Trop. plant pathol. (2017) 42:273–283
DOI 10.1007/s40858-017-0145-5

ORIGINAL ARTICLE

Etiology of root rot and wilt disease of chickpea in Brazil

Daiana M. Q. Azevedo [1,3] · Fernando S. Rocha [1] · Cândido A. Costa [1] · Ludwig H. Pfenning [2] · Sarah S. da Costa [2] · Maruzanete P. Melo [2] · Josiane G. da Silva [1,3] · Maria de Fátima G. Fernanades [1]

Received: 14 June 2016 / Accepted: 1 March 2017 / Published online: 23 March 2017

Abstract Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato has become the main limiting factor for chickpea (*Cicer arietinum* L.) production around the world. Although the cultivation of this legume is recent in Brazil, there are reports on *Fusarium* spp. occurrence causing crop losses. Fourteen isolates obtained from roots of chickpea plants showing wilt and yellowing symptoms in Brazil were evaluated through phylogenetic analysis of the *EF*-1α region, morphological markers and pathogenicity tests. Three isolates were clustered within a distinct lineage from those already described for the FSSC. The remaining 11 isolates were clustered within the FOSC, in a different clade from *F. oxysporum* f. sp. *ciceris*. All isolates were pathogenic but showed differences in aggressiveness. Isolates of the different complexes elicited the same symptoms: yellowing, wilt and root rot of chickpea plants. Morphological markers allowed differentiating isolates from distinct complexes but not differentiating between lineages.

Keywords *Cicer arietinum* L · *Fusarium oxysporum* · *Fusarium solani* · Fusariosis · Pathogenicity · Translation elongation factor1-α

Section Editor: Olinto L. Pereira

✉ Fernando S. Rocha
rochafsplant@yahoo.com.br

[1] Universidade Federal de Minas Gerais, Montes Claros, MG 39.404-547, Brazil

[2] Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, MG 37200-000, Brazil

[3] Present address: Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, MG 36570-000, Brazil

Introduction

Chickpea, *c* L., is a legume crop of great importance worldwide, especially within African and Asian countries due to the nutritional value of its seeds. This legume has been increasingly used as an alternative to animal protein, mostly within developing countries, and has become an important dietary component for people that are vegetarian by choice or due to economic reasons (Wood and Grusak 2007; Jukanti et al. 2012).

There are more than 170 reported plant pathogens associated with chickpea (Nene et al. 1996), among which *Fusarium oxysporum* (Padwick) f. sp. *ciceris* Matuo & Sato is considered as the most important pathogen. This fungus causes vascular wilt or Fusarium wilt, limiting chickpea production (Jiménez-Díaz et al. 2015). The disease has already being reported in production regions from Asia, Africa, Southern Europe and The Americas (Chand and Khirbat 2009). Black root rot caused by *Fusarium solani*, although considered of a secondary importance, also causes significant losses in production in areas where the fungus is present in the soil (Nene et al. 2012).

Fusarium oxysporum is considered as being a complex of different species indistinguishable by only morphological markers (Lievens et al. 2008). This complex comprehends nonpathogenic and pathogenic isolates designated as *formae speciales*, characterized based in their host specificity (Armstrong and Armstrong 1981). Members of various phylogenetic lineages of the *Fusarium oxysporum* species complex (FOSC) are known to cause important plant diseases such as vascular wilt in various plants of economic interest (Di Pietro et al. 2003).

Results of studies with multigenic analysis, AFLP and vegetative compatibility group analysis showed that *formae speciales* do not represent phylogenetic groups, evidencing

that isolates of a determined *formae specialis* may have distinct evolutionary origin (Baayen et al. 2000). Studies performed in Brazil with de *F. oxysporum* f. sp. *malvacearum* and *F. oxysporum* f. sp. *phaseolis* isolates evidenced that isolates of both *formae speciales* are polyphyletic (Silva et al. 2014).

Fusarium solani is a widely used name for what is nowadays named *Fusarium solani* species complex - FSSC (O'Donnell 2000). Members of the FSSC include various names that correspond to approximately 50 phylogenetic lineages (O'Donnell et al. 2008). Within the *Fusarium solani* complex are included various species causing soybean sudden death syndrome (Aoki et al. 2005; Costa et al. 2016). *Fusarium azukicola* was described in azuki beans, causing root rot (Aoki et al. 2012). In large soybean producing centers as Unites States, Brazil and Argentina, outbreaks of red rot were reported as having species belonging to the FSSC as their etiological agents (Scandiani et al. 2004; O'Donnell et al. 2010; Aoki et al. 2005).

The pathogenic association of *F. oxysporum* f. sp. *ciceris* with chickpea plants was reported in 1985 in plantations from the cerrado region in the Distrito Federal, Brazil (Sharma and Cerkauskas 1985). Recently, reports of the occurrence of fusariosis were made in the states of Goiás and Minas Gerais, however, no research using modern molecular techniques was done in order to confirm the etiological agent (Unpublished data).

Therefore, due to the absence of studies concerning the characterization of *Fusarium* species associated with chickpea in Brazil, we aimed to identify species associated to the etiology of the chickpea wilt by using the concept of morphological and phylogenetic species, as well as pathogenicity tests.

Materials and methods

Fusarium isolates

Isolates of *Fusarium* were obtained from root and stem tissue fragments from plants of different chickpea cultivars exhibiting yellowing, wilt and vascular discoloration symptoms. Fragments were externally disinfected in alcohol 70% for 1 min, sodium hypochlorite 1% for 1 min and three consecutive washes in sterilized distilled water. Then, fragments were transferred to Petri dishes containing water-agar (AA) medium. After four days of incubation at 25 °C and photoperiod of 12 h, fragments from the periphery of typical *Fusarium* colonies were transferred to potato dextrose agar (PDA) medium in order to obtain pure cultures. Monosporic cultures were obtained from pure colonies before being cryopreserved according Smith and Onions (1994) and stored at Coleção Micológica de Lavras (CML) at Universidade Federal de Lavras (UFLA), Lavras, Minas Gerais, Brazil (http://www.dfp.ufla.br/cml). Fourteen isolates were studied, being 13 originated from plants from the county of Cristalina - GO, Brazil and one isolate from the county of Montes Claros – MG, Brazil (Table 1).

DNA extraction, PCR amplification and phylogenetic analysis

Isolates were grown in 2% malt extract broth medium for three days at 100 rpm and had their DNA extracted with the Wizard® Genomic DNA Purification (Promega) kit, following manufacturer's instructions. PCR amplifications were performed using a GoTaq Colorless Master Mix (Promega) kit in a My Cycler (Bio-Rad) thermocycler. Partial sequences of the gene *EF-1α* were amplified using primers Ef1-F (5′-ATGG GTAAGGAGGACAAGAC-3′) and Ef2-R (5′-GGAA GTACCAGTGATCATGTT-3′), described by O'Donnell et al. (1998). PCR conditions were one cycle of 90 s at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 62 °C and 1 min at 72 °C; followed by one cycle of 5 min at 72 °C and a 4 °C soak (O'Donnell et al. 2008). PCR products were purified using a Wizard®SV Gel kit and PCR Clean-up System (Promega) and sequenced by Macrogen Inc. Consensus sequences were assembled from forward and reverse sequences using SeqAssem (Hepperle 2004). Edited sequences were compared with the GenBank database, from the National Center for Biotechnology Information – NCBI, using the Basic Local Alignment Search Tool – BLAST program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). Phylogenetic analyses were performed for each species found, based in the results from BLAST. Sequences of the isolates of *F. solani* were compared with the sequences of phylogenetic lineages of the FSSC (O'Donnell et al. 2008). Isolates of *F. oxysporum* were compared with the sequences of the phylogenetic lineages of FOSC (O'Donnell et al. 2009) and sequences of *F. oxysporum* f. sp. *ciceris* - NRRL 32153, NRRL 32154, NRRL 32155, NRRL 32156 (Gurjar et al. 2009), Foc 11, Foc 19, Foc 122 and Foc 155 (Dubey et al. 2014). Multiple alignments of sequences were generated using the ClustalW as implemented by the software Mega 6 (Tamura et al. 2013). The Maximum Parsimony (MP) phylogenetic analysis was performed on MEGA 6 (Tamura et al. 2013), using the Close-Neighbor-Interchange algorithm. Clade support was inferred from 1000 bootstrap replications. A complementary Bayesian inference (BI) analysis employing a Markov Chain Monte Carlo method was performed. MrModeltest 2.3 (Nylander 2004) was used to determine the best nucleotide substitution model for each data set. According Akaike Information Criterion (AIC) were selected K80 + G for the FOSC dataset and GTR + G for FOSC dataset. Markov chains were run for 10.000.000 generations and sampled every 100th generation. From the resulting 100.000 trees 25% was discarded and the remaining 75.000 trees were used for the calculation of posterior probabilities of

Table 1 Strains and GenBank acession numbers of *Fusarium* spp. of the *Fusarium oxysporum* species complex (FOSC) and *Fusarium solani* species complex (FSSC) used to generate the phylogram in this study

Species	CML Code	Other code[1]	Host/Substrate	Origin	GenBank acession EF-1α
F. oxysporum	2865	FCA 01	*Cicer arietinum*	Brazil, Cristalina, GO	KX901671
F. oxysporum	2866	FCA 02	*Cicer arietinum*	Brazil, Montes Claros MG	KX901672
F. oxysporum	2867	FCA 07	*Cicer arietinum*	Brazil, Cristalina, GO	KX901673
F. oxysporum	2869	FCA 11	*Cicer arietinum*	Brazil, Cristalina, GO	KX901674
F. oxysporum	2871	FCA 13	*Cicer arietinum*	Brazil, Cristalina, GO	KX901675
F. oxysporum	2872	FCA 15	*Cicer arietinum*	Brazil, Cristalina, GO	KX901676
F. oxysporum	2874	FCA 21	*Cicer arietinum*	Brazil, Cristalina, GO	KX901677
F. oxysporum	2875	FCA 22	*Cicer arietinum*	Brazil, Cristalina, GO	KX901678
F. oxysporum	2876	FCA 24	*Cicer arietinum*	Brazil, Cristalina, GO	KX901679
F. oxysporum	2877	FCA 26	*Cicer arietinum*	Brazil, Cristalina, GO	KX901680
F. oxysporum	2878	FCA 27	*Cicer arietinum*	Brazil, Cristalina, GO	KX901681
F. oxysporum f. sp. *ciceris* race 1		NRRL 32153	*Cicer arietinum*	India	FJ538240
F. oxysporum f. sp. *ciceris* race 2		NRRL 32154	*Cicer arietinum*	India	FJ538241
F. oxysporum f. sp. *ciceris* race 3		NRRL 32155	*Cicer arietinum*	India	FJ538242
F. oxysporum f. sp. *ciceris* race 4		NRRL 32156	*Cicer arietinum*	India	FJ538243
F. oxysporum f. sp. *ciceris* race 5		Foc 11	*Cicer arietinum*	India	JN231164
F. oxysporum f. sp. *ciceris* race 6		Foc 155	*Cicer arietinum*	India	JN231179
F. oxysporum f. sp. *ciceris* race 7		Foc 122	*Cicer arietinum*	India	JN231143
F. oxysporum f. sp. *ciceris* race 8		Foc 19	*Cicer arietinum*	India	JN231169
F. oxysporum f. sp. *batatas*		NRRL 38289	*Ipomoea batatas*	-	FJ985368
F. oxysporum f. sp. *passiflorae*		NRRL 38273	*Passiflora edulis*	Australia	FJ985362
F. foetens		NRRL 38302	*Pinus radiata* seedling	-	FJ985444
F. solani	2868	FCA 10	*Cicer arietinum*	Brazil, Cristalina, GO	KX901682
F. solani	2870	FCA 12	*Cicer arietinum*	Brazil, Cristalina, GO	KX901683
F. solani	2873	FCA 19	*Cicer arietinum*	Brazil, Cristalina, GO	KX901684
F. iludens		NRRL 22090	*Beilschmiedia tawa*	New Zealand	AF178326
F. plagianthi		NRRL 22632	*Hoheria glabrata*	New Zealand	AF178354
F. tucumaniae		NRRL 31096	*Glycine max*	Argentina	GU170636
F. virguliforme		NRRL 22825	*Glycine max*	Indiana	EF408437
F. brasiliense		NRRL 22743	*Glycine max*	Brazil	AY320145
F. crassistipitatum		NRRL 31949	*Glycine max*	Brazil	AY320161
F. cuneirostrum		NRRL 31104	*Phaseolus vulgaris*	Japan	AY320159
F. phaseoli		NRRL 22276	*Phaseolus vulgaris*	United States	AY220186
F. petroliphilum 1-a		NRRL 28546	Human eye	United States	DQ246887
F. keratoplasticum T 2-ss		FRC S-2477	Sink drain	United States	JN235712
F. falciforme CBS 475.67 T 3 + 4-ccc		CBS 475.67	Human	Puerto Rico	DQ247188
Fusarium sp. 5-c		NRRL 32492	Human	United States	DQ246990
Fusarium sp. 5-h		NRRL 28679	Human	Cuba	DQ246912
F. ensiforme clade 6-a		NRRL 43489	Human eye	United States	DQ790484
F. ensiforme clade 7-a		NRRL 43502	Human eye	United States	DQ790488
Neocosmospora sp. 8-a		NRRL 43467	Human eye	United States	EF452940
Fusarium sp. 9-a		NRRL 32755	Turtle	United States	DQ247073
F. solani f. sp. *cucurbitae* MP-I 10-b		NRRL 22098	Cucurbit	United States	AF178327
F. solani f. sp. *pisi* MP-VI 11-a		NRRL 22278	*Pisum sativum*	United States	AF178337
F. ensiforme clade 12-d		NRRL 32309	Human	United States	DQ246937
F. solani f. sp. *robiniae* MP-VII 13-b		NRRL 22586	*Robinea pseudoacacia*	United States	AF178353
F. ensiforme clade 15-a		NRRL 28009	Human eye	United States	DQ246869

Table 1 (continued)

Species	CML Code	Other code[1]	Host/Substrate	Origin	GenBank acession EF-1α
F. lichenicola 16-b		NRRL 32434	Human	Germany	DQ246977
F. solani f. sp. *mori* MP-III 17-a		NRRL 22157	*Morus alba*	Japan	AF178359
F. ensiforme clade 18-a		NRRL 31158	Human	United States	DQ246916
F. ambrosium 19-a		NRRL 20438	*Camellia sinensis*	India	AF178332
Fusarium sp. 20-a		NRRL 22608	Human	United States	DQ246838
F. striatum 21-a		NRRL 22101	Cotton cloth	Panama	AF178333
F. solani f. sp. *xanthoxyli* MP-IV 22-a		NRRL 22163	*Xanthoxylum piperitum*	Japan	AF178328
F. solani f. sp. *batatas* MP-II 23-a		NRRL 22400	*Ipomoea batatas*	United States	AF178343
Fusarium sp. 24-a		NRRL 22389	*Liriodendron tulipifera*	United States	AF178340
F. ensiforme clade 25-a		NRRL 31169	Human	United States	DQ246923
F. ensiforme clade 26-a		NRRL 28541	Human	Connecticut	DQ246882
F. ensiforme clade 27-a		NRRL 37625	Human	Netherlands	FJ240353
F. ensiforme clade 28-a		NRRL 32437	Human	Switzerland	DQ246979
F. ensiforme clade 29-a		NRRL 28008	Human eye	United States	DQ246868
Fusarium sp. 30-a		NRRL 22579	Bark	Indonesia	AF178352
F. solani f. sp. *piperis* 31-a		NRRL 22570	*Piper nigrum*	Brazil	AF178360
Fusarium sp. 32-a		NRRL 22178	Dicot tree	Venezuela	AF178334
Fusarium sp. 33-a		NRRL 22354	Bark	French Guiana	AF178338
F. pseudensiforme 33		NRRL 46517	-	-	KC691555
Fusarium sp. 34-a		NRRL 46703	Nematode	Spain	HM347126
Fusarium sp. 35-a		NRRL 46707	Human eye	Brazil	HM347127
Fusarium sp. 38		FRC S1950	Soil	Australia	DQ247507

[1] Culture collection abbreviations: *CBS* Centraalbureau voor Schimmelcultures - Fungal Biodiversity Center, Utrecht, The Netherlands; *CML* Coleção Micológica de Lavras, Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; NRRL, The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, Illinois, USA; FCA, Phytopathology Laboratory Culture Collection, Instituto de Ciências Agrárias, Universidade Federal de Minas Gerais, Montes Claros, Minas Gerais, Brazil; FRC S, Fusarium Research Center, The Pennsylvania State University, State College, PA, USA

branches. The BI phylogenetic analyses were performed using the CIPRES webportal (Miller et al. 2010). The sequences generated in this study were deposited in the GenBank (http://www.ncbi.nlm.nih.gov) (Table 1) and the alignments were deposited in the TreeBASE under accession number 19949 (http://purl.org/phylo/treebase/phylows/study/TB2:S19949).

Evaluation of morphological markers

Morphological characterization was performed according to the characters described by Gerlach and Niremberg (1982) and Leslie and Summerell (2006). Isolates were cultivated in Petri dishes containing PDA medium, incubated at 25 °C in the dark for 4 days, with three replicates used to evaluate the mycelium growth rate. Colony colour and formation of aerial mycelium were observed after 14 days of incubation in PDA medium at 20 °C in a 12 h photoperiod under near UV and white fluorescent bulbs. In synthetic nutrient-poor agar medium (SNA), under the same previously mentioned conditions, were observed microconidial and macroconidial shape and septation, presence and color of sporodochia, arrangement of conidiogenous cells and presence or absence of chlamydospores. For each characterized structure, 30 measures were registered under a light microscope at 40 x magnification.

Pathogenicity test

The pathogenicity test was performed in a greenhouse from the Instituto de Ciências Agrárias from Universidade Federal de Minas Gerais Campus Montes Claros-MG. Isolates of *Fusarium* were cultivated in Petri dishes containing PDA medium and incubated for 15 days in the darkness at 25 °C temperature. Seven days old chickpea plants cv. Cícero, previously produced in polypropylene trays filled with commercial substrate Plantmax (Plantmax Sementes Ltda), had their roots wounded with a scalpel and then were immersed 10 min in 200 mL of a conidia suspension (1×10^6 conidia.mL^{-1}) of each *Fusarium* isolate. Then, plants were transferred to 700 mL pots containing autoclaved substrate 1:1 (soil:sand). Twenty plants (replicates) were inoculated for each isolate.

The control treatment consisted of wounded plants immersed in sterilized distilled water. The experiment was performed under a completely random design. Plants showing symptoms of wilt or yellowing were considered diseased. Disease incidence was evaluated during 42 days (6 weeks). Isolates aggressiveness was determined by the increase of weekly incidence, during the whole period of evaluation. The pathogen was recovered in order to complete Kock's postulates and confirm pathogenicity. The pathogenicity test was repeated twice. Aggressiveness was determined based on the incidence observed during the first test.

Results

Phylogenetic analysis

Phylogenetic trees were generated for each species complex, FOSC and FSSC. First the analysis of MP and BI were performed for all 22 partial sequences of the TEF-1α gene from *F. oxysporum* which contained 442 characters, being 378 conserved, 63 variable and within these, 24 were informative. In the resulting phylogram, the isolates of *F. oxysporum* (CML 2865, CML 2866, CML 2867, CML 2869, CML 2871, CML 2872, CML 2874, CML 2875, CML 2876, CML 2877 and CML 2878) made a distinct lineage within FOSC, strongly supported by both methods, clustering in a clade separated from *F. oxysporum* f. sp. *ciceris* isolates (Fig. 1).

Maximum parsimony and BI analysis was performed with 48 partial sequences of TEF-1α gene from *F. solani* contained 480 characters, being 304 conserved and 171 variable, with 103 being informative within these. The topology of the generated tree showed that isolates CML 2868, CML 2870 and CML 2873 did not cluster with any already described lineages for FSSC, constituting a clade strongly supported with a *bootstrap* of 97% and posterior probability equal to one, having the *holotype* of *F. falciforme* CBS 475–67 lineage FSSC 3 + 4 as a sister group (Fig. 2).

Evaluation of morphological markers

Within the 14 characterized *Fusarium* isolates, 11 isolates showed typical morphological markers for *F. oxysporum* and three for *F. solani* (Table 2).

Isolates of *F. oxysporum* on PDA showed sparse and cotton-like aerial mycelium, white or cream in color, with pigmentation at the colony's bottom ranging from violet to cream. Growth rate ranged from 9.7 to 14.4 mm/day after 4 days at 25 °C in the dark. On SNA were observed microconidia aggregated in false heads on short phialides in the aerial mycelium (Fig. 3a). Presence of simple and short monophialides, 4–32 μm length (Fig. 3c). Microconidia cylindrical to kidney shaped, with 0–2 septs, 3–20 × 1–3 μm (Fig. 3b). Macroconidia falcate to straight in shape, with curved apical cell and evident basal foot cell, 17–55 × 2–5 μm, 3–7 septs, (Fig. 3d). All isolates showed globose chlamydospores, single or in chains, intercalary or terminal, with smooth or rough walls, 3–13.5 μm wide (Fig. 3e).

Isolates of *F. solani* showed white colored cotton-like aerial mycelium, with brown pigmentation at the colony's bottom. Colonies on PDA showed mycelial growth rate varying from 9.9 to 10.7 mm/day. On SNA were observed microconidia produced in false heads on long monophialides, 22.5–172.5 μm length (Fig. 4a and b). Microconidia globose, fusiform and reniform in shape, 0–2 septate, 3–27 × 1.5–5 μm (Fig. 4c). All *F. solani* isolates showed abundant sporodochia with cream

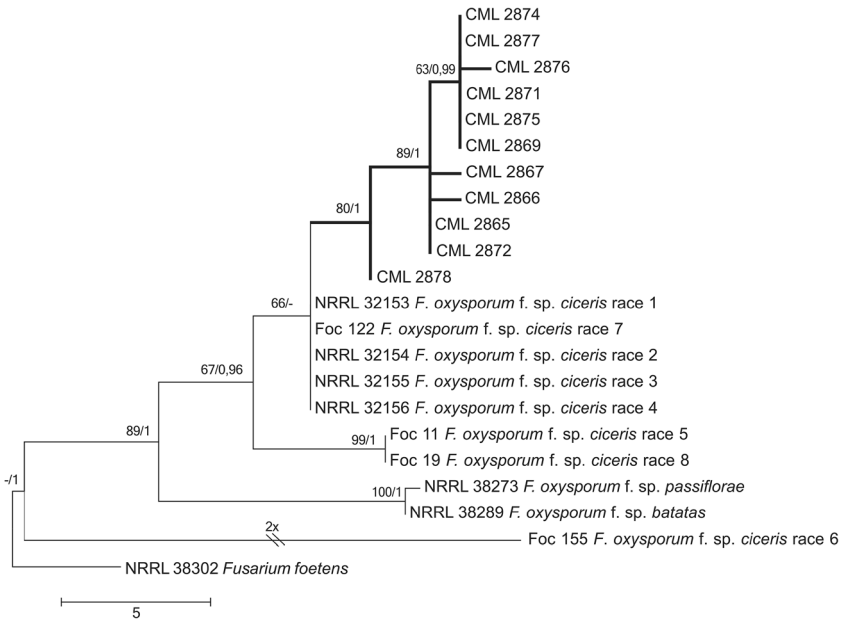

Fig. 1 Phylogram of maximum parsimony for *EF-1α* gene from lineages of the *Fusarium oxysporum* complex, including isolates from chickpea from Brazil. Length of the branches is shown by the scale in the tree base. Maximum parsimony bootstrap values ≥50% (1000 replicates) and posterior probabilities ≥0.90 are above the nodes. This tree has *F. foetens* NRRL38302 as root

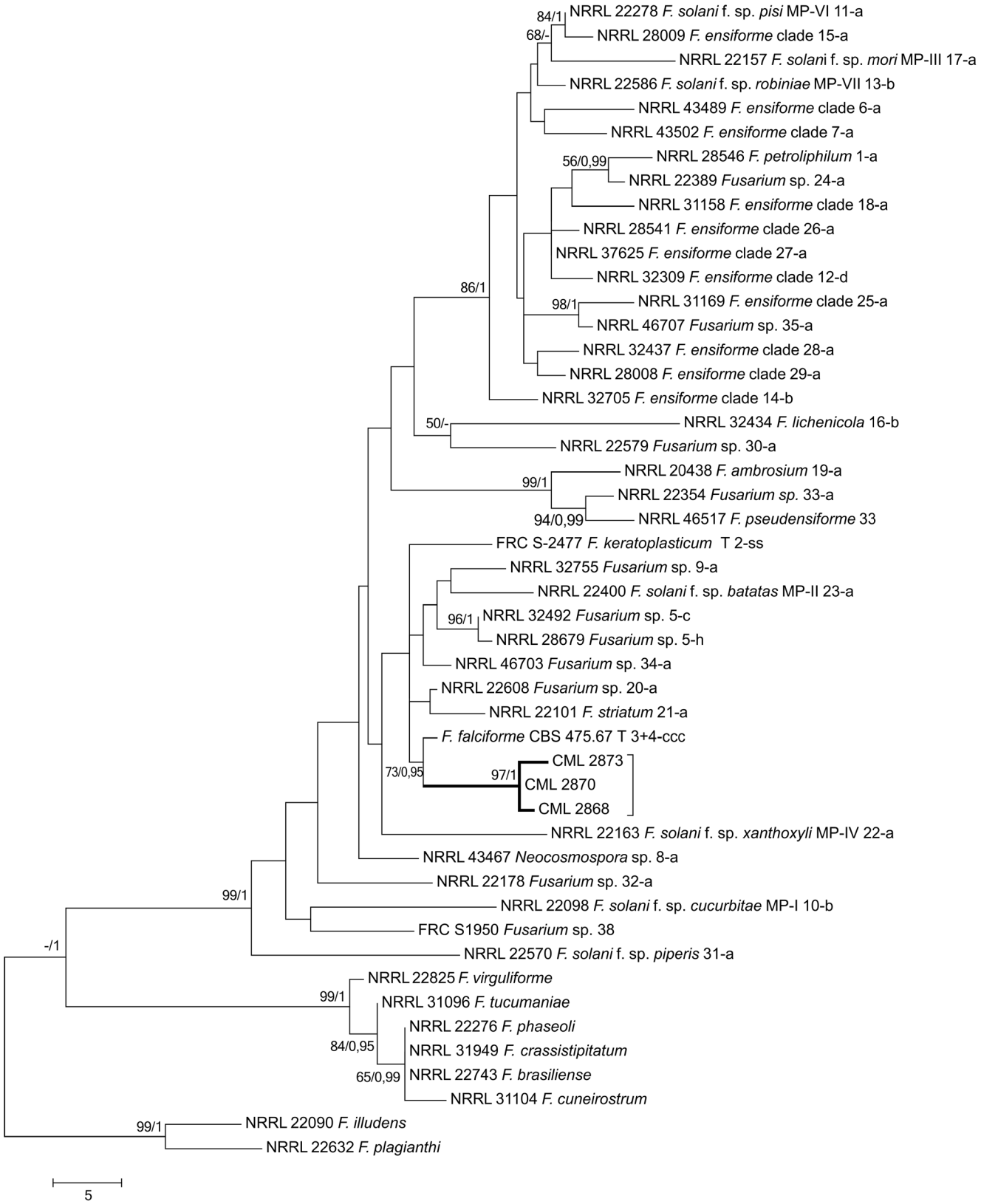

Fig. 2 Phylogram of maximum parsimony for *EF-1α* gene from lineages of the *Fusarium solani* complex, including isolates from chickpea from Brazil. Length of the branches is shown by the scale in the tree base. Maximum parsimony bootstrap values ≥50% (1000 replicates) and posterior probabilities ≥0.90 are above the nodes. This tree has *F. plagianthi* NRRL22632 and *F. iludens* NRRL22090 as root

Table 2 Morphological characterization of *Fusarium oxysporum* and *Fusarium solani* isolates from chickpea plants from Brazil

Isolates	[1]Mycelial Growth rate (mm/day)	[2]Color of the culture	[3]Macroconidia		[3]Microconidia		[3]Chlamidospores
		Front/back	Lenght/width (μm)	Septs	Comp/width (μm)	Septs	Diameter (μm)
F. oxysporum							
CML 2865	11.1	White/Violet	18–47 × 2–4	3–7	4–16 × 1.5–3	0–1	5–9.5
CML 2866	12.1	White/Violet	19–34 × 2–4	2–4	5–18 × 1.5–3	0–1	5–11
CML 2867	11.7	White/Violet	27–47 × 2.5–5	3–6	4–10 × 1.5–2	0–1	5–8.75
CML 2869	12.8	Cream/Cream	29–42 × 3.5–4	3–5	3–12 × 1.5–2	0–1	5.5–10
CML 2871	13.0	White/Violet	22–55 × 2.5–4	3–7	4–16 × 1.5–3	0–1	5.5–9.5
CML 2872	12.9	Cream/ Violet	27–36 × 3–4	2–5	3–19 × 1.5–2.5	0–1	5–9.5
CML 2874	10.5	White/Violet	31–53 × 3–4	3–6	5–20 × 1.5–3	0–1	3–12
CML 2875	9.7	White/Violet	25–55 × 2.5–4	3–5	4.5–17 × 1.5–3	0–1	3.5–10
CML 2876	13.2	White/Cream	23–46 × 2–3.5	3–5	4–17 × 1.5–2.5	0–1	4.5–10
CML 2877	14.4	White/Violet	27–42 × 3–3.5	3–5	3–16 × 1–2.5	0–1	5–13.5
CML 2878	10.6	White/Violet	17–31 × 3–4	3	4–16 × 1.5–3	0–1	4–15
F. solani							
CML 2868	10.1	White/Brown	21–50 × 4–5	3–5	3–26 × 1.5–5	0–2	4–8.5
CML 2870	10.7	White/Brown	23–45 × 4–5	3–4	6–24 × 2–4	0–2	5–10.5
CML 2873	9.9	White/Brown	22–51 × 3.5–5	3–5	7–27 × 2–4	0–2	5–10.5

[1] Obtained 4 days after growing on PDA at 25 °C in the dark

[2] Obtained 14 days after incubation on PDA medium at 20 °C in a 12 h photoperiod under near UV and white fluorescent bulbs

[3] Characterized 14 days after incubation on PDA medium at 20 °C in a 12 h photoperiod under near UV and white fluorescent bulbs

to pale orange coloration. Macroconidia showed cylindrical shape, with slight curved and rounded apical cell and slightly protruding basal foot cell, 21–51 × 3.5–5 μm, 3–5 septate (Fig. 4d). Chlamydospores were observed in pairs or in chains, globose shape, rough-walled, 4–10.5 μm wide (Fig. 3f and g).

Pathogenicity test

All *Fusarium* isolates studied were pathogenic to chickpea. All Infected plants showed rapid yellowing starting from the leaf blade with later wilt and plant death. *F. solani* and *F. oxysporum* isolates triggered similar aerial symptoms (Fig. 3f and 4h). Regarding to the radicular system, roots of all infected plants were rotted or showed blackened tissue (Fig. 3g- h and 4i). Some plants infected with *F. oxysporum* showed darkening of the vascular tissues, but always associated to root rot (Fig. 3o and p). Plants infected with *F. solani* don't showed darkening of vascular tissues. Isolates varied in aggressiveness according to the velocity they caused disease symptoms (Fig. 5). In general, *F. oxysporum* isolates were more aggressive when compared to *F. solani* isolates. Isolate CML 2878 was more aggressive and caused high disease incidence since the second week after inoculation, while isolates CML 2865, CML 2866, CML 2869, CML 2872 and CML 2873 were less aggressive. Isolates CML 2867, CML 2868, CML CML 2870, CML 2871, CML 2874, CML 2875, CML 2876 and CML 2877 showed an intermediary aggressiveness (Fig. 5). The highest disease incidence was observed in plants inoculated with the isolate CML 2877.

Fungi were recovered from symptomatic plants and showed the same morphological characteristics from the originally inoculated isolates, thus confirming their pathogenicity. Control plants showed no symptoms at all. Inoculations were performed twice, showing similar results.

Discussion

The presence of *Fusarium oxysporum* f.sp. *ciceris* infecting chickpea plants in the cerrado's areas from the Distrito Federal was reported in Brazil (Sharma and Cerkauskas 1985). However, the identification was based only in morphological markers, which are not enough to make distinctions between species within the FOSC. In the present study, isolates identified as belonging to the FOSC formed a clade with highly supported, clearly separated from *F. oxysporum* f. sp. *ciceris*, which is a monophyletic origin group (Jiménez-Gasco et al. 2002; Demers et al. 2014). This result corroborates the information that different FOSC phylogenetic lineages are able to parasite the same host. Phylogenetic analysis of *Fusarium oxysporum* f. sp. *phaseoli* and *Fusarium oxysporum* f. sp. *malvacearum* isolates confirmed that both populations are constituted by distinct phylogenetic lineages (Silva et al. 2014). Recently, phylogenetic studies evidenced that

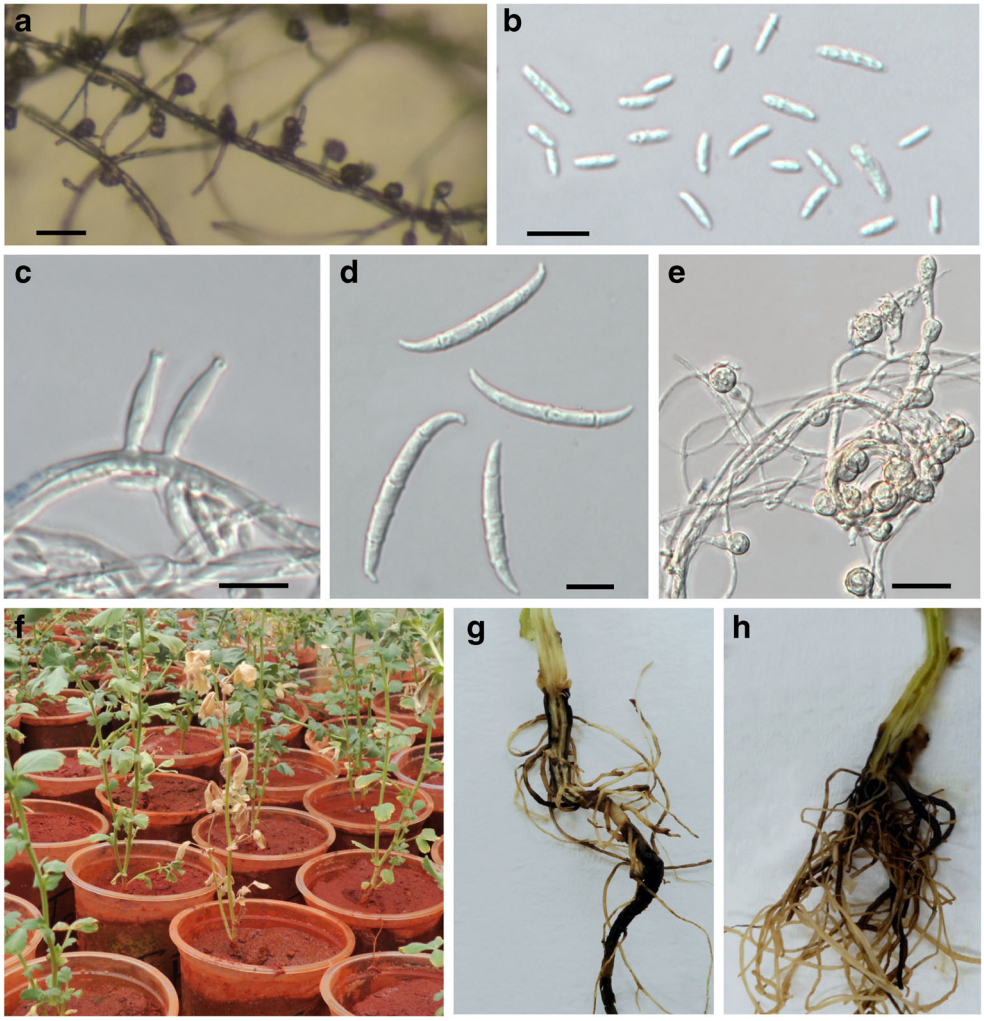

Fig. 3 Morphological characteristics of *Fusarium oxysporum* isolates: **a** False heads on short phialides, bar = 50 μm; **b** Microconidia, bar = 10 μm; **c** Simple and short monophialides, bar = 10 μm; **d** Macroconidia, bar = 10 μm; **e** Chlamidospores, bar = 20 μm. Symptoms triggered by *F. oxysporum* isolates in chickpea plants: **f** Yellowing; **g**-**h** Root rot; and darkening of the vascular tissues

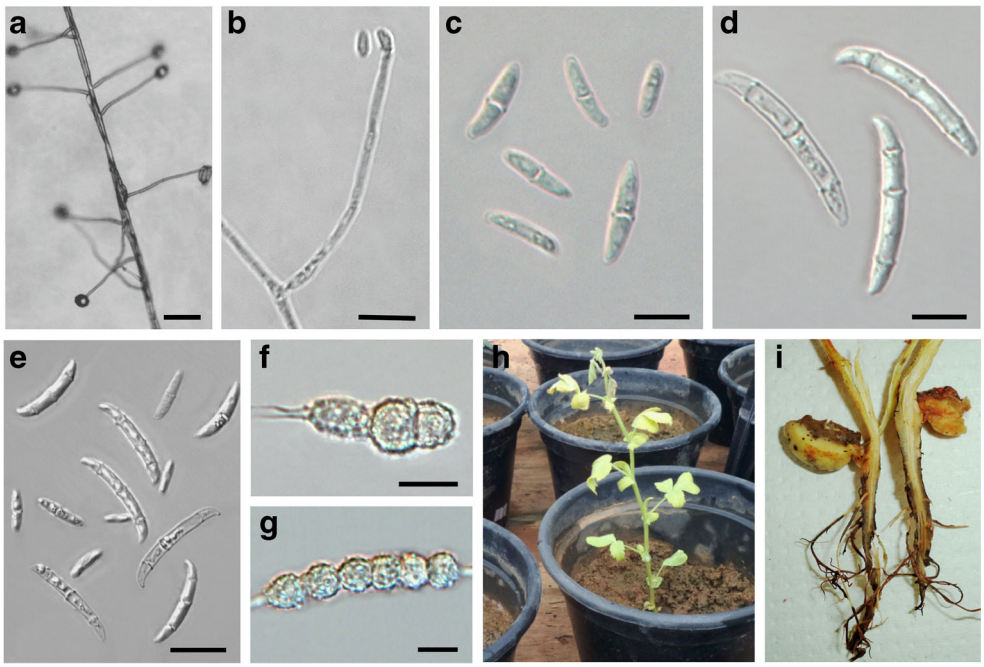

Fig. 4 Morphological characteristics of *Fusarium solani* isolates: **a** False heads on long phialides, bar = 50 μm; **b** Long monophialide, bar = 20 μm; **c** Microconidia, bar =10 μm; **d** Macroconidia, bar = 10 μm; **e** Macro and Microconidia, bar = 20 μm; **f** Chlamidospores, bar = 20 μm; **g** Chain of chlamidospores, bar = 10 μm. Symptoms triggered by *F. solani* isolates in chickpea plants: **h** Yellowing; **i** Root rot

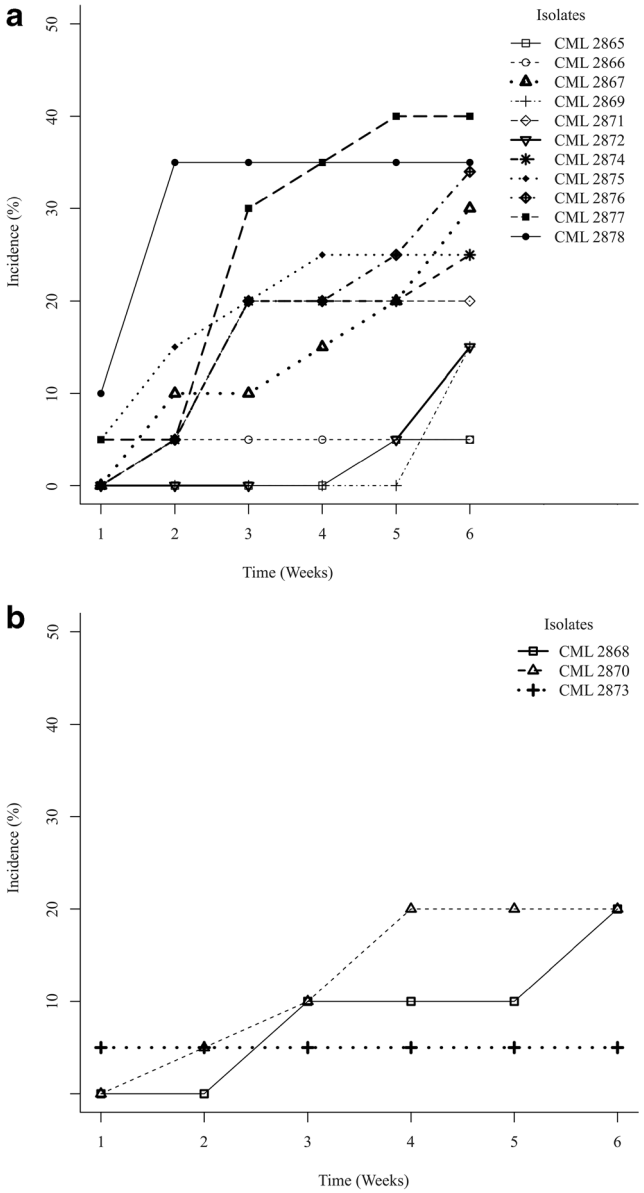

Fig. 5 Disease incidence (wilted) caused by *Fusarium oxysporum* (**a**) and *Fusarium solani* (**b**) isolates on chickpea plants cv. Cícero for six weeks after inoculation

Fusarium oxysporum f. sp. *lycopersi*, agent of tomato wilt, actually consists of four distinct phylogenetic lineages (Nirmaladevi et al. 2016). These information shows that *formae specialis* is an obsolete taxonomical terminology and may exist many species of the FOSC to be described.

After phylogenetic analysis, *F. solani* isolates (CML 2868, CML 2870 and CML 2873) clustered together forming a strongly supported clade, having as a brother group the *F. falciforme* CBS 475–67 *holotype*, lineage FSSC 3 + 4 (Fig. 2). *F. falciforme* consists of a diverse group not yet resolved phylogenetically, associated to soils and human infections (Zhang et al. 2006; O'Donnell et al. 2008; Nalim et al. 2011). The isolates of *Fusarium solani* studied had no phylogenetic relation with the main species causing root rot in various legumes. Species triggering root rot in legumes crops are included in clade 2 which gathers pathogens causing red rot in soy bean and beans (Aoki et al. 2005; Covert et al. 2007; Aoki et al. 2012). Recently, *Fusarium paranaense* Costa & Pfenning was described as being pathogenic to soy bean, however, this species belongs to clade three (Costa et al. 2016).

The morphological characteristics evaluated allowed to differentiate between FOSC and FSSC but were not sufficient to differentiate lineages within each complex. The limited number of existent morphological characters is often not enough to express the great diversity within the genus *Fusarium* (Leslie and Summerell 2006), once individuals sharing the same morphology may belong to distinct phylogenetic species (O'Donnell 2000). *Fusarium tucumaniae* Aoki, member of the FSSC, shows distinct markers from the others species of the FSSC, such as macroconidia morphology and production of blue colored sporodochia (Aoki et al. 2005). Recently, *Fusarium secorum* Secor, a new member from the *Fusarium fujikuroi* complex was described. Based on morphological markers, this fungus was previously named *Fusarium oxysporum* f. sp. *betae* (Secor et al. 2014). *Fusarium foetens* Schroers and *Fusarium oxysporum* are distinct species, however they share similar morphological markers (Schroers et al. 2004). These observations evidence the importance for using the phylogenetic concept to identify fungi from the genus *Fusarium.*

Isolates of both complexes were pathogenic and triggered similar aerial symptoms of yellowing and root rot in chickpea plants. But only *F. oxysporum* isolates triggered darkening of the vascular tissues. Studies performed in California-USA, showed that although chickpea plants inoculated with *F. oxysporum* f. sp. *ciceris* and *F. solani* had similar symptomatology in the aerial portion of the plant, *F. oxysporum* f. sp. *ciceris* caused vascular discoloration and *F. solani* black root rot without vascular discoloration (Westerlund et al. 1974). In a different study, Trapero-Casas and Jiménez-Díaz (1985) reported that *F. oxysporum* isolates caused leaf yellowing and wilt associated to vascular discoloration, as well as rotting in roots and in the lap of the chickpea plants. Isolates of *F. oxysporum* from various *formae speciales* when infecting plant tissues shows systemic colonization, observed by the darkening of conducting vessels tissues (Leslie and Summerell 2006). While species from FSSC show local colonization, mainly causing rot roots, specifically in leguminous. Due to tissues colonization, the plants exhibit reflexes symptoms of yellowing (Aoki et al. 2005; Aoki et al. 2012).

Differences observed in aggressiveness from isolates may be a result of genetic variability within the population, once isolates were obtained from different chickpea cultivars. For *F. oxysporum* f. sp. *ciceris*, differences in aggressiveness between isolates may be explained by the existence of different pathotypes and races of the pathogen (Jiménez-Gasco et al.

2004). Studies must be performed to evaluating the pathogenic capacity of the isolates to different cultivars of chickpea. Future research is needed to exploit the diversity of *Fusarium* obtained from different geographical regions from Brazil, aiming to confirm the existence of different phylogenetic species associated to fusariosis in this culture.

Acknowledgements The first author acknowledges the financial support provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES/PNPD. Authors are grateful to Osmar Artiaga for kindly allowing collect samples in his chickpea production field. We would like to express our sincere thanks to all team members from the Laboratório de Fitopatologia-UFMG and Labroratório de Ecologia e Sistemática de Fungos-UFLA for the voluntary help. Special thanks to Edson Luis Rezende and Everson for their support and technical assistance.

References

Aoki T, O'Donnell K, Scandiani MM (2005) Sudden death syndrome of soybean in South America is caused by four species of *Fusarium*: *Fusarium brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F. tucumaniae*, and *F. virguliforme*. Mycoscience 46:162–183

Aoki T, Tanaka F, Suga H, Hyakumachi M, Scandiani MM, O' Donnel K (2012) *Fusarium azukicola sp.* nov., an exotic azuki bean root-rot pathogen in Hikkaido, Japan. Mycologia 104:1068–1084

Armstrong GM, Armstrong JK (1981) *Formae speciales* and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson PE, Toussoun TA, Cook RJ (eds) *Fusarium*: diseases, biology, and taxonomy. Pennsylvania State University Press, University Park, PA, pp 391–399

Baayen RP, O'Donnell K, Bonants PJM, Cigelnik E, Kroon LPNM, Roebroeck EJA, Waalwijk C (2000) Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nommonophyletic *Formae Speciales* causing wilt and rot disease. Phytophatology 90:891–900

Chand H, Khirbat SK (2009) Chickpea wilt and its management-a review. Agric Rev 30:1–12

Costa SS, Matos KS, Tessmann DJ, Seixas CDS, Pfenning LH (2016) *Fusarium paranaense* sp. nov., a member of the *Fusarium solani* species complex causes root rot on soybean in Brazil. Fungal Biol 120:51–60

Covert SF, Aoki T, O'Donnell K, Starkey D, Holliday A, Geiser DM, Cheung F, Town C, Strom A, Juba J, Scandiani M, Yang XB (2007) Sexual reproduction in the soybean sudden death syndrome pathogen *Fusarium tucumaniae*. Fungal Genet Biol 44:799–807

Demers JE, Garzón CD, Jiménez-Gasco MDM (2014) Striking genetic similarity races of *Fusarium oxysporum* f. Sp. *ciceris* confirms a monophyletic origin and clonal evolution of the chickpea vascular wilt pathogen. Eur J Plant Pathol 139:303–318

Di Pietro AMP, Caracuel ZM, Delgado-Jarana J, Roncero MIG (2003) *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt pathogen. Mol Plant Pathol 4:315–325

Dubey SC, Priyanka K, Singh V (2014) Phylogenetic relationship between different race representative populations of *Fusarium oxysporum* f. Sp. *ciceris* in respect of translation elongation factor-1α, β-tubulin, and internal transcribed spacer region genes. Arch Microbiol 196:445–452

Gerlach W, Niremberg H (1982) The genus *Fusarium*: a pictorial atlas. Biologischen Bundesanstalt fur Land-und Forstwirtschaft, Berlin

Gurjar GS, Barve MP, Giri AP, Gupta VS (2009) Identification of Indian pathogenic races of *Fusarium oxysporum* f. Sp. *ciceris* with gene specific, ITS and random markers. Mycologia 101:484–495

Hepperle D (2004) SeqAssem©: a sequence analysis tool conting assembler and trace data visualization tool for molecular sequences: win32-version. Sequentx, Klein Raden

Jiménez-Díaz RM, Castillo P, Jiménez-Gasco MM, Landa BB, Navas-Cortés JA (2015) *Fusarium* wilt of chickpeas: biology, ecology and management. Crop Prot 73:16–27

Jiménez-Gasco MM, Milgroom MG, Jiménez-Díaz RM (2002) Gene genealogies support *Fusarium oxysporum* f. Sp. *ciceris* as a monophyletic group. Plant Pathol 51:72–77

Jiménez-Gasco MM, Navas-Cortes J, Jiménez-Díaz RM (2004) The *Fusarium oxysporum* f. Sp. *ciceris*/*Cicer arietinum*: a case study of the evolution of plant-pathogenic fungi into races and pathotypes. Int Microbiol 7:95–104

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br J Nutr 108:11–26

Leslie JF, Summerell BA (2006) The *Fusarium* laboratory manual. Blackwell Publishing Asia, Australia

Lievens B, Rep M, Thomma BPHJ (2008) Recent developments in the molecular discrimination of *formae speciales* of *Fusarium oxysporum*. Pest Manag Sci 64:781–788

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE). New Orleans, pp. 1–8

Nalim FA, Samuels GJ, Wijesundera RL, Geiser DM (2011) New species from the *Fusarium solani* species complex derived from perithecia and soil in the old world tropics. Mycologia 103:1302–1330

Nene YL, Sheila YK, and Sharma SB (1996) A world list of chickpea and pigeonpea pathogens. 5th edn. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. (Semi-formal publication.), 27p

Nene YL, Reddy MV, Haware MP, Ghanekar AM, Amin KS, Pande S, Sharma M (2012) Field Diagnosis of Chickpea Diseases and their Control, Information Bulletin No. 28. (revised). International Crops Research Institute for the Semi-Arid Tropics

Nirmaladevi D, Venkataramana M, Srivastava RK, Uppalapati SR, Gupta VK, Yli-Mattila T, Clement Tsui KM, Srinivas C, Niranjana SR, Chandra NS (2016) Molecular phylogeny, pathogenicity and toxigenicity of *Fusarium oxysporum* f.sp. *lycopersici*. Sci Rep 6:21367

Nylander JAA (2004) MrModeltest ver. 2.3. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

O'Donnell K (2000) Molecular phylogeny of the *Nectria haematococca–Fusarium solani* species complex. Mycologia 92:919–938

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci 95:2044–2049

O'Donnell K, Sutton DA, Fothergill A, Mccarthy D, Rinaldi MG, Brandt ME, Zhang N, Geiser DM (2008) Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J Clin Microbiol 46:2477–2490

O'Donnell K, Gueidan C, Sink S, Johnston PR, Crous PW, Glenn A, Riley R, Zitomer NC, Colyer P, Waalwijk C, Van Der Lee T, Moretti A, Kang S, Kim HS, Geiser DM, Juba JH, Baayen RP, Cromey MG, Bithell S, Sutton DA, Skovgaard KR, Kistler P, Elliott HC, Davis M, Sarver MBAJ (2009) A two- locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. Fungal Genet Biol 46: 936–948

O'Donnell K, Sink S, Scandiani MM, Luque A, Colletto A, Biasoli M, Lenzi L, Salas G, González V, Ploper LD, Formento N, Pioli RN, Aoki T, Yang XB, Sarver BAJ (2010) Soybean sudden death

syndrome species diversity within north and South America revealed by multilocus genotyping. Phytopathology 100:58–71

Scandiani M, Ruberti D, O'Donnell, Aoki T, Pioli R, Giorda L, Luque A, Biasoli M (2004) Recent outbreak of soybean sudden death syndrome caused by *Fusarium virguliforme* and *F. tucumaniae* in Argentina. Plant Dis 88:1044

Schroers HJ, Baayen RP, Meffert JP, Gruyter J, Hooftman M, O'Donnell K (2004) *Fusarium foetens,* a new species pathogenic to begonia elatior hybrids (*Begonia* x *hiemalis*) and the sister taxon of the *Fusarium oxysporum* species complex. Mycologia 96:393–406

Secor GA, Rivera- Varas V, Christ DS, Mathew FM, Khan MF, Varrelmann M, Bolton MD (2014) Characterization of *Fusarium secorum*, a new species causing *Fusarium* yellowing decline of sugar beet in north Central USA. Fungal Biol 118:764–775

Sharma RD, Cerkauskas RF (1985) Interação entre *Meloidogyne javanica* e *Fusarium oxysporum* f. sp. *ciceris* sobre o grão-de-bico. Nematol Bras 9:113–121

Silva FP, Vechiato MH, Harakava R (2014) EF-1 gene and IGS sequencing of *Fusarium oxysporum* f.Sp. *vasinfectum* and *F. oxysporum* f.Sp. *phaseoli* reveals polyphyletic origin of strains. Trop Plant Pathol 39:64–73

Smith D, Onions AHS (1994) The preservation and maintenance of living fungi. Kew: Commonwealth Mycological Institute

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Trapero-Casas A, Jiménez-Díaz RM (1985) Fungal wilt and root rot diseases of chickpea in southern Spain. Phytopathology 75:1146–1151

Westerlund FV, Campbell RN, Kimble KA (1974) Fungal root rots wilt of chickpea in California. Phytopathology 64:432–436

Wood JA, Grusak MA (2007) Nutritional value of chickpea. In: Redden RR, Chen W, Sharma B (eds) Yadav SS. CAB International, Chickpea Breeding and Management, pp 101–142

Zhang N, O'Donnell K, Sutton DA, Nalim FA, Summerbell RC, Padhye AA, Geiser DM (2006) Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in environment. J Clin Microbiol 44:2186–2190