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Revista de Ciencias Biológicas y de la Salud
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IDENTIFICATION AND CHARACTERIZATION OF *Fusarium spp*. FROM MUSKMELON IN NORTHWEST MEXICO

IDENTIFICACIÓN Y CARACTERIZACIÓN DE *Fusarium spp* EN MELÓN CULTIVADO EN EL NOROESTE DE MÉXICO

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ABSTRACT

Muskmelon *(Cucumis melo* L.) is a primary crop of Mexico. Nevertheless, the fruit has a high susceptibility to postharvest fungal diseases. *Fusarium* species are one of the main causes of diseases that limit production of muskmelon. The objective of this study was to characterize and identify by taxonomic keys and molecular markers species of *Fusarium* related to rot of muskmelon var. Reticulatus in Northwest Mexico. To identify the causative agent, fruits were collected from cultivated fields. The isolated fungi were inoculated on muskmelon to determine its pathogenicity. Morphological analyses as well as molecular techniques confirmed that the pathogen was the fungus *Fusarium proliferatum*.

Keywords: Muskmelon, *Fusarium* spp., *Fusarium proliferatum,* Molecular identification.

RESUMEN

El melón (*Cucumis melo* L.) es uno de los principales cultivos en México. Sin embargo, la fruta tiene una alta susceptibilidad a enfermedades fúngicas a nivel de poscosecha. Algunas especies de *Fusarium* son las causantes de enfermedades que limitan la producción de melón. Los objetivos de este estudio fueron caracterizar e identificar por medio de claves taxonómicas y marcadores moleculares las especies de *Fusarium* relacionadas con la pudrición de melón var. Reticulatus en el noroeste de México. Para identificar al agente causante, se colectaron frutos de campo provenientes de huertas comerciales. Los hongos aislados se inocularon en melón para determinar su patogenicidad. El análisis morfológico y las técnicas moleculares confirmaron que el patógeno fue el hongo *Fusarium proliferatum*.

Palabras clave: Melón, *Fusarium* spp ., *Fusarium proliferatum*, Identificación molecular.

INTRODUCTION

Muskmelon *(Cucumis melo* L.) is one of the most economically important fruit crops of Mexico with production close to 560,000 tons produced in approximately 22,000 ha (Vargas-Gonzalez *et al*., 2016). The Northwestern region of Mexico stands out as the most important producer of muskmelon var. Inodorus and Reticulatus (Arellano *et al*., 2017). However, despite its economic importance, production of this crop is limited because the fruit has a high susceptibility to fungal diseases, causing a reduction in postharvest life and consequently decreasing commercialization availability (Guo *et al*., 2007). The most common pathogens of muskmelon are *Alternaria cucumerina*, *A. Alternaria, Fusarium* spp., *Sphaerotheca fuliginea, Trichothecium roseum,* among others (Bi *et al*., 2006; Chew-Madinaveitia *et al*., 2008; Wang *et al*., 2010).

It has been identified that the postharvest rot caused by *Fusarium* species is one of the major diseases limiting production of muskmelon (Yuan *et al*., 2013; Mahdikhani and Davoodi, 2016). Furthermore, *Fusarium* spp. produce toxins that affect not only the quality of melons but of all food in general, as well as causing human health problems (Leslie and Summerell, 2006; Sui *et al*., 2014). *Fusarium* species are generally identified based on phenotypic characteristics (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al*., 1994), however, some species of this plant pathogen can be morphologically similar, which can confuse and make identification through traditional methods difficult (Summerell *et al.,* 2010; Aoki *et al*., 2014).

The use of molecular techniques to complement the identification of *Fusarium* spp. facilitates phylogenetic identification and improves accuracy in identifying different species (Li *et al*., 2016; Wang *et al*., 2016; Araujo *et al*., 2017). Among the most common PCR based techniques used for identification of *Fusarium* spp. are Random Amplified Polymorphic DNA (RAPD) (Singha *et al*., 2016), Restriction Fragment Length Polymorphism (RFLP) (Zarrin *et al*., 2016), Internal Transcript Spacer (ITS) (Ghaffar *et al*., 2016), Intergenic Space (IGS) (Peltomaa *et al*., 2016), Elongation Factor (TEF-1-α) (Arif *et al*., 2012), tubulin (β-Tub) (Wang *et al*., 2014), and Inter-Simple Sequence Repeat (ISSR) (Moncrief *et al*., 2016).

Timely identification of any causal agent of disease permits establishing the best management plan for crop plants and a possible improvement in selection programs

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Recibido: 08 de febrero de 2018
Aceptado: 29 de marzo de 2018

to find the best plant tolerant varieties to diseases (Cunniffe *et al*., 2015; Martinelli *et al*., 2015; Ray *et al*., 2017). Therefore, the objective of this study was to (i) characterize the aggressiveness of the various isolates of *Fusarium* spp. on fruits of muskmelon var. Reticulatus and (ii) determine their identity by molecular markers.

MATERIALS AND METHODS

Isolation of *Fusarium* spp

The fungi were isolated from rot caused by *Fusarium* spp. in fruits of muskmelon var. Reticulatus that were cultivated in a commercial orchard located in Todos Santos, Baja California Sur, Mexico at latitude: 23.45° and longitude: -110.23°. Isolates were performed from the infected part of the fruit, taking segments that were 0.5 cm^2 which were disinfected by washing with 1% (v/v) sodium hypochlorite for 3 min and then washed two times with sterile distilled water. Segments were deposited in Petri dishes with potato dextrose agar medium (PDA, at a dose of 39 g/L) mixed with 0.5% Bactrol 2 × (streptomycin at 18.5%, oxytetracycline hydrochloride at 2%). All culture dishes were incubated at 28°C for 10 days. Pure cultures were obtained from hyphal tips and each pure fungus isolated was maintained on PDA Petri dishes and slants at 4°C.

Pathogenicity test

Muskmelon var. Reticulatus fruit was selected after reaching commercial maturity and disinfected by washing with 1% (v/v) sodium hypochlorite for 3 min and washed with sterile distilled water. Four equidistant 2-mm wounds in diameter were performed in each fruit, and inoculated with 10 µL of a suspension adjusted to 1×10^6 spores/mL of each phytopathogenic fungus isolated. As control, a fruit group was wounded and inoculated only with sterile distilled water. Five fruits were used per treatment and the experiment was repeated twice. The muskmelons were deposited in sterilized plastic containers and stored at 27°C and 90% RH for 7 days to determine disease incidence (incidence (%) = Fi/Tf (100) where Fi = number of infected fruit and Tf = total fruit) and lesion size in mm. Each phytopathogenic fungus of the damaged fruit was re-isolated in Petri dishes with PDA, to confirm Koch's postulates.

Micrographs

Tissue sample of 0.5 cm^2 were collected from fruit inoculated with each phytopathogenic fungi and fixed in glutaraldehyde at 2% (v/v) for 5 min. After that, each sample was washed with sterile distilled water and partially dehydrated in an ethanol gradient (30, 50, 70, 80, 95 and 100%) for 20 min. Critical point drying was carried out with CO_2 and samples were subjected to a bath of gold coating. Samples were examined by scanning electron microscopy (SEM) (Hitachi®, S-3000N).

Morphological identification

The fungi isolates were cultured in PDA and carnation leaf agar (CLA, at a dose of 10 g/mL) at 28°C for 7 days (Schroers *et al*., 2016). The morphological characteristics of each fungus were determined using taxonomic keys described by Booth (1971), Gerlach and Nirenberg (1982) and Nelson *et al*. (1994).

Molecular identification

The fungi isolates were cultured in potato dextrose broth (PDB, at a dose of 39 g/L) at 27°C for 7 days. DNA extraction was performed following the method of Ochoa *et al*. (2007). For identification of each fungus, the region ITS1-5.8S-ITS4 of rRNA was amplified using primers ITS1 (5'-TCCGTAGGTGAACCCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). For the amplification of the tubulin (β-Tub), primers BT3 (5'-CGTCTAGAGGTACCCATACCGGCA-3') and BT5 (5'-GCTCTAGACTGCTTTCTGGCAGACC-3') (Tooley *et al*., 2001). Finally, the amplification of the elongation factor (TEF-1-α) with primers EF-1α (5'-ATGGGTAAGGAAGACAAGAC-3') and EF2 (5'-GGAAGTACCAGTGATCATGTT-3') (O'Donnell *et al*., 1998). The PCR protocol was performed as follows: mix reaction (25 µL) consisted of 1X Buffer, 1.5 mM $MgCl_2$, 1 µM of each set of primers, 0.2 mM dNTPs, 1 µL of DNA and 0.04 U μL^{-1} Platinum Taq polymerase (Invitrogen). All reactions were performed in a thermocycler (iCycler Bio-Rad, Model T100) with the following parameters: preheating 3 min at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, 30 sec alignment at 58, 52 and 55°C for ITS, EF1-α and BT, respectively; an extension for 45s at 72°C and a final extension of 72°C for 10 min. The PCR products were separated by electrophoresis in agarose gel 1% and visualized under UV trans-illuminator. All PCR products were purified with the QIAquick (Qiagen) kit and sequenced (Genewiz, New Jersey, USA). The ITS1-5.8S-ITS4, β-Tub and TEF-1-α regions of each fungus isolated were compared to sequences from type strains held in GenBank DNA database using Basic Local Alignment Search Tool BLAST.

Phylogenetic analysis

To study the evolutionary relationship between different members of the species complex *F. proliferatum,* a phylogenetic analysis using MEGA 6.0 (Tamura *et al*., 2013) was performed. ITS marker for a selection of relevant sequences was performed in the literature and obtained by a BLAST search. The method used was maximum parsimony. Clade was inferred based on 1000 bootstrap replicates. Strains of *Fusarium oxysporum* f. sp. *melonis* (AY354393.1) is representative of complex species in the genus that cause rot in melon. *Fusarium foetens* (KF467433.1) was used as the out-group.

Statistical analysis

The data were processed by one-way analysis of variance (ANOVA). Statistical data analyses were performed using the software program Statistica 6.0 (StatSoft software package, Tulsa, OK), and the post hoc least significant difference Fisher test ($p \leq .05$) was used for comparison of the means. Data for disease incidence of fruit were transformed into the arcsine square root values to normalize distribution before analysis of variance.

RESULTS

Pathogenicity of *Fusarium* spp

Five fungi were isolated from muskmelon fruit with rot and catalogued as FMP1, FMP2, FMP3, FMP4 and FMP7 (Table 1). Fruit rot was evident by the second day after inoculation with each fungus. The fruits showed color changes from dark brown, with thickening of tissue, dry fluffy exocarp was observed, to white coloration and slightly pink. At the advanced stages of rot, cracks or fissures formed on the shell with abundant white aerial mycelium. These symptoms correspond to rot caused by *Fusarium* spp. in muskmelon. In the pathogenicity test, all fungus produced 100% disease incidence. As for the lesion diameter, the muskmelons inoculated with FMP2 strain showed a greater lesion size.

Table 1. Pathogenicity test of Fusarium spp. on muskmelon var. Reticulatus.
Tabla 1. Prueba de patogenicidad de Fusarium spp. en melón var. Reticulatus.

Strain	Disease incidence	Lesion size (mm)
FMP1	100% a*	17.78 d
FMP2	100% a	27.05 a
FMP3	100% a	19.65 c
FMP4	100% a	15.14 e
FMP7	100% a	21.86 b

*Means followed by the same letter in the column do not significantly differ from each other according to according to least significant difference test ($p < .05$).

Morphological identification of *Fusarium* spp

Five days after incubation, all fungus development white mycelium that covered the entire plate and produced a violet pigment in PDA and CLA medium. The fungi produced hyaline mycelium, white color, dense and aerial, slim macroconidia with a length of 22-29 µm and a width of 4.3-6.5 µm, with a curved apical cell with 3-5 septa. Microconidia had a length of 6.78 to 8.44 µm and a width of 2.43 to 3.12 µm, without septa forming any aggregates. Chlamydospores were observed (Fig. 1). These characteristics observed in the fungi isolated confirm the identification of *Fusarium* spp.

Molecular identification and phylogenetic analysis

PCR products of approximately ≈ 600, 500 and 800 bp in size for ITS1-5.8S-ITS4, β-Tub and TEF-1-α regions were obtained, respectively. All fungi insolated (FMP1, FMP2, FMP3, FMP7 and FMP4) were identified as *F. proliferatum*. The phylogenetic tree obtained based on the sequences of *F. proliferatum* within the ITS1-5.8S-ITS4 region after alignment, showed that fungi isolated belong to the same clade (Fig. 2). In comparing these sequences with others of the same species, the strains FMP1, FMP2, FMP3, FMP7 and FMP4 form a distinct pathogenic clade to the consensus sequences, indicating that the phytopathogenic fungi isolated are species-specific.

DISCUSSION

Although *F. proliferatum* have been reported as a pathogen of maize (Nguyen *et al*., 2016), soy (Chang *et al*., 2015), garlic (Dugan *et al*., 2003), tomato (Gao *et al*., 2016), pineapple (Stępień *et al*., 2011), onion (Carrieri *et al*., 2013), among others, this is the first report of *F. proliferatum* infecting muskmelon var. Reticulatus in Northwestern Mexico. The identification of genus and species of *Fusarium* spp. was carried out through phenotypic characteristics and different molecular markers (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al*., 1994). Even though the molecular identification of fungi is based on genomic regions as the internal transcribed spacer region (ITS) (Schoch *et al*., 2012; Blaalid

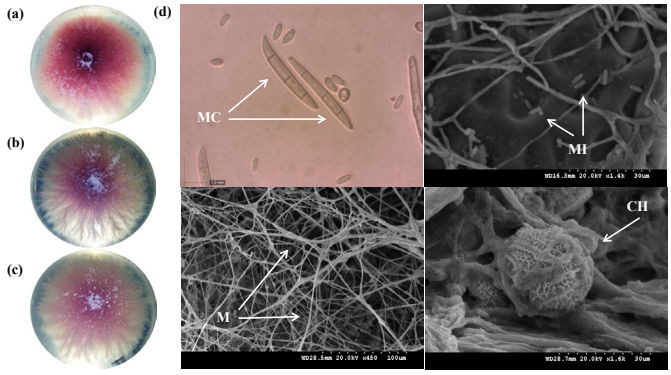

Figure 1. Identification of *Fusarium* spp. Morphological characteristics of FMP1 strain (a), FMP3 strain (b) and FMP7 strain (c) of *Fusarium* cultured in PDA at 28°C for 7 days. Microscopic characteristic of FMP1 strain of *Fusarium* (d), macroconidia [MC], microconidia [MI], mycelium [M] and chlamydospore [CH].
Figura 1. Identificación de *Fusarium* spp. Características morfológicas de la cepa FMP1(a), FMP3 (b) y FMP7 (c) de *Fusarium* cultivado en PDA a 28° C por 7 días. Característica microscópica de la cepa FMP1 de *Fusarium* (d), macroconidios [MC], microconidios [MI], micelio [M] y clamidosporas [CH].

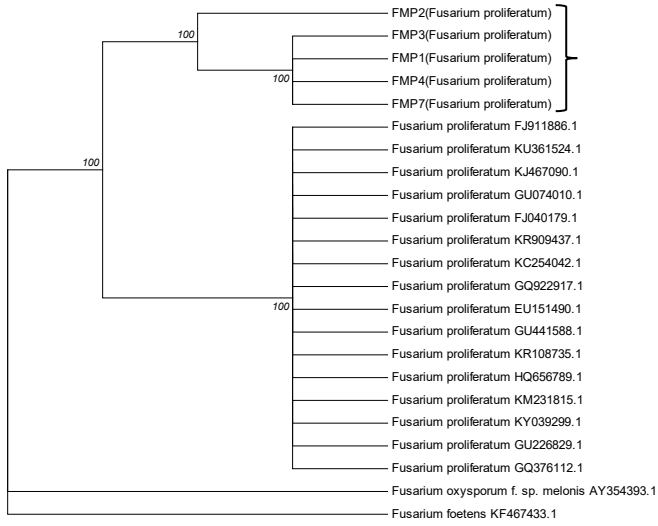

Figure 2. Maximum-parsimony phylogram of FMP1, FMP2, FMP3, FMP4 and FMP7 nucleotide fragments obtained from *Fusarium proliferatum* strains in comparison with corresponding *Fusarium* spp. sequences from the database (indicated by accession numbers). Bootstrap values (n = 1000 repetitions).
Figura 2. Filograma de máxima parsimonia de fragmentos de nucleótidos de FMP1, FMP2, FMP3, FMP4 y FMP7 obtenidos a partir de cepas de *Fusarium proliferatum* en comparación con secuencias de la base de datos de *Fusarium* spp. (indicadas por los números de acceso). Valores de Bootstrap (n = 1000 repeticiones).

et al., 2013), many species of *Fusarium* contain orthologous regions making the identification unreliable through the use of only the ITS region. Some coding regions of nuclear genes have elongation factor 1-α (TEF-1-α) and tubulin (β-Tub), that allow for identification of *Fusarium* species because they contain only a single copy in its genus and it has high polymorphism even in species relatively close genetically (Geiser *et al*., 2004; Kroon *et al*., 2004). Regions such as ITS, TEF-1-α and β- Tub, are currently more utilized for identifying the various species of *Fusarium* (O'Donnell *et al*., 2015; Stakheev *et al.,* 2016; Araújo *et al.,* 2017).

The results of pathogenicity tests showed that *F. proliferatum* is a fungus capable of infecting muskmelons on average within two days, so its quick and timely identification, will allow for better monitoring and optimization strategies for disease control (Araujo *et al*., 2017). Early and accurate detection of phytopathogenic through phenotypic taxonomy methods and PCR techniques are used routinely in plant pathology laboratories, improves the quantity and quality of food through the implementation of various strategies to control plant diseases (Sanzani *et al*., 2014). Although rot caused by *Fusarium* spp. has been controlled with synthetic fungicides, the use of biocontrol agents and plant extracts can be an efficient alternative for the control of various species of *Fusarium* (Zhao *et al*., 2013; Gopi and Thangavelu, 2014; Vargas-Gonzalez *et al*., 2016).

ACKNOWLEDGMENT

The authors are thankful and recognize CONACYT for providing financial support with SEP-CONACYT project 181972, Problemas Nacionales project 2015-01-352 and the CONACYT fellowship grant awarded to T. Rivas Garcia; and we also recognize the help provided by Ernesto Diaz for his excellent technical assistance and to M. Córdoba-Matson for editorial services. **Dr. Luis Guillermo Hernandez Montiel and Dr. Bernardo Murillo Amador are co-director of the doctoral thesis of the student Tomas Rivas Garcia.**

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