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Development of chickpea near-isogenic lines for fusarium wilt

P. Castro · F. Pistón · E. Madrid · T. Millán · J. Gil · J. Rubio

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Abstract Four pairs of near-isogenic lines (NILs) of chickpea with resistance/susceptibility to *Fusarium oxysporum* f. sp. *ciceris* (*Foc*) have been developed in this study. These lines were produced by searching in advanced recombinant inbred lines (RILs) that are segregating for *Foc* race 5 based on a phenotypic screening. The sequence tagged microsatellite (STMS) marker TA59, closely linked to wilt resistance genes on linkage group 2 (LG2) of the chickpea map, was used to assist the selection of resistant or susceptible genotypes. The NILs were also characterized for disease reaction to *Foc* races 1A, 2, 3 and 4. Resistance, susceptibility and slow wilting reactions were found in these NILs. Our results suggest that more than one gene controls the resistance to race 5. Combination of the major gene *foc-5* linked to TA59 with other gene/s appears to be required to complete resistance, and the absence of these unknown genes leads to slow wilting reactions. The independent differential responses to races 2 and 3 observed in three NILs could be explained as recombination events. This result suggests that *foc-2* and *foc-3* are delimiting points at opposite ends of a genomic region that includes the remaining *foc* genes and the TA59 marker. This set of NILs has great potential for studying the genetics and mechanisms of wilt resistance. In addition, the NIL RIP8-94-11 can be used as differential line for *Foc* race 3; it showed a clear resistance reaction to race 3 and susceptibility to the other *Foc* races.

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P. Castro (✉) · J. Rubio
Área de Mejora y Biotecnología, IFAPA,
Centro 'Alameda del Obispo', Apdo. 3092,
14080 Córdoba, Spain
e-mail: patriciar.castro@juntadeandalucia.es

F. Pistón · E. Madrid · T. Millán · J. Gil
Dpto de Genética, Universidad de Córdoba,
Campus de Rabanales Edificio C5 2a planta,
14071 Córdoba, Spain

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *ciceris* (Padwick) Matuto & K. Sato, is the major soil-borne fungus affecting chickpea (*Cicer arietinum,* L.), the third most important cultivated grain legume in the world after soybean and beans (FAOSTAT 2009). The disease has been reported from almost all of the world's chickpea growing areas (Nene et al. 1989; Halila and Strange 1996; Sharma and Muehlbauer 2007). Annual yield losses from this disease have been estimated to range from 10 to 15% (Jalali and Chand 1992), but fusarium wilt epidemics can be devastating to individual crops and cause 100% loss under favorable conditions (Halila and Strange 1996; Navas-Cortés et al. 2000). Persistence of the pathogen in soil and its capacity to survive there for years, even in the absence of host plants (Haware et al. 1996), renders its control difficult. The most economic, effective and eco-friendly method of controlling chickpea wilt is by use of resistant cultivars, the effectiveness of which is limited by the existence of different races of pathogens. Moreover, evaluation of a large number of germplasm accessions, varieties and breeding lines for resistance to specific races of the pathogen is tedious, laborious, expensive, time consuming and is affected by inoculum load and environmental conditions (Jiménez-Gasco et al. 2001; Landa et al. 2001).

To date, eight pathogenic races of *F. oxysporum* f. sp. *ciceris* (*Foc*) (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6), identified

by their effects on a set of chickpea cultivars, have been described (Jimenez-Diaz et al. 1993; Sharma et al. 2005; Sharma and Muehlbauer 2007). Races 1A, 2, 3 and 4 have only been reported in India (Haware and Nene 1982), whereas races 0, 1B/C, 5 and 6 are found mainly in the Mediterranean region and the USA (Sharma and Muehlbauer 2007). In addition to the pathogenic variability of the fungus, two distinct types, referred to as yellowing and wilting syndromes, have been distinguished based on the symptomatology of infected plants (Trapero-Casas and Jimenez-Diaz 1985). The yellowing syndrome induces progressive foliar yellowing with vascular discoloration, while the wilting type induces severe and fast chlorosis, flaccidity and vascular discoloration. Races 1A, 2, 3, 4, 5 and 6 induce the wilting syndrome that causes more economically important losses, while races 0 and 1B/C induce yellowing syndrome (Haware and Nene 1982; Jimenez-Diaz et al. 1993; Kelly et al. 1994). Although yellowing and wilting syndromes have been considered as race specific, evidence is emerging that both syndromes can be caused by a single pathogen race, depending on the genotype of the host (Sharma and Muehlbauer 2007).

In addition, it should be noted that some susceptible chickpea lines exhibit a phenomenon known as late wilting, which is characterized by an extended latent period (the time between inoculation and first appearance of visible wilt symptoms) followed by a normal rate of disease development (Upadhyaya et al. 1983a). Late wilting has been described in response to both races 1 (Upadhyaya et al. 1983a, b; Singh et al. 1987a, b) and 2 (Gumber et al. 1995). Sharma et al. (2005) have reported another phenomenon called slow wilting, where the first signs of disease appear at about the same time as in other susceptible lines, but disease incidence increases slowly over time. The disease usually progresses to reach a final intermediate rate of incidence. The occurrence of slow wilting has been reported in response to races 2 and 3 (Sharma et al. 2005; Sharma and Muehlbauer 2007), race 5 (Cobos et al. 2009) and more recently to race 0 (Halila et al. 2009b).

Early studies on the genetics of resistance to *Foc* showed that resistance to race 1 was governed by three independent genes (*h1*, *h2*, *H3*). Late wilting is controlled by any one of these three genes, and complete resistance occurs when a combination of any of the two late wilting genes are present (*h1h2*, *h1H3*, *h2H3*) (Upadhyaya et al. 1983b; Singh et al. 1987a, b; Sharma and Muehlbauer 2007). Moreover, a different genetic system also based on three independent genes was found to confer resistance to race 2 (Gumber et al. 1995; Kumar 1998). In this case, a combination of two of these in the recessive form confers resistance, whereas when only one of these genes is recessive late wilting occurs. Two genes were reported to control resistance to race 4 (Tullu et al. 1999) and to race 0 (Rubio et al. 2003), whereas only one gene was reported for races 3 and 5 (Tekeoglu et al. 2000; Sharma et al. 2004). Until now, the genetics of resistance to races 1B/C and 6 has remained unknown. Similarly, the genetics of the slow wilting reaction has not been studied but it has been suggested that it is controlled by minor genes (Sharma and Muehlbauer 2007).

In previous studies, resistance genes to races 0, 1, 2, 3, 4 and 5 (*foc-0, foc-1*, *foc-2*, *foc-3*, *foc-4* and *foc-5*) have been found to form a cluster located on linkage group 2 (LG2) of the chickpea map (Ratnaparkhe et al. 1998a, b; Tullu et al. 1998; Tekeoglu et al. 2000; Winter et al. 2000; Sharma et al. 2004, 2005; Sharma and Muehlbauer 2007; Cobos et al. 2009; Gowda et al. 2009; Halila et al. 2009a, b). This linkage group is considered to be a hotspot for fusarium wilt resistance genes (Millán et al. 2006; Sharma and Muehlbauer 2007). However, a higher density of markers in the area of these genes is still necessary to detect polymorphisms for marker-assisted selection (MAS) in different genetic backgrounds and to perform fine mapping that could contribute to the understanding of the molecular aspects of resistance.

Near-isogenic lines (NILs) have the advantage that only a small target region of the genome is segregating; consequently, the genetic background noise can be eliminated. They have been widely used to produce fine maps of genomic regions related to genes or QTLs (quantitative trait loci) for agronomic traits. For instance, they have been used in maize (Koester et al. 1993; Graham et al. 1997), tomato (Brouwer and St. Clair 2004), soybean (Muehlbauer et al. 1991), rice (Yu et al. 1991) and lettuce (Paran et al. 1991). Thus, NILs could be ideal for localizing the exact position in LG2 of individual wilt resistance genes in chickpea and developing expression studies to identify candidate genes that control the resistance reaction.

NILs have been traditionally generated by consecutive backcrossing, followed by self pollination. But this method is laborious and time consuming, even if the process is shortened using marker-assisted selection (Yamamoto et al. 1998, 2000; Li et al. 2004; Fan et al. 2006). Alternative methods based on searching directly for NILs in advanced recombinant inbred lines (RILs) have been reported. In these methods, the selection of RILs that are heterozygous in a small genomic region is based on a phenotypic screening (Rajesh et al. 2002; Zhang et al. 2006) or on a molecular marker screening (Tuinstra et al. 1997).

In our chickpea breeding program, we have developed RIL populations segregating for *Foc* race 5 that may be useful for developing NILs. So far, in chickpea no NILs with resistance to *Foc* have been reported. Given this, the aim of this research was the development of chickpea NILs for *F. oxysporum* f. sp. *ciceris* by searching in segregating RIL populations and using sequence-tagged microsatellite (STMS) markers closely linked to wilt resistance genes.

Materials and methods

Plant materials

Three $F_{6:8}$ RIL populations (RIP), derived by single seed descent from inter- and intra-specific crosses that segregate for *F. oxysporum* f. sp. *ciceris* race 5, were used in this study for NIL development. These RIL populations will be referred to as RIP12, RIP8 and RIP5 and were derived from ICCL81001 × Cr5-9, ILC3279 × WR315 and WR315 × ILC3279, respectively. The parental line ICCL81001 is a kabuli type, selected from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), and is resistant to *F. oxysporum* f. sp. *ciceris* (Kumar and Haware 1983). Cr5-9 is a local selection from *Cicer reticulatum* ILWC36 maintained by the International Centre for Agricultural Research in the Dry Areas (ICARDA), and is susceptible to all races of fusarium wilt. ILC3279 is a kabuli line from the former Soviet Union maintained by ICARDA, and is susceptible to wilt. WR315 is a desi landrace from central India maintained by ICRISAT, which is resistant to all races of fusarium wilt. These RIPs have previously been evaluated for reaction to *Foc* race 5 under controlled conditions by our chickpea breeding group (Iruela et al. 2007; Cobos et al. 2009).

Six $F_{6:8}$ RILs that showed an intermediate level of response to *Foc* race 5 resistance were chosen from any of the three RIPs (RIP12-6, RIP12-10, RIP12-54, RIP12-68, RIP5-62 and RIP8-94; the last number refers to line number within a specific RIL population). Twenty seeds from each of these were sown in the field. Seedlings were genotyped for STMS TA59, which is closely linked to resistance genes on LG2 (Sharma and Muehlbauer 2007) to detect whether there was segregation within each RIL.

For DNA extraction, about 100 mg of young leaf tissue was excised, frozen immediately in liquid nitrogen and stored at −80°C. DNA was isolated using the DNAzol® method (Invitrogen), and TA59 marker analysis was performed according to Winter et al. (1999). Amplification was carried out in 10 µl reaction volumes containing: 30 ng of plant genomic DNA, buffer (50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 250 µM of dNTPs, 0.3 µM of TA59 and 0.25 units of Taq DNA polymerase (Promega). After denaturing the DNA for 2 min at 94°C, the reaction mixture was subjected to 35 cycles with the following temperature profile: 20 s at 94°C, 50 s at 55°C and 50 s at 60°C, followed by a final extension at 60°C for 5 min. PCR products were separated on 10% non-denaturing polyacrylamide gel and stained with ethidium bromide.

Evaluation of resistance to *Foc*

Four plants, two resistant and two susceptible, according to TA59 genotype, were selected from each one of the segregating RILs. Progenies of those plants were evaluated for wilt reaction to race 5 under controlled conditions to confirm their expected reaction. Parental lines (ICCL81001, Cr5-9, ILC3279 and WR315) were included in the evaluation as susceptible and resistant controls. Plant material was sown in trays (41 × 56 × 12 cm) filled with perlite, five lines per tray and ten seeds per line. Colonized filter paper cultures of *Foc* race 5 were cultured in PDB (potato dextrose broth, 24 g l^{-1}) at 25°C at 100 rpm under continuous fluorescent light for 1 week to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheesecloth to remove the mycelium. The spore suspension was then pelleted by centrifugation at low speed (3,000 rpm) for 3 min. After that, the supernatant was discarded and the concentration of spores was adjusted to 1×10^6 spores ml^{-1}. Plants were inoculated at the three to four node stage following the method described by Bhatti (1990) and the inoculated plants were grown under a temperature regime of 25 and 22°C (12 h:12 h) and 12 h photoperiod under fluorescent light. The plants were watered daily and supplied with nutrient solution once a week after inoculation. Disease incidence, scored as percentage of dead plants, was recorded weekly from weeks 2 to 5 after inoculation. The disease incidence data were converted to categorical data in the following way: 0–10% wilting = resistance, 11–89% wilting = intermediate, >90% wilting = susceptible (Sharma et al. 2005).

Pairs of NILs showing a differential reaction to *Foc* race 5 were also evaluated for *Foc* races 1A, 2, 3 and 4 following the procedure described above for *Foc-5*. Race-specific differential lines (CRIL1-17, CRIL1-53, CRIL1-94, 'Sanford') were employed in each assay as controls (Sharma et al. 2005). *Foc* isolates (races 1A, 2, 3, 4 and 5) were kindly provided by Dr. Chen (Washington State University, Pullman, USA).

In order to confirm the results, the evaluation for *Foc* races 1A, 2, 3, 4 and 5 was repeated as described above with the selected pairs of NILs. In this second experiment, plants were grown in plastic pots with ten seeds per NIL.

Results

Alleles associated with resistance and susceptibility in STMS TA59 were denoted as *a* and *b*, respectively. One out of the six RILs initially selected for NIL development was eliminated (RIP12-10) because there was no segregation for TA59 in the progeny suggesting that RIP12-10 could be non-segregating for wilt race 5. In the remaining five RILs analyzed, plants carrying homozygous alleles associated with resistance or susceptibility were found, as well as heterozygous plants (Table 1).

Table 1 Frequency distribution of genotypes (*aa*, *ab*, *bb*) according to TA59 locus, linked to fusarium wilt resistance genes, in recombinant inbred lines (RILs) selected by their intermediate disease reaction to wilt race 5 (*Foc*5) in three RIL populations (RIP)

Cross	RIL	Genotype for TA59		
		aa	*ab*	*bb*
ICCL81001 × Cr5-9 (RIP12)	RIP12-6	9	3	7
	RIP12-54	4	4	11
	RIP12-68	10	4	2
ILC3279 × WR315 (RIP8)	RIP8-94	3	2	13
WR315 × ILC3279 (RIP5)	RIP5-62	4	1	15

Evaluation of resistance to *Foc* race 5

According to the data obtained with the TA59 marker, two plants genotyped as *aa* and another two as *bb* were selected from each RIL. Their progenies were evaluated with *Foc* race 5 to contrast genotypic and phenotypic results. Disease reaction to *Foc* race 5 of the 20 chickpea progenies selected and their genotypic results for TA59 are shown in Table 2. Characteristic wilt symptoms appeared 13–15 days after inoculation, the moment at which disease incidence scoring was initiated. Three weeks after inoculation, susceptible parental lines (Cr5-9; ILC3279) wilted and died, and resistant parental lines (ICCL81001; WR315) showed no symptoms of wilt. Among the 20 progenies evaluated with *Foc* race 5, 3 of them (RIP12-54-3, RIP8-94-5 and RIP8-94-20; last number referring to the selected plant number within line) were resistant, 12 were susceptible and 5 displayed an intermediate reaction. In the five progenies showing intermediate reaction (RIP12-6-12, RIP12-68-3, RIP12-68-4, RIP5-62-10 and RIP5-62-20), the first signs of disease appeared at the same time as in the susceptible parental lines. However, disease incidence increased slowly over time, with final levels of mortality ranging from 75 to 88% 5 weeks after inoculation. Accordingly, the response of the five progenies that showed an intermediate reaction was considered to be slow wilting. In contrast, susceptible progenies completely wilted (100%) 15–20 days after inoculation.

With regard to the relationship between TA59 marker and phenotypic disease reaction, it was confirmed that the ten genotypes selected as *bb* for TA59 were susceptible to *Foc* race 5 (Table 2). However, among the ten selected plants that were homozygous for the allele associated with resistance (*aa*), three (RIP12-54-3, RIP8-94-5 and RIP8-94-20) were evaluated through their progenies as resistant, five (RIP12-6-12, RIP12-68-3, RIP12-68-4, RIP5-62-10, RIP5-62-20) as slow wilting and two (RIP12-6-17 and RIP12-54-14) as susceptible (Table 2), suggesting that there may have been recombination events within the targeted region. These results with regard to slow wilting reveal that together with the major gene *foc-5*, other genes could be implicated in the resistance reaction. The absence of these other genes could lead to the slow wilting response.

In line with these results, we propose a set of four pairs of putative NILs differing with regard to *Foc* race 5 disease reaction. These sets comprise: RIP12-6-4 and RIP12-6-12 (susceptible and slow wilting, respectively); RIP12-54-13 and RIP12-54-3 (susceptible and resistant, respectively); RIP12-68-2 and RIP12-68-3 (susceptible and slow wilting, respectively), all of them derived from the interspecific cross, ICCL81001 × Cr5-9, and the pair, RIP8-94-11 and RIP8-94-5 (susceptible and resistant, respectively), from the intraspecific cross ILC3279 × WR315. It is noteworthy that the two NILs comprising each pair were identical to each other in terms of morphological traits. Putative NILs from line RIP5-62 were not selected due to the lack of seed availability. The set of putative NILs were evaluated for *Foc* race 5 in the second experiment (repeat) and all the lines showed the same reaction in both experiments confirming the phenotype of this set of NILs with regard to *Foc* race 5 reaction (Table 3).

Characterization of the NILs for *Foc* races 1A, 2, 3 and 4

The final disease reaction of the four pairs of chickpea NILs to *Foc* races 1A, 2, 3 and 4 are shown in Table 3. The onset of visual wilt symptoms in these NILs to the four *Foc* races varied between 2 and 3 weeks after inoculation. The susceptible control (Cr5-9) manifested 100% mortality 2 weeks after inoculation. Differential line CRIL1-53 took 5 weeks after inoculation with race 1A to die completely in the first experiment, while, in the second one, it manifested an intermediate reaction 5 weeks after inoculation. CRIL1-53 has been described as resistant to race 2 (Sharma et al. 2005), but in our experiments it showed an intermediate reaction. The remaining lines that were employed as controls for each race showed the expected reactions. Generally, the same disease reaction was obtained in the two experiments for the four pairs of selected NILs. Only RIP12-6-12 exhibited different reactions between experiments for races 2 and 4. It displayed a slow wilting reaction to race 2 in the first experiment, but was susceptible in the second one. With regard to race 4, it was resistant and slow wilting in the first and second experiment, respectively (Table 3). Differential responses to the five races tested were found in three of the four pairs of NILs (Table 3). Only the NIL pair, RIP12-54-3 and RIP12-54-13 (resistant and susceptible, respectively), showed the same reaction to all *Foc* races used in this study.

When the phenotypic reactions to *Foc* isolates of each NIL were compared with the genotypic results (TA59), the

Table 2 Reaction to *Fusarium oxysporum* f. sp. *ciceris* race 5 (*Foc5*) of progenies derived from plants selected by their genotypic results for TA59 marker within recombinant inbred lines (RILs) with intermediate disease reaction to *Foc5*

Cross	RIL	Plant	Reaction to *Foc5*	TA59 genotype
81001 × Cr5-9 (RIP12)	6	4	S (100%)	*bb*
		6	S (100%)	*bb*
		12	I (75%)	*aa*
		17	S (100%)	*aa*
	54	2	S (100%)	*bb*
		13	S (100%)	*bb*
		3	R (0%)	*aa*
		14	S (100%)	*aa*
	68	2	S (100%)	*bb*
		17	S (100%)	*bb*
		3	I (80%)	*aa*
		4	I (80%)	*aa*
WR315 × ILC3279 (RIP5)	62	12	S (100%)	*bb*
		15	S (100%)	*bb*
		10	I (88%)	*aa*
		20	I (63%)	*aa*
ILC3279 × WR315 (RIP8)	94	6	S (100%)	*bb*
		11	S (100%)	*bb*
		5	R (0%)	*aa*
		20	R (0%)	*aa*
Parental lines				
ICC81001			R (0%)	*aa*
Cr5-9			S (100%)	*bb*
ILC3279			S (100%)	*bb*
WR315			R (0%)	*aa*

Disease incidence (%) in parentheses

S susceptible, *R* resistant, *I* intermediate, *a* allele associated with the resistance, *b* allele associated with the susceptibility

lines that were susceptible to all five races (RIP12-6-4, RIP12-54-13, RIP12-68-2) were homozygotic for the allele *b*, associated with the susceptibility. RIP8-94-11, which was susceptible to all races except race 3, also carried the allele associated with susceptibility, suggesting a recombination event between TA59 marker and race 3 gene. The remaining lines homozygotic for allele *a*, associated with the resistance, in general showed resistance or slow wilting except RIP12-68-3, which was susceptible to race 2 (Table 3).

Discussion

Near-isogenic lines (NILs) differ only in a small target region of the genome and are identical with regard to the remainder of the genome. These lines have been widely used to produce fine maps of genomic regions related to genes or QTL (Muehlbauer et al. 1991; Graham et al. 1997; Brouwer and St. Clair 2004) and to conduct studies on the effects, expression and isolation of genes (Zhou et al. 2005). They have also been employed for studying the genetics of pathotype-specific resistance in crop plants (Mackill and Bonman 1992). To date, the only chickpea NILs reported were those developed by Rubio et al. (1998) for the double-podding trait. In the current study, we have developed a set of four pairs of NILs, which differ in their *Fusarium oxysporum* f. sp. *ciceris* reactions, by searching in advanced RILs that are segregating for *Foc* race 5 based on a phenotypic screening. STMS marker TA59 was used to assist the selection of resistant or susceptible genotypes. This marker has been mapped in LG2, closely linked to wilt resistance genes (Winter et al. 2000; Iruela et al. 2007; Cobos et al. 2009; Halila et al. 2009a). In previous studies, resistance genes to races 0, 1, 2, 3, 4 and 5 of wilt (*foc-0*$_2$, *foc-1*, *foc-2*, *foc-3*, *foc-4* and *foc-5*) have been found to form a cluster located on this genomic region (Ratnaparkhe et al. 1998a, b; Tullu et al. 1998; Tekeoglu et al. 2000; Winter et al. 2000; Sharma et al. 2004, 2005; Sharma and Muehlbauer 2007; Cobos et al. 2009; Gowda et al. 2009; Halila et al. 2009a, b). Specifically, TA59 was tightly linked to genes *foc-1* to *foc-5* (Sharma and Muehlbauer 2007). Recently, it has been reported that this marker is also linked to the locus *Foc0*$_2$/*foc0*$_2$ (Halila et al. 2009a, b). Hence this marker allows us to tag genes for wilt resistance simultaneously, assisting NIL development for *Foc* resistance. The benefits of using this marker were that it facilitated the search for putative segregating lines and reduced

Table 3 Disease reaction of near-isogenic lines (NILs) for *Fusarium oxysporum* f. sp. *ciceris* (*Foc*) races 1A, 2, 3, 4 and 5 and their genotypic results for TA59 marker

NILs	*Fusarium oxysporum* f. sp. *ciceris* reaction					TA59 genotype
	Race 1A	Race 2	Race 3	Race 4	Race 5	
RIP12-6-4	S	S	S	S	S	*bb*
RIP12-6-12	SW	SW/S[a]	R	R/SW[a]	SW	*aa*
RIP12-54-3	R	R	R	R	R	*aa*
RIP12-54-13	S	S	S	S	S	*bb*
RIP12-68-2	S	S	S	S	S	*bb*
RIP12-68-3	SW	S	R	SW	SW	*aa*
RIP8-94-5	R	R	R	R	R	*aa*
RIP8-94-11	S	S	R	S	S	*bb*
Differential lines						
Cr5-9	S	S	S	S	S	
CRIL1-17	R	R	R	S	R	
CRIL1-53	S/I[a]	I	R	R	R	
CRIL1-94	R	S	R	I	I	
'Sanford'	R	S	S	S	S	

For *Foc* races 1A, 2, 3, 4 and 5: *R* resistant, *S* susceptible, *I* intermediate, *SW* slow wilting

a allele associated with the resistance, *b* allele associated with the susceptibility

[a] Disease reaction differing in experiments 1 and 2

the sample size for disease testing. In our case, only four plants per segregating RIL were sufficient to obtain NILs for both resistant and susceptible reactions.

Resistance, susceptibility and slow wilting reactions have been found in this study. The inheritance of resistance to race 5 has been analyzed in several studies, which found it to be monogenic (Tekeoglu et al. 2000; Sharma et al. 2005; Iruela et al. 2007). These studies used ICC4958 (Tekeoglu et al. 2000) and WR315 (Sharma et al. 2005; Iruela et al. 2007) as the source of resistance. Cobos et al. (2009) suggested that more than one gene controls resistance to *Foc* race 5 in a population derived from ICCL81001 × Cr5-9; this was the first report of a slow wilting response to this race. The genetics of slow wilting resistance in chickpea has not yet been determined, but it has been proposed that the process is related to minor genes (Sharma et al. 2005; Sharma and Muehlbauer 2007). According to our results, two groups of NILs (RIP12-6 and RIP12-68) carrying ICCL81001 as the source of resistance and one (RIP5-62) carrying WR315 as the source of resistance showed a slow wilting response (Table 2). Therefore, our results are consistent with Cobos et al. (2009), suggesting that more than one gene controls the resistance to race 5. Moreover, the lines that were either resistant or slow wilting carried the TA59 allele linked to the major gene *foc-5*, suggesting that the major gene for resistance could be present in these lines. These results also imply that a combination of the major gene *foc-5* with other gene/s could be required to complete resistance; nevertheless, the absence of these unknown genes leads to slow wilting reactions. In this study, similar reactions were found to races 1A, 2, 3 and 4 in the four pairs of selected NILs. With the elucidation of slow wilting resistance in chickpea, according to Sharma and Muehlbauer (2007), it should be considered that wilt management involves two types of host resistance, vertical resistance and slow wilting resistance. The four pairs of NILs developed in this study could be useful test subjects with which to study these two types of host resistance.

Homozygous NILs with TA59 alleles derived from susceptible parents showed susceptibility reaction, except NIL RIP8-94-11 which was resistant to race 3. Similarly, homozygous NILs with TA59 alleles from resistant parents displayed resistance/slow wilting reaction, except NIL RIP12-68-3 which was susceptible to race 2. Consequently, it should be considered that recombination events took place during the development of the NILs in comparison with original parents.

Previous studies indicated two clusters of fusarium wilt resistance genes on LG2: one that contained *foc-1* and *foc-4* and the other comprising *foc-3* and *foc-5* (Winter et al. 2000; Benko-Iseppon et al. 2003; Millán et al. 2006). Sharma and Muehlbauer (2005) also subdivided the resistance gene cluster into two sub-clusters with *foc-4*, *foc-2* and *foc-3* forming one and *foc-5* and *foc-1* comprising another sub-cluster. However, Gowda et al. (2009) reported that the closest genes were *foc-1* and *foc-2* (6.8 cM), while *foc-1* was flanked on the other side by *foc-3* at a distance of 22 cM. In our study, differential response to races 2 and 3 was found in three pairs of NILs. This could be explained by recombination events of *foc-2* and *foc-3* with respect to other genes and the TA59 marker (Table 3). If these two genes were forming a cluster with respect to the others, the recombination should affect them simultaneously. However, the performance of *foc-2* and *foc-3* is independent. This finding suggests that these genes are delimiting points at opposite ends of a genomic region that includes the remaining *foc* genes and TA59 marker.

The set of NILs developed in the current study is a valuable genetic stock, which has great potential for the study of the genetics and mechanisms of wilt resistance. It could be used for screening of molecular markers tightly linked to wilt resistance genes. An advantage of these lines is that the phenotypic variation observed between pairs of NILs can be assigned directly to the restricted target region of genome that differs between them. Furthermore, these NILs are also ideal for use in gene expression studies. In particular, this set may provide a starting point for unraveling the functional genes underlying wilt resistance loci and possibly be useful for positional cloning.

Moreover, the fact that RIP8-94-5 and RIP12-54-3 are resistant to the five *Foc* races is very useful for future resistance breeding programs. These lines have multiple race resistance to *Fusarium oxysporum* f.sp. *ciceris*, and can therefore enhance the durability of wilt resistance. In addition, RIP8-94-11 could be used as a differential line for identification of race 3, because it shows a clear-cut resistance reaction to race 3 and susceptibility to the other four races.

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