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Morphological and molecular characterization of *Fusarium incarnatum* associated to the malformation of mango (*Mangifera indica*) in Sinaloa, Mexico

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ABSTRACT

Background/Objective. The malformation in mango is one of the main diseases to attack this crop, and therefore limits its worldwide production. The aim of this study was to identify, morphologically and molecularly, the phytopathogen associated to the malformation of mango in Sinaloa, Mexico.

Materials and methods. In 2018, samples showing malformation symptoms, such as compacted panicles and the eruption of multiple vegetative shoots, were collected from mango orchards in Sinaloa. Based on morphological characteristics, the isolates were identified as *Fusarium incarnatum*. The molecular identification was carried out by amplifying the partial sequence of the elongation factor gene (TEF-1α) and conducting a phylogenetic analysis. Pathogenicity tests were performed on mango plants through artificial inoculation.

Results. Symptoms on the inoculated plants were observed seven weeks after inoculation, with a disease severity scale rating between 2 and 4; no symptoms were observed in the control plants. The pathogen was re-isolated from symptomatic tissue and identified based on morphology, thus fulfilling Koch's postulates.

Conclusion. *Fusarium incarnatum* is repored for the first time as associated with mango malformation in Sinaloa, Mexico.

Keywords: Elongation factor, *Fusarium*, Koch's postulates

INTRODUCTION

The mango (*Mangifera indica*) is the most important fruit tree in the world's tropical and subtropical regions. Mexico is one of the main producers globally; in 2022, it was fifth in terms of surface and production, contributing 2,485,545 t (FAO, 2024), whereas Sinaloa contributed 407,831.14 t in 2022, placing itself in second place (SIAP, 2024). However, this production is affected by destructive diseases. However, this production is affected by the "mango malformation disease," "witches' broom" vegetative and floral proliferation (VFP), first reported in India in 1891 (Krishnan *et al*., 2009). This disease affects vegetative and floral shoots, with notable severity and inefficient control, occurring in most varieties and cultivated areas (Ploetz, 2007). The withered inflorescences that remain on the tree cause the disease to spread to healthy buds (Gamliel-Atinsky *et al.*, 2009).

The mango crop is affected by 83 different diseases and disorders, out of which 52 fungal and three bacterial diseases stand out, along with the fact that it displays phytoparasitic nematodes of the tree and the fruit that cause losses (Pernezny and Simone, 2000). The *Fusarium* genus described by Link (Link, 1809) belongs to the family of the Nectriaceae, which covers over 100 species, most of which are plant pathogens. Among the symptoms caused are wilting, blight and rot in different crops of economic importance and in natural ecosystems (Ma *et al*., 2013).

Several species have been described in *Fusarium* as causing diseases in mango. *F. decemcellulare* was reported as a causal agent of malformation in Venezuela and regressive death in China (Castellano and Guanipa, 2004; Qi *et al*., 2013); *F. proliferatum* causing malformation and foliar spot in China (Zhan *et al*., 2010; Omar *et al*., 2018); malformation has been reported in Pakistan associated to *F. nivale* (Khaskheli *et al*., 2008); in Senegal and Spain, caused by *F. tupiense* (Lamine *et al*., 2012; Crespo *et al*., 2016); in the Dominican Republic, associated to *F. pseudocircinatum* (García-López *et al*., 2016). In Mexico, the causal agents were reported as *F. subglutinans* (Mora, 2000), *F. mexicanum* (Otero-Colina *et al.*, 2010), *F. pseudocircinatum* (Freeman *et al*., 2014), *F. neocosmosporiellum* and *F. proliferatum* (Molina-Cárdenas *et al*., 2021; 2023). Due to the importance of the disease, it is suggested that more *Fusarium* species may cause malformation. For this reason, the aim of the present study was to morphologically and molecularly identify the phytopathogenic agent associated to the malformation of mango in Sinaloa, Mexico.

In 2018, mango trees with floral and vegetative malformations were observed in commercial orchards, and 25 tissue samples were gathered from trees with symptoms such as the swelling of buds, eruption of multiple buds ending in cluster-like apices and compacted panicles, in the municipal areas of Culiacán (6 samples) located at 24º 19' 53.6'' LN 107º 21' 59.2'' LW, Navolato (3 samples) 24° 46' 08.6" LN 107° 33' 10.0" LW, El Rosario (10 samples) 22° 54' 40.9" LN 105° 53' 45.3 "LW and Escuinapa (6 samples) 22° 53' 52.9 "LN 105° 48' 30.9" LW in Sinaloa. The samples were labeled and transported to the Phytoprotection laboratory in the School of Agronomy (UAS) to be analyzed. The pathogen was isolated by cutting small pieces of the samples with symptoms that sere superficially disinfested with 2% sodium hypochlorite for three minutes and 70% ethanol for three minutes. Subsequently, they were rinsed twice for three minutes with sterilized distilled water. The pieces were placed on sterile absorbent paper to remove excess moisture. They were planted in Petri dishes with Potato Dextrose

Agar (PDA) culture medium and incubated at 25 ºC. To obtain the monosporic cultures, the methodology proposed by Hansen and Smith (1932) was used, in which a piece of the mycelium was placed in an Eppendorf tube with 1 mL of sterile distilled water and, with serial dilutions, a spore was chosen and placed in a new dish with PDA culture medium.

For the morphological characterization, the methodology proposed by Leslie and Summerell (2006) was followed. For this, the isolates were planted in PDA at 25 ºC in the dark for 10 days, to observe the formation of aerial mycelia and the color of the colony. Subsequently, it was transferred to a clover leaf (CLO) culture medium under the conditions mentioned above. Using an optic microscope, the length and width of the microconidia and macroconidia (n=50) were observed, along with the number of septa, shape, the layout of conidiogenic cells in monophialides and polyphialides, the presence or lack of sporodochia and chlamydospores. To measure the fungal structures (n=50), an optic microscope with a camera built into the DinoCapture version 2.0 software was used.

For the molecular characterization, two monosporic isolates (95VESIN and 121FRSIN) were chosen out of a total of eight isolates that were characterized by morphology. The mycelia of the isolates were gathered by scraping the surface of the cultures grown in PDA, previously incubated for a week at 25 ºC. One hundred milligrams of fungal mycelium from each isolate were ground in liquid nitrogen, and genomic DNA was extracted using the method described by Ausubel *et al*. (2003). The DNA quality and concentration were estimated using a Thermo Scientific NanoDropT^{TM} 1000 spectrophotometer (Fisher Scientific).

The DNA extracted from the *Fusarium* isolates was analyzed using endpoint PCR using the primers EF 1 (D) ATGGGTAAGGA(A/G)GACAAGAC, EF 2 (R)GGA(G/A)GTACCAGT(G/C)ATCATGTT (O´Donell *et al*., 1998; Geiser *et al*., 2004). The mixture of the final reaction was of 25 µL containing 100 ng of mold DNA, an equimolar mixture of dNTPs and 25 nM $MgCl_2$, 2.1 U de ADN Taq Polymerase and 40 pmol of each oligonucleotide (Bioline, TN, EE. UU.).

The products amplified by PCR (DNA of the TEF-1α gene) were purified and sequenced by Macrogen Inc. (Soul, South Korea). The sequences were used to search for similarities and verify their identity in a comparison with the sequences in the GenBank NCBI (National Center for Biotechnology Information) data base (http://www.ncbi.nlm.nih.gobierno) using the BLAST (Basic Local Alignment Search Tool) program and in BLAST in Fusarium-ID. The species were identified based on a sequence identity of 99 to 100%. For the phylogenetic analysis, the sequences of the TEF-1α gene of the isolates were aligned with sequences of references obtained from the GenBank and the phylogenetic relations between the *Fusarium* monosporic cultures were inferred based on the alignment of the gene nucleotide sequences. The tree was built with the neighbor-joining method, based on distances determined with the method by Jukes and Cantor (Erickson, 2010) using 1,000 bootstrap replicates.

The ability of eight isolates from the municipal areas of Culiacán (161VCSIN, 163VCSIN and 166VCSIN), Navolato (170FNSIN), El Rosario (39VRSIN, 121FRSIN and 134VRSIN) and Escuinapa (95VESIN) was evaluated and characterized by the morphology as *Fusarium incarnatum* to cause the malformation disease by artificial inoculations in five healthy creole mango plants per isolate, aged four months, contained in polyethylene bags with sterile soil, kept under greenhouse conditions for 12 months. The inoculum was prepared by planting the isolates in CLO under cold fluorescent white light for 14 days at 25 ºC to stimulate sporulation (Freeman *et al*., 1999). The

pathogenicity tests were performed from 2019 to 2021. The apical buds were inoculated, along with five nodes per plant, injecting 20 µL of the conidial suspension (1 x 10^6 conidia mL^{-1}) and five nodes per plant were inoculated. The control plants were inoculated with sterile distilled water. The pathogenicity assays were performed twice. Th severity of the disease was evaluated using the scale proposed by Iqbal *et al*. (2006) (Table 1) with modifications, 0 being an asymptomatic plant and 5, a plant with more severe symptoms.

Table 1. Scale of severity of the malformation disease proposed by Iqbal *et al*. (2006).

Value	Symptoms
0	No symptoms
1	Bud swelling for vegetative and floral malformations
2	Eruption of multiple shoots (vegetative) or short and thick rachis (floral)
3	Clustered shoots with shortened internodes (vegetative) or thickened peduncles (floral)
4	Small scaly leaves (vegetative) or flowers (floral)
5	Bunchy apex (vegetative) or compact deformed panicle (floral)

The isolates were morphologically characterized as *F. incarnatum* (Leslie and Summerell, 2006; Xia *et al*., 2019) and in a PDA culture medium, they displayed a white-to-brown cottonlike mycelium, which turned dark brown in time. In a CLA culture medium, the microconidia (n=50) were pyriform, with 0 -1 septum, measuring 7.4-30.9 x 3.8-8.3 µm. The macroconidia (n=50) were relatively slim, with a curve in the base and apex cells, from 3 to 5 septa, measuring 41.4-92.7 x 4.2-10.1 µm. Brown sporodochia and alternated and lone chlamydospores were observed (Figure 1).

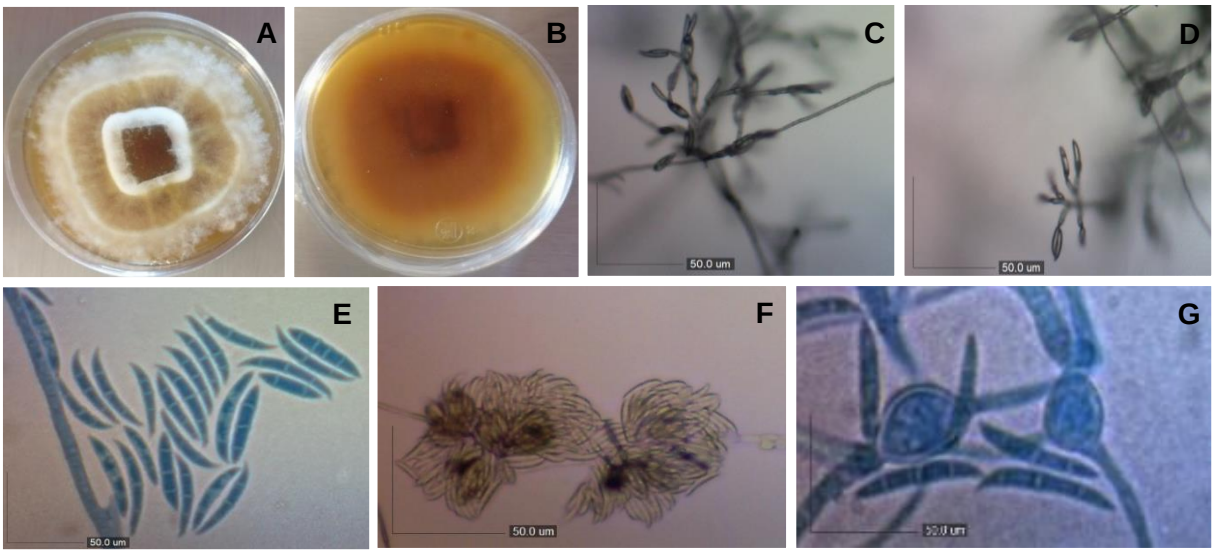

Figure 1. A-G. Morphological characteristics of *Fusarium incarnatum*. A and B) Culture in PDA; C and D) *In situ* microconidia; E) Microconidia and macroconidia; F) Brown sporodochia; G) Alternated and lone chlamidospores in a CLA culture medium. Bars= 50 µm.

The eight isolates named 39VRSIN, 95VESIN, 121FRSIN, 134VRSIN, 161VCSIN, 163VCSIN, 166VCSIN and 170FNSIN induced symptoms, with a range in the disease severity scale between 2 and 4. Symptoms such as swelling, multiple shoots, shortened internode and scale leaves were observed between seven and thirteen weeks after inoculation (Figure 2). Out of the symptoms, the fungus was isolated and identified with the same morphological characteristics described previously for *Fusarium incarnatum*, thus complying with Koch's postulates.

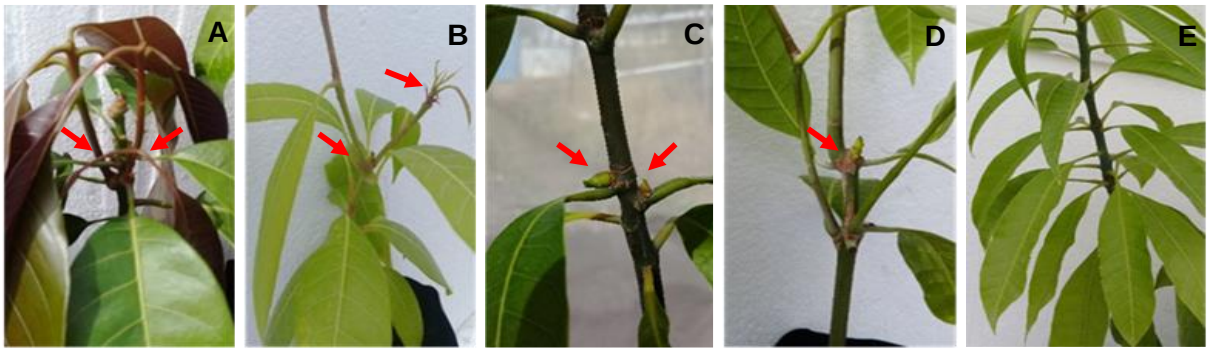

Figure 2. A–D) Symptoms of malformation in common mango plants inoculated with *Fusarium incarnatum* (95VESIN and 121FRSIN); E) Control mango plant.

F. incarnatum has been previously reported by Gai *et al*. (2016) as causing blight and wilting in the peanut plant (*Arachis hypogaea*), with symptoms observed 6 days after inoculation (Thirumalaisamy *et al*., 2018), as well as causing rot in the moringa fruit (*Moringa oleifera*) (Ekabote *et al*., 2023). In turn, a species of the FIESC complex was reported for the first time as causing the rot of the cantaloupe fruit (*Cucumis melo*) in Taiwan (Ahmen *et al*., 2023).

The lack of phytosanitary regulations in the movement of vegetative material from mango nurseries to different regions within a country could contribute to the dissemination of this pathogen (Zheng and Ploetz, 2002). In addition, it is a fungus that can survive in the ground, since it forms resistance structures called chlamidospores. In the absence of the host and when it has a host plant and under favorable conditions, infection can begin on the roots or aerial parts of the plants, being favored through the water or air (Ma *et al*., 2013).

The sequences of isolations (95VESIN and 121FRSIN) were deposited in the GenBank data base with accession numbers MK932800 and MK932804, respectively. The analysis of the sequences in the GenBank and Fusarium-ID data bases showed that the sequences were aligned to species in the *Fusarium incarnatum-equiseti* complex (FIESC). In the GenBank, both isolates displayed a homology of 99 to 100% with *Fusarium incarnatum* and *F. pernabucanum*. In the Fusarium-ID they displayed a similarity of 99.84% with the sequences (MK289625 and MK289588) recorded as *Fusarium pernambucanum*.

The phylogram obtained with the sequences under study and other sequences registered in the GenBank was divided into three groups. In the first one, the sequences recorded in this study, highlighted in bold, formed a cluster aligned with *F. incarnatum*

reported from other countries, such as China and India. The remaining two groups represent outgroups (Figure 3).

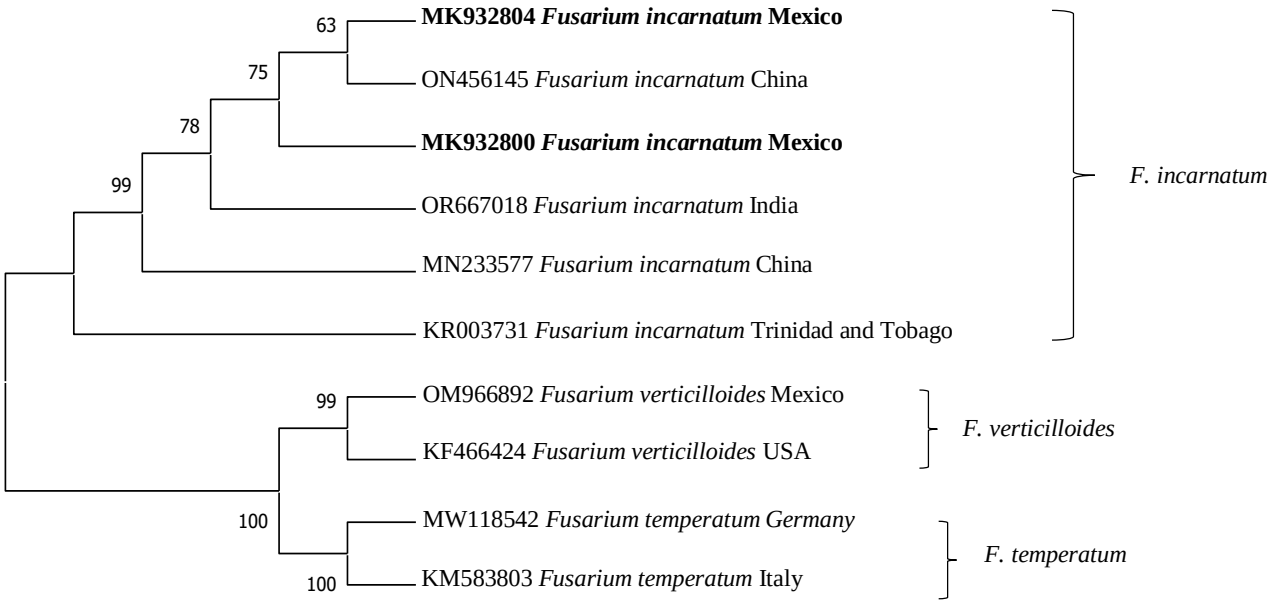

Figure 3. Phylogram inferred by Neighbor Joining for partial sequenced of the TEF-1α gene of *Fusarium* species. The values in the nodes represent bootstrap scores based on 1000 replicates. Sequences MK932800 and MK932804 originate from the present study.

Fusarium incarnatum is reported for the first time as causing malformation in mango, in Sinaloa, Mexico. Therefore, future investigations are crucial to understand the pathogen-host relation, since it is a new pathogen in a new crop, and to be able to design adequate control strategies for the disease.

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