the identification of novel pathogenic fungi isolated from plantains (*Musa* sp. AAB) growing on plantations on the Pacific coast of Oaxaca, a state of the Southeastern Mexico that contributes to the overall local production of plantains. Isolates were classified based on the sequencing of the known markers *Translation Elongation Factor 1-α* (*TEF 1-α*) and the *Nuclear Ribosomal Intergenic Spacer Region* (*IGS*) [12]. Analysis of the *rDNA Internal Transcribed Spacer 2* (*ITS2*) indicated that each species complex carries at least one sequence variant of this spacer. *Foc* and related organisms were identified in this search, and its virulence towards *Musa* sp. AAB was demonstrated. The isolation of fungi that do not belong to the FOSC suggested that Fusarium wilt in plantains is not restricted to *Foc*. PCR-based amplification of *Secreted in Xylem* (*SIX*) genes in these novel isolates corresponds to the pool of *SIX* genes detected in previously reported *Foc*1 and *Foc*2 strains. As in other phytopathogenic *F. oxysporum* isolates, mechanisms of horizontal transfer of *SIX* genes might facilitate adaptations required to colonize plantains.

METHODS

Isolation of fungi

Two samples from plantations of *Musa* sp. AAB cv. Macho (Subgroup 'Plantain') and *Musa* sp. AAB cv. Manzano (subgroup Silk) were obtained at the municipality of Villa de Tututepec de Melchor Ocampo, in the region Pacific coast region of Oaxaca, Mexico (16° 08′N, 97° 36′ W). Both cultivars are susceptible to Panama disease [13]. Pseudostems and leaves from plants showing symptoms of wilting were harvested. Tissue samples were washed with sterile water and cut to obtain square-shaped pieces of approximately 0.5 × 0.5 mm. Squares were subjected to a superficial asepsis with 3% sodium hypochlorite for 1 min, and then, were rinsed with sterile distilled water. They were placed in Potato Dextrose Agar (PDA) plates and incubated for 1 week at room temperature. Visible independent mycelia were subsequently transferred into fresh PDA plates until morphologically unique and independent isolates were obtained. The name and source of isolates are shown in Table 1. Isolates showing mycelium reminiscent to the *Fusarium* genus were analyzed by lactophenol-cotton blue staining and optical microscopy.

Polymerase chain reaction

Mycelia from the isolates grown in PDA were harvested, frozen in liquid nitrogen and ground with a mortar and pestle. DNA extraction was performed according to a published protocol [14] and the integrity of samples was confirmed by 1% agarose electrophoresis coupled with ethidium bromide staining and UV radiation. DNA from each isolate was diluted and used as template to amplify a fragment of *TEF 1-α* gene and the complete *IGS* region according to established PCR conditions [12] by using Recombinant *Taq* polymerase (Invitrogen). The complete *ITS* that encompasses the *ITS1*, *5.8S rDNA* and *ITS2* was also amplified [15] by using *Pfu* polymerase (Agilent). The oligonucleotides are enlisted in Table 2.

Cloning of *TEF1-α* and *IGS*

Once the PCR products of *TEF 1-α* and *IGS* were confirmed by electrophoresis, the amplicons were cloned into pGEM T-Easy (Promega) by following manufacturer's protocol. Chemically competent *E. coli* DH10B cells were transformed with the ligation reactions. Positive transformed cells were selected in LB plates supplemented with 100 µg /mL ampicillin and 40 µL of 50mg/mL X-Gal. Cells carrying the expected plasmids were grown overnight in LB medium supplemented with 100 µg/mL ampicillin. Plasmid purification was performed using the alkaline lysis method, and

Table 1: *Fusarium* sp. isolates identified in symptomatic plantains in the coast of Oaxaca.

Name	Source	Part of the Plant
M1a	*Musa* sp. AAB cv. Macho	Leaf
M5	*Musa* sp. AAB cv. Manzano	Pseudostem
M7	*Musa* sp. AAB cv. Macho	Leaf
M104	*Musa* sp. AAB cv. Manzano	Pseudostem
M108	*Musa* sp. AAB cv. Manzano	Pseudostem

Table 2: List of oligonucleotide sequences used on this study.

Template	Oligonucleotide pairs	Tm (°C)	Amplicon Size (bp)
TEF 1-α	ef1: 5'-ATGGGTAAGGARGACAAGAC-3'	53	~700
	ef2: 5'-GGARGTACCAGTSATCATGTT-3'		
IGS	iNL11: 5'-AGGCTTCGGCTTAGCGTCTTAG-3'	62	~2200
	iCNS1: 5'-TTTCGCAGTGAGGTCGGCAG-3'		
ITS	ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'	55	~540
	ITS4: 5'- TCCTCCGCTTATTGATATGC -3'		
SIX1a	SIX1F: 5'-CCCTCTCAATCCTTGGGTTT-3'	58	153
	SIX1R: 5'-TAGTGTCATTCCACGGCAAA-3'		
SIX6	SIX6F: 5'-GACMTATGACCGCTCCGTYTG-3'	58	197
	SIX6R: 5'-GGGWMGTTTTCCACGAGACAAG-3'		
SIX9	SIX9F: 5'-CTTCTCCCGAAGCTTCTCCT-3'	58	164
	SIX9R: 5'-TTGGAAGCCCAGTTGTAAGG-3'		
SIX13	SIX13F: 5'-CGATGGAGTAAATGGGGAAA-3'	58	196
	SIX13R: 5'-TTGTAAACTGTCCCGTGCTG-3'		

successful cloning was confirmed by restriction digestion with *Eco*RI.

DNA sequencing

DNA was sequenced by capillary electrophoresis at Macrogen Inc, Korea (https://dna.macrogen.com/eng/). Plasmids carrying the *TEF 1-α* and *IGS* clones were sequenced using the T7 and SP6 universal primers. Primer walking was necessary to obtain the complete sequences of *IGS*. The complete *ITS* PCR products were sequenced by using the oligonucleotides used for amplification. Elecropherograms were analyzed in Chromas Version 2.6.5, and sequences were deposited in GeneBank (https://www.ncbi.nlm.nih.gov/nucleotide/). List of the accession numbers of *TEF 1-α* and *IGS* are included in the Table 3. The *ITS* sequences are deposited in GeneBank with the accession numbers MK250065 to MK250069.

Table 3: List of sequences of *TEF* 1-α and *IGS* used to phylogenetic analysis.

ID/NRRL	Host	TEF	IGS
M1a	*Musa* sp. AAB Macho	MG018806	MG193546
M5	*Musa* sp. AAB Manzano	MG018805	MG193545
M7	*Musa* sp. AAB Macho	MG018802	MG193542
M104	*Musa* sp. AAB Manzano	MG018803	MG193543
M108	*Musa* sp. AAB Manzano	MG018804	MG193544
NRRL5883	Wheat	AF107883.1, FD_00001_EF-1a	FD_00001_rDNA-IGS
NRRL22172	Maize	AF160262.1,	FD_01185_IGS-2733
VI01096	Barley	AJ543567.1	AY250995.1
VI01087	Bread wheat	AJ543570.1	AY253668.1
NRRL13999	Sugarcane	AF160278.1	FD_01170_IGS-2733
NRRL22016	Corn	AF160289.1	FD_01160_IGS-2733
NRRL25226	Mango	AF160281	GU737449.1
NRRL47473	Mango	GU737416.1	GU737473.1
NRRL36266	Cyclamen	FJ985339.1	FJ985572.1
NRRL26033	Tomato	AF008507.1	FJ985484.1
NRRL26203	Tomato	AF008501.1	FJ985487.1
NRRL25603	*Musa* sp. AAA Cavendish	AF008487.1	FJ985480.1
NRRL25607	*Musa* sp. ABB Bluggoe	AF008489.1	FJ985469.1
NRRL25609	*Musa* sp. ABB Harare	AF008490.1	FJ985481.1
NRRL26022	*Musa* sp. ABB Pisang Awak	AF008491.1	FJ985482.1
NRRL26029	*Musa* sp. AAB Silk	AF008493.1	FJ985483.1
NRRL36114	*Musa* sp. ABB Pisang Manurung	FJ985328.1	FJ985561.1
NRRL36118	*Musa* sp. ABB Pisang Awak	FJ985330.1	FJ985563.1
NRRL31649	*Musa* sp.	FD_01770_EF-1a	FD_01770_IGS-2733
TR2	*Musa* sp. ABB Bluggoe	KC889020.1	KC869389.1
NRRL26406	Muskmelon	AF008504.1	FD_01193_EF-IGS
NRRL26960	Carnation	AF246839.1	FJ985514.1
NRRL38302	Pinus	GU170559.1	FJ985679.1
NRRL25375	Human	AY527521.1	AY527718.1

Alignment and phylogenetic analysis:

Sequences of *TEF 1-α* and *IGS* from the isolates reported here, and reference sequences retrieved form GeneBank and *Fusarium*-ID [16] (ESM1) were aligned separately by using the ClustalW algorithm. Phylogeny of concatenated alignments were subject to Maximum Likelihood (ML) method with 1000 bootstrap replicates by using PhyML 3.0 [17]. The phylogenetic tree was built in MEGA6 [18]. The Clustal W algorithm was also used to align the *ITS2* sequences of all isolates together with previously reported sequences of *F. oxysporum* NRRL22902 (U34566.1), *F. subglutinans* NRRL22034 (GQ167235.1). These reference sequences displayed 100% identity with the sequences of the isolates. The *ITS2* of *F. fujikuroi* NRRL13566 (AY249382.1) was included as reference of *ITS2* Type II [15].

In vitro propagation of plants

Micropropagation of *Musa* sp. AAB cv. Manzano plants was established by following reported procedures [19,20]. Briefly, axenic plants were produced after surface disinfection of flower buds incubated in MS medium [21] supplemented with 5mg/L Benzyl amino purine (BAP) and 5 g/L Phytagel as gelling agent. Direct organogenesis was induced after 8 weeks of incubation. The shoots were then transferred into MS medium supplemented with 0.5 mg/L BAP and Phytagel. Newly emerging shoots were excised and transferred into solid MS supplemented with 0.1 mg/L of Indole-3-acetic acid (IAA) to induce rooting. After 4 weeks, rooted plantlets were transferred into plastic containers with vermiculite rinsed with 25% MS solution without sucrose. Under these conditions, plants were grown and acclimatized until reach a 6-leaf stage, then, they are harvested and used for infection experiments. All procedures of plant tissue culture were run in a growth chamber at 26°C with a 16 h light and 8 h darkness photoperiod.

Pathogenicity assays

The experimental design was based on the aggressiveness test previously reported [10], it was performed twice. Healthy plants generated as described above were harvested, and their roots were thoroughly washed with distilled water. Every *Fusarium* isolate was grown in PDA at 28°C for 1 week. Spores were then collected with sterile distilled water and adjusted to a concentration of 10^6 spores/ml. Inoculation with every *Fusarium* isolate was performed by submerging the roots of five independent plants in the spore solutions for 30 seconds. After inoculations, plants were planted in plastic bags containing a mixture of peat:vermiculite 1:1. Symptom development and severity in the leaves was monitored and scored based on previous reported criteria [22].

Detection of *SIX* genes

DNA from fungal isolates was used as a template to amplifying *SIX* genes commonly present in *Foc* [23]. Oligonucleotides were

designed according to *SIX* gene sequences available in GeneBank (Table 2). PCR was performed by using the qARTA *Taq* polymerase following the manufacturer's recommendations. All PCR reactions started with a 5 min denaturation at 95°C step, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. After amplification, reactions were analyzed by electrophoresis in 2.5% agarose gels stained with ethidium bromide under UV illumination. Amplification of the *TEF 1-α* was used as a positive control.

RESULTS

Fusarium isolates from the coast of Oaxaca belong to two distinct species complexes

Inspection of fungal diversity coming from symptomatic plants resulted in the isolation of five independent morphotypes (Table 1). Phylogenetic analysis of the markers *TEF 1-α* and *IGS* was performed to examine the genetic diversity of such isolates (Figure 1). A ML tree revealed that isolates M1a and M5 were closely related to *Foc* NRRL25609, an isolate from *Musa* sp. ABB classified as *Foc*2. It belonged to the clonal lineage VIII and vegetative compatibility group (VCG) 01214 [24,25]. In accordance with this finding, most of the isolates recently identified in Puerto Rico also belonged to the VCG 0124 [9]. The isolate M104 was not related to the known races of *Foc*, instead, it was more related to the pathogens of tomato NRRL26033 and NRRL26203 [26]. All members of FOSC in this analysis are clustered together, including *F. foetens*, which were included as a reference of another member of FOSC. This phylogenetic analysis revealed that *F. sacchari* and *F. verticillioides* were species assigned to M7 and M108, respectively. Both are members of the *F. fujikuroi* species complex (FFSC). M7 is quite similar to NRRL31649, also isolated form *Musa* sp., and *F. sacchari* NRRL13999 isolated from sugar cane. The phylogenetic analysis of M108 reveals that *F. verticillioides* NRRL22172 is the most related species. Due to evident polymorphisms in the sequences of *ITS2*, that intervenes the 5.8S and 18S ribosomal genes, the *ITS2* sequences are grouped into two divergent types in the *Fusarium* genus. Members of FOSC primarily carry variants of the *ITS2* Type I, although some members FFSC have also copies of this Type, which might be consequence of interspecies hybridization or gene duplication events [15]. The complete *ITS* of the isolates was sequenced and aligned together with reference sequences of *ITS2* Type I and Type II. All the isolates presented here carried at least one copy of the *ITS2* Type I (Figure 2). It is worth to mention that none of the identified isolates was related to the TR4. Phylogenetic analysis presented here is supported by lactophenol cotton blue staining and optical microscopy observations, which revealed the production of characteristic structures such as chlamydospores and false heads in the isolates from FOSC. The FFSC isolate M7 produced septate mesoconidia, the isolate M108 produced chains of microconidia reminiscent of *F. verticillioides* (Supplementary Information). Taken together, morphology and phylogenetic analysis illustrate that fungi from FOSC and FFSC were found in the organs of plantains displaying symptoms of *Fusarium* wilt.

Fusarium isolates are virulent in *Musa* sp. AAB cv. Manzano

A pathogenicity assay was performed in order to confirm whether the identified *Fusarium* spp. caused symptoms related to Panama disease. Acclimatized plantains obtained by a micropropagation method were infected by submerging their roots in a spore solution.

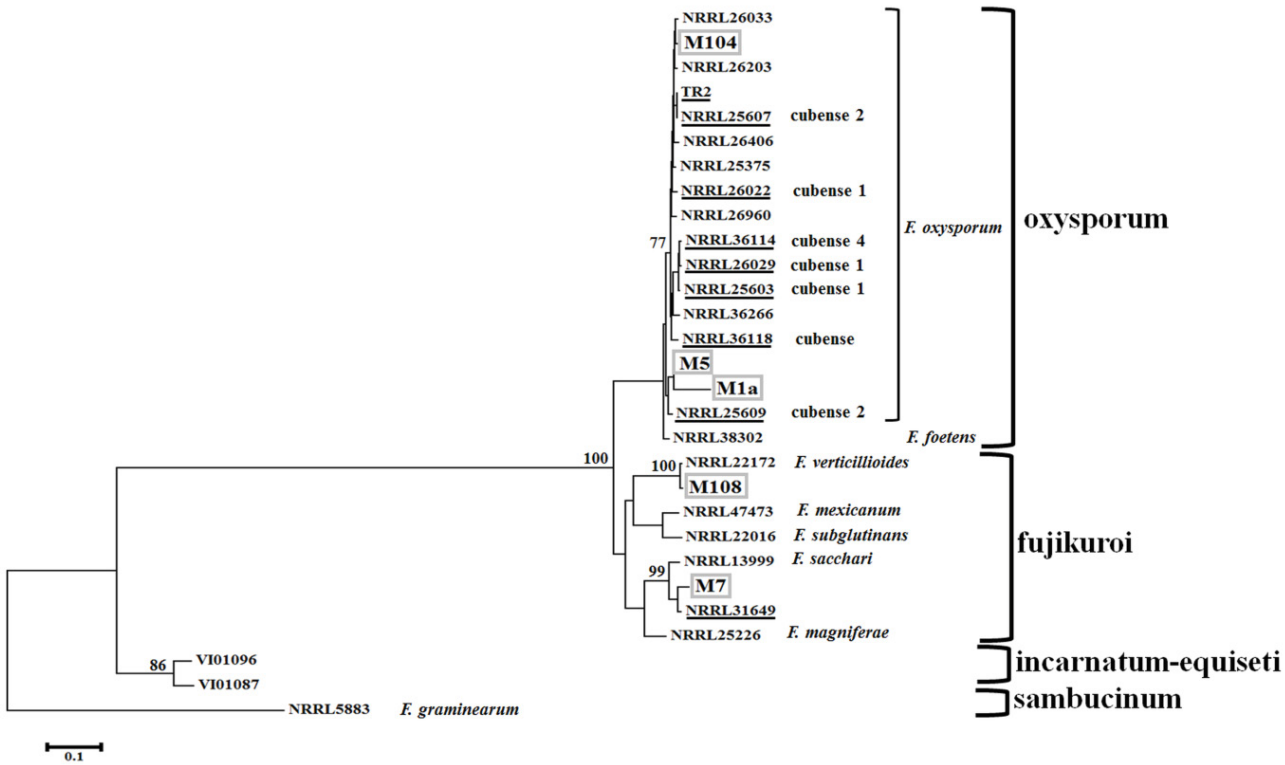
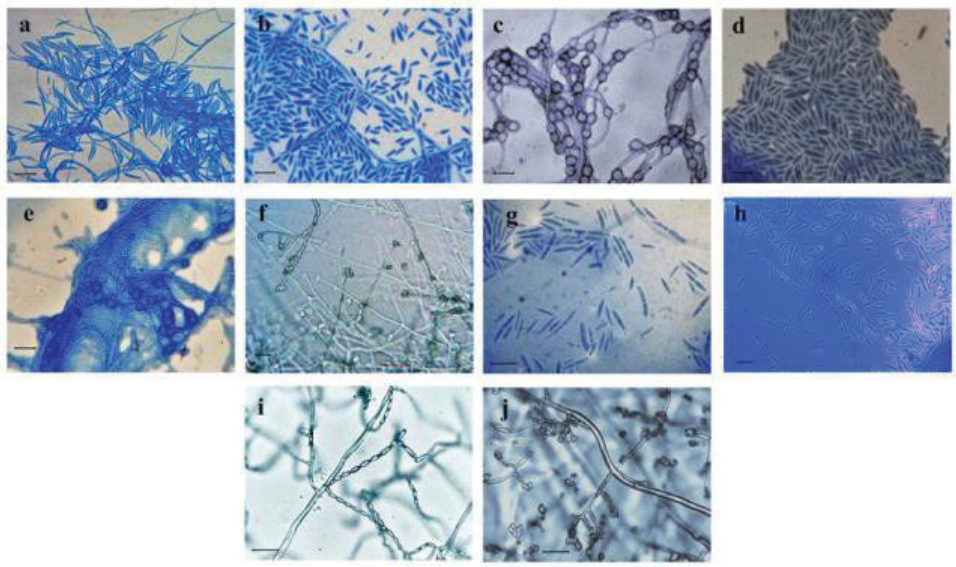


Figure 1: ML phylogenetic tree based on the sequences of *TEF1-α* and *IGS* reveals that the isolates M1a, M5, and M104 are members of the FOSC, while M7 and M108 belong to the FFSC. Banana or plantain pathogens are underlined. Reference sequences from members of other two complexes were included. Bootstrap values above 70% are based on 1000 replicates and indicated at the internodes. The three is rooted with *Fusarium graminearum* NRRL5883.



Supplementary information. Representative optical microscopy observations. Slightly falcate macroconidia generally of 3 to 5 septa with curved and conical apical cells **(a)** and microconidia **(b)** from the isolate M1a. Chlamydospores **(c)** and microconidia **(d)** form the isolate M5. Chlamydospores **(e)** and microconidia grouped in false heads **(f)** from the isolate M104. Mesoconidia with one or two septa **(g)** and microconidia **(h)** from isolate the M7.Microconidia emerging in chains **(i)** and forming small aggregates in the isolate M108 **(j)**.
a, c, e, f, h scale bar = 20 µm; **b, d, g, i, j** scale bar = 10 µm.

Figure 2: Partial alignment of *ITS2* sequences reveals the presence of *ITS2* Type I in the *Fusarium* isolates from the coast of Oaxaca. The sequences of *F. oxysporum* NRRL22092 and *Fusarium subglutinans* NRRL22034 are used as a reference for the *ITS2* Type I sequences. The sequence of *F. fujikuroi* NRRL13566 was included as a reference for *ITS2* Type II.

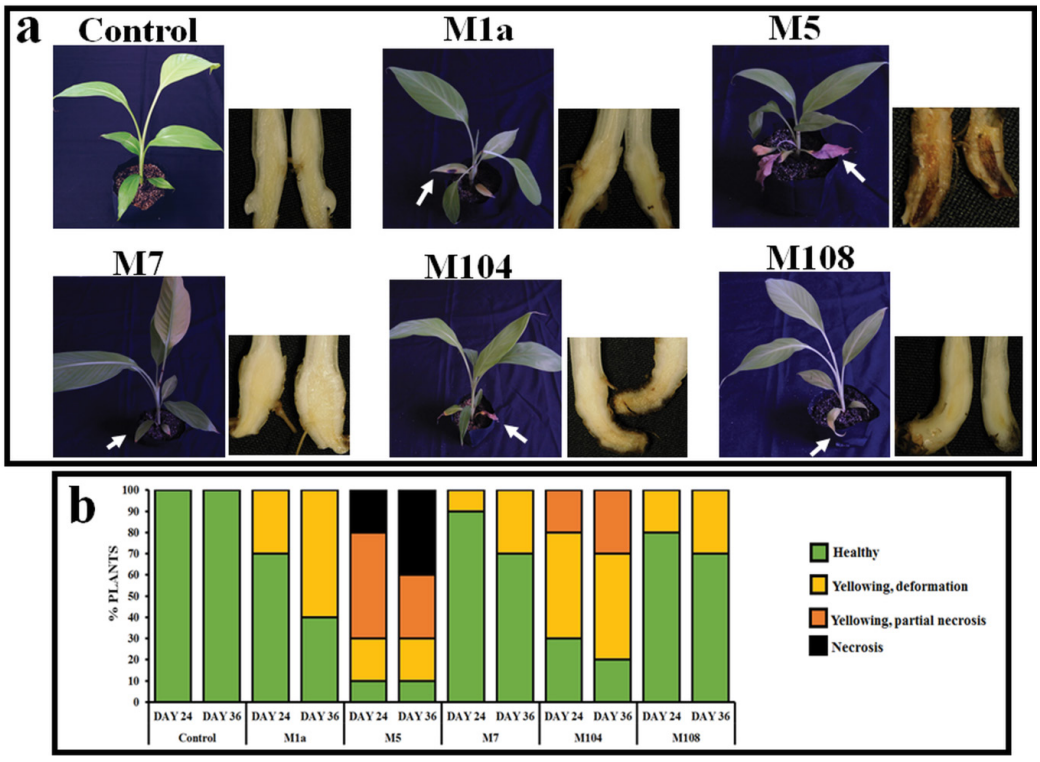


Figure 3: Plant-pathogen assays exhibited different degrees of virulence. **a)** Emergence of Fusarium wilt in leaves was detected in plants of *Musa* sp. AAB cv. Manzano 2 months after inoculation. White arrows indicate diseased tissue. A cut corm of the inoculated plant is placed at the right. **b)** Monitoring of the symptoms 24 and 36 days post inoculation reveals the severity of the disease caused by each isolates. The colour code is shown next to the graphic.

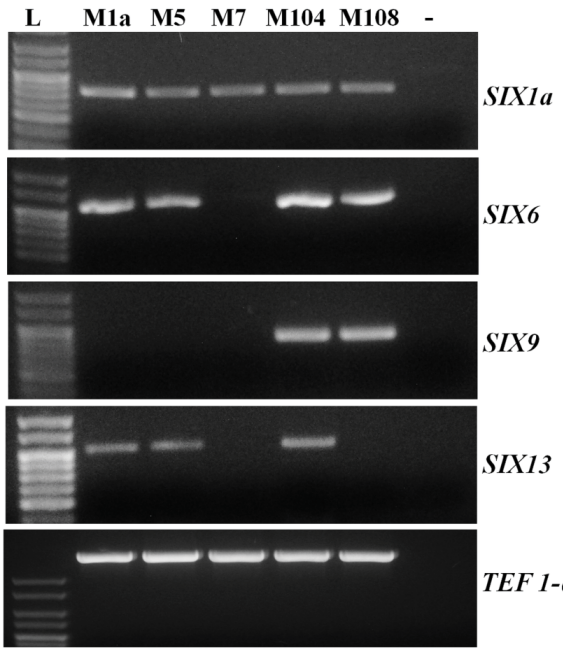


Figure 4: PCR-based detection of *SIX* genes in the *Fusarium* isolates from the coast of Oaxaca. DNA from the isolates was used as template to amplify *SIX1a*, *SIX6*, *SIX9* and *SIX13*, as well as the positive control *TEF 1-α*. The name of the isolate, DNA ladder (L), and negative control without DNA (-) are indicates at the top.

Five plants were used per each treatment, and the experiment was performed twice. Every isolate caused symptoms both in leaves and corms of inoculated plants (Figure 3a); however, the emergence of wilting was dependent on the isolate, and the M5 was one of the FOSC that produced symptoms in leaves sooner, and provoked more severe disease by the end of the experiments (Figure 3b). Yellowing and necrosis caused by isolates of FFSC were weaker, and leaf deformation was detectable.

Amplification of *SIX* genes commonly found in the causal agents of Panama disease

A critical event during colonization the xylem vessels and establishment of the *Fusarium* wilt is the secretion of small and cysteine-rich proteins known as 'Secreted in Xylem' (SIX), which were initially identified in the sap of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* (*Fol*) [22]. So far, there have been 14 *SIX* genes identified in *F. oxysporum*. Nevertheless, every *forma specialis* has a specific combination of such genes, which contributes to disease establishment across a narrow host range [27]. *SIX1a*, *SIX6*, *SIX9* and *SIX13* are often found in isolates of the three *Foc* races. Additional copies of *SIX1* (*SIX1b* and *SIX1c*), *SIX2* and *SIX8* are specific to the race 4 [23]. To get initial molecular evidences regarding the pathogenicity of these isolates, oligonucleotides were designed to amplify the known *SIX* genes present in *Foc*. The amplification products are presented in the Figure 4. The *SIX1a* amplicon was detected in all the isolates regardless the species complex to belong. The *SIX6* amplicon is detected in M1a, M5, M104 and M108. *SIX9* was detected in isolate M104 and M108, and *SIX13* was amplified in isolates M1a, M5 and M104, which were classified as *F. oxysporum*. M1a and M5 are phylogenetically related, and shared the same combination of the *SIX* amplicons. However, M5 was the most virulent in our conditions; thus, virulence did not correlate with the presence or lack of these genes. Despite the *SIX* genes are found in virulent isolates of *F. oxysporum*, we did amplify selected *SIX* genes in isolates M7 and M108, both of them belong to the FFSC. We also designed oligonucleotides to amplify *SIX2* and *SIX8*, which are typically detected in the race 4. However, there was no amplification detected in any isolate examined in this research (data not shown).

DISCUSSION

The FOSC encompasses saprophyte constituents of the rhizosphere and soil borne phytopathogenic fungi, including *Foc* [28]. The lack of sexual reproduction in *F. oxysporum* might be compensated by anastomosis which enables horizontal gene transfer (HGT) processes, that could originate over 70 *formae especiales* distributed in many clonal lineages [29]. In the case of *Foc*, 23 clonal lineages have been reported, they could emerge from events of HGT events that carried over the genetic information required to parasitize *Musa* spp.

Although there are reports of *Foc* in Latin America, we barely know about outbreaks of this pathogen on plantations in Mexico. In order to investigate the genetic diversity of the causal agents of Panama disease, we sampled symptomatic plantains on the coast of Oaxaca, Mexico. After this focused analysis, we demonstrated that the isolates M1a and M5 are two *Foc* individuals related to NRRL25609, a reference *Foc2* strain isolated from *Musa* sp. ABB. In this context, the genome B of plantains such as *Musa* sp. cv. Manzano might encode for factors that facilitate the colonization of *Foc2*. In spite of the close phylogenetic relationship between M5 and M1a, wilting symptoms produced by both isolates are contrasting, being the M5 the most virulent. Variations in the content and regulation of virulence genes might explain the difference in the symptoms caused by M1a and M5. The ML phylogeny of concatenated *TEF 1-α* and *IGS* inferred a close relationship between the virulent isolate M104 and the tomato pathogens NRRL26033, NRRL26023, that belong to a lineage unrelated to *Foc* [24]. Recently, a wide analysis of the diversity of *Foc* in Indonesia –the centre of origin of banana- demonstrated that *Fusarium* wilt is not limited to FOSC, since isolates form FFSC and other species complexes are virulent against Gros Michel and Cavendish [30]. *F. verticillioides* is a well-known pathogen of maize, but some isolates have been identified in rotten banana fruits produced in Mexico [31]. In this work, we identified isolates *F. verticillioides* M108 and *F. sacchari* M7, both of which are members of the FFSC. Leaf symptoms caused by the isolates of M108 and M7 are minimal, but it demonstrates that individuals of the FFSC are part of the diversity of *Fusarium* in plantains AAB in the coast of Oaxaca, Mexico. Their presence in symptomatic plants might be facilitated by virulent organisms such as *Foc* that suppress the plant immune system to permit the growth of weak pathogens. *Fusarium sacchari* was also recovered form symptomatic banana plants in Puerto Rico [9], but no symptoms were produced.

Whole genome sequencing of the *F. oxysporum* model strains has revealed the presence of core and lineage specific (LS) chromosomes [32,33]. LS chromosomes are also known as conditionally dispensable (CD) chromosomes or pathogenicity chromosomes, they are unique to each virulent *F. oxysporum* isolate and were acquired by HGT [34]. LS chromosomes carry genes encoding virulence factors such as the *SIX* genes, their own transcriptional regulators and the majority of transposable elements (TE) in the whole genome [33,35,36]. Each LS chromosome was likely outcome from "copy-paste" or "cut-paste" mechanisms promoted

by the TE and transposases encoding within [34,35]. After DNA rearrangements, specific combinations of *SIX* and other genes are formed, and they provide the ability to colonize the roots and suppress the plant's immune response. The natural selection of every fungus was probably facilitated when the genetic information carried on the LS chromosomes conferred an adaptive advantage. Specific combination of *SIX* genes might have a more critical role during the invasion of banana plants. For example, homologs of *SIX1* are frequently identified in *Foc* isolates, suggesting that homologs of SIX1 protein play a prominent role in causing disease, regardless the race, plant host and geographic origin [8,37]. Homologs of *SIX1* are early expressed *in planta* both in *Foc*1 and *Foc*4 [38], furthermore, the deletion of *SIX1a* compromises the virulence of TR4 towards Cavendish bananas [39]. SIX1a might target a critical pathway conserved in plants, whose disturbance is necessary to set the Fusarium wilt, since deletion of *SIX1* also attenuates the virulence of *Fol* and *F. oxysporum* f.sp. *conglutinans* [22,40]. In accordance with the reports mentioned above, PCR analyses presented here showed that all the isolates possess a copy of *SIX1a*. The *SIX6* gene is also detected in isolates from the coast of Oaxaca. Analysis if *SIX6* of *Fol* suggests a role in suppressing cell death [41] and it might have the same role in *Musa* pathogens. *SIX9* was detected in the isolates M104 and M108, and it was not detected in the isolate M5, for which inoculation displayed the more severe symptoms. The *SIX9* gene has been detected in various isolates of *Foc*1, *Foc*2 and *Foc*4 and other *formae especiales* [23]. However, its role is unknown. The phylogenetic analysis mentioned above revealed that M1a, M5 and M104 are members of FOSC, M1a and M5 are closely related, but M104 derives from a different lineage. This observation might imply that M104 acquired its virulence via an HGT mechanism in a manner unrelated that of M1a and M5. Sequencing of *SIX* genes in our isolates will be necessary to elucidate the basis of their virulence. *SIX13* is found in pathogens of tomato, cucurbits and banana [27]. In our case it was exclusively amplified in members of the FOSC.

SIX genes have been also identified in species from FFSC that infect flower bulbs, such as *F. proliferatum* and *F. agapanthi* [42]. Those *SIX* genes might be acquired by inter-species HGT from FOSC to FFSC [42]. The amplicons of *SIX* genes in the isolates M7 and M108 presented in this work are hints of HGT. This potential mechanism might have led to the selection of novel virulent strains, or at least, it might have permitted endophytic growth of rhizosphere inhabitant fungi. The potential interspecies hybridization that led the conservation of nonorthologous copies of *ITS2* suggests the possibility of the interspecies acquisition of virulence factors, that shape the high genetic diversity of virulent isolates, such as those in Indonesia [30].

CONCLUSION

Taken together, this work contributes in the identification of novel FOSC and FFSC isolates from the coast of Oaxaca that displayed differential aggressiveness towards *Musa* sp. AAB cv. Manzano. The ability to cause wilting symptoms and the potential translocation into the plant might be facilitated by the profile of *SIX* genes. Therefore, we consider the isolates identified here are candidates for Next Generation Sequencing in order to decipher the complete arsenal of virulence factors, the biogenesis and structure of potential LS chromosomes as well as their transfer among *Fusarium* populations.

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