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First morphogenetic identification of *Fusarium solani* isolated from orange fruit in Egypt

Primera identificación morfogenética de *Fusarium solani* obtenido de naranjas en Egipto

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Abstract. Losses due to postharvest decay may occur at any time during postharvest handling, from harvest to consumption affecting the produce quality and quantity. Accurate identification of the pathogen causing postharvest disease is essential to the selection of an appropriate disease control approach. Nine isolates of Fusarium recovered from orange fruit were identified as *Fusarium solani*. The fungus is involved with fruit decay. The obtained cultures were purified and grown on potato-dextrose agar (PDA), malt yeast agar (MYA), and Czapek's nutrient media (CNM) under light for identification. A pathogenicity test was carried out to fulfil Koch's postulates. The pathogen could only enter ripe orange fruit through wounds and cracks causing the rot disease. The identification of the fungal isolates was confirmed to be *F. solani* by DNA sequencing, which was 99 to 100% homologous to those deposited in the GenBank. The identity of nine fungal isolates was confirmed to be *F. solani* by DNA sequencing of the internal transcribed spacer (ITS) rDNA region (GenBank Accession Nos. DQ486874 to DQ486881 and KC758879). To our knowledge, this is the first morphogenetic identification of *F. solani* isolated from orange fruit in Egypt.

Keywords: Fusarium fruit rot; Orange; Pathogenicity; ITS.

Resumen. Las pérdidas por pudrición post-cosecha pueden ocurrir en cualquier momento durante el manipuleo desde la cosecha hasta su consumo, afectando la cantidad y calidad del producto. Es necesaria una identificación exacta del patógeno causante de la enfermedad luego de la cosecha para seleccionar un enfoque de control apropiado de la enfermedad. Nueve aislados de *Fusarium*, obtenidos de naranjas, fueron identificados como *Fusarium solani*. El hongo está estrechamente asociado con la pérdida del fruto. Los cultivos obtenidos fueron purificados y crecieron en agar papa-dextrosa (PDA), agar de levadura de cerveza (MYA), y medio nutritivo Czapek (CNM) bajo condiciones de luz para la identificación del cultivo. Se realizó una prueba de patogenicidad para cumplir con los postulados de Koch. El patógeno solo podría entrar en las naranjas maduras a través de heridas y rajaduras, y causar la pudrición del fruto. Se confirmó que los aislados del hongo, por secuenciación de ADN, pertenecían a *F. solani*. Estos fueron 99 a 100% homólogos a los depositados en el Banco de Genes. Se confirmó que la identidad de los nueve aislados del hongo pertenecía a *F. solani* por secuenciación de ADN del espaciador de transcripción interno (ITS) de la región de ADNr (Registros en el Banco de Genes Nros. DQ486874 a DQ486881 y KC758879). De acuerdo a nuestro conocimiento, éste es el primer registro de *F. solani* como causante de la pudrición de naranjas en Egipto.

Palabras clave: Pudrición del fruto por Fusarium; Naranja; Patogenicidad; ITS.

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INTRODUCTION

In Egypt climate is suitable for the production of citrus fruit especially orange, which accounts for over half of the total fruit production. Export amount of the fresh and dried citrus fruit especially orange reached to 2.18% of the total export amount of the world. Orange harvested area in Egypt reached 118731 hectares in 2012 producing around 2786397 tons of orange fruits (Faostat, 2013). The economic losses due to fungal infection in fruits and vegetables during the postharvest chain are variable and not well documented. They usually reach anywhere from 30 to 50%, and on some occasions rots can lead to total loss of the product (Smilanick et al., 2006; Youssef et al., 2012). Postharvest diseases can cause serious losses of citrus fruits both in terms of quantity and quality. Some pathogens can cause decay to stored citrus fruit but their incidence is generally low (Snowdon, 1990; Youssef et al., 2010). Some of these pathogens infect produce before harvest and then remain latent until conditions become more favorable for disease development after harvest. The predominant pathogens causing the most important postharvest disease of fruits worldwide according to Poppe et al. (2003) are *Penicillium digitatum*, *Aspergillus niger* and *Fusarium* sp., respectively. Orange fruits infected with *Fusarium* rot disease have no market value (Schiffmann-Nadel et al., 1987). As mentioned before, *Fusarium* is one of the pathogenic fungi associated with rot of tropical fruits (Zakaria et al., 2012). *Fusarium* rot of tropical fruits could present a potential health risk as many *Fusarium* species are known to produce mycotoxins under suitable conditions (Abd-Elsalam, 2009). Newly recognized species are isolated in mycogeographical studies, an indication of the degree of diversity in *Fusarium* that remains to be discovered worldwide. This paper describes morphological, pathological and molecular characteristics of nine isolates of *F. solani* recovered from orange fruits showing rot symptoms. The pathogenicity of fungal isolates was also confirmed on commercial orange fruits. To date, *F. solani* had never been reported to occur as a contaminating fungus on orange fruit produced in Egypt.

MATERIALS AND METHODS

The fungal isolates used in the current study were recovered from Navel orange fruits collected from a commercial hypermarket in Egypt. Fruit surfaces were disinfected in 70% ethanol. Pieces (0.5 × 0.5 cm) of the orange fruit cuticles (flavedo and albedo) that showed symptoms were disinfected by dipping into a 10% sodium hypochlorite solution for 4 min, followed by rinsing three times with sterile distilled water. Excess water in pieces was eliminated by dabbing on sterile tissue paper. The pieces were then placed on the surface of potato dextrose agar (PDA) and incubated at 28 °C for 3 days. The fungal mat from each piece was transferred to fresh PDA medium. After 7 days, only the predominant *Fusarium* sp. produced typical lesions, which were brown, water soaked, and approximately 3 cm in diameter. The single-spore isolation technique using conidial suspension was used to obtain pure cultures. Cultures were maintained on PDA medium at 4 °C and then stored as spore suspensions in 15% glycerol at -80 °C for further investigation.

For cultural characterization, colonies of *F. solani* isolates were grown on (PDA), malt yeast agar (MYA), and Czapek's nutrient media (CNM). Identification of Fusarium isolates was based on the methods described in The Fusarium Laboratory Manual (Leslie & Summerell, 2006). Cultures were examined microscopically under low magnification (100-200X) to study morphological features of the aerial mycelia. When sporulation was observed in the cultures, agar blocks containing conidial structures were mounted on a microscopic slide with a drop of sterile water and examined at 400X.

Orange fruit cuticles (flavedo and albedo) were surface disinfected with 70% ethanol followed by three rinses with sterile distilled water. Thereafter, 0.4-cm-diameter agar plugs of the isolates were inserted into wounds made with a sterile, one cm-diameter borer to 3 mm depth. Sterile PDA plugs served as negative controls. After inoculation, fruits were placed in sealed, clear, plastic bags. The fruits were incubated at approximately 25 °C and evaluated after seven days. *Fusarium solani* was consistently re-isolated from the affected tissue, fulfilling Koch's postulates. The whole experiment was conducted twice. Mycelium was harvested from well-developed PDA cultures and DNA extracted using a SDS procedure (Abd-Elsalam et al., 2011). The primer pair ITS1/ITS4 (White et al., 1990) was used for PCR using parameters previously described by Abd-Elsalam et al. (2003). A commercial sequencing facility (Macrogen, Seoul, Korea) was used to generate *Fusarium* sequences. The construction of a Neighbor-joining (NJ) tree was based on the Jukes and Cantor model (Jukes & Cantor, 1969). It was obtained from an analysis of the rDNA-ITS sequences of 9 *F. solani* that were isolated from orange fruits.

RESULTS AND DISCUSSION

Some species of the genus *Fusarium* can cause orange fruit rot during storage (Utkhede & Mathur, 2003). Moreover, *Fusarium* is one of the most heterogeneous and difficult fungal genera to identify morphologically. The aim of this study was to identify *Fusarium* species from rotten orange fruits, based on combined morphological and pathological characteristics, and molecular data.

The resulting cultures were purified and grown on potato-dextrose agar (PDA), malt yeast agar (MYA), and Czapek's nutrient media (CNM) under light for cultural identification. Colonies on PDA were fast-growing with white, fluffy, aerial mycelia (Fig. 1); hyphae were septate and hyaline; conidiophores were unbranched; and microconidia were abundant, thin-walled, hyaline, and ovoid. Microconidia, formed on long

monophialides, were one to three-cells, "2,4-5 x 9,1-15,8 µm" long, and occurred in false heads (Fig. 2).

Initially, the lesions were water soaked. A cross section of the symptomatic fruit rind revealed a dry, brown, spongy rot with a light brown halo. Lesions finally became soft and wet, causing infected fruits to collapse. Masses of white mycelium surrounded advanced lesions. After 7 days, only the predominant *Fusarium* sp. produced typical lesions, which were brown, water soaked, and approximately 3 cm in diameter. Inoculated fruits developed brown, water soaked lesions that expanded from the initial wound site over a period of approximately 12 days after inoculation (Fig. 3). The same fungus was reisolated from each of the symptomatic fruits; control fruits remained asymptomatic and no fungus was isolated from the control fruits. Pathogenicity tests showed typical symptoms of Fusarium rot in most of the inoculated wounded orange fruits. In this respect, *F. solani*, as the dominant cause of Fusarium rot in stored orange fruits is a typical wound parasite (Schiffmann-Nadel et al., 1987).

Fig. 1. Colony morphology of *F. solani* on three cultural media. A: potato-dextrose agar (PDA); B: malt yeast agar (MYA); C: Czapek's nutrient media (CNM) at 25 °C after 5 days.
Fig. 1. Morfología de las colonias de *F. Solani* en tres medios de cultivo. A: agar de papa-dextrosa (PDA); B: agar de levadura de cerveza (MYA); C: medio nutritivo de Czapek (CNM) a 25 °C después de 5 días.

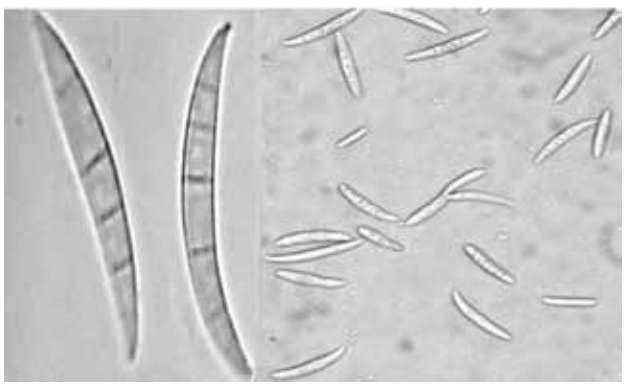

Fig. 2. *Fusarium solani* (Mart.) produces oval to kidney-shaped and generally single-cell microconidia. Macroconidia are stout and generally falcate. The apical cell is blunt and rounded, and the basal cell may be distinctly foot-shaped, notched, or rounded.
Fig. 2. .*Fusarium solani* (Mart.) produce microconidios de forma oval o de riñón, y generalmente de una célula. Los macroconidios son sólidos y generalmente curvados. La célula apical es afilada y redondeada, y la célula basal puede tener indistintamente forma de pie, dentada o redondeada.

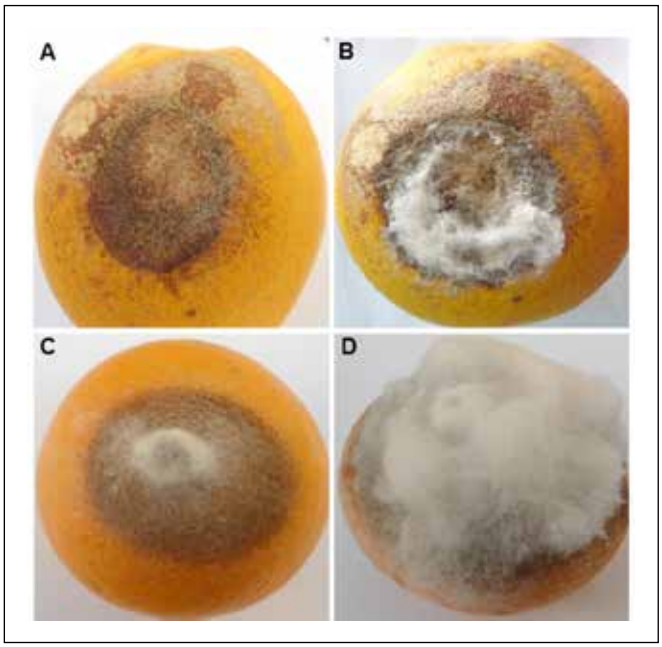

Fig. 3. Disease symptoms and pathogenicity test. A and B: Naturally infected orange fruit with *F. solani.* C: Symptoms developed on *in vitro* inoculated orange fruit. D: Severe infection after 12 days of inoculation.
Fig. 3. Síntomas de la enfermedad y prueba de patogenicidad. A y B: Naranja infectada naturalmente con *F. solani*. C: Síntomas desarrollados en naranja inoculada in vitro. D: Infección severa luego de 12 días de inoculación.

PCR amplicons of approximately 550-600 bp were obtained from both isolates and sequenced. Sequences of amplicons were identical and the sequence was submitted to GenBank (Accession No. KC758879.). The DNA sequence was 99% identical to *F. solani* isolates (DQ486874 to DQ486881). The pathogen was identified as *F. solani* based upon colony and conidial morphology (Leslie & Summerell, 2006). The identification was confirmed by comparison of ITS (internal transcribed spacer) sequences. The ITS region of rDNA was amplified by polymerase chain reaction (PCR) with primers ITS1/ITS4 and

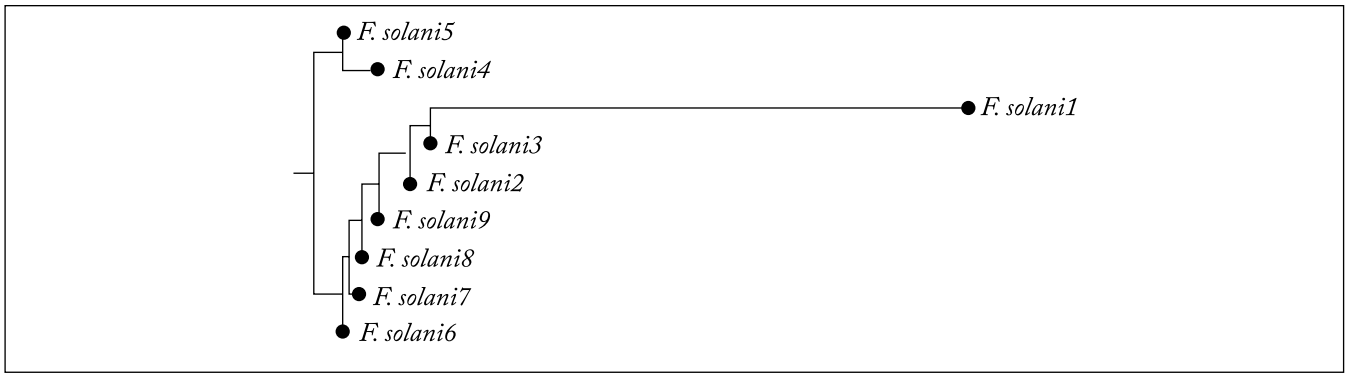

Fig. 4. Neighbor-joining tree showing the phylogenetic kinship among nine *F. solani* isolates derived from internal transcribed sequence data. Bootstrap values were 100% between individual taxa (1000 iterations).
Fig. 4. Árbol de unión de vecinos mostrando el parentesco filogenético entre los nueve aislados de *F. solani* derivados de datos de secuencia transcriptos internos. Los valores de Bootstrap fueron 100% entre taxones individuales (1000 repeticiones).

sequenced (Arruda et al., 2005). Phylogenetic analysis grouped the *F. solani* isolates into two clades (Fig. 4). Major clade comprised 77.7% of the isolates, while minor clade comprising 23.3%. BLAST analysis of the sequence obtained showed a 100% homology with several isolatesed *F. solani* in the GenBank database. Based on these morpho biometrical and cultural characteristics, the fungus was identified as *F. solani* (Demicri & Maden, 2006; Leslie & Summerell, 2006). Based on morphological characteristics, pathogenicity tests, and molecular investigations the pathogen was identified as *F. solani*. In conclusion, the presence of Fusarium infection in Egyptian citrus industry may represent a threat for long-term storage and hence exportation future.

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