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Pathogenic and genetic characterization of Iranian isolates of *Fusarium oxysporum* f. sp. *Lentis* by ISSR analysis

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Lentis (*Lens culinaris* Medik.) is an important widely cultivated food legume crop in Iran. The wilt disease caused by *Fusarium oxysporum* f.sp. *lentis* has been observed in high incidence rates during recent years(2007-2008) and has caused high losses in some of the cultivated areas. In order to study the pathogenicity and genetic diversity of *Fusarium oxysporum* f. sp. *Lentis* (Fol) isolates. Forty five isolates of *Fusarium oxysporum* f. sp. *Lentis* isolated from wilted lentis plants collected from different lentis growing areas in Iran, with two isolates from ICARDA were investigated. A pathogenicity test were performed for all isolates. Results showed that the *Fol* isolates differ in their aggressiveness on the susceptible lines could be grouped into 3 categories based on bayaa scales. The amount of genetic variation were evaluated by ISSR technique, using six primers, all of them could show the diversity between isolates. The most diversity was observed by primers $(CAG)_5$ and ISSR10 (93%) and the minimal diversity by ISSR02 (50%). Six ISSR primers produced 156 fragments that the number of fragment was variable, ranging from 6 to 17 fragments in each profile. The analysis showed that *F. oxysporum f .sp. lentis* isolates could be differentiated into 6 groups at 74% similarity, and that could partially separate isolates based on their geographical regions. But could not found any correlation between this marker and pathogenesis grouping.

Key words: ISSR, *Lens culinaris*, *Fusarium* wilt, genetic diversity, pathogenicity, Iran

Introduction

Fusarium wilt of lentis is an important disease reported in every continent where lentis is grown except Australia (Beniwal *et al*., 1993; Tosi and Cappelli, 2001). The disease may cause complete crop failure under favorable conditions for disease development, and can be the major limiting factor for lentis cultivation in certain areas (Chaudhary and Amarjit, 2002). The causal fungi of

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vascular wilt of lentis is *Fusarium oxysporum* Schlecht. Emend. Snyder & Hansen f. sp. *lentis* Vasudeva and Srinivasan. *Fusarium* wilt usually occurs near or at reproductive stages of crop growth. Symptoms include wilting of top leaves that resemble water deficiency, stunting of plants, shrinking and curling of leaves from the lower part of the plants that progressively move up the stems of the infected plant. Plants finally become completely yellow and die. Root symptoms include reduced growth with marked brown discoloration, tap root tips that are damaged and proliferation of secondary roots above the area of tap root injury. Discoloration of vascular tissue in the lower stem may not always be visible. General symptoms at the seedling stage include seed rot and sudden drooping more like wilting and damping off (Khare, 1980).

Although its sexual state has not been found, it is generally believed to belong to the *Hypocreales* of *Ascomycetes*. *F. oxysporum* f. sp. *lentis* is a soilborne pathogen, although seed infestation and infection is common. The chlamydospores can survive in soil either in dormant form or saprophytically for several years without a suitable host (Yadav *et al*., 2007). In the recent years, several types of molecular marker systems such as RAPD (random amplified polymorphic DNA) amplified fragment length polymorphism (AFLP), and PCR RFLP (restriction fragment length polymorphism), have been increasingly used to study the variability in pathogenic populations of FOl (Belabid, 2004).

Term of microsatellites was used first by (Litt and Luty, 1989). Microsatellites as primers in PCR amplifications (ISSRs). This technique enables amplification of genomic DNA, provides information about many loci simultaneously, and has been successfully used to identify variability within fungal species (Thanos *et al.*, 1996; Zeze *et al*., 1997). ISSR analysis is a simpler procedure than AFLP analysis because there is no need for an adapter ligation step. In addition, ISSRs appear to be more stable than RAPDs because they have longer primer sequences and use a higher annealing temperature during PCR (McCall *et al.*, 2004). The primers for the repeat regions can be designed outside of the region or within the repeats. Microsatellites and minisatellite primers are reported to be more effective since the sequences are usually dispersed throughout the genome. Just as with repeat sequence probes, variability due to high frequency of change in the sequences may reduce the effectiveness of the method in clustering moderately related isolates (Mbofung, 2006).

The purpose of this study was to identify genetic variation of Iranian isolates of wilt-causing pathogen of lentis, *F. oxysporum f .sp. lentis* (FOl) using ISSR marker to determine whether or not the observed variations could represent intraspecific relationships in *Fusarium oxysporum*. In a previous

research, ISSR marker has been used for determining of genetic variations between several populations of *F. oxysporum* f. sp. *cicer* (Bayraktar and Dolar, 2008) and up to our knowledge, this is the first study using ISSR marker for studying of genetic variation between forms of *F. oxysporum* f. sp. *lentis*.

Materials and methods

Fungal isolates

Forty five isolate of *Fusarium oxysporum* were obtained from wilt-infected lentis plants collected from different region of Iran during 2008–2009 and in addition two isolates from ICARDA (International Center for Agricultural Research in the Dry Areas) (Fig. 1). The fungus was isolated from stem and crown wilted Lentis. The isolates were identified according the main identification keys of *Fusarium*. Identified isolates were single spored and was stored for short time at SNA, but for perennial time then stored in tube containing sand at 4°C. Detail of the *F. oxysporum f .sp. lentis* isolates presented in Table 1.

Fig. 1. *F. oxysporum* f. sp. *lentis* isolates collected from diverse agro-ecological regions in Iran include: (1) Ardebil, (2) East Azerbaijan, (3) West Azerbaijan, (4) North Khorasan, (5) Khorasan razavi, (6) South Khorasan and (7) Ilam provinces.

Pathogenicity test

The pathogenicity of 47 isolates of FOl were tested in the greenhouse on a very susceptible lentis line (ILL 4605) by root dip method. The pathogenicity test was carried out on the Lentis cultivar (ILL 4605), highly susceptible to fusarium wilt. Seeds of (ILL 4605) were surface sterilized using 5% sodium hypochlorite for 3min, washed in sterile water and germinated for ten days in pots containing sterilized perlit. The ten days old seedlings were carefully uprooted and the roots

were washed under tap water to clean excess soil. Root tips around 0.7 cm long were cut off to facilitate the accession of the pathogen into the roots. The roots of the seedlings were then dipped in the inoculums of each isolate (5×10^6 conidiuml-1) for 5 min to enable conidia to cohere to the roots. Inoculated seedlings were transplanted in pre-irrigated sterile soil:sand:pit:perlit (1:1:1:1) in pots and incubated at 25 ± 3ºC. Control plants were dipped in the sterile water for 5 minute and then planted in pots. Twenty-thirty days after inoculation all isolates based on aggressiveness were grouped using 1-9 scale (Bayaa *et al*., 1995) with minor changes. Low virulent isolates (1-3 scale value), moderately virulent isolates (3.1-6), and highly virulent isolates (6.1-9) based on Bayaa's scale. Statistical analysis of data was performed with software (Minitab version 15.1) and SAS (version9.1).

DNA extraction

Potato dextrose broth (PDB) medium was prepared. This medium was inoculated with a bit of the fungus (*Fusarium oxysporum* f.sp. *lentis*) from a 48 hrs fresh culture. The inoculated plates/flusks were incubated on a shaker at room temperature (25±1˚C) for four days. Then they were placed under florescent light at 25±2˚C with alternation of 12/12 hours light and darkness for ten days. DNA was extracted by the Ctyldimethyle Ammonium Bromide (CTAB) method with minor modification. DNA was quantified using a spectrophotometer (eppendorf).The quality of the extracted DNA was visually checked on 1.5% agarose gels.

ISSR

PCR reaction was performed in Eppendorf Gradient (Germany) thermocycler. ISSR-PCR was performed in a total volume of 25 µl of reaction containing 10 mM of Tris-HCl (pH 8.8), 50 mM of KCl, 2.5 mM of MgCl2, 0.24 µM of primer, 0.2 mM of dNTPs, and 1 U of Taq polymerase (MBI, Fermantase). Amplification was performed as follows: initial denaturation (95ºC/5 min) followed by 40 cycles of denaturing (94ºC/30s), annealing (60, 58, 59, 54, 52 and 57) for different primers 1 min. (Table 2) and extension (72˚C/30s) and a final elongation step (72ºC/6 min). Product of PCR amplification were separated by horizontal agarose gel electrophoresis on 1.5% agarose in 1x TBE buffer (0.1 M Tris, 0.05M boric acid and 0.001M Ethylene Diamine Tetra Acetate (EDTA)), at 80V for 180 min and stained with Ethidium bromide at 0.5mg ml^- and photographed under UV Translaminator with Gel Doc. Lourmat T-5×20-2A. A 1kbp ladder (Gene Ruler^{TM}, Fermantas, France) was used as a molecular size standard. All PCR assays were repeated at least twice.

Table 1. Isolates of *Fusarium oxysporum* f.sp. *lentis* collected from different geographical regions and disease development.

No.	Isolate	origin	state	Disease development
1	Fol1	Goytapeh	Ardebil	H*
2	Fol2	Goytapeh	Ardebil	L***
3	Fol3	Goytapeh	Ardebil	H
4	Fol4	Jafarabad	Ardebil	M**
5	Fol5	Ruhkandi	Ardebil	L
6	Fol6	Goytapeh	Ardebil	L
7	Fol7	Goytapeh	Ardebil	M
8	Fol8	Bilesavar	Ardebil	L
9	Fol9	Goytapeh	Ardebil	M
10	Fol10	Ruhkandi	Ardebil	M
11	Fol11	Ruhkandi	Ardebil	H
12	Fol12	Goytapeh	Ardebil	H
13	Fol13	Ptilgan	Ardebil	H
14	Fol14	Bilesavar	Ardebil	H
15	Fol15	Goytapeh	Ardebil	L
16	Fol16	Ilam	Ilm	L
17	Fol17	Ilam	Ilam	L
18	Fol18	Ilam	Ilam	L
19	Fol19	Ilam	Ilam	M
20	Fol20	Ilam	Ilam	M
21	Fol21	ICARDA	ICARDA	L
22	Fol22	ICARDA	ICARDA	L
23	Fol 23	Oshnavie	W. Azerbaijan	M
24	Fol24	Kigal	E. Azerbaijan	M
25	Fol25	Varzeghan	E. Azerbaijan	M
26	Fol26	Dizaj	E. Azerbaijan	L
27	Fol27	Kigal	E. Azerbaijan	M
28	Fol28	Dizaj	E. Azerbaijan	M
29	Fol29	Agbolagh	E. Azerbaijan	M
30	Fol30	Varzeghan	E. Azerbaijan	H
31	Fol31	Tokhomdel	E. Azerbaijan	L
32	Fol32	Varzeghan	E. Azerbaijan	H
33	Fol33	Kigal	E. Azerbaijan	H
34	Fol34	Dizaj	E. Azerbaijan	H
35	Fol35	Dizaj	E. Azerbaijan	L
36	Fol36	Dizaj	E. Azerbaijan	L
37	Fol37	Ahar	E. Azerbaijan	H
38	Fol38	Ahar	E. Azerbaijan	L
39	Fol39	Ahar	E. Azerbaijan	M
40	Fol40	Ahar	E. Azerbaijan	H
41	Fol41	Ahar	E. Azerbaijan	H
42	Fol42	Khorasan	Khorasan	H
43	Fol43	Khorasan	Khorasan	H
44	Fol44	Khorasan	Khorasan	L
45	Fol45	Khorasan	Khorasan	H
46	Fol46	Hashtrud	E. Azerbaijan	L
47	Fol47	Hashtrud	E. Azerbaijan	L

*, ** and *** are High, Moderate and Low virulence respectively.

Table 2. List, sequence, annealing temperature, number of total bands and percent of polymorphism of the primers for amplifying DNA probes used in ISSR analysis of *F. oxysporum* f.sp. *lentis*.

primer	sequence	TA(°C)	Polymorphism (%)	Amplified Fragments
$(CAG)_5$	5ó - CAGCAGCAGCAGCAG - 3ó	58	93	15
$(GAC)_5$	5ó - GACGACGACGACGAC - 3ó	60	90	10
$(GTG)_5$	5ó - GTGGTGGTGGTGGTG - 3ó	64	88	17
PCMS	5'-GTCGTCGTCGTCGTCGTCGTC-3'	57	86	7
ISSR02	5'-ACTGACTGACTGACTG-3'	52	50	6
ISSR10	5'-CACCACCACCACCAC-3'	54	93	15

Data analysis

Resemblance of each profile for each primer was made scored as present (1) or absent (0) of ISSR fragments of the same molecular weight. A binary matrix combined all the data records for all isolates. The numerical taxonomic software package NTSYS-pc, version 2.0 was used to order the isolates by unweighted paired group method with arithmetic averages (UPGMA) using the SAHN (Sequential Agglomerative Hierarical Nested) cluster analysis module based on Dice's coefficient of similarity.

Results

Pathogenicity test

Typical symptoms of the fungus after inoculation on susceptible cultivar (ILL 4605) appeared in the leaflet tip of wilt contaminated then progress down, and finally the wilt of total plant is the common symptoms of this disease. The leaflets breaches did not fall prematurely and remained on the stem. Thereafter the stems yellow and wilted from the tip down and started to progress and finally near 25 days after the death occurs in sensitive plant.

Most of the *Fusarium oxysporum* isolates, were tested in greenhouse had little aggressivness that includes 18 of the isolates. 16 of the isolates are grouped in high aggressiveness and finally, 13 of them are grouped in moderate aggressiveness (Table 1). The isolates mainly collected from Ardebil and Khorasan provinces showed 34.04% of them had a severe aggressive and caused wilting and death of plants in less than 3 week. Isolates with modereate pathogenesis (27.65%), caused wilting of plants and their deaths occurs little late to the severe pathogenesis group and their deaths happened about a month.

All provinces had a representatives in this group except Khorasan province. Finally, third group, of isolates with low pathogenicity sharing from all provinces even the isolates from ICARDA were classified in this group.

ISSR analysis

To assess genetic variability within the *F. oxysporum f .sp. lentis* 47 isolates were analyzed (Table 1). PCR amplification using six selected primers resulted in the production of 70 different ISSR fragment in UPGMA analysis. The number of fragment was variable, ranging from 6 to 17 fragments in each profile. The size of fragments ranged from 150 to 3500 bp for all isolates (Fig. 2). Also showed Principle coordinates analysis of 47 isolates of *F. oxysporum* f. sp. *Lentis* (Fig. 4). The analysis showed that *F. oxysporum f .sp. lentis* isolates could be differentiated into 6 groups at 74% similarity (Fig. 3).

Cluster I included 32 isolates from Ardebil, one isolates from West Azerbaijan (W.Az), five isolates from Ilam, four isolates from Mashhad (Khorasan) and seven isolates from different regions of East Azerbaijan (E.Az). Cluster II comprised three isolates from Ahar (East Azerbaijan) however two isolates of Ahar is grouped in Cluster 1. Cluster III contained two isolates from ICARDA, cluster IV comprised five isolates from East Azerbaijan. Cluster V included five isolates from E.Az, cluster VI contained two isolates from E.Az, (Dizaj village).

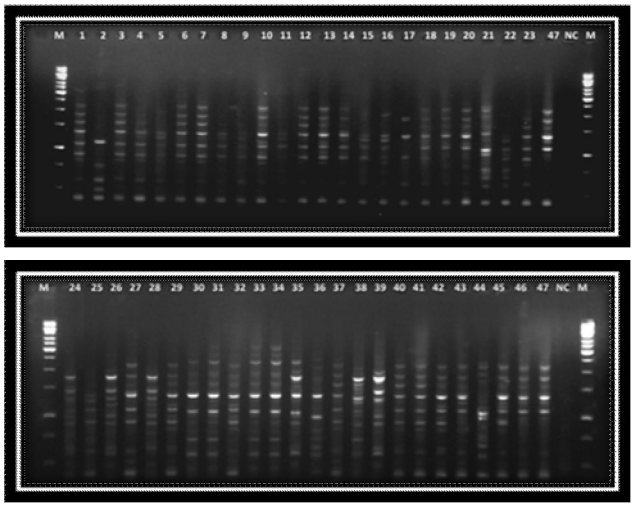

Fig. 2. PCR fingerprintig patterns from genomic DNA of *F. oxysporum* f. sp. *lentis* from different geographic regions of Iran with primer GTG.

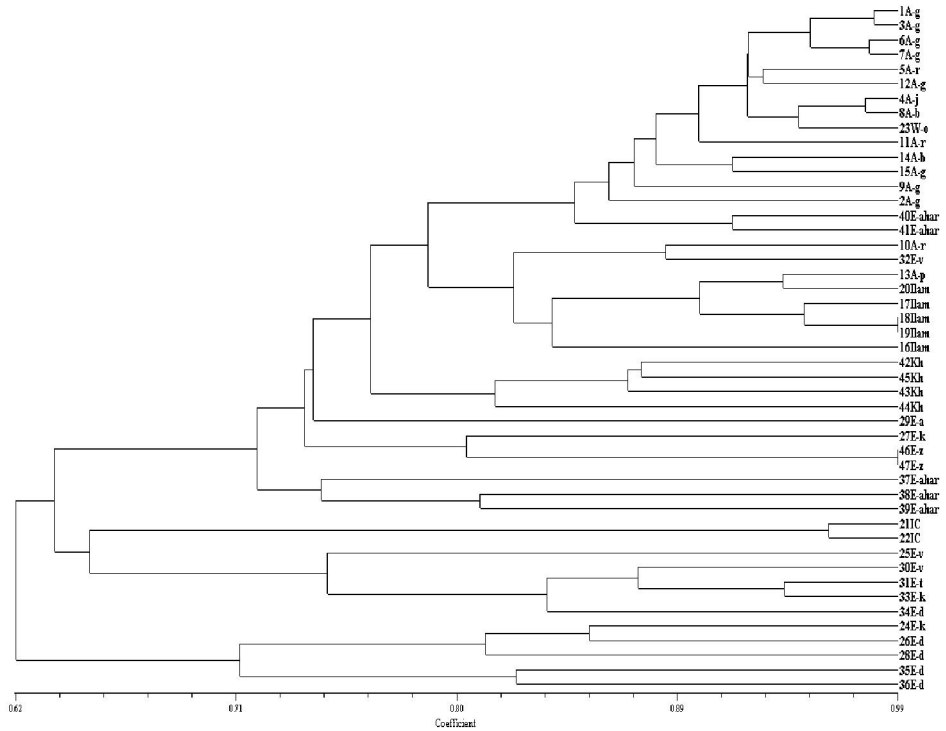

Fig. 3. Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 47 *Fusarium oxysporum* f. sp. *lentis* isolates listed in Table 1. Genetic similarity was obtained by ISSR marker, using the Dice similarity coefficient.

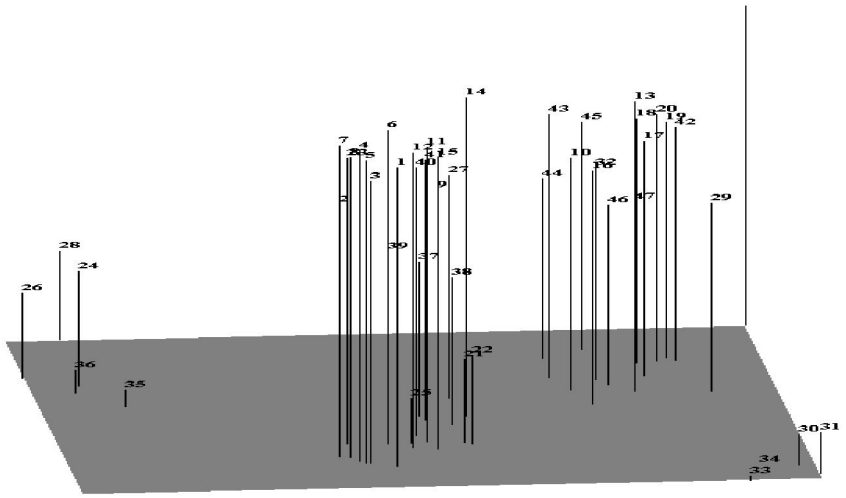

Fig. 4. Principle coordinates analysis of 47 isolates of *F. oxysporum* f. sp. *lentis*.

Discussion

The results show that most isolates of aggressiveness were related to Ardebil and Khorasan provinces. These two provinces, particularly Ardebil is the center of lentis or lentil production. The most steadily and continuously cultivated lines in these areas are very sensitive to this disease. Of course there is a possibility of transmitting externally via lentis seeds from province to province. The possibility of externally transmission previously had been proven (Setti and Bouznad, 1998; Belabid and Fortas, 2002). The results obtained for molecular studies with ISSR markers showed very high similarities between the isolate population of fungus, were seen in different areas of Iran and also reviewing two isolates of the ICARDA it seems that this fungus probably is not physiological race (Bayaa *et al*., 1995; Belabid and Fortas, 2002). Although this fungus has no physiological race as isolates in different areas showed large differences in power of pathogenesis (Belabid and Fortas, 2002).

Genetic characterization of *F. oxysporum* f. sp. *lentis* is essential for the efficient management of *Fusarium* wilt through use of resistant cultivars in *Lentis* growing areas. However, an understanding of occurrence, distribution, and genetic relatedness of such pathogenic variants is a necessary for developing effective and efficient integrated disease management. The results showed that there were a high level of genetic variation among the isolates of *F. oxysporum* f. sp. *lentis* in Iran. Cluster analysis at 74% similarity separated the *F.oxysporum* f. sp. *lentis* isolates into six distinct groups. Considering this study, this the first report of genetic diversity of *F. oxysporum f .sp. lentis* with ISSR markers. Groups done as much as could reasonably isolates according to geographic areas will separate. Even two isolates of ICARDA showed 62% similarity with Iranian isolates. However, this marker could not separate isolates according to pathogenesis. In other words, any correlation between this marker and pathogenesis grouping could not be found. This study indicate that the reliability of the ISSR markers with other such as RAPD or PCR-RFLP as a tools for addressing questions on geographical distribution of population of this pathogen. In conclusion, isolates of *F. oxysporum* f. sp. *Lentis* are different from each other and have existing high level of genetic variation.

ISSR analyses were very useful in assessing the intra- and interspecific diversity of *F. oxysporum* (Bayractar and Dolar, 2008). In addition to contributing to the understanding of the diseases caused by these pathogens and improving crop productivity, these results will be useful for developing integrated strategies for disease management and breeding programs.

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