

Occurrence, Identification, and Pathogenicity of *Fusarium* spp. Associated with Tomato Wilt in Mexico

Micah R. ISAAC¹, Santos G. LEYVA-MIR²,
Jaime SAHAGÚN-CASTELLANOS¹, Kamila CÂMARA-CORREIA³,
Juan M. TOVAR-PEDRAZA², Juan E. RODRÍGUEZ-PÉREZ^{1*}

¹Universidad Autónoma Chapingo, Departamento de Fitotecnia, Km. 38.5 Carretera México-Texcoco, CP 56230 Chapingo, Estado de México, México; mickie50@hotmail.com; jsahagunc@yahoo.com.mx; erodriguezx@yahoo.com.mx (*corresponding author)

²Universidad Autónoma Chapingo, Departamento de Parasitología Agrícola, Km. 38.5 Carretera México-Texcoco, CP 56230 Chapingo, Estado de México, México; lsantos@correo.chapingo.mx jmtovar@colpos.mx

³Universidade Federal do Cariri, Centro de Ciências Agrárias e da Biodiversidade 63133-610 Crato, Ceará, Brazil; kamila.correia@ufca.edu.br

Abstract

Fusarium wilt is considered as one of the most important diseases that affects tomato (*Solanum lycopersicum* L.) cultivation. The objective of this study was to identify and characterize *Fusarium* species with the potential to cause tomato wilt using morphological and molecular approaches, in order to generate the necessary information to achieve effective control of this disease. *Fusarium* isolates were found associated with commercial grown cultivars with disease incidence ranging from 10 to 85%. Forty isolates were identified by morphological characteristics as *Fusarium oxysporum* (38) and as *Fusarium* sp. (2). The isolates were evaluated for their pathogenicity on healthy tomato seedlings, which presented root rot at 20–35 days after inoculation. Fifteen of the most pathogenic isolates were analyzed with the internal transcribed spacer (ITS) region of DNA and the partial sequence of the translation elongation factor 1 α (EF-1 α). Isolates associated with tomato wilt, were identified molecularly as *Fusarium oxysporum* (13), *Fusarium circinatum* (1), and *Fusarium andiyazi* (1). Both analysis revealed that the mayor agent of tomato wilt in Mexico was *F. oxysporum*. This finding provides relevant information on tomato wilt in Mexico to decide the proper control methods for the pathogen.

Keywords: characterization; *Fusarium oxysporum* complex; genotypes; ITS and EF-1 α ; phylogenetic tree; tomato

Introduction

Tomato (*Solanum lycopersicum* L.) is a high-value horticultural crop worldwide. The overall tomato production estimates 161 million ton and total production area of 8.5 million ha, in 2015. Mexico has shown remarkable development in tomato production and has been recognized as the second largest tomato exporter (FAOSTAT, 2015). However, tomato production has been tremendously limited by a high number of biotic and abiotic factors (Parke and Grünwald, 2012), among them, fungal soil-borne pathogens such as *Fusarium oxysporum* f. sp. *radicis-lycopersici*, first observed in 1969 in Japan (Sato and Araki, 1974), and *Fusarium oxysporum* f. sp. *lycopersici*, described over 100 years ago in the UK (Masse, 1895), causes tomato wilting (Inami *et al.*, 2014), resulting in low

yields and high economic losses (Arie *et al.*, 2007; Panthee and Chen, 2010), exceeding 50% in production systems in Mexico (Apodaca *et al.*, 2004).

Adequate identification of this pathogen is necessary to apply appropriate measures to reduce its negative effects (Takken and Rep, 2010). Morphological characteristics based on shape and size microconidia and macroconidia, phialides and the formation of chlamydospores (Rodrigues and Menezes, 2005), are the main form of identification and taxonomic description of *Fusarium* species (Leslie and Summerell, 2006). Additionally, modern molecular methods such as PCR and DNA sequence analysis of internal transcribed spacer (ITS) region and the translation elongation factor 1 α (EF-1 α), are faster and more reliable to obtain proper identification (El-Kazzaz *et al.*, 2008; Singha, 2016).

Hence, the objectives of the present study were to determine the occurrence and incidence of *Fusarium* isolates in field and greenhouse conditions, in the central growing region of Mexico. Identify and characterize selected *Fusarium* isolates which causes tomato wilting, using morphological characteristics and sequence analysis of internal transcribed spacer (ITS) region of rDNA and the translation elongation factor 1 α (EF-1 α), and to verify the pathogenicity in four commercial tomato varieties. Despite some studies which have addressed these problems (Leyva-Mir et al., 2013), scarce information is available concerning occurrence, growth rates and phylogenetic relation of this pathogens found in fields and greenhouses production in Mexico. Thus, the finding in this study would provide relevant information on *Fusarium* species that affects tomato production in Mexico, to help decide the proper control methods to improve fruit yields.

Materials and Methods

Sampling of plant tissue and isolation of fungi

In 2014 and 2015, samples of tomato plants showing symptoms of wilting and chlorosis were obtained from a total of 12 different geographical locations in central Mexico, distributed in the states of Morelos, Puebla, and Tlaxcala (Fig. 1).

Symptomatic fragments were taken from the infected parts of the plant showing necrosis, dark brown and/or reddish coloration, sterilized in a 5% sodium hypochlorite solution, rinsed with sterilized water and placed on potato dextrose agar (PDA) (Difco, USA) supplemented with 0.5 g L⁻¹ of streptomycin sulphate (Sigma-Aldrich, USA) and 1 ml L⁻¹ of lactic acid. The Petri dishes were incubated at 26 °C for 3-5 days. Fungal colonies were transferred to fresh PDA medium and one colony per sample was re-isolated using a single spore (Leslie and Summerell, 2006). Forty of the isolates were stored in glycerol at 15% in cryogenic tubes at -80 °C for further investigations. The isolates used in this study were deposited in the Culture Collection of Phytopathogenic Fungi at the Universidad Autónoma Chapingo as UACH-202 to UACH-241. The disease incidence (%) of stem and root infections was calculated as the ratio of total number of infected plants divided by the

total number of examined plants, multiplied by 100.

Morphological characterization

For the morphological identification, pure cultures obtained from a single spore of each isolate were grown on PDA to examine the mycelium growth and appearance (Leslie and Summerell, 2006). To differentiate among isolates the presence or absence of chlamyospores, formation of sporodochia, width and length (μ m) of fifty random macroconidia and one hundred microconidia, were measured with the Motic Image Plus v. 4.0 (Motic Group®, China). Isolates were grown on PDA and carnation leaf agar (CLA) medium. Wet chambers were used to observe the development of monophialides and/or polyphialides (Rodrigues and Menezes, 2005). Species of *Fusarium* were identified using the keys and descriptions reported by Booth (1971), Nelson et al. (1983), and Leslie and Summerell (2006). For characterization on culture medium, a 5-mm disc in diameter of each single spore isolate previously grown for 5-day-old on PDA medium, were placed at the center of each Petri dish and were incubated at 26 \pm 2 °C under darkness.

Pathogenicity test

The pathogenicity of 40 *Fusarium* isolates was verified on healthy 35-days-old tomato seedlings of four commercial tomato cultivars ('Rio Grande', 'Moctezuma F1', 'Floradade', and 'DRW 7744 F1') under greenhouse conditions. Conidial suspension of each fungal isolate was prepared using sterile distilled water and conidia of 7-days-old colonies to inoculate each plant. The concentration of the conidial suspension was adjusted to 10⁶ spores mL⁻¹ including macroconidia and microconidia, using a hemocytometer. The inoculated plants were transplanted in styrofoam cups (1L) as described by Shahnazi et al. (2012). Data collection were carried out at 5, 10, 15, 20, 25, 30, and 35 days after inoculation (DAI). To determine the severity a visual scale was designed based on the percentage of affected plants, where 1= no symptoms; 2= initial symptoms or 10-20% chlorosis of leaves; 3= 20-50% chlorosis of leaves; 4= >50% chlorosis of leaves and initial symptoms flaccidity of the top leaves; 5= completely or the major part of the plant wilted or death.

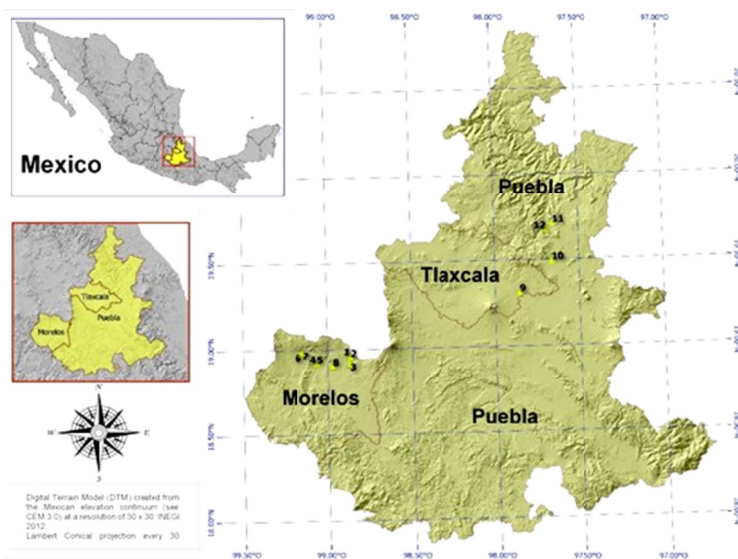


Fig. 1. Tomato growing areas sampled in central Mexico (Morelos, Puebla and Tlaxcala), in the growing season of 2014 and 2015

Dry weight of the plants following oven drying at 80 °C for 48 h, along with internal lesion was registered. The experimental design of pathogenicity test was a completely randomized block with four replicates. The experimental unit was five styrofoam cups. Area under the disease progress curve (AUDPC) was calculated according to Campbell and Madden (1990). The AUDPC values were used to classify the level of pathogenicity among the different *Fusarium* species. Analysis of variance (ANOVA) of the data was performed with the SAS v. 9.3 (2012). The mean values of the treatments were compared using the Tukey test ($p \leq 0.05$).

Radial growth rate

The radial growth (cm) of the fungal isolates was recorded in 24 h intervals during seven days. The experiment was performed twice, each one with three replicates. The radial growth of the isolates was estimated with the respective slopes of the simple linear regressions, obtained using the time (days) as independent variable and radial growth as the dependent variable (Lamrani, 2009). The radial growth was classified as: fast ($\beta_1 > 12$), moderate ($7 \leq \beta_1 \leq 12$) and slow growth ($\beta_1 < 7$). The final radial growth measurement and area under disease progress curve (AUDPC), were calculated and submitted to an analysis of variance procedure. The mean values of the isolates were compared with the Tukey test ($P \leq 0.05$).

DNA extraction, PCR amplification and sequencing

DNA extraction was performed only for 15 isolates with seven-day-old mycelial colony growth on PDA medium in Petri dishes at 26 °C. Mycelium and conidia of each isolate was scrapped and ground to a fine powder under liquid nitrogen using a mortar and pestle. DNA extraction was done by using The DNeasy Plant Mini Kit (Qiagen®, USA) according to the manufacturer's instructions. DNA was visualized on 1 % agarose gels, with TAE 1 X buffer and 5 µL of the PCR product at 90 Volts. The gel was analysed in a Transilluminator Gel Doc-It™ 300 (UVP®, UK) and stored at -20 °C. For *Fusarium* species identification, the internal transcribed spacer (ITS) region of DNA was amplified using the primers ITS5/ ITS4 (White *et al.*, 1990), and the partial sequence of the translation elongation factor 1 α (EF-1 α) gene using the primers EF1-728F/EF1-986R (O'Donnell *et al.*, 1998). PCR products were prepared to a final volume of 50 µL, containing PCR buffer (1x), 2.5 µL MgCl₂, 0.2 mM of 1 µL dNTP, 0.8 µL of each primer, 1U of DNA polymerase (Promega®, USA) and 100 ng of DNA and was carried out in a C-1000™ Thermal Cycler (Biorad®, USA). The amplified products were sent to MACROGEN (Korea) for sequencing.

Phylogenetic analysis

The phylogeny was reconstructed by analyses from sequences of the ITS region and EF-1 α gene. Forward and reverse sequences were assembled using the Staden Package (Staden *et al.*, 1998). Sequences generated in this study were supplemented with additional sequences obtained from GenBank, based on BLASTn searches and the literature. Sequences of the isolates were aligned using ClustalX v. 1.81 (Thompson *et al.*, 1997) and manual adjusted. Individual

alignments of ITS and EF-1 α dataset were analysed with Maximum Likelihood and Maximum Parsimony using MEGA5 (Tamura *et al.*, 2011) with partial deletion of gaps, substitution models proposed by this program and 1000 bootstrap replicates. The obtained sequences were deposited in the NCBI database (GenBank accession numbers: ITS, MG557855–MG557869; EF-1 α MG557870–MG557884).

Results and Discussion

Field and greenhouse sampling

Fusarium wilting was observed in the 120 samples (Fig. 1) obtained from tomato plants (two to five months old) of twelve locations in three states of Mexico (Fig. 2a-b) in field and greenhouse conditions; typical symptoms were more prominent in the field. The disease incidence varied from 10-85%; the highest was observed in Apanquetzalco, Morelos (82%), grown in the field. Meanwhile, the lowest was observed in a greenhouse located in Tlaxcala, with 12%. 'Pony Express' was the most affected cultivar followed by 'Tisey-DRD8551', 'Serengheti', and 'Reserva', with intermediate incidence; while 'Cid' and 'Moctezuma' were less affected (Table 1). Those results were similar to those reported by Hernández-Martínez *et al.* (2014), indicating that Tomato wilt is one of the most devastating disease, resulting in 40-70% in economic losses around the world.

The severity and incidence of this pathogen is conditioned according to the geographical location, climatic factors, cultural practices (Daami-Remadi, 2006), and physiology of the host plant (Tivoli *et al.*, 1986). According to Nirmaladevi *et al.* (2016) Tomato wilt occurs in most cultivated soils and is isolated mainly from contaminated plants tissues. Many of these plants grown in these soils may become infected to some degree during their life cycle, suggesting their potential involvement in the spread and incidence of the disease (Rajput *et al.*, 2008). Our results confirms that observation. The effect of Tomato wilt is most apparent during the flowering stage when the plant and its productivity are more sensitive to stress (Gargouri-Kammoun *et al.*, 2009; Panthee and Chen, 2010; Siddique *et al.*, 2014).

The fields where tomato plants were cultivated are often subjected to crop rotation of other Gramineae (millet, corn, sorghum, rice and sugarcane), and Solanaceous crops (potato, pepper, and eggplant), suggesting the source of transmission of *Fusarium* spp. (Trabelsi *et al.*, 2017). Thus, these crops can increase the pathogen population in the soil in a very efficient way (Cai *et al.*, 2011). However, wilting caused by *Fusarium* spp. in Mexico has not been fully documented, despite its significant impact on commercial production and yields of tomato plants. This could be explained because Mexico is the centre of domestication for tomato, indicating the widespread variability of different tomato cultivars (Marin-Montes *et al.*, 2016), therefore, increasing the distribution, variability and evolution of *Fusarium* spp. (Inami *et al.*, 2014).

Morphological characteristics and growth rate of the *Fusarium* species

The *Fusarium* isolates exhibited colonies on PDA

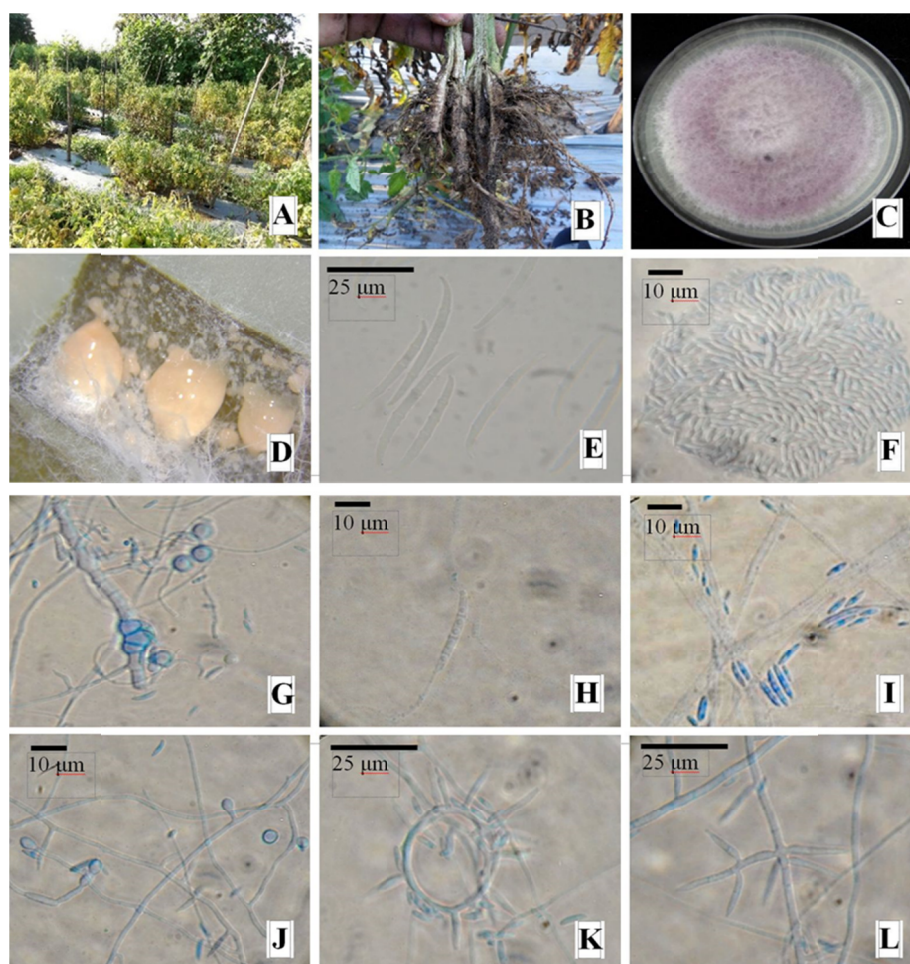


Fig. 2. Symptoms, colony, and morphological characteristics of *Fusarium* spp. (A). Yellowing symptoms of tomato plants in open field conditions. (B). Dark lesions and discoloration of tomato stems. (C). Violet colour of *Fusarium oxysporum* colony grown in PDA medium. (D). Mass of sporodochia *F. oxysporum* (E). Macroconidia of *F. oxysporum*. (F). Mass of microconidia of *F. oxysporum*. (G). Pseudochlamydospores of *Fusarium andiyazi*. (H). Macroconidia of *F. andiyazi*. (I). Microconidia of *F. andiyazi*. (J). Chlamydospores of *F. oxysporum*. (K). Coiled hyphae of *Fusarium circinatum*. (L). Monophialides of *F. circinatum*

Table 1. Mean incidence of tomato (*Solanum lycopersicum* L.) wilting in different field and greenhouse systems in Central Mexico

| State | Place of sample collection | GPS Coordinates | Altitude (msl) | Production System type | Plant age (months) | Tomato cultivar | Tomato growth type | % Mean incidence |
|----------|----------------------------|----------------------|----------------|------------------------|--------------------|------------------|--------------------|------------------|
| Morelos | Atlatlahucan | 20.971°N 51.372°E | 1817 | Field | 2.5 | 'Srengheti' | Determinate | 27 |
| Morelos | Atlatlahucan | 20.959°N 51.419°E | 1785 | Field | 2 | 'Pony Express' | Determinate | 22 |
| Morelos | Yecapixtla | 20.917°N 51.388°E | 1774 | Field | 3.5 | 'Tisey-DRD 8551' | Determinate | 20.83 |
| Morelos | Almícingo | 20.922°N 49.281°E | 1254 | Field | 4 | 'Rio Grande' | Determinate | 46.67 |
| Morelos | Apanquetzalco | 20.923°N 49.325°E | 1250 | Field | 3 | 'Pony Express' | Determinate | 82 |
| Morelos | Santa Catarina | 20.981°N 48.245°E | 1676 | Field | 3 | 'Tisey-DRD 8551' | Determinate | 26 |
| Morelos | Santa Catarina | 20.993°N 48.451°E | 1746 | Field | 3 | 'Pony Express' | Determinate | 29.1 |
| Morelos | Oaxtepec | 20.904°N 50.312°E | 1250 | Field | 3 | 'Pony Express' | Determinate | 39.44 |
| Tlaxcala | Huamantla | 21.360°N 62.026°E | 2422 | Greenhouse | 4 | 'Cid' | Indeterminate | 12 |
| Puebla | Libres | 21.562°N 64.044°E | 2376 | Greenhouse | 5 | 'Moctezuma' | Indeterminate | 27 |
| Puebla | San Miguel | 21.802°N 64.107°E | 2107 | Greenhouse | 4 | 'Cid' | Indeterminate | 18 |
| Puebla | Zautla | 21.761°N 63.703°E | 2037 | Greenhouse | 4 | 'Reserva' | Indeterminate | 29 |

medium that were red, white, purple, brown and pink in color. The colonies showed diversity according to the morphological characteristics based on macroconidia, microconidia, chlamydo-spores and phialides (Fig. 2 e-l), described by Nelson *et al.* (1983) and Leslie and Summerell (2006). Thirty-eight isolates were classified as *Fusarium oxysporum* and two as *Fusarium* sp. (Isolates 1 and 2) which varied in shape and size and were similar to those defined by Zainudin *et al.* (2010).

Mycelia of *Fusarium oxysporum* isolates were sparse to abundant, while the colours ranged from white to pink, and often pale violet. Macroconidia presented were also sparse to abundant, are formed from monophialides on branched conidiophores or on the surface of sporodochia (Fig. 2d), ranging from orange to brown. Three-septate macroconidia were most common although four thin walled septate were also presented. The macroconidia measured 26.5 to 36.1×2.6 to $4.6 \mu\text{m}$. The microconidia presented 0-septate, oval, elliptical or reniform (kidney-shaped), and were formed abundantly in false heads on short monophialides. The microconidia measured 3.4 to 12×2 to $3.3 \mu\text{m}$. Chlamydo-spores were formed abundantly in hyphae, singly or in pairs, terminally or on an intercalary basis presenting both smooth and rough walls. In carnation leaf agar (CLA) medium some of the isolates presented chlamydo-spores after 21 days. The *Fusarium oxysporum* isolates exhibited a high level of diversity presenting a wide range of colours and morphological structures. All 38 isolates were consistent with reports by Leslie and Summerell (2006). This species is considered as the principal causal agent of tomato wilt and *Fusarium* crown rot (Tanyolac and Akkale, 2010) in vegetable plants.

The isolate classified as *Fusarium* sp. (Isolate 1), exhibited abundant aerial mycelium, the color of the colony was initially white then turned to red and violet. The macroconidia were straight or slightly curved, with 3-4 septate, 38.6 to 46×2.7 to $3.4 \mu\text{m}$ (Fig. 2h). Sporodochia were rarely observed on CLA medium. The microconidia were oval, with a flat base, without septa, of 9.2 to 10.9×2.6 to $2.8 \mu\text{m}$, formed in monophialide in conidiophores sometimes branched, in false heads or long chains of more than 12 conidia. After 3-4 weeks in PDA medium, pseudochlamydo-spores in hyphae with smooth walls, and in short chains were observed (Fig. 2g). These observations were similar to those of *Fusarium andiyazi* which have been

associated with sorghum, in Africa and USA (Marasas *et al.*, 2001), with rice bakanae, in Asia and Africa (Wulff *et al.*, 2010), and with corn in Siria (Madamia *et al.*, 2013). *Fusarium andiyazi* is a major pathogen of sorghum (Klittich *et al.*, 1997; Marasas *et al.*, 2001) and can be distinguished from other *Fusarium* spp., mainly *Fusarium verticillioides*, *Fusarium thapsinum*, and *Fusarium musae*, based on its production of pseudochlamydo-spores in PDA medium (Van Hove *et al.*, 2011). These structures are distinguishable from true chlamydo-spores because they have no surface ornamentation and they are not thick- or double-walled (Leslie and Summerell 2006). According to Klaasen and Nelson (1998), pseudochlamydo-spores differ from 'swollen hyphal cells' because they do not have transverse septa (Marasas *et al.*, 2001).

On the other hand, the isolate classified as *Fusarium* sp. (Isolate 2) presented aerial mycelium was white, or slightly violet. The colony exhibited growth rate relatively rapidly. In CLA, coiled hyphae (Fig. 2k) were observed, but these characters are shared with species like *F. mexicanum*, *F. pseudocircinatum*, *F. sterilihyphosum*, and *F. tupaense* (Nirenberg and O'Donnell, 1998; Britz *et al.*, 2002; Lima *et al.*, 2012). Macroconidia were typically 3-septate, with slightly curved walls, measuring 32 to 42×3.3 to $3.8 \mu\text{m}$. Microconidia were typically single-celled, ovoid (or nearly oval), were borne in false heads on aerial polyphialides. The microconidia measured 7.6 to 11.8×1.8 to $3.5 \mu\text{m}$. Presented proliferation of microconidiophores, coupled with a slight twisting of the aerial mycelium on which they are borne, differentiating the morphology of the colony. Chlamydo-spores were absent. These observations were similar to those of *Fusarium circinatum* (Leslie and Summerell 2006), which was recently reported in association with grass species as symptomless hosts in the proximity of *Pinus* stands (Sweet and Gordon, 2012) in which it has the ability to survive in alternative hosts which is relevant for establishment and subsequent dissemination of the pathogen to new areas. The fungus is also associated with significant losses in yield and productivity, making it one of the most important limitations to commercial forestry (Wingfield *et al.*, 2008; Mitchell *et al.*, 2011). According to Steenkamp *et al.* (2014), it is widely believed that Central America and Mexico represents the centre of origin of *F. circinatum* this based on the high levels of

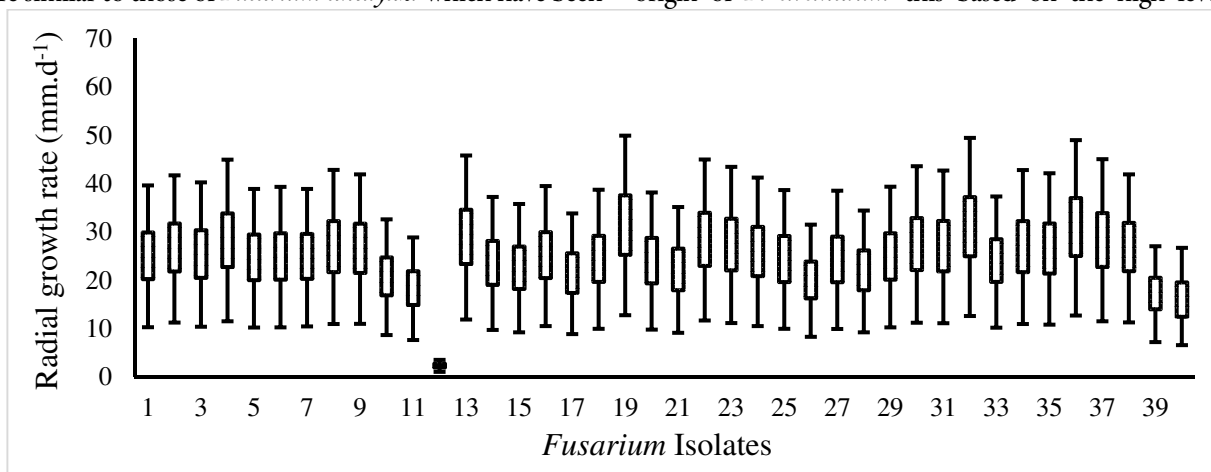


Fig. 3. Radial growth rate values ($\beta_1, \text{cm} \cdot \text{d}^{-1}$) of 40 *Fusarium* isolates (lines represent the 95% confidence intervals)

genetic diversity among species and the wide number of *Pinus* trees produced in Mexico, generating coevolution of both (Britz *et al.*, 2005).

The radial growth (β_1 , mm d⁻¹) for the 40 *Fusarium* isolates, obtained by linear regression in a period of five to seven days in PDA medium (Fig. 3), showed R²'s above 0.8 and all the β_1 values were different to zero ($P < 0.01$). Three isolates (UACH-216, UACH-234, and UACH-238) showed fast growth (12.0-12.3 mm d⁻¹) which reached maximum growth on the 5th day of incubation; while 34 isolates exhibited moderate growth (7.6-11.2 mm d⁻¹), and three (UACH-213, UACH-241, and UACH-212) presented slow growth (0.8-6.9 mm d⁻¹). The isolates with maximum growth covered the Petri dish by a 100% on the 7th day; while the isolates with minimal growth only covered by 50%. Chehri *et al.* (2015) mentioned that the growth rate of *Fusarium solani* were 5.5-8.6 mm d⁻¹, this were slightly under the results obtained in the study 6.9-12 mm d⁻¹ (Fig. 3), this probably due to difference in temperature and light intensity used during the experiment. According to Brock *et al.* (1994) and Trabelsi *et al.* (2017), fungal growth under natural conditions is very slow due to the low intake of substrates and to the variation of nutrient distribution among microbial habitats. Growth also depends on other factors such the antagonistic interactions (Giurgiu *et al.*, 2017) with other species like *Trichoderma* spp. competing for the space and nutrients (Marzano *et al.*, 2013), drought and moisture conditions, stress caused by nutrient depletion, and changes in temperature and soil pH (Trabelsi *et al.*, 2017). This helps to explain the capacity of the *Fusarium* species in adapting and growing under a different and wide range of environmental conditions.

Pathogenicity test and the response of the tomato cultivars

The pathogenicity test showed considerably differences among isolates in terms of their ability to cause disease as mentioned by Joshi *et al.* (2013). In this study, 15 isolates were highly pathogenic, 15 showed moderate pathogenicity, and 10 were weak. Symptoms were presented 21-35 days after inoculation in which the leaves presented yellowing, flaccidity and wilting. Necrosis and a brownish discoloration were also observed on roots and stem as well as a reddish coloration of vascular tissues as shown in Fig. 2. Furthermore, the inoculated fungi were re-isolated from plants that showed wilting symptoms, while no symptoms were found on control plants, for this reason the Koch's postulates were confirmed. ANOVA results of disease severity of *Fusarium* spp. were variable where AUDPC, internal lesion, dry weight, and fresh weight values indicated highly significant differences among the inoculated isolates

and cultivars (Table 2) as well as for their interaction ($P \leq 0.01$). The means comparison for cultivars indicated that 'Moctezuma' showed higher resistance to *Fusarium* by presenting the lowest AUDPC, and higher fresh and dry weight (Table 2); although the internal lesion was high. In contrast, 'Rio Grande', 'Floradade', and '7744' presented similar performance showing high susceptibility. According to Steinkellner *et al.* (2005) the pathogenicity of *Fusarium* in tomato could be affected by the *Fusarium* race, internal factor such as enzymes, growth-regulating compounds, toxins, gummosis; and environmental and growing conditions among them nutrition, phenological stage, and cultural management. Jiménez-Fernández *et al.* (2010) mentioned *F. oxysporum* are responsible for wilt diseases on many plants of economic importance other than tomato. Pathogenic strains show high levels of host specificity and are classified on this basis into *formae speciales* and races. Ajit *et al.* (2006) also reported that *F. oxysporum* has approximately 80 *formae speciales* divided into pathotypes specific to species, and subdivided into races specific to cultivar within a species.

Molecular characterization

Molecular characterization based on analyses of ITS and EF-1 α sequences confirmed the findings of the morphological observations, hence the importance of combining both morphological and genetic data for fungal identification (Correia *et al.*, 2013). *Fusarium* isolates associated with tomato wilt formed three genetic clusters based on (EF-1 α) analysis (Fig. 4). The phylogenetic tree resulting in the formation of three major clusters. According to Aoki *et al.* (2014), primers used in any study must be specific for the correct amplification of *Fusarium* spp. This situation proves that the majority of the *Fusarium* isolates pertains to the *Fusarium oxysporum* complex, one as *Fusarium andiyazi*, and one as *Fusarium circinatum*. The last two isolates forms part of the *Gibberella fujikuroi* complex (Fig. 3). Several studies have shown that phylogenetic species criterion is most appropriate and congruent (O'Donnell *et al.*, 1998; Cai *et al.*, 2011; Bashyal and Aggarwal, 2013).

For example, phylogenetic analyses have revealed the existence of several cryptic species (Steenkamp *et al.*, 2002) such as *Fusarium subglutinans*, using the genes translation elongation factor 1 α gene (EF-1 α) and the DNA sequences of ITS regions for specie recognition, thus, offering a finer resolution, separating strains of most *Fusarium* complex species (Hsuan *et al.*, 2011; Irzykowska *et al.*, 2012). The identification of the genera *Fusarium* on a species level is essential because species are used as the basic units of analysis

Table 2. Means comparisons of four varieties of tomatoes during pathogenicity test

| Variety | Severity (AUDPC) | Internal lesion (cm) | Dry weight (g) | Fresh weight (g) | | | | |
|--------------|------------------|----------------------|----------------|------------------|--------|---|---------|---|
| 'Rio Grande' | 948.05 | a | 32.112 | b | 1.627 | c | 86.219 | c |
| 'Moctezuma' | 439.02 | d | 39.005 | a | 4.2751 | a | 210.098 | a |
| 'Floradade' | 833.66 | b | 32.372 | b | 1.4255 | c | 83.96 | c |
| '7744' | 712.93 | c | 33.501 | b | 2.6434 | b | 138.935 | b |
| HSD | 15.892 | | 3.2628 | | 0.2901 | | 15.053 | |

Note: HSD: Honest significant difference
Different letters in each column indicates significant difference (Tukey, $P < 0.05$).

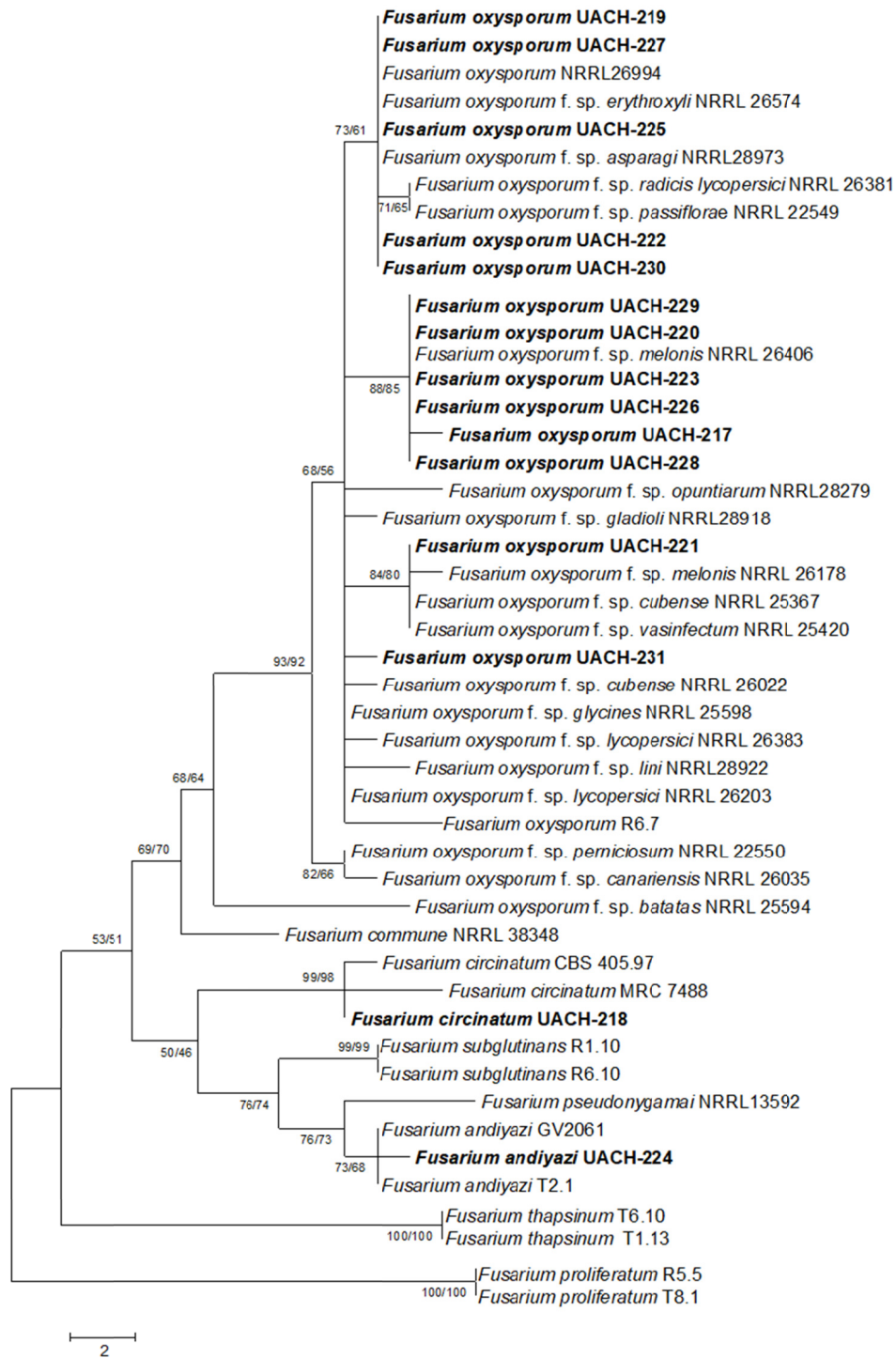


Fig. 4. Maximum parsimony tree generated from sequence analysis of the EF-1 α gene dataset. Designated outgroup taxa are *Fusarium thapsinum* and *F. proliferatum*. Bootstrap support values for maximum parsimony (left) and maximum likelihood (right) are indicated at the nodes. The isolates in this study are in boldface

Table 3. Results of pathogenicity test of *Fusarium* spp. Isolates

| County | Code | Severity (AUDPC) | Severity | Internal lesion (cm) | Dry weight (g) | Fresh weight (g) | | | | |
|----------------------|----------|------------------|----------|----------------------|----------------|------------------|-----|-----|------|-----|
| Atlatlahucan | UACH-202 | 400 | op | Weak | 38.8 | c-k | 1.7 | h-k | 85 | ijk |
| Santa Catarina Monte | UACH-203 | 985 | d-f | High | 12.4 | op | 1.4 | k | 68.5 | jk |
| Apanquetzalco | UACH-204 | 415 | op | Weak | 24.4 | k-p | 1.7 | h-k | 87 | h-k |
| Atlatlahucan | UACH-205 | 760 | f-i | High | 17.6 | nop | 1.7 | h-k | 86 | h-k |

| | | | | | | | | | | |
|----------------|----------|------|-----|----------|------|-----|--------|-----|--------|-----|
| Atlatlahucan | UACH-206 | 880 | efg | High | 25.6 | j-o | 1.7 | h-k | 95 | e-k |
| San Miguel | UACH-207 | 428 | op | Weak | 41.1 | b-i | 1.8 | g-k | 85 | ijk |
| Libres | UACH-208 | 548 | i-n | Moderate | 48 | b-e | 2.8 | c-j | 138 | b-j |
| San Miguel | UACH-209 | 350 | p | Weak | 51.8 | a-d | 3.4 | b-e | 190 | b |
| Zuautla | UACH-210 | 843 | e-h | High | 25.1 | k-o | 1.7 | h-k | 90.5 | f-k |
| Santa Catarina | UACH-211 | 765 | f-g | High | 46.2 | c-g | 3.5 | b-e | 190 | b |
| Oaxtepec | UACH-212 | 828 | e-h | High | 36.4 | d-l | 1.5 | jk | 81 | ijk |
| Apanquetzalco | UACH-213 | 465 | k-p | Moderate | 45.1 | b-g | 4.5 | ab | 185 | b |
| San Miguel | UACH-214 | 783 | f-h | High | 22.1 | l-p | 2.3 | e-k | 133 | b-k |
| San Miguel | UACH-215 | 1395 | ab | High | 12.4 | op | 1.4 | jk | 76 | ijk |
| Yecapixtla | UACH-216 | 435 | n-p | Weak | 26.4 | l-o | 1.6 | h-k | 102 | c-k |
| Huamantla | UACH-217 | 605 | g-p | Moderate | 52.3 | abc | 3 | c-h | 163 | b-f |
| Atlatlahucan | UACH-218 | 478 | j-l | Moderate | 67.1 | a | 3.2 | b-f | 172.9 | bc |
| Almencingo | UACH-219 | 470 | l-p | Moderate | 36.7 | c-l | 3.5 | b-d | 200 | b |
| Huamantla | UACH-220 | 1698 | a | High | 50.3 | cbd | 3.7 | bcd | 200 | b |
| Huamantla | UACH-221 | 640 | h-o | Moderate | 31.7 | fl | 3.1 | b-g | 162 | b-g |
| Almencingo | UACH-222 | 590 | i-p | Moderate | 46.6 | b-f | 3 | c-h | 170 | bcd |
| Santa Catarina | UACH-223 | 710 | fl | Moderate | 24.4 | k-p | 1.8 | f-k | 107 | c-k |
| Huamantla | UACH-224 | 1218 | bcd | High | 29.2 | h-l | 1.8 | f-k | 97.8 | f-k |
| Apanquetzalco | UACH-225 | 743 | fl | Moderate | 27.8 | i-l | 2.4 | d-k | 129 | b-k |
| Apanquetzalco | UACH-226 | 663 | j-l | Moderate | 29.7 | i-n | 3.4 | b-e | 161 | b-g |
| Apanquetzalco | UACH-227 | 1073 | c-d | High | 29.8 | i-n | 1.4 | kj | 79 | ijk |
| Apanquetzalco | UACH-228 | 730 | fl | Moderate | 26.6 | j-o | 2.5 | c-k | 147 | b-i |
| Apanquetzalco | UACH-229 | 973 | def | High | 24.7 | k-p | 1.6 | ijk | 103.3 | c-k |
| Libres | UACH-230 | 715 | fl | Moderate | 34.3 | e-m | 1.8 | g-k | 83.6 | ijk |
| Libres | UACH-231 | 1268 | bc | High | 33.9 | f-l | 1.9 | f-k | 99 | d-k |
| Apanquetzalco | UACH-232 | 650 | h-l | Moderate | 30.5 | i-m | 2.1 | e-k | 133 | b-k |
| Apanquetzalco | UACH-233 | 753 | fj | Moderate | 25 | k-o | 1.7 | h-k | 106 | c-k |
| Huamantla | UACH-234 | 1430 | ab | High | 51.4 | a-d | 1.7 | h-k | 85.2 | ijk |
| Oaxtepec | UACH-235 | 603 | h-p | Moderate | 37.5 | c-k | 3.6 | bcd | 183 | b |
| Huamantla | UACH-236 | 735 | fl | Moderate | 33.5 | f-n | 1.5 | jk | 89.5 | g-k |
| Oaxtepec | UACH-237 | 748 | fl | Moderate | 27.9 | i-n | 2.7 | c-k | 159 | b-h |
| Oaxtepec | UACH-238 | 745 | fl | Moderate | 57.1 | ab | 3.9 | bc | 173 | bc |
| Oaxtepec | UACH-239 | 655 | g-l | Moderate | 50.5 | bcd | 3.4 | b-e | 165.2 | b-e |
| Huamantla | UACH-240 | 403 | op | Weak | 18.5 | m-p | 1.4 | k | 65 | jk |
| Apanquetzalco | UACH-241 | 473 | k-p | Moderate | 42.9 | b-h | 1.7 | h-k | 64 | k |
| Control | | 30 | q | | 8.89 | p | 5.6 | a | 333 | a |
| HSD | | 277 | | | 15.9 | | 1.4136 | | 73.362 | |

Note: HSD: Honest significant difference. Different letters in each column indicates significant difference (Tukey, $p < 0.05$).

and for global biodiversity assessments (Sites and Marshall, 2004) based on monophyletic grouping at higher levels and discover lineages at lower levels.

Conclusions

In conclusion, in the present study three *Fusarium* spp. (*Fusarium oxysporum*, *Fusarium andiyazi*, and *Fusarium circinatum*) associated with wilting disease of tomato in Mexico were identified, which will be very helpful information for developing new strategies for the adequate control of this pathogen. It was not find any relationship between the identified *Fusarium* species with the sampled areas and the production system; this shows the high capacity of this pathogen to affect tomato established under any condition due to the genetic variability of *Fusarium*, which affected the high variability of the infection period and growth rate as observed in this study. This situation can

also explain the presence of the different species such as the *Fusarium oxysporum* complex, *Fusarium andiyazi*, and *Fusarium circinatum* in tomato; this is of significant important to continue monitoring and evaluating crop diseases development to avoid high losses in tomato production.

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