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Molecular and Pathogenic Characterization of *Fusarium redolens*, a New Causal Agent of Fusarium Yellows in Chickpea

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Abstract

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The association of *Fusarium redolens* with wilting-like symptoms in chickpea in Lebanon, Morocco, Pakistan, and Spain is reported for the first time, together with the molecular and pathogenic characterization of isolates of the pathogen from chickpea of diverse geographic origin. Maximum parsimony analysis of sequences of the translation elongation factor 1α (TEF-1α) gene grouped all *F. redolens* isolates from chickpea in the same main clade. Pathogenicity assays using three chickpea cultivars and isolates from different geographic origins indicated that *F. redolens* is mildly virulent on chickpea. Moreover, infection of chickpea by *F. redolens* induces a disease syndrome similar to that caused by the yellowing pathotype of *F. oxysporum* f. sp. *ciceris,* including leaf yellowing and necrosis that develop upward from the stem base, and premature senescence of the plant. In contrast, *F. redolens* does not cause discoloration of the vascular tissues in chickpea but does cause brown necrotic lesions in the tap root and necrosis of lateral roots. *F. redolens* is not easily differentiated from *F. oxysporum* f. sp. *ciceris* using morphology-based diagnosis, and the two species cause similar symptoms on chickpea; therefore, the use of molecular protocols should help to avoid misdiagnoses of Fusarium yellows in chickpea.

Chickpea (*Cicer arietinum* L.) is an important source of human food and animal feed that also helps in the management of soil fertility, particularly in dry areas. In the European Union, chickpea production is concentrated in the Mediterranean Basin, with Spain being the main producer (16). In Spain, most chickpea crops are located in the Córdoba and Sevilla provinces of Andalusia (45).

Chickpea can be severely affected by several diseases of diverse etiology, among which Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* Matuo & K. Sato, is the most important soilborne disease limiting chickpea production in the Mediterranean Basin and the Indian subcontinent (26,39,47). Fusarium wilt-affected chickpea can show an early wilting syndrome a few weeks after sowing or a late wilting one later in the season. Early wilt is characterized by flaccidity of individual leaves followed by a dull-green discoloration, desiccation, and collapse of the entire plant; whereas late-wilted plants exhibit drooping of the petioles and leaflets, followed by yellowing and necrosis of foliage (27,29). Although both syndromes are a direct consequence of vascular infection by the pathogen, they may also be caused by different plant pathogens and, as a consequence, be misidentified as Fusarium wilt if wrongly diagnosed. For instance, several plant viruses can induce leaf yellowing and flaccidity in chickpea (29,35,51). Similarly, plants showing leaf yellowing and necrosis are frequently found in chickpea crops affected by the wilt and root rot disease complex that has been reported in California, India, and southern Spain associated with infections by *F. oxysporum* f. sp. *ciceris, F. solani* f. sp. *pisi* (F.R. Jones) W.C. Snyder & H.N. Hansen, *F. solani* f. sp. *eumartii* (C.W. Carp.) W.C. Snyder & H.N. Hansen (= *F. eumartii* C.W. Carp.), and *Macrophomina phaseolina* (Tassi) Goid. (23,37,51,59,63). In those cases, as well as in chickpea plants affected by abiotic stresses, isolations in pure culture often yield nonpathogenic isolates of *F. oxysporum* or other *Fusarium* spp. with morphology similar to that of the *F. oxysporum* species-complex (1,36,59; R. M. Jiménez-Díaz and B. B. Landa, *unpublished results*). Consequently, isolating *F. oxysporum*-like fungi from chickpea showing wilt-like symptoms, even if performed from upper stem tissues, does not guarantee recovery of *F. oxysporum* f. sp. *ciceris* and can lead to misdiagnosis of Fusarium wilt if isolates are not further characterized by morphological or molecular examinations.

Typically, morphology-based diagnoses of *Fusarium* spp. are hugely challenging due mainly to the use of different taxonomic systems, the reduced number of mycologists with adequate experience for identifying these fungi, and the lack of sufficiently informative morphological features. Moreover, the morphology-based identification of *Fusarium* spp. relies on a limited number of taxonomic characters of minor differences in morphology and varying importance in different *Fusarium* spp. (42). In addition, the microscopic traits are sensitive to the influence of environmental conditions, and their plasticity and intergradation make them prone to misinterpretation (42). For example, differentiating *F. redolens* from *F. oxysporum* can sometimes be especially difficult because it is based mainly on size differences of their macroconidia (22) and is further complicated by the presence of intermediate conidial forms (2). For these reasons, the taxonomic position of *F. redolens* has always been problematic. Thus, Booth (10) treated this fungus as a variety of *F. oxysporum*, whereas Nelson et al. (50) considered *F. redolens* a synonym of *F. oxysporum*. However, use of DNA-based methodologies made it recently possible to clearly differentiate between these two species and also revealed that *F. redolens* and the *F. oxysporum* species complex lack a sister group relationship (4,17,53,61,62).

As in the *F. oxysporum* complex, *F. redolens* harbors isolates that can cause a wide range of diseases, including cortical rot, seedling damping-off, and wilt diseases (10,20,21). Furthermore, differentiating between diseases caused by *F. redolens* and *F. oxysporum* has also been complicated because they can induce symp-

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toms similar to those that occur with asparagus crown and root rot associated with decline of the crop (65). Similarly, *F. redolens* occurs in association with *F. oxysporum* f. sp. *pisi* in wilt-affected pea but each of these fungi can cause wilting in this plant separately and induce symptoms indistinguishable from one another (10).

Currently, *F. redolens* and *F. oxysporum* are most easily diagnosed based on restriction fragment length polymorphism (RFLP) patterns of their rRNA internal transcribed spacer (ITS) regions (61,62). However, this ITS-RFLP assay does not differentiate *F. redolens* from *F. hostae,* a close relative (5,19). That technique is also expensive and technically demanding because it calls for the use of three restriction enzymes. More recently, development of species-specific polymerase chain reaction (PCR) assays for the identification of *F. hostae*, *F. redolens*, and *F. oxysporum* have facilitated their diagnosis and helped to sort out some isolates originally misidentified (5,15,31,46).

This study was conducted to comparatively determine the nature of *Fusarium* spp. associated with leaf yellowing in chickpea in different countries. The association of *F. redolens* with chickpea showing wilting-like symptoms in Spain and other countries is reported for the first time. Furthermore, chickpea *F. redolens* isolates were characterized molecularly and pathogenically and then positioned in relation to other *Fusarium* spp. infecting chickpea, other *F. redolens* isolates infecting different hosts, and the *F. oxysporum*-complex clade.

Materials and Methods

***Fusarium* isolates from chickpea.** A wilt-like syndrome was observed affecting chickpea crops during disease surveys in 12 commercial fields in Córdoba, Jaén, Sevilla, and Granada provinces at Andalusia, southern Spain. Affected plants showed yellowing or wilting and brown discoloration of vascular tissues. Pieces (5 cm long) were sampled from the uppermost main stem of diseased plants and used for isolations. Tissues were surface disinfested (0.5% NaOCl, 2 min), cut into 5-mm-long pieces, plated onto *Fusarium*-selective V8 juice-oxgall-pentachloronitrobenzene agar (11), and incubated for 7 days at 25 ± 1°C and a 12-h photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1}. Examination of cultures indicated that profuse growth of a fungus morphologically similar to *F. oxysporum* (49) occurred from the vascular ring at the cut ends of the stem pieces, suggesting vascular infection of the affected plants. In total, 84 *Fusarium* spp. isolates were recovered and subsequently characterized to species using molecular protocols (see below). Additionally, 80 chickpea isolates of *Fusarium* spp. provided by scientists around the world were used in the study (Tables 1 and 2). Those latter isolates had originally been assigned to the forma specialis *ciceris* by the source laboratory but we consistently failed to amplify the 1,500-bp *F. oxysporum* f. sp. *ciceris* specific-PCR marker (32) (see below).

All the above referred isolates are deposited in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (IAS-CSIC), Córdoba, Spain. All cultures were single spored before storage in sterile soil tubes at 4°C and in 35% sterile glycerol in water at –80ºC for long-term storage.

Fungal growth and DNA extraction. A small agar piece from actively growing *Fusarium* cultures in potato-dextrose agar (PDA) (Difco Laboratories, Detroit) was placed onto a film of sterile cellophane layered over a plate of PDA and incubated for 3 to 4 days at 25 ± 1°C and a 12-h photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1}. Then, mycelia growing over the cellophane surface were scraped off using a sterile scalpel, lyophilized, and stored at –20°C until used.

Fungal DNA was extracted from 50 mg of lyophilized mycelium using the G-SpinTM IIp Plant Genomic DNA extraction kit (Intron Biotechnology, Korea) and the Fast Prep System Bio 101 (Qbiogene, Illkirch, France), according to Jiménez-Fernández et al. (31). DNA quality was assessed by gel electrophoresis and staining with ethidium bromide. All DNA samples were accurately quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) and a Tecan Safire fluorospectrophotometer (Tecan Spain, Barcelona, Spain) (38). DNA was diluted with sterile, ultrapure water (SUW) to 20 ng/μl.

Molecular characterization of *Fusarium* spp. isolates. *Fusarium* spp. isolates from chickpea in the study were characterized as *F. oxysporum* f. sp. *ciceris* or *F. oxysporum* by means of specific-PCR markers developed by Jiménez-Gasco and Jiménez-Díaz (32) (FOC-PCR), and Jiménez-Fernández et al. (31) (FO-PCR), respectively. A *F. redolens*-diagnostic, specific-PCR assay developed by Bogale et al. (9) was used to confirm results of phylogenetic analysis of the *Fusarium* spp. translation elongation factor 1α (TEF-1α) gene (see below). The set of primers LR3 (5′-CCGTGTTTCAAG ACGGG-3′) (64) and CS33 (5′-CGAATCTTTGAACGCACATTG-3′) (60) that amplify a 900-bp region of the large subunit of the rRNA was included in the assays as an internal positive control for successful PCR amplification (Fig. 1).

All *Fusarium* isolates that were diagnosed as *F. redolens* were further characterized by RFLP assays of the ribosomal intergenic spacer region (IGS) as previously described (13,14). Profiles yielded from these assays were compared with the RFLP-IGS profiles of 15 *F. oxysporum* f. sp. *ciceris* isolates representative of the eight pathogenic races (i.e., races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) of this pathogen (30) and three isolates of *F. oxysporum* nonpathogenic to chickpea (Table 1). To generate the RFLP-IGS amplicons, a fragment of the IGS sequence of the isolates was amplified using primers PNFo (5′-CCCGCCTGGCTGCGTCCGACTC-3′) and PN22 (5′-CAAGCATATGACTACTG GC-3′). The amplicons were then digested with the restriction enzymes *Alu*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Scr*FI, and *Xho*I (13,14). DNA banding patterns were separated by electrophoresis using ReadyAgarose gels, 3% Tris-acetate-EDTA plus ethidium bromide (Bio-Rad, Madrid, Spain), visualized under UV light, and digitalized. Then, images were converted, normalized, and combined using Bionumerics 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

For those *Fusarium* spp. isolates from chickpea that could not be molecularly characterized as *F. oxysporum* f. sp. *ciceris* or *F. oxysporum*, the translation elongation factor 1-alpha (TEF-1α) gene was amplified using primers EF1 (5′-ATGGGTAAGGA(A/G) GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C) ATCATG-TT-3′) (54). Then, the amplified TEF-1α sequences were purified using a gel extraction kit (Geneclean turbo; Qbiogene), quantified as described for fungal DNA, and used for direct DNA sequencing using both primers and a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, UK) according to the manufacturer's instructions, on a capillary sequencer (ABI Model 3100 genetic analyzer; Applied Biosystems at STABVIDA (Monte da Caparica, Portugal) sequencing facilities. The TEF-1α sequences of *Fusarium* spp. were deposited in GenBank (accessions GU126796 and HQ731047 to HQ731068; Table 2). A BLAST search was done against sequences in the FUSARIUM-ID v.1.0 database (http://fusarium.cbio.psu.edu) that represent a phylogenetically diverse selection of TEF-1α sequences from the genus *Fusarium* to characterize unidentified *Fusarium* isolates (18), as well as against the GenBank database.

For phylogenetic analysis, *F. redolens* sequences were edited and aligned with representative homologous sequences from FUSARIUM-ID and GenBank databases, as well as *Fusarium* TEF-1α sequences from Baayen et al. (5), Jiménez-Gasco et al. (33), and Gurjar et al. (24) using Bionumerics 6.1 software. These studies were selected because they include the largest number of TEF-1α sequences of *F. redolens* from different hosts or of *Fusarium* spp. isolates from chickpea crops ever reported. A phylogenetic tree was generated with the maximum-parsimony method using Bionumerics 6.1. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal trees. *F. beomiforme* strain NRRL25174 was used as an outgroup.

Pathogenicity assay. Seven *F. redolens* isolates were arbitrarily selected from those in the study and used for pathogenicity assays

on chickpea. Inocula were produced on autoclaved cornmeal-sand (CMS) in flasks incubated at 25 ± 1°C and a 12-h photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1} for 2 weeks, as described before (39,40). Inoculum of *F. oxysporum* f. sp. *ciceris* race 0 isolate 7802 was also similarly increased and included in the assay for comparative purposes. The infested CMS substrates were mixed thoroughly with an autoclaved soil mixture (clay loam/sand/peat at 1:1:1, vol/vol/vol) at a rate of 1:12 (wt/wt) to reach an inoculum density of approximately 10^5 CFU/g of soil for each of the tested and reference isolates. Noninfested CMS mixed with the autoclaved soil mixture at the same rate as above served as the control.

Three Kabuli (ram-head shape, beige seed) chickpea cultivars—'P-2245' and 'PV-60' (<30 g per 100 seeds) and 'PV-61' (>44 g per 100 seeds)—were used in the study. All three cultivars are susceptible to different races of *F. oxysporum* f. sp. *ciceris* (40). Seed were surface disinfested in 2% NaOCl for 3 min, germinated, selected for uniformity (length of radicle = 1 to 2 cm), and sown into 15-cm-diameter clay pots (four plants per pot) filled with the CMS-soil mixture of an isolate. Control plants were grown in the noninfested CMS-soil mixture. Plants were incubated in Conviron MTR26 growth chambers (Conviron Ltd., Winnipeg, Canada) adjusted to 25 ± 0.2ºC, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 μE m^{-2} s^{-1}. Plants were watered as needed and fertilized weekly with 100 ml of 0.1% hydro-sol fertilizer solution (20-5-32 of N-P-K + micronutrients; Haifa Chemicals, Ltd., Haifa, Israel). The experiment consisted of a two-way factorial treatment design with *Fusarium* isolates and chickpea cultivars as factors. There were five replicated pots (four plants per pot) in a complete block design per treatment combination. The experiment was repeated once.

Disease assessment and data analysis. The incidence and severity of disease reactions were assessed at 2- to 3-day intervals until the end of the experiment, 50 days after inoculation. The severity of symptoms on individual plants was rated on a 0-to-4 scale according to the percentage of foliage with yellowing or necrosis (0 = 0, 1 = 1 to 33, 2 = 34 to 66, and 3 = 67 to 100%; and 4 = dead plant) (40,48). Incidence of foliar symptoms (*I*; 0-to-1 scale), and severity data (*S*; 0-to-4 scale) were used to calculate a disease intensity index (DII) by the equation DII = ($I \times S$)/4 (40,48). Disease progress curves were obtained from the accumulated DII over time in days from the date of inoculation. Disease development was characterized by three variables associated with disease progress curve: (i) disease onset (DO), established as the

Table 1. Isolates of *Fusarium* spp. characterized molecularly by restriction fragment length polymorphism of intergenic spacer region of rDNA, with geographic origin, year of isolation, and source

***Fusarium* spp., isolate**[a]	**Origin**	**Year of isolation**	**Source**[b]
Fusarium oxysporum f. sp. *ciceris*			
Race 0			
7802	Córdoba, Spain	1978	DPC (IAS-CSIC)
9605	Tunisia	1996	CA, ICARDA
L96-5	Lebanon	1996	CA, ICARDA
Race 1B/C			
USA-1987-W17	California, United States	1987	DPC (IAS-CSIC)
9602	Tunisia	1996	HMH, INRA
Race 1A			
8272	Jaén, Spain	1982	DPC (IAS-CSIC)
7989	Hyderabad, India	1979	MPH, ICRISAT
Race 2			
8605	Kanpur, India	1986	MPH, ICRISAT
1992 R2N	India	1986	MPH, ICRISAT
Race 3			
8606	India	1986	MPH, ICRISAT
1992 R3N	Gurdaspur, India	1986	MPH, ICRISAT
Race 4			
8607	India	1986	MPH, ICRISAT
1992 R4N	Jabalpur, India	1986	MPH, ICRISAT
Race 5			
USA W6-1	California, United States	1988	DPC (IAS-CSIC)
8012	Córdoba, Spain	1980	DPC (IAS-CSIC)
Race 6			
Tonini	California, United States	1988	DPC (IAS-CSIC)
8924	Sevilla, Spain	1989	DPC (IAS-CSIC)
F. oxysporum, nonpathogenic			
Fo-90105	Córdoba, Spain	1990	DPC (IAS-CSIC)
Fo-8250	Jaén, Spain	1982	DPC (IAS-CSIC)
Fo-506	Tarquinia, Italy	1989	DPC (IAS-CSIC)
F. redolens			
7982	Córdoba, Spain	1979	DPC (IAS-CSIC)
L96-13	Lebanon	1996	DPC (IAS-CSIC)
34.92*	Morocco	1996	CA, ICARDA
9914	Pakistan	1999	SA, NIAB
164-B3	'Castro', Córdoba, Spain	2004	This study
165-B	'Las Fuentes', Córdoba, Spain	2004	This study
167-A2,	'Monterrite 1', Córdoba, Spain	2004	This study
167-B	'Monterrite 2', Córdoba, Spain	2004	This study
181-2A, 181-3A	'Montefrío', Granada, Spain	2004	This study

[a] Race of *Fusarium oxysporum* f. sp. *ciceris* was determined by pathogenicity tests on differential chickpea lines before polymerase chain reaction assays (28,32). *F. oxysporum* isolates listed as nonpathogenic are not pathogenic to chickpea; * = only DNA was available for this isolate.

[b] Isolates from Italy, Spain, and the United States (California) were obtained from the fungal culture collection of Departamento de Protección de Cultivos (DPC), Institute of Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Córdoba, Spain. *F. oxysporum* f. sp. *ciceris* isolates from India were provided by M. P. Haware (MPH), ICRISAT, Hyderabad, India. Isolates from Tunisia were provided by M. H. Halila (MHH), Institute Nacionale de la Recherche Agronomique, Ariana, Tunisia. Isolates from Lebanon were provided by C. Akem (CA), ICARDA, Aleppo, Syria. Isolate *F. redolens* 9914 was provided by S. Alam (SA), Plant Health Group, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

time in days taken for disease severity > 0; (ii) final disease severity (DII_{final}) = disease severity observed at the final date of disease assessment; and (iii) the standardized area under the disease severity progress curve (SAUDPC), calculated by trapezoidal integration standardized for the duration of disease development in days (43). In addition, at the end of experiments, isolations were made from all plants showing a severity score <4 to determine the occurrence and extent of infection by the tested *Fusarium* isolates. Isolations were done as described above, from the lower four stem internodes and the main root (four segments).

The effects of *F. redolens* isolates and chickpea cultivars on DO, DII_{final}, and SAUDPC as well as on the extent of root and stem colonization by the fungus were determined by multivariate analysis of variance (MANOVA) and univariate standard ANOVA using the general linear model procedure of SAS (Statistical Analysis System v. 9.2; SAS Institute, Cary, NC). Replications within experiments were considered random effects in the analysis. Linear single-degree-of-freedom contrasts were computed to test the effect of selected experimental treatment combinations at $P < 0.05$. In addition, a multivariate principal components analysis was performed. A factor loading was considered significant when it was larger than 0.65. The analysis was performed using the PRINCOMP procedure of SAS software. The three disease curve elements (DO, DII_{final}, and SAUDPC) and extent of root and stem colonization by the fungus were used to characterize each *F. redolens* isolate–chickpea cultivar combination.

Results

Molecular characterization of *Fusarium* spp. isolates. Six *Fusarium* isolates (164-B3, 165-B, 167-A2, 167-B, 181-2A, and 181-3A), 7.1% of the 84 obtained from the 12 surveyed chickpea fields in southern Spain, could not be assigned to *F. oxysporum* f. sp. *ciceris* or *F. oxysporum* using the FOC-PCR (32) and FO-PCR (31) protocols, respectively (Table 1; Fig.1). Conversely, those six isolates were later characterized as *F. redolens* by sequencing of the TEF-1α gene and BLASTn analysis using the FUSARIUM-ID database (Fig. 2; Table 1). The identity of the six isolates as *F. redolens* was further confirmed by means of the *F. redolens*-specific-PCR (FR-PCR) assay developed by Bogale et al. (9; Fig. 1).

As a follow-up of the study, we characterized a set of 80 *Fusarium* spp. isolates from chickpea included in a worldwide culture collection that originally had been assigned to *F. oxysporum* f. sp. *ciceris* by source laboratories but could not be identified to this forma specialis using a specific FOC-PCR protocol (32) (*data not shown*). Use of the specific FO-PCR and FR-PCR protocols and TEF-1α gene sequencing and BLASTn analysis against the FUSARIUM-ID database led the 80 *Fusarium* spp. isolates to be identified as follows: *F. redolens*, 4 isolates (5.0%; isolates 9914I, L96-13, 34.92, and 7982; Table 1; Fig. 2); *F. oxysporum*-complex, 64 isolates (80.0%); *F. solani*-complex, 8 isolates (10.0%); *F. proliferatum*, 1 isolate; *Neocosmospora vasinfecta*, 1 isolate; and an unidentified *Fusarium* sp. that was close to *F. biseptatum* and *F. delphinoides* (Table 2). Interestingly, one of the *F. redolens* isolates (isolate 7982) obtained from wilted chickpea in a field in southern Spain in 1979 was originally assigned to the yellowing pathotype of *F. oxysporum* f. sp. *ciceris* (34; Fig. 1; Table 1).

Amplification of the IGS sequence of 15 isolates of *F. oxysporum* f. sp. *ciceris*, 3 isolates of nonpathogenic *F. oxysporum*, and 10 *F. redolens* isolates using primers PN22 and PNFo yielded a DNA fragment of approximately 1,700 bp (Table 1). Digestion of the amplified fragments with each of seven restriction enzymes produced two to four different restriction profiles per PCR product, depending upon the enzyme used. Four different combinations of restriction profiles, representing four IGS types, were identified among the assayed isolates (*data not shown*). All *F. oxysporum* f. sp. *ciceris* isolates produced identical IGS type (RFLP-IGS I) regardless of pathogenic race and geographical origin. Also, all *F. redolens* isolates yielded a single IGS type (RFLP-IGS II). The

Table 2. *Fusarium* spp. isolates originating from chickpea in different countries originally misidentified as *Fusarium oxysporum* and their most related database accessions based on translation elongation factor 1-alpha (TEF-1α) gene and species assignment

				Sequence comparison to FUSARIUM-ID v. 1.0[a]		Sequence comparison to GenBank (BLASTn)[b]	
Species	**Isolate**	**GenBank[c]**	**Origin**	**Most-related**	**Similarity (%)**	**Most-related**	**Similarity (%)**
Neocosmospora vasinfecta	3Pak	HQ731049	Pakistan	NRRL 43467	99.4 (644/648)	AY381148	100 (647/647)
Fusarium spp.	56.93	HQ731047	Morocco	NRRL 53289*	92.0 (555/603)	EU926322**	90.5 (645/713)
	…	…	…	NRRL 36186*	91.9 (559/608)	EU926307**	90.3 (635/703)
	…	…	…	NRRL 34027***	91.9 (559/608)	…	…
F. solani	cc18F	HQ731048	Israel	NRRL 22278	99.9 (675/676)	AB294872	90.5 (732/737)
	…	…	…	…	…	DQ452423	99.1 (730/737)
F. solani	cc19Y	HQ731050	Israel	NRRL 32343	99.4 (667/671)	AB498982	99.9 (693/694)
F. solani	cc43A	HQ731051	Israel	NRRL 32343	99.4 (667/671)	AB498982	99.9 (693/694)
F. solani	cc41W	HQ731052	Israel	NRRL 28555	99.7 (668/670)	GU170631	99.0 (708/715)
F. solani	2205I	HQ731053	Pakistan	NRRL 25388	99.9 (682/683)	EU925653	99.6 (717/720)
F. solani	cc40A	HQ731054	Israel	NRRL 32720	99.6 (666/669)	GU170630	99.3 (705/710)
F. solani	cc20B	HQ731055	Israel	NRRL 32720	99.6 (666/669)	GU170630	99.3 (705/710)
F. solani	cc61C	HQ731056	Israel	NRRL 32720	100 (666/669)	GU170630	99.9 (709/710)
F. oxysporum	808	HQ731057	Italy	NRRL 38608	100 (681/681)	DQ837688	100 (691/691)
F. oxysporum	817	HQ731058	Italy	NRRL 39464	99.9 (685/686)	DQ837680	99.7 (696/698)
F. proliferatum	9009	HQ731059	Spain	FD_01389	99.26 (671/676)	FJ538244	100 (678/678)
F. redolens	9914I	HQ731060	Pakistan	FD_01103	100 (665/665)	GU250584	100 (703/703)
F. redolens	34.92	HQ731063	Morocco	FD_01103	100 (665/665)	GU250584	100 (703/703)
F. redolens	7982	HQ731061	Spain	FD_01103	100 (665/665)	GU250584	100 (696/696)
F. redolens	165-B1	HQ731062	Spain	FD_01103	99.84 (663/664)	GU250584	99.9 (663/664)
F. redolens	164-B3	HQ731065	Spain	FD_01103	100 (665/665)	GU250584	100 (678/678)
F. redolens	167-A2	GU126796	Spain	FD_01103	100 (665/665)	GU250584	100 (657/657)
F. redolens	167-B	HQ731068	Spain	FD_01103	100 (665/665)	GU250584	100 (692/692)
F. redolens	181-3a	HQ731066	Spain	FD_01103	100 (663/663)	GU250584	100 (649/649)
F. redolens	181-2A	HQ731067	Spain	FD_01103	99.84 (664/665)	GU250584	99.9 (693/694)
F. redolens	L96-13	HQ731064	Lebanon	FD_01101	99.84 (663/664)	GU250581	99.7 (701/703)

[a] Most related sequences from FUSARIUM-ID v.1.0 database at http://fusarium.cbio.psu.edu based on the translation elongation factor-1α; Similarity = sequence similarity.

[b] Most related sequences from GenBank database performed with BLASTn 2.2.25; * = *Gibberella fujikuroi*-complex, ** = *F. biseptatum*, and *** = *F. dimerum* based on the translation elongation factor-1α; Similarity = sequence similarity.

[c] GenBank accession number.

three nonpathogenic *F. oxysporum* isolates from chickpea showed two different IGS types; with isolates 90105 and 8250 from Spain sharing the same RFLP-IGS III type and isolate 506 from Italy being the only one representative of RFLP-IGS type IV (*data not shown*).

PCR assays using primers EF1 and EF2 yielded a TEF-1α gene fragment of approximately 700 bp from all *Fusarium* spp. isolates tested. Maximum parsimony analysis using 690 characters that included TEF-1α sequences from the tested *Fusarium* isolates together with those from Baayen et al. (5), Jiménez-Gasco et al. (33), and Gurjar et al. (24) yielded a tree with a topology similar to that described by Baayen et al. (5) for the *Fusarium* spp. common to this latter study and the present one (Fig. 2). Nine of the *Fusarium* isolates in our study clustered with *N. vasinfecta* isolates within the *F. solani* complex (100% bootstrap support). *Fusarium* sp. isolate 56.93 clustered (100% bootstrap support) with *F. biseptatum* and *F. delphinoides*, two species within the *F. dimerum* species group (57). Those 10 isolates and the different species above mentioned clustered independently (100% bootstrap support) to the remaining *Fusarium* isolates included in the study. A well-supported *F. hostae–F. redolens* clade (79% bootstrap support) was resolved as a sister to the remaining ingroup taxa. All *F. redolens* isolates fell within a main clade, within which several subclades could be identified. In those subclades, all *F. redolens* isolates from chickpea in Morocco, Pakistan, and Spain clustered together, with the exception of isolate L96-13 from Lebanon that grouped in a different subclade. Of the remaining clades, the basal clade (86% bootstrap) was the most phylogenetically diverse and included a *F. nisikadoi–F. miscanthi* clade (98% bootstrap), as well as the *Gibberella fujikuroi* and *F. oxysporum* complexes.

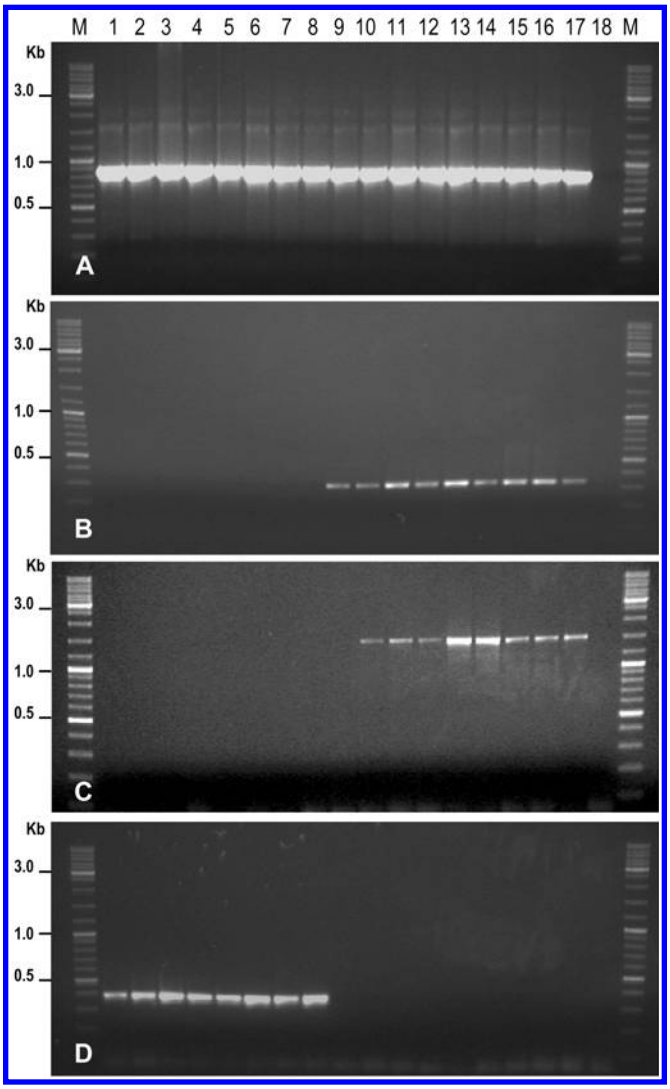

Fig. 1. Gel electrophoresis of DNA amplicons from polymerase chain reaction (PCR) assays using: **A,** primer set LR3 (64) and CS33 (60) that amplify a 900-bp region of the large subunit of the rRNA and was included as an internal positive control for successful PCR amplification; **B,** *Fusarium oxysporum*-specific primers FOF1 and FOR1 (30); **C,** *F. oxysporum* f. sp. *ciceris*-specific primers Foc0-12f and Foc0-12r (30); and **D,** *F. redolens*-specific primers Redolens-F and Redolens-R (9). Lane M, Gene-ruler DNA ladder mix (Fermentas, St. Leon-Rot, Germany); lanes 1–8, *F. redolens* isolates 9914I, 7982, 165-B1, 164-B3, 167-A2, 167-B, 181-2A, and L96-13, respectively; lane 9, *F. oxysporum* isolate 90105; lanes 9–17, *F. oxysporum* f. sp. *ciceris* isolates Foc-7802 (race 0), USA-1987-W17 (race 1B/C), 7989 (race 1A), 8605 (race 2), 8606 (race 3), 8607 (race 4), 8012 (race 5), and 8924 (race 6) , respectively; and lane 18, negative control (water).

Several subclades could also be differentiated in the *F. oxysporum* complex. All *F. oxysporum* f. sp. *ciceris* isolates from our culture collection showed identical TEF-1α sequence (33) and grouped within a subclade with *F. oxysporum* f. sp. *ciceris* isolates from India (24). Other chickpea *F. oxysporum* isolates referred to as nonpathogenic to chickpea and isolate NRRL-32158 from India (referred to as *F. oxysporum* f. sp. *ciceris*; 24) grouped with pathogenic *F. oxysporum* from other formae speciales in a different subclade. Finally, isolate 9009 from chickpea was included in the *G. fujikuroi* complex with another *F. proliferatum* isolate from chickpea in India (Fig. 2).

Pathogenic characterization of isolates on chickpea cultivars. All seven *F. redolens* isolates tested were pathogenic to the three chickpea cultivars in the study. Disease symptoms consisted of foliar yellowing and necrosis. Foliar yellowing developed at the stem base by 13 days after inoculation and progressed upward along the plant, with severely infected plants senescing prematurely. Leaflets first showed dull green discoloration that later turned yellowish, and they occasionally became necrotic and fell off by the end of the experiments. Examination of affected plants at the end of the experiment indicated that there was no discoloration in the vascular tissues. However, these plants showed 2- to 5-mm-long brown, necrotic lesions in their tap root, necrosis of lateral roots, and, occasionally, collar stem necrosis. Conversely, infection by race 0 isolate Foc-7802 of *F. oxysporum* f. sp. *ciceris* induced progressive foliar yellowing with vascular discoloration but no symptoms in the root system and only on P-2245. For this latter combination, disease reaction was more severe (i.e., shorter DO and higher DII_{final} and SAUDPC; $F > 90.24$, $P < 0.0001$) than that caused by any of the seven tested *F. redolens* isolates. Isolations from affected plants indicated that isolates of *F. redolens* grew within the cortex along the plant stem to a variable extent. Conversely, *F. oxysporum* f. sp. *ciceris* race 0 caused vascular infections and colonized the plant stem to a larger extent ($F > 90.24$, $P < 0.0001$) compared with that by *F. redolens* isolates. There were no significant differences ($F = 2.97$, $P = 0.0946$) in the extent of root colonization by isolates of the two fungal species. A maximum 100% disease incidence occurred in all *F. redolens* isolate–chickpea cultivar combinations, except for isolate 7982 in P-2245 and PV-61 that reached 95 and 75% disease incidence, respectively, and isolates L96-13 and 9914I that caused 95 and 85% disease incidence, respectively, on PV-61 (Fig. 3A). Larger differences were observed for the other disease progress curve elements and plant colonization estimates used to characterize disease reaction (Figs. 3–5).

Overall, differences in disease progression and the extent of chickpea root and stem colonization were related primarily to *F. redolens* isolate and chickpea cultivar and, to a lesser extent, to their interaction (Figs. 3–5; Table 3). Thus, MANOVA analysis using disease progress curve elements (DO, DII_{final}, and SAUDPC) and variables associated with plant infection (root and stem colonization) revealed a significant effect of *F. redolens* isolates (Wilks' $\lambda = 0.1431$, $P < 0.0001$), chickpea genotypes (Wilks' $\lambda = 0.2036$, $P < 0.0001$), and the interaction between these two factors (Wilks' $\lambda = 0.2987$, $P = 0.0003$) (Table 3). Eigenvectors of characteristic roots in MANOVA analysis indicated that DII_{final} and SAUDPC were the dependent variables with the greatest influence, while extent of root and stem colonization showed intermediate weights and DO

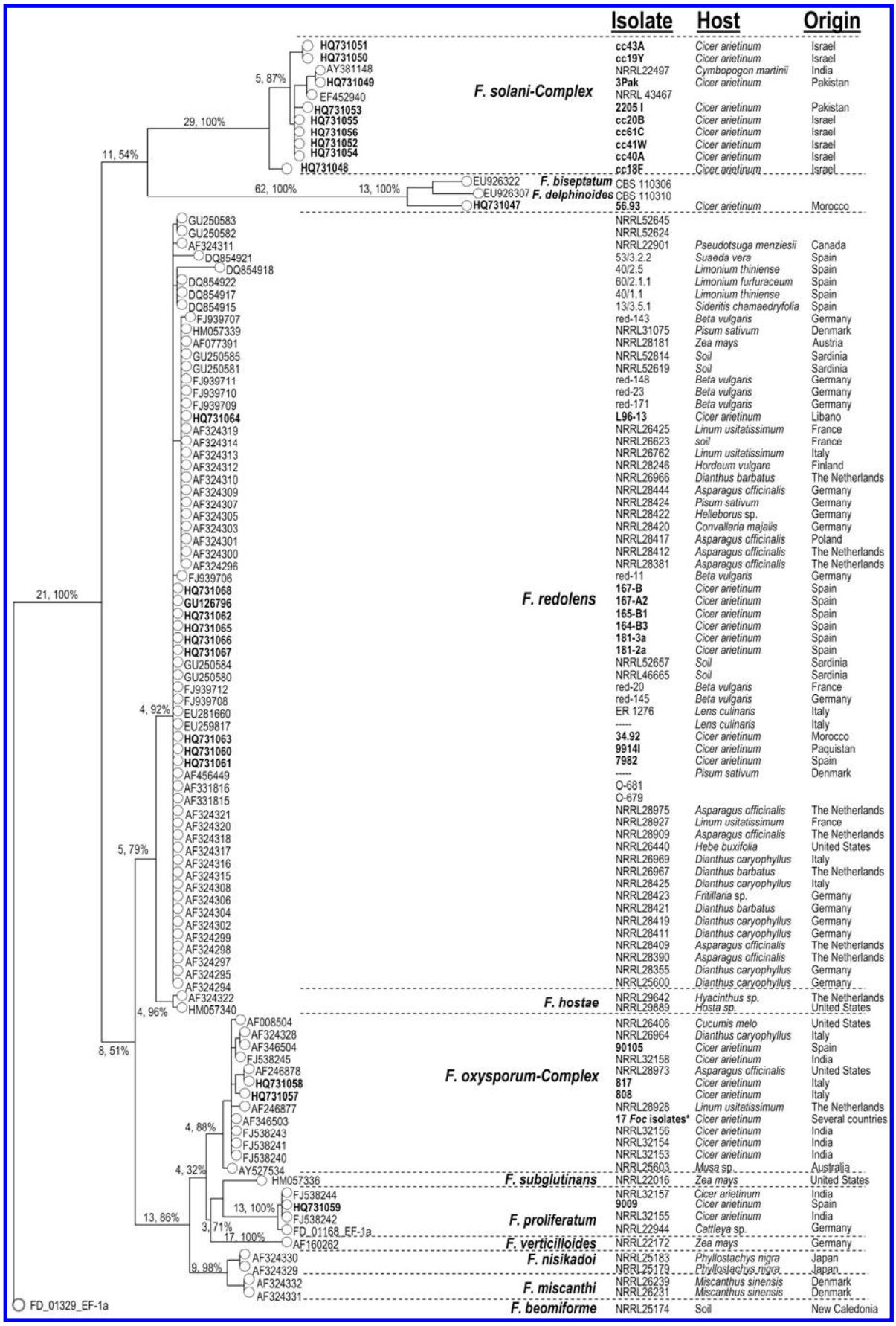

Fig. 2. Maximum parsimony phylogram inferred from the translation elongation factor-1α sequences of *Fusarium redolens, F. oxysporum, F. oxysporum* f. sp. *ciceris*, and other *Fusarium* spp. isolates from chickpea derived from this study (in bold) as well as 23 GenBank accession sequences. A sequence of *F. beomiforme* (FUSARIUM-ID database accession FD_01329) was used to root the tree by the outgroup method. Brach length and bootstrap values >50% from 1,000 maximum parsimony replications implemented in Bionumerics 6.1 are indicated in the branches.

had the lowest weights (*data not shown*). Similarly to MANOVA, overall, *F. redolens* isolates and chickpea genotypes accounted for a larger amount of the variation explained by the ANOVA model (38.8 and 37.1%, respectively) compared with that accounted for their interaction (24.0%) (Table 3).

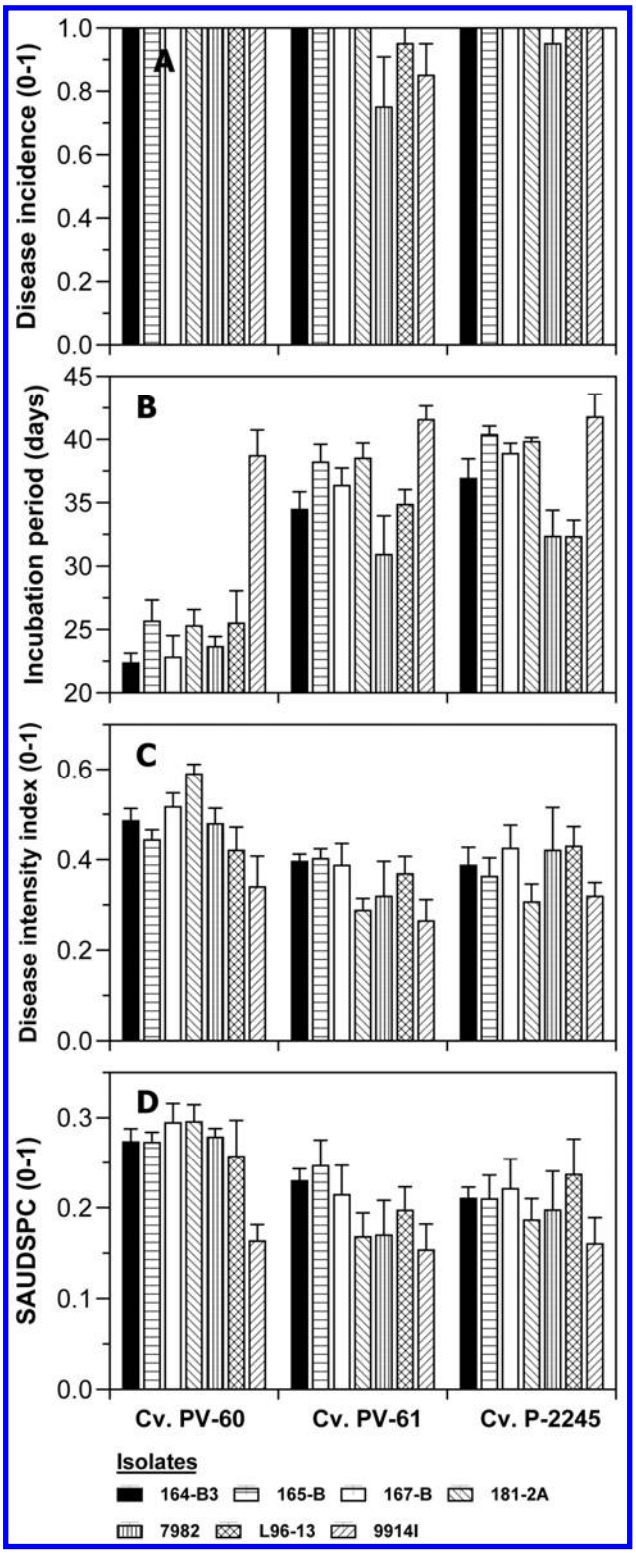

Fig. 3. Reaction of chickpea cultivars ('PV-60', 'PV-61', and 'P-2245') grown in soil artificially infested with seven *Fusarium redolens* isolates based on **A,** disease incidence; **B,** disease onset (number of days until disease intensity index > 0); **C,** disease intensity index on the last day of assessment; and **D,** SAUDPC = area under disease progress curve standardized by duration time in days of the epidemic. Each bar is the mean of five replicated pots with four plants per pot. Error bars indicate the standard error of the mean.

Time to DO was lower on chickpea PV-60 regardless of *F. redolens* isolates, except for isolate 9914I that showed the longest DO in all three chickpea cultivars. The time to appearance of first symptom by isolate 9914I was estimated to be 38.7 ± 2.03 days on PV-60, 41.6 ± 1.10 days on PV-61, and 41.8 ± 1.80 days on P-2245 (Figs. 3B and 4). In the remaining *F. redolens* isolate–chickpea cultivar combinations, time to DO ranged from 22.4 to 25.5 days on PV-60 and from 30.9 to 39.8 days on PV-61 and P-2245 (Fig. 3B).

Disease intensity, estimated by the DII_{final} and SAUDPC, was determined primarily by the chickpea cultivar ($F > 12.03$, $P < 0.0001$) and, to a lesser extent, by pathogen isolate ($F > 2.61$, $P < 0.0226$), with no significant interaction ($F < 1.53$, $P > 0.1339$) (Table 3). Thus, regardless of the *F. redolens* isolate, both DII_{final} and SAUDPC were significantly higher ($P < 0.05$) on chickpea PV-60 and decreased in PV-61 and P-2245, which showed no significant differences ($P \geq 0.05$) between them (Table 3; Figs. 3C and D and 4). Across *F. redolens* isolates, DII_{final} and SAUDPC showed, in general, a narrow range of variation for the different isolates, which was estimated at 0.39 to 0.44 and 0.22 to 0.24, respectively, with the exception of isolate 9914I that reached much lower ($P < 0.05$) DII_{final} and SAUDPC values (Figs. 3C and D and 4).

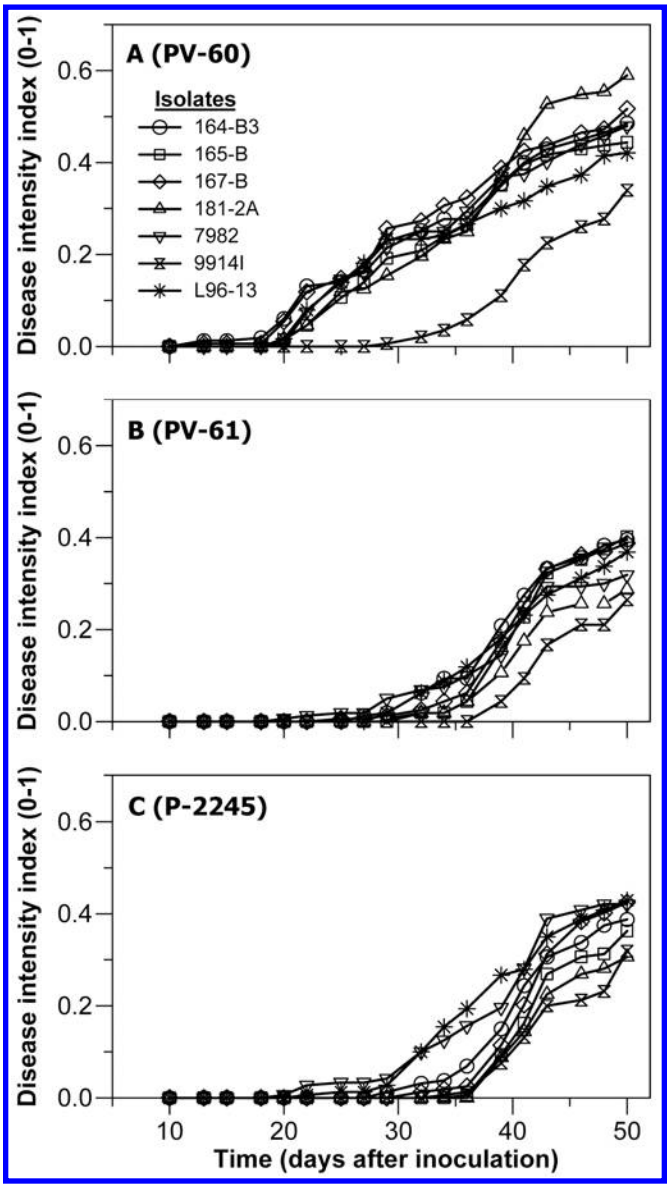

Fig. 4. Disease progress curves in three chickpea cultivars **A, '**PV-60'; **B, '**PV-61'; and **C, '**P2245' grown in soil artificially infested with different *Fusarium redolens* isolates. Each point represents the mean disease intensity index of 20 plants (4 plants in each of five replicated pots).

The extent of root colonization was determined mainly by the *F. redolens* isolate (F = 19.11, P < 0.0001; Table 3). Overall, isolates 164-B3, 165-B, 167-B, 181-2A, and 9914I colonized chickpea roots of all three cultivars extensively, ranging from 3.20 to 3.50 cm, but the extent of colonization was lower for isolates L96-14 (2.66 to 3.12 cm) and 7982 (1.18 to 2.55 cm) (Fig. 5). In contrast, the extent of stem colonization was determined mainly by chickpea cultivar (F = 11.40, P < 0.0001; Table 3). Overall, the stem was colonized to a larger extent in P-2245 and PV-60 than in PV-61 (P < 0.05; Fig. 5). Isolate 181-2A colonized the stem more extensively (P < 0.05) in PV-60 (5.90 ± 0.28 cm) and PV-61 (5.45 ± 0.61 cm) while isolate 7982 showed the highest (6.63 ± 0.57 cm) and lowest (2.78 ± 0.63 cm) level of colonization (P < 0.05) in P-2245 and PV-61, respectively. The remaining five *F. redolens* isolates showed intermediate colonization levels, though close within cultivars (P ≥ 0.05; Fig. 5).

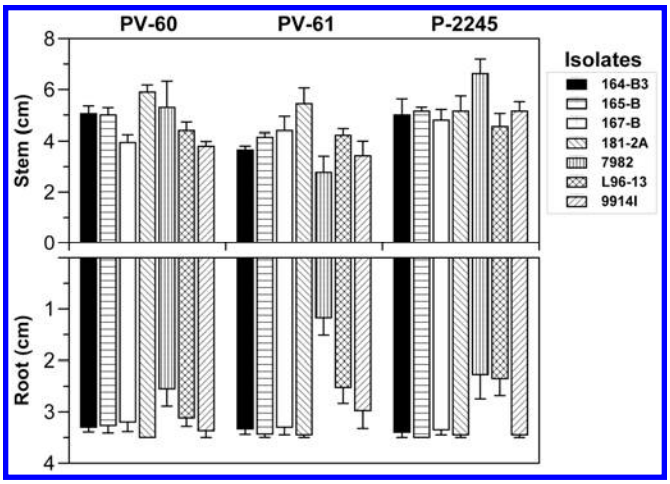

Fig. 5. Extent of stem (upper panel) and root (lower panel) colonization of three chickpea cultivars ('PV-60', 'PV-61', and 'P-2245') grown in soil infested with seven *Fusarium redolens* isolates. Each bar is the mean of 20 plants (4 plants in each of five replicated pots). Error bars indicate the standard error of the mean.

Grouping *F. redolens* isolates by their geographic origin indicated that isolate L96-13 from Lebanon and isolates from southern Spain (164-B3, 165-B, 167-B, 181-2A, and 7982) did not differ (F < 3.51, P > 0.2815) in any of the disease progress curve and plant colonization associated variables, except for a significantly lower extent of root colonization (F = 11.65, P = 0.0020) and DO (F = 12.84, P = 0.0013) by isolate L96-13, although only on P-2245 (Table 3; Figs. 3 and 5). In contrast, infection by isolate 9914I from Pakistan was associated with a significant delay of symptoms in all three cultivars (F > 7.56, P < 0.0103) compared with the set of Spanish isolates above, and a lower disease intensity (F > 13.92, P < 0.0009) and stem colonization (F = 5.86, P = 0.0222) on PV-60 only (Table 3; Figs. 3 and 5). Within the group of isolates from Spain, those originating in Córdoba province (central-west Andalusia) were as virulent as isolate 181-2A originating in Granada province (southeast Andalusia), except for a significantly higher DII_{final} (F = 5.88, P = 0.0220) in PV-60 and higher extent of stem and root colonization (F > 4.22, P < 0.0492) in PV-60 and PV-61 by the latter (Table 3; Figs. 3 and 5).

To summarize the effects of main experimental factors and their interactions on disease reaction, additional multivariate analysis of principal components (PCs) was conducted. In this analysis, the first two PCs accounted for 82.1% of the total variance and are presented in Figure 6. As a result, the dimensionality of the three curve elements and two plant colonization variables was effectively reduced to two descriptive variables, PC1 and PC2. PC1 accounted for 55.7% of the variation and was dominated by high negative weights (–0.88) for DO and high positive weights (>0.96) for DII_{final} and SAUDPC and, therefore, represents the position of disease reaction development over time and the amount of disease developed. PC2 accounted for 36.4% of the variation and was dominated by high positive weighs (>0.66) for the extent of root and stem colonization by the pathogen (Fig. 5). According to position of disease reactions projected on the X (PC1)-Y (PC2) plane in Figure 6, symptoms were delayed from right to left and the over-

Table 3. Effects of infection by *Fusarium redolens* isolate, chickpea cultivar, and their interactions on disease progress curve elements on disease development and extent of plant colonization

			Disease progress curve elements[a]						Plant colonization[b]			
	MANOVA[c]		DO		DII_{final}		SAUDPC		Root		Stem	
Source of variation[d]	Wilks' λ	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*	*F*	*P* > *F*
Isolate	0.1431	<0.0001	17.79	<0.0001	2.62	0.0225	3.51	0.0038	19.11	<0.0001	2.72	0.0184
Cultivar	0.2036	<0.0001	109.09	<0.0001	13.20	<0.0001	12.04	<0.0001	3.79	0.0265	11.40	<0.0001
Isolate × cultivar	0.2987	0.0003	2.98	0.0017	1.52	0.1340	0.86	0.5860	2.23	0.0171	2.51	0.0073
Contrasts												
PV-60												
Spain vs. L96-13	0.8393	0.4855	0.74	0.3962	3.51	0.0713	1.21	0.2815	0.06	0.8135	1.56	0.2218
Spain vs. 9914I	0.2734	<0.0001	66.48	<0.0001	13.92	0.0009	25.96	<0.0001	1.08	0.3082	5.86	0.0222
Spain: Co vs. Gr	0.5982	0.0229	0.81	0.3771	5.88	0.0220	0.45	0.5078	4.43	0.0444	4.23	0.0491
PV-61												
Spain vs. L96-13	0.8227	0.4201	0.21	0.6534	0.05	0.8303	0.07	0.7894	2.68	0.1125	0.08	0.7851
Spain vs. 9914I	0.6694	0.0697	10.40	0.0032	3.79	0.0616	2.79	0.1059	0.02	0.8855	1.68	0.2049
Spain: Co vs. Gr	0.3937	0.0003	3.61	0.0679	3.25	0.0824	2.17	0.1517	6.17	0.0192	10.81	0.0027
P2245												
Spain vs. L96-13	0.4494	0.0011	12.84	0.0013	0.72	0.4049	0.88	0.3562	11.65	0.0020	2.21	0.1481
Spain vs. 9914I	0.6763	0.0770	7.56	0.0103	1.14	0.2939	1.73	0.1994	1.06	0.3118	0.13	0.7180
Spain: Co vs. Gr	0.6941	0.0983	3.12	0.0880	2.48	0.1263	0.45	0.5079	1.59	0.2175	0.20	0.6584

[a] Disease intensity index (DII) was calculated based on the incidence and severity of symptoms recorded at 2- to 3-day intervals. Disease onset (DO) was estimated as the number of days to initial symptoms; DII_{final} = disease intensity index at the final date of disease assessment and SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method and standardized for the epidemic duration. Data are the means of five replicated pots, each with four plants.

[b] Inoculated plants were incubated for 50 days in a growth chamber. To determine the occurrence of plant infection, isolations were made from all plants showing a severity score ≤4 within a 0-to-4 rating scale, from four stem internodes starting from the hypocotyl, and from the main root (four segments).

[c] Wilks' λ statistic and associated probability (P) for the hypothesis of overall *F. redolens* and chickpea cultivar effects after multivariate analysis of variance (MANOVA).

[d] Linear single-degree-of-freedom contrasts computed to test the effect of selected treatment combinations at a significance level of P < 0.05. Contrast = contrasts by isolate within cultivars: *F. redolens* isolates were grouped according to their geographic origin; Spain = isolates 164-B3, 165-B, 167-B, 181-2A, and 7982; Co = Córdoba, isolates 164-B3, 165-B, 167-B, and 7982; Gr = Granada, isolate 181-2A.

all disease intensity increased along the *x*-axis. Similarly, the extent of plant colonization increased from bottom to top along the *y*-axis. Overall, disease reactions were located according to chickpea cultivars; hence, reactions developed on PV-61 and P-2245 were located at the left side and in the middle part while those developed on PV-60 were located at the right side (Fig. 6). Within each cultivar, lower differences occurred among *F. redolens* isolates. Isolate 9914I induced the most delayed reaction and lowest disease intensity (i.e., lowest DII_{final} and SAUDPC) regardless of chickpea cultivar, with colonization level increasing in PV61, PV-60, and P-2245, in that order. Isolates 181-2A and 9914I induced reactions of similar disease intensity and plant colonization levels in small-seeded PV-61 and P-2245. Conversely, isolate 181-2A induced the most severe disease reaction (i.e., lowest DO and highest DII_{final} and SAUDPC) and extent of colonization in PV-60. Isolates 164-B, 165-B, and 167-B induced reactions of intermediate disease intensity in PV-61 and P-2245 but the extent of colonization was larger in P-2245. Finally, all remaining *F. redolens* isolates, except for isolate 9914I, caused a severe disease reaction in PV-60, which was associated with moderate (167-B, 7982, and L96-13) or high (164-B, 165-B, and 181-2A) extent of colonization of the plant (Fig. 6).

Discussion

Use of morphological traits for differentiating among morphologically similar *Fusarium* spp., such as *F. oxysporum* and *F. redolens*, may lead to the wrong pathogen identification. However, accurate identification of pathogenic *Fusarium* spp. and *F. oxysporum* formae speciales is crucial for the efficient management of Fusarium diseases, particularly if resistant cultivars are one of the few and most effective control measures, as exemplified by Fusarium wilt of chickpea (28,39,40). In this study, use of previously developed specific-PCR protocols (9,31,32) and pathogenicity assays demonstrated for the first time that *F. redolens* is pathogenic on chickpea, causing root necrosis and acropetal foliar yellowing. Furthermore, these PCR protocols allowed the exact and quick differentiation among *F. oxysporum* sensu lato, *F. redolens*, and *F. oxysporum* f. sp. *ciceris* isolated from chickpea showing a wilt-like symptom. We demonstrated that isolating *F. oxysporum*-like *Fusarium* spp. from chickpea showing wilting-like symptoms, even if performed from upper stem tissues, does not necessarily guarantee recovery of *F. oxysporum* f. sp. *ciceris* and can lead to misidentification if the fungal isolates are not further characterized.

Indeed, by using the specific-PCR protocols mentioned above and sequencing of the TEF-1α gene, we showed that several isolates of *Fusarium* spp. provided by scientists around the world had originally been miss-assigned to the forma specialis *ciceris* by the source laboratory. Those isolates consistently failed to amplify a *F. oxysporum* f. sp. *ciceris*-specific marker (32) but were identified as *F. redolens*, *F. oxysporum*-complex, *F. solani*-complex, *F. proliferatum*, *N. vasinfecta*, or unknown *Fusarium* sp. Such misidentification could be attributed to different circumstances. (i) Isolations in pure culture from Fusarium-wilt-affected chickpea does not necessarily result in *F. oxysporum* f. sp. *ciceris* but often yielded nonpathogenic isolates of *F. oxysporum* or other saprophytic *Fusarium* spp. with morphology similar to that of the *F. oxysporum* species-complex (36,59; R. M. Jiménez-Díaz and B. B. Landa, *unpublished results*). Without further characterization, this may have led to storage of isolates wrongly assumed as *F. oxysporum* f. sp. *ciceris* because of their association with wilting symptoms. (ii) Lack of extensive experience in the identification of *Fusarium* spp. may have led to overlooking morphological features and microscopic traits key for differentiating morphologically close species. (iii) Storage of bulk isolates while disregarding the need of single-sporing cultures before storage can result in storing a mixture of hidden species. Finally, (iv) eventual contamination of single-spore cultures during storage of isolates which remain undetected if they are not checked further. All these facts stress the need for carefully assuring that a *Fusarium* sp. isolate induces exactly the same vascular infection and disease symptoms on race differentials compared with a previously described *F. oxysporum* f. sp. *ciceris* race. As an example, the previously described race 3 of the Fusarium wilt pathogen was recently and erroneously redescribed as *F. proliferatum* (24).

In this present study, the newly reported symptomatic infection of chickpea showing wilting-like symptoms in Spain by *F. redolens* was demonstrated to occur also in other chickpea-producing countries, including Lebanon, Morocco, and Pakistan. Here, we demonstrated that *F. redolens* isolates were previously misdiagnosed as *F. oxysporum* f. sp. *ciceris,* and characterized them molecularly by means of different molecular protocols. Indeed, use of these protocols in our laboratory has facilitated proper identification of chickpea *Fusarium* isolates and helped to sort out some isolates originally miss-assigned to a wrong species, as happened for isolate 7982, originally assigned to the yellowing pathotype of *F. oxysporum* f. sp. *ciceris*. The differentiation between *F. redolens* and *F. oxysporum* f. sp. *ciceris* was further strengthened by their distinct IGS type in RFLP analysis with seven restriction enzymes, which also showed that all *F. oxysporum* f. sp. *ciceris* isolates representative of the eight described pathogenic races have identical RFLP-IGS restriction patterns regardless of their geographic origin or race. It should be emphasized that all *F. oxysporum* f. sp. *ciceris* isolates used in this study and additional ones from other studies, including race 3 isolates, share an identical sequence of the ITS region of rDNA (D. J. Fernández and B. B. Landa, *unpublished data*) and a 1.5-kb sequence-characterized amplified region-specific sequence of the forma specialis *ciceris* (GenBank accession AF492451; 32), which supports the hypothesis that each of those eight races forms a monophyletic lineage (33) and that race 3 of the Fusarium wilting pathogen on chickpea should be named *F. oxysporum* f. sp. *ciceris* and not *F. proliferatum*, as was erroneously claimed (24).

A similar scenario of misidentifications of *F. redolens* isolates has been described for other formae speciales of *F. oxysporum*, including f. sp. *dianthi* (9), f. sp. *lini* (5), and f. sp. *hebae* (5). Particularly interesting is the situation of *F. oxysporum* f. sp. *dianthi* and *F. redolens* f. sp. *dianthi*, for which classification of vegetative compatibility groups (VCGs) and races of carnation isolates of *F. oxysporum* sensu lato has been hampered in the past by controversies concerning the distinction of *F. redolens* from *F. oxysporum* (2,21,50). Thus, Baayen and Kleijn (3) originally described five VCGs among isolates belonging to *F. oxysporum* f. sp. *dianthi* but two of them were recently demonstrated to be *F. redolens* f. sp. *dianthi* (7).

In addition to distinctiveness between *F. redolens* and *F. oxysporum* indicated by specific-PCR and RFLP-IGS assays, results

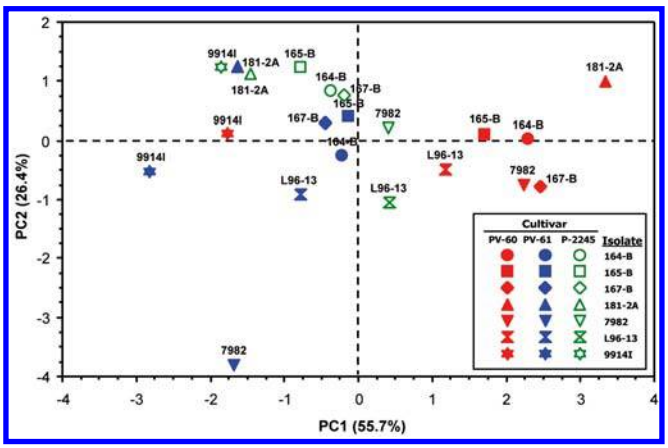

Fig. 6. Projection of factor scores on the plane of principal component (PCs) 1 and 2 from PC analysis for disease developed on three chickpea cultivars ('PV-60', 'PV-61', and 'P-2245') grown in soil infested with seven isolates of *Fusarium redolens*. Disease intensity (PC1) increased along the *x*-axis (most disease on right) and plant colonization (PC2) on the *y*-axis was most extensive at the top.

from phylogenetic analysis of the TEF-1α gene sequence clearly indicate that *F. redolens* and *F. oxysporum* from chickpea lack a sister group relationship (Fig. 2). Previous phylogenetic analyses of sequences of TEF-1α, the nuclear ITS region and large subunit 28S rDNA, the mitochondrial small subunit rDNA, and β-tubulin have also showed that the *F. oxysporum* complex, the *G. fujikuroi* complex, and the *F. miscanthi–F. nisikadoi* clade form a monophyletic group independent from the *F. redolens–F. hostae* clade (2,17,19,52,53,61,62). As for the *F. oxysporum* complex, *F. redolens* forms a species complex that includes isolates from different hosts and countries bearing the ability to cause several diseases on different crops (10,20,21). Thus, host specificity in some *F. redolens* isolates has led to grouping them into formae speciales as was done in the *F. oxysporum* complex; that is, *F. redolens* f. sp. *dianthi* Gerlach (20), *F. redolens* f. sp. *spinaciae* (Sherb.) Subram. (58), and *F. redolens* f. sp. *asparagi* Baayen (6). It would be worthwhile developing further pathogenicity tests with *F. redolens* isolates from chickpea on other plant species to determine whether or not they show pathogenic specificity on this legume.

In the phylogenetic analysis of TEF-1α in our study, the grouping of *F. redolens* isolates within a subclade was not correlated with their geographic origin or host source. This is in agreement with other studies where host preference in *F. redolens* grouped into formae speciales did not appear to correlate with subclades resolved by the molecular phylogeny, which may indicate that members of those *F. redolens* formae speciales may have independent evolutionary origins (5). In our study, all *F. redolens* isolates from chickpea from three different countries showed a low genetic diversity in TEF-1α sequence and grouped together in one subclade, with the exception of isolate L96-13 from Lebanon. This clustering could support the hypothesis that pathogenicity to chickpea has developed recently in *F. redolens* (i.e., the host plant has not yet developed specific resistance against the pathogen and the latter has not needed to evolve).

F. redolens has been referred as an important pathogen on different crops, including asparagus (65), carnation (7,44), pelargonium (55), rose (66), and tomato (25); and as a weak pathogen on spinach (41) and some legumes. *F. redolens* has been referred as a pathogen causing foot rot on pea and bean (12), necrotic taproot lesions and lateral root necrosis on soybean (8), and wilt and root rot on lentil (56); however, to our knowledge, pathogenicity to chickpea has not been reported yet. In this present study, we report differences in virulence among seven chickpea *F. redolens* isolates from three countries on three chickpea cultivars (P-2245, PV-60, and PV-61) commonly grown in Spain. All tested *F. redolens* isolates induced disease symptoms similar to those reported before for the yellowing-inducing isolates of *F. oxysporum* f. sp. *ciceris* (27,29) but in the absence of vascular infection characteristic of the latter. Symptoms induced by *F. redolens* include foliar yellowing that started 13 days after inoculation and gradually progressed upward on the plants, which was associated with cortical collar and lateral root necrosis. Interestingly, similar symptoms were described by Trapero-Casas and Jiménez-Díaz (59) in chickpea inoculated with what they named reddish-colony-forming *F. oxysporum* isolated from chickpea plants affected by the 'Seca' disease complex in southern Spain. With such a name, those authors aimed to distinguish the reddish *F. oxysporum* isolates from the typical salmon-cream-colored colonies of vascular-infecting *F. oxysporum* f. sp. *ciceris* isolates. We noted that some of the *F. redolens* from Spain in this study formed reddish-colored colonies (*data not shown*), which could imply that isolates that Trapero-Casas and Jiménez-Díaz (59) characterized as *F. oxysporum* using only morphology-based diagnoses could actually be *F. redolens*. As reported by Trapero-Casas and Jiménez-Díaz (59) for reddish *F. oxysporum* isolates, *F. redolens* isolates in our study induced extensive foliar yellowing but failed to cause death of inoculated plants. The opposite has been observed for the yellowing-inducing isolate Foc-7802 of *F. oxysporum* f. sp. *ciceris* which killed all plants by 50 days after inoculation. All these considerations would suggest that *F. redolens* is a weak pathogen on chickpea, substantiating the conclusion drawn by Trapero-Casas and Jiménez-Díaz (59) in their work about reddish *F. oxysporum* isolates. Although *F. redolens* has been usually referred to as a weak pathogen on different pulses, including pea, bean, and soybean (8,12), it has been recently described as a highly virulent on lentil (56).

Finally, because several pathogenic forms of *F. oxysporum* and *F. redolens* may cause similar symptoms on a host plant, as has been shown on several crops (for example, asparagus [6,65], broad bean and pea [10], carnation [21], lentil [56], and spinach [41]) and chickpea (this study), our results emphasize the importance of assuring the accurate identification and distinction between these two species in a geographical area for which suitable molecular protocols are available. This approach would facilitate the development of resistant germplasm for these crops, if available, and better the understanding of the etiology and epidemiology of the diseases caused by the different formae speciales of *F. oxysporum* and *F. redolens*.

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