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Phylogenetics and histology provide insight into damping-off infections of 'Poblano' pepper seedlings caused by *Fusarium* wilt in greenhouses

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Abstract

The 'Poblano' pepper crop is economically important in Mexico and throughout the world as it is used as a hot spice in food. The cultivated area of the 'Poblano' pepper crop is decreasing yearly for many reasons, among them a wilt disease commonly associated with *Fusarium* spp. This disease is a problem of field and greenhouse production plants. Moreover, it is not clear whether the pathogens that cause wilt in mature plants are the same as those involved in the damping-off symptoms and death of pepper seedlings in greenhouses. For this reason, the aim of the present study was to identify the causal agent of damping-off in pepper during seedling production, establish its relationship with the causal agent of wilting in mature plants, and determine whether histological damage in seedlings occurs. Isolates were recovered from the crown rot and stem base of 4-month-old infected 'Poblano' mature pepper plants and were identified using morphological and phylogenetic approaches. *Fusarium oxysporum* and *F. solani* were isolated from the crown rot and base stem, respectively. A pathogenicity test showed that both species caused damping-off in pepper seedlings. Histological studies with inoculated seedlings of both isolates showed several changes in the external cortex, epidermal cells, endodermis, Casparian strips, cell size, and xylem wall. Casparian strip rupture resulted in permeability loss and regulatory activity to maintain the cellular equilibrium inside the vascular bundles. Hence, according to these findings, producers should avoid seedling contamination by infected mature plants because the aggressiveness of *Fusarium* isolates can cause rapid seedling mortality.

Keywords *Capsicum annuum* · Casparian strips · Crown rot · Canker · Histopathology · Identification

Introduction

The 'Poblano' pepper (*Capsicum annum* L.) is Mexico's major native pepper cultivar. The 'Poblano' pepper has high commercial value as a hot spice and is in strong demand in international commodity markets (FAOSTAT 2015). Pepper growers face a number of production problems that, if mitigated, would greatly increase their commercial returns. Statistical data show that although sown areas have increased by approximately 10% (SIAP 2017), the reduction in the percentage of harvested area has not changed since 2006 (36,200 ha), 2010 (34,144 ha), and 2016 (32,627 ha) (FAOSTAT 2017).

Many factors have been considered to contribute to yield losses, such as the incidence of biotic diseases, among them, vascular wilt caused by *Fusarium* spp. This disease occurs when susceptible cultivars are grown in infected native soil using a continuous monoculture. In this case, the 'state of alert' has been observed to be a novel mechanism in plants that is involved in the interaction of plants with soil microbiota and drives plant responses (Chialva et al. 2018). In addition, high temperature and moisture stimulate fungal development and contribute to the spread of this disease over large planted areas (Di Pietro et al. 2003; Gordon 2017).

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Species of *Fusarium* are considered to cause a soilborne fungal disease (Yadeta and Thomma 2013); plants are infected when vegetative or propagative structures enter through the roots, causing yellowing and wilting, which often appear later during the growing season and are first observed on the lower leaves (Goodman et al. 1986; Beckman 1987). These symptoms progress up the plant as the fungus spreads within its host; a brown discoloration of the conducting vascular system just beneath the epidermis is often observed. The discoloration can extend from the roots up the stem through the branches and into the leafstalks of the plant (Michielse and Rep 2009; Sundaramoorthy et al. 2012). As the disease progresses, younger leaves are also affected and the plant prematurely drops its fruit or eventually dies, significantly reducing fruit production (Perez-Hernandez et al. 2014).

The severe outbreaks of most of the *Fusarium* vascular wilt pathogens are caused by *F. oxysporum* Schlechtendal, which contains pathogenic and non-pathogenic strains that are morphologically indistinguishable (Booth 1971; Lievens et al. 2008). Tropical race 4 has been mentioned in the literature as historically economically damaging to the banana industry (Dale et al. 2017), and races 1 and 4 have been mentioned as being damaging to other commercially important plants, including basil (Rekah et al. 2000) and cotton (Wang et al. 2018). On the other hand, *F. solani* (Martius) Saccardo has also been implicated as a causal agent of chilli wilt disease and as a pathogen of a large number of solanaceous plants (Zhang et al. 2007; Sundaramoorthy et al. 2012).

During the production of many solanaceous plants, seed germination is conducted in trays that are maintained in a greenhouse for later transplanting. During this period, several affectations are observed in seedling development that lead to rapid mortality. Therefore, there is little information regarding which *Fusarium* spp. result in damping-off symptoms in pepper seedlings under greenhouse production and whether the root tissue or the conducting vessels are colonised by species-specific or other phytopathogens. The purpose of this study was to identify the causal agents of damping-off in pepper seedlings in greenhouse and establish their relationship with those that cause crown rot and necrotic symptoms in the stem base of 'Poblano' adult pepper plants. Additionally, we sought to determine whether damage occurs in seedlings at a histological level.

Materials and methods

Plant material and fungal isolates

During spring/summer from 2007 to 2009, 'Poblano' mature pepper plants with wilt symptoms were recovered from commercial fields in the Puebla State of southeastern Mexico. Stems and roots with necrotic tissues were separated and washed in running water. Afterwards, the exterior portion was disinfested by immersion for 1 min in a 1.5% *w*/*v* aqueous sodium hypochlorite solution and rinsed three times with sterile distilled water. Pieces of the crown rot and stem base were excised and dipped in 25% aqueous ethyl alcohol (*v*/*v*) for 30 s and finally rinsed twice in sterile distilled water. The samples were then surface dried in a sterile flow chamber at room temperature. Small samples (1–3 cm) that displayed a reddish brown, rusty looking discolouration in the interior of the crown and stem were excised using a sterile scalpel and placed in Petri dishes containing potato dextrose agar (PDA) (BD Becton Dickinson, NJ, USA). When mycelium growth and macro and microconidia were evident, the conidia were removed by adding 10 mL of sterile distilled water to each Petri dish. This volume was placed into a test tube from which 1 mL was transferred to a new tube containing 9 mL of sterile distilled water to create a 10-fold serial dilution. From this, 10^{-5} to 10^{-7} conidia per millilitre were plated onto water agar (WA) medium. After 3 days, single conidia that had germinated were transferred to a Petri dish containing PDA amended with kanamycin (Sigma-Aldrich, MO, USA) at 50 mg L^{-1} to obtain pure cultures. Petri dishes were maintained for 10 days for pathogenicity tests and genomic DNA extraction (Table 1).

DNA extraction

The mycelium was removed using a spatula from the surface of the Petri dish and crushed in a mortar with 1 mL of lysis solution (10-mM TRIS base HCL pH 8.0, Triton × 100 0.5%) and 10 μL of proteinase K (Sigma-Aldrich, MO, USA) (10 mg/mL). Then, 600 μL of each sample was transferred to a 2.0-mL Eppendorf tube for DNA extraction in accordance

Table 1 Species of *Fusarium* isolated from mature plants of 'Poblano' pepper showing wilt symptoms from 2007 to 2009 in Puebla State, Mexico

Isolate	GenBank accession number ITS-28SrDNA	Isolated from	Colony colour
Fusarium solani M15	EU082095	Stem base	Cream
Fusarium solani M14	EU082096	Stem base	Cream
Fusarium solani M4	EU082097	Stem base	Cream
Fusarium solani M2	EU082098	Stem base	Cream
Fusarium solani M18	EU082099	Stem base	Cream
Fusarium solani M11	EU082100	Stem base	Cream
Fusarium solani M10	EU082101	Stem base	Cream
Fusarium solani M5	EU082102	Stem base	Cream
Fusarium oxysporum M3	EU082103	Crown rot	Purple
Fusarium oxysporum M6	EU082104	Crown rot	Purple
Fusarium oxysporum M17	EU082105	Crown rot	Purple

with the protocol of Doyle and Doyle (1990) with very few modifications. DNA from each tube was suspended in 50 μL of TE buffer (10-mM Tris-HCl, pH 8, 1-mM EDTA), and the concentration was quantified using spectrophotometry in a Nanodrop 2000C (Thermo Scientific, MA, USA). DNA quality was estimated by the ratio of $A_{280/260}$ and $A_{230/260}$, and then, DNA was diluted to 20 ng μL^{-1} for PCR.

PCR amplifications of the ITS region of rDNA and a partial sequence of the TEF-1α gene

PCR using the ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Guadet et al. 1989) set of primers was used to amplify a partial sequence of 18S rDNA, the complete sequence of the 5.8S rRNA gene, and the internally transcribed spacer 1 and 2 regions plus domains D1 and D2 of the nuclear large subunit (LSU) of a partial sequence of the rDNA gene. A fragment of 1100 base pairs (bp) was expected, including 500 bp corresponding to the end of the 18S rRNA gene, entire ITS region, and approximately 600 bp of the conserved domains in the 28S rRNA gene. In the case of the translation elongation factor 1-α (TEF1) partial sequence, the EF1-a (5′-ATGGGTAAGGARGACAAGAC-3′) and EF2-a (5′-GGARGTACCAGTSATCATGTT-3′) primers were used (O'Donnell et al. 1998), and an ~ 700-bp product was expected.

PCR was prepared in 0.2-mL Eppendorf tubes with a final volume of 15 μL containing 5× Go *Taq* DNA Reaction buffer, 2 μM of each dNTPs, 10 pmol of each primer, and 2 units of Go *Taq* DNA (Promega, WI, USA). Finally, 100 ng of DNA was added to each tube. PCR was performed using an initial denaturing hold at 95 °C for 4 min, 35 denaturing cycles at 95 °C for 1 min, annealing at 56 °C (ITS-28S rDNA) and 53 °C (TEF-1 α) for 1 min, extension at 72 °C for 2 min, followed by a single final extension cycle at 72 °C for 10 min. PCR was performed in a Peltier Thermal Cycler DNA Engine PTC-200 (BioRad, CA, USA), and the PCR products were verified by loading 5 μL on a 1.5% agarose electrophoresis gel (Seakem, CA, USA), which was stained as previously described. The remaining PCR amplified products were purified using the QIAquick PCR purification kit (Qiagen, CA, USA) following the manufacturer's instructions. To confirm that there were no misreads in any of the strands, forward and reverse primers were used in individual wells for sequencing with the Big-Dye Terminator version 3.1 Cycle Sequencing kit in an Applied Biosystems model 3130 automated DNA analyser (Applied BioSystems, CA, USA).

Phylogenetic analyses

Sequences corresponding to both strands of the ITS entire region, 28S rDNA gene and TEF1 (partial sequence) were assembled independently and edited using BioEdit software version 7.0.5 (Hall 1999). A consensus sequence of each isolate was created for each gene or rDNA region and partial TEF1 and later submitted to the BLASTN 2.2.19 (Altschul et al. 1997) and *Fusarium* ID databases (Geiser et al. 2004).

Phylogenetic reconstruction for all of the sequences was performed with Bayesian inference using MrBayes v3.2.2 (Huelsenbeck and Ronquist 2001), and a 1-M generation post-stationarity was run in concatenated analyses. Markov Chain Monte Carlo (MCMC) analysis used four chains, one cold and three heated chains, and started from a random tree topology. The sample frequency was set at 1 in 1000 for the combined analysis. The consensus tree and subsequent Bayesian inference probabilities for the most complex model of evolution available for nucleotides (GTR + gamma distribution + invariant positions) were obtained (Ronquist and Huelsenbeck 2003).

The accession numbers of the *F. oxysporum* and *F. solani* mentioned in MycoBank (http://www.mycobank.org/) and deposited in the NCBI-GenBank database (Benson et al. 2012) were downloaded and included for comparison along with the sequences obtained in this study (Table 1). For construction of the phylogenetic tree, the sequence of *Phoma herbarum* (accession number EU082106) was designated as the out-group (Fig. 3).

Additionally, to determine to which *Fusarium* species complex the isolates obtained in this study belonged, the sequences described by O'Donnell and collaborators (2016) were used to construct a new phylogenetic tree based on the amplification of the TEF1 partial gene (Table 2). A phylogenetic tree was created in the same manner as previously described (Fig. 4).

Morphological and cultural characteristics

Small portions of ~ 5 mm^2 plugs of agar containing mycelium from each species of *Fusarium* were transferred onto Petri dishes containing PDA medium. To study the morphological features of these species, each isolate was grown on Carnation Leaf Agar (CLA) medium (Fisher et al. 1982), and the dishes were incubated at 24 °C with 12 h of white light and 12 h of UV light (365-nm General Electric 40W F40SL, CT, USA) for 15 days. Fifty cells were examined for each isolate to identify fungi at the genus level, and the descriptions of Nelson et al. (1983), Burgess et al. (1994) and Leslie and Summerell (2006) were used.

Inoculum preparation for pathogenicity tests

Isolates were increased using the oat glume method (Llop et al. 2000). Oat glumes were sterilised in a flask (twice for 45 min). Petri dishes with mycelium from each isolate grown on PDA were incubated under previously described

Table 2 The *Fusarium* species complex and their accession numbers corresponding to partial sequences of translation elongation factor 1-α

GenBank accession number	Identified as	Isolate	Species complex	Origin
KC808211	*Fusarium* sp. 3+4d culture-collection	NRRL 54989	FSSC	TX, USA
KC808191	*Fusarium* sp. 3+4eee culture-collection	NRRL 54964	FSSC	TN, USA
KC808201	*Fusarium* sp. 3+4eeee culture-collection	NRRL 54978	FSSC	FL, USA
KC808209	*Fusarium* sp. 3+4eeee culture-collection	NRRL 54987	FSSC	FL, USA
KC808212	*Fusarium* sp. 3+4eeee culture-collection	NRRL 54991	FSSC	FL, USA
KC808218	*Fusarium* sp. 3+4eeee culture-collection	NRRL 62543	FSSC	FL, USA
KC808193	*Fusarium* sp. 3+4ffff culture-collection	NRRL 54966	FSSC	GA, USA
KC808206	*Fusarium* sp. 3+4gggg culture-collection	NRRL 54983	FSSC	FL, USA
KC808203	*Fusarium* sp. 3+4uuu culture-collection	NRRL 54980	FSSC	FL, USA
KC808189	*Fusarium keratoplasticum* culture-collection	NRRL 54162	FSSC	AL, USA
KC808199	*Fusarium keratoplasticum* culture-collection	NRRL 54975	FSSC	FL, USA
KC808216	*Fusarium keratoplasticum* culture-collection	NRRL 54998	FSSC	CA, USA
KC808217	*Fusarium keratoplasticum* culture-collection	NRRL 54999	FSSC	CA, USA
KC808219	*Fusarium keratoplasticum* culture-collection	NRRL 62544	FSSC	CA, USA
KC808192	*Fusarium keratoplasticum* culture-collection	NRRL 54965	FSSC	MA, USA
KC808207	*Fusarium petroliphilum* culture-collection	NRRL 54985	FSSC	GA, USA
KC808208	*Fusarium petroliphilum* culture-collection	NRRL 54986	FSSC	GA, USA
KC808194	*Fusarium* sp. 5 culture-collection	NRRL 54969	FSSC	CA, USA
KC808204	*Fusarium* sp. 5 culture-collection	NRRL 54981	FSSC	CA, USA
KC808198	*Fusarium* sp. 12a culture-collection	NRRL 54974	FSSC	MA, USA
KC808195	*Fusarium* sp. 12b culture-collection	NRRL 54970	FSSC	CT, USA
KC808202	*Fusarium* sp. 12f culture-collection	NRRL 54979	FSSC	MA, USA
KC808220	*Fusarium* sp. 12f culture-collection	NRRL 62549	FSSC	MD, USA
KC808196	*Fusarium* sp. 12h culture-collection	NRRL 54971	FSSC	FL, USA
KC808205	*Fusarium* sp. 12h culture-collection	NRRL 54982	FSSC	MA, USA
KC808197	*Fusarium* sp. 20 culture-collection	NRRL 54972	FSSC	FL, USA
KC808213	*Fusarium* sp. 43a culture-collection	NRRL 54992	FSSC	GA, USA
KC808214	*Fusarium* sp. 43a culture-collection	NRRL 54993	FSSC	GA, USA
JQ743207	*Fusarium* sp.	NRRL 54720	FSSC	GA, USA
DQ246861	*Fusarium* sp.	NRRL 25392	FSSC	NY, USA
KR935898	*Fusarium solani* M2	CPO 3.01	FSSC	Puebla, Mexico
KR935897	*Fusarium solani* M4	CPO 3.02	FSSC	Puebla, Mexico
KR935896	*Fusarium solani* M5	CPO 3.03	FSSC	Puebla, Mexico
KR935888	*Fusarium solani* M10	CPO 3.04	FSSC	Puebla, Mexico
KR935889	*Fusarium solani* M11	CPO 3.05	FSSC	Puebla, Mexico
KR935890	*Fusarium solani* M14	CPO 3.06	FSSC	Puebla, Mexico
KR935891	*Fusarium solani* M15	CPO 3.07	FSSC	Puebla, Mexico
KR935892	*Fusarium solani* M18	CPO 3.08	FSSC	Puebla, Mexico
KR935893	*Fusarium oxysporum* M3	CPO 3.09	FOSC	Puebla, Mexico
KR935894	*Fusarium oxysporum* M6	CPO 3.010	FOSC	Puebla, Mexico
KR935895	*Fusarium oxysporum* M17	CPO 3.011	FOSC	Puebla, Mexico
KC808225	*Fusarium oxysporum* ST-33 culture-collection	NRRL 54984	FOSC	Massachusetts, USA
KC808229	*Fusarium oxysporum* ST-33 culture-collection	NRRL 62542	FOSC	TX, USA
GQ505420	*Fusarium* sp.	NRRL 36147	FTSC	Unknown
GQ505432	*Fusarium* sp.	NRRL 45994	FTSC	TX, USA
GQ505433	*Fusarium flocciferum*	NRRL 45999	FTSC	CA, USA
EU926344	*Fusarium dimerum*	NRRL 37039	FDSC	Slovenia

Fusarium selected isolates to determine the species complex of isolates obtained in this study were based on O'Donnell and collaborators (2016), *FSSC*, *Fusarium solani* species complex; *FOSC*, *Fusarium oxysporum* species complex; *FTSC*, *Fusarium tricinctum* species complex; and *FDSC*, *Fusarium dimerum* species complex

conditions. The mycelium from each isolate were cut into pieces that were 1 cm^2 in area and mixed in flasks of suspended oat glumes. The flasks were maintained at room temperature (*c*. 25 °C) for 3 weeks until colonisation of whole glumes was observed. Conidia were harvested by washing the glumes with rubbing in 250 mL of sterile distilled water.

Pathogenicity tests in the greenhouse

One-hundred seeds of the 'Poblano' pepper cv. San Luis were germinated in peat moss for 10 days and then transplanted during the two-leaf stage to a sterilised potting mix (3 peat:1 vermiculite, by volume) in a 12-cm-diameter × 15-cm-deep plastic pot. They were maintained in the greenhouse during the experiments, with temperatures ranging from 20 to 25 °C for 30 days. The seedlings were then inoculated with a preparation of 10^5 conidia mL^{-1} according to the protocol of Punja and Parker (2000). Four groups of 25 seedlings each were inoculated with (a) *F. oxysporum* (FO) M3 isolate, (b) *F. solani* (FS) M5 isolate, (c) FO + FS jointly, or (d) sterile distilled water (control). Seedlings were distributed at random in the greenhouse. To identify the most aggressive isolates for the pathogenicity test, M3 and M5 were selected in a preliminary study performed for the same pepper cultivar (data not shown). Disease severity was scored on a scale as follows: S0 = healthy plants, S1 = canker at the base of stem, S2 = base lesions joined into a single canker, and S3 = dead plants.

Evaluation was initiated at 5 days after the first symptoms of damping-off were observed on the stem base and ended after 35 days when the plants died. *Fusarium* spp. were re-isolated from inoculated seedlings to fulfil Koch's postulates, and their identities were confirmed through molecular and morphological approaches. Pathogenicity tests were conducted three times.

Statistical analysis

Based on data of severity (scale 0 to 3) obtained from four replicates of 25 plants each, a Kruskal-Wallis test was performed providing the nature of the data (ratings) to determine whether there were significant differences in the severity of the inoculated pathogens. In addition, the incidence (proportion of symptomatic plants in relation to the total plants) and seedling mortality, i.e. those that were in category 3 at the end of the experiment, were determined. Control plants were not inoculated.

Histopathological test of the stem base and crown rot

The transition regions of the crown rot were selected for this study. The seedlings were analysed during stage 1 to maintain the integrity of symptomatic vegetal tissue during sample processing. Although cutting was attempted in tissue during the posterior stages, a clear observation of the regions was not possible. Three treatments were utilised (FO-M3, FS-M5, or FO-M3 + FS-M5 isolates) in addition to a control, and the crown rot areas with necrotic symptoms were fixed in FAA solution (ethanol:glacial acetic acid:formaldehyde:water in a 50:5:10:35 proportion) for 48 h. Each sample was gradually dehydrated in ethanol and ethylene and then processed for paraffin infiltration according to Zavaleta-Mancera and Engleman (1994). Cross sections that were 20 μm in thickness were obtained using a rotary microtome Spencer 820 (American Optical Company, USA) and stained with safranin 'O' (0.05% safranin 'O', 13% NH_2SO4, 0.01% phenol in water) and Fast Green FCF (0.12% FCF Green in 95% ethanol) (Ruzin 1999). The sections were observed at × 40 magnification using a compound light microscope (Zeiss Axiosiop plus, Oberkochen, Germany) and were photographed using an Axiocam MRc 5 digital camera (Zeiss, Oberkochen, Germany).

Results

Isolated fungi

Eleven isolates recovered from the stem base and crown rots of infected 'Poblano' pepper adult plants with typical symptoms of yellowing or wilting were selected for this study. Three colonies grown in PDA medium showed similarity to *F. oxysporum*; they had grown to less than 6 cm in diameter after 8 days and had abundant aerial mycelium forming a convex shape, and the isolates were pale pink to deep purple and magenta in colour with a cottony texture. The macroconidia had an average of one to five septa with conidial dimensions of 23–54 × 3–4.5 μm; they were half-moon shaped and slightly curved, with delicate, fine walls. Their apical cells were sharp, and their basal cells had an upright form, although sometimes both extremities were sharp (Fig. 1a).

In addition, eight colonies grown in the same medium resembled *F. solani*; they had grown to approximately 6 cm in diameter after 10 days and had aerial mycelium with a concave shape, smooth texture, and white to pale yellow colour. The colour of the colony beneath the surface layer varied from cream to light blue or clear coffee. The macroconidia had three to four septa with dimensions of 32–55 × 3–5.0 μm and were slightly curved, and in many, the terminal cell was slightly crushed (Fig. 1b).

Sequence analyses

As expected, ITS5/NL4 primer amplification yielded a fragment of 1100 base pairs (bp) (Fig. 2a) and the EF1-a/EF2-a yielded a fragment of ~ 700 bp (Fig. 2b) for all isolates. The results from BLASTN (https://blast.ncbi.nlm.nih.gov/Blast) and *Fusarium* ID (http://www.fusariumdb.org/) confirmed that three of the isolates that caused wilt symptoms in

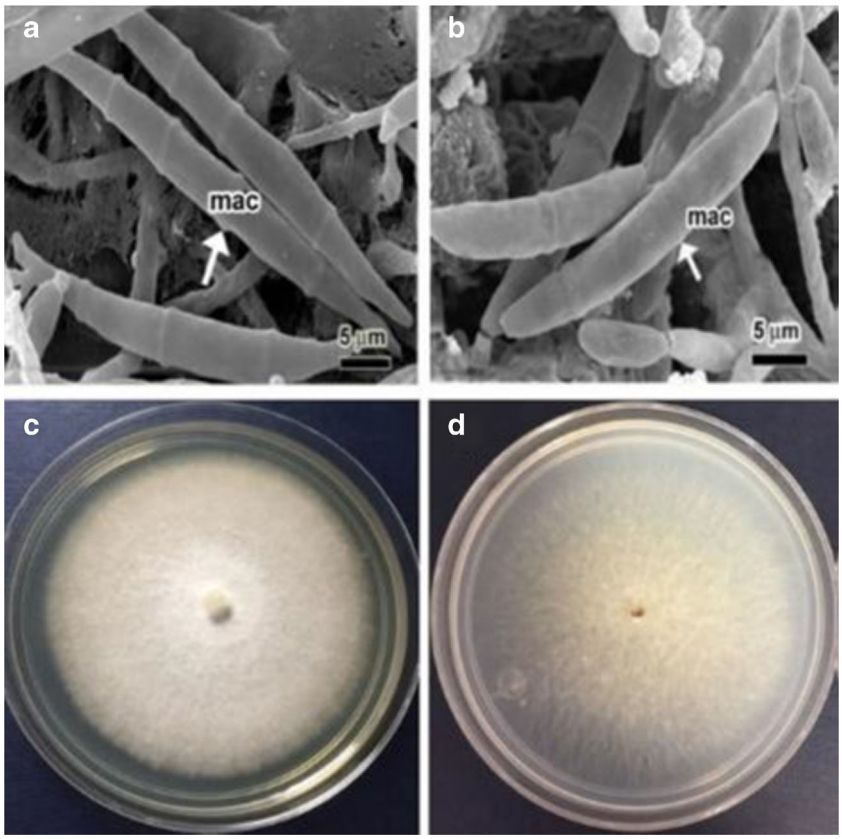


Fig. 1 Scanning electron microscope images of **a** *Fusarium oxysporum* and **b** *F. solani*. mac: macroconidia with two or three divisions. **c** *F. oxysporum* had colonies less than 6 cm in diameter after 8 days. **d** *F. solani* grew more slowly and was less than 6 cm in diameter after 10 days

'Poblano' mature pepper plants were *F. oxysporum* and eight isolates were *F. solani* (Table 1).

The ITS rDNA regions and nLSU rDNA revealed that the nucleotide sequence identities ranged from 99 to 100% for both species. The sequences of *F. oxysporum* and *F. solani* obtained in this study were deposited in GenBank at NCBI under the accession numbers EU082095-EU082105 (Table 1). The amplified sequences of TEF1 partial sequences are listed in Table 2.

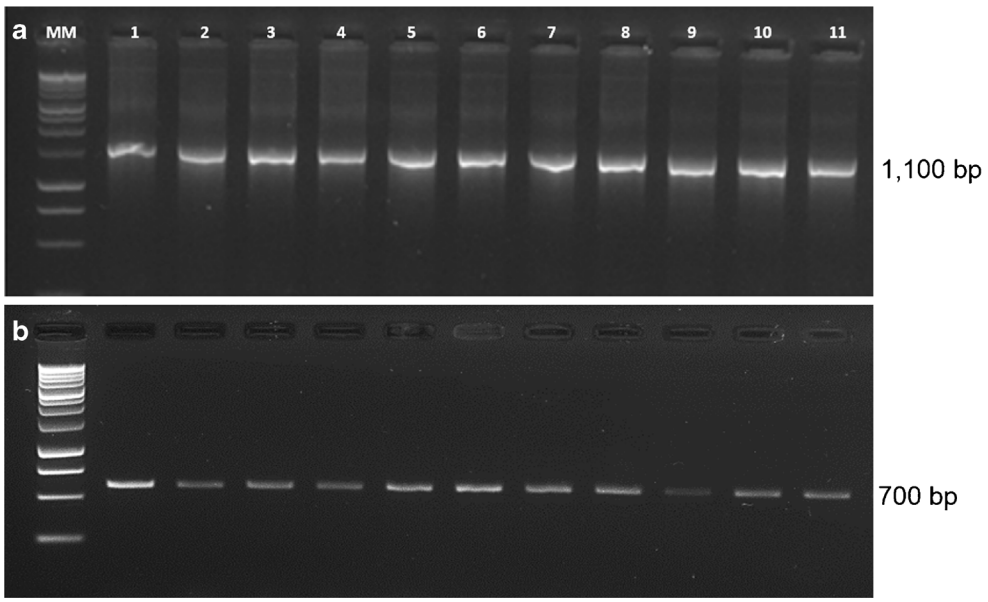


Fig. 2 PCR amplifications of **a** the internal transcribed spacer (ITS) complete region including large subunit rDNA partial sequence, and **b** translation elongation 1-α partial sequences. MM, molecular marker 1Kb; bp, base pair

Phylogenetic reconstruction

For phylogenetic reconstruction sequences, the *F. solani* and *F. oxysporum* that were downloaded from Genbank formed two different clades. In the first clade, the sequences identified in this study as *F. solani* were grouped with accession no. EU029589, which corresponds to the S-0900 isolate from sorghum in the USA, and had a maximum identity of 100%. Other subgroups were observed in the same clade and belonged to sequences with a maximum identity of 99% from humans in Brazil, India, and the USA; in all cases, they were reported as a causal agent of rot in vegetables. The second clade group of sequences belonged to *F. oxysporum*; they were clustered with sequence KM030315, which corresponds to the NRRL 66023 isolate from the stem of a pre-silk corn plant in the USA. The sequence of *Phoma herbarum* included in this study was clearly clustered in a different clade and considered to be an out-group (Fig. 3).

The *Fusarium* species complex of each isolate obtained in the present work was clearly determined by grouping and was consistent with those mentioned by O'Donnell et al. (2016), who studied *Fusarium* recovered from veterinary sources in the USA. Isolates of *F. oxysporum* and *F. solani* corresponded to the *Fusarium oxysporum* species complex (FOSC) and the *Fusarium solani* species complex (FSSC) (Fig. 4), respectively.

Once the identity of the isolates was confirmed, they were deposited in the 'Colección of microorganismos from Centro Nacional de Recursos Genéticos' with numbers CM-CNRG 0424 and CM-CNRG 0425, respectively. This collection is a member of the World Data Collection for Microorganism (WDCM) under number WDCM 1006.

Pathogenicity test

Typical wilt symptoms were observed in 'Poblano' pepper seedlings inoculated with *F. oxysporum*, *F. solani*, or both. Necrotic tissues in the stem and crown rot began as small spots with a brown dark colour that were 2 mm in diameter, and later, they extended to the whole stem base to form a single black canker until the plant died 35 days after inoculation (dai). Necrotic spots were first observed five dai. The control seedlings did not develop symptoms. All inoculated seedlings (100%) were infected. No obvious differences in symptom severity were observed for *F. oxysporum* or *F. solani* at the cankers in the stem base and crown rot (Table 3).

The results suggest that *F. oxysporum* and *F. solani* are both pathogens of 'Poblano' pepper seedlings, causing both necrosis of the stem base and crown rot.

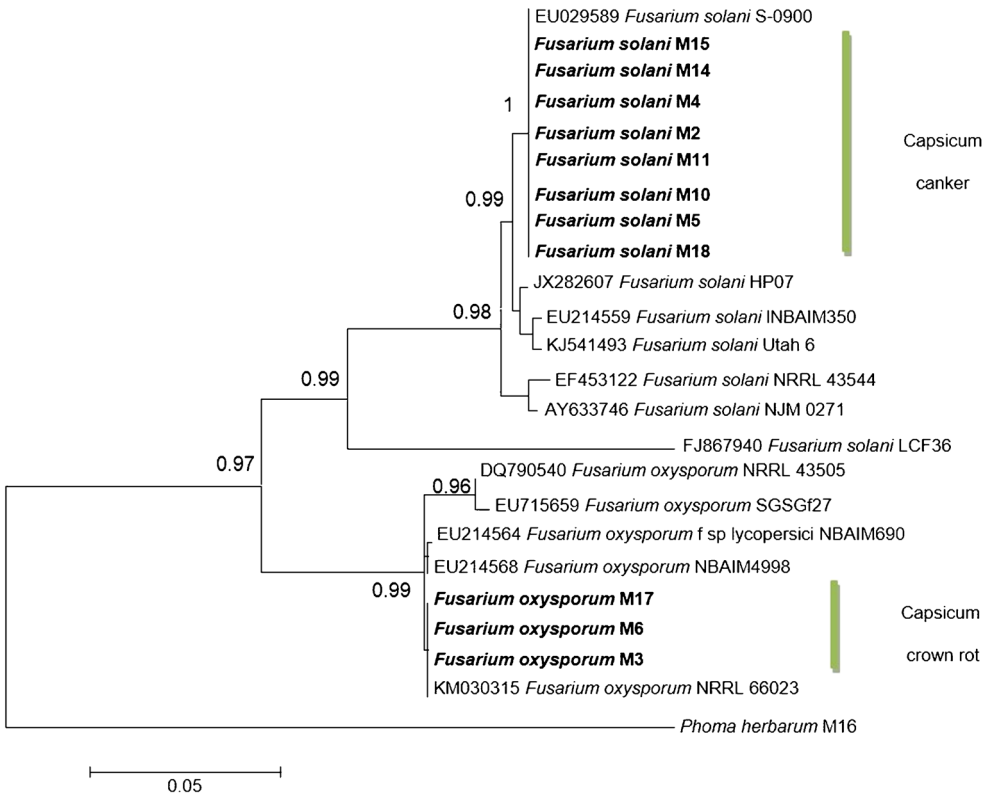
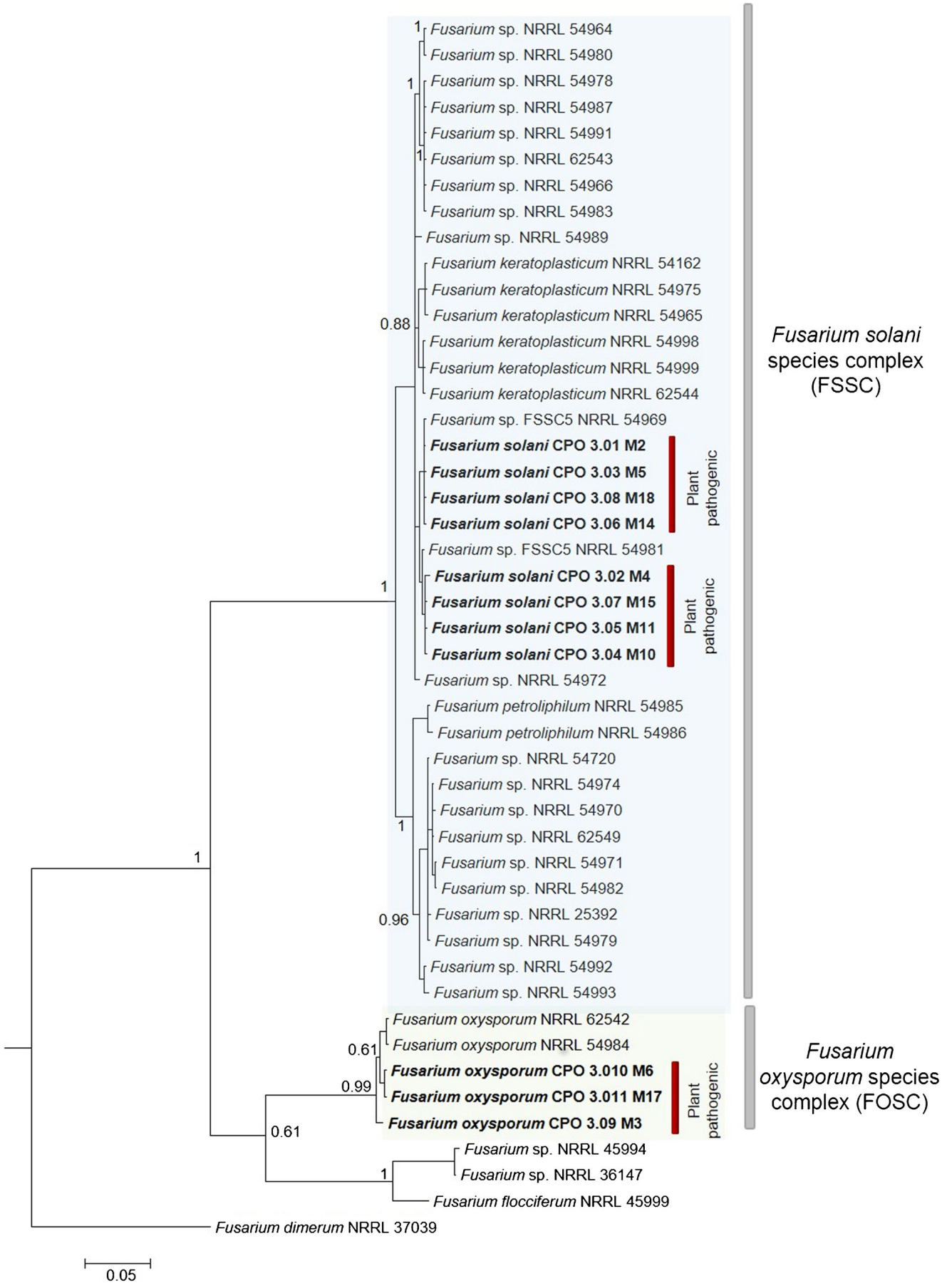


Fig. 3 Phylogenetic tree constructed with concatenated Bayesian inference using sequences belonging to the internal transcribed spacer (ITS) complete region, large subunit rDNA partial sequence, and translation elongation 1-α partial sequences. Amplifications corresponding to *Fusarium oxysporum* and *F. solani* were recovered from 'Poblano' pepper cv. San Luis plants that were decaying, yellowing, or wilting. The Bayesian inference posterior probability (> 0.95) is indicated below the internodes. The tree is rooted on a sequence of *Phoma herbarum* EU082106


Fusarium sp. NRRL 54964
Fusarium sp. NRRL 54980
Fusarium sp. NRRL 54978
Fusarium sp. NRRL 54987
Fusarium sp. NRRL 54991
Fusarium sp. NRRL 62543
Fusarium sp. NRRL 54966
Fusarium sp. NRRL 54983
Fusarium sp. NRRL 54989
Fusarium keratoplasticum NRRL 54162
Fusarium keratoplasticum NRRL 54975
Fusarium keratoplasticum NRRL 54965
Fusarium keratoplasticum NRRL 54998
Fusarium keratoplasticum NRRL 54999
Fusarium keratoplasticum NRRL 62544
Fusarium sp. FSSC5 NRRL 54969
Fusarium solani CPO 3.01 M2
Fusarium solani CPO 3.03 M5
Fusarium solani CPO 3.08 M18
Fusarium solani CPO 3.06 M14
Plant pathogenic
Fusarium sp. FSSC5 NRRL 54981
Fusarium solani CPO 3.02 M4
Fusarium solani CPO 3.07 M15
Fusarium solani CPO 3.05 M11
Fusarium solani CPO 3.04 M10
Plant pathogenic
Fusarium sp. NRRL 54972
Fusarium petroliphilum NRRL 54985
Fusarium petroliphilum NRRL 54986
Fusarium sp. NRRL 54720
Fusarium sp. NRRL 54974
Fusarium sp. NRRL 54970
Fusarium sp. NRRL 62549
Fusarium sp. NRRL 54971
Fusarium sp. NRRL 54982
Fusarium sp. NRRL 25392
Fusarium sp. NRRL 54979
Fusarium sp. NRRL 54992
Fusarium sp. NRRL 54993
Fusarium oxysporum NRRL 62542
Fusarium oxysporum NRRL 54984
Fusarium oxysporum CPO 3.010 M6
Fusarium oxysporum CPO 3.011 M17
Fusarium oxysporum CPO 3.09 M3
Plant pathogenic
Fusarium sp. NRRL 45994
Fusarium sp. NRRL 36147
Fusarium flocciferum NRRL 45999
Fusarium dimerum NRRL 37039
1
1
1
1
0.88
1
1
0.96
1
0.61
0.61
0.99
1
0.05
Fusarium solani species complex (FSSC)
Fusarium oxysporum species complex (FOSC)

◀ **Fig. 4** Phylogenetic tree constructed with Bayesian inference using translation elongation factor 1-α partial sequences of *Fusarium* isolates belonging to four *Fusarium* species complexes. Plant pathogenic isolates were clustered with *Fusarium solani* species complex (FSSC) and *Fusarium oxysporum* species complex (FOSC). The bar scale represents the number of substitutions per site

Seedling mortality caused by *Fusarium* spp. in inoculated pepper seedlings

For the mortality variable, the data indicated that while the percentage of mortality in seedlings inoculated with the combination of *F. oxysporum* and *F. solani* (FOS) was slightly less than those of FO and FS 49, 54, and 56%, respectively, there were no significant differences between the three combinations, but there was a significant difference with the control ($F = 135.4$, $P < 0.001$).

Considering this evaluated variable, the data indicate that both FO, FS, and the combined inoculation of both resulted in a similar level of mortality, whether alone or in a mixed inoculation pathogenicity.

Histopathological observations

In control seedlings, the parenchyma of the cortex, epidermal, endodermal tissue, and vessels of the vascular cylinder were intact (Fig. 5a, b). Light micrograph cross sections (Fig. 5) of the crown rot of 'Poblano' pepper seedlings inoculated with *F. oxysporum* showed two microconidia around the xylem vessels; one had penetrated a vascular vessel of the xylem but was impeded by the thickening of this structure (Fig. 5c) due to the defence response of the plant to the pathogen. In another sample with an annular disturbance, intercellular growth of the hyphae and thickening of the Casparian strips (Fig. 5d) were observed. Seedlings inoculated with *F. solani* had necrosis of the epidermal cells, the presence of macroconidium (mac, Fig. 5f) and collapse of the subepidermal parenchyma (Fig. 5e). We also observed necrosis of the epidermis and external layer of the cortex (Fig. 5f), intercellular growing hyphae, and partial destruction of the Casparian strips. Inoculated seedlings with both pathogens had a vascular cylinder without damage due to the presence of pathogens, although they had localised damage to the endodermal tissue of the Casparian strips and necrosis at the epidermis (Fig. 5g). Plants inoculated with both pathogens *F. oxysporum* and *F. solani* (Fig. 5h) had an epidermal layer with necrotic tissue (NT) and abnormal development of parenchymatic cells.

Table 3 Severity of disease in 'Poblano' pepper seedlings inoculated in a greenhouse with *Fusarium oxysporum*, *F. solani*, and their mixture after 35-day post-inoculation

Isolates	Seedling numbers (*n*/25)			
	S0	S1	S2	S3
F. oxysporum M3	2	3	6	14
F. solani M5	1	2	8	14
F. oxysporum M3–*F. solani* M5	1	2	10	12
Control	25	0	0	0

Symptom severity based on the following scale: S0, healthy plants; S1, canker at the base of stem; S2, base lesions joined into a single large spot (canker); and S3, dead plant.

Discussion

Vascular wilt is among the most destructive plant diseases that occurs in annual crops (Michielse and Rep 2009; Ferniah et al. 2014). Cultural, chemical, and biological measures to control vascular wilt are required to reduce its devastating effects, as the causative agents of this disease are considered to be soilborne pathogen fungi (Yadeta and Thomma 2013). The precise identities of the causal agents of wilting that affect 'Poblano' mature pepper plants in field production, as well as damping-off in pepper seedlings in greenhouses in Mexico, are not known. Different fungal species have been reported in related cultivars, including *Fusarium* spp. (Schumann and D'Arcy 2009), *Fusarium lateritium* (Vasquez-Lopez et al. 2009), *F. oxysporum* (Lievens et al. 2008; Perez-Hernandez et al. 2014), and *F. solani–F. oxysporum* together (Fayzalla et al. 2008), but not *F. solani* alone (Figs. 6 and 7).

In the present research, phylogenetic reconstruction based on amplification of conserved ribosomal operon genes and translation elongation factor 1-α found only two species of the *Fusarium* genus involved in these symptoms. *F. oxysporum* was isolated from crown rot and *F. solani* was recovered from rotten tissues at the stem base. They clustered in clades that are phylogenetically different, but together form part of a larger monophyletic group with strong statistical support (Geiser et al. 2013). In both cases, there was general plant decay, including wilt and root rot. These symptoms in mature plants were attributed to a reduction in water flow from the root to the aerial parts of the plants because of the obstruction or blockage of the xylem vessel with fungal hyphae. Fungi colonise cortical cells from which the hyphae migrate intercellularly toward vascular parenchyma cells and invade the xylem vessels, where conidiospores are produced and disseminated (Di Pietro et al. 2003; Schumann and D'Arcy 2009). The results of this study indicated that the isolates recovered from the mature plants were capable of causing damping-off in seedlings under greenhouse conditions. In this tissue, penetrating fungal hyphae and their differentiation

Fig. 5 Severity symptoms of 'Poblano' pepper seedlings inoculated with *Fusarium* spp. **a** Stage 1, canker at the base of the stem and yellowing; **b** Stage 2, whole necrotic crown; **c** Stage 3, dead plants; and **d** control plant, no symptoms

cause damage mainly in the external cell layers in the root cortex and in internal layers comprising the endodermis (Genre et al. 2008), which is characterised by broken Casparian strips (Fig. 5). Endodermal cell walls perform an important function as apoplastic barriers, like the suberin lamellae in the roots of vascular plants (Schreiber and Franke 2011; Robbins II et al. 2014). The Casparian strips are a tangential deposition of a complex mixture of biopolymers in the cell wall of the endodermis, including carbohydrates, lignin, suberin, and structural proteins. These cell wall structures offer not only a significant barrier to apoplastic water flow but also a physical barrier to the movement of microorganisms, and it is well recognised that cell walls are degraded by enzymes secreted by *Fusarium* spp. and other pathogens during the infection process (Jones et al. 1972). Polygalacturonases have been isolated from *Fusarium* plant pathogens; the enzyme endoPGs macerates plant tissue by depolymerising homogalacturonan, a major component of the plant cell wall

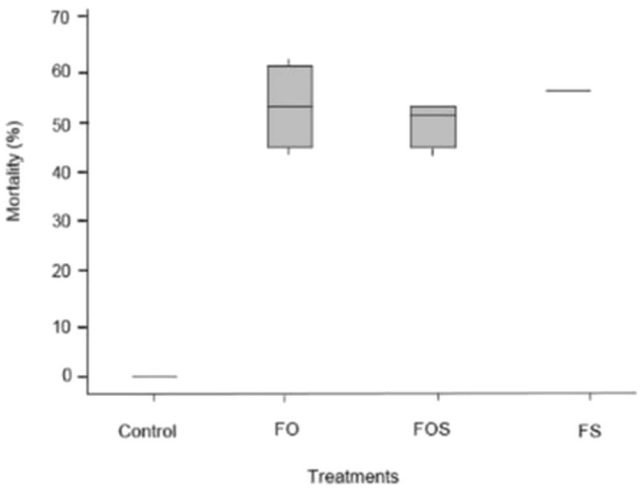


Fig. 6 Seedling mortality percentage of *Capsicum annuum* cv 'Poblano' inoculated with *Fusarium oxysporum* (FO), *F. solani* (FS), and *F. oxysporum* and *F. solani* jointly (FOS). There were no significant differences between the two isolates, but there was a significant difference with the control ($F = 135.4$, $P < 0.001$). No synergistic effect was observed with joint inoculation

(Collmer and Keen 1986). Recent work on hormone signalling, propagation of calcium waves, and plant-fungal symbiosis has provided evidence that supports the hypothesis that the endodermis acts as a signalling centre (Robbins II et al. 2014).

It is known that *F. oxysporum* secretes fusaric acid (Singh et al. 2017), polygalacturonase, cellulase, α- and β-galactosidases, α- L-arabinofuranosidase, and β-xylosidase (Jones et al. 1972). *F. solani* is able to oxidise α, β-unsaturated alcohols, such as coniferyl alcohol, to the corresponding aldehyde, as well as the non-esterified side chain of dehydroconiferyl alcohol. *F. solani* is also able to degrade lignin compounds (Iwahara et al. 1980). However, degradation of chemically distinct lignified cell walls is not easy (Schreiber and Franke 2011). The primary function of the Casparian strip is to force water through a symplastic pathway as it enters the stele. In this manner, the Casparian strip controls the ion movement into the vascular system. In consideration of this feature, the function of the conductive elements is the most important feature for maintaining equilibrium in the vascular bundles and the union between adjacent cells. Rupture of this strip causes an alteration in the regular process of water movement and ions from the exterior to the interior of the plant and also alters the interior concentration of oxygen, creating significant gaps in the diffusion barrier and causing cells remain separated from each other (Robbins II et al. 2014). In addition, the plant cell wall represents a major barrier to the penetration and spread of microorganisms. It has been observed that pathogens produce cell-wall-degrading enzymes during the development of vascular wilt disease, penetrate different layers of the root cortex to the vascular system, and colonise the host by spreading upward through xylem vessels (Beckman 1987).

Light micrographs of seedlings inoculated with either *F. oxysporum*-M3 or *F. solani*-M5 show damage to the epidermis and parenchyma cells. They also show significant damage to the Casparian strips. Similarly, seedlings inoculated with both of these pathogens had vascular and epidermal damage, including to the surrounding epidermal cells. In all cases, we clearly observed damage to the

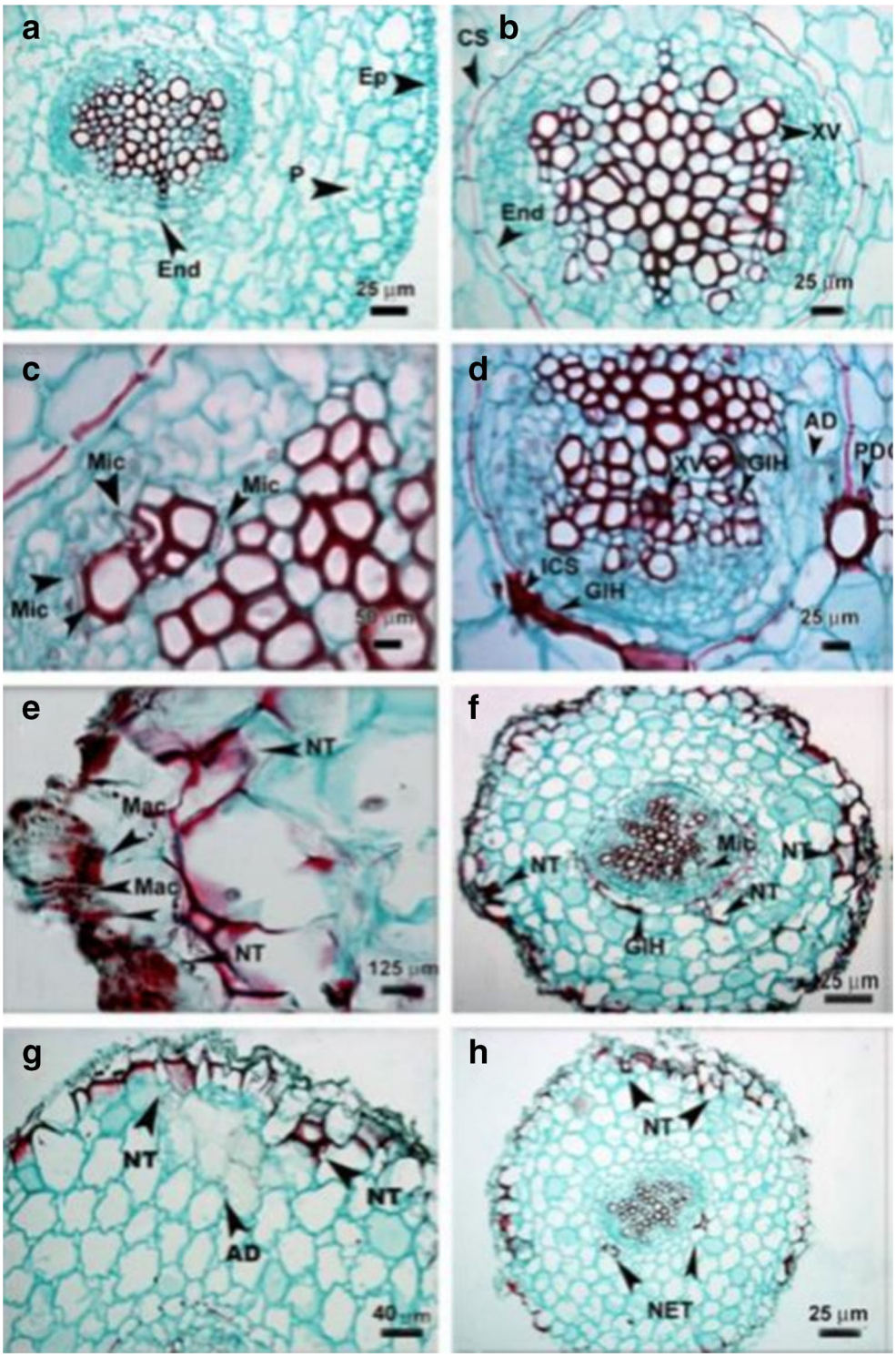


Fig. 7 Histology of *Fusarium*-infected crown rot. **a**–**b** Controls. **c**–**d** Crown rot of inoculated plant with *Fusarium oxysporum* displaying microconidium around the xylem vessels, necrotic cells in the epidermis, and disturbance of the Casparian strips. **e**–**f** Plants inoculated with *Fusarium solani* species complex. **g**–**h** Plants inoculated with both pathogens *F. oxysporum* and *F. solani*. ICs, increased Casparian strips; GIH, grow intercellular hyphae; AD, abnormal development; PD, peripheral destruction of the endodermal and Casparian strip tissue; XVO, xylem vessel obstruction; NT, necrotic tissue; Mac, macroconidium; Mic, microconidium

aforementioned structures. These results were unexpected in that there are no reports of which we are aware that show the breakdown of Casparian strips in pepper seedlings infected by *Fusarium* species. By contrast, light micrographs of the stem base of control seedlings (Fig. 5a) confirmed that there were healthy cells in the epidermis, parenchyma, endodermis, and xylem.

During the initial stages of the interaction, fungal pathogens must sense stimuli from the plant and respond with appropriate morphogenetic and biochemical changes. The general conditions of the host genotypes, environmental factors, and virulence of the pathogen can determine the speed and severity at which the symptoms develop (Roncero et al. 2003), as was observed in seedlings inoculated with *F. oxysporum*

and *F. solani* both together and independently in the present study.

The fact that the fungus identified on rockwool cubes of the transplants in the greenhouse requires confirmation to substantiate this result indicates that maintaining a clean transplant production area is important. No fungicides were labelled for *Fusarium* stem rot in the greenhouse. Growers of 'Poblano' peppers are very concerned about the increasing incidence of fungal diseases that result in severe yield and economic losses due to a reduction in cultivated area. Lastly, observation of damage to the Casparian strips provides an opportunity for further research to determine if this is the mechanism that is responsible for the gradual death and ripening of 'Poblano' pepper plants and the prompt death of their seedlings. Additionally, we should be able to ascertain whether, with better management of fungicide applications and biological or cultural strategies, producers will be able to control these two *Fusarium* species that cause harm in 'Poblano' pepper production in Mexico. Successful management of wilt diseases will be very important to ensure future economic viability of pepper production seedlings and pepper fruits for international trade.

Conclusions

Fusarium oxysporum and *F. solani* are two species of *Fusarium* that are responsible for wilting in 'Poblano' pepper mature plants and damping-off in seedlings during their growth in greenhouses. In mature plants, they only colonise the crown or base stem. Histological studies performed in inoculated seedlings with the isolates both separately and jointly showed necrotic cells of the external cortex, collapse of epidermal cells, necrotic endodermis, Casparian strip rupture, and changes in the cell size and wall thickness of the xylem during stage 1 of infection when mycelial growth was absent inside the vascular tissue of the pepper seedlings. Moreover, Casparian strip rupture caused by *F. oxysporum* and *F. solani* resulted in permeability loss and regulatory activity to maintain cellular equilibrium. To address these issues, pepper growers producing seedlings must take special care during seedling production in greenhouses because the aggressiveness of these isolates leads to rapid seedling death.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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