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Phylogeny and pathogenicity of soilborne fungi associated with wilt disease complex of tomatillo (*Physalis ixocarpa*) in northern Sinaloa, Mexico

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Abstract Wilt disease complex is one of the most important diseases of tomatillo (*Physalis ixocarpa*) in the production areas of Mexico. Disease symptoms include wilting, poor growth, discoloration of vascular tissues, root rot, and death of plants. The aims of this study were to identify the fungi associated with wilt disease complex of tomatillo by the combination of phylogenetic analyses and morphological characterization, as well as to determine their pathogenicity and virulence on tomatillo seedlings. A total of 88 fungal isolates were obtained from symptomatic plants from 19 tomatillo fields distributed in northern Sinaloa, Mexico. Subsequently, a subset of 37 isolates representing the range of geographic origin was selected for further morphological and molecular characterization as well as pathogenicity tests. Phylogenetic analyses using Maximum Likelihood were used to identify 15 isolates of *Rhizoctonia* (ITS sequence data), 14 isolates of *Fusarium* (EF-1α sequence data), five isolates of *Macrophomina* (ITS, EF-1α, BT, and ACT sequence dataset) and three isolates of *Neocosmospora* (EF-1α sequence data) to species level. Pathogenicity tests were performed on tomatillo seedlings (cv. Gran Esmeralda) under greenhouse conditions. Phylogenetic analyses of 37 fungal isolates allowed the identification of *Rhizoctonia solani* AG 4-HGI (40.5%), *Fusarium oxysporum* (29.8%), *Macrophomina phaseolina* (13.5%), *F. nygamai* (8.1%) and *Neocosmospora falciformis* (8.1%). All fungal species were found to be pathogenic on tomatillo seedlings but a significant difference in disease severity was observed. To our knowledge, *F. nygamai, M. phaseolina* and *N. falciformis* were recorded for the first time infecting tomatillo in Mexico and worldwide.

Keywords *Fusarium* · *Rhizoctonia* · *Macrophomina* · *Neocosmospora* · Pathogenicity · Phylogeny

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Introduction

The tomatillo (*Physalis ixocarpa* Brot. ex Horm.), also called husk tomato or green tomato, is one of the most

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widely cultivated horticultural crops in Mexico, and more than 60% of the production is concentrated in the Mexican states of Sinaloa, Zacatecas, Jalisco, Puebla, Michoacán, and Sonora (SIAP 2018). In 2017, Sinaloa was the first largest producer of tomatillo in Mexico with a production of 150,697 t (SIAP 2018) with the main production area in this state is located in the northern region, including the municipalities of Guasave, Ahome, and El Fuerte.

In Mexico, the tomatillo plants are commonly affected by several plant pathogens including fungi (Apodaca-Sánchez et al. 2008), viruses (Méndez-Lozano et al. 2001; De La Torre-Almaráz et al. 2003; Gámez-Jiménez et al. 2009; González-Pacheco and Silva-Rosales 2013), and phytoplasmas (Santos-Cervantes et al. 2006; Mauricio-Castillo et al. 2018). The most important fungal diseases found in tomatillo fields in Mexico are white smut (*Entyloma australe*), white mold (*Sclerotinia sclerotiorum*) (Apodaca-Sánchez et al. 2008), leaf spot (*Cercospora physalidis*) (Félix-Gastélum et al. 2007; Apodaca-Sánchez et al. 2008), powdery mildew (*Podosphaera xanthii*) (Félix-Gastélum et al. 2007), leaf blight (*Alternaria* sp.) (Soto et al. 1998), and wilting (complex of soilborne fungi) (Apodaca-Sánchez et al. 2008).

Tomatillo wilt is a serious disease that commonly occurs wherever the crop is cultivated in Mexico and has been associated with a complex of soilborne fungi, such as *Fusarium oxysporum*, *Neocosmospora solani* (syn. *Fusarium solani*), *Rhizoctonia solani*, *Pythium* sp., *Macrophomina phaseolina* and *Sclerotium rolfsii.* The first visible symptom of the disease is yellowing of the foliage that is followed by poor growth, wilting, and death of plants. On roots, a light to dark brown rot is observed, which sometimes extends towards the neck and base of the stem. In diseased plants, the number and size of fruits are smaller, and they fall easily. The disease can occur at any stage of crop development, with economic losses that can exceed 50%. This disease is very important because tomatillo cultivars are highly sensitive to this fungal complex and the disease management is difficult and expensive using cultural and chemical methods (Apodaca-Sánchez et al. 2008).

This study aimed to identify the fungi associated with wilt disease complex of tomatillo in Sinaloa by the combination of phylogenetic analyses and morphological characterization, as well as to determine their pathogenicity and virulence on tomatillo seedlings.

Materials and methods

Sample collection

During several surveys carried out during 2016 and 2017 seasons, a total of 95 plants showing wilt and root rot symptoms were collected from 19 commercial tomatillo fields distributed in the main production area in northern Sinaloa (Ahome, El Fuerte, and Guasave municipalities), Mexico.

Isolation, purification, and conservation of fungi

For isolation, pieces of roots (5 · 5 mm) were taken from the margin between necrotic and healthy tissues. Their surface was sterilized by dipping in 2% sodium hypochlorite solution (NaOCl) for 1 min, rinsed two times with sterile distilled water and dried on sterilized paper. The pieces were placed in Petri plates with potato dextrose agar (PDA) (Difco, Detroit, MI, USA). The plates were incubated at 25 ºC for 3 days in darkness. Then, mycelial plugs (5 mm in diameter) from the edge of fungal colonies were aseptically transferred to fresh PDA. For isolates morphologically resembling *Rhizoctonia* and *Macrophomina*, pure cultures were obtained by transferring hyphal tips from the colony margin onto fresh PDA and incubated at 25 °C in the dark. For *Fusarium*-like colonies, single germinated conidia were removed and transferred to a new Petri plate containing PDA. Cultures of each isolate were deposited in the Culture Collection of Phytopathogenic Fungi of the Faculty of Agronomy of Fuerte Valley (FAVF) at the Sinaloa Autonomous University (Juan Jose Rios, Sinaloa, Mexico).

Morphological characterization

A total of 37 isolates (15 of *Rhizoctonia*, 14 of *Fusarium*, five of *Macrophomina*, and three of *Neocosmospora*) were selected as representatives. *Fusarium* and *Neocosmospora* isolates were incubated at 25 °C with a 12-h photoperiod for 12 days on PDA and Carnation Leaf Agar (CLA) to examine the shape and size of macro, microconidia, and chlamydospores (Leslie and Summerell 2006) using a Leica TCS-SP5X confocal microscope (Leica, Germany). For *Rhizoctonia* isolates, young hyphae (5-days-old) of each isolate were stained with Safranin O + 3% KOH (Sneh et al. 1991) and the number of nuclei was determined for 20 cells.

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For *Macrophomina* isolates, slides containing fungal structures (mycelium and sclerotia) were stained with lactophenol cotton blue and observed under a light microscope.

DNA extraction, PCR amplification, and sequencing

Molecular identification was performed for 37 fungal isolates. Aerial mycelium from a 6-day-old culture was scraped from the medium using a sterile spatula and was placed in 2 mL microtubes. Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the protocols provided by the manufacturer. DNA concentrations were quantified using a NanoDrop One (Thermo Fisher Scientific, Madison, WI, USA) and the samples were diluted to 100 ng μL^{-1} for polymerase chain reaction (PCR).

For *Rhizoctonia* isolates ($n = 15$), the internal transcribed spacer (ITS) region of ribosomal DNA was amplified. For *Fusarium* ($n = 14$) and *Neocosmospora* ($n = 03$) isolates, part of the translation elongation factor 1-alpha (EF-1α) gene was amplified. For *Macrophomina* isolates ($n = 05$), the ITS region and part of EF-1α, β-tubulin (BT2), and actin (ACT) genes were amplified. PCR amplifications were conducted in a Bio-Rad CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA), differently programmed for each primer set (Table 1). The PCR products were separated by electrophoresis in 1% agarose gels stained with ethidium bromide and the images were documented with the Gel Doc XR + System (Bio-Rad Laboratories, Hercules, CA, USA). The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and both strands were sequenced by Macrogen (Macrogen Inc., Seoul, Korea) using the same primers that were used for amplification. All sequences generated in this study were deposited in GenBank (Table 2).

Phylogenetic analysis

To determine the identity of the 37 isolates to species level, phylogenetic analyses were conducted. The phylogeny was reconstructed by analyses from ITS sequences for *Rhizoctonia*, EF-1α sequences for *Fusarium*, ITS and EF-1α sequences for *Neocosmospora*, and ITS, EF-1α, BT2, and ACT sequences for *Macrophomina*. For the last two genera, phylogenetic reconstructions were performed using the concatenated sequences of the respective genes obtained with the SequenceMatrix (Vaidya et al. 2011). Forward and reverse sequences were assembled using the Staden Package (Staden et al. 1998). Multiple sequence alignments for each locus were independently performed using ClustalX v.1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and various alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, divergent delay sequences = 25%). The alignments were manually adjusted when necessary. The events of indels (gaps) were considered in the phylogenetic analysis (Young and Healy 2003). Nucleotide sequences of reference isolates for each genus obtained from GenBank were included in the analyses. The evolutionary history was inferred by using the Maximum Likelihood method and performed using the MEGA 7 (Kumar et al. 2016).

Pathogenicity and virulence tests

Pathogenicity tests involving fungal isolates of *Rhizoctonia* ($n = 15$), *Fusarium* ($n = 14$), *Macrophomina* ($n = 05$), and *Neocosmospora* ($n = 03$) were performed on tomatillo seedlings (cv. Gran Esmeralda) under greenhouse conditions. Tomatillo seeds were surface disinfested with 2% NaOCl solution for 1 min, rinsed two times with sterile distilled water and dried on sterilized paper. Seeds were germinated for four days on wet filter paper in glass Petri dishes at room temperature. Two germinated seeds were planted into 500 mL plastic pots containing 250 g of a sterilized substrate composed of sand + peat moss (1:3) and inoculated with four mycelial plugs (5 mm in diameter) removed from the margin of a 5-day-old PDA culture. Four non-colonized agar plugs were used on control plants. The pots were incubated under greenhouse conditions at 20–30 °C. Pots were irrigated when necessary to avoid dryness and fertilized with complex NPK solution after planting and every week. Three replicates (pots) arranged in a completely randomized design were used per each isolate. Twenty-five days after inoculation, plants were removed from pots, the roots were gently washed under running tap water and dried with paper towels. The virulence of each isolate was assessed by measuring disease severity using Correll et al. (1986) scale with some modifications, where: 0 = no

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Table 1 Primers used in this study, with PCR conditions and references

Fungus	Primers/Code	Reference	PCR conditions
Rhizoctonia	ITS5 / ITS4	White et al. 1990	35 cycles; 30 s at 94 °C, 1 min at 54 °C, 1 min at 72 °C
Fusarium	EF1-728F / EF1-986R	Carbone and Kohn 1999	35 cycles; 30 s at 94 °C, 1 min at 52 °C, 1 min at 72 °C
Neocosmospora	ITS5 / ITS4	White et al. 1990	35 cycles; 30 s at 94 °C, 1 min at 54 °C, 1 min at 72 °C
	EF1-728F / EF1-986R	Carbone and Kohn 1999	35 cycles; 30 s at 94 °C, 1 min at 52 °C, 1 min at 72 °C
Macrophomina	ITS5 / ITS4	Alves et al. 2008	35 cycles; 30 s at 94 °C, 1 min at 54 °C, 1 min at 72 °C
	EF1-688F / EF1-1251R	Alves et al. 2008	35 cycles; 30 s at 94 °C, 1 min at 52 °C, 1 min at 72 °C
	Bt2A / Bt2B	Glass and Donaldson 1995	35 cycles; 30 s at 94 °C, 1 min at 52 °C, 1 min at 72 °C
	ACT-512F/ACT-783R	Carbone and Kohn 1999	35 cycles; 30 s at 94 °C, 1 min at 57 °C, 1 min at 72 °C

discoloration; 1 = slight discoloration of the vascular tissue in roots; 2 = extensive discoloration of the vascular tissue in roots; 3 = slight discoloration of vascular tissue and cortex; 4 = extensive discoloration of vascular tissue and cortex; 5 = root completely necrotic or dead seedling. The entire experiment was done twice, obtaining similar results in both tests. Differences in virulence caused by soilborne fungi were determined by nonparametric analysis of variance (ANOVA) with Kruskal-Wallis test and rank average values were compared by Conover test.

Results

Fungal isolation

A total of 88 fungal isolates belonging to the genera *Fusarium* ($n = 39$), *Rhizoctonia* ($n = 21$), *Macrophomina* ($n = 16$), and *Neocosmospora* ($n = 12$) were obtained from diseased roots of tomatillo plants grown in 19 commercial fields in northern Sinaloa. Subsequently, 37 isolates were selected as representatives of the genera and geographical regions and were included in the morphological characterization, phylogenetic analysis, and pathogenicity tests.

Morphological characterization

Based on morphological features, 15 isolates were tentatively identified as *Rhizoctonia solani*, 11 belonging to *Fusarium oxysporum* species complex (FOSC), five as *Macrophomina* sp., three belonging to *Fusarium solani* species complex (FSSC), and three belonging to *Fusarium fujikuroi* species complex (FFSC).

Isolates belonging to FOSC exhibited cottony colonies with pigmentation varying from white to purple or violet on PDA. On CLA, macroconidia were falcate, hyaline, 3 to 4–septate and measuring 23.4–34.7 × 2.9–5.1 μm. On CLA, microconidia were hyaline, oval to ellipsoidal, aseptate or 1–septate, of 8.4–17.6 × 3.1–5.1 μm, and abundantly formed in false heads on short monophialides (Fig. 1).

Isolates belonging to FFSC exhibited cottony colonies with pigmentation that was white with a violet mass in the center on PDA. Macroconidia were falcate to almost straight, hyaline, 3–4-septate and measuring 20.5–52.7 × 3.1–4.9 μm. Microconidia were oval to elliptical, hyaline, aseptate or 1–septate, of 3.9–13.3 × 2.6–4.7 μm, and developed in false heads on short monophialides (Fig. 1).

Isolates belonging to FSSC showed cottony colonies with pigmentation varying from white to cream on PDA. On CLA, macroconidia were falcate, hyaline, with pointed apexes, with 3 to 4 septa, measuring 23.1–37.1 × 4.8–5.8 μm. On CLA, microconidia were oval, ellipsoid or reniform, hyaline, with rounded apexes, with 0 to 1 septate, measuring 7.9–21.1 × 2.9–5.8 μm, and arranged in false heads at the tips of long monophialides (Fig. 1).

All *R. solani* isolates were found to be multinucleate (5–10 nuclei per cell). On PDA, colonies were light brown to brown with mycelial growth of 2.4 cm day^{-1}. Hyphae showed a right-angle branching pattern, with a constriction at the base and adjacent septa. Colonies of *Macrophomina* isolates were olivaceous grey and with numerous dark oblong sclerotia on PDA.

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Table 2 GenBank accession numbers of DNA sequences of fungal isolates included in the phylogenetic analyses. Newly deposited sequences are shown in bold

Species	Isolate code[a]	Host	Location	GenBank accession number			
				ITS	EF1	BT	ACT
B. dothidea	CMW8000	*Prunus* sp.	Switzerland	AY236949	AY236898	-----	-----
F. oxysporum	FAVF145	*Physalis ixocarpa*	Mexico	-----	**MN270358**	-----	-----
F. oxysporum	FAVF146	*Physalis ixocarpa*	Mexico	-----	**MN270362**	-----	-----
F. oxysporum	FAVF144	*Physalis ixocarpa*	Mexico	-----	**MN270361**	-----	-----
F. oxysporum	FAVF143	*Physalis ixocarpa*	Mexico	-----	**MN270360**	-----	-----
F. oxysporum	FAVF142	*Physalis ixocarpa*	Mexico	-----	**MN270359**	-----	-----
F. oxysporum	FAVF141	*Physalis ixocarpa*	Mexico	-----	**MN270357**	-----	-----
F. oxysporum	FAVF140	*Physalis ixocarpa*	Mexico	-----	**MN270356**	-----	-----
F. oxysporum	NRRL26406	*Cucumis melo*	-----	-----	AF008504	-----	-----
F. oxysporum	FAVF130	*Physalis ixocarpa*	Mexico	-----	**MN270352**	-----	-----
F. oxysporum	FAVF131	*Physalis ixocarpa*	Mexico	-----	**MN270353**	-----	-----
F. oxysporum	FAVF135	*Physalis ixocarpa*	Mexico	-----	**MN270354**	-----	-----
F. oxysporum	NRRL38445	*Solanum lycopersicum*	USA	-----	FJ985395	-----	-----
F. oxysporum	NRRL26381	*Solanum lycopersicum*	-----	-----	AF008510	-----	-----
F. oxysporum	FAVF137	*Physalis ixocarpa*	Mexico	-----	**MN270355**	-----	-----
F. pseudocircinatum	NRRL22946	Unknow	-----	-----	AF160271	-----	-----
F. nygamai	FRC M1374	Sorghum	Australia	-----	AY337445	-----	-----
F. nygamai	FAVF138	*Physalis ixocarpa*	Mexico	-----	**MN270365**	-----	-----
F. nygamai	NRRL52708	Unknow	-----	-----	JF740790	-----	-----
F. oxysporum	FAVF132	*Physalis ixocarpa*	Mexico	-----	**MN270363**	-----	-----
F. oxysporum	FAVF134	*Physalis ixocarpa*	Mexico	-----	**MN270364**	-----	-----
G. cicatricum	CBS125552	*Buxus sempervirens*	Slovenia	HQ728145	HM626644	-----	-----
N. ambrosium	NRRL20438	*Camellia sinensis*	India	AF178397	AF178332	-----	-----
N. ambrosium	NRRL22346	*Camellia sinensis*	India	EU329669	FJ240350	-----	-----
N. cyanescens	CBS518.82	Human foot	Netherlands	EU329684	FJ240353	-----	-----
N. falciformis	FAVF133	*Physalis ixocarpa*	Mexico	**MT151681**	**MN270366**	-----	-----
N. falciformis	FAVF136	*Physalis ixocarpa*	Mexico	**MT151682**	**MN270367**	-----	-----
N. falciformis	FAVF139	*Physalis ixocarpa*	Mexico	**MT151683**	**MN270368**	-----	-----
N. falciformis	NRRL32542	Human eye	India	-----	DQ247008	-----	-----
N. falciformis	CBS475.67	Human	Puerto Rico	MG18993	LT906669	-----	-----
N. falciformis	CBS318.73	*Trichosanthes dioica*	India	JX435208	JX435158	-----	-----
N. falciformis	NRRL43441	Human eye	USA	DQ790522	DQ790478	-----	-----
N. falciformis	NRRL32928	Human eye	USA	DQ094630	DQ247176	-----	-----
N. illudens	NRRL22090	*Beilschmiedia tawa*	New Zealand	AF178393	AF178326	-----	-----
N. keratoplastica	CBS490.63	Human	Japan	-----	LT906670	-----	-----
N. keratoplastica	NRRL43373	Contact lens	Malaysia	EF453072	EF452920	-----	-----
N. keratoplastica	NRRL43458	Human	Singapore	EU329686	DQ790511	-----	-----
N. lichenicola	NRRL34123	Human eye	India	DQ094645	DQ247192	-----	-----
N. macrospora	CBS142424	*Citrus sinensis*	Italy	LT746266	LT746218	-----	-----
N. macrospora	CPC28192	*Citrus sinensis*	Italy	LT746267	LT746219	-----	-----
N. perseae	CBS144142	*Persea americana*	Italy	LT991940	LT991902	-----	-----
N. perseae	CBS144143	*Persea americana*	Italy	LT991941	LT991903	-----	-----

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Table 2 (continued)

Species	Isolate code[a]	Host	Location	GenBank accession number			
				ITS	EF1	BT	ACT
N. plagianthi	NRRL22632	*Hoheria glabrata*	New Zealand	AF178417	AF178354	-----	-----
N. pseudensiformis	NRRL22354	Bark	French Guiana	AF178402	AF178338	-----	-----
N. solani	NRRL32741	human eye	USA	DQ094522	DQ247061	-----	-----
N. solani	CBS140079	*Solanum tuberosum*	Slovenia	KT313633	KT313611	-----	-----
N. suttoniana	CBS124892	Human nail	Gabon	JX435189	JX435139	-----	-----
N. suttoniana	NRRL32316	Human corneal ulcer	USA	DQ094413	DQ246944	-----	-----
N. suttoniana	CBS143214	Human wound	USA	DQ094617	DQ247163	-----	-----
N. suttoniana	NRRL28001	Human skin	USA	DQ094348	DQ246866	-----	-----
N. vasinfecta	CBS130182	Human	USA	EF453092	EF452940	-----	-----
R. solani AG4 HGI	FAVF125	*Physalis ixocarpa*	Mexico	**MN264610**	-----	-----	-----
R. solani AG4 HGI	FAVF124	*Physalis ixocarpa*	Mexico	**MN264611**	-----	-----	-----
R. solani AG4 HGI	FAVF128	*Physalis ixocarpa*	Mexico	**MN264609**	-----	-----	-----
R. solani AG4 HGI	FAVF120	*Physalis ixocarpa*	Mexico	**MN264608**	-----	-----	-----
R. solani AG4 HGI	FAVF118	*Physalis ixocarpa*	Mexico	**MN264607**	-----	-----	-----
R. solani AG4 HGI	FAVF116	*Physalis ixocarpa*	Mexico	**MN264606**	-----	-----	-----
R. solani AG4 HGI	FAVF115	*Physalis ixocarpa*	Mexico	**MN264605**	-----	-----	-----
R. solani AG4 HGI	FAVF126	*Physalis ixocarpa*	Mexico	**MN264612**	-----	-----	-----
R. solani AG4 HGI	FAVF127	*Physalis ixocarpa*	Mexico	**MN264613**	-----	-----	-----
R. solani AG4 HGI	FAVF129	*Physalis ixocarpa*	Mexico	**MN264614**	-----	-----	-----
R. solani 4 HGI	FAVF117	*Physalis ixocarpa*	Mexico	**MN264600**	-----	-----	-----
R. solani 4 HGI	FAVF119	*Physalis ixocarpa*	Mexico	**MN264601**	-----	-----	-----
R. solani 4 HGI	FAVF121	*Physalis ixocarpa*	Mexico	**MN264602**	-----	-----	-----
R. solani 4 HGI	FAVF122	*Physalis ixocarpa*	Mexico	**MN264603**	-----	-----	-----
R. solani 4 HGI	FAVF123	*Physalis ixocarpa*	Mexico	**MN264604**	-----	-----	-----
R. solani 4 HGI	CIMAP:Pyr	Pyrethrum	India	KU253633	-----	-----	-----
R. solani 4 HGI	NPC-03-1	*Beta vulgaris*	China	EF679777	-----	-----	-----
R. solani 4 HGII	F5	*Beta vulgaris*	USA	FJ492064	-----	-----	-----
R. solani 4 HGII	F6	*Beta vulgaris*	USA	FJ492065	-----	-----	-----
R. solani 4 HGIII	Rs20	Strawberry	Israel	DQ102449	-----	-----	-----
R. solani 4 HGIII	Rh–81	*Solanum tuberosum*	South Africa	KF712285	-----	-----	-----
Athelia rolfsii	FSR-052	Lily	Taiwan	AY684917	-----	-----	-----
M. phaseolina	FAVF147	*Physalis ixocarpa*	Mexico	**MN268731**	**MN270369**	**MN270374**	**MN270379**
M. phaseolina	FAVF148	*Physalis ixocarpa*	Mexico	**MN268732**	**MN270370**	**MN270375**	**MN270380**
M. phaseolina	FAVF149	*Physalis ixocarpa*	Mexico	**MN268733**	**MN270371**	**MN270376**	**MN270381**
M. phaseolina	CPC21420	*Vigna unguiculata*	Senegal	KF951717	KF952088	KF952178	KF951857
M. phaseolina	CPC21416	*Arachis hypogaea*	Senegal	KF951715	KF952086	KF952176	KF951855
M. phaseolina	FAVF151	*Physalis ixocarpa*	Mexico	**MN268735**	**MN270373**	**MN270378**	**MN270383**
M. phaseolina	CBS205.47	*Phaseolus vulgaris*	Italy	KF951622	KF951997	-----	KF951804
M. phaseolina	FAVF150	*Physalis ixocarpa*	Mexico	**MN268734**	**MN270372**	**MN270377**	**MN270382**
M. pseudophaseolina	CPC21403	*Arachis hypogaea*	Senegal	KF951790	KF952152	KF952232	KF951917
M. pseudophaseolina	CPC21417	*Arachis hypogaea*	Senegal	KF951791	KF952153	KF952233	KF951918
M. pseudophaseolina	CPC21502	*Hibiscus sabdarifa*	Senegal	KF951797	KF952159	KF952239	KF951924
M. euphorbicola	CMM4045	*Jatropha gossypifolia*	Brazil	KU058928	KU058898	MF457657	MF457654

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Table 2 (continued)

Species	Isolate code[a]	Host	Location	GenBank accession number			
				ITS	EF1	BT	ACT
M. euphorbicola	CMM4134	*Ricinus communis*	Brazil	KU058936	KU058906	MF457658	MF457655
M. euphorbicola	CMM4145	*Ricinus communis*	Brazil	KU058937	KU058907	MF457659	MF457656
M. vaccinii	CGMCC 3.19503	*Vaccinium* spp.	China	MK687450	MK687426	MK687434	MK687442
M. vaccinii	CGMCC 3.19506	*Vaccinium* spp	China	MK687453	MK687429	MK687437	MK687445
M. vaccinii	CGMCC 3.19510	*Vaccinium* spp.	China	MK687457	MK687433	MK687441	MK687449

[a] CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CMM = Culture Collection of Phytopathogenic Fungi 'Professora Maria Menezes', Universidade Federal Rural de Pernambuco, Recife, Brazil; FAVF = Culture Collection of Phytopathogenic Fungi of the Faculty of Agronomy of Fuerte Valley, Sinaloa Autonomous University, Sinaloa, Mexico; NRRL = Agricultural Research Service Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL, USA); CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CGMCC = China General Microbiological Culture Collection Center, Beijing, China

Phylogenetic analyses

The phylogenetic analyses inferred under the Maximum Likelihood criterion provided sufficient information to distinguish *Fusarium, Rhizoctonia, Neocosmospora*, and *Macrophomina* species associated with tomatillo wilt in northern Sinaloa, Mexico. The alignment characteristics and statistics are summarized in Table 3.

The *Fusarium* isolates dataset included 23 taxa, including three outgroup taxa, *Neocosmospora lichenicola* NRRL 34123, *N. falciformis* NRRL 32542 and *N. falciformis* NRRL 32928. Maximum likelihood analysis using the EF-1α gene sequences resulted in three well-supported clades, corresponding to the *Fusarium* species described previously (Fig. 2). The first clade with 11 isolates clustered with *Fusarium oxysporum* [Agricultural Research Service Culture Collection, Philadelphia, USA (NRRL) 26406, 26381, and 38445], with 100% bootstrap support. The second clade with three isolates clustered with *Fusarium nygamai* [NRRL52708 and Fusarium Research Center, Pennsylvania State University, USA (FRC) M1374], with 99% bootstrap support.

In the phylogenetic tree generated with the sequences of the ITS region and the EF-1α gene of the *Neocosmospora* isolates, composed of 29 taxa (including 1 outgroup taxon, *Geejayessia cicatricum* NRRL 22316), the three isolates obtained in that study grouped with isolates of *N. falciformis* (NRRL32542, CBS 47567 and CBS 31873) with 85% bootstrap support (Fig. 3).

The datasets used for phylogenetic analysis of the *Rhizoctonia* isolates consisted of 75 taxa for *R. solani* and 76 taxa for binucleate *Rhizoctonia* (including one outgroup taxon, *Athelia rolfsii* FSR-052). Phylogenetic analysis of the ITS region showed that all the isolates obtained in this study belong to the *R. solani* AG4-HGI anastomosis group (Fig. 4).

For the phylogenetic analysis of *Macrophomina* isolates, the combined ITS, EF-1α, BT, and ACT datasets consisted of 15 taxa, including one outgroup taxon, *Botryosphaeria dothidea* CMW8000. The alignment contained 1883 characters, of which 309 corresponded with the EF-1α gene, 674 corresponded with the BT gene, 288 corresponded with the ACT gene, and 611 corresponded with the ITS region. The five *Macrophomina* isolates (FAVF147, FAVF148, FAVF149, FAVF150, and FAVF140) clustered with *M. phaseolina* [Culture Collection of Pedro Crous, housed at Utrecht, The Netherlands (CPC) 21416 and 21420, and Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) 20547, 26381, and 38445], with 99% bootstrap support (Fig. 5).

Distribution of fungal species

Rhizoctonia solani AG 4–HGI was the most frequently identified species (40.5%) followed by *F. oxysporum* (29.8%), *M. phaseolina* (13.5%), *N. falciformis* (8.1%), and *F. nygamai* (8.1%) from

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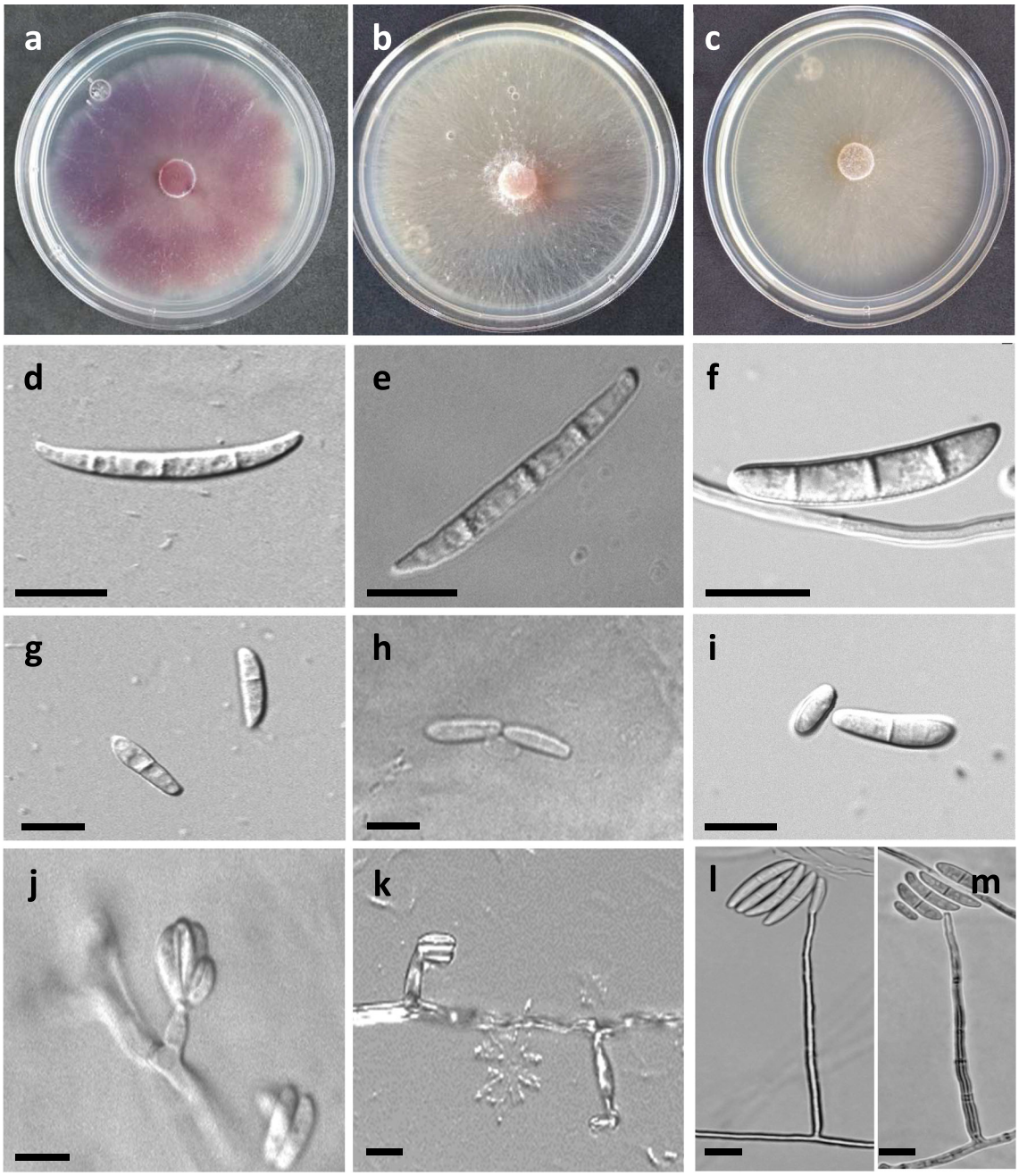

Fig. 1 Morphology of *Fusarium oxysporum* (a, d, g and j), *F. nygamai* (b, e, h and k), and *Neocosmospora falciformis* (c, f, i, l and m). **a–c.** Surface of colony growth on PDA after 7 days; **d–f.** Macroconidia; **g–i.** Microconidia; **j–m.** Monophialides. Scale bar = 10 μm

the 37 fungal isolates obtained from tomatillo symptomatic samples. The distribution of fungal species varied among the populations obtained from tomatillo fields in the three municipalities located in northern Sinaloa, Mexico. *Fusarium oxysporum, N. falciformis*, and *M. phaseolina* were found in tomatillo fields from the three municipalities. *Fusarium nygamai* was only recorded in the Ahome and El

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Table 3 Characteristics of the data partitions used for phylogenetic analyses in this study

Genus	Data Partition[a]	ML evolutionary model[b]	Number of characters			
			Total	Conserved	Variable	Informative
Fusarium	EF-1α	GTR + G + I	698	501	182	141
Neocosmospora	EF-1α	GTR + G + I	739	497	205	99
	ITS	GTR	551	442	97	48
Macrophomina	ACT	GTR + G + I	288	249	37	7
	BT	GTR + G + I	672	607	58	27
	EF-1α	GTR + G + I	309	216	85	33
	ITS	GTR + G + I	611	568	39	5
Rhizoctonia	ITS	T92 + I	792	514	247	65

[a] EF-1α: Translation elongation factor 1-alpha. ITS: Internal transcribed spacer regions of the rDNA and 5.8S region. ACT: Actin. BT: Beta-tubulin;

[b] ML: Maximum-likelihood. GTR: Generalized time-reversible. G: Gamma distributed rate variation among sites. I: Proportion of invariable sites. T92: Tamura 3-parameter

Fuerte populations, whereas, *R. solani* was only identified in the Ahome and Guasave populations. The Ahome population had all fungal species identified in this study (Fig. 6).

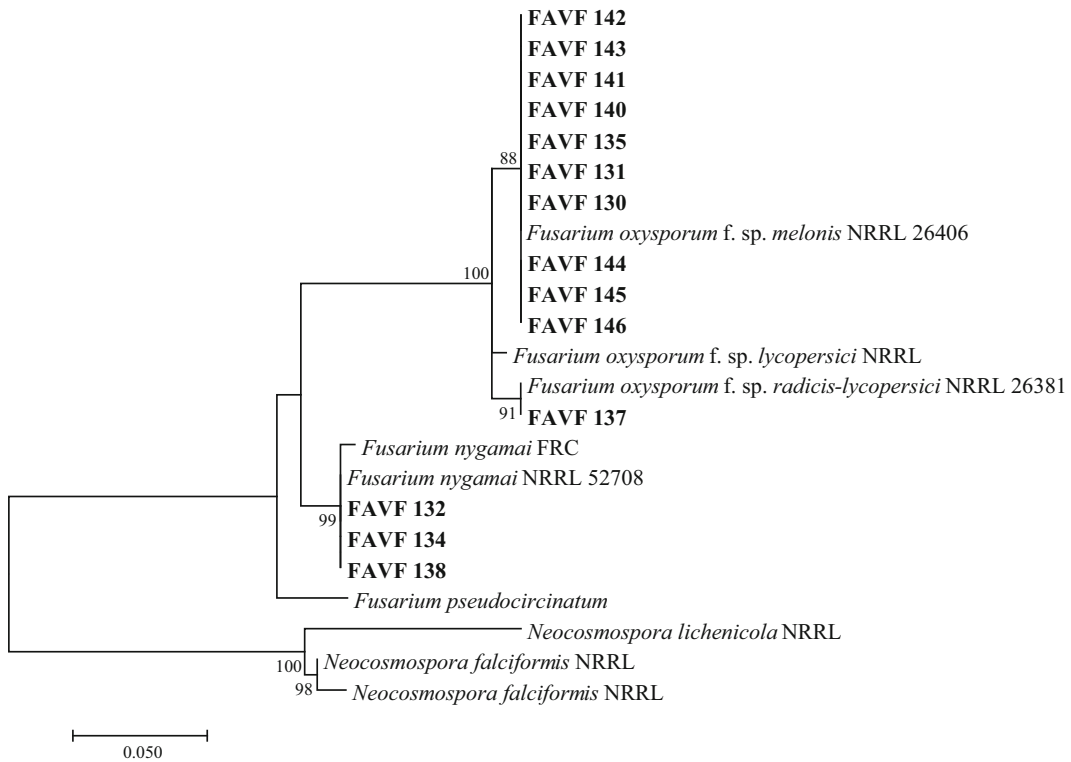

Fig. 2 Maximum Likelihood tree generated from sequence analysis of the EF-1α gene dataset of *Fusarium* species. Bootstrap support values for Maximum Likelihood are indicated at the nodes. The isolates in this study are in bold. *Neocosmospora lichenichola* strain NRRL 34123, *N. falciformis* strain NRRL 32542 and *N. falciformis* strain NRRL 32928 are used as outgroups. The scale bar indicates the number of expected changes per site

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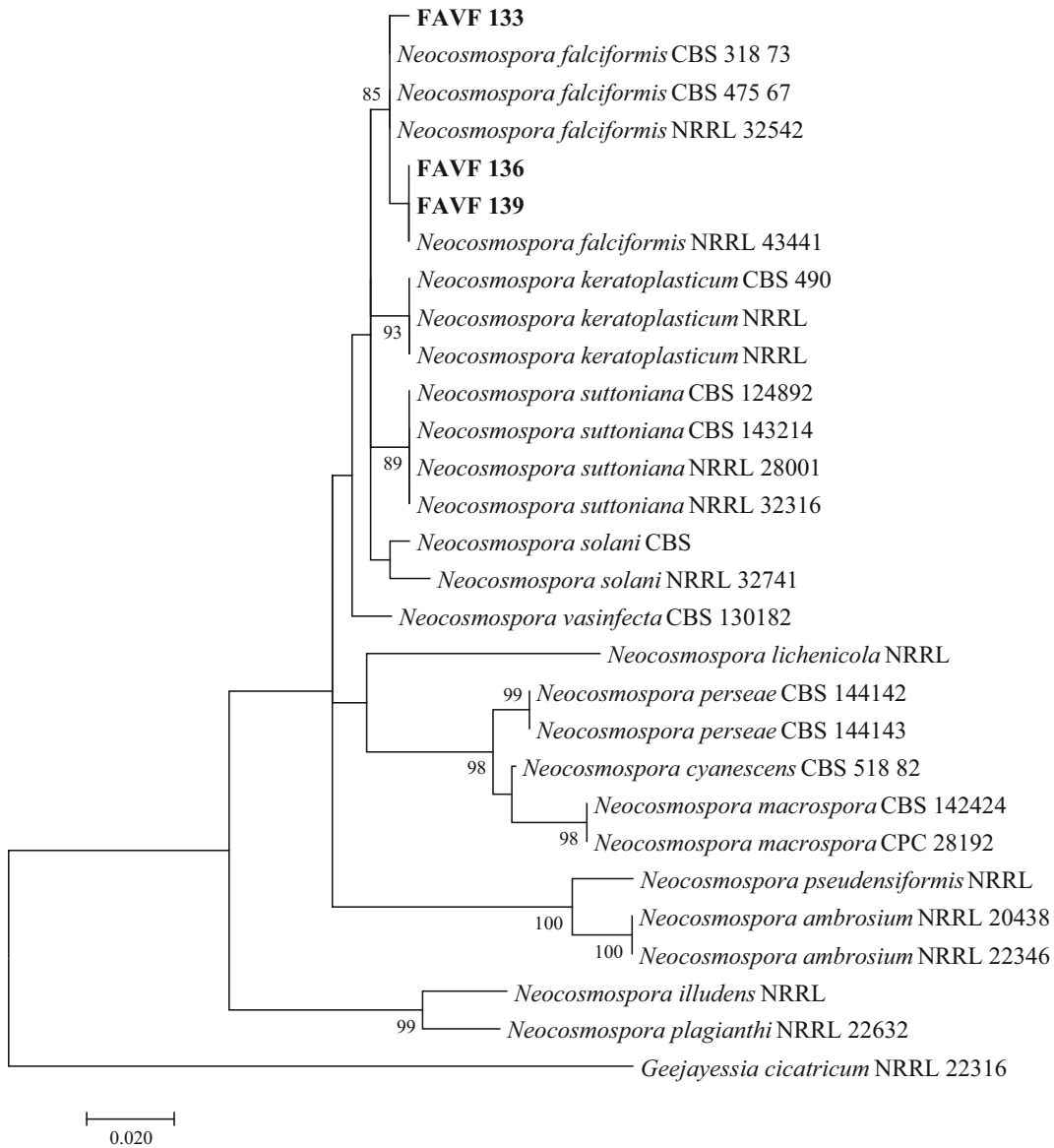

Fig. 3 Maximum Likelihood tree generated from sequence analysis of the EF-1α gene and ITS region dataset of *Neocosmospora* species. Bootstrap support values for Maximum Likelihood are indicated at the nodes. The isolates in this study are in bold. *Geejayessia cicatricum* strain NRRL 22316 is used as outgroup. The scale bar indicates the number of expected changes per site

Pathogenicity tests

All isolates of *Fusarium, Rhizoctonia*, *Macrophomina*, and *Neocosmospora* were pathogenic in tomatillo seedlings. Inoculated seedlings developed symptoms of root rot, poor growth, discoloration of vascular tissues, wilting, and death of plants whereas control plants remained symptomless. Fungal colonies were re-isolated from all symptomatic plants and were found to be morphologically similar to the original isolates inoculated on tomatillo seedlings, thus fulfilling Koch's postulates. Additionally, there were significant differences ($H = 72.04$, $P \leq 0.05$) in virulence between the isolates of each species, although the disease was evident in all the fungal species analyzed, only 17 isolates were different from the control (Fig. 7). *Rhizoctonia*

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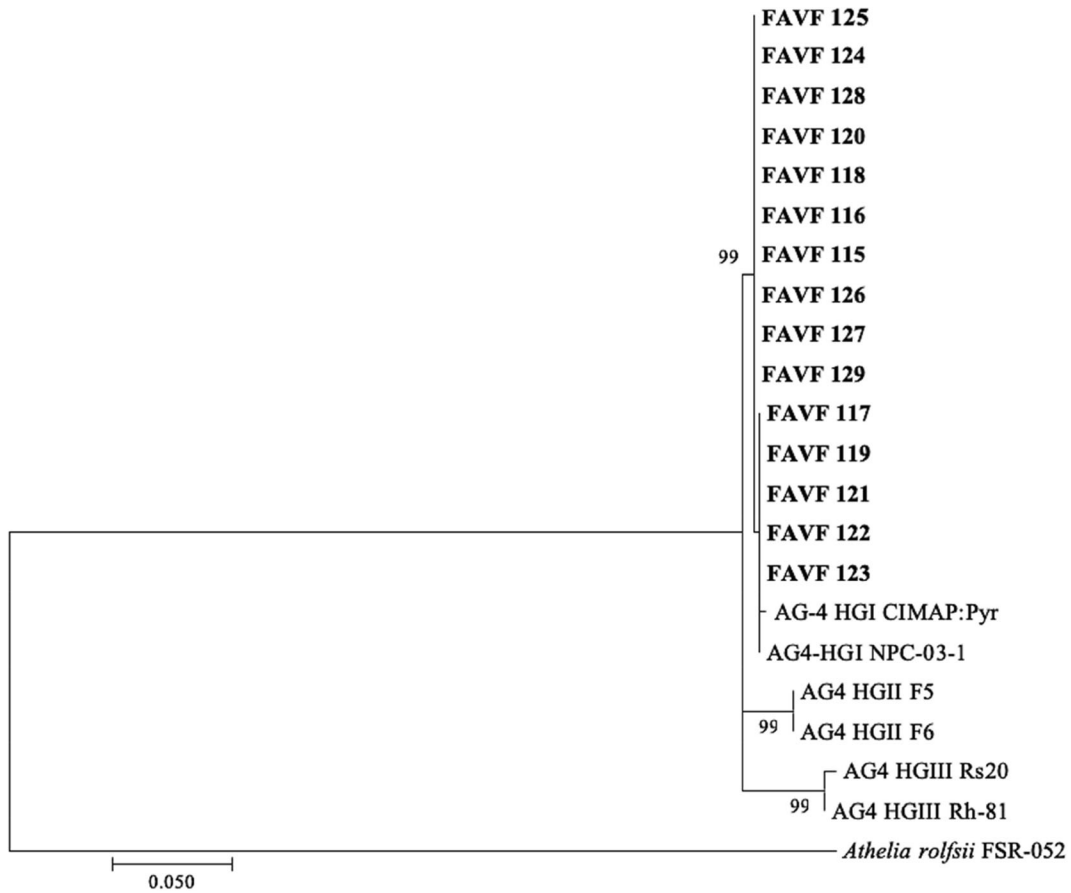

Fig. 4 Maximum Likelihood tree generated from sequence analysis of the ITS region of *Rhizoctonia solani* AG4**.** Bootstrap support values for Maximum Likelihood are indicated at the nodes. The isolates in this study are in bold. *Athelia rolfsii* strain FSR-052 is used as outgroup. The scale bar indicates the number of expected changes per site

solani isolates were the most virulent ($H = 36.29$, $P \leq 0.05$), followed by the rest of the fungal species (Fig. 8). The pathogenicity data of *F. nygamai* and *N. falcifomis* were verified using the Mann-Whitney-Wilcoxon test and in both cases their virulence with respect to the control was identified ($P \leq 0.05$).

Discussion

Based on phylogenetic analysis of the ITS region from 15 *Rhizoctonia* isolates, it was determined that all of them belonged to *R. solani* AG 4-HGI. These isolates were obtained from tomatillo fields located in the municipalities of Ahome and Guasave, Sinaloa. *Rhizoctonia solani* is a soil borne fungus with a wide range of hosts and cosmopolitan distribution (Koike et al. 2007). In Mexico, the presence of *R. solani* on tomatillo has been reported, however, its anastomosis group has not been determined (Apodaca-Sánchez et al. 2008). Predominance of *R. solani* AG 4-HGI isolates associated with tomatillo plants in Sinaloa agrees with previous reports indicating a wide distribution of this anastomosis group in Mexico. Also, the anastomosis group AG-4 was identified as the most common in the rhizosphere of grapevine (*Vitis vinifera*) on the coast of Hermosillo in the state of Sonora, Mexico (Meza-Moller et al. 2007, 2011). Similarly, Montero-Tavera et al. (2013) determined that anastomosis group AG-4 was widely distributed in fields of chili pepper (*Capsicum annuum*) in the Mexican states of Zacatecas, San Luis Potosí, Durango, Guanajuato, Chihuahua, Colima, and Querétaro. Furthermore, Elias-Medina et al. (1997) reported the presence of this anastomosis group in plants of chili pepper

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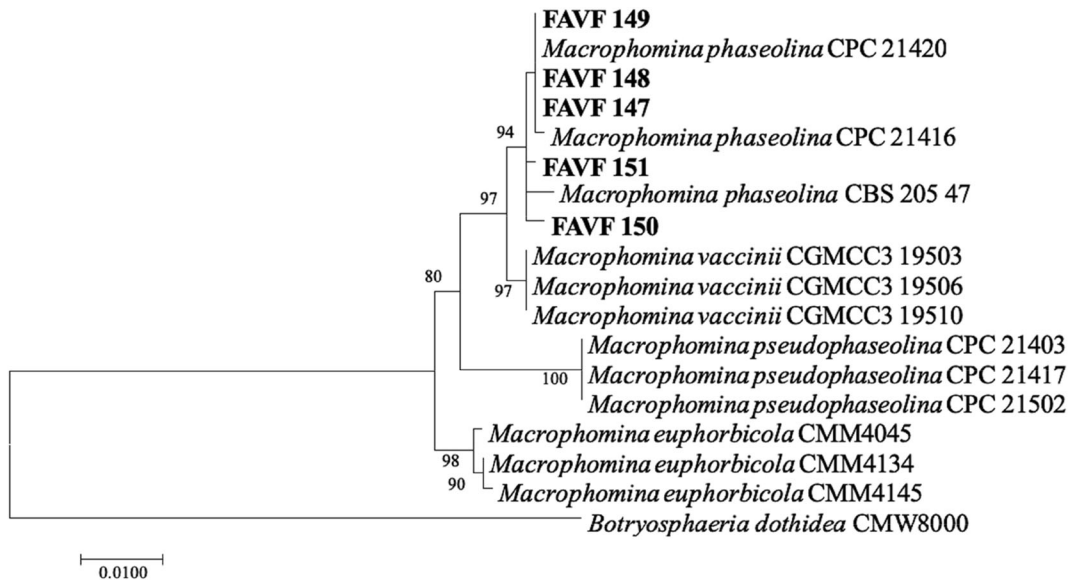

Fig. 5 Maximum Likelihood tree generated from the combined ITS, EF-1α, BT, and ACT sequence alignment of *Macrophomina* species. Bootstrap support values for Maximum Likelihood are indicated at the nodes. The isolates in this study are in bold. *Botryosphaeria dothidea* strain CMW8000 is used as outgroup. The scale bar indicates the number of expected changes per site

and common bean (*Phaseolus vulgaris*) in the State of Mexico and San Luis Potosí, respectively. In contrast, Virgen-Calleros et al. (2000) found that anastomosis group AG-4 was the second most frequently isolated after anastomosis group AG-3 in potato fields (*Solanum tuberosum*) in Guanajuato, Mexico.

In the current study, 14 *Fusarium* isolates were identified through phylogenetic analysis of EF-1α sequences data, determining that 11 isolates belonged to *F. oxysporum* and three isolates to *F. nygamai*. Regarding *F. oxysporum*, this species has been previously reported causing wilt symptoms on tomatillo plants in Sinaloa (Apodaca-Sánchez et al. 2008) and State of Mexico (Gómez-Camacho et al. 2006). Similarly, this fungus has been reported as the causal agent of vascular wilt, which is the most devastating disease that affects cape gooseberry (*Physalis peruviana*) in Colombia (Urrea et al. 2011; Enciso-Rodríguez et al. 2013; Osorio-Guarín et al. 2016). Soto et al. (1998) found differences in susceptibility of 95 genotypes of tomatillo in Mexico due to natural infection by *Fusarium* sp. under field conditions. However, further studies should evaluate the response of tomatillo genotypes produced in Mexico to the infection by each fungal species associated with symptoms of the disease, in order to identify the levels of resistance and tolerance to these pathogens.

On the other hand, *F. nygamai* has been mainly found in hosts of the Poaceae, including *Pennisetum typhoides, Sorghum bicolor*, *Zea mays* and *Saccharum officinarum*. Concerning Solanaceous hosts, *F. nygamai* only has been reported in tobacco (*Nicotiana tabacum*) (Farr and Rossman 2019). In Mexico, *F. nygamai* was associated with root necrosis on sugarcane (*Saccharum officinarum*) in the state of Morelos (Martínez-Fernández et al. 2015). Likewise, Leyva-Madrigal et al. (2015) reported that *F. nygamai* was the most prevalent and one of the most aggressive species causing root rot on maize in northern Sinaloa. In the current study, *F. nygamai* isolates were significantly less virulent and less abundant on tomatillo plants as compared with *F. oxysporum* isolates. The magnitude of change in severity shown by *F. nygamai* and *N. falcifomis* did not allow the identification of their pathogenicity with respect to the control in the analysis carried out using Kruskal-Wallis test, so only for the purpose of providing evidence for the pathogenic capacity of these species the Mann-Whitney-Wilcoxon test was performed. To the best of our knowledge, this is the first report of *F. nygamai* isolates associated with wilt symptoms in tomatillo in Mexico and worldwide.

Based on EF-1α sequences, three *Neocosmospora* isolates included in this study were identified as *N. falciformi*s (syn. *F. falciforme*). This fungus is included in the *Fusarium solani* species complex which has more than 15 phylogenetic species (Sandoval-Denis and Crous 2018). Recently, *N. falciformis* has been

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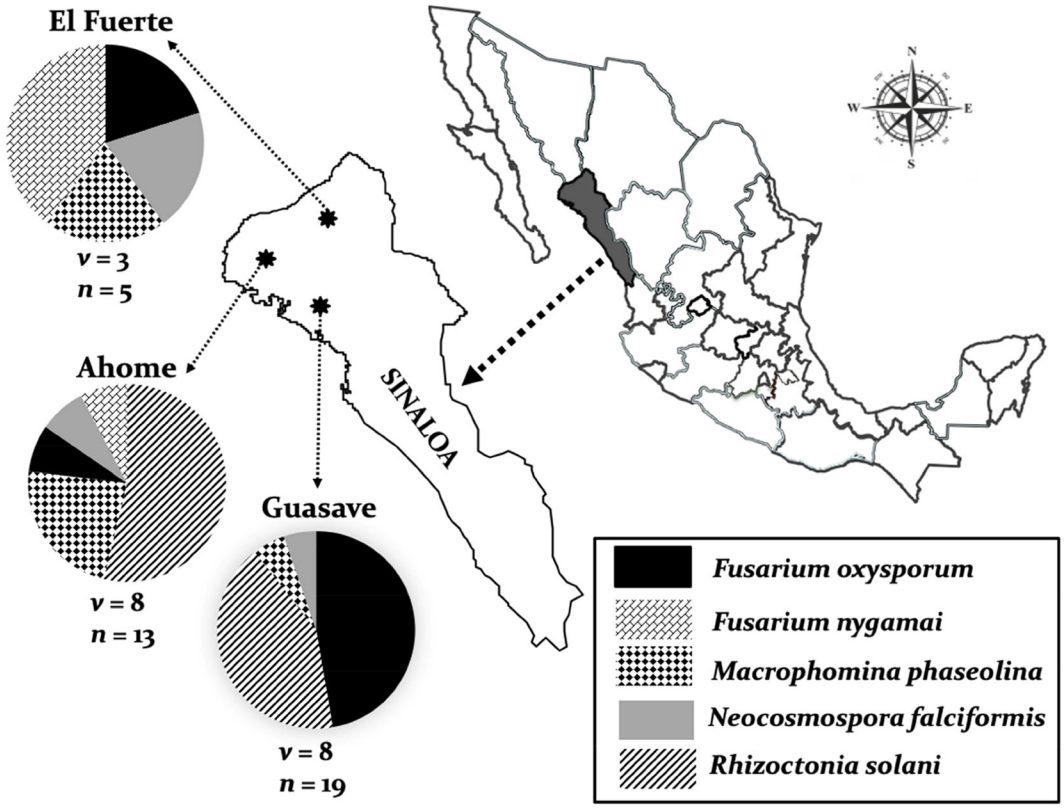

Fig. 6 Collection sites of fungal species associated with tomatillo wilt in three municipalities distributed in the state of Sinaloa, Mexico. Circles represent association frequency of each fungal species in each population sampled, "*v*" is the number of tomatillo fields sampled in each population and "*n*" is the number of isolates analyzed in each population

reported causing root rot on lima bean (*Phaseolus lunatus*) in Brazil (Sousa et al. 2017), basal rot on onions (*Allium cepa*) (Tirado-Ramírez et al. 2018), root rot on watermelon (*Citrullus lanatus*) (Rentería-Martínez et al. 2018) and tomato wilt (*Solanum lycopersicum*) (Vega-Gutiérrez et al. 2019a) in Mexico. It has also been reported causing root and stem rot on papaya in Mexico (Vega-Gutiérrez et al. 2019b) and India (Gupta et al. 2019). However, this is the first report of *N. falciformis* infecting *P. ixocarpa* in Mexico and worldwide.

The phylogenetic analysis generated with sequence data (ITS, EF-1α, BT, and ACT) indicated that all *Macrophomina* isolates tested in this work belonged to *M. phaseolina.* The genus *Macrophomina* belongs to the family Botryosphaeriaceae and it has four phylogenetic species: *M. phaseolina, M. pseudophaseolina, M. euphorbiicola*, and *M. vaccinii* (Machado et al. 2019; Zhao et al. 2019). Previous studies showed that *M. phaseolina* has been associated with *Physalis peruviana* (Shivas 1989) in Australia and Pakistan (Ahmad et al. 1997) and on *Physalis minima* in Australia (Fuhlbohm et al. 2012). In Mexico, *M. phaseolina* has been detected on a wide range of host plants from different families (Muñoz-Cabañas et al. 2005) and with a wide distribution in arid, tropical and subtropical regions (Mayek-Pérez et al. 2001), but has not been previously reported on plants of the genus *Physalis*. Thus, this is the first report of *M. phaseolina* infecting *P. ixocarpa* in Mexico and worldwide.

Our results of pathogenicity tests revealed that isolates of *R. solani* were more virulent in tomatillo plants as compared with *F. oxysporum, M. phaseolina*, *N. falciformis*, and *F. nygamai* isolates. In a similar study, Güney and Güldür (2018) found no significative differences in severity of foliar symptoms and root rot induced by *R. solani, F. oxysporum, M. phaseolina* and *N. solani* on artificially inoculated chili pepper plants. Other authors like Datnoff and Sinclair (1988) found that severity in root rot of soybean (*Glycine max*) plants inoculated with *F. oxysporum* and *R. solani* was not

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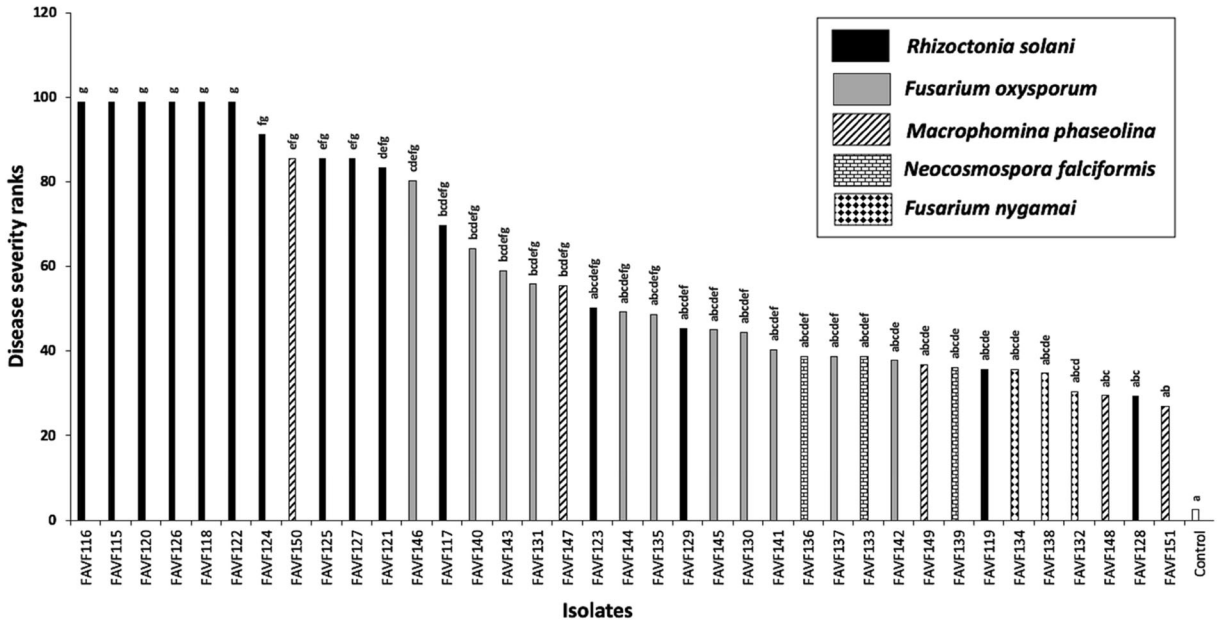

Fig. 7 Mean disease severity caused by 37 fungal isolates associated with tomatillo wilt in northern Sinaloa, Mexico, 30 days after inoculation with mycelium colonized agar plugs onto roots of *Physalis ixocarpa* seedlings. Isolates with the same letter do not differ significantly ($P \leq 0.05$)

statistically different. Nevertheless, their results demonstrated an additive interaction with severity of the disease increasing with the presence of both pathogens. Finally, Navarrete-Maya et al. (2009) stated that the virulence of *R. solani* was less severe compared to that of *Fusarium* spp. in common bean plants under field

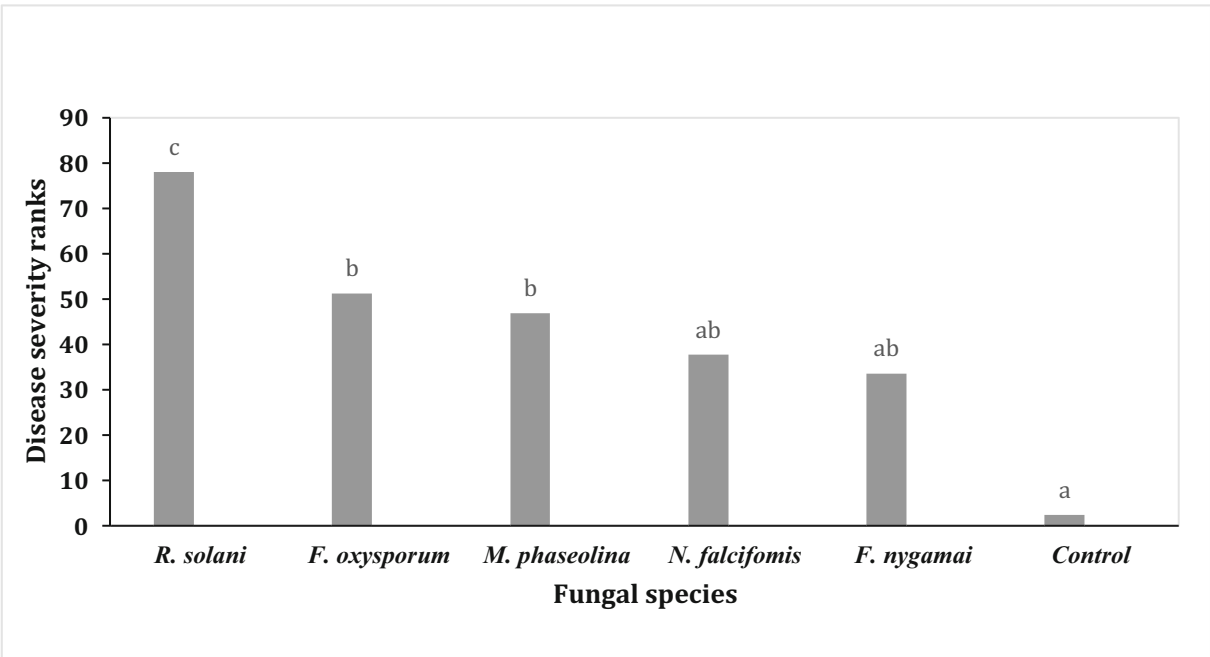

Fig. 8 Mean disease severity caused by five fungal species associated with tomatillo wilt in northern Sinaloa, Mexico, 30 days after inoculation with mycelium colonized agar plugs onto roots of *Physalis ixocarpa* seedlings. Bars above columns are the standard errors of the means. Fungal species with the same letter do not differ significantly ($P \leq 0.05$)

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conditions, but in a controlled environment, the virulence of *R. solani* was more severe.

This work provides new information about identification and pathogenicity of soilborne fungi associated with tomatillo wilt in northern Sinaloa, Mexico. It also reported for the first time the presence of *M. phaseolina, N. falciformis* and *F. nygamai* isolates associated with this disease. These findings are part of further epidemiological studies that will be used to establish effective management strategies of tomatillo wilt in commercial fields of Mexico.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Human and animals rights No human or animal was involved in this research by the authors.

Informed consent All authors have reviewed the manuscript and approved its submission to the European Journal of Plant Pathology.

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