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Pathogenic and Genetic Characterization of *Fusarium oxysporum* F. sp. *lentis* by RAPD and IGS Analysis in Khorasan Province

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Abstract: 33 isolates of *Fusarium oxysporum* were obtained from wilted lentil plants collected from different lentil growing areas In Northern and Razavi Khorasan provinces. Pathogenecity tests were performed for all isolates on the susceptible lines. Results indicated that 27 isolates were pathogenic. Then formae special were carried out on different plants and pathogenic isolates were determined as *Fusrarium oxysporum* f.sp. *lentis*. The amount of genetic variation was evaluated by polymerase chain reaction (PCR) amplification with a set of 14 RAPD primer and IGS primer pairs. Genetic similarity between each of the isolates was calculated by using the Dice similarity coefficient and cluster analysis was used to generate a dendrogram showing relationship between them. Result obtained indicate that there is no apparent correlation with geographic origin and pathogenecity score of the isolates.

Key woeds: Lentil · Pathogenecity · Formae specials · RAPD · IGS

INTRODUCTION

Fusarium wilt of lentil, caused by *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen f.sp. *lentis* Vasudeva and Srinvasan (*Fol*) is a devastating disease in most countries where lentil is grown. *Fol* has great variability [1]. Isolates of the fungus have been differentiated on the basis of their nutritional requirements [2, 3] temperature [4] their sensitivity to fungicide [5] morphology and virulence [6, 7]. The pathogen persists in the soil as chlamydospore that can remain viable for several seasons [8]. As a result of the persistence of the pathogen in the soil, host-plant resistance is the best means to control lentil-vascular wilt [9]. Isolates with the same or similar host ranges are assigned to a forma specials and more than 70 formae specials have been described [10, 11]. Several analytical methods are available to enable identification genetic diversity in *Fol* population. Random Amplified polymorphic DNA (RAPD) used in detecting intra specific polymorphisms among fungi [12]. RAPD used for detection nonpathogenic isolates of *F. oxysporum* from pathogenic isolates of *F. o.* f.sp. *dianthi*, *F. o.* f.sp. *vasinfectum*, *F. o.* f.sp. *ciceris* and *albedinis* [13]. This marker used for identification of genetic diversity isolates of *F. o.* f.sp. *lentis*, showed that limited genetic variability observed among isolates would be expected for pathogen that became widespread relatively quickly as a result of an increase in production of the host plant i.e. distribution by seed. Also this genetic variation had no correlation between RAPD data with geographic location or vigor of pathogenicity [9]. PCR-RFLP is a technique that used widespread for screening genetic variation of *F. oxysporum* population. Based on analysis of IGS region, correlation exist between pathogenic races *F. o.* f.sp. *melonis* and nonpathogenic isolates of F.oxysporum. Studies were carried out based on IGS-region to detecte variations of intra specific between isolates of *F. oxysporum* on two host plant, tomato and common bean. For common bean, two types of polymorphism in IGS region were introdced, first the size of the IGS fragment amplified with primers CNS1 and CNL12 was in some of isolates 2.55 Kbp and in others 2.6 Kbp. Second level of polymorphism following analysis of restriction enzyme fragments that introduced 6 haplotype [14]. In tomato found only one fragment 2.6 Kbp that amplified with primers described and 5 haplotype detected [15].

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MATERIALS AND METHODS

33 isolates of *F. oxysporum* were obtained from wilted lentil plants collected from fields in major lentil producing areas In Northern and Razavi Khorasan provinces during 2006-2007. The fungus was isolated from the stem and crown of the wilted plants. The isolates collected were identified according to identification keys of *F. oxysporum* [16], then pathogenicity test carried out. All the isolates were grown in a 100 ml Erlenmeyer flasks containing 50 ml liquid culture of potato-dextrose Broth. Each flasks were inoculated with a mycelia plug (5 mm diameter) taken from a 5-days old culture raised on PDA medium. The cultures were shaken with 120 rpm, for 3 days, then spore suspensions were adjusted to 1×10^6 microconidia/ml. Pathogenicity test was carried out in the greenhouse on Lentil line (ILL4605) that is susceptible to *F. oxysporum*. This cultivar was obtained from the Lintel International Fusarium wilt Nursery of the International Center for Agricultural Research in the Dry Areas (ICARDA) Aleppo-Syria. Seed were surface-sterilized using 0.5% sodium hypochlorite for 5 min, rinsed in sterile water and put in plastic pots for germination. After 3-4 days, germinated seeds were sown in plastic pots containing perlit. The 15 days old seedlings were carefully uprooted and their roots dipped into inoculums for 2 min then sowed in plastic pots containing sterile soil. The disease severity was recorded starting from the 5th day after inoculation and continued for 8 weeks using a 1-9 scale [17] 1, no symptoms; 3, yellowing of the basal leaves only; 5, yellowing of 50% of the foliage; 7, complete yellowing of the foliage, flaccidity of the top leaves and partial drying; 9, whole plant or part of the plant wilted and/or dried. After the final score, reisolation of the fungus from the stem just above the crown was performed. Next the forma specials was defined by inoculation of the pathogen isolates on different plants for example chickpea, bean, tomato, melon and eggplant.

Mycelium Production and DNA Extraction: Fungal isolates grown on PDB in Erlenmeyer flasks, after 3 days shaking, incubated under light for 12-14 days. DNA was extracted by modification CTAB that described by Adopa *et al.* [18]. DNA was quantified using a spectrophotometer. The quality of the extracted DNA was visually checked on 1% agarose gels.

RAPD Analysis: The reaction mixture (25µl) contained; 10x PCR buffer, 50mM $MgCl_2$, 10mM each of dNTPs, 5u/µ Taq DNA polymerase, 10µm each of primer and 20 ng of extracted DNA. The mixtures were subjected to the following conditions: hold at 94°C for 3 min for starting, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2min and a final hold for 5 min. RAPD carried out with 14 primer (Table1). The PCR products together with marker (Lambda DNA/ *Hind* III *Eco*RI) were resolved by gel electrophoresis on 1.5% agarose gel in 0.5X Tris-Boric acid-EDTA buffer at 75 V (3 V cm^{-1}) for 3 hours, then visualized by ethidium bromide and photography under UV light.

Table 1: Sequence of primers used in RAPD and IGS

RAPD	Rco 8	5'-GGATGTCGAA-3'
	UBC 199	5'-GCTCCCCCAC-3'
	UDC 82	5'-GGGCCCGAG-3'
	OPK 19	5'-CTCCTGCCAA-3'
	UBC 53	5'-CTCCCTGAGA-3'
	RCO 9	5'-GATAACGCAC-3'
	UBC 300	5'-GGCTAGGGCG3'
	OPK 15	5'-CTCCTGCCAA-3'
	UBC 83	5'-GGGCTCGTGG-3'
	UBC 222	5'-AAGCCTCCCC-3'
	UBC 228	5'-GCTGGGCCGA-3'
	UDC 6	5'-CCTGGGCCTA-3'
	OPA-02	5'-TGCCGAGCTG-3'
	OPB-11	5'-GTAGACCCGT-3'
IGS	CLN12	CTGAACGCCTCTTAAGTCCAG
	IGS2	AATGAGCCATTCGCAGTTC

IGS-RFLP Analysis: The IGS region of the ribosomal DNA was amplified by using primers CLN12 and IGS2 (Table1). Amplification reactions were performed in a total volume of 50µl containing 10x PCR buffer, 50mM $MgCl_2$, 10mM each of dNTPs, 5u/µ Taq DNA polymerase, 10Pmol each of primers and 50ng of template DNA. The amplification conditions included an initial denaturation step of 94°C for 4min that was followed by 35 amplification cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), extention (72°C for 3 min) and a final extention for 10 min. The size of the amplified IGS fragments were estimated by comparison to a Lambda DNA/ *Hind* III *Eco*RI digest in 1% agarose gels.

Restriction Digestion: PCR products were individually digested with the restriction enzymes *Eco*RI, RsaI and HaeIII acording to the manufacturer 's directions (Fermentas). These enzymes were subjected to electrophoresis at 96 V (4 V cm^{-1}) in 1.5% agarose gels and stained with ethidium bromide.

Data Analysis: Comparison of each profile for primers was carried out on the basis of the presence versus absence (1/0) of RAPD and IGS products of the same molecular

weight. Base on molecular weight fragments were scored by software Lab Works™ V.3. The numerical taxonomic software package NTSYS-pc version 2.02 [19] was used to order the isolates by unweighted paired group method with arithmetic averages (UPGMA) based on Dice's similarity coefficient [20].

RESULTS AND DISCUSSION

Pathogenicity Test: Symptoms of disease depend to vigor of pathogenicity of isolates. From 33 isolates, 27 isolates were pathogenic and 6 isolates were nonpathogenic on ILL4605 (Table 2). At the first, symptoms of disease appeared as yellowing and wilting from apical of down lower leaves, then developed to upward parts and finally the whole plant dried.

Sometimes drying of leaves started very early and plant died in seedling stage. In flowering step, flowers dried and in pod-filling stage, seed of lentil didn't form.

Host Range: 27 isolates that caused pathogenicity on ILL4605 line, didn't 'show any disease symptom on bean and chickpea (Fabaceae), tomato and eggplant (Solanaceae) melon (Cucurbitaceae). Thus those isolates caused wilting in lentil identified as forme speciale *F.o.* f.sp. *lentis*.

Molecular Result: Polymorphism and genetic similarity between *Fol* isolates was revealed by RAPD and PCR-RFLP markers. RAPD analysis of DNA from 20 *Fol* isolates using the primers listed in Table 1 amplified 12 to 27 regions of DNA. In total introduced 230 polymorphic fragment that maximum polymorphism depended to primer UBC199 and minimum to primer RCO8.

Table 2: Isolates of *Fusarium oxysporum* obtained from wilted lentil plants in this study, their origin and score of pathogenicity on line 4605

Number	Isolates	Geographic origin	Score of Pathogenicity
1	Mp8	Pardis of college	9.0
2	Ka3F1	Maneh and samalgan	9.0
3	PaF1	Bojnord	9.0
4	Kb4F1	Bardaskan	1.0
5	Ra1F1	Bojnord	6.0
6	Gl1F5	Bojnord	9.0
7	Gl2F4	Bojnord	6.8
8	KdF3	Bardaskan	7.6
9	Mp4	Pardis of college	5.0
10	Mp16	Pardis of college	8.6
11	Gl2F2	Bojnord	6.4
12	Ka1F1	Maneh and samalgan	8.2
13	Ho3F2	Bardaskan	1.0
14	Ra2F1	Bojnord	1.0
15	Za1F1	Maneh and samalgan	6.2
16	Ho3F3	Bardaskan	6.8
17	Mp11	Pardis of college	1.0
18	Mp7	Pardis of college	1.0
19	NzF7	Bardaskan	6.2
20	KoF1	Bardaskan	7.4
21	Za2F3	Maneh and samalgan	9.0
22	Gl1F4	Bojnord	7.8
23	Mp5	Pardis of college	8.2
24	PaF2	Bojnord	8.2
25	Kb2F1	Bardaskan	7.8
26	Mp2	Pardis of college	8.2
27	Za1F3	Maneh and samalgan	7.8
28	Mp13	Pardis of college	9.0
29	Mp17	Pardis of college	7.4
30	Sa1F3	Garmkhan	7.4
31	Ra2F4	Bardaskan	7.0
32	Mp12	Pardis of college	1.0
33	Mp14	Pardis of college	3.0

The size of amplification DNA fragments genenrated with the RAPD primers ranged from 0.3 - 4.7 Kbp. Base on number of polymorphic fragments, separated 146 genotype from genomes of isolates that in between primers OPA-02 showed maximum variation (Fig. 1).

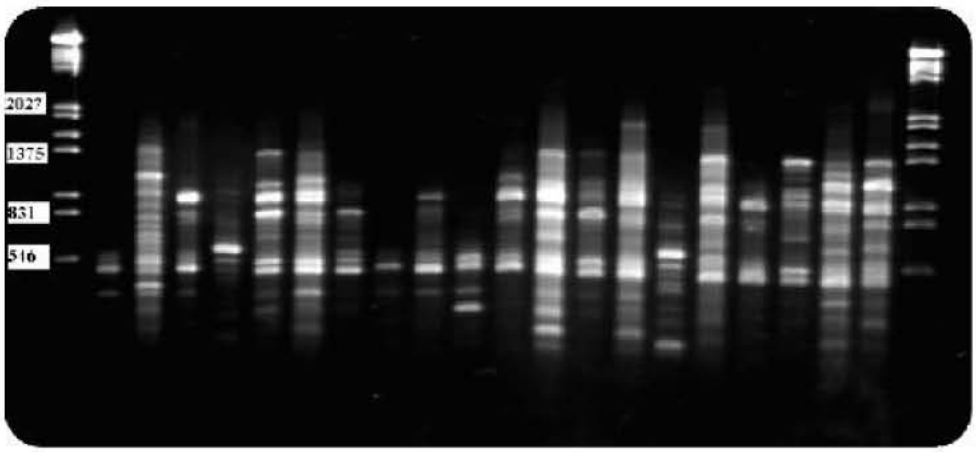

Fig. 1: Representation RAPD profile *Fusarium oxysporum f. sp. lentis*. Amplification of the genomic was performed using primer OPA-02

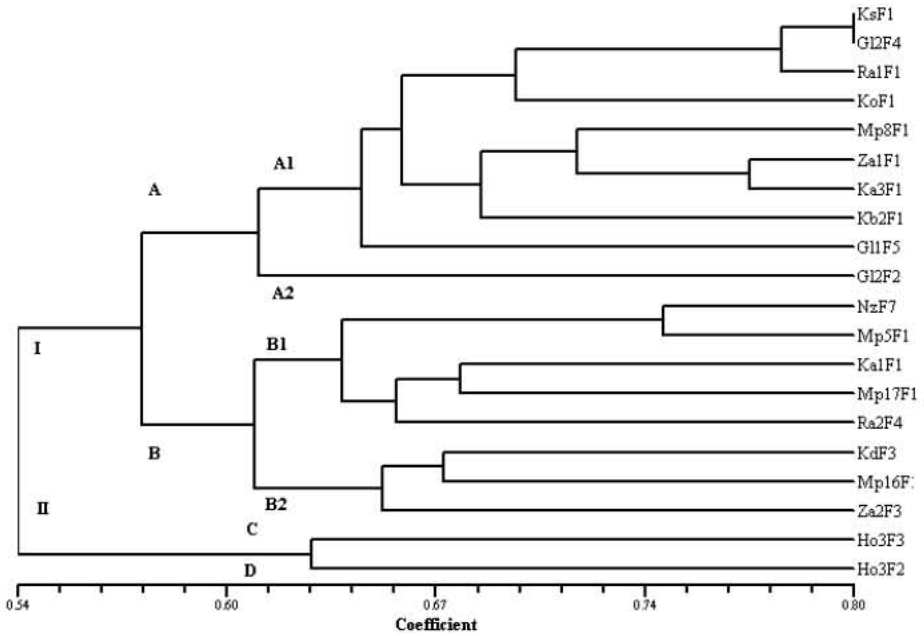

Fig. 2: Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 20 *Fusarium oxysporum f.sp. lentis* isolates listed in Table 2. Genetic similarity obtained by RAPD marker.

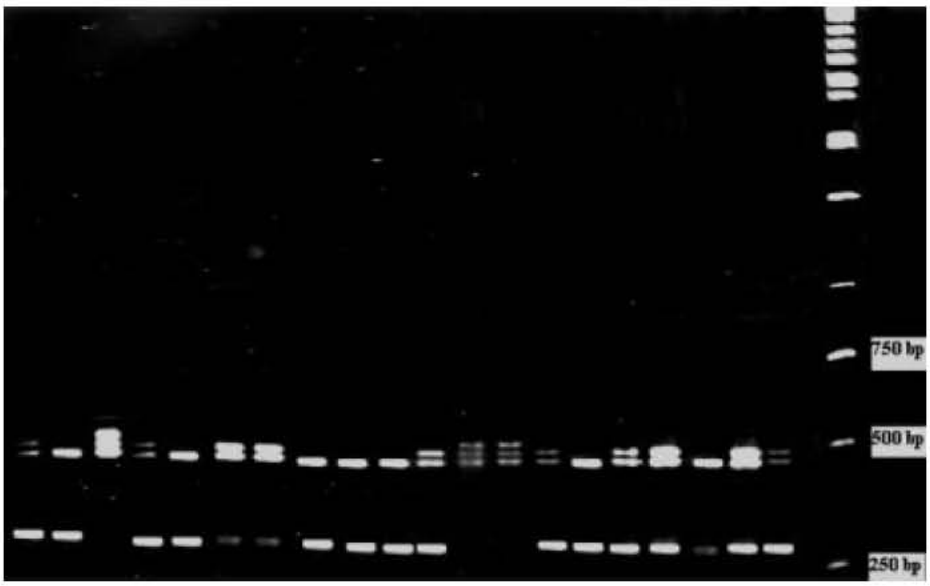

Fig. 3: Representation IGS profile *Fusarium oxysporum* f.sp. *lentis*. Restriction of the rDNA was performed using primer HaeIII

Dendrogram of RAPD divided two clusters I and II based on similarity value 0.54, each of them are separated again in two clusters A and B with similarity value of 0.57, each of them are separated again into two other cluster at similarity value of 0.61 (Fig. 2).

Between isolates that were in these clusters there was no apparent correlation with climatic area and pathogenicity score of isolates. Cluster II divided in to two cluster C and D at similarity value of 0.63 and each of them included one isolate only that corresponded to the same climatic area and field, but Ho3F2 isolate was nonpathogenic versus ILL4605 wherase Ho3F3 was pathogenic. Only 4 primers (UBC83, OPA-02, OPB-11 and OPK19) from 14 primer showed variation, maybe this

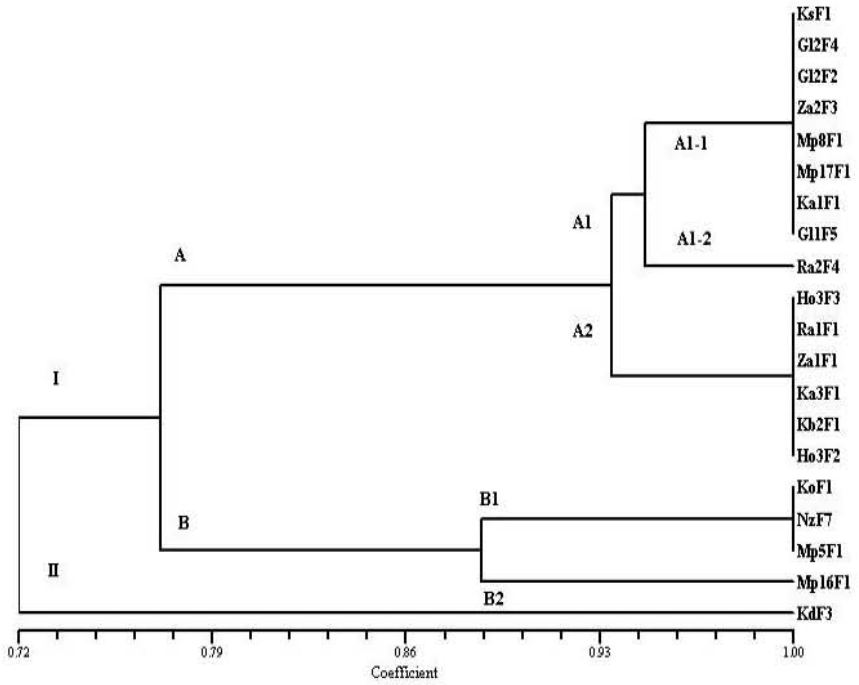

Fig. 4: Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 20 *Fusarium oxysporum* f.sp. *lentis* isolates listed in Table 2. Genetic similarity obtained by IGS marker

difference in amplification fragment corresponded to pathogenicity/ nonpathogenicity of this two isolates. IGS amplified a fragment of DNA, this fragment was 2.6 Kbp in total of 20 isolate. Restriction with 2 enzymes HaeIII (Fig. 3) and RsaI caused 3-4 bands and *Eco*RI showed 2 bands, one of them 0.4 Kbp and the other 2 Kbp also one of isolates didn 't have restrict region of *Eco*RI.

This discrepancy could have been caused by failure to detect very small fragment or by co-migrating fragments that couldn't be resolved. The IGS profile separated two cluster I and II at similarity value 0.72 (Fig. 4). Cluster II include only one isolate that *Eco*RI couldn't restrict this isolate. Cluster I divided into two groups of A and B at similarity value 0.77. Each of them is separated again into smaller clusters.

Groupe A1 and B1 isolates were from the same climatic area of the Northern Khorasan and Razavi provinces but groupe A2 couldn't separate climatic area, maybe if we used more restriction enzymes, climatic areas could be separated.

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