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Study on pathogenic and genetic diversity of *Alternaria alternata* isolated from citrus hybrids of Iran, based on RAPD-PCR technique

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ABSTRACT

Different pathotypes of Alternaria alternata (Fr.:Fr.) Kessl, cause various diseases in citrus including Alternaria brown spot of tangerines, leaf spot in rough lemon and Alternaria black rot on citrus fruits. In order to study genetical structure of A. alternata populations, 36 isolates with the highest diversity in morphological traits and pathogenicity test were chosen from 87 isolates collected from infected leaves, fruits and twigs of various citrus gardens of Iran. Genetic diversity was analyzed based on RAPD-PCR using 15 random 10mer primers. These primers were among 35 random primers, based on clarity, repeatability and the number of polymorphic bands. Cluster analysis of DNA fragments was performed using NTSYSpc V2.2 based on UPGMA method and Jacard coefficient. Results of pathogenicity test showed obvious diversity in Alternaria isolates and they were classified as three groups: severe, moderate and low pathogenic. Cluster analysis of RAPD fragments of all primers revealed that isolates were classified in five groups at 85% similarity level. Molecular diversity of isolates was highly related to geographical region, host and partly virulence of isolates. This is the first study about genetic diversity of Iranian isolates of A. alternata fungus using RAPD molecular marker in its populations.

Keywords: *Alternaria alternata*, citrus, genetical diversity, RAPD, PCR.

INTRODUCTION

Alternaria fungus has about one hundred species which can be found in various places all over the world. Many of them are important pathogens of plants and cause important economical disease in wide range of hosts. Some of them live Saprophytic and are main part of fungi population in soil and dead or dying plant tissues [11-12-13]. Polyphagous nature and ability of them in producing toxic and carcinogenic materials indicates that alternaria is potentially hazardous to human and animals health [4-7]. This pathogen has a wide range (more than 380) of hosts in Iran including citrus, pistachio, apple, pear, tobacco, tomato, and beens [15]. *Alternaria alternata* has an important place among species of this genus, because of wide range of hosts including garden plants, field crops, vegetables, and ornamentals. This fungus is one of the important pathogens of citrus which cause brown spot by tangerine pathotype, leaf spot by rough lemon pathotype, and black rot of harvested fruits.

Alternaria pathotypes which cause brown spot in Tangelo and leaf spot in rough lemon are distinguished from black rot disease of citrus fruits by their toxin production and attack to aerial organs [5-6-1]. Population of this pathogen shows high diversity in various citrus species. This pathogen attacks to all aerial organs of plants, especially fruits, and causes considerable loss in years with suitable weather conditions for disease [1]. Pathogen causes leaves defoliation in sensitive varieties and acts as a limiting factor of these varieties production in humid regions. Even in arid regions, stained skin of citrus fruits significantly reduces marketing of them [15]. Brown spot is now the most important disease of tangerines and their hybrids in world. In Florida it attacks to Tangelo mandarine mainly, but causes also losses in other varieties like Tangor Murcott, Nova, Lee and Page [9]. Leaf spot causes on rough lemon and Rangpur lime [16] and black rot which is called the rottenness of fruits, causes loss of navel oranges mainly [16].

For controlling this pathogen, various methods including chemical control like using different fungicides [3-14-15], cultural and physical practices have been used but none of them have been completely successful in this way.

Then, to adopt an appropriate controlling method, accurate recognition of disease and comprehensive information about population dynamic of pathogen is essential and lack of information has led to non-efficient mentioned methods. Wide studies are conducting in world about genetical diversity of this pathogen populations [6-8-9-16]

In a pathogen population, high genetical diversity increases resistant appearance of pathogen to fungicides. On the other hand, resistance stability or tolerance stability of host varieties are not predictable without knowing genetical structure of pathogen. Then, with correct information about genetical structure of population and good management in breeding plans, appropriate cultivars can be produced for various regions or different pathogen populations which this will be led to tolerance stability in these regions. One of the other positive features of this study is pathogen tracking, by using genetical pattern of fungus isolates; we can study distribution of it. Studying diversity of *A. alternata* populations has been focused in world permanently, but there are no many studies extant on genetical diversity and distribution of this fungus in our country in spite of its importance and damages. Since genetical study of pathogens will be led to determining genetical diversity of them and their adaption to different methods of pathogen management. These studies will eventually improve methods of sustainable management. Considering mentioned facts, determining the genetical diversity of Iranian *A.alternata* populations on citrus seems very necessary.

MATERIALS AND METHODS

Pathogen isolation

Eighty seven isolates of *A. Alternata* were used in this study. Among them, 25 isolates were received from fungi collection of Iranian Institute of Citrus Researches (Ramsar, Iran). These were collected from main citrus gardens of country, during years 1995 to 2006. 62 isolates were collected from various regions of Mazandaran, Gilan, Golestan, and Fars provinces during years 2006 to 2011.

Pathogenicity test

After single sporing and morphological identification of isolates, evaluating pathogenicity of isolates was done using Peever et al. Method [8] in a completely randomized design with four replications for each isolate. Pathogenicity test was conducted on detach leaves of Page mandarin which is one of the most important varieties in north region of Iran.

For each isolate of *A. alternata*, four young healthy leaves were selected and transferred to laboratory. Leaves were washed by water and were dried by tissue paper. Detached leaves were placed in microcentrifuge tubes filled with water by inserting petioles into water and sealing with parafilm. In this method, suspension of conidia (10^5 conidia /ml) was prepared from 5-7 days old PDA cultivated isolates. For regulating concentration of suspension, the number of conidia per one drop of it was counted by hemacytometer slide.

After inoculation, leaves were incubated for seven days at 25 °C. Determining the virulence severity of isolates was done also according to Peever et al. [8], following numbers have been defined: 0 = without disease symptomse, 1= 1-20 % leaf spot, 2= 20-40% leaf spot, 3= 40-60% leaf spot, 4= 60-80% leaf spot, 5= full leaf spot and leaf death.

Study of genetic diversity

In order to determine genetical diversity of fungi isolates, 15 randomize 10 oligomer primers were used in RAPD-PCR (table1). Preparing mycelia mass of pathogen isolates was done in 2-YEG liquid medium (2g of yeast extract plus 10 g glucose in one liter of water). Three discs (5mm) were taken from margins of 5 days old pathogen colonies and transferred to Erlenmeyer flasks which had 50 ml of 2-YEG liquid medium. After that, they incubated at 25°C and after five days, mycelia mass was collected.

Table1- specifications of random primers used in RAPD-PCR reaction

No	Primer	Sequence
1	UBC 203	CACGGCGAGT
2	UBC 208	ACGGCCGACC
3	UBC 211	GAAGCGCGAT
4	UBC 213	CAGCGAACTA
5	UBC 214	CATGTGCTTG
6	UBC 283	CGGCCACCGT
7	UBC 285	GGGCGCCTAG
8	UBC 286	CGGAGCCGGC
9	Takapoozist 1	CGTTGGATGC
10	Takapoozist 2	CCAGACAAGC
11	Takapoozist 7	CCGGCCTTAG
12	Takapoozist 8	CCTGGGCCTC
13	Takapoozist 9	CCTGGGCTGG
14	Takapoozist 10	CCGGCCCCAA
15	CinnaGene 9	CCTGGGCCT<A>

DNA Extraction

The total DNA of isolates were extracted from any age of leaves according to Peever et al. (2002) with some modification: Briefly, lyophilized, powdered mycelium (approximately 50 µg) was extracted using a lysis buffer containing 50 mM EDTA, 100 mM Tris buffer (pH 8), and 3% sodium dodecyl sulfate for 30 to 45 min at 65°C. Mycelium was pelleted by centrifugation (1200 gn for 15 min) and the supernatant precipitated with 8 M potassium acetate at –20°C for 15 min. Following centrifugation, the supernatant was subjected to two rounds of phenol/chloroform isoamyl alcohol (25:24:1) extraction and one round of chloroform/isoamyl alcohol (24:1) extraction. The final supernatant was precipitated with two volumes of 100% EtOH and 0.5 M NaCl. Pellets were resuspended in water, precipitated again in 14% polyethylene glycol and 1 M NaCl, and resuspended in 50 µl of Tris-EDTA buffer (10 mM Tris and 1 mM EDTA). RNA was digested with 20 µg of RNase A (Sigma Chemical Co., St. Louis) per ml at 37°C for 3 h. DNA concentrations were estimated visually in ethidium bromide-stained agarose gels by comparing band intensity with known quantities of phage lambda DNA (Cinagen, Iran). At least three independent DNA extractions were made from each of several selected isolates to verify the repeatability of the polymerase chain reactions (PCRs) among different extractions.

RAPD-PCR (Randomly Amplified Polymorphism DNA-PCR) reaction

Polymerase chain reaction was performed in total reaction volume of 25 µl containing 1× PCR buffer, 200 µM of dNTPs; 2 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase; 100 ng primer; 50 ng DNA extract. Mixture of PCR reaction was prepared and after pouring in tubes, they were placed in thermo cycler machine. The following temperature cycle was used: an initial denaturation step of 5 min at 94 °C followed by 39 cycles of 40 sec at 94 °C, 40 sec at 34 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C [10]. 35 primers were studied in RAPD-PCR test at first. Primary study was done by six isolates to determine if there is identification place, and in this survey, 15 primers which showed better DNA polymorphism were selected. PCR reaction for 36 isolates- which had the most diversity in morphology and pathogenicity test- was done by 15 selected primers (table 1). Evaluating of PCR product was done on agars gel (1.2%). Nine micro liters of each reactions product plus one micro litter loading buffer (6X from 0.25% Boromo phenol blue and 40% w/v of sucrose) were electrophored at 2.5 volts/cm and then was visualized by staining with ethidium bromide [18]. RAPD bands were observed under UV lamps and were shot. Execution buffer of electrophoresis machine was TAE (0.04M Tris-acetate and 0.001 M Na_2EDTA). To data analysis of electrophoresis results, existence or none existence of each band was recorded in Excel as one and zero, respectively. Cluster analysis of isolates (by UPGMA method) was conducted in MVSP software.

RESULTS

Pathogenicity test

Pathogenicity of 33 isolates of *A. Alternata* (table2) was evaluated according to disease appearance (color changing and necrosis) on page tangerine leaves. After three days, leaves were removed from spore suspension and were graded from zero to five scales. With cultivating sterilized pieces of leaves (by sodium hypochlorite 0.5%) in PDA cultivation medium, presence of pathogen on them was certified. The results of this separation were *A. Alternata* cultivations which were morphologically similar to samples used for infection. No fungi isolate was separated from control plants (fig. 1).

Table2- characteristics of *A.alternata* selected isolates collected from various citrus cultivation regions of Iran

	Host name	Sampling date	Geographical region	Isolate code		Host name	Sampling date	Geographical region	Isolate code
1	Page tangerine	2007	Ramsar	NPR1	19	Thomson orange	2007	Chalous	Npmr19
2	Fortune tangerine	2007	Ramsar	NFR2	20	Page tangerine	2010	Nowshahr	PTCH20
3	Thomson orange	2007	Jouybar	PTJ3	21	Thomson orange	2009	Soleimanabad	PTNO21
4	Page tangerine	2010	Katra	Npkgh4	22	Thomson orange	2011	Amol	PTSO22
5	Thomson orange	2009	Abassabad	Ptab5	23	Thomson orange	2010	Saari	PTA23
6	Page tangerine	1998	Katra	Npksh6	24	Thomson orange	2010	Ghaemshahr	PTSA24
7	Kiwi	2007	Katra	Nmtk7	25	Thomson orange	2001	Babol	Ptgh25
8	Page tangerine	1998	Tonekabon	Npkh8	26	Thomson orange	2003	Rahimabad	PTB26
9	Page tangerine	2007	Katra	Npkh9	27	Thomson orange	2011	Rasht	Ptrh27
10	Mineola tangelo	2007	Ghaemshahr	Nmtk10	28	Thomson orange	2010	Babol	PTR28
11	Thomson orange	2011	Ramsar	PTGH11	29	Thomson orange	2006	Tonekabon	PTB29
12	Page tangerine	1997	Ramsar	NPR12	30	Thomson orange	2008	Jeeroft	PTT30
13	Thomson orange	1995	Chaboksar	PTR13	31	Thomson orange	2007	Jahrom	NMJ31
14	Thomson orange	2009	Ramsar	Ptch14	32	Local tangerine	2007	Jahrom	Lsh32
15	Thomson orange	2010	Ramsar	PTR15	33	Sweet lemon	2007	Jahrom	NAJ33
16	Tangerine	1999	Ramsar	NOR16	34	Sour orange	2010	Gorgan	PMJ34
17	Orlando tangelo	2007	Babol	NUR17	35	Local orange	2007	Tonekabon	NMJ35
18	Younesi tangerine	2007	Ramsar	PTB13	36	Sour orange	2007	Jahrom	NAJ36

Figure 1: Pathogenicity test of *A. Alternata* isolates on Page leaves in laboratory; A) inoculated citrus leaves with spore suspension filled by sterilized distilled water. B) Comparing disease symptoms on leaves one week after inoculation and grading them according to symptoms severity (B1: zero level: without symptoms; B2: level 1: leaf spot 1-20%; B3: level 2: leaf spot 20-40%; B4: level 3: leaf spot 40-60%; B5: level 4: leaf spot 60-80%; B6: level 5: complete leaf spot and leaf death).

Cluster analysis of isolates using average linkage between groups in Squared Euclidean Distance in SPSS software, divided them in three groups: **A**) low pathogenic isolates, **B**) moderate pathogenic isolates, and **C**) high pathogenic isolates (fig. 2).

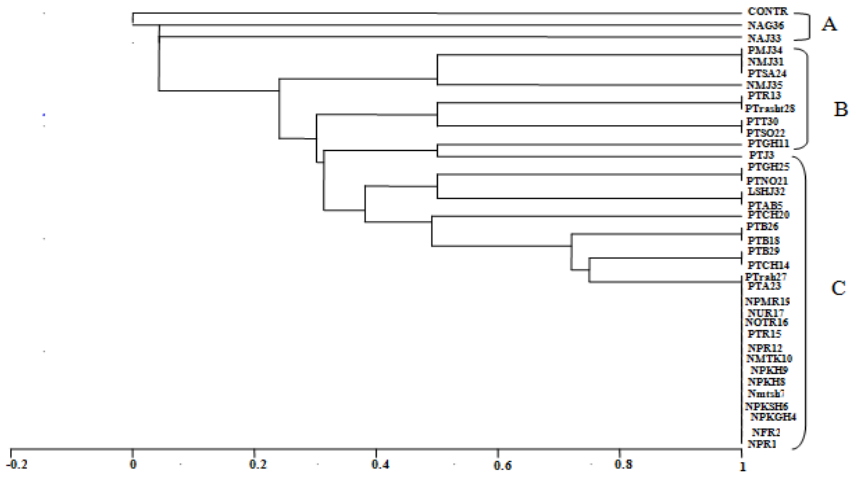

Figure 2: cluster analysis of *Alternaria alternata* isolates according to disease severity of *in vivo* test. Group A: low pathogenic isolates, group B: moderate pathogenic isolates, and group C: high pathogenic isolates.

RAPD-PCR analysis

All 15 used primers of this study showed good polymorphism among studied isolates. The sizes of obtained bands were estimated about 120-3000 bp. Among them, UBC-208 and UBC-285 primers showed the highest polymorphism and number of amplified fragment (fig. 3). According to cluster analysis, isolates were classified in five groups. The first group (A) had 13 isolates of *A. Alternata* which had been collected from infected orange trees of Iran north. The second group (B) had five isolates which all were belonged to south regions of Iran. All isolates related to Tangelo tangerine which were different in pathogenicity, were located in third group (C). Considerable point of this study was kiwi isolate locating in a separate group (D) according to RPD-PCR test. The last group (E) includes isolates of Tangelo tangerine. These isolates had been collected from citrus gardens of Iran north and showed different level of pathogenicity on page tangerine. The results of this study show that separation of isolates in RAPD-PCR reaction is completely related to host and geographical region and has a weak relationship with their pathogenicity (fig. 4).

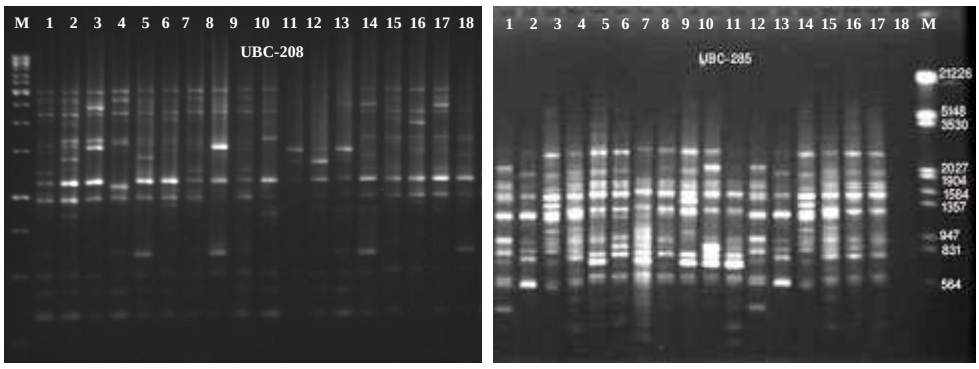

Figure 3: Band figures of amplified fragments of *A. alternata* isolates using UBC-208 and UBC-285 primers in Agars gel (1.2%). Isolates from 1 to 18, respectively:

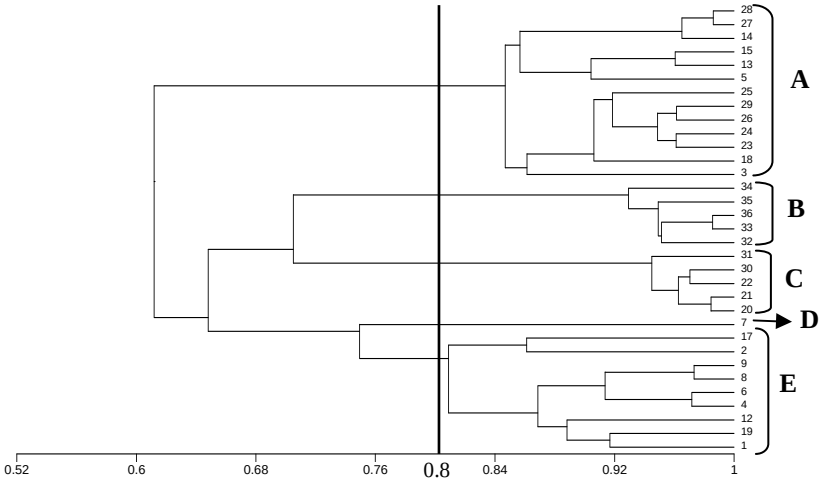

Figure 4: Cluster analysis of *A. alternata* isolates using RAPD-PCR. Group A: orange isolates; group B: isolates from south region of Iran; group C: tangelo isolates; group D: kiwi isolate; group E: isolates of Tangelo hybrids.

DISCUSSION

One of the most important factors in plant protection is sufficient information about genetical structure of pathogen populations and more information will certainly be effective in selecting efficient strategies of control [19]. On the other hand, pathogens populations are developing because they must adapt to environmental changes like crop rotation, tolerant varieties, fungicides and irrigation. Then, for effective control strategies we should focus on populations in place of singular specific cases [16].

Al. altrnata pathogen is a fungus with wide distribution on various plants which has attracted plant protection specialist since 80 years ago [2]. In this study, 35 isolates of *A. alternata* from citrus cultivars and one from kiwi as a foreigner group were studied. In pathogenicity assay, they shown highly variability and were classified in three pathogenicity groups: low, moderate, and high pathogenic. This study was conducted for clarifying structure of Iranian citrus populations of *A. Alternata* from genetical diversity aspect, and also for evaluating RAPD-PCR polymorphism in these populations. In this study, UBC-211 primer had the lowest polymorphism whereas UBC-208 and UBC-256 showed the highest polymorphism among 15 used random primers. Also, these primers could present significant relationship with pathogenicity of isolates. Grouping isolates by Jackard coefficient in MVSP software showed that host diversity and geographical region caused genetical distance between isolates of this fungus, so that isolates from southern regions of Iran were located in a separate clade, isolates from northern regions were also located in three groups considering their host and kiwi isolate as located in a separate group.

In conclusion, the result of this study showed that this fungus has a relative high diversity, so effective factors in its creation and distribution must be studied. Although mutation, mating system, and gene flow have been known as main reasons of genetic diversity, it seems that interaction between pathogen and Host -which high diversity of hosts cause high diversity in pathogen- and lack of tolerant host pressure plays role in no controlled diversity of this pathogen population. Obtained results confirm previous reports about documentary genetical diversity in this fungus [9]. Since using resistant varieties is the most effective and safe method in plant disease managment, and first step in evaluating susceptibility of varieties is knowledge of pathogen population's structure [17], this study was conducted about structure of pathogen population via studying diversity in pathogenicity and genetics of *A. alternata* isolates. Results showed that because of extant diversity in pathogenicity, each fungus isolate cannot be used for evaluating resistance of citrus varieties.

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