Genetical diversity analysis of Iranian Fusarium oxysporum f.sp. melonis by PCR - RAPD marker

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ABSTRACT: Fusariums wilt of melon, Fusarium oxysporum f.sp. melonis is one of the most important diseases, causing tremendous losses in melon growing areas in Iran. Searching for resistant sources needs detail knowledge of the pathogen. Thus, genetical diversity of the pathogen were assessed on various isolates through out of important melon's growing areas, using differential standard hosts and molecular marker PCR - RAPD. The results indicated that, eighty percent of the isolates are pathogenic to the melon crops. Forma specials proving tests on seedlings of melon, cucumber, watermelon and pea showed typical symptoms of the disease on melon plants, confirming the only forma specials of melon. For the race identification tests, differential hosts were used, out of which, the existence of race one was approved. PCR - RAPD profile showed a polymorphism among populations of the pathogen and cluster analysis divided them into seven genotypic groups accordingly.

Keywords: Melon, Fusarium wilt, forma specials, PCR - RAPD, races

INTRODUCTION

Fusariums wilt of melon caused by Fusarium oxysporum Schlechtend. Emend. W.C. Snyder & H.N. Hans. f. sp melonis Leach & Currence inflict considerable yield loss annually (Burger et al., 2003). Because of the high ranges of host specify, Snyder and Hansen identified more than 150 form specials for F. oxysporum (Burgess and Summerell., 1994). Form specials are divided to races according to the disease symptoms of different cultivars of a plant species. In F. oxysporum f.sp. melonis, four common races exist worldwide and have been designed as 0, 1, 2, and 1, 2 (Shahriyari and Emamjome, 2003). Due to the persistent nature of this pathogen, the disease is best managed with wilt-resistant cultivars. However as resistant cultivars are utilized, new virulent populations (physiological races) may develop in specific locations. For this reason, race and genetic diversity determination in pathogen is important. Specific differential hosts used for physiological races determination ( Udupa et al., 1998).

Differential hosts have specific resistance genes that overcome specific virulent genes in pathogen and causes different reaction against it. 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 have more susceptibility and accuracy in identification of the fungus. RAPD, RFLP, AFLP and isozyme are markers that have been used to investigate the population diversity of F.oxysporum f.sp. melonis (Omamor et al., 2006). 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 (Thangaraj et al., 2011, Cruz et al.,1999).

The overall objective of this study was to determine race and or races and genetic diversity of F. o. f.sp. melonis of Melons (Cucumis melo.L) in the major melon growing areas in Iran.
MATERIALS AND METHODS

Collection of F. o. f.sp. melonis. Sampling was carried out from melon fields in Northern and Razavi Khorasan provinces (its geographical coordinates are 35° 42' 0" North, 47° 16' 0" East) including Torbat jaam, Sarakhs, Taibad, Khaaf, Kashmar, Farouj, Torbat heidariye areas in the years 2004-2005. Infected plants were collected randomly on the basis of symptoms such as damping-off, yellowing, wilting and excretory gum from stems.

For isolating 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 fungus to produce sporodochium, carnation leaf agar (CLA) culture medium was used. For measurement of colony diameter, isolates were cultured on acid free PDA and keep in dark for 72 hours at 25°C. Fusarium oxysporum was recognized on the basis of the Nelson identification keys with attention to morphological characters (Zink and Thomac, 1990).

Pathogenicity tests
Artificial inoculation was performed under controlled greenhouse condition (temp: 25°C at day and 17°C at night; photoperiod: 12h light/ 12h dark). The used inoculums was a fungal suspension containing 10⁶ conidia ml⁻¹ collected from 5-10 days' old PDA cultures of F. oxysporum f.sp. melonis isolates. Standard cv Charentais T was used in this experiment (Perchepied and Pitrat, 2004). After removing the seedling, roots were washed in tap water and pruned to about half of their length, inoculated for 7-10 minutes with conidial suspension and then transplanted to pots filled with equal ratio of sand, soil and humus. Recording symptoms continued every day from first symptom appearance on the plants to the 21 day after inoculation. Symptoms were divided to six groups named as 0 to 5. Group 0 contained no symptoms, group1: wilting and yellowing in cotyledons, group2: wilting and yellowing in the two first leaves, group3: wilting and yellowing in two first leaves, group 4: wilting and yellowing symptoms in the three or more first leaves and group5: with damping off symptoms (Perchepied and Pitrat, 2004).

Form special 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) and weed Porslane (Portulaca oleracea ) which was prevalent in melon fields.

Root dipping method used for inoculating and fungal suspension containing 10⁶ conidia ml⁻¹ in according to the method that mentioned in pathogenicity tests. Recording of symptoms continued to the 21 days after inoculation (Perchepied and Pitrat, 2004).

Physiological race determination
Differential hosts that used for this test were Charentais T, Charentais Fom1, Charentais Fom2, Margot and Isabelle which were sent by Dr Gordon at Davis University and Dr Pitrat at Institute National de la Recherche Agronomique. INRA Institute in France.

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 Trimethyl Ammonium Bromide (CTAB) protocol and in RAPD-PCR 10 primers were used. The primers were VBC83, VBC199, VBC228, VBC222, VBC300, VBC53, VBC82, OPK19, OPK15 and VBC6 and their sequences were GGG-CTC-GTG-G, GCT-CCC-CCA-C, GCT-GGG-CGG-A, AAG-CCT-CCC-C, GCC-TAG-GGC-G, CTC-CCT-GAG-C, GGG-CCC-GAG-G, CTC-CTG-CCA-C, CCT-GGG-CCT-A and AAG-CCT-CCC-C respectively. Optimization of RAPD-PCR included, applying some aggregative materials such as Triton (5 ml), increasing the quantity of MgCl₂ 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 to 44 that caused better results and improvement in PCR reaction (Fonseca et al., 2008).

RAPD analysis
Molecular weight of bands was measured with UVIGEL software. Size marker Lambda DNA/Hind 111, Ecor 1 digest having 1 kbp weight was used. For genetic distance calculation and cluster analysis POPGEN, Minitab and JMP software were used respectively (Mahmood et al., 2002, Cumagun et al., 2007).
RESULTS AND DISCUSSION

In pathogenicity test, 15 isolates were known as virulent. Damping-off created by Fth1, Ft1 and Fs2 isolates. High and medium level of wilting and yellowing established by Fk1, Fkh1, Fs3, Ft3, Fth2 and Fk3, Ff3, Ft1 and Fs5, respectively. The others did not create any symptoms of disease. These results confirm the findings by Burger et al., (2003) indicating that, Fusarium oxysporum f.sp. melonis is highly pathogenic.

Distinguished symptoms such as damping-off and vascular wilting were established only on melon plants. In race determination test, all isolates were virulent on susceptible cv. Charentaise T that dose not 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 Fom1 resistant gene, therefore indicating the presence of race 1 in the region which is in agreement with others reports (Shahriyari and Emamjome, 2003).

Table 1. Reaction of isolates to F. oxysporum f. sp. melonis in Race Determination and Formae Special tests

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>Name of isolate</th>
<th>Region</th>
<th>Average of Disease Severity (%)</th>
<th>differential hosts used in Race Determination test</th>
<th>Results of formae special</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fm1</td>
<td>Mashhad</td>
<td>40.00</td>
<td>Charentais T, Charentais Fom-1, Charentais Fom-2</td>
<td>Melon cv. Charentais T, Melon cv. Khatooni, Watermelon cv. Crimson sweet, Cucumber cv. Super dominoes, Peac Jam porslane</td>
</tr>
<tr>
<td>2</td>
<td>Fth1</td>
<td>Torbat heidariy</td>
<td>100.00</td>
<td>100.00, 100.00</td>
<td>75.00, 75.00, 50.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>3</td>
<td>Fh2</td>
<td>Torbat heidariy</td>
<td>81.25</td>
<td>75.00, 75.00</td>
<td>75.00, 75.00, 50.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>4</td>
<td>Fk1</td>
<td>Kashmir</td>
<td>81.25</td>
<td>50.00, 50.00</td>
<td>75.00, 25.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>5</td>
<td>Fs</td>
<td>Kashmar</td>
<td>40.00</td>
<td>25.00, 25.00</td>
<td>25.00, 10.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>6</td>
<td>Fk3</td>
<td>Kashmar</td>
<td>62.50</td>
<td>25.00, 50.00</td>
<td>50.00, 25.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>7</td>
<td>Fkh1</td>
<td>Khaf</td>
<td>81.25</td>
<td>50.00, 25.00</td>
<td>75.00, 50.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>8</td>
<td>Ft1</td>
<td>Torbat jaam</td>
<td>100.00</td>
<td>75.00, 50.00</td>
<td>100.00, 75.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>9</td>
<td>Ft2</td>
<td>Torbat jaam</td>
<td>81.25</td>
<td>50.00, 25.00</td>
<td>75.00, 50.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>10</td>
<td>Fs</td>
<td>Sarakhs</td>
<td>62.50</td>
<td>75.00, 50.00</td>
<td>50.00, 10.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>11</td>
<td>Fs</td>
<td>Sarakhs</td>
<td>100.00</td>
<td>100.00, 75.00</td>
<td>100.00, 75.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>12</td>
<td>Fs</td>
<td>Sarakhs</td>
<td>81.25</td>
<td>75.00, 75.00</td>
<td>75.00, 75.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>13</td>
<td>Fs</td>
<td>Sarakhs</td>
<td>40.00</td>
<td>50.00, 50.00</td>
<td>25.00, 25.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>14</td>
<td>Fs</td>
<td>Sarakhs</td>
<td>62.50</td>
<td>10.00, 10.00</td>
<td>50.00, 25.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
<tr>
<td>15</td>
<td>Fs</td>
<td>Farouj</td>
<td>62.50</td>
<td>25.00, 25.00</td>
<td>50.00, 50.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00</td>
</tr>
</tbody>
</table>

*All the results are in percent(%)*

Pathogen can be isolated in all plant growth stages, specially in seedling and fruit ripening, whereas temperature differences in these two stages was about 12º C at sampling regions. The lack of disease symptoms in other mentioned crops except melon, corroborates the host specificity toward Cucumis melo and proves the existence of F. oxysporum f.sp. melonis which is in agreement (Udupa et al., 1998) findings.

Amplification of F. o f. sp. melonis DNA using the RAPD technique produced clear, reproducible and polymorphic bands that allowed the characterization of isolates 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. Therefore, it is recommended to use molecular and biochemical markers that have more susceptibility and accuracy in identification of the fungus, because there are some limitations in using morphological factors for grouping of isolates, including environmental factors (Omamor et al., 2006).

In cluster analysis on the basis of genetic distance, the shortest distance observed among (Fk1,Fkh1), (Fs1 & Fs2) and (Fth1 & Fm1) isolates, from Kashmir, Khaaf, Sarakhs, Torbat Heidariy and Mashhad respectively. Whereas the maximum genetic distance was between Fm1 and Ft1 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, obtained (Figure 3). These findings showed that there are variations among the isolates, which are very distinct, therefore in spite of this fact that, there are some problems such as, disability in repetition, susceptibility to condition of reaction and long time optimization, RAPD-PCR can be used for the distinguishing of
the isolates of *F. oxysporum* f.sp. *melonis*, as was used previously by others. (Thangaraj et al., 2011; Cruz et al., 1999).

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

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

Groups A and E each had 4 members. Group B and F had 2 members and the others had only one member. 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 an exact factor 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.

This fact shows that geographical region factor cannot be used for this purpose. 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.

A potential application of the results obtained in this study is the development of a molecular marker, such as a sequenced characterized amplified region (SCAR), to distinguish F.o.m isolates. A natural marker such as a RAPD could be used for diagnostic purposes, thus more research is needed to identity polymorphic genes with alleles specific to individual formae speciales which should lead to a greater understanding of the F. oxysporum complex.

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