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New Lines of Chickpea Against *Fusarium Oxysporum* f. sp. *Ciceris* Wilt

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Abstract: Problem statement: In Mexico, 70 and 20% of chickpea is produced in Sinaloa and Sonora, respectively. In Sonora wilting by *Fusarium Oxysporum* f. sp. *Ciceris* (FOC) causes losses of up to 60%, while in other parts of the world ranged from 12-15% annually. The aim of this study was to evaluate the resistance of new lines of chickpea obtained through breeding programs against FOC wilt. **Approach:** In order to evaluate the resistance of new chickpea lines: Hoga-012, Hoga-490-2 and Hoga-508, including the two most important commercial cultivars in Mexico: Blanco Sinaloa-92 and Costa-2004 and as control two cultivars: JG-62 (susceptible) and WR-315 (resistant), a pathogen city test was performed with races 0 and 5 of FOC. Plants were evaluated based on leaf and root damage during 50 days, using a hedonic scale of five levels (0-4). **Results:** New chickpea lines as well as commercial cultivars were susceptible to races 0, 5 of FOC. Changes ($P<0.05$) were observed on wilting by effect of the main factors and the interaction of factors. Cultivar JG-62 showed susceptibility to all races, while WR-315 was resistant. In all treatments it was proved that wilt was caused by races of FOC. **Conclusion:** New lines of chickpea and commercial cultivars did not show resistance to FOC races isolated in chickpea fields of Sonora. Thus, it should be continued in the search for resistant genotypes through breeding programs to assist in controlling the disease.

Key words: Vascular-fusariosis, resistant genotypes, commercial cultivars, chickpea lines, *Fusarium oxysporum*, lines against, resistant genotypes

INTRODUCTION

The cultivation of chickpea (*Cicer arietinum* L.) is affected by diseases such as wilt or vascular-fusariosis caused by *Fusarium oxysporum* f. sp. *ciceris* Matuo and K. Sato with significant losses in production. In Sonora, Mexico, losses of up to 60% are recorded (Gomez, 2004), while in Spain ranged from 12-15% annually (Landa *et al*., 2004). The management of the disease is complex and the use of resistant cultivars seems to be the most practical and economically efficient control measure (Jimenez-Diaz *et al*., 1991). However, plant resistance to the pathogen varies regionally and therefore; the improved varieties are evaluated through multiple trials. Also the knowledge about the behavior of population of the pathogen is essential to design a program of effective improvement and reduce the high losses caused by this disease (Sivaramakrishnan *et al*., 2002).

Lines of chickpea type Kabuli of high yield have been developed with partial or complete resistance to *Fusarium oxysporum* Schltdl. wilt and *Didymella rabiei* (Kovatsch.) Arx (Navas-Cortes *et al*., 1998). However, the effectiveness of resistance to vascular-fusariosis can be limited by the occurrence of pathogenic strains, which differ in pathogenicity and virulence (Jimenez-Gasco *et al*., 2005). Eight races of *F. oxysporum* f. sp. *ciceris* (FOC) have now been identified: 0, 1A, 1B/C, 2, 3, 4, 5 and 6 (Jimenez-Gasco and Jimenez-Diaz, 2003). Races 0, 1A, 1B/C, 5 and 6 have been recorded in Spain and California, United States, while races 2, 3 and 4 in India, the latter three being the most virulent (Haware and Nene, 1982; Jimenez-Diaz and Alcala-Jimenez, 1994; Halila and Strange, 1996). For Mexico: Sinaloa and Sonora, the authors of this study determined 4 strains, being pathotypes of yellowing (R0, R1B/C) and wilting (R5, R6). FOC morphological variability was high and is not determined by the geographic region of crop fields or the physical and chemical properties of soil (Arvayo-Ortiz *et al*., 2011). This study was the first record of strains of FOC by specific PCR for Mexico.

Strain 0 is the least virulent and strain 1B/C induces progressive leaf yellowing compared to severe

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wilting of strains 1A to 6. It also has been observed that races 0 and 1B/C are differentially pathogenic on the cultivar JG-62, despite sharing the same path type, while the strains 1B/C and 1A, belonging to different path types are moderately or highly virulent on the cultivar C104 (Jimenez-Gasco *et al*., 2004). The yellowing path type of FOC is less virulent than wilting, but may also be differences in virulence between strains of the same path type (Jimenez-Gasco *et al*., 2005; Ahmad *et al*., 2010). Thus, the hypothesis of this study was that the FOC strains isolated from chickpea fields of Sonora are pathogenic for new lines of chickpea. The aim of this study was to evaluate the resistance of new lines of genetically improved chickpea with strains 0 and 5 of *Fusarium oxysporum* f. sp. *ciceris* isolated in chickpea fields of La Costa de Hermosillo and Valle del Yaqui, Sonora, Mexico.

MATERIALS AND METHODS

Preparation of inocula: The inoculums was prepared from six strains (S1-S6) obtained previously by the authors (Arvayo-Ortiz *et al*., 2011), from the most important chickpea regions of Sonora: La Costa de Hermosillo and Valle del Yaqui (Table 1). Three strains belonged to race 0 (yellowing path type) and three to race 5 (wilting path type) of *Fusarium oxysporum* f. sp. *ciceris* (FOC), which are deposited in the Fungal Biotechnology Laboratory of the CIAD. The strains were previously identified by specific PCR with primers for FOC (Table 2) and amplifying a 1500 bp fragment of the positive strains FOC. Then there was a specific PCR for race 0 and 5 (Table 2), following the methodology proposed by Jimenez-Gasco and Jimenez-Diaz (2003). Genomic DNA was obtained using the commercial kit of ZR Fungal Zymo Research. The primers were purchased through Eurofins MWG Operon.

PCR conditions were: 94°C/10 min^{-1}; 36 cycles of 94°C/1 min; 58°C/1 min and 72°C/1 min; a polymerization cycle at 72°C/5 min and a storage temperature of 4°C. Electrophoresis was done on 1.0% agarose gel in Tris-borate-EDTA buffer (TBE buffer) and samples were dyed with etydium bromide to visualize the amplified DNA, using a UV transilluminator and photographs were taken with a Polaroid camera (Kodak).

The inocula were multiplied in 50 mL of potato-dextrose agar, in an orbital shaker at 120 rpm and 25°C for 7-10 days, under fluorescent light for 12 h the liquid culture was filtered through a double layer of sterile gauze. Conidial suspensions were measured with a hemacytometer and adjusted to a concentration of 4×10^6 spores mL^{-1}. The inocula were increased in a mixture of sand and cornmeal (9:1, w/w), sterilized twice for 1 h at 121°C, homogeneously mixed and incubated for 15 days at 25°C with 33% relative humidity and constant fluorescent light (Nene and Haware, 1980; Trapero-Casas and Jimenez-Diaz, 1985).

Lines of chickpea and inoculation: The new lines were obtained by breeding through hybridization of single crosses, backcrosses and multiple crosses between genotypes of swineherd chickpeas type Desi with commercial varieties such as Kabuli of high yield and with FOC wilt resistance, in the Instituto Nacional de Investigaciones Forestales, Agricolas and Pecuarias after 15 years of investigation (INIFAP, 2008).

The chickpea seeds of L1 = Blanco Sinaloa-92 (commercial cultivar), L2 = Costa-2004 (commercial cultivar), L3 = Hoga-012 (new line), L4 = Hoga-490-2 (new line), L5 = Hoga-08 (new line), L6 = JG-62 (susceptible cultivar), positive control) (Navas-Cortes *et al*., 2000) and L7 = WR-315 (resistant cultivar, negative control) (Sharma *et al*., 2005), were pre-germinated in trays with sand sterilized twice for 1 h at 121°C.

Table 1: Isolates and races of *Fusarium oxysporum* f. sp. *ciceris* used in the pathogenicity tests

Number of Isolate and strain	Race	Chickpea field of origin	Latitude	Longitude
150 = S1	R5	Bloque 217, Valle del Yaqui	27º30’31.9	110º10’56.8
315 = S2	R0	Bloque 213, Valle del Yaqui	27º29’37.4	110º09’26.9
174 = S3	R0	Esperanza, La Costa de Hermosillo	28º47’40.9	111º36’13.6
324 = S4	R5	Bloque 215, Valle del Yaqui	27º29’22.3	110º09’48.9
500 = S5	R0	Santa Lucia, La Costa de Hermosillo	28º42’39.5	111º33’20.6
501 = S6	R5	Tinajita, La Costa de Hermosillo	28º45’52.5	111º20’09.9

Table 2: Primers used in the analysis of strains of FOC

Primers	Sequence of the primer (5´-3´)	Race	Size of the fragment (Kb)
FOC-f	GGCGTTTCGCAGCCTTACAATGAAG	FOC	1.5
FOC-r	GACTCCTTTTTCCCGAGGTAGGTCAGAT		
FOC-0f	GGAGAGCAGGACAGCAAAGACTA	R0	0.9
FOC-0r	GGAGAGCAGCTACCCTAGATACACC		
FOC-5f	GGAAGCTTGGCATGACATAC	R5	0.9
FOC-5r	AAGCTTGGGCACCCTCTT		

Inocula at a concentration of 4×106 UFC/g of soil (Kaiser *et al*., 1994) were placed in plastic pots of 1 L, containing soil-sand-peat at the same rate and sterilized twice for 1 h at 121°C and mixed homogeneously.

Pre-germinated plants for 4 days from certified pathogen-free seed, whose phytosanitary quality was confirmed *in vitro* in PDA plates with seeds of chickpea of different cultivars, were transplanted to the inoculated pots (three plants per pot and three pots per treatment) and were placed in the soil under natural conditions of light and darkness. 441 plants were evaluated daily with 9 control plants per experimental line, which were not inoculated with the pathogen. The initial irrigation was of 200 mL and from the second day the irrigation was 100 mL daily. Average temperatures during the pathogen city trial were 20 and 25°C in March and April 2010, respectively (Whether Channel, 2010).

Evaluation of the disease and identification of the causal agent: The progress of the disease was evaluated every 5 days between 10 and 50 days after inoculation. A hedonic scales of five levels was used to evaluate symptomatology of plants, where: 0 = without symptoms, 1 = slight choruses, 2 = moderate choruses, 3 = severe choruses o severe wilt and 4 = dead of the plant, according to the scale of Cai *et al*. (2003). At the end of the test and to check the damage caused by the pathogen, plants were removed from the pots, washed with tap water, dried on study towels and damage to leaves, stem base and root were observed. Cuts were made from the root and stem base of 1-2 cm, which were placed in 50% ethyl alcohol for 30 sec, sodium hypochlorite at 2% for 2 min, were washed twice with sterile distilled water the excess of water was removed, five samples were placed in Agar-Dextrose-Potato (PDA), they were incubated for 7-10 days at 25°C and it was confirmed Koch's postulate of causality, comparing the macro-and microscopic characteristics of the colonies, with the strains previously characterized morphologically (Nelson *et al*., 1983; Burgess, 1994) and genetically (Jimenez-Gasco and Jimenez-Diaz, 2003) and inoculated in different cultivars.

Statistical analysis and experimental design: For the statistical analysis of wilting, the results were adjusted to a completely randomized design with a factorial arrangement for three factors, being the A factor the lines of chickpea with 7 levels, identified from L1 to L7, the B factor, the FOC races with 6 levels (S1-S6) and the C factor, evaluation days with 9 levels, day 10-50 with intervals of 5 days. An analysis of variance was performed by the general linear models procedure, fitting a model that included the main effects of the factors and their double interactions.

The original values of the variable wilt, being ordinal data showed no normality, so some transformations of the variable were tested being the natural logarithm (Log) which allowed for obtaining their adjustment to the normal by the test Martinez-Iglewicz. Significances were estimated with the terms of the model to a probability level of 0.05 in the Type I error and comparison of means was performed by Tukey's multiple range test. Graphs were constructed of the variable for two-way interactions that were significant. All statistical procedures were performed in the statistical package NCSS (Hintze, 2007).

RESULTS

The time of exposure to the pathogen was the most determining factor as to the damage by wilting in the plant. The symptoms began on day 10 with yellowing in three plants of JG-62 with R0, showing wilting at 20 days and death at day 30. However, other plants of this line died up to 45 days. In the other lines (L1, L2, L3, L4 and L5), the symptoms showed yellowing on day 15, changing to wilt as time of exposure to the races of the pathogen passes. Complete wilting of the plants occurred on the days 45-50. The performance of L2, L3 and L4 was similar between them. Instead WR-315 (L7) was the strongest, only showed slight damage against R0 and R5.

With respect to exposure time of chickpea lines against the pathogen, significant differences ($P<0.05$) were observed between all times, indicating that as time passes the damage is more severe and as a result, the death of the plant. As for the virulence between strains significant differences ($p<0$-05) were observed between the S6 (R5) and the other strains; as well as between S4 (R5) and the remaining strains, except for the S2 (R0) and the S1 (R5) and S6 (R5), S2 (R0) and S4 (R5). Thus, the S1 (R5) and S6 (R5) were the most pathogenic. On the contrary, there were no significant differences ($p>0.05$) between S3 and S5, both of the R0. In regard to the FOC strain-by-time interaction (Fig. 1), important changes ($p<0.05$) in wilting were observed, the most virulent strains for chickpea lines the S1 (R5), S3 (R0), S6 (R5), S2 (R0), S5 (R0) and S4 (R5) with wilting 4, 3.7, 3.5, 3.3, 3.2 y 3.2 respectively. According to the degree of virulence, those that affected earlier the plants were S6 and S3 followed by the S1, S2, S5 and S4.

As to chickpea lines there were differences ($P<0.05$) between L7 with respect to the other lines, as well as between L1, L6 lines.

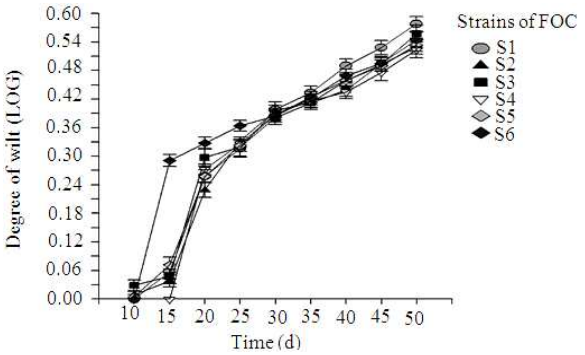

Fig. 1: Changes observed in wilt (mean ± standard error of LOG of wilt), due to the FOC strain × time interaction. Strains of FOC: ⬭ S1 (R5), ▲ S2 (R0), ■ S3 (R0), ▽ S4 (R5), ◇ S5 (R0) and ◆ S6 (R5). S: Strain. R: Race

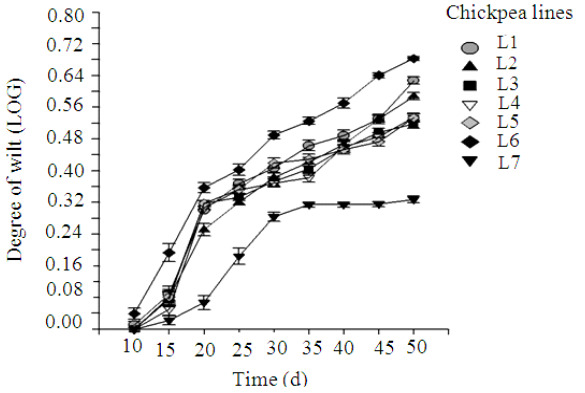

Fig. 2: Behavior of wilt (mean ± standard error of LOG of wilting) over time for each experimental line (chickpea line x time interaction). Chickpea lines: ⬭ L1 (BS-92), ▲ L2 (Costa-2004), ■ L3 (Hoga-012), ▽ L4 (Hoga-490-2), ◇ L5 (Hoga-508), ◆ L6 (JG-62) and ▼ L7 (WR-315). L: Line

There were no differences ($p>0.05$) between L2, L3, L4 and L5, indicating that the lines of chickpea most affected by FOC were L6 and L1, while L7 was the least damaged. Among the new lines, L4 was the least affected than the other lines. In the chickpea line-by-time exposure interaction (Fig. 2), L6 in time 10, showed differences ($p<0.05$) with the rest of the lines, being the most susceptible where the symptoms began earlier than in the other lines and a greater degree of wilting. Of the new lines, the least susceptible line was the L4 (Hoga-490-2), which on day 15 showed differences ($p<0.05$) only with L6. Likewise, at 20 days, L2 showed differences ($p<0.05$) with all lines except L7 and on day 25, L7 showed dissimilarity with the other lines except L1.

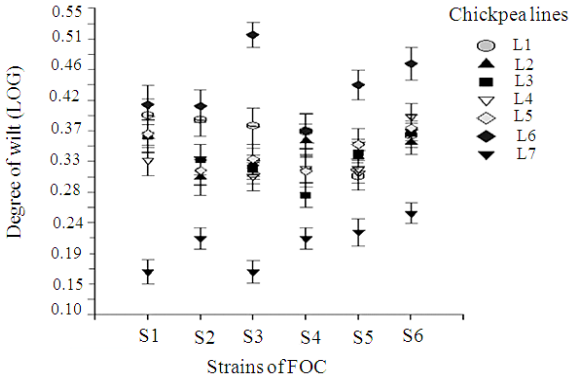

Fig. 3: Mean values ± standard error of LOG of wilting for the FOC strain x chickpea line interaction at 50 days. Chickpea lines: ⬭ L1 (BS-92), ▲ L2 (Costa-2004), ■ L3 (Hoga-012), ▽ L4 (Hoga-490-2), ◇ L5 (Hoga-508), ◆L6 (JG-62) and ▼ L7 (WR-315). L: Line. FOC races: R0 (S2, S3 and S5) and R5 (S1, S4 and S6)

To the chickpea line-by-FOC strain interaction it was observed that the lines L3, L4, L5, were susceptible to both races, being the damage more severe in L6 (JG-62), that presented a value of 4 (death), While L7 line (WR-315) was the most resistant with a mean value of wilt of 1.5 to the Races 0 (R0) and 5 (R5) of FOC. Most lines were susceptible ($p<0.05$) to Races 0 (R0) and 5 (R5) FOC (Fig. 3). R5 induces rapid wilt in susceptible cultivars, being occasionally non-pathogenic on WR-315. To a lesser degree, L7 is affected also especially with S6 (R5) and S5 (R0), whereas L6, was very susceptible to S3 (R0) and also to S6 (R5). In addition for L2, the most virulent strains were S1 (R5) and S4 (R5) (Fig. 3 and 4), while S2 (R0) and S3 (R0) were the least virulent strains. For lines 3 and 5, the most virulent strains were S1 (R5) and S6 (R5), while for L4; the most virulent was S6 (R5) and the least one, S3 (R0).

Thus, as to exposure times of each of the chickpea lines against races 0 and 5, L7 showed difference ($p<0.05$) with respect to the other lines, being the most resistant. Lines 2, 3 and 5 showed similar behavior among them, but all were affected by the races of FOC (Fig. 2-4). Conversely, L7 (WR-315) showed more resistance to the pathogen, with a maximum degree of wilting of 1-2 (Fig. 2 and 3). All lines were affected with strains of R0 (yellowing) and R5 (wilting) except L7 (WR-315), being resistant to R0 and R5, while L6 (JG-62) was very susceptible to those races.

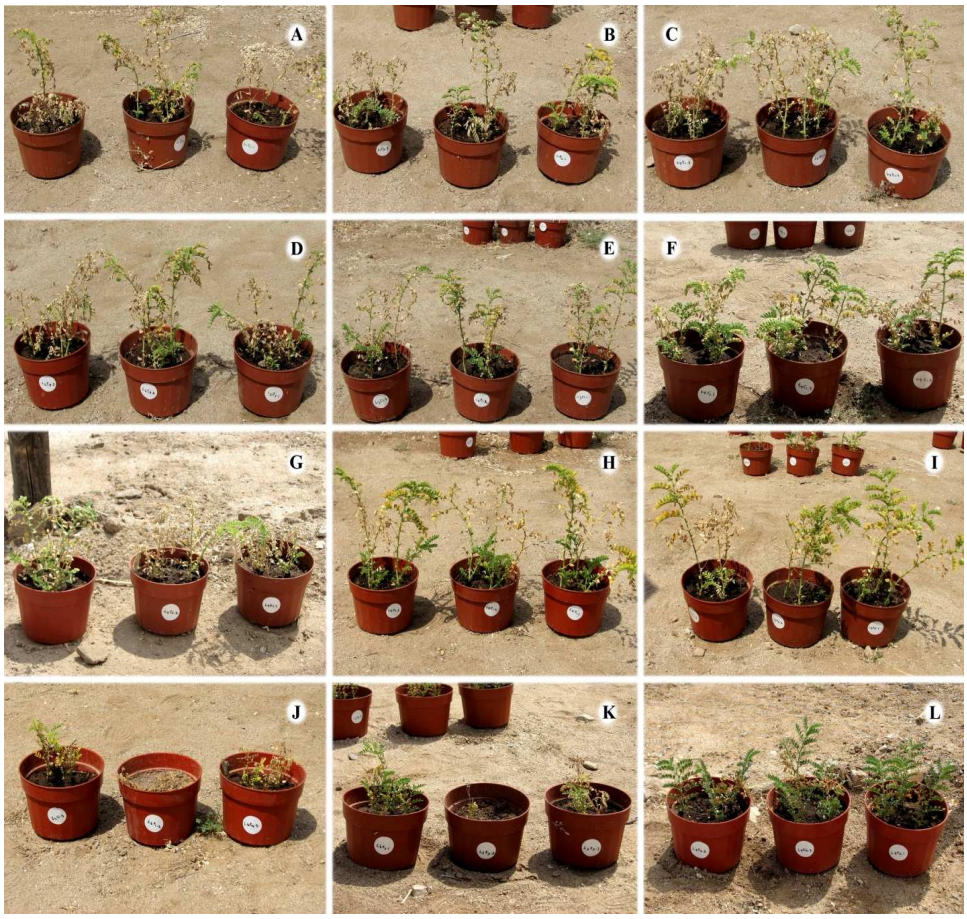

Fig. 4: Behavior of lines and cultivars of chickpea at 40 days compared to R0 and R5 of FOC isolated in northwestern Mexico. A and B: Cultivar BS-92 (L1) with wilt by R5 (S1), as well as yellowing and wilting by R0 (S3). C and D: Cultivar Costa-2004 (L2) with wilt by R5 (S1 and S4). E: L3 (Hoga-012) with wilt by R5 (S1). F and G: L4 (Hoga-490-2) less susceptible to wilt by R5 (S1), but more susceptible to wilt by R5 (S6). H and I: L5 (Hoga-508) with yellowing by R5 and R0 (S1 and S5). J and K: L6 (JG-62) highly susceptible to R5 and R0 (S1 and S3). L: L7 (WR-315) resistant to wilt by R5 (S6)

DISCUSSION

It was confirmed that R0 and R5 were the cause of the vascular-fusariosis in chickpea lines and that the damage began with yellowing, moving gradually to complete wilting. Moreover, one would expect that the greatest damage always occurs as a result of R5 of wilting, which was not observed in this study. Results indicate that the most virulent strain for most of new lines was S1 (R5), belonging to the pathotype of wilt. However, S4 also from race 5 did not show the same degree of virulence in all lines, which may be due to the fact that the response of the lines is different to a same race or by the variability between strains. This was consistent with Jimenez-Gasco *et al*. (2001), who confirm that the cultivars moderately susceptible to R5 develop a slow and progressive leaf yellowing that can be differed from yellowing caused by the R0 line in susceptible cultivars.

Most Kabuli and Desichickpeas grew in the Mediterranean region and the Indian subcontinent, respectively, are resistant to R0. Races 2, 3 and 4 are the most virulent of the eight races described and identified only in India (Halila and Strange, 1996; Haware and Nene, 1982; Jimenez-Diaz and Alcala-Jimenez, 1994). The R0 is the least pathogenic of all races of FOC and occasionally may not be pathogenic to cultivar JG-62 (Trapero-Casas and Jimenez-Diaz, 1985). In contrast to this, in our study JG-62 was susceptible to R0 isolated from chickpea fields of Sonora.

Trapero-Casas (1983) observed that after 40 days, all plants showed symptoms in all isolated-cultivar combinations, except for WR-315. In our study WR-315 (L7) was the strongest, only showed slight damage against R0 and R5. Navas-Cortes *et al*. (2000) observed that the races of FOC differ in pathogen city and virulence, depending on the susceptibility of the cultivar. Other factors favoring the development of FOC are high temperature, amount of inoculums and excess soil water (Navas-Cortes *et al*., 2000; Maya, 2002). In this study the temperature ranged between 20 and 25°C, while the amount of initial inoculums was the same for both races of yellow and wilting. Thus, it is assumed that the damage in the plants could be due to the susceptibility of these lines to races 0 and 5 of FOC of this geographic region.

Sharma *et al*. (2005) investigated the genetic resistance of WR-315 against the races 1A, 2, 3, 4 and 5 of FOC and suggested that resistance is monogenic. Subsequently, molecular marker studies indicated the presence of quantitative loci affecting resistance to chickpea wilt (Gowda *et al*., 2009). Shinde *et al*. (2010) obtained recombinant inbred lines based on the resistant genotype WR-315, susceptible to early wilt JG-62 and susceptible to late wilt BG-256. The development of wilt in recombinant lines confirmed the participation of various genes and, secondarily, genes for resistance to this disease. Also, they concluded that both the resistance and wilt is polygenic and that may have genes with secondary effects which modify the response to the disease.

The behavior of R0 and R5 of FOC in chickpea lines was not dissimilar to what was expected, due to the fact that R0 also caused wilt in some lines specifically JG-62 (L6) and yellow in others. Similar results were observed by Tekeoglu *et al*. (2000) and Kaiser *et al*. (1994) with R0; causing wilting instead of leaf yellowing, similar that induced by R5 in *C. reticulatum* (PI 489777) and in susceptible differential lines (RILs). *Cicer reticulatum* Ladizinsky (chickpea pea parent) introduced plant death within 30 days of inoculation with R0, whereas ICCC-4958 was resistant.

In Mexico, as part of the strategy a greater number of genotypes should be generated based on high biotechnology such as molecular markers for genes involved in the disease resistance and production of glucanase and chitanase enzymes, to reduce significantly their time to obtain and increase in resistance. For example, in Pakistan where FOC wilt is also a devastating disease of chickpea, Ahmad *et al*. (2010) evaluated 321 genotypes and 82 of them showed resistance both in the seedling stage and reproduction. Also, to evaluate different cultural practices, e.g., in a study conducted with late sowing in the Valle del Mayo, Sonora with four varieties and four experimental lines, the variety Progreso-95 had low incidence of disease and high yield but with lower grain size (Padilla *et al*., 2008), also to consider the different races of FOC and abiotic conditions where it grows (Arvayo-Ortiz *et al*., 2011). These criteria will contribute to face the disease with a high possibility of success.

CONCLUSION

The new lines of chickpea Hoga-012, Hoga-490-2 and Hoga-508, as well as the commercial cultivars Blanco Sinaloa-92 and Costa-2004, did not show resistance to races 0 and 5 of *Fusarium oxysporum* f. sp. *ciceris*, isolated in chickpea fields of Sonora, thus it should be continued in the search for resistant genotypes through breeding programs to assist in controlling the disease.

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