Molecular identification of *Fusarium* species isolated from transgenic insect-resistant cotton plants in Mexicali valley, Baja California

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ABSTRACT. Cotton production in the Mexicali valley is adversely affected by wilt and root rot disease associated with *Fusarium* species. In the present study, we sought to isolate and identify the *Fusarium* species in the rhizosphere of transgenic insect-resistant cotton plants grown in the Mexicali valley. Our analyses isolated four native fungi from the rhizosphere of cotton plants, namely, T-ICA01, T-ICA03, T-ICA04, and T-ICA08. These fungal isolates were categorized as belonging to *Fusarium solani* using their phenotypic characteristics and ITS region sequence data. Examination of the infection index showed that T-ICA03 and T-ICA04 caused systemic colonization (90%) of seeds followed by the occurrence of radicle and coleoptile decay. In contrast, T-ICA08 strain was less pathogenic against seed tissues (40%) in comparison to the other strains isolated. Our study

showed that in transgenic insect-resistant cotton the disease “Fusarium wilt” is caused by the fungus, *F. solani*. Future studies are necessary to characterize the *F. solani* populations to determine whether phenological stages might influence the genetic diversity of the fungal populations present.

Key words: Mexicali valley; DNA; *Fusarium;* Cotton; Disease

INTRODUCTION

Mexicali valley is located in the District of Rural Development 002, which covers the municipality of Mexicali, Baja California, and the municipality of San Luis Rio Colorado, Sonora (Mendez-Trujillo et al., 2013). It covers an arable and irrigated area of 210,930 hectares (ha) of which 184,283 ha belong to Mexicali and 26,648 ha to San Luis Rio Colorado. Over 15,000 people are employed in agriculture in the Mexicali valley producing mainly cotton (Vargas-Bejarano et al., 2012). Cotton (*Gossypium hirsutum* L.) is the most economically important crop in the global textile industry and constitutes more than half of all textile fiber consumption worldwide (Karademir et al., 2011). Transgenic Bollgard (BG) cotton has been grown in Mexico since 1996; this strain contains the Cry 1Ac toxin of *Bacillus thuringiensis kurstaki* (Nava et al., 2002). In recent years, adoption of BG cotton has increased in Mexicali valley as it gives a higher yield and offers better pest control with reduced insecticide application (Terán-Vargas et al., 2005). However, seedling disease is a problem in cotton; in particular, wilt and root rot disease causes substantial losses to farmers (Hillocks, 1992; Abd-Elsalam, 2003). The fungi most commonly associated with cotton root rots and wilt diseases are *Fusarium* spp (Abo-Elyousr and El-Hendawy, 2008). These pathogens cause root rot and damping-off symptoms in cotton, and affect yield and fiber quality (Wang et al., 2009). *Fusarium* spp also cause seedling disease complex in cotton plants (Chimbekujwo, 2000). Infection of cotton plants with *Fusarium* spp results in seed rot, pre- and post-emergence damping-off, and seedling root rot that combine to cause reduced seedling vigor (Abd-Elsalam et al., 2007; Wang et al., 2009). Recent studies have shown that transgenic plants can have unintended characteristics, such as a decrease in disease resistance in comparison to conventional lines (Li et al., 2009). In Mexico, studies on the *Fusarium* species associated with transgenic insect-resistant cotton are scarce. The aim of this study was therefore to isolate the *Fusarium* species associated with transgenic insect-resistant cotton plants in the Mexicali valley; the identities of the species were established using a phylogenetic approach.

MATERIAL AND METHODS

Soil sample collection and isolation of fungi

Rhizosphere soil samples were collected from three different sites of transgenic insect-resistant cotton growing in fields of the Mexicali valley, Baja California, during August-November 2013 (Figure 1). Approximately, 100 g rhizosphere soil adhering to roots of cotton plants were placed in sterilized plastic bags and transported to the laboratory at room temperature for the isolation of microorganisms. A 10 g sample of rhizosphere soil was placed into a 250-mL Erlenmeyer flask containing 90 mL sterile distilled water and shaken (100 rpm) for 15 min. Then, serial dilutions

were made and 0.1 ml aliquots (10^3-10^5) were spread on plates containing potato dextrose agar (PDA). The PDA plates were incubated for 7 days at 30°C and morphologically different colonies that appeared on the medium were isolated and sub-cultured for further analysis.

Figure 1. Images of the sites of collection of rhizosphere soil samples from a transgenic insect-resistant cotton crop in the Mexicali valley.

Molecular identification of fungal isolates

Total DNAs from four native fungi (T-ICA01, T-ICA03, T-ICA04, and T-ICA08) were extracted using the method of Gonzalez-Mendoza et al. (2010) with minor modifications. Each DNA was amplified by PCR with Taq DNA polymerase following the manufacturer instructions (Invitrogen, Carlsbad, CA, USA). PCR was performed using 2 µL DNA (20 ng) as PCR template. The primers for the amplification were ITS 4 forward (TCCTCCGCTTATTGATATGC) and ITS 5 reverse (GGAAGTAAAAGTCGTAACAAGG). The PCR amplification was carried out using the following protocol: 94°C for 5 min (1 cycle), 54°C for 40 s and 72°C for 1 min (30 cycles).

To confirm the quality of the PCR, amplification products were run on 1% Tris acetate EDTA agarose gels and bands were visualized by staining with ethidium bromide. Images were captured and stored with the Multidoc-It Digital Imaging system (UVP). The PCR products were purified using 'Purelink® PCR Purification' kit (Invitrogen) and were sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems).

Phylogenetic analysis of fungal isolates

The four sequences obtained were then compared with other DNA sequences using the BLAST program of the National Center for Biotechnology Information (Altschul et al., 1997). Sequences with high similarity were recovered from GenBank, and a phylogenetic neighbor-joining tree that included the isolates obtained and their closest relatives was constructed using MEGA 4.0.

Pathogenicity assays in cotton seeds

Conidia of the four fungal isolates (T-ICA01, T-ICA03, T-ICA04, and T-ICA08) were produced on PDA culture plates at room temperature for 5 days. The conidia were collected by

flooding the plates with 10 mL water and dislodging the conidia using a soft brush. Conidia from each fungal isolate were collected in a beaker and the conidial suspension was adjusted to 1 x 10^6 conidia/mL. Transgenic cotton seeds (Bollgardll®) were disinfected with 5% sodium hypochlorite and rinsed thoroughly with several changes of sterile water. The seeds were then inoculated by immersion in a 50-mL suspension of conidia for 30 min. The inoculated seeds were placed on the surface of SNA plates and were incubated at 25° ± 2°C for 7 days. Disease symptoms on the seeds were classified using the 4 categories proposed by Venturini et al. (2013): 0 = no mycelia on the seed surface or well-developed root/coleoptile with negligible decay symptoms; 1= 25% seed surface covered with mycelia or root/coleoptiles showing decay symptoms; 2 = 26-50% seed surface covered with mycelia or root/coleoptile showing decay symptoms; 3 = 51-80% seed surface covered with mycelia or root/coleoptile showing decay symptoms; and 4 = 81-99% seed surface covered with mycelia or root/coleoptile showing decay symptoms. The disease levels were converted to a percentage infection index (I%) using the formula of Townsend and Heuberger (1943). The assays were carried out on three replicates groups of 10 seeds for each fungus isolated.

RESULTS AND DISCUSSION

Diseases caused by soil pathogenic fungi are a limiting factor in cotton production and are one of the main causes of reductions in the planting area in the Mexicali valley. In the present study, phylogenetic analysis using the ITS marker revealed that the T-ICA01, T-ICA03, T-ICA04, and T-ICA08 strains formed a stable clade with members of the genus *Fusarium***,** with sequence similarity of 99% for the ITS region. The neighbor-joining method was employed to construct a phylogenetic tree to illustrate the relationships between the ITS region sequences of the isolated strains and other *Fusarium* species (Figure 2). As a consequence, the 4 strains were identified as *Fusarium solani* and their sequences were deposited in GenBank with accession Nos. KJ620374 (T-ICA01), KJ620372 (T-ICA03), KJ620371 (T-ICA04), and KJ620375 (T-ICA08). Our studies showed that *Fusarium solani* was the most common of *Fusarium* species in the rhizosphere soils around transgenic insect-resistant cotton plants in the Mexicali valley. Similar results were reported by Chimbekujwo (2000) and Wang et al. (2004) who found that *F. solani* is common in soils of Nigeria and Australia after the planting of cotton.

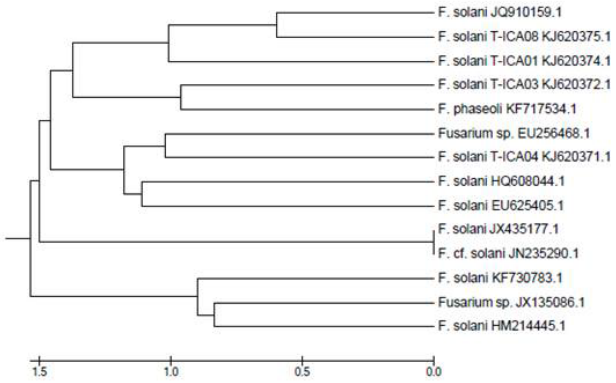

Figure 2. Phylogenetic tree based on ITS sequences, constructed using the neighbor-joining method, showing a close relationship between the four strains and the nearest relatives of the genus *Fusarium solani*. Only values greater than 90% are shown. The scale represents percentage sequence divergence.

We also showed from the I% that T-ICA03 and T-ICA04 caused systemic colonization (90%) after 2-3 days of inoculation, and was followed by the appearance of radicle/coleoptile decay (Figure 3). In contrast, the T-ICA08 strain obtained from site 2 was less pathogenic against seed tissues (40%) compared to fungal isolates from the other site. The attenuation of resistance in transgenic insect-resistant cotton to different diseases, especially to those caused by *Fusarium* species has been widely reported (Li et al., 2009). Li et al. (2013) found that root exudates from transgenic lines stimulated spore germination and mycelial growth of *Fusarium oxysporum* compared with the root exudates from their parental lines. Additionally, Yuan et al. (2002) found that the contents of sugar and amino acids in the root exudates of disease-susceptible cotton varieties were more abundant than that in disease resistant cotton. These findings suggest that root exudates might initiate and influence biological and physical interactions between the roots of transgenic cotton plants and *Fusarium* species. Finally, our study showed that in transgenic insect-resistant cotton the disease "Fusarium wilt" is caused by the fungus, *F. solani*. Future studies are necessary to evaluate the character of the *F. solani* populations in order to clarify whether phenological stage may also have an effect on the genetic diversity of the fungal populations observed.

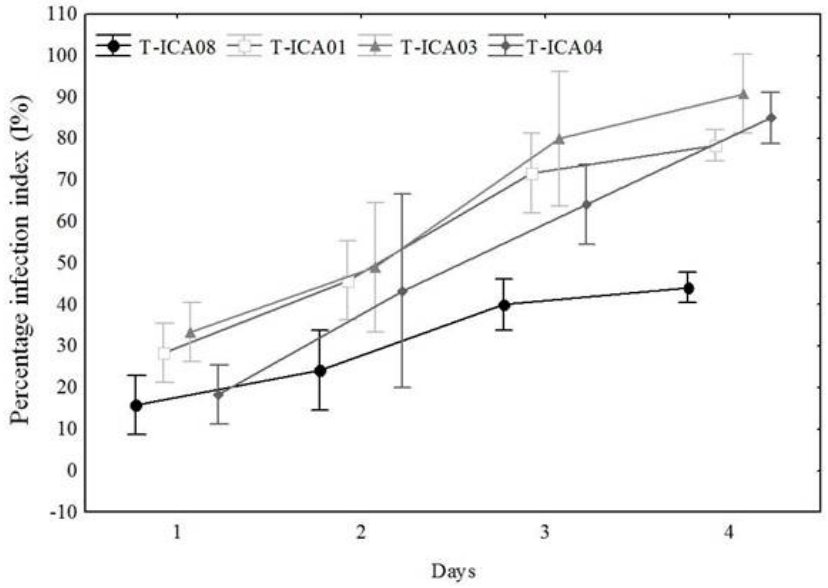

Figure 3. Percentage infection index (I%) of seeds of Bollgard transgenic cotton after inoculation with *Fusarium solani*.

Conflicts of interest

The authors declare no conflict of interest.

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