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Physiological Race and Genetic Diversity Determination of *Fusarium oxysporum* f.sp. *melonis* by Differential Hosts and Molecular Marker Rapd in Northern and Razavi Khorasan Provinces

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Abstract: Fusarium wilt of melon, caused by *Fusarium oxysporum* f.sp. *melonis* is one of the most important diseases of this crop, which annually causes tremendous losses. Because of the importance of this disease in Northern and Razavi Khorasan provinces, race identification and genetic diversity of the pathogen was investigated by using the differential standard hosts cultivars and molecular marker RAPD. Total 15 from 20 isolates which obtained from 52 fields located at different geographical regions were identified as pathogenic in pathogenicity test. In formae speciales test, seedling of melon, cucumber, watermelon and pea were used and typical symptoms of disease observed on melon plants only, therefore *F. oxysporum* f.sp. *melonis* was identified as the cause of wilting disease in melon. At race determination test, differential hosts were used and the existence of race 1 in the regions was proved. The result of RAPD-PCR bands showed a polymorphism among population of this fungus. Cluster analysis showed that there are 7 genotypic groups in *Fusarium oxysporum* f.sp. *melonis* population which designated as A to G.

Key words: Melon, fusarium wilt, *Fusarium oxysporum* f.sp. *melonis*, RAPD-PCR, polymorphism

INTRODUCTION

Melon (*Cucumis melo* L.) is grown in many parts of Iran. Northern and Razavi Khorasan provinces are the two major area with more than 45000 ha under cultivation. Fusarium wilt of melon caused by *Fusarium oxysporum* f.sp *melonis* induces considerable yield loss annually (Jahan Bakhsh, 1998). With attention to the high level of host specify, Snyder and Hansen identified more than 150, formae speciales for *F. oxysporum* (Sar Pelleh and Bani Hashemi, 2001). In *F. oxysporum* f.sp. *melonis*, 4 common races exist worldwide and have been designated as 0, 1, 2 and 1, 2 (Risser *et al*., 1976). Due to the nature of this pathogen, the disease is best managed with wilt-resistant cultivars (Zink and Thomac, 1990). However, as resistant cultivars are utilized, new virulent populations (physiological races) may develop. For this reason, race and genetic diversity determination in pathogen population is important. Specific differential hosts that are used for physiological races determination have specific resistance genes that overcome specific virulent genes in pathogen (Zink and Thomac, 1990).

There are some limitations in using morphological factors for grouping of isolates, including environmental factors. Therefore, it is recommended to use molecular and biochemical markers that are more reliable in identification of genetic diversity in this fungus. RAPD, RFLP, AFLP and Isozymes are markers that have been used to investigate the population diversity of *F. oxysporum* f.sp. *melonis* (Shokoohi *et al*., 2004). In spite of this fact that there are some problems such as, disability in repetition, susceptibility to condition of reaction and long time optimatization, RAPD-PCR is used broadly. In this research, race identification and genetic diversity determination of *F. oxysporum* f.sp. *melonis* population was carried out.

MATERIALS AND METHODS

In order to obtain isolates of *F. oxysporum* f.sp. *melonis*, sampling was carried out from melon fields in Northern and Razavi Khorasan provinces in 2004-2005 years. These areas were included: Torbat jaam, Sarakhs, Taibad, Khaaf, Kashmar, Farouj and Torbat heidariye. Infected plants were collected on the basis of symptoms such as damping-off, yellowing, wilting and excretory gum from stem.

For isolation of the pathogen, Potato Dextrose Agar (PDA) culture medium and for single sporing, Water Agar (WA) culture medium were used. In order to force the

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fungus to produce sporodochium, Carnation Leaf Agar (CLA) culture medium was employed. For measurement of colony diameter, isolates were cultured on acid free PDA and kept in dark for 72 h at 25°C. *Fusarium oxysporum* was recognized on the basis of Nelson identification key with attention to morphological characters (Nelson *et al.*, 1983).

Pathogenicity tests: Artificial inoculation was performed under greenhouse condition (temp: 25°C at day and 17°C at night; photoperiod: 12 h light/12 h dark). The used inoculum was fungal suspension containing 10^6 conidia mL^{-1} collected from 5-days-old PDA cultures of *F. oxyporum* f.sp. *melonis* isolates. Standard cv Charentais T, was used in this experiment (Burger *et al.*, 2003). After removing the seedling, roots were washed in tap water and pruned to about half of their length, inoculated for 7-10 min with conidial suspension and then transplanted to pots filled with equal ratio of sand, soil and humus. Recording symptoms was carried out every day from first symptom appearance on the plants to the 21 day after inoculation. Symptoms were divided to 5 groups (Perchepied and Pitrat, 2004) (Table 1).

Formae speciales test: The plants used for this test were melon (*Cucumis melo* L), standard cv. (Charentais T), cv. (Khatooni), cucumber cv. (Super Dominous), watermelon cv. (Crimpson Sweet), pea cv. (Jam). Root dipping method used for inoculation with fungal suspension containing 10^6 conidia mL^{-1} according to the method that mentioned in pathogenicity tests. Recording of symptoms continued up to the 21 days after inoculation (Burger *et al.*, 2003).

Physiological race determination: Differential hosts that used for this test were Charentais T, Charentais Fom 1, Charentais Fom 2, Margot and Isabelle which were sent by Dr. Tom Gordon at Davis University and Dr. Michael Pitrat at Institute National de la Research Agronomique, INRA Institute in France. Root dipping method was used in this test as pathogenicity and formae speciales tests.

RAPD-PCR test: Isolates of *F. oxysporum* f. sp. *melonis* were cultured on potato dextrose broth (PDB) medium and maintained for 3-4 days on shaker with 120 rpm and at 20-22°C. For DNA extraction Cetyl Trimetyl Ammonium Bromide (CTAB) protocol and in RAPD-PCR 10 primers were used (Table 2). Optimatization of RAPD-PCR included, applying some aggregative materials such as Triton (5 mL), increasing the quantity of $MgCl_2$ from 1 to 2 mL and decreasing DNA from 4 to 3 mL, increasing the cycle number in denaturation, annealing and extension phase from 40-44 that caused better results and improvement in PCR reaction.

Table 1: Grouping of symptoms in pathogenicity test

Code of virulence	symptoms
1	Without symptom
2	Wilting and yellowing in cotyledons
3	Wilting and yellowing in the two first leaves
4	Wilting and yellowing in three or more first leaves
5	Damping-off

Table 2: Sequence of primers used in RAPD-PCR test

Primer	Sequence
VBC83	GGG-CTC-GTG-G
VBC199	GCT-CCC-CCA-C
VBC228	GCT-GGG-CCG-A
VBC222	AAG-CCT-CCC-
VBC300	GGC-TAG-GGC-G
VBC53	CTC-CCT-GAG-C
VBC82	GGG-CCC-GAG-G
OPK19	CTC-CTG -CCA-C
OPK15	CCT-GGG-CCT-A
VDC6	AAG-CCT-CCC-C

RAPD analysis: Molecular weight of bands were measured with UVIGEL software. Size marker Lambda DNA/Hind Ø, Ecor 1 digest having 1 kbp weight was used. For genetic distance calculation and cluster analysis POPGEN, Minitab and JMP software were employed respectively.

RESULTS AND DISCUSSION

In pathogenicity test 15 isolates were known as virulent (Table 3). Damping-off created by Fth_1, Ft_1 and Fs_2 isolates. Medium and High level of wilting and yellowing established by Fk_3, Ff_3, Fs_1, Fs_5 and Fk_1, Fkh_1, Fs_3, Ft_2, Fth_2, isolated, respectively. The others did not show any symptoms.

Distinguished symptoms such as damping-off and vascular wilting was established only in melon plants confirming that the pathogenic isolates are *F. oxysporum* f.sp. *melonis* (Table 4).

In race determination test, all isolates were virulent on susceptible cv. Charentaise T that dose not have any resistance gene and on cv. Charentais Fom1 that has the Fom-1 resistance gene. The other differential hosts did not show any symptoms. In other words, the isolates were only able to overcome Fom 1 resistant gene, therefore indicating the presence of race 1 in the region (Table 5).

Pathogen was isolated in all plant growth stages, specially in seedling and fruit ripening, whereas temperature differences in these 2 stages was about 12 ^{PP}C at sampling regions. The lack of disease symptoms in other mentioned crops except melon, indicates the host specificity toward *Cucumis melo* and proves the existence of *F. oxysporum* f.sp. *melonis*. Amplification of

Table 3: Grouping of isolates in pathogenicity test

Isolate	Symptom	Part of plant	Fields	Region
Fm$_1$	2	Stem	Amaanrghan	Mashhad
Fm$_2$	1	Stem	Mashhad	Mashhad
Fh$_1$	1	Crown	Dizbad	Mashhad
Fth$_1$	5	Crown	Fath abad	Torbat
Fth$_2$	4	Stem	Momen abad	Torbat
Fta$_1$	1	Crown	Taibad	Taibad
Fk$_1$	4	Stem	Ghochd	Kashmar
Fk$_2$	2	Stem	Kashmar	Kashmar
Fk$_3$	3	Stem	[illegible]	Kashmar
Fkh$_1$	4	Crown	Khaaf	Khaaf
Ft$_1$	5	Crown	Haji abad	Torbat jaam
Ft$_2$	4	Crown	Kate shamshir	Torbat jaam
Fs$_1$	3	Stem	Sarakhs	Sarakhs
Fs$_2$	5	Stem	Sarakhs	Sarakhs
Fs$_3$	4	Stem	Ali abad	Sarakhs
Fs$_4$	2	Root	Sarakhs	Sarakhs
Fs$_5$	3	Stem	Sarakhs	Sarakhs
Ffn$_1$	1	Stem	Fariman	Fariman
Ff$_2$	1	Crown	Farouj	Farouj
Ff$_3$	3	Stem	Farouj	Farouj

Isolate naming is on the basis of the first letter of the name of fungus, the first letter of the sampling place and the number of isolate. For example in Fm$_1$: F= Fusarium, m= Mashhad, 1= Number of isolate

Table 4: Reaction of different cultivars to *F. oxysporum* f. sp. *Melonis*

Isolate	Jamm	Super dominous	Crimps on sweet	Khatooni	Charentais T
Fm$_1$	1	1	1	2	2
Fth$_1$	1	1	1	5	5
Fth$_2$	1	1	1	3	4
Fk$_1$	1	1	1	2	4
Fk$_2$	1	1	1	2	2
Fk$_3$	1	1	1	3	3
Fkh$_1$	1	1	1	3	4
Ft$_1$	1	1	1	4	5
Ft$_2$	1	1	1	3	4
Fs$_1$	1	1	1	2	3
Fs$_2$	1	1	1	4	5
Fs$_3$	1	1	1	4	4
Fs$_4$	1	1	1	2	2
Fs$_5$	1	1	1	2	3
Ff$_3$	1	1	1	3	3

Table 5: Race determination and reaction of differential hosts to isolates of *F. oxysporm* f. sp. *Melonis*

Isolate	Charentais T	Charentais Fom-1	Charentais Fom-2	Margot	Isabelle
Fm$_1$	2	2	1	1	1
Fth$_1$	4	3	1	1	1
Fth$_2$	4	4	1	1	1
Fk$_1$	3	3	1	1	1
Fk$_2$	2	2	1	1	1
Fk$_3$	2	3	1	1	1
Fkh$_1$	3	2	1	1	1
Ft$_1$	4	3	1	1	1
Ft$_2$	3	2	1	1	1
Fs$_1$	4	3	1	1	1
Fs$_2$	5	4	1	1	1
Fs$_3$	4	4	1	1	1
Fs$_4$	3	3	1	1	1
Fs$_5$	2	2	1	1	1
Ff$_3$	2	2	1	1	1

F. oxysporum f.sp. *melonis* DNA using the RAPD technique produced clear, reproducible and polymorphic bands that allowed the characterization of isolates

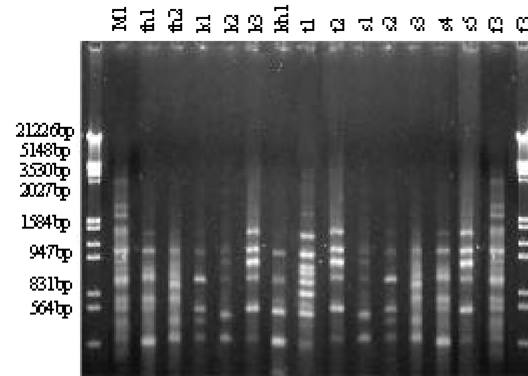

Fig. 1: The bands that have been created by using primer OPK 19 in RAPD-PCR test

examined in this study. Cluster analysis of the RAPD banding pattern data, however, revealed a substantial amount of genetic diversity among all the isolates examined (Fig. 1 and 2). In cluster analysis on the basis of genetic distance, the shortest distance observed among (Fk$_1$ and Fkh$_1$), (Fs$_1$ and Fs$_2$) and (Fth$_1$ and Fm$_1$) isolates, from Kashmar, Khaaf, Sarakhs, Torbat heidariye and Mashhad, respectively. Whereas the maximum genetic distance was between Fm$_1$ and Ft$_1$ from Mashhad and Torbat jaam, indicating the least genetic similarity among these isolates. Based on 60% similarity, 7 genotypic groups were classified designated as A to G (Fig. 3).

Groups A and E each had 4 members. Group B and F had 2 members and the others had only one member (Fig. 3). Isolates from Mashhad, Torbat heidariye, Kashmar and Khaaf were in group A. Sarakhs and Torbat jaam isolates were in group E. Group B included isolates from Torbat heidariye and Kashmar and group F included Sarakhs and Farouj isolates. In spite of A, B, E and F members being derived from different geographical regions, all of them settled in one genotypic group. The high amount of genetic diversity resulted in virtually no clusters that clearly identified geographical origin or pathogenicity of the isolates. Therefore, geographical region can not be used for determining genetic diversity as a suitable measure because it is affected by mutation and selective force of host. Analysis of cluster showed 7 genotypic groups. With attention to this fact that all pathogenic isolates belonged to race 1, we can say that there are high level of genetic diversity in this race.

Isolates with same morphological and microscopical characteristics have been placed in different groups, therefore morphological characters is not an assured factor for distinguishing the isolates.

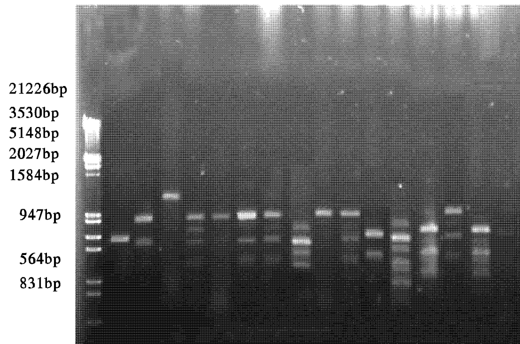

Fig. 2: The bands that have been created by using primer VBC 222 in RAPD-PCR test

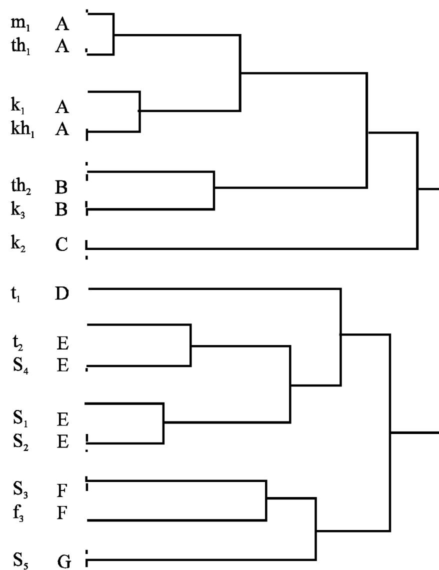

Fig. 3: Dendrogram of combinated data of primers, depicted by JMP software and create 7 genotypic groups

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