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Using SCAR Molecular Marker to Detect Resistance Genes to *Fusarium oxysporum* f. sp. Ciceris in Chickpea Cultivars and Lines

Somayeh Farahani*, Bahar Morid**, Mojdeh Maleki* and Siamak Saberi***
**Department of Plant Protection, College of Agriculture,*
Varamin-Pishva Branch, Islamic Azad University, Varamin, IRAN.
***Department of Plant Protection, College of Agriculture,*
Takestan Branch, Islamic Azad University, Takestan, IRAN.
****Department of Agronomy, College of Agriculture,*
Varamin-Pishva Branch, Islamic Azad University, Varamin, IRAN.

(Corresponding author: Somayeh Farahani)
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ABSTRACT. The occurrence of fusarium wilt disease is reported from 32 countries around the world. It is estimated that this disease results in 10-90% crop yield loss annually. It is difficult to control this disease because of the ability of the pathogen to survive in soil for several years, even without the host. The most effective method for controlling fusarium wilt is the cultivation of resistant chickpea cultivars. In this experiment, SCAR molecular marker was used to detect chickpea genotypes resistant to the disease. The DNA of 42 chickpea genotypes was extracted by CTAB method. After that, polymerase chain reaction was conducted by CS-27 and OPM-20 molecular markers. Results showed that 41 genotypes out of the 42 tested genotypes were sensitive to all five races of the pathogen. The only resistant genotype was Flip 06-152c which was resistant to the five races. Finally, the pathogenesis test confirms all results obtained in the genetic studies.

Keywords: chickpea fusarium wilt disease, SCAR.

INTRODUCTION

Chickpea fusarium wilt is induced by *Fusarium oxysporum* f. sp. ciceris (Jalali and Chand, 1992; Dubey et al., 2007) and it was reported for the first time from India (Jimenez-Diaz et al., 1992; Singh, 2003). Nowadays, fusarium wilt is reported to infect chickpea fields all around the world including India, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey, the United States and the countries of USSR (Westerland et al., 1974; Trampero-Casas and Jemenez-Diaz, 1985). In Iran, *F. lateritium* f. *ciceris* was the first pathogen which was reported to induce fusarium wilt (Manouchehri and Mesri Alamdari, 1966).

Studies indicate that 19% of the chickpea fields in North West of Iran were infested to the disease in 1999 and the severity of this disease was 5-60% (Akem, 1988). The severity of this disease in all over the world is estimated to be 10-90% (Jimenez-Diaz et al., 1989; Singh and Reddy 1991). The epidemics of fusarium wilt significantly damages crop yield and under suitable conditions for pathogen development, crop yield loss may reach to 100% (Halila and Strange, 1996; Landa *et al.*, 2004). The fungus causing fusarium wilt disease is a soil-borne pathogen which lives in seeds and dead plant materials in the form of chlamydospore. It can survive in soil for more than five years (Singh, 2003).

Nowadays, application of chemical pesticides is limited because of their environmental pollutions and health risks. So, using genetic resistance and cultivating resistant genotypes is the most suitable and possible method for control of fusarium wilt disease. Selecting the resistant genotypes by phenotypical methods is a complicated and time consuming method. So, using DNA based molecular markers is a major tool for the selection of the resistant cultivars; facilitating the process (Lindhout, 2002; Tanksley *et al.*, 1992).

Eight races of the pathogen of fusarium wilt disease are diagnosed (0, 1A, 1B/C, 2, 3, 4, 5 and 6) by pathogenesis tests on 10 chickpea cultivars (Haware and Nene, 1982; Jimenez-Diaz *et al.*, 1994). 1A, 2, 3, 4, 5 and 6 races results in the wilting; 0 and 1B/C races induce chlorosis in plants (Jimenez-Diaz et al., 1991). Moreover, two separate clusters are diagnosed for fusarium wilt disease. The first one is a linkage group (LG2 chromosome F or G) which is a gene cluster including five gens (foc-1, foc-2, foc-3, foc-4 and foc-5) and gens that are effective on resistance to pathotype of fusarium wilt.

Another one is LG3 chromosome C or D which includes gens that are effective on resistance to chlorosis pathotype (race zero). Gens that are effective on resistance to 6 and 1B/C races are not yet located (Sharma and Muehlbauer, 2007).

The objective of this experiment was to evaluate the resistance or sensitivity of 42 chickpea genotypes against the pathogen of fusarium wilt disease by using SCAR molecular marker.

MATERIALS AND METHODS

A. Chickpea seedling production for DNA extraction

In this experiment, 42 cultivars, lines and masses of chickpea were used. Their names and places they were collected from are listed in Table 1. From each genotype, five seeds were cultivated and their leaves were used for DNA extraction at five leaves stage.

Table 1: The studied genotypes and their origin.

No.	Genotype	Origin
1	Azad Cultivar	Dryland Agricultural Research Institute
2	Arman Cultivar	Dryland Agricultural Research Institute
3	KC 215474 (Razavi Khorasan – Neyshabur)	Seed and Plant Improvement Institute
4	Hashem Cultivar	Dryland Agricultural Research Institute
5	KC 216193 (Tehran - Karaj)	Seed and Plant Improvement Institute
6	KC 215950 (Razavi Khorasan – Quchan)	Seed and Plant Improvement Institute
7	KC 215920 (Razavi Khorasan – Quchan)	Seed and Plant Improvement Institute
8	KC 215079 (Mazandaran - Sari)	Seed and Plant Improvement Institute
9	KC 215887 (East Azerbaijan - Ahar)	Seed and Plant Improvement Institute
10	Bioniz Cultivar	Dryland Agricultural Research Institute
11	KC 216084 (East Azerbaijan - Mugan)	Seed and Plant Improvement Institute
12	Flip 97-102c	Dryland Agricultural Research Institute
13	KC 216313 (East Azerbaijan - Ardabil)	Seed and Plant Improvement Institute
14	ILC 482 Line	Dryland Agricultural Research Institute
15	KC 215377 (Razavi Khorasan – Neyshabur)	Seed and Plant Improvement Institute
16	Ilam Local Cultivar (Jam)	Dryland Agricultural Research Institute
17	KC 216194 (Tehran – Karaj)	Seed and Plant Improvement Institute
18	KC 215004 (Markazi - Saveh)	Seed and Plant Improvement Institute
19	KC 216223 (Bakhtaran - Bakhtaran)	Seed and Plant Improvement Institute
20	Flip 97-116c	Dryland Agricultural Research Institute
21	KC 215437 (Razavi Khorasan – Quchan)	Seed and Plant Improvement Institute
22	KC 215909 (East Azerbaijan - Ahar)	Seed and Plant Improvement Institute
23	KC 215002 (Markazi - Saveh)	Seed and Plant Improvement Institute
24	KC 216364 (East Azerbaijan - Ardabil)	Seed and Plant Improvement Institute
25	KC 216195 (Isfahan - Isfahan)	Seed and Plant Improvement Institute
26	KC 216228 (East Azerbaijan - Ardabil)	Seed and Plant Improvement Institute
27	Khoram-Abad Local Cultivar	Lorestan Agriculture Research Institute
28	KC 215543 (Fars - Shiraz)	Seed and Plant Improvement Institute
29	Flip 97-109c	Dryland Agricultural Research Institute
30	KC 215858 (Razavi Khorasan – Quchan)	Seed and Plant Improvement Institute
31	Flip08-90c	Dryland Agricultural Research Institute
32	Flip05-77c	Dryland Agricultural Research Institute
33	Flip07-177c	Dryland Agricultural Research Institute

34	Flip06-152c	Dryland Agricultural Research Institute
35	Flip08-93c	Dryland Agricultural Research Institute
36	Flip07-123c	Dryland Agricultural Research Institute
37	Flip07-216c	Dryland Agricultural Research Institute
38	Flip03-28c	Dryland Agricultural Research Institute
39	Flip07-197c	Dryland Agricultural Research Institute
40	Flip08-81c	Dryland Agricultural Research Institute
41	Flip02-04c	Dryland Agricultural Research Institute
42	Flip05-183c	Dryland Agricultural Research Institute

DNA extraction: The CTAB method, with a little modification, was used for DNA extraction (Ausubel *et al.*, 1994). DNA was extracted from 0.05 mg of the fresh leaves of chickpea (Fig. 1).

PCR test: In order to detect the resistant chickpea cultivars to 1, 2, 3, 4 and 5 races of the fungus, a SCAR marker named CS-27 was used. The components of polymerase chain reaction are listed in Table 2. Temperature programming included an initial denaturation of 95°C for 5 min and after that 35 cycles with denaturation of 94°C for 20 s, connection in 65°C for 40 s and expansion in 72°C for 40 s which was finished with a final expansion in 72°C for 8 min. The amplified DNA fragments were electrophoresed on 2% Agarose gel. GelRed was used to stain the gels. At the end, the samples were subjected to UV and photographed (Fig. 2).

On the other hand, in order to detect the resistant chickpea cultivars to race 5 of the fungus, OMP-20 marker was used. The components of polymerase chain reaction are listed in Table 3. Temperature programming included an initial denaturation of 94°C for 5 min and after that 35 cycles with denaturation of 94°C for 50 s, the connection was in 30°C for 50 s and expansion in 72°C for 105 s which was finished with a final expansion in 72°C for 10 min. The amplified DNA fragments were electrophoresed on 2% Agarose gel and the gels were stained using GelRed. At the end, samples were subjected to UV and photographed (Fig. 3).

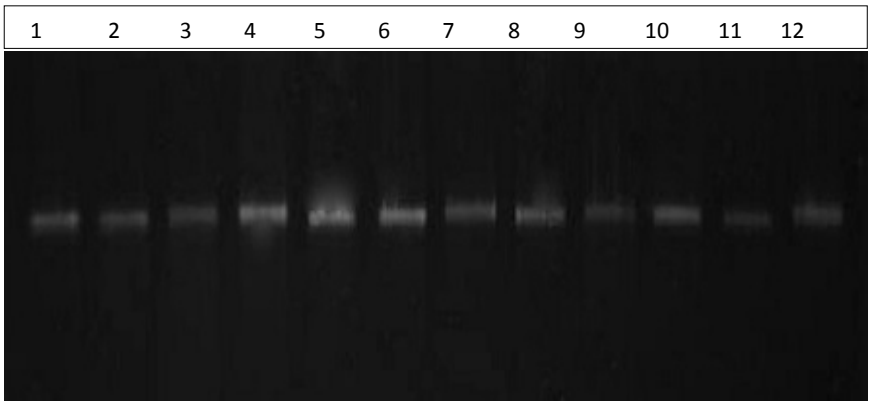

Fig. 1. The quality of the obtained DNA by electrophoresis on 0.8% concentration Agarose gel.

Table 2: Components of polymerase chain reaction using CS-27 marker.

Components of the reaction	Amount used (µl)
PCR buffer (10X)	2.5 µl
$MgCl_2$ (50 mm)	0.9 µl
dNTP (10 mm)	0.5 µl
Primer F (10 pmol/ml)	1 µl
Primer R (10 pmol/ml)	1 µl
Taq DNA polymerase (5 unit/ml)	0.25 µl
DNA	2 µl
ddH_2O	16.85 µl

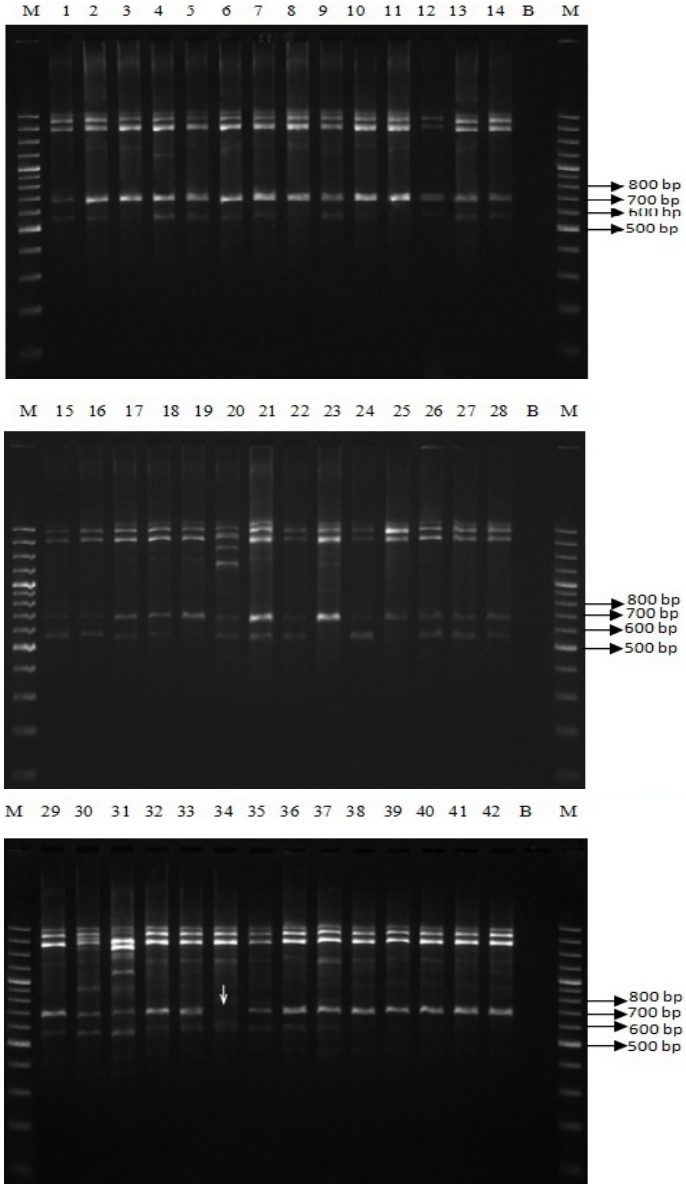

Fig. 2. Cultivars number according to the table of genotypes name (Table 1). M: 100 bp marker (#SM0323, Manufacturer: Fermentase). B: Blank

Table 3: Components of polymerase chain reaction using OMP-20 marker.

Components of the reaction	Amount used (µl)
PCR buffer (10X)	2.5 µl
$MgCl_2$ (50 mm)	0.75 µl
dNTP (10 mm)	0.5 µl
Primer F (10 pmol/ml)	1 µl
Primer R (10 pmol/ml)	1 µl
Taq DNA polymerase (5 unit/ml)	0.25 µl
DNA	2 µl
ddH_2O	17 µl

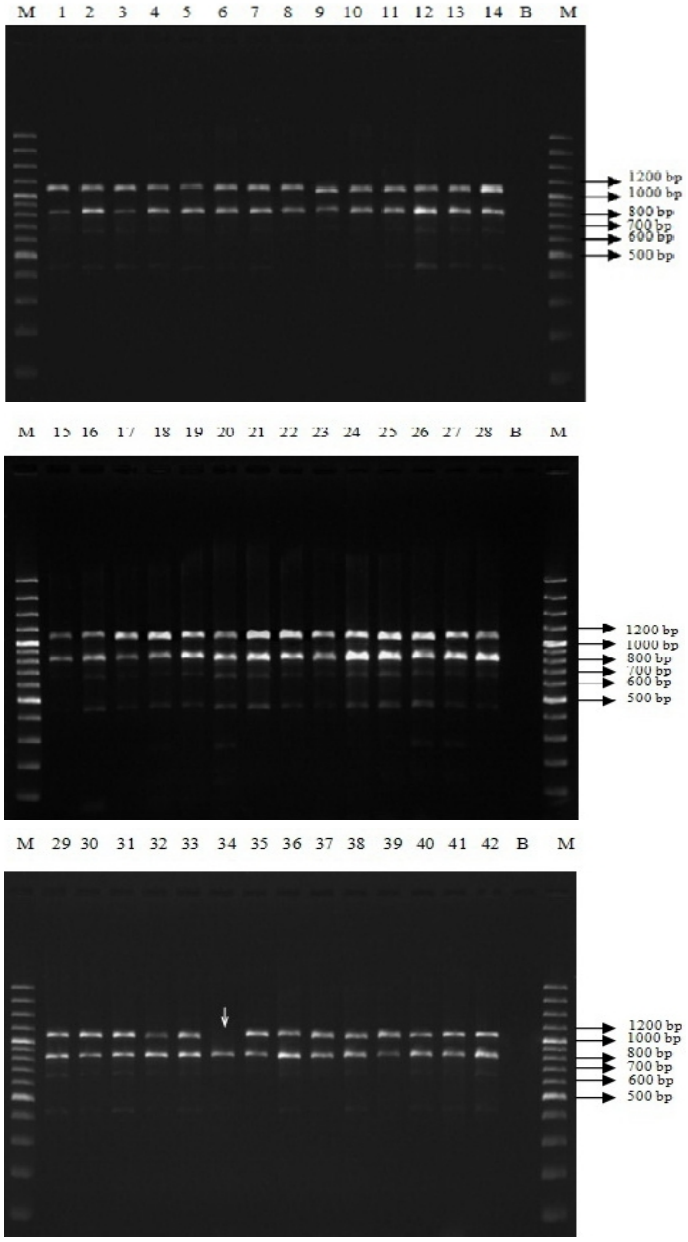

Fig. 3. Cultivars number according to the table of genotypes name (Table 1).
M: 100 bp marker (#SM0323, Manufacturer: Fermentase). B: Blank.

Pathogenesis test: The inoculum was obtained from 0, 1, 2 and 5 races which was previously isolated and detected using molecular primers (Beigi, 2013). Each race was cultured in a 250 ml flask containing 50 ml of PDB culture medium and was held for seven days in a shaker-incubator (125 rpm, 25°C, under 12 h of fluorescent light daily). The liquid culture was passed through two-layer sterile gauze. Then, the number of spores was counted with hemocytometer lam and the concentration of the suspension was adjusted on 4 × 106 cfu/g. A mixture of sand and corn flour (9:1 weight ratio) was produced and sterilized two times; each time 1 h in 121°C. After that, the suspension of spore was added to this mixture and was held for 15 days in 25°C under fluorescent light (Haware *et al.*, 1980; Trampero-Casas and Jimenez-Diaz, 1985).

B. Chickpea genotypes and their inoculation

Sixteen cultivars and lines of chickpea were studied in this experiment (Table 4). For germination, chickpea seeds were placed in trays containing the sterilized sand mixture. Sands were sterilized two times; each time 1 h in 121°C. The inoculum with the population of 4 × 106 cfu/g was added to the soil of 0.5 L plastic pots. Pots contained soil, sand and peat, which were sterilized two times; each time 1 h in 121°C (Kaiser *et al.*, 1994).

Ten seeds of each cultivar were located on PDA culture medium in order to prevent any infestation. Three normal and pre-germinated seeds were planted in each pot. Three replications were considered for each treatment. For each genotype, three pots were considered as the control (without inoculation).

The first irrigation was conducted using 100 ml water and in the following days it was conducted using 50 ml water every day. The pots were held in greenhouse with the normal light condition and 20-25°C temperature.

C. Evaluation of the symptoms

The development of the disease was monitored every five days from the day 10 to 50. As the result, plants were categorized into five types (Table 5).

At the end of the experiment, to verify the symptoms of pathogen, plants were harvested from the pots and the results were compared with Koch principles. Nelson identification key was used to identify the samples (Nelson *et al.* (1983).

Table 4: The genotypes used in pathogenesis test.

No.	Genotype	No.	Genotype	No.	Genotype	No.	Genotype
1	Azad Cultivar	5	KC 215887	9	KC 216194	13	KC 216228
2	Arman Cultivar	6	Bioniz Cultivar	10	KC 216223	14	Khoram-Abad Local Cultivar
3	Hashem Cultivar	7	KC 216084	11	KC 215002	15	KC 215858
4	KC 215079	8	Ilam Local Cultivar (Jam)	12	KC 216195	16	Flip06-152c

Table 5: The development of the disease.

Symptom	Rank	Diagnosis
No symptom	0	Resistant
Mild chlorosis	1	Semi-resistant
Moderate chlorosis	2	Semi-Sensitive
Chlorosis or severe wilting	3	Sensitive
Plant death	4	Highly-sensitive

RESULTS AND DISCUSSION

A. Results of PCR

The CS-27 marker produces a band with the weight of 700 bp in sensitive genotypes to races 1, 2, 3, 4 and 5 of the pathogen. In addition to this band, the CS-27 marker produces another index band weighting 565 bp in genotypes sensitive to race 3 (Sharma and Muehlbauer, 2007). Results indicated that 41 genotypes out of the 42 tested genotypes had 700 bp band which shows their sensitivity to races 1, 2, 3, 4 and 5. Moreover, the sensitive genotypes had the 565 bp band which proves their sensitivity to the race 3. According to the findings of Benko-Iseppon *et al.* (2003), chickpea genotypes which form a 1100 bp band with OP-M20 marker, are sensitive to the race 5. Results of our experiment also indicated that 41 genotypes out of the 42 tested genotypes had this band; representing their sensitivity to race 5. Among all genotypes, only Flip06-152c line had none of these bands and was resistant to all five races of the pathogen.

B. Results of pathogenesis test

Result of grading the 16 genotypes based on their resistance to the pathogen is given in Table 6. The genotypes varied from highly sensitive to resistant.

One of the most important and cost effective methods to control the damages of fusarium wilt is development of the resistant cultivars. Developing the resistant cultivars is a more suitable method with higher advantages compared with the chemical or other methods of pest management. The main advantages include the reduction of labor, costs and time and the protection of environmental health. The genetic resistance is induced by the presence of resistant genes in plant genome.

Table 6: The results of pathogenesis test.

No.	Genotype	Race 0		Race 2		Race 1/A	
1	Azad Cultivar	Highly-Sensitive	4	Highly-Sensitive	4	Highly-Sensitive	4
2	Arman Cultivar	Sensitive	3	Highly-Sensitive	4	Highly-Sensitive	4
3	Hashem Cultivar	Sensitive	3	Highly-Sensitive	4	Highly-Sensitive	4
4	KC215079	Highly-Sensitive	4	Sensitive	3	Sensitive	3
5	KC215887	Sensitive	3	Sensitive	3	Sensitive	3
6	Bioniz Cultivar	Sensitive	3	Sensitive	3	Highly-Sensitive	4
7	KC216084	Semi-resistant	1	Sensitive	3	Sensitive	3
8	Jam Cultivar	Sensitive	3	Sensitive	3	Sensitive	3
9	KC216194	Semi-Sensitive	2	Semi-Sensitive	2	Semi-Sensitive	2
10	KC216223	Semi-Sensitive	2	Semi-Sensitive	2	Semi-Sensitive	2
11	KC215002	Semi-resistant	1	Highly-Sensitive	4	Highly-Sensitive	4
12	KC216195	Semi-resistant	1	Semi-Sensitive	2	Highly-Sensitive	4
13	KC216228	Semi-resistant	1	Highly-Sensitive	4	Semi-Sensitive	2
14	Khoram-Abad Cultivar	Sensitive	3	Semi-resistant	1	Semi-resistant	1
15	KC215858	Semi-Sensitive	2	Highly-Sensitive	4	Highly-Sensitive	4
16	Flip06-152c	Resistant	0	Resistant	0	Resistant	0

So, identification of the resistant genes in chickpea cultivars is the most important step in developing commercial resistant cultivars. Different methods are introduced for evaluation of the genes which are responsible for chickpea resistance to fusarium wilt disease and development of the resistant cultivars. The classic methods require the evaluation of a large number of genotypes. This method is a phenotypical method which is difficult, expensive and is affected by the environmental factors (Landa *et al.*, 2004; Huttle *et al.*, 2002; Gumber *et al.*, 1995).

RAPD markers help to identify the resistant genes easily and quickly, and do not require to be informed of the genome sequence, so they under attention of researchers. In addition, DAN is easily amplified in this method and there is no need to use isotopes (Zhang *et al.*, 2005). This method has been successful in producing markers in different organisms and to create the genome maps. RAPD marker provides a large number of multi positional markers which can effectively differentiate samples (Singh *et al.*, 2011). RAPD markers are useful for marker-based genotype selection when they are strongly linked to a gene resistant to a disease or other trait and it can be transferred from a single dominant line to a large number of populations (Brown and Myers, 2001).

Results indicated that among the 42 tested genotypes, Flip 06-152c line was the only genotype which was resistant to 1, 2, 3, 4 and 5 races. The results of pathogenesis test (phenotypical studies) also confirmed the results of molecular markers (genotypic studies). So, cultivation of this line is recommended for areas infested to the fungus; the cultivation of sensitive cultivars, lines and masses must be prevented. These findings are in agreement with the findings of Haji-Allahverdipoor *et al.* (2011).

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