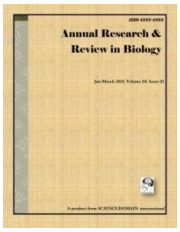

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Characterization and Identification of *Fusarium* Species Complexes Affecting Chickpea with Total Protein Profiles, EF-1α Gen

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Fusarium oxysporum* Schl. is a cosmopolitan fungus that causes severe damage to many important crops. This fungus is the causal agent of chickpea fusariosis, a very important disease in this crop, resulting in 10 to 60% of annual losses. These symptoms have generally been associated with *Fusarium oxysporum* f. sp. *ciceris* races.
Aims: In the present study, 41 *Fusarium* isolates from chickpea plants with symptoms of yellowing and wilting were characterized, differentiated and separated into species complexes from the Bajío zone in Mexico.
Methodology: The *Fusarium* isolates were characterized with total protein profile and pathogenicity tests from differential chickpea plants. The total protein profiles were associated with the *Fusarium* species complexes previously reported for these same isolates.
Result and conclusion: The protein profiles were different among the fungi isolates and formed four groups corresponding to three *Fusarium species* complexes. The isolates also showed clustering tendencies according to the collection zone. The differential plants

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showed different reaction degrees. The results indicate that yellowing and wilting pathotypes in chickpea from the Bajío zone were produced by three *Fusarium* complexes and not just for the *Fusarium oxysporum* f. sp. *ciceris* races.

Keywords: Cicer arietinum L.; biochemical markers; molecular markers; differential plants.

1. INTRODUCTION

Fusarium oxysporum Schl. (*Fo*) f. sp. *ciceris* has been reported as the causal agent of fusariosis in chickpea (*Cicer arietinum* (L.) [1], where eight races have been identified (0, 1A, 1B/C, 2, 3, 4, 5 and 6). Races 1A, 2, 3 and 4 were first identified in India [2]. Races 0, 1A, 1B/C, 5 and 6 have been found in California (USA) and Spain; races 0 and 1B/C in Syria, Tunisia and Turkey; races 0, 1A and 6 in Israel; races 1A and 6 in Morocco; race 0 in Lebanon [3]; and race 0, 1B/C, 5 and 6 in Mexico [4].

The identification of species in *Fusarium* genus has been based traditionally on morphological characters; however, many species have no difference in their mycelia, macroconidia and microconidia. For this purpose, molecular techniques based on phylogenetic analysis of specific genes have been used. O'Donnell et al. [5-6] pointed out that many species of *Fusarium* correspond to cryptic species known as species complexes.

Special attention has been given to biochemical markers, especially total protein profiles, generated through electrophoretic SDS-PAGE, which provide valid evidence to detect intra and inter-specific variations [7,8]. For example, the detection of esterase banding patterns overcome all limitations and provide additional information for fungal characterization [9]. It was found that this particular analysis is a useful tool for differentiating between *Fusarium* species, as well as different formae speciales of *Fo* collected from various geographic regions [10,11,12]. By other side, differential chickpea genotypes react to different isolates showing a specific reaction (susceptibility and tolerance at different levels). These behaviors help to differentiate among *Fo* races [2].

In Mexico, the *Fusarium* species causing fusariosis in chickpea area scarcely known. Luna et al. [13] analyzed the variation in several *Fusarium* isolates from chickpea using RAPDs markers. Lopez et al. [14], based on EF-1α gene phylogenetic analysis, found the presence of three *Fusarium* species complexes: *Fusarium oxysporum*, *Fusarium solani*, *and Giberella fujikuroi*. However, the wilting and yellowing pathotypes in chickpea are normally associated with *Fo* f. sp. *ciceri*s races [3]. The wide genetic variability observed previously in Bajío isolates [13] opens the possibility that they correspond to more than one specie. For these reasons, in the present study, biochemical markers and pathogenicity tests were used to understand the behavior of these same *Fusarium* isolates, previously studied by Luna et al. [13]. Also, this study was also supplemented with molecular identification that Lopez et al. [14] performed for the same isolates.

2. MATERIALS AND METHODS

2.1 Origin of Strains

The study was performed with 41 *Fo* monosporic isolates (Table 1) previously isolated by Luna et al. [13]. All the isolates were obtained from necrotic roots of chickpea plants with

yellowing or wilting symptoms from producer localities in the Bajío zone (Guanajuato and Michoacán), Mexico (Fig. 1).

2.2 Isolate Activation

To prevent mutations and to reduce the loss of pathogenicity under laboratory conditions, the -5ºC water-agar stored strains were activated using Czapek culture medium [15]. Subsequently the isolates were transferred to Richard's medium [16] for a rapid mycelium growth. All the isolates were exposed to a photoperiod of 12h during 5 days, or until the mycelium covered 3/4 of the Petri dish.

2.3 Pigmentation

This was an important character considered in this study. The isolates preserved in water-agar were grown in Potato Dextrose Agar (PDA) medium, incubated at 25ºC, and monitored for 4 weeks to observe the development and color of their mycelia.

2.4 Total Proteins Extraction

Fusarium isolates were grown in Czapek culture medium; the mycelia were harvested, ground with liquid nitrogen, and transferred to an Eppendorf tube containing 0.5mL of extraction solution (Tris-glicina 0.01M pH 8.9) [17]. Later, the samples were centrifuged at 1400g during 45 min at 4ºC. Crude extracted proteins were recovered in new Eppendorf tubes as clear supernatants, and were stored at -20ºC.

2.5 Total Protein Quantification and Electrophoresis

Protein quantifications were carried out according to Lowry et al. [18]. The proteins were diluted to 20µg final concentration in 25µL of Laemmli buffer (1.0mL of Tris-glycine 0.025M pH 8.3, 0.8mL of glycerol, 1.6mL 10% SDS, 0.4mL of β-mercaptoethanol, 1mL of 0.05% bromophenol blue) [19], and denatured for 3 min at 80ºC; finally, they were immersed in ice to keep the peptides separate.

Electrophoresis containing SDS were performed using the discontinuous system described by Laemmli [19]. Electrophoresis were carried out in a Minive Basic model vertical camera (Amersham Pharmacia Biotech NY, USA), using 0.25mg of total protein for each isolate at 125 volts for 5h. Gels were placed in a staining solution (Coomassie blue 0.2%, methanol 45%, glacial acetic acid 10%) for 12h, and subsequently washed with discoloration solution (50% methanol, 7% glacial acetic acid) until the protein bands were visible. Three gel replicates were performed from the same protein extract.

Fig. 1. Geographic area of *Fusarium* strains site of collected. MC: Calvario, Michoacán (Mich); MS:Singuio, Mich.; MP:La Purísima, Mich.; MI: INIFAP, Mich.; MM:Morelia, Mich.; MCu: Cuitzeo, Mich. GP: Puquichapio, Guanajuato (Gto); GVS: Valle de Santiago, Gto.; GYr: Yuriria, Gto.; GSa: Salvatierra, Gto. [13]

Only consistent and reproducible protein bands were used for the analysis. The molecular weight of each protein band was calculated using a Benchmark® marker. Protein profiles were analyzed Kodak Digital Science program DV.2.03. Each band was considered as an independent character; all detected bands were visually coded as absent ("0") or present ("1") among genotypes to construct a binary matrix. Similarity of the isolates was calculated using the Nei and Li/Dice coefficient. The clusters were performed using the arithmetic averages with the Unweighted Pair Group Method (UPGMA) [16]. The phenogram was constructed using the bootstrapping method with 1000 repetitions. The analysis was performed with the Free Tree software Version 0.9.1.50, and the tree was displayed on Tree View 1.6.6 software.

2.6 Differential Chickpea Genotype inoculation

The isolates MC2, MS10, MP14, MM21, MM23, MM25, GVS48, GYr55, MS8, MM22, MCu37, GP41, GP42, GP43, GYr57, MP16, GSa62 and MM30 (Table 2) were inoculated into three seedlings of eight chickpea differential genotypes (CPS-1, JG-74, K-850, BG-212, JG-62, WR-315, Chaffa and Annigeri) provided by ICRISAT (International Crops Research Institute for the Semi-Arid-Tropics, Patancheru, India). Non-inoculated seedlings of each genotype were considered as negative controls. All the seedlings were kept for 40 days under greenhouse conditions at 25-35ºC, and with a soil humidity of field capacity, according to Haware and Nene [2].

Table 1. Monosporic *Fusarium oxysporum* isolates used for the extraction of total proteins

Code	State	Location	EF-1α gene accession number*	Code	State	Location	EF-1α gene accession number*
MC1	Michoacán	El Calvario	NR	MM32	Michoacán	Morelia	KC113020
MC2	Michoacán	El Calvario	EU091074	MCu35	Michoacán	Cuitzeo	EU091050
MC3	Michoacán	El Calvario	KC113012	MCu36	Michoacán	Cuitzeo	NR
MC4	Michoacán	El Calvario	NR	MCu37	Michoacán	Cuitzeo	EU091051
MS6	Michoacán	Singuio	KC113013	MCu38	Michoacán	Cuitzeo	EU091052
MS8	Michoacán	Singuio	EU091041	GP41	Guanajuato	Puquichapio	EU091053
MS10	Michoacán	Singuio	EU091043	GP42	Guanajuato	Puquichapio	EU091054
MP12	Michoacán	La Purísima	KC113014	GP43	Guanajuato	Puquichapio	EU091055
MP14	Michoacán	La Purísima	EU091073	GVS47	Guanajuato	Valle de Santiago	EU091057
MP15	Michoacán	La Purísima	EU091044	GVS48	Guanajuato	Valle de Santiago	EU091058
MP16	Michoacán	La Purísima	KC113037	GYr50	Guanajuato	Yuriria	KC113033
MI17	Michoacán	El calvario	NR	GYr51	Guanajuato	Yuriria	KC113034
MM21	Michoacán	Morelia	EU091045	GYr52	Guanajuato	Yuriria	KC113022
MM22	Michoacán	Morelia	EU091046	GYr54	Guanajuato	Yuriria	KC113023
MM23	Michoacán	Morelia	EU091047	GYr55	Guanajuato	Yuriria	EU091060
MM25	Michoacán	Morelia	EU091072	GYr57	Guanajuato	Yuriria	EU091061
MM26	Michoacán	Morelia	EU091048	GSa59	Guanajuato	Salvatierra	KC113040
MM27	Michoacán	Morelia	KC113038	GSa60	Guanajuato	Salvatierra	EU091063
MM29	Michoacán	Morelia	KC113018	GSa62	Guanajuato	Salvatierra	EU091064
MM30	Michoacán	Morelia	KC113019	GSa63	Guanajuato	Salvatierra	KC113024
MM31	Michoacán	Morelia	EU091049				

**NCBI: National Center for Biotechnology Information; NR: Not reported*

The isolates were grown in Czapek medium with12h of light during two weeks at 25ºC. The inoculum was prepared with distilled water, adjusting the concentration to $1x10^6$ conidia·mL^{-1} in a hemacytometer [20-21]. The seedlings were submerged in the inoculum suspension for 1 min and transplanted to plastic pots (6x30cm) with sterile substrate (2 soil/ 1 dried leaves) [21-22]. The experiment was carried out for 64 days, recording data every 10 days for 40 days. The data were employed to assess the pathogen-host reaction. The scale used [2] is based on the presence or absence of symptoms (yellowing or wilting) in acropetala progression according to the following percentages: 0-20%=Resistant; 21-50%=Moderately susceptible; 51% or more= Susceptible. The percentage was obtained considering the total number of plant leaves with symptoms.

In another study, the EF-1α gene (translation elongation factor 1-alpha gene) was analyzed in 37 of the 41 isolates considered in the present study; The data are available in the database of the National Center for Biotechnology Information. The EF-1α gene allowed defining the species and complexes to which the isolates belonged [23], as well as the intra- and inter- specific variation [5].

3. RESULTS AND DISCUSSION

3.1 Protein Extraction and Separation in SDS-PAGE

The method of Tris-glycine pH 8.9 protein referred by Suseelendra et al. [17] provided an adequate quantity and quality measurement of total proteins, in order to establish differences between fungi isolates on SDS-PAGE (Fig. 2). The conditions used for the electrophoresis allowed an appropriate resolution of the proteins that were visualized as bands. 112 polymorphic bands were resolved in SDS-PAGE analysis varying in a range of molecular weight from 2 to 260 kilodaltons (KDas).

The 41 isolates were separated into 4 groups (1, 2, 3, 4) in the phenogram (Fig. 3). In the first group, there were 18 isolates; in the second group, 15 isolates; in the third group, 3 isolates; and in the last group, 5 isolates. These results indicated that the *Fusarium* strains considered in this study showed differences in their protein profiles, even if they have been collected from the same plant or from nearby plants (Fig. 1). The isolates were also grouped based on their geographical origin for example; Yuriria, Guanajuato (GYr50, GYr54 and GYr55); El Calvario, Michoacán (MC1, MC2 and MC3); and Morelia, Michoacán (MM27, MM29, MM30, MM31 and MM32) (Fig. 1). Similar behavior was reported by Satija [24] and Ghafoor et al. [25-26], who reported polymorphism in proteins in *Cicer arietinum* L. and *Vigna mungo* according to the geographical origin of the germplasm studied.

The MP14 and MP16 isolates that Luna et al. [13] reported as identical using RAPD markers proved to be different in the total protein profiles from the present study. In the phenogram, those isolates were respectively separated in groups 1 and 3, but they were collected in the same area. On the other hand, the MM25 isolate, also located by Luna et al. [13] in the same groups MP14 and MP16, were also located in this study in a different group. In Fig. 2, these three isolates show few different protein bands (Fig. 1). Identification of isolates using the EF-1α gene made it possible to locate the isolates in different *Fusarium* species complexes; for example, particularly the isolates MP14 and MP16 were located in the FIESC complex (*Fusarium incarnatum-equiseti* species complex), and the MM25 isolate belongs to the GFC complex (*Gibberella fujikuroi* species complex) [5]. Even when the DNA analysis reported by Luna et al. [12] suggests that these three isolates are very similar, the color and

shape of the mycelium and the total protein profiles allowed differentiating each one (Figs. 2 and 3).

The remaining 38 isolates used in the study were located in the FOSC complex. Isolates GSV48 and GSA60, were identified also as *Fo* f. sp. *ciceris* by comparing their EF-1α gene sequences in the database. Particularly, this fungus specie with its special formae, is referred to as being the main cause of the yellowing and wilting chickpea symptoms.

The different shapes and pigmentations of the mycelia isolates were distributed throughout the phenogram, but they were not associated with specific groups. In this study, the genetic and biochemical variations of the *Fusarium* isolates are intimately linked to their physiology (pigmentation, mycelium shapes and pathogenicity level). However, all these variations can respond to events like punctual, minimal mutations or the presence of transposable elements that occur in time and space, favoring the genotypes by selection as described by Kistler [27] and Daboussi and Langin [22]. According to Ainsworth et al. [28], the genetic variation of *Fusarium* does not only come from an exchange between chromosomes, but also from variations of the mitotic cycle. Mainly, under lab conditions [14], the parasexual cycle increases their infective capacity, because it allows the asexual recombination of genetic material amongst the hypha nuclei.

Generally, the mycelium of *Fo* is variable, and we can find two types in this study's isolates: the first type with abundant aerial mycelium (for example: the GYr51, GYr57 and GP43 isolates referred in this work), and the second type with cottony-like mycelium (for example: the MM25, MM30 and Gyr52 isolates). In both cases, the color ranges from white to pink, sometimes with a purple or violet dye, being more intense on the agar surface [29]. We observed a wide range of colorations in the different isolates (Fig. 3), even if they share the same complex. For this reason, the color of the mycelium found in this study was important; because this character has been associated with the *Fo* species or races identification *Fo* [4].

3.2 Reaction of the Differential Chickpea Plants to *Fusarium* Isolates

Based on the similarity groups separated in the phenogram, some representative isolates were selected for each group in order to evaluate their pathogenicity response in differential plants (Table 2). The interaction response was diverse, showing some resistance until susceptibility condition.

Table 2. Response of differential plants to the inoculation with several *Fusarium* isolates obtained from chickpea plants with yellowing and wilting symptoms

Isolate Variety	MC 2	MS 10	MP 14	MM 21	MM 23	MM 25	GVS 48	GYr 55	MS 8	MM 22	MCu 37	GP 41	GP 42	GP 43	GYr 57	MP 16	GSa 62	MM 30
CPS-1	S	M	M	M	S	S	M	M	M	S	M	M	S	M	S	R	S	S
JG-74	R	-	R	-	R	R	-	R	-	-	-	R	-	-	M	R	R	R
BG-212	R	R	R	R	R	M	R	R	R	R	R	R	R	R	R	R	R	R
JG-62	M	R	M	M	R	S	S	R	R	M	R	R	S	R	R	M	M	M
Annigeri	R	-	R	-	R	M	-	R	-	-	-	R	-	-	R	R	M	M
K-850	R	R	R	R	R	R	R	M	M	S	R	R	-	R	R	R	R	R
Chaffa	-	-	M	-	M	-	-	-	-	-	-	M	-	-	M	M	M	M
WR-315	R	R	M	R	M	M	M	M	R	R	MS	M	R	R	R	R	R	M

R= Resistant (0 –20 % of mortality); M= Moderately susceptible (21 – 50% of mortality); S=Susceptible (more than 50% of mortality)

The more evident symptoms were observed between 8 and 10 days after inoculation in the differential CPS-1 and JG-74 genotypes (susceptible varieties). The BG-212, K-850 and WR-315 genotypes showed greater resistance, and the CPS-1 genotype was the most susceptible of them. The Gyr57, which belongs to the FOSC complex isolate, was the unique fungus that caused a wilting symptom. The GVS48, Gsa62, MP16, MA30, GP42S, MM25 and MA22 isolates showed both symptoms. The MM25 showed it belonged to the GFC, the MP16 isolate to the FIESC, and the rest to the FOSC complexes, respectively.

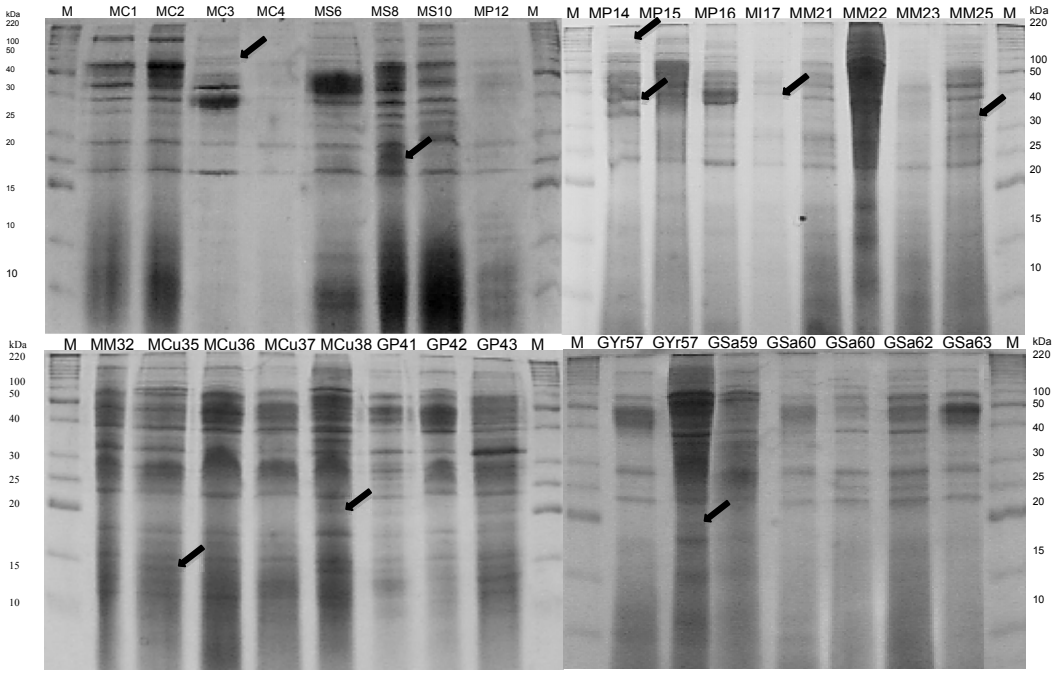

Fig. 2. Total protein profiles of 29 *Fusarium* spp. isolates. M: Molecular Weight Marker Benchmark®. Black arrows indicate some polymorphic band proteins

Fusarium isolates used in this study allow assuming the variable infective capacity of these species complexes. Mexico is recognized as a mega-diverse place in climate and crop species, so it is relatively easy to also find different pathogens associated to different crops. The present study presents evidence that different species of *Fusarium* complexes that damage the chickpea crop share genes that can cause yellowing or wilting (same symptomatology shown by other plants affected by *Fusarium* spp.), and that these pathotypes are not only exclusive to the different *Fo* f. sp. *ciceris* strains [4]. The severity of damage in chickpea by the different complexes reported point to the necessity for the development and use of resistant varieties in geographic areas such as the Bajío, Mexico. This area is characterized by presenting appropriate conditions for the development of different agricultural crops. Knowing the set of genes involved in the damage may provide information for the important control of this cosmopolitan fungi group, as well as for the development of resistant varieties.

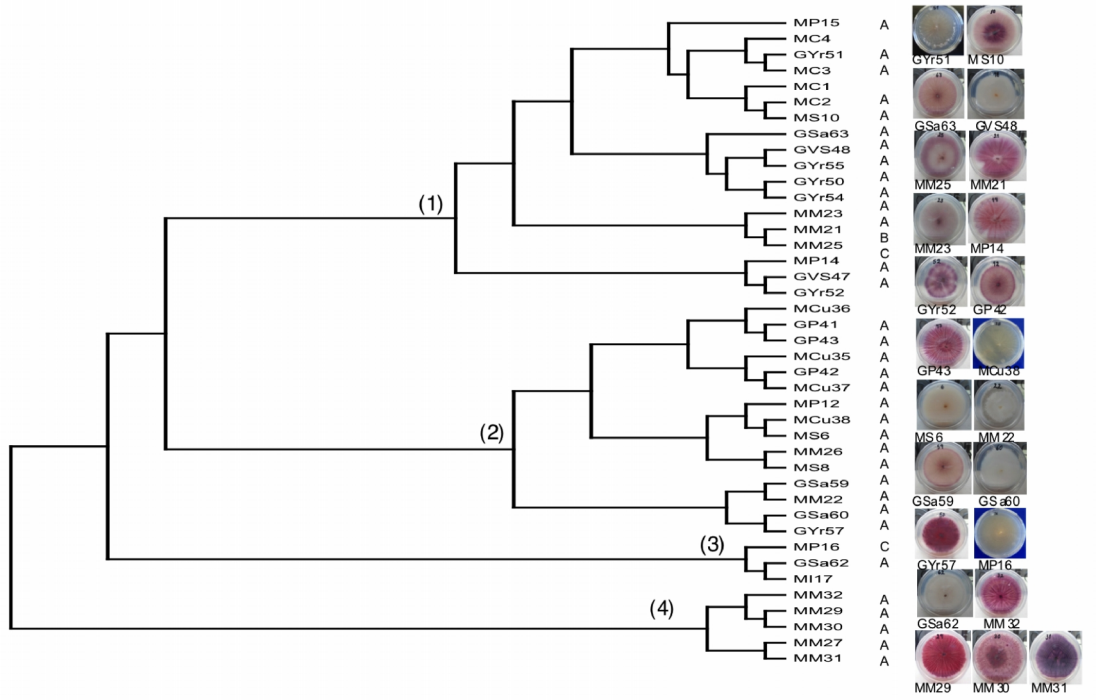

Fig. 3. Phenogram generated from the total protein analysis using UPGMA and Nei and Li/Dice coefficient, and a bootstrap analysis with 1000 repetitions. A: FOSC=*Fusarium oxysporum* species complex, B: GFC=*Gibberella fujikuroi* species complex and C:FIESC=*Fusarium incarnatum-equiseti* species complex. The Petri dishes show the mycelium type and the color of each representative *Fusarium* complex

4. CONCLUSION

The analysis of total proteins allowed characterizing and differentiating the *Fusarium* isolates, all separated from chickpea plants presenting wilting and yellowing symptoms. The *Fusarium* protein profile groups were related to the isolates originally from the same collected place; at the same time, they maintained a close relationship with the genomic species complexes of *Fusarium oxysporum* (FOCS), *Gibberella fujikuroi* (GFC) and *Fusarium incarnatum-equiseti* (FIESC). The use of differential genotypes allowed making a distinction between different reactions from all fungi isolates. Nevertheless, all isolates reported in the different races of *Fo* f. sp. *ciceris* exhibited the same symptoms of wilting and yellowing pathotypes. The combination of several techniques, like the ones used in this study, allows us to obtain better and more results that can help us to differentiate and to identify each fungus isolate more exactly.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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