INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 1, p. 29-35, 2015

RESEARCH PAPER OPEN ACCESS

Detection of resistance genes to *Fusarium* wilt disease in chickpea cultivars and lines using molecular markers

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Key words: Chickpea, *Fusarium* wilt, Molecular markers, Race zero, RAPD.

http://dx.doi.org/10.12692/ijb/6.1.29-35 Article published on January 05, 2015

Abstract

Fusarium wilt is reported to infect crops in over 32 countries all around the world; reducing 10-90% of annual crop yield. The ability of the pathogen to survive in soil for several years, even without the host, makes it really difficult to control. However, using chickpea cultivars resistant to Fusarium wilt is the most effective and environmentally friendly method of preventing yield loss. Phenotypic evaluation of germplasm and improved lines to find resistance to specific race of the pathogen is a time consuming and expensive method which is affected by the amount of inoculum and environmental conditions. In this experiment, molecular marker RAPD was used to detect chickpea genotypes containing the resistance gene. The DNA of 42 chickpea genotypes was extracted by CTAB method. Then, polymerase chain reaction was conducted using OPG-20 marker. At the end, results were confirmed by the pathogenesis test. Results indicated that 40 genotypes out of the 42 were sensitive to race zero and only KC216194 mass and Jam cultivar were resistant to race zero. The pathogenesis test confirmed the results of genetic evaluations.

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Introduction

Chickpea Fusarium wilt is induced by *Fusarium oxysporum* f. sp. ciceris (Jalali and Chand, 1992). This disease was first reported in India (Jimenez-Diaz *et al.*, 1992; Singh, 2003). Today, it is reported from all chickpea producing countries around the world such as India, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey, USA and the countries of the USSR (Westerlund *et al.*, 1974). In Iran, this disease was first reported to be caused by *F. lateritium* f. ciceris (Manouchehri and Mesri Alamdari, 1966).

Studies indicate that 19% of chickpea fields in North West of Iran were infested with the disease in 1999; the severity of the disease was 5-60% (Akem, 1998). This is evaluated to be 10-90% worldwide (Jimenez-Diaz *et al.*, 1989; Singh and Reddy 1991). The epidemic of Fusarium wilt sometimes causes significant damages to crop yield and in cases with suitable conditions for the pathogen development, the damage reaches 100% (Halila and Strange, 1996).

The fungus is a soil-borne pathogen which lives in the form of chlamydospore in seeds and dead plant materials and can survive in soil for more than five years (Singh, 2003). Today, there is an effort to reduce the application of chemical pesticides in order to eliminate their environmental risks and their effect on human health. So, genetic resistance and cultivation of resistant cultivars is the most important method to control Fusarium wilt disease. The phenotypical selection of resistant cultivars is complicated and time consuming method; using DNA based markers in breeding programs of commercial crops facilitate the selection of the resistant cultivars (Lindhout, 2002; Tanksley *et al.*, 1992). RAPD technique is a powerful detection tool to select all main FOC races in Mediterranean area (Jimenez-Gasco *et al.*, 2001).

The objective of this experiment was to evaluate the resistance or sensitivity of 42 chickpea cultivars to race zero of the pathogen using RAPD marker.

Materials and methods

Chickpea seedling production for DNA extraction

In this research, 42 cultivars, lines and masses of chickpea were studied; their names and places of collection are listed in table 1. Five seeds of each genotype were planted and their leaves were used for DNA extraction at five leaves stage.

DNA extraction from plant tissue

For DNA extraction from plant tissue, CTAB method was used with little modifications (Ausubel *et al.*, 1994). DNA was extracted from 0.05 mg fresh tissue of plants leaves.

PCR test

To do this, OPG-20 marker was used. Components of polymerase chain reaction are listed in table 2. Temperature programming included an initial denaturation in 94°C for 5 min followed by 35 cycles with denaturation in 94°C for 50 s, connection in 30°C for 50 s and extension in 72°C for 105 s which was finished with a terminal extension in 72°C for 10 min. Amplified DNA fragments were electrophoresed on 2% Agarose gel. Gels were stained with GelRed. Then, samples were subjected to UV and photographed.

Pathogenesis test

The inoculum was obtained from race zero 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 in a shaker-incubator (125 rpm, 25°C, under 12 h of fluorescent light daily) for seven days.

The liquid culture was passed through two-layer sterile gauze and the number of spores was counted with hemocytometer lam and the concentration of the suspension was set on 4×10^6 cfu/g. A mixture of sand and corn flour (9:1 ratio) was produced and sterilized two times; each time 1 h in 121°C. Then, 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).

Chickpea genotypes and their inoculation

In this experiment, 16 cultivars and lines of chickpea were studied (Table 3). Seeds were placed in trays containing the sterilized sand mixture (sterilized two times; each time 1 h in 121°C), in order to germinate. The inoculum with the population of 4×10^6 cfu/g was added to the soil of 0.5 L plastic pots containing 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. Normal and pre-germinated seeds were planted in the pots. Three seeds were planted in each pot and three replications were considered for each treatment. For each genotype, three pots were considered as the control (without inoculation).

The first irrigation was conducted with 100 ml water and in the following days it was conducted with 50 ml water. Pots were held in greenhouse with the normal light and in 20-25°C.

Symptoms assay

Development of the disease was evaluated every five days from the day 10 to 50 and plants were categorized into resistant (without any symptoms) and sensitive (showing chlorosis) groups.

At the end of the experiment, to make sure of the damages of pathogen, plants were harvested from the pots and the results were compared with Koch principles. For detection, Nelson *et al.* (1983) identification key was used.

Results

DNA quality measurement

The DNA quality obtained by electrophoresis on 8.0% concentration Agarose gel (Fig. 1).

Table 1. Names and the places of collection of the genotypes.

No.	Genotype	Obtained from
1	Azad cultivar	Iranian Dryland Agricultural Research Institute
2	Arman cultivar	Iranian Dryland Agricultural Research Institute
3	KC 215474 (Khorasan Razavi, Neishaboor, Iran)	Iranian Seed and Plant Improvement Institute
4	Hashem cultivar	Iranian Dryland Agricultural Research Institute
5	KC 216193 (Karaj, Tehran, Iran)	Iranian Seed and Plant Improvement Institute
6	KC 215950 (Ghoochan, Khorasan Razavi, Iran)	Iranian Seed and Plant Improvement Institute
7	KC 215920 (Ghoochan, Khorasan Razavi, Iran)	Iranian Seed and Plant Improvement Institute
8	KC 215079 (Sari, Mazandaran, Iran)	Iranian Seed and Plant Improvement Institute
9	KC 215887 (Ahar, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
10	Bionizh cultivar	Iranian Dryland Agricultural Research Institute
11	KC 216084 (Moghan, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
12	Flip 97-102c	Iranian Dryland Agricultural Research Institute
13	KC 216313 (Ardebil, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
14	ILC 482 line	Iranian Dryland Agricultural Research Institute
15	KC 215377 (Khorasan Razavi, Neishaboor, Iran)	Iranian Seed and Plant Improvement Institute
16	Native to Ilam (Jam)	Iranian Dryland Agricultural Research Institute
17	KC 216194 (Karaj, Tehran, Iran)	Iranian Seed and Plant Improvement Institute
18	KC 215004 (Saveh, Markazi, Iran)	Iranian Seed and Plant Improvement Institute
19	KC 216223 (Bakhtaran, Iran)	Iranian Seed and Plant Improvement Institute
20	Flip 97-116c	Iranian Dryland Agricultural Research Institute
21	KC 215437 (Ghoochan, Khorasan Razavi, Iran)	Iranian Seed and Plant Improvement Institute
22	KC 215909 (Ahar, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
23	KC 215002 (Saveh, Markazi, Iran)	Iranian Seed and Plant Improvement Institute
24	KC 216364 (Ardebil, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
25	KC 216195 (Isfahan, Iran)	Iranian Seed and Plant Improvement Institute
26	KC 216228 (Ardebil, East Azerbaijan, Iran)	Iranian Seed and Plant Improvement Institute
27	Khorram Abad native mass	Khorram Abad Agricultural Research Institute, Iran
28	KC 215543 (Shiraz, Fars, Iran)	Iranian Seed and Plant Improvement Institute
29	Flip 97-109c	Iranian Dryland Agricultural Research Institute
30	KC 215858 (Ghoochan, Khorasan Razavi, Iran)	Iranian Seed and Plant Improvement Institute
31	Flip08-90c	Iranian Dryland Agricultural Research Institute
32	Flip05-77c	Iranian Dryland Agricultural Research Institute

33	Flip07-177c	Iranian Dryland Agricultural Research Institute
34	Flip06-152c	Iranian Dryland Agricultural Research Institute
35	Flip08-93c	Iranian Dryland Agricultural Research Institute
36	Flip07-123c	Iranian Dryland Agricultural Research Institute
37	Flip07-216c	Iranian Dryland Agricultural Research Institute
38	Flip03-28c	Iranian Dryland Agricultural Research Institute
39	Flip07-197c	Iranian Dryland Agricultural Research Institute
40	Flip08-81c	Iranian Dryland Agricultural Research Institute
41	Flip02-04c	Iranian Dryland Agricultural Research Institute
42	Flip05-183c	Iranian Dryland Agricultural Research Institute

Table 2. Components of polymerase chain reaction by OPG-20 marker.

Harvested (µl)	Components of the reaction
2.5 µl	PCR buffer (10X)
0.9 µl	$MgCl_2$ (50 mm)
0.6 µl	dNTP (10 mm)
1.2 µl	Primer (10 pmol/ml)
0.25 µl	*Taq* DNA polymerase (5 unit/ml)
2 µl	DNA
17.55 µl	ddH_2O

Table 3. Genotypes used in pathogenesis test.

No.	Genotype	No.	Genotype	No.	Genotype	No.	Genotype
1	Azad cv.	5	KC 215887	9	KC 216194	13	KC 216228
2	Arman cv.	6	Bionizh cultivar	10	KC 216223	14	Khorram Abad native mass
3	Hashem cv.	7	KC 216084	11	KC 215002	15	KC 215858
4	KC 215079	8	Native to Ilam (Jam)	12	KC 216195	16	Flip06-152c

PCR results

According to Cobos *et al.* (2005), chickpea genotypes which are resistant to the race zero of *Fusarium oxysporum* f. sp. ciceris produce an index band weighting 600 bp. Results of our experiment showed that only Jam cultivar and KC 216194 mass produced the 600 base pair, which is the indicator of presence of resistance gene. In other 40 genotypes, the 600 bp index band was not observed; meaning that the genotypes are sensitive to the disease (Fig. 2, 3 and 4).

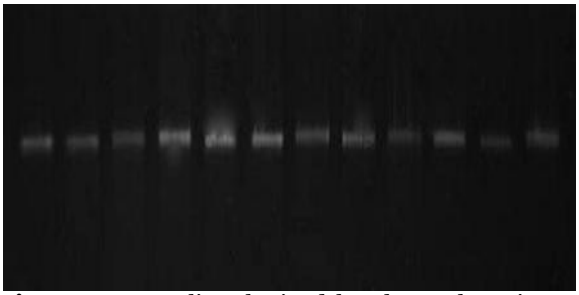

Fig. 1. DNA quality obtained by electrophoresis on 8.0% concentration Agarose gel.

Pathogenesis test results

The race zero of the pathogen results in chlorosis symptoms in sensitive plants; so, the genotypes were categorized in two groups. In the first group, genotypes were resistant and showed no symptom (Jam cultivar and KC 216194 mass), and all other genotypes were sensitive to the pathogen and were categorized in the second group.

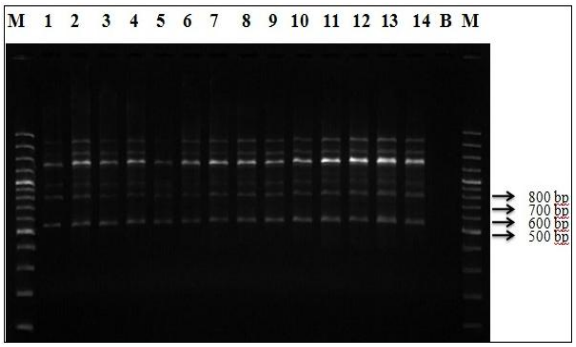

Fig. 2. Cultivars number according to the table of genotypes name.

M: marker 100 bp (#SM0323 by Fermentase Co.)

B: Blank

Discussion

Inducing genetic resistance to Fusarium wilt diseases in chickpea plants is the most important and cost-

effective method of controlling the disease. This method has many advantages compared with other methods such as the application of chemical pesticides because it reduces the expenses, labor and time. Genetic resistance is caused by the presence of genes in plant genome which are resistant to the disease. Detection of the resistant gene in resistant chickpea cultivar is the most important step to reach commercial cultivars resistant to Fusarium wilt. For this purpose, various methods are developed yet. Classic methods require the evaluation of a large number of genotypes; these methods are expensive and time consuming (Landa *et al*., 2004; Huttle *et al*., 2002; Gumber *et al*., 1995).

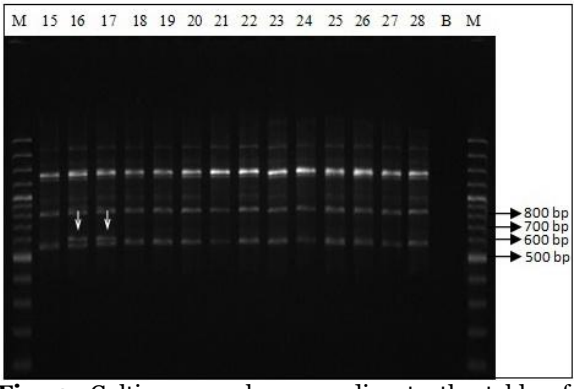

Fig. 3. Cultivars number according to the table of genotypes name.
M: marker 100 bp (#SM0323 by Fermentase Co.)
B: Blank.

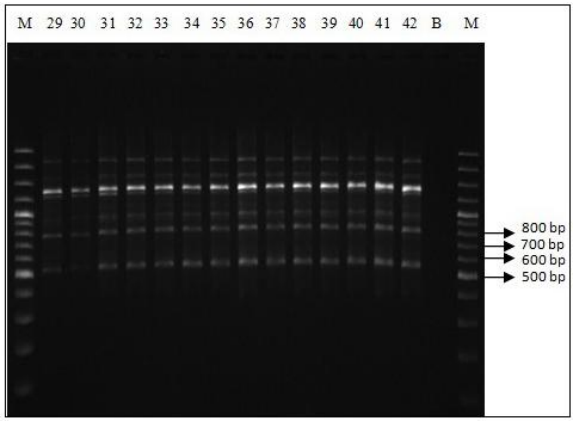

Fig. 4. Cultivars number according to the table of genotypes name.
M: marker 100 bp (#SM0323 by Fermentase Co.)
B: Blank.

Using RAPD markers is a more commonly applied method because it enables an easy and fast detection and does not need to know the genome sequence. In addition, in this method DNA is easily amplified and there is no need to use isotopes (Zhang *et al*., 2005). This method has been successfully used to make markers in various organisms and also to create the genome maps. RAPD marker provides a large number of multi positional markers which can distinct samples from each other with high clarity (Singh *et al*., 2011). Using RAPD marker to select genotypes based on marker is a useful method when it is extremely related 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).

Conclusion

Among the 42 studied genotypes, only KC 216194 mass and Jam cultivar were resistant to the race zero of the pathogen. Results of pathogenesis test (phenotypic study) confirmed the results of molecular marker (genotypic study). So, the two resistant genotypes are recommended for cultivation in areas infested with the race zero of the pathogen; in these areas farmers must avoid planting the sensitive genotypes.

References

Akem C. 1998. Survey on chickpea diseases in Iran. ICARDA.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1994. Current protocols in molecular biology. New York: John Wiley and Sons Inc.
http://dx.doi.org/10.1002/0471142727

Beigi A. 2013. Studying the genotypic variation and determining the races causing *Fusarium oxysporum* f.sp ciceris in Ilam province, Iran. MSc thesis, Tehran University, Tehran, Iran.

Brown RN, Myers JR. 2001. RAPD markers linked to morphological and disease resistance traits in squash. Cucurbit Genetics Cooperative Report **24,** 91-93.

Cobos MJ, Fernandez MJ, Rubio J, Kharrat M, Moreno MT, Gil J, Milla`n T. 2005. A linkage map of chickpea (*Cicer arietinum* L.) based on populations from Kabuli 9 Desi crosses: location of genes for resistance to Fusarium wilt race 0. Theoretical and Applied Genetics **110,** 1347-1353.

Gumber RK, Kumar J, Haware MP. 1995. Inheritance of resistance to Fusarium wilt in chickpea. Plant Breeding **114,** 277-279. http://dx.doi.org/10.1111/j.14390523.1995.tb00814.x

Halila MH, Strange RN. 1996. Identification of the causal agent of wilt of chickpea in Tunisia as *Fusarium oxysporum* f.sp. *ciceris* race 0. Phytopathology **86,** 67-74.

Haware MP, Kumar J, Reddy MV. 1980. Disease resistance in Kabuli-desi chickpea introgression. In: Haware MP, Kumar J, Reddy MV, eds. Proceeding of the International Workshop on Chickpea Improvement. ICRISAT, Hyderabad, India, 67-69.

Huttle B, Santra D, Muehlbauer FJ, Kahle G. 2002. Resistance gene analogs of chickpea: isolation, genetic mapping and association with Fusarium resistance gene cluster. Theoretical and Applied Genetics **105,** 479-490.

Jalali M, Chand L. 1992. Races of *Fusarium oxysporum* f. sp. *ciceris*. Plant Disease **66,** 809-810.

Jimenez-Diaz RM, Trapero-Casas AT, Cubero JI. 1992. Importance of chickpea soil-borne diseases in the Mediterranean basin. In: Singh KB, Saxena MC, eds. Disease Resistance Breeding in Chickpea. Syria: ICARDA, Aleppo.

Jimenez-Diaz RM, Basallote-Ureba MJ, Rappoport H. 1998. Colonization and pathogenesis in chickpea infected by races of *Fusarium oxysporum* f.sp. *ciceris*. In: Tjamos EC, Beckmann C, eds. Vascular wilt diseases of Pl.s., vol. H**28,** NATO ASI Series. Germany: Springer-Verlag, 113-121.

Jimenez-Gasco MM, Perez-Artes E, Jimenez-Diaz RM. 2001. Identification of pathogenic races 0, 1B/C, 5 and 6 of *Fusarium oxysporum* f.sp. *ciceri* with random amplified polymorphic DNA. European Journal of Plant Pathology **107**, 237-248.

Kaiser WJ, Alcala-Jimenez AR, Herves-Vargas A, Trapero-Casas JL, Jimenez-Diaz RM. 1994. Screening of wild cicer species for resistance to races 0 and 5 of *Fusarium oxysporum* f. sp. ciceris. Plant Disease **78,** 962-967.

Landa B, Navas-Cortes JA, Jimenez-Diaz RM. 2004. Integrated management of Fusarium wilt of chickpea with sowing date, host resistance and biological control. Phytopathology **94**, 946-960. http://dx.doi.org/10.1094/PHYTO.2004.94.9.946

Lindhout P. 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. Euphytica **124,** 217-226.

Manouchehri A, Mesri Alamdari Y. 1966. Iranian chickpea Fusarium wilt disease. Iranian Journal of Plant Diseases **3,** 1-11.

Nelson PE, Toussoun TA, Marasas WFO. 1983. Fusarium species: an illustrated manual for identification. USA: Pennsylvania State University Press, University Park.

Singh SD. 2003. Soil borne diseases of chickpea. Indian: ICRICAT, Patancheru Pradesh.

Singh KB, Reddy MV. 1991. Advances in disease-resistance breeding in chickpea. Advances in Agronomy **49,** 191-222.

Singh M, Chaudhuri I, Mandal SK, Chaudhuri RK. 2001. Development of RAPD markers linked to Fusarium wilt resistance gene in Castor Bean (*Ricinus communis* L). Genetic Engineering and Biotechnology Journal **28,** 1-9.

Tanksley SD, Ganal MW, Prince JP, de Vicente

MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Martin S, Messeguer GB, Miller RJC, Paterson AH, Pineda O, Ro MS, Wing RA, Wu W, Young ND. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics **132,** 1141-1160.

Trampero-Casas AT, Jimenez-Diaz RM. 1985. Fungal wilt and root rot of chickpea in southern Spain. Phytopathology **75,** 1146-1151.

Westerlund FV, Campbell RN, Kimble KA. 1974. Fungal root rot and wilt of chickpea in California. Phytopathology **64,** 632-635.

Zhang HY, Liu X, He Z. 2005. Random amplified DNA polymorphism of *Nicotiana tabacum* L. cultivars. Biologia Plantarum **49,** 605-607. http://dx.doi.org/10.1007/s10535-005-0056-z