Pathogenic, Morphological, and Phylogenetic Characterization of *Fusarium solani* f. sp. *cucurbitae* Isolates From Cucurbits in Almería Province, Spain

Ana Pérez-Hernández,^{1,†} Liliana O. Rocha,² Elena Porcel-Rodríguez,¹ Brett A. Summerell,³ Edward C. Y. Liew,³ and Julio M. Gómez-Vázquez¹

¹ IFAPA Centro La Mojonera, La Mojonera, Almería 04745, Spain

² Departamento de Ciencia de los Alimentos, Universidade Estadual de Campinas, Campinas, SP 13.083-862, Brazil

³ The Royal Botanic Gardens and Domain Trust, Sydney, NSW 2000, Australia

Abstract

Fusarium solani f. sp. *cucurbitae* (syn. *Neocosmosporium cucurbitae*) is one of the most devastating soilborne pathogens affecting the production of cucurbits worldwide. Since its first detection in Almería Province in Spain in the spring of 2007, it has become one of the main soilborne pathogens affecting zucchini production. It has also been reported on melon, watermelon, and squash rootstocks in Spain, representing a high risk of dissemination in the area. The objectives of this study were to investigate the incidence and distribution of this disease in southeastern Spain and characterize isolates collected over 5 years. These strains were characterized on the basis of greenhouse aggressiveness assays on a range of cucurbit hosts, morphological characteristics, and elongation factor 1- α and RNA polymerase II second largest subunit phylogenies. All pathogenic isolates were highly

aggressive on zucchini plants, causing a high mortality rate a few weeks after inoculation. The rest of the cucurbit hosts showed differential susceptibility to the pathogen, with cucumber being the least susceptible. Plants belonging to other families remained asymptomatic. Morphological characterization revealed the formation of verticillate monophialides and chlamydospores forming long chains, characteristics not described for this forma specialis. Phylogenetic studies of both the individual loci and combined datasets revealed that all pathogenic isolates clustered together with strong monophyletic support, nested within clade 3 in the *F. solani* species complex.

Keywords: disease development and spread, epidemiology, fungi, pathogen diversity

Zucchini (*Cucurbita pepo* L.) is intensively cropped in the province of Almería, the main vegetable production area in Spain. In 2016, zucchini was produced on 11,081 ha in Spain, of which 7,490 ha were for greenhouse production in Almería, producing 434,195 metric tons of this crop. This production accounts for 74.7% of the total production in Spain, making it one of the most economically important crops for this region (MAPA 2017).

In 2007, a new fungal disease of zucchini was detected in the province of Almería. The first symptom observed was a dark green coloration of the younger leaves followed by wilt, crown rot, and eventual plant death. The causal agent was *Fusarium solani* (Mart) Sacc f. sp. *cucurbitae* W. C. Snyder and H. N. Hansen (Gómez et al. 2008), which is host specific to cucurbits (Boughalleb et al. 2007; Messiaen et al. 1995; Paternotte 1987). This pathogen has been reported worldwide, causing disease in field production in pumpkin (Elmer et al. 2007), muskmelon (Champaco et al. 1993), cucumber (Bourbous

et al. 1997), watermelon, and rootstock hybrids of *Cucurbita maxima* $\times$ *Cucurbita moschata* (Armengol et al. 2000) among other hosts.

In Almería, zucchini crops have been considered for years to be highly resistant to soilborne fungal diseases. Until this study, aside from *F. solani* f. sp. *cucurbitae*, only *Phytophthora capsici* Leonian has been detected as the causal agent of a soilborne disease in adult plants in this area (Gómez et al. 2013). Furthermore, since its detection, *Fusarium* crown, foot, and fruit rot of zucchini has been spreading to other cucurbits, such as melon (Gómez et al. 2014) and watermelon (Porcel 2013), becoming an important threat to the cucurbit production in this region.

Several methods can be used for the control of *Fusarium* crown rot, such as fungicide applications with prochloraz, carbendazim, and thiophanate-methyl; crop rotation with noncucurbits for at least 2 years; or soil solarization or biosolarization (Pérez-Hernández et al. 2017).

In the decades since 2000, molecular tools have revolutionized the understanding of fungal taxonomy, including that of the genus *Fusarium*. Phylogenetic studies have demonstrated that several species actually correspond to species complexes composed of different morphologically indistinguishable cryptic species. *F. solani*, known as the *F. solani* species complex (FSSC), comprises three major clades encompassing >60 phylogenetic species (Nalim et al. 2011; O'Donnell 2000; O'Donnell et al. 2008; Short et al. 2013; Zhang et al. 2006). Members of clades 1 and 2 are known exclusively from diseased or dead plants, whereas members of clade 3 are frequently isolated from soil and as saprotrophs in other environments, causing diseases in immunocompromised humans (Zhang et al. 2006). In addition, clades 1 and 2 are composed of isolates limited to specific geographical origins (New Zealand and South America, respectively), whereas clade 3 includes most of the commonly encountered FSSC isolates associated with plant diseases and soil, with a cosmopolitan distribution (O'Donnell 2000). Lombard et al. (2015) proposed that the FSSC be redefined as *Neocosmospora* species based on morphology of the group, especially the sexual structures, and DNA phylogenetic placement. Geiser et al. (2004) argued that such a change was unnecessary, maintaining that the taxonomy better

†Corresponding author: A. Pérez-Hernández; anaperezh@gmail.com

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serves the community needs, especially with respect to plant biosecurity and human health, by retaining the species complex within the genus *Fusarium*. Additionally, Summerell (2019) argued for the retention of the genus *Fusarium* for the species complex until such a time that there is consensus of view. Consequently, we have retained the use of the genus *Fusarium* for these fungi in this study.

Sandoval-Denis et al. (2019) have monographed the FSSC (as the genus *Neocosmospora*) and described *F. solani* f. sp. *cucurbitae* as *Neocosmospora cucurbitae* based on morphology and DNA phylogeny. Based on the available data, we refer to the fungus as *F. solani* f. sp. *cucurbitae* throughout this paper.

F. solani f. sp. *cucurbitae* (syn. *N. cucurbitae*) was first described by Snyder and Hansen (1941) in 1941 as the causal agent of crown and foot rot of cucurbits. In 1961, Tousson and Snyder (1961) divided this forma specialis into two races on the basis of their ability to produce crown and foot rot in cucurbit plants, designated as *F. solani* f. sp. *cucurbitae* race 1, or only able to produce fruit rot, designated as *F. solani* f. sp. *cucurbitae* race 2. In 1973, Matuo and Snyder (1973) conducted fertility crosses between the different forma speciales described within *F. solani*, demonstrating that both races correspond to different biological species assigned to different mating populations MP-I and MP-V, respectively. Some decades later, a comprehensive phylogenetic study (O'Donnell 2000) revealed that both races of *F. solani* f. sp. *cucurbitae* were polyphyletic, therefore representing two distinct phylogenetic species. Several years later, given the large amount of phylogenetic species lacking proper descriptions and Latin binomials in addition to the need to facilitate better communication, a haplotype nomenclatural system was introduced in which each phylogenetic species received a numerical identifier, with unique sequence types within species designated by lower case letters (Chang et al. 2006; O'Donnell et al. 2008). In this study, race 1 was assigned to the phylogenetic species FSSC 10, with two haplotypes FSSC 10a and FSSC 10b, whereas race 2 was assigned to FSSC 1. Mehl and Epstein (2007) and Zhang et al. (2006) demonstrated that race 2 was conspecific with the phylogenetic species FSSC 1, which was the most common phylogenetic species isolated in hospital environments and one of the predominant species in the 2005 to 2006 outbreaks of mycotic keratitis in the United States and Southeast Asia associated with contact lens wear (Chang et al. 2006; Khor et al. 2006). Additional studies have confirmed this hypothesis, and in 2013, the former forma specialis was elevated to the species rank, *Fusarium petrophilum* stat. nov. (Short et al. 2013).

In this context, the aim of this study was to investigate the incidence and distribution of *Fusarium* crown and foot rot of cucurbits in Almería Province and characterize a collection of *F. solani* f. sp. *cucurbitae* isolates obtained over the years from different hosts. These isolates were analyzed on the basis of their pathogenicity to zucchini and other cucurbit hosts; a detailed description of their morphological characteristics; and their elongation factor 1- α (*EF1- α*) and RNA polymerase II second largest subunit (*RPB2*) phylogenetic relationships within the FSSC.

Materials and Methods

Disease survey. To assess the incidence and geographical distribution of *Fusarium* crown and foot rot of zucchini in the province of Almería, isolates were collected in zucchini commercial greenhouses situated in different growing areas. Crops with plants showing foot rot, wilt, or death were visited in collaboration with the Production and Plant Health Laboratory, Almería, and agricultural technicians. In addition, five cucumber greenhouses, one watermelon greenhouse, and one melon greenhouse with plants showing similar disease symptoms were included in this survey.

In each of the greenhouses visited, the percentage of plants showing any of the abovementioned symptoms was estimated, and plant samples were collected to diagnose the causal agent of the disease and obtain a collection of isolates.

Collection of isolates. Basal stem samples were surface disinfected, placed on Komada medium (Komada 1975), potato dextrose agar (PDA) medium (Rapilly 1968), and P5ARP (Jeffers and Martin 1986); and incubated at 25°C for 2 to 3 days. Colonies were obtained

and subcultured on PDA or lima bean agar (Calvert et al. 1960). In addition, to detect possible chlamydospores or perithecia of *Chalara elegans* or *Monosporascus cannonballus*, roots were observed under a stereoscopic microscope. The isolates classified as *F. solani* according to their morphological characteristics were maintained as single-spore cultures in the IFAPA (Instituto de Investigación y Formación Agraria y, Pesquera de Andalucía) Centro La Mojonera. Among a collection of a total of 83 *Fusarium* spp. isolates, 22 of them were selected for this study, and the isolate PCI-711 was deposited with the accession number CECT20831 in the Colección Española de Cultivos Tipo, Parque Científico de la Universidad de Valencia, Paterna, Spain.

Production of pathogen inoculum. For the pathogenicity tests, cultures of each isolate were grown on 90-mm PDA plates and incubated under indirect natural light at 25°C between 15 and 20 days until the plates were completely colonized. Conidial suspensions were then prepared by scraping the surface of the plates with a sterile loop and recovered by washing the plates with autoclaved water. The inoculum was then filtered through a 0.5-mm mesh to remove large portions of mycelia and adjusted with a hemocytometer to an approximate concentration of 1.5×10^3 conidia ml⁻¹. Fifty milliliters of inoculum was poured around the stem base of each plant.

Plant growth and maintenance. The first pathogenicity trial of the fungus on various horticultural crops was conducted in an 80-m², multispan polymethyl methacrylate-covered greenhouse equipped with ridge and side windows in a semicontrolled environment with a cooling system as well as automated climate control and fertigation. The second pathogenicity trial on various horticultural crops was conducted in a 970-m², multispan polyethylene-covered greenhouse to maintain the plants for a longer period of time along with the pathogenicity trials on zucchini plants. The greenhouse was equipped with ridge and side windows as well as automated climate control and fertigation. All greenhouses were located in the IFAPA Centro La Mojonera, Almería Province, Spain (36°47'17", -2°42'14"). Fertigation was managed with a programmable system based on the leaching fraction, which was maintained close to 20%. Temperature for all crops was measured inside the bags of perlite and in the greenhouse environment above the plant canopies with P-108 temperature probes for 10-min intervals and recorded with a Campbell CR10X datalogger (Campbell Scientific, Inc., Logan, UT).

Pathogenicity trials on zucchini plants and fruit. The pathogenicity of the isolates used in this study was tested on both zucchini plants and fruit. To evaluate the pathogenicity on zucchini plants, two trials were conducted: one commenced during the third week of February 2012 (first trial), and the other commenced during the third week of May 2012 (second trial). The experiments were arranged as a randomized complete block design with four replicates. Each experimental unit within a block consisted of four bags of perlite with 3 plants per bag, totaling 48 plants per isolate in each trial. An uninoculated control was included for each block. Seeds of the cultivar Cónsul (Seminis Vegetable Seeds, St. Louis, MO) were sown in the bags, and the plants were inoculated at the two- to three-true leaf stage as previously described. Plants were monitored twice a week for the presence of disease symptoms. Disease incidence (DI) and mortality (M) were calculated as the percentages of symptomatic plants per isolate at the end of the experiment. In addition, disease severity was assessed by using a 0 to 2 rating scale, where 0 = plant without symptoms, 1 = aerial disease symptoms, and 2 = plant death. These data were plotted against time for each experiment, and the development of the disease was evaluated by calculating the area under the disease progress curve (AUDPC) using the trapezoidal method (Madden et al. 2007). The relative area under the disease progress curve (RAUDPC) was calculated as the proportion of the maximum possible AUDPC. Plants were maintained in the greenhouse over periods of 65 and 53 days for the first and second trials, respectively. At the end of the experiments, 10% of the symptomatic plants were randomly selected for pathogen reisolation to fulfill Koch's postulates.

To evaluate the pathogenicity of *F. solani* isolates on zucchini fruit, two consecutive trials were conducted. The experiments were

arranged in a randomized complete block design with four replicates. Each experimental unit within a block consisted of one zucchini fruit with three wounds. Multiple fruits of approximately 200 g were surface disinfected by spraying with 70% ethanol on the surface and allowed to dry. Wounds of a depth of approximately 5 mm were made in the fruit surface with a cork borer; 7-mm PDA plugs, excised from the actively growing margins of 5-day-old synthetic nutrient-poor agar (SNA) cultures of each isolate, were placed mycelium side down into the wounds. Three wounds per fruit and four fruits were inoculated with each isolate. Two fruits per block were inoculated with uncolonized PDA plugs to serve as negative controls. To maintain a humid environment, the fruits were placed on shelves and covered with unsealed plastic bags to allow air flow. Fruits were incubated in the laboratory at approximately 25°C. After 7 days of incubation, the diameter of the lesion produced on the fruit was obtained from an average of two perpendicular measures after subtracting the diameter of the wound.

Pathogenicity on other horticultural crops. After verifying the pathogenicity of 21 *Fusarium* isolates on both zucchini plants and fruit, two representative isolates were selected to study the pathogenicity of *F. solani* f. sp. *cucurbitae* to other horticultural crops. Two trials were conducted: one commenced during the first week of April 2013 (first trial), and the other commenced during the second week of April 2014 (second trial). The experiments were arranged in a factorial design in three blocks, with each block comprising six plants. Factors studied were inoculation with the isolate PCI-511 obtained from zucchini, inoculation with the isolate PCI-1621 obtained from watermelon, and noninoculated controls; the following horticultural species were evaluated: melon (*Cucumis melo*) cultivar Amarillo Canario Jardiver; watermelon (*Citrullus lanatus*) cultivar Mariola; tomato (*Solanum lycopersici*) cultivar Pasadena; eggplant (*Solanum melongena*) cultivar Alegría; pea (*Pisum sativum*) cultivar Altesse; lettuce (*Lactuca sativa*) cultivar Romana Jardiver (Ramiro Arnedo S.A., Calahorra, Spain); cucumber cultivars Borja (Enza Zaden Beheer, B.V., Enkhuizen, the Netherlands), Marketmore, and Pepinillo Jardiver (Ramiro Arnedo S.A.); zucchini (*Cucurbita pepo*) cultivar Cónsul (Seminis Vegetable Seeds, Woodland, CA); squash rootstock (*Cucurbita maxima* × *Cucurbita moschata*) cultivars RS-841 (Akira Seeds S.L., Sant Boi de Llobregat, Spain), Hércules, and Titán (Ramiro Arnedo S.A.); pepper (*Capsicum annuum*) cultivar Airen (Syngenta Seeds Inc., Boise, ID); snow pea (*Pisum sativum* sp. *arvense*) cultivar Capuchino (Semillas Batlle S.A., Molins de Rei, Spain); and green bean (*Phaseolus vulgaris*) cultivar Emerite (Vilmorin, La Méritré, France). In the first trial, plants were sown in 1-liter pots with vermiculite and inoculated when the cotyledons were completely opened as previously described. In the second trial, seeds were sown in 32-liter perlite bags to maintain the plants for a longer period of time. Plants were monitored twice a week for the presence of disease symptoms, and the parameters DI, M, and RAUDPC were calculated per treatment as previously described. At the end of the experiments, 10% of the symptomatic plants per species were randomly selected for pathogen reisolation.

Determination of mycelial growth rates and morphological characteristics. Cardinal growth rates were determined by measuring the in vitro growth of four representative isolates (PCI-341, PCI-511, PCI-731, and PCI-1621) at different temperatures. Mycelial plugs with a diameter of 7 mm were excised from the actively growing margins of 4-day-old PDA cultures and placed mycelium side down in the center of 90-mm PDA plates, sealed with laboratory film, and incubated for 6 days in complete darkness at each of the following temperatures: 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35°C. Mycelial growth data were obtained by calculating the average of two perpendicular measures of the colony diameter after 6 days of growth. Rates were calculated as mean values per day. Four repetitions of each temperature-isolate were included; this experiment was performed twice.

Morphological characteristics of the isolates were examined based on the criteria of Leslie and Summerell (2006). Single-spore isolates were grown using SNA (Nirenberg 1976) for 10 days at 25°C, and subsequently, 7-mm plugs from actively growing edges of each

culture were subcultured in plates with PDA, carnation leaf-piece agar (CLA) (Leslie and Summerell 2006), and SNA in 90-mm plastic petri dishes. Six repetitions per isolate were included, and the plates were incubated for 10 days at 25°C under indirect natural light or continuous Blacklight Blue (F40 BLB) fluorescent light. Colony color and morphology together with the morphology and type of conidiogenous cells were recorded from cultures grown on PDA plates under natural indirect light. The morphology, size, and shape of 30 randomly selected microconidia formed on SNA plates and the presence or absence and shape of 30 chlamydospores were determined under an Olympus BX41 microscope and a ColorView IIIu camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). The production of sporodochia on CLA was induced on subcultures incubated at 25°C under continuous BLB light and checked within 15 days of incubation. The length and width of 30 randomly selected macroconidia of each septation class on CLA and the number of septa were measured and recorded. The reported measurements consisted of the minimum and maximum values in brackets as well as the mean values plus and minus the standard deviation.

Genomic DNA extraction, PCR amplification, and sequencing. *Fusarium* cultures were grown on PDA for 5 days. Genomic DNA was extracted using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions. The PCR amplifications of the isolates were performed for part of the translation *EF1-α* using the primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTAC CAGT(G/C)ATCATGTT-3') (Carbone and Kohn 1999) and *RPB2* using the primers 5f2 (5'-GGGGWGAYCAGAAGAAGGC-3') (Reeb et al. 2004) and 7cr (5'-CCCATRGCTTGTYRCCCAT-3') (Liu et al. 1999). Conditions for amplification for the *EF1-α* gene region were an initial denaturation step of 3 min at 95°C followed by 30 cycles of denaturation (95°C for 30 s), annealing (53°C for 30 s), and elongation (72°C for 45 s). The final elongation step was conducted at 72°C for 7 min. For the *RPB2* loci, amplification consisted of 5 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C; 5 cycles of 45 s at 94°C, 45 s at 58°C, and 2 min at 72°C; and 30 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C (Woudenberg et al. 2013). The PCR amplicons were sequenced in both directions at the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales, Australia, or by the Sequencing Service, Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain.

Phylogenetic analyses. Sequences obtained were edited manually in Geneious software v.5.3.6 (Drummond et al. 2011), and their classification as the genus *Fusarium* was confirmed through the BLAST in the NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the *Fusarium*-ID v. 1.0 database (Geiser et al. 2004). The sequences were aligned with reference sequences from the FSSC obtained from GenBank (Table 1) with ClustalW v.1.83 plug in within Geneious. The alignments were edited, and polymorphisms were confirmed by re-examining the chromatograms. Sequences generated in this study were deposited in GenBank (Table 2). Phylogenetic analyses were performed using PAUP 4.0b10 (Swofford 2002) on the single and combined *EF1-α* and *RPB2* datasets. Unweighted parsimony analysis was conducted using the heuristic search option with 1,000 random addition sequences and tree bisection reconnection branch swapping. Gaps were treated as missing data. The consistency index (CI) and the retention index (RI) were calculated to indicate the amount of homoplasy present. Clade stability was assessed via maximum parsimony bootstrap proportions in PAUP 4.0b10 using 1,000 heuristic search replications with random sequence addition. The *EF1-α* and *RPB2* datasets were rooted with *Fusarium thapsinum* NRRL22045. Phylogenetic trees were visualized and edited using FigTree v.1.4 (Rambaut 2013).

Statistical analyses. Kruskal–Wallis nonparametric analysis of variance (ANOVA) tests were conducted for each of the experiments, of which the DI expressed as the percentage of symptomatic plants per treatment, M, and RAUDPC were analyzed with Statistix 9.0 (Tallahassee, FL) owing to data nonnormality and variance heterogeneity. The different repetitions of the experiments were

included in the model and assayed for differences. In cases where ANOVA revealed no significant differences between experiments, data were pooled prior to analyses. In all cases, if the ANOVA results were significant for the main effects, treatment means were compared using all-pairwise comparison tests ($P \leq 0.05$).

Results

Disease survey. A total of 26 zucchini greenhouses, 5 cucumber greenhouses, 1 melon greenhouse, and 1 watermelon greenhouse with diseased plants were surveyed where symptoms similar to those caused by *F. solani* f. sp. *cucurbitae* were observed, mainly leaf wilt, brown rot lesions at the base of the stem near the soil level, and plant death (Supplementary Fig. S1). In addition, 25 zucchini samples from the Production and Plant Health Laboratory with similar disease

symptoms were included in this study. *F. solani* was present in 92.3% of the zucchini greenhouses surveyed and 100% of the zucchini samples received by the Plant Health Laboratory of Almería. Several of the affected greenhouses were located in the municipality of Las Norias de Daza, where the disease was first observed in zucchini (Gómez et al. 2008). In addition, the DI was observed throughout the province in at least five different municipalities. The DI among the different greenhouses visited was variable. In some greenhouses, the disease affected a small number of plants distributed in one or few rows. In most of the cases, the percentage of affected plants ranged between 5 and 30%. In two greenhouses, between 50 and 60% of the plants showed crown and foot rot symptoms. In most of the cases, the disease was observed in spring and summer crops, but in some cases, it was detected in autumn crops in greenhouses that had been soil disinfested during summer, a common practice

Table 1. Reference strains obtained from the NCBI GenBank database used in the phylogenetic analysis of *Fusarium solani* f. sp. *cucurbitae* isolates

Species	Collection ^y	GenBank accession number ^z		Host	Origin
		<i>EF1-α</i>	<i>RPB2</i>		
<i>Fusarium thapsinum</i> (outgroup)	NRRL22045	AF160270	JX171600	<i>Sorghum bicolor</i>	South Africa
<i>Fusarium virguliforme</i>	NRRL22825	AF178357	GU170615	<i>Glycine max</i>	United States
<i>Fusarium tucumaniae</i>	NRRL31096	GU170600	EU329557	<i>Glycine max</i>	Argentina
<i>Fusarium striatum</i>	NRRL22101	AF178333	EU329490	<i>Gossypium</i> sp.	Panama
<i>F. solani</i> sensu stricto	NRRL25388	DQ246857	EU329535	Human eye	India
<i>F. solani</i> sensu stricto	NRRL32737	DQ247056	EU329606	Human eye	United States
<i>F. solani</i> f. sp. <i>xanthoxyli</i>	NRRL22163	AF178328	EU329496	<i>Xanthoxylum piperitum</i>	Japan
<i>F. solani</i> f. sp. <i>robiniae</i>	NRRL22586	AF178353	EU329516	<i>Robinea pseudoacacia</i>	United States
<i>F. solani</i> f. sp. <i>robiniae</i>	NRRL22161	AF178330	EU329494	<i>Robinea pseudoacacia</i>	Japan
<i>F. solani</i> f. sp. <i>robiniae</i>	NRRL22162	DQ247599	EU329495	<i>Robinea pseudoacacia</i>	Japan
<i>F. solani</i> f. sp. <i>piperis</i>	NRRL22570	AF178360	EU329513	<i>Piper nigrum</i>	Brazil
<i>F. solani</i> f. sp. <i>mori</i>	NRRL22230	AF178358	EU329499	<i>Morus alba</i>	Japan
<i>F. solani</i> f. sp. <i>mori</i>	NRRL22157	AF178359	EU329493	<i>Morus alba</i>	Japan
<i>F. solani</i> f. sp. <i>cucurbitae</i>	NRRL22098	AF178327	EU329489	<i>Cucurbita</i> spp.	United States
<i>F. solani</i> f. sp. <i>cucurbitae</i>	NRRL22153	AF178346	EU329492	<i>Cucurbita</i> spp.	United States
<i>F. solani</i> f. sp. <i>batatas</i>	NRRL22400	NRRL22400	EU329509	<i>Ipomoea batatas</i>	United States
<i>F. solani</i> 9a	NRRL32755	DQ247072	EU329613	Turtle	United States
<i>F. solani</i> 6i	NRRL28553	DQ246894	EU329548	Human	United States
<i>F. solani</i> 6g	NRRL28016	DQ246873	EF470140	Human	United States
<i>F. solani</i> 6e	NRRL32849	DQ247155	EU329628	Human eye	United States
<i>F. solani</i> 6d	NRRL32785	DQ247094	EU329618	Human	United States
<i>F. solani</i> 6a	NRRL43489	DQ790484	DQ790572	Human eye	United States
<i>F. solani</i> 34a	NRRL46703	HM347126	EU329661	Nematode	Spain
<i>F. solani</i> 25a	NRRL22389	AF178340	EU329506	<i>Liriodendron tulipifera</i>	United States
<i>F. solani</i> 22a	NRRL32751	DQ247069	EU329611	Human eye	United States
<i>F. solani</i> 20a	NRRL22608	DQ247622	EU329517	Human	United States
<i>F. solani</i> 14a	NRRL22611	DQ246840	EU329518	Human eye	United States
<i>F. solani</i> 12d	NRRL32309	DQ246937	EU329571	Human	United States
<i>F. solani</i> 12a	NRRL22642	DQ246844	EU329522	<i>Fenneropenaeus</i> spp.	Japan
<i>Fusarium plagianthi</i>	NRRL22632	AF178354	JX171614	<i>Hoheria glabrata</i>	New Zealand
<i>Fusarium pisi</i>	NRRL22820	AF178355	EU329532	<i>Pisum sativum</i>	United States
<i>Fusarium pisi</i>	NRRL22278	AF178337	EU329501	<i>Pisum sativum</i>	United States
<i>Fusarium pisi</i>	NRRL45880	FJ240352	JX171655	<i>Pisum sativum</i>	United States
<i>Fusarium phaseoli</i>	CBS265.50	HE647964	KM232375	<i>Phaseolus</i> sp.	United States
<i>Fusarium petrophilum</i>	NRRL28546	DQ246887	HM347152	Human eye	United States
<i>Fusarium petrophilum</i>	NRRL43812	EF453054	EF470093	Contact lens solution	United States
<i>Fusarium petrophilum</i>	NRRL32856	DQ247161	EU329629	Hospital	United States
<i>Fusarium neocosmosporiellum</i>	NRRL22166	AF178350	EU329497	<i>Heterodermis glycinis</i>	United States
<i>Fusarium neocosmosporiellum</i>	NRRL43467	EF469979	EF469979	Human eye	United States
<i>Fusarium neocosmosporiellum</i>	NRRL22436	AF178348	EU329511	Soil	South Africa
<i>Fusarium lichenicola</i>	NRRL34123	DQ247191	EU329635	Human eye	India
<i>Fusarium keratoplasticum</i>	NRRL43433	DQ790473	DQ790561	Human eye	United States
<i>Fusarium keratoplasticum</i>	NRRL43445	DQ790479	DQ790567	Human eye	United States
<i>Fusarium illudens</i>	NRRL22090	AF178326	AF178392	<i>Beilshmidtia tawa</i>	New Zealand
<i>Fusarium falciforme</i>	NRRL43441	DQ790478	DQ790566	Human eye	United States
<i>Fusarium euwallaceae</i>	NRRL54722	JQ038007	JQ038028	<i>Persea americana</i>	Israel
<i>Fusarium ambrosium</i>	NRRL20438	AF178332	JX171584	<i>Camellia sinensis</i>	India

^y CBS, Centraalbureau voor Schimmelcultures Fungal Biodiversity Center, Utrecht, the Netherlands; NRRL, Agricultural Research Service Culture Collection, Peoria, IL.

^z *EF1-α*, elongation factor 1-α; *RPB2*, RNA polymerase II second largest subunit.

in the growing area. In 7.7% of the greenhouses surveyed, the disease symptoms were associated with *Phytophthora capsici*. *F. solani* was also detected in the melon and watermelon greenhouses but not in any of the cucumber greenhouses inspected, where the disease symptoms were associated with *Monosporascus cannonballus* (data not shown).

Pathogenicity on zucchini plants and fruit. In the pathogenicity trials of *F. solani* on zucchini plants, all isolates tested, except PCI-2121, were pathogenic to zucchini, reproducing the crown and foot rot symptoms (Supplementary Fig. S1). The first disease symptom observed was plant wilt observed at 16 and 9 days after inoculation (DAI) for the first and second trials, respectively. In the first trial, the most virulent isolates causing visible disease symptoms were PCI-511, PCI-711, PCI-1641, and PCI-2211, and the less virulent ones were PCI-341 and PCI-1911, which showed the first diseased plants by 22 DAI. By 27 DAI, all of the pathogenic isolates had caused disease in at least 8% of the plants, and by 41 DAI, only 2% of the plants inoculated with PCI-1121 and 4% of the plants inoculated with PCI-1911 remained asymptomatic. Three isolates showed a 100% M rate by 41 DAI, namely PCI-1731, PCI-1811, and PCI-2111 (Table 2). In this trial, temperatures ranged from 6.4 to 34.9°C with a mean of 18.1°C in the greenhouse environment and from 14.4 to 28.7°C with a mean of 21.4°C in the substrate bags.

In the second trial, the most virulent isolates causing disease symptoms were PCI-511, PCI-731, and PCI-1731, and the least virulent one was PCI-1121, which caused visible disease symptoms at 17 DAI. At 27 DAI, all of the pathogenic isolates had caused disease in at least 6% of the plants, and by 34 DAI, all of the inoculated

plants, except one plant inoculated with PCI-341 and another plant inoculated with PCI-1121, were diseased. By this date, all isolates had caused a 100% M rate, except PCI-341, PCI-1121, PCI-1431, PCI-1911, and PCI-2111. In this trial, temperatures ranged from 18.5 to 37.1°C with a mean of 27.4°C in the greenhouse environment and from 23.5 to 37.1°C with a mean of 29.7°C in the substrate bags.

In both trials, the isolate PCI-1731 was the most aggressive, showing an RAUDPC significantly higher than the rest and M rates of 100%, and it was followed by the isolate PCI-1721; both were obtained from watermelon. In contrast, the isolates PCI-1121 and PCI-341 from zucchini produced the lowest RAUDPC. Noninoculated controls and plants inoculated with the isolate PCI-2121 remained asymptomatic (DI = 0%). In both trials, the pathogen was successfully recovered from all plants selected for reisolation.

In fruits, all of the isolates, including PCI-2121, caused lesions, which consisted of brown, water-soaked rot that sometimes formed white mycelium on the surface. The fruit lesion growth rate ranged from 0.9 to 2.64 mm day⁻¹, corresponding to isolates PCI-341 and PCI-2121, respectively, the latter being the only isolate not pathogenic on zucchini stems. Fruits inoculated with uncolonized PDA plugs showed no symptoms (Table 2). *F. solani* isolates were successfully recovered from the lesions.

Pathogenicity on other horticultural crops. In the first pathogenicity trial on the various horticultural crops, all plants belonging to different plant families except cucurbits remained asymptomatic (data not shown). All species in the cucurbit family except cucumber showed aerial disease symptoms (Table 3). The first disease symptoms were observed at 11 DAI when melon, zucchini, and the three

Table 2. Collection details, GenBank accession numbers for the elongation factor 1- α (*EF1- α*) and RNA polymerase II second largest subunit (*RPB2*) loci, and final disease parameters of the pathogenicity tests performed in zucchini plants and fruits of the strains of *Fusarium solani* species complex examined in this study

Isolate	Host	Year	Disease parameters								
			GenBank accession number	Zucchini plants ^x						Zucchini fruits ^y	
				First trial			Second trial				
				DI (%)	M (%)	RAUDPC	DI (%)	M (%)	RAUDPC		
Fruit lesion (mm·day ⁻¹)											
PCI-341	Zucchini	2007	MK048138	MK048118	100 a	54.17 a	0.28 ef	97.92 a	97.92 a	0.42 fgh	0.95 ± 0.28 kl
PCI-511	Zucchini	2009	KF372878	MK048117	100 a	85.42 a	0.50 bcde	100 a	100 a	0.76 ab	0.86 ± 0.12 l
PCI-571	Zucchini	2009	MK048152	MK048116	100 a	91.67 a	0.58 abc	100 a	100 a	0.69 abcde	1.13 ± 0.20 jk
PCI-711	Melon	2009	KC711040	MK048120	100 a	95.83 a	0.55 abcd	100 a	100 a	0.75 abc	1.73 ± 0.49 cd
PCI-731	Melon	2009	KC711041	MK048119	100 a	95.83 a	0.58 abc	100 a	100 a	0.77 ab	1.54 ± 0.34 de
PCI-1021	Zucchini	2010	MK048151	MK048132	100 a	93.75 a	0.51 bcde	100 a	100 a	0.70 abcd	1.24 ± 0.27 hij
PCI-1111	Zucchini	2011	MK048150	MK048123	100 a	83.33 a	0.53 bcde	100 a	100 a	0.81 ab	1.80 ± 0.43 bc
PCI-1121	Zucchini	2011	MK048149	MK048122	97.92 a	39.58 b	0.27 ef	97.92 a	70.83 b	0.28 gh	1.18 ± 0.27 ij
PCI-1421	Zucchini	2011	MK048148	MK048115	100 a	97.92 a	0.66 ab	100 a	100 a	0.69 abcd	1.15 ± 0.18 j
PCI-1431	Zucchini	2011	MK048147	MK048114	100 a	89.58 a	0.59 abc	100 a	95.83 a	0.51 defgh	1.14 ± 0.19 j
PCI-1511	Zucchini	2011	MK048136	MK048131	100 a	95.83 a	0.56 abcd	100 a	100 a	0.72 abcd	1.96 ± 0.32 b
PCI-1521	Zucchini	2011	MK048146	MK048129	100 a	72.92 a	0.45 cdef	100 a	100 a	0.55 cdefgh	1.08 ± 0.24 jk
PCI-1621	Zucchini	2011	MK048145	MK048124	100 a	93.75 a	0.53 bcde	100 a	100 a	0.61 bcdefg	1.34 ± 0.28 fghi
PCI-1721	Watermelon	2011	MK048144	MK048125	100 a	97.92 a	0.70 ab	100 a	100 a	0.81 ab	0.90 ± 0.20 l
PCI-1731	Watermelon	2011	MK048143	MK048126	100 a	100 a	0.73 a	100 a	100 a	0.84 a	1.18 ± 0.25 ij
PCI-1811	Zucchini	2011	MK048142	MK048127	100 a	100 a	0.56 abcd	100 a	100 a	0.62 bcdefg	1.11 ± 0.21 jk
PCI-1821	Zucchini	2011	MK048141	MK048128	100 a	95.83 a	0.56 abcd	100 a	100 a	0.71 abcd	1.76 ± 0.49 c
PCI-1911	Zucchini	2011	MK048140	MK048130	95.83 a	60.42 a	0.35 def	100 a	89.58 c	0.47 efgh	1.51 ± 0.27 ef
PCI-2111	Zucchini	2011	MK048139	MK048133	100 a	100 a	0.60 abc	100 a	95.83 a	0.64 abcdef	1.43 ± 0.19 efg
PCI-2121	Zucchini	2011	MK048134	MK048110	0 b	0 b	0 f	0 b	0 d	0 h	2.64 ± 0.33 a
PCI-2211	Zucchini	2012	MK048137	MK048113	100 a	95.83 a	0.65 ab	100 a	100 a	0.61 bcdefg	1.41 ± 0.23 efgh
PCI-2321	Zucchini	2012	MK048135	MK048121	100 a	97.91 a	0.64 ab	100 a	100 a	0.70 abcd	1.25 ± 0.24 ghij
PCI-25F1	Asparagus	2008	MK048154	MK048108	n/a ^z	n/a	n/a	n/a	n/a	n/a	n/a
PCI-25F2	Pepper	2008	MK048156	MK048112	n/a	n/a	n/a	n/a	n/a	n/a	n/a
PCI-25F3	Green bean	2008	MK048155	MK048109	n/a	n/a	n/a	n/a	n/a	n/a	n/a
PCI-25F4	Orange tree	2009	MK048153	MK048111	n/a	n/a	n/a	n/a	n/a	n/a	n/a

^x Disease parameters obtained at the end of the two trials conducted expressed as the percentage of plants at the end of the experiment. Values in columns followed by different letters indicate significant differences according to the Kruskal–Wallis nonparametric analysis of variance ($P < 0.05$). DI, disease incidence; M, mortality; RAUDPC, relative area under the disease progress curve expressed as the proportion of the maximum potential area under the disease progress curve assessed by plotting the disease rates per plant on the basis of a 0 to 2 scale versus time with the trapezoidal method (Madden et al. 2007).

^y Measurements of lesions caused in zucchini fruits after 7 days expressed as millimeters·day⁻¹. Values in columns followed by different letters indicate significant differences according to the least significant difference method ($P < 0.05$). Data of the two experiments were pooled prior to analyses.

^z n/a, not available.

rootstock hybrids showed some diseased plants. Three days later at 14 DAI, there was at least one wilted plant per cucurbit species except cucumber. Plants were maintained up to 31 DAI owing to the height of the cucumber plants, which did not allow us to maintain the crop for a longer time. By that time, the species most affected was zucchini followed by melon, watermelon, and the three rootstock hybrids. Cucumber plants were asymptomatic because no visible wilting or crown rot was observed (Table 3). A detailed evaluation

of the root system revealed necrosis in the root system of 100% of the plants, indicating the ability of the isolates to cause disease in this species. Temperatures in this trial ranged from 9 to 39.7°C with a mean of 21.5°C in the greenhouse environment and from 9.4 to 34°C with a mean of 21.2°C in the pots.

In the second trial, such as was observed in the previous one, only plants belonging to the cucurbit family showed disease symptoms, with the isolates being nonpathogenic to the other families. The first

Table 3. Disease parameter values for nine cucurbit species and cultivars tested for susceptibility to *Fusarium solani* f. sp. *cucurbitae* isolates PCI-511 and PCI-1621 in greenhouse experiments during the two trials conducted

Species	Trial 2013 ^y			Trial 2014		
	DI (%)	M (%)	RAUDPC	DI (%)	M (%)	RAUDPC
<i>Cucumis sativus</i> 'Borja'	0	0 c	0 c	91.6	80.5	0.37 c
<i>Cucumis sativus</i> 'Marketmore'	0	0 c	0 c	88.9	77.8	0.26 c
<i>Cucumis sativus</i> 'Pepinillo Jardiver'	n/a ^z	n/a	n/a	88.9	80.6	0.24 c
<i>Cucumis melo</i> 'Amarillo Jardiver'	97.2	97.2 a	0.64 a	100	100	0.77 ab
<i>Cucurbita pepo</i> 'Cónsul'	100	97.2 a	0.62 a	100	100	0.88 a
<i>Citrullus lanatus</i> 'Mariola'	97.2	63.9 b	0.30 b	100	100	0.70 b
<i>Cucurbita maxima</i> × <i>Cucurbita moschata</i> 'Hércules'	94.4	44.4 b	0.41 b	100	91.7	0.71 ab
<i>Cucurbita maxima</i> × <i>Cucurbita moschata</i> 'Titán'	86.1	16.7 b	0.26 b	100	83.3	0.66 b
<i>Cucurbita maxima</i> × <i>Cucurbita moschata</i> 'RS-841'	80	23.3 b	0.24 b	100	72.2	0.66 b

^y Values in columns followed by different letters indicate significant differences according to the Kruskal–Wallis nonparametric analysis of variance ($P < 0.05$). DI, disease incidence expressed as the percentage of the total number of plants; M, mortality; RAUDPC, relative area under the disease progress curve expressed as the proportion of the maximum potential area under the disease progress curve assessed by plotting the disease rates per plant on the basis of a 0 to 2 scale versus time with the trapezoidal method (Madden et al. 2007).

^z n/a, not available.

Fig. 1. Effect of *Fusarium solani* f. sp. *cucurbitae* inoculation with the isolates PCI-511 and PCI-1621 on different cucurbit species and hybrids. Data correspond to the final relative area under the disease progress curve (RAUDPC) calculated as the proportion of the maximum possible area under the disease progress curve assessed by plotting the rating on the basis of a 0 to 2 scale versus time, with the trapezoidal method (Madden et al. 2007) calculated 60 days after the inoculation in the second trial to evaluate the pathogenicity of *F. solani* f. sp. *cucurbitae* to several horticultural crops.

disease symptoms were observed at 8 DAI, and as in the previous year, all species except cucumber and watermelon were the most susceptible. The first watermelon plants showed wilt symptoms at 12 DAI; the cucumber cultivars Marketmore and Borja displayed the first wilted plants at 26 DAI, whereas the cultivar Pepinillo Jardiver displayed symptoms at 29 DAI. By 29 DAI, the DI for zucchini and

the three *Cucurbita* hybrids was 100%; melon and watermelon displayed 91.7 and 86.1% DI, respectively; and the cucumber cultivars Marketmore, Borja, and Pepinillo Jardiver displayed 13.9, 41.7, and 11.1% DI, respectively. Plants were maintained in the greenhouse for a total of 60 DAI when the DI for all of the cucurbits tested, except cucumber, was 100%. M for melon, zucchini, and watermelon was

Fig. 2. Morphological description of *Fusarium solani* f. sp. *cucurbitae*. **A and D**, Growth on potato dextrose agar (PDA). **B and C**, Yellow sporodochia after 10 days on pieces of carnation leaves on carnation leaf-piece agar (CLA). **E to G**, Long and branched conidiophores producing zero- to one-septate microconidia. **H and I**, Macroconidia produced in CLA. **J and K**, Microconidia produced in PDA. **L and M**, Long chains of intercalary chlamydospores produced in PDA. **N**, Terminal chlamydospores produced in PDA. Scale bars: **H, L, and M**, 50 μ m; **G, I, and N**, 20 μ m; **J and K**, 10 μ m.

100%, and the M for the rest of the cucurbits was between 91.7 and 72.2% (Table 3). In this trial, temperatures ranged from 12.3 to 39.5°C with a mean of 23.6°C in the greenhouse environment and from 21.3 to 35.4°C with a mean of 27.5°C in the substrate bags.

When comparing the ability of the two isolates to cause disease on the different cucurbit hosts, a higher aggressiveness was observed in PCI-1621 compared with PCI-511 in both experiments. In the first trial by 11 DAI and in second trial by 8 DAI, the isolate PCI-511 had only caused visible disease in zucchini, whereas PCI-1621 had caused disease symptoms in melon, zucchini, and the three *Cucurbita* hybrids. In watermelon in the first trial, one plant inoculated with PCI-511 showed symptoms at 14 DAI, and no other plants were symptomatic until 21 DAI when several plants inoculated with both isolates were wilted. In the second trial, the first disease symptoms were observed in watermelon plants by 12 DAI, and in cucumber, they were observed by 26 and 29 DAI. In both cases, they were produced by PCI-1621, whereas in all cases, plants inoculated with PCI-511 remained asymptomatic by those dates. The final RAUDPC data per species and isolate of the second experiment are presented in Figure 1.

Morphology. Culture characteristics. Colonies on PDA showed radial growth rates of 8 to 8.5 mm day⁻¹ at 23°C and 7.8 to 9.1 mm day⁻¹ at 29°C in the dark, reaching a diameter of 51 to 57 mm after 6 days at 26°C. Colony surface was white and cottony with white to straw aerial mycelium and sporulation abundant; colony margins were regular to slightly undulate and reverse white to pale straw (Fig. 2).

Cardinal temperatures for growth. Temperatures were a minimum of 8°C, a maximum of 35°C, and an optimum of 23 to 29°C (Fig. 3).

Sporulation abundant from conidiophores formed on aerial mycelium, on the agar surface, and from sporodochia. Conidiophores were straight, long, solitary, and simple or branched one to several times

verticillately or sympodially, with each branch bearing a single terminal monophialide. Microconidia formed on aerial conidiophores, clustered in false heads at the tip of monophialides, hyaline, oval or obovoid with a truncate base, symmetrical or gently bent dorso-ventrally, smooth and thin walled, 0–(1) septate. Zero-septate conidia were (5.8) 7.0–10.4 (12.9) × (3.1) 3.9–5.3 (5.9) μm. One-septate conidia (11.1) 12.7–17.5 (21.0) × (3.9) 4.8–6.3 (6.7) μm. Cream-colored sporodochia formed abundantly on the surface of carnation leaves. Sporodochial macroconidia showed slight to moderate dorsal curvature, elongate and slender, apical cell blunt or papillate, sporadically hooked, more or less equally sized than the adjacent cell, basal cell barely or distinctly notched, (3) 4–5 (6–7) septate, hyaline, thin and smooth walled. Three-septate conidia were (34.3) 37.2–46.5 (48.7) × (4.1) 4.5–5.6 (6) μm. Four-septate conidia were (45.9) 49.4–58.8 (64.7) × (3.4) 4.5–5.8 (6.8) μm. Five-septate conidia were (55) 57.9–64.1 (67.6) × (3.9) 4.6–5.8 (6.3) μm. Six-septate conidia were (68.6) 74.6–90.7 (101.5) × (4.1) 4.8–6 (7.1) μm. Overall was (34.3) 44.2–75.7 (101.5) × (3.4) 4.6–5.8 (7.1) μm. Chlamydospores were abundant and rapidly formed on agar media (approximately 10 days), hyaline to pale brown, spherical to subspherical (5.9) 8.3–10.8 (12) μm in diameter, solitary in pairs or in chains, terminal or intercalary, and smooth and thick walled (Fig. 2).

Phylogeny. Phylogenetic analyses inferred from the *EF1-α* and *RPB2* datasets resolved the position of all of the isolates included in this study within the FSSC clade 3 in relation to currently recognized monophyletic species. The single and combined *EF1-α* and *RPB2* datasets included 76 strains. The *EF1-α* tree consisted of 436 characters, of which 142 characters were parsimony informative (CI = 0.67, RI = 0.89) (Supplementary Fig. S2); the *RPB2* tree consisted of 555 characters, of which 165 characters were parsimony informative (Supplementary Fig. S3) (CI = 0.59, RI = 0.85), and the combined *EF1-α* and *RPB2* dataset consisted of 1,011 nucleotides,

Fig. 3. Growth rates per day on potato dextrose agar of isolates PCI-341, PCI-511, PCI-731, and PCI-1621 measured after 6 days of incubation in constant dark at temperatures ranging from 8 to 35°C in 3°C intervals and expressed in millimeters·day⁻¹.

of which 307 were parsimony informative (Fig. 4) (CI = 0.61, RI = 0.86). Phylogenetic analysis resulted in the 92 most parsimonious trees. No major topological differences were detected between Bayesian, neighbor-joining, and maximum parsimony phylogenetic inferences (data not shown).

In the three inferences, all of the isolates pathogenic to zucchini clustered together with the only two available *F. solani* f. sp. *cucurbitae* sequences in GenBank, NRRL22098, and NRRL22153, forming a monophyletic group with 100% bootstrap support. The single *RPB2* and combined *EF1-α* and *RPB2* datasets generated two *F. solani* f. sp. *cucurbitae* lineages, one of them containing eight isolates from Spain that are grouped with isolates NRRL22098 and NRRL22153 and another one containing isolates exclusively from Spain; however, only the second lineage presented bootstrap support. The isolate NRRL22570, corresponding to *F. solani* f. sp. *piperis*, was resolved as the sister group of the *F. solani* f. sp. *cucurbitae* group.

The remaining isolates included in this study, nonpathogenic to zucchini, were also placed in the FSSC clade 3; however, their positions varied. According to the combined loci phylogeny, the isolate PCI-2121, pathogenic on zucchini fruit, was placed in the *Fusarium keratoplasticum* lineage with a bootstrap value of 81%. The isolates PCI-25F1 and PCI-25F3 isolated from asparagus and bean, respectively, were placed in the *Fusarium falciforme* lineage with a bootstrap value of 81%. The isolate PCI-25F2, isolated from pepper (*Capsicum annuum*), was clustered in the *Fusarium striatum* lineage with 100% bootstrap value. The isolate PCI-25F4, obtained from orange tree roots, clustered with *F. solani* sensu stricto strains NRRL25388 and NRRL32737; bootstrap support was only observed for the combined *EF1-α* and *RPB2* datasets (Fig. 4).

Discussion

The results from our study indicate that *F. solani* f. sp. *cucurbitae* is the main soilborne fungus affecting adult zucchini plants in the province of Almería, being the problem of less importance in melon and watermelon crops. *F. solani* f. sp. *cucurbitae* was detected in 88.4% of the greenhouses surveyed. The disease caused by this fungus frequently associated with greenhouses dedicated to zucchini monoculture because this is a common practice in the area. The symptoms observed both in the commercial greenhouses as well as in the artificial inoculations reproduced the typical brown crown rot described by previous authors (Bourbos et al. 1997; Champaco et al. 1993; Doidge and Kresfelder 1932; Fantino et al. 1989; García-Jiménez et al. 1997; Mehl and Epstein 2007; Snyder and Hansen 1941). *Phytophthora capsici* was also detected, causing disease in adult zucchini plants (Gómez et al. 2013). In all of the diseased cucumber plants found in commercial greenhouses, perithecia of *Monosporascus cannonballus* were consistently observed and isolated; however, after several pathogenicity tests on adult plants grown in perlite bags, the symptoms could not be reproduced (data not shown).

With respect to pathogenicity, all of the *F. solani* f. sp. *cucurbitae* isolates studied were highly virulent to zucchini. The isolate PCI-2121 was not pathogenic, and it was identified based on phylogenetic analysis as *F. keratoplasticum*. In our trials, almost 100% of the plants inoculated with *F. solani* f. sp. *cucurbitae* showed disease symptoms relatively early (by 44 and 38 DAI) in the first and second trials, respectively. In addition, large numbers of plants were dead by those dates, with M rates of 67 to 100% in the first trial and 73 to 100% in the second trial. The two trials were conducted consecutively in winter and spring 2013. Disease development was favored in the second trial, most likely because of the

Fig. 4. One of the most parsimonious trees of the *Fusarium solani* species complex inferred from the elongation factor 1- α (*EF1-α*) + RNA polymerase II second largest subunit (*RPB2*) combined gene sequences. The dataset comprised 1,011 bp of aligned sequence data. The phylogram was rooted with *Fusarium thapsinum* NRRL22045. Bold lines indicate bootstrap values >70% based on 1,000 pseudoreplicates. CI, consistency index; PIC, parsimony informative; RI, retention index. * Lineage composed of *F. solani* f. sp. *cucurbitae* isolates from Spain (bootstrap 70.9%). ** Lineage clustering isolates from Spain and the U.S.A. (no bootstrap support).

higher temperatures. In the first experiment, the average temperature measured in the substrate bags was 21.4°C; however, in the second experiment, the mean temperature in the substrate bags was 29.7°C, which is closer to the optimal temperature range assessed in our *in vitro* experiments (i.e., between 26 and 29°C). These results are in agreement with those of Miguel (2001). Another study performed previously to compare different *F. solani* isolates obtained from sweet pepper plants and *F. solani* f. sp. *cucurbitae* reported the highest growth rate at 30°C (Paternotte 1987). In our morphological studies, the daily growth at 23°C was between 8 and 8.5 mm day⁻¹ on PDA. Camilo (2001) reported a daily growth of 6.11 to 6.22 mm day⁻¹ at the same temperature; however, these data differ from the description in the work by Sandoval-Denis et al. (2019), who observed an average daily growth of 2.4 to 4.2 mm day⁻¹ at 24°C.

In addition to studying the pathogenicity on whole zucchini plants, zucchini fruits cultivar Cónsul were inoculated with the same strains to study the ability of the fungus to cause fruit rot. In this case, all isolates were able to produce visible fruit rot. In both trials, PCI-2121 caused the highest growth in the zucchini fruits, being significantly higher than the rot produced by the *F. solani* f. sp. *cucurbitae* strains. Although fruit rot was observed in laboratory experiments, these symptoms were not observed in the diseased plants in the field, probably because the fruits are normally harvested in the immature state and because of the lack of contact between the fruit and soil.

Our phylogenetic inference placed the isolate PCI-2121 in the *F. keratoplasticum* lineage, an opportunistic pathogen causing human eye infections (Chang et al. 2006). In 2018, Šišić et al. (2018) recovered *F. keratoplasticum* from naturally diseased pea, showing its potential pathogenicity to this plant species. This has clinical importance because plant debris and soil may be a potential source of infection for farmers and crop workers (Šišić et al. 2018). *F. keratoplasticum* commonly occurs in plumbing biofilms and is among the most frequent etiological agents of fusarial keratitis together with *F. petrophilum* (Short et al. 2013). Most known *F. keratoplasticum* and *F. petrophilum* isolates have been obtained from plumbing, infected humans, or animals (Mehl and Epstein 2008; Short et al. 2011, 2013; Zhang et al. 2006); however, *F. petrophilum* was considered to be *F. solani* f. sp. *cucurbitae* race 2 because of its pathogenicity to cucurbit fruit in naturally infested fields until it was reclassified in 2013. In addition, *F. keratoplasticum* was isolated from zucchini in this study and also, from pea, indicating the plasticity of this FSSC member to colonize different environments and the potential threat to growers and field workers.

The greenhouse data on the host range of *F. solani* f. sp. *cucurbitae* are in agreement with previous reports that limit the host range of this forma specialis to cucurbits (Miguel 2001; Paternotte 1987; Tousson and Snyder 1961). In our trials, all plants evaluated belonging to the cucurbit family were susceptible to the pathogen. The rest of the plants belonging to other families were asymptomatic, and the fungus could not be recovered from these plants. Within the cucurbit species tested, differences in susceptibility were observed. Based on previous trials performed with zucchini, we conducted a first host range experiment in pots placed on benches, inoculating the plants in the seedling stage. The *F. solani* f. sp. *cucurbitae* isolates produced visible crown rot a few weeks after inoculation in several melon, watermelon, zucchini, and *Cucurbita* rootstock hybrids. Under the same conditions, cucumber plants were able to remain asymptomatic, but rot was observed below the soil level when the plants were removed and the root system was evaluated for symptoms. This led us to repeat the trial with the same design but in perlite bags to maintain the plants in the greenhouse for a longer period. In the second trial, the same tendency was observed, with melon, zucchini, and watermelon plants being the most susceptible plants closely followed by the *Cucurbita* hybrids. Cucumber plants remained asymptomatic for a longer period of time compared with the rest of the cucurbits. At 26 DAI, the first wilted cucumber plants were observed, and by the end of the experiment, 20 to 31% of the plants had remained asymptomatic, indicating that, although this species is also a suitable host for *Fusarium* crown and foot rot, it is a less susceptible host for the disease.

The phylogeny inferred from the combined *EF1-α* and *RPB2* datasets as well as the single-gene phylogenies placed our isolates within clade 3 of the FSSC as previously reported in other studies (Chang et al. 2006; O'Donnell 2000; O'Donnell et al. 2008; Sandoval-Denis et al. 2019; Zhang et al. 2006). The isolates characterized as *F. solani* f. sp. *cucurbitae* were clustered together with NRRL22098 and NRRL22153 with 100% bootstrap support, suggesting that they correspond to the same phylogenetic species, with *F. solani* f. sp. *piperis* as the ancestral group as previously reported in other FSSC phylogenies (O'Donnell 2000; O'Donnell et al. 2008; Short et al. 2013; Šišić et al. 2018). The combined *EF1-α* and *RPB2* datasets separated the *F. solani* f. sp. *cucurbitae* into two lineages, one of them containing only Spanish isolates, with identical haplotypes (bootstrap 85.2% for the *RPB2* and 70.9% for the combined dataset). The other lineage contains the rest of the isolates together with NRRL22098 and NRRL22153; these isolates have previously been identified as pathogenic to cucurbits (Elmer et al. 2007; O'Donnell 2000). This group is more diverse, containing different haplotypes, and it did not receive bootstrap support. Our inference suggests that the first lineage could have been diverged in our region after the introduction of *F. solani* f. sp. *cucurbitae*. To resolve this, more loci should be included for the phylogenetic inference, and a wider disease survey conducted in different regions of Spain would shed light on this hypothesis.

With respect to morphology, our study reveals some variations to the traditional description of *F. solani*. Although *F. solani* produces relatively wide, straight, stout, and robust macroconidia, mostly three or four septate (Schroers et al. 2016), the *F. solani* f. sp. *cucurbitae* isolates produce relatively long and slender macroconidia, mostly five septate measuring >55 µm and less frequently, six septate measuring >75 µm. These results are concordant with the observations made by Paternotte (1987), who found that conidia from *F. solani* f. sp. *cucurbitae* were up to five septate and more slender than conidia of *F. solani* from pepper, which were up to three septate. These observations are in agreement with the description of Sandoval-Denis et al. (2019), who described the macroconidia as long and narrow. Sandoval-Denis et al. (2019), however, reported macroconidia up to nine septate, and we have never observed macroconidia containing more than seven septa in any of the isolates included in this study.

In addition, in our *F. solani* f. sp. *cucurbitae* isolates, the conidiophores were often verticillate, which is not common in the *F. solani* morphological description (Leslie and Summerell 2006; Nelson et al. 1994; Schroers et al. 2016), and this was not reported by Sandoval-Denis et al. (2019), who described conidiophores rarely sparingly branched. We also observed chlamydospores forming long chains, which is a characteristic that has never been described for *F. solani* f. sp. *cucurbitae*. Although our chlamydospores ranged between 5.9 and 12 µm in diameter, Sandoval-Denis et al. (2019) described smaller chlamydospores ranging between 4 to 5 and 9 µm in diameter.

Fusarium taxonomy has been controversial since the first description of this fungus by Link in 1908. The initial species descriptions were based exclusively on morphological or physiological characteristics, and the use of new approaches in the last decades, such as sexual compatibility or phylogenetic analysis, has challenged the morphological species description of the genus *Fusarium* because it has been demonstrated that many of those descriptions correspond to species complexes composed of distinct species that are morphologically indistinguishable (Leslie and Summerell 2006; Nelson et al. 1994; Zhang et al. 2006). The forma specialis nomenclature is an informal taxonomic rank that is also controversial (Armstrong and Armstrong 1981; Jacobson and Gordon 1991; Rossman and Palm-Hernández 2008). In several cases, species have been described and assigned to a forma specialis without proper host range tests, and this taxonomic classification provides no insights into the phylogeny or the evolutionary history of the pathogen (Al-Hatmi et al. 2018; Fourie et al. 2011; Šišić et al. 2018). This fact leads us to consider that there were two races of *F. solani* f. sp. *cucurbitae* for decades until vegetative compatibility and phylogeny demonstrated

that they correspond to two very distinct species (Matuo and Snyder 1973; Mehl and Epstein 2007; O'Donnell 2000; Zhang et al. 2006). In 2018, Šišić et al. (2018) reevaluated *F. solani* f. sp. *pisi* and assigned it to *Fusarium pisi* comb. nov. Šišić et al. (2018) confirmed the pathogenicity of this species to 33 hosts corresponding to 10 legume genera, which is in agreement with previous studies, expanding the host range of this forma *specialis* to chickpea (*Cicer auriantum* L.) and other nonlegume hosts (Matuo and Snyder 1972; Westerlund et al. 1974). However, in 2012, Kolander et al. (2012) expanded the host range of *Fusarium virguliforme* (formerly *F. solani* f. sp. *glycines*) to several legumes, and the host range of *Fusarium oxysporum* f. sp. *chrysanthemi* was expanded in 2018 to orange coneflower (*Rudbeckia fulgida*) (Matić et al. 2018). Šišić et al. (2018) stress the confusion that the forma *specialis* nomenclature has caused in several cases and challenge this nomenclatural system, stating that it might deserve revision and formal taxonomic treatment. Similarly, several authors stress the necessity for proper species descriptions and nomenclatures to designate cryptic speciation within the FSSC (Debourgogne et al. 2010; O'Donnell 2000; Wang et al. 2011; Zhang et al. 2006).

Based on the phylogenetic data and the morphological differences between *F. solani* and *F. solani* f. sp. *cucurbitae*, the definition of the latter to species rank within the FSSC by Sandoval-Denis et al. (2019) is supported.

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