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Original Paper

Molecular and pathogenic characterization of Iranian isolates associated with leaf spot disease of potato

Armita Pourarian[1], Mehdi Nasr Esfahani[2], Mehdi Sadravi[1]
[1]*Yasuj University Plant Protection Department, Yasuj, Iran*
[2]*Isfahan Center for Agricultural and Natural Resources Research and Education, (AREEO), Plant Protection Research Department, Isfahan, Iran*

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Alternaria alternata (Fr.) Keissler is one of the main causal agents of leaf spot on potato in Iran and worldwide. In this study, random amplified polymorphic DNA (RAPD) and pathogenicity assay were employed to analyze 28 *A. alternata* isolates obtained from potato plants. The isolates were collected from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces. Cluster analysis of genotypes produced by RAPD marker, using UPGMA method indicated that the isolates have been clustered into different groups with no correlation to geographical origins of the isolates. Pathogenicity assay indicated that all *A. alternata* isolates were pathogenic on potato; however, virulence variability was observed among the isolates. The findings revealed that because of extant diversity in pathogenicity and genetics of *A. alternata* isolates, a single isolate should not be used for evaluating resistance of potato.

Keywords: *Alternaria alternata*, pathogenicity, RAPD, *Solanum tuberosum* L.

1 Introduction

Alternaria alternata (Fr.) Keissler has been isolated from a wide range of foods including fresh fruits and vegetables, nuts and cereals (Andersen et al., 2005). *Alternaria alternata* is a common saprobe found on many plants and other substrata worldwide. This species is also an opportunistic pathogen affecting many agricultural crops in field and during postharvest storage of vegetables and fruits. The fungus causes brown necrotic lesions on foliage and black pit of potato (Droby et al., 1984). Previous reports have shown that *A. alternata* could destroy more than 20% of potato production (Rotem, 1994; Van der Waals et al., 2003). In Iran, the two species *A. alternata* and *A. solani* (Ellis & Martin) has been reported as the causal agent of leaf spot on potato, which *A. alternata* is the dominant species (Nasr Esfahani and Ansaripour, 2006).

The efficacy of control strategy on the plant pathogen populations are inhibited by limited information on genetic variability (McDonald and Linde, 2002). The most common adopted effort is the use of fungicides and resistant cultivars. Therefore, understanding genetic variations within the pathogen populations is imperative and should be considered as one of the first steps for the delineation of disease management programs (McDonald and Linde, 2002).

In Iran, no comprehensive study has been done to determine the genetic diversity among *A. alternata* isolates causing leaf spot of potato in different geographical regions. Therefore, the objective of this study was to estimate the genetic and virulence variability among *A. alternata* isolates obtained from potato plants in main potato growing regions of Iran.

2 Materials and methods

2.1 Fungal isolates

Twenty-eight monoclonal isolates of *A. alternata* were obtained from the Plant Pathology Laboratory, Isfahan Research Center for Agriculture and Natural Resources, Isfahan, Iran (Table 1). The isolates were collected from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces in 2010. Previous study has confirmed that all 28 isolates belonged to *A. alternata* based on morphological characteristics (Van der Waals et al., 2011). Conidia of all isolates were produced in chains on conidiophores, and presented the shapes of inverted pears with short beaks, dark

***Corresponding Author:** Mehdi Nasr Esfahani, Isfahan Center for Agricultural and Natural Resources Research and Education, (AREEO), Plant Protection Research Department, Isfahan, Iran
e-mail: mne2011@gmail.com

brown, with dimensions of 20–50 × 9–18 µm, and 2–6 (4) transversal and 1–4 (2) longitudinal septa.

2.2 Fungal DNA extraction

Mycelium of each isolate was grown in 50 ml volumes of potato dextrose broth in 250 ml flasks incubated on a rotary shaker (150 rpm) at 25 ±2 °C for 3 days. The mycelium was harvested by filtration and washed with sterile-distilled water. Total genomic DNA was extracted from all isolates using a modification of the CTAB method described by Talbot (2001). The mycelium was ground into a fine powder under liquid nitrogen and suspended in 500 µl extraction buffer. The slurry was incubated for 30 minutes at 65 °C in 1.5 ml micro centrifuge tubes. DNA samples were purified with equal volumes of Chloroform: Iso-amyl Alcohol (24 : 1) mixture, and precipitated with Iso-propanol. The tubes were centrifuged at 13000 rpm (Eppendorf Centrifuge) for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in ddH_2O or TE buffer (pH 8.0) and stored at -20 °C for further use. A NanoDrop ND-1000 spectrophotometer (LMS Co., Ltd., Tokio, Japan) was used to check the quality and concentration of genomic DNA.

Table 1 Origins and virulence variability of *Alternaria alternata* isolates used in this study

No.*	Isolate	Species	Location	State	Year	Percent Disease Severity (*PDS*)**
1	Aa-H1	*Alternaria alternata*	Asad Abad	Hamedan	2010	23.83 ab
2	Aa-H2	*A. alternata*	Shirin Su	Hamedan	2010	48.00 ab
3	Aa-H3	*A. alternata*	Kabodar Ahang	Hamedan	2010	49.50 ab
4	Aa-H4	*A. alternata*	Bahar and Saleh Abad	Hamedan	2010	45.33 ab
5	Aa-H5	*A. alternata*	Hamedan	Hamedan	2010	67.50 ab
6	Aa-H6	*A. alternata*	Qabaq Tappeh	Hamedan	2010	12.50 b
7	Aa-H7	*A. alternata*	Famenin	Hamedan	2010	31.67 ab
8	Aa-A1	*A. alternata*	Agha Bagher Village	Ardebil	2010	16.50 b
9	Aa-A2	*A. alternata*	Khalifeh Lu Village	Ardebil	2010	28.83 ab
10	Aa-A3	*A. alternata*	Yunjalu Village	Ardebil	2010	52.33 ab
11	Aa-A4	*A. alternata*	Tupraghlu Village	Ardebil	2010	17.67 b
12	Aa-A5	*A. alternata*	Samian Village	Ardebil	2010	44.67 ab
13	Aa-A6	*A. alternata*	Soltan Abad	Ardebil	2010	42.83 ab
14	Aa-A7	*A. alternata*	Ardebil	Ardebil	2010	37.67 ab
15	Aa-F1	*A. alternata*	Kushk Mola Village	Fars	2010	48.88 ab
16	Aa-F2	*A. alternata*	Boroj Village	Fars	2010	30.67 ab
17	Aa-F3	*A. alternata*	Dariun Village	Fars	2010	36.50 ab
18	Aa-F4	*A. alternata*	Deh Bid	Fars	2010	69.17 a
19	Aa-F5	*A. alternata*	Shirin Abad Village	Fars	2010	50.00 ab
20	Aa-F6	*A. alternata*	Hasan Abad Village	Fars	2010	38.00 ab
21	Aa-F7	*A. alternata*	Bovanat Village	Fars	2010	36.00 ab
22	Aa-I1	*A. alternata*	Chadegan	Isfahan	2010	46.00 ab
23	Aa-I2	*A. alternata*	Daran	Isfahan	2010	52.50 ab
24	Aa-I3	*A. alternata*	Rozveh	Isfahan	2010	55.00 ab
25	Aa-I4	*A. alternata*	Semirom	Isfahan	2010	27.17 ab
26	Aa-I5	*A. alternata*	Golpayegan	Isfahan	2010	43.33 ab
27	Aa-I6	*A. alternata*	Mehdi Abad	Isfahan	2010	38.00 ab
28	Aa-I7	*A. alternata*	Nisian	Isfahan	2010	49.33 ab

* – *Alternaria alternata* isolates causing leaf spot of potato; ** – values followed by the same letter in the column did not differ significantly (0.05 level) in Duncan's multiple range test

2.3 RAPD analysis

Six primers OPA-16, OPC-06, OPC-08, OPP-16, OPP-19 and OPX-12 (Operon Technologies Inc., Alameda, CA) with high polymorphism and reproductive profiles were chosen among 15 primers to perform RAPD analysis on *A. alternata* isolates based on the results of initial screening against a set of representative isolates (Table 2). PCR amplification of RAPD loci was carried out in a 25 ml containing 0.5 µM primer, 2.5 µl of a 10× buffer (200 mM Tris-HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Taq DNA polymerase and 2 µl of DNA template (10 ng). RAPD analysis was carried out as described by Nasehi et al. (2014). PCR amplification was conducted in a thermocycler programmed with the following parameters: 45 cycles of 94 °C for 1 min (denaturation), 35 °C for 1.5 min (annealing) and 72 °C for 2 min (extension) with the initial denaturing of 94 °C for 4 min and final extension of 72 °C for 10 min. All PCR were performed in three replications to confirm the consistency of amplification.

Table 2 RAPD primers utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates collected from potato plants

Primera	Sequence (5´-3´)
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-13	CAGCACCCAC
OPA-16*	AGCCAGCGAA
OPB-17	AGGGAACGAG
OPB-18	CCACAGCAGT
OPC-06*	GAACGGACTC
OPC-08*	TGGACCGGTG
OPE-01	CCCAAGGTCC
OPP-16*	CCAAGCTGCC
OPP-17	TGACCCGCCT
OPP-18	GGCTTGGCCT
OPP-19*	GGGAAGGACA
OPX-12*	TCGCCAGCCA
OPX-14	ACAGGTGCTG

a Primers with an asterisk (*) were utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates. These primers with high polymorphism and reproductive profiles were chosen among all primers based on the results of initial screening against a set of representative isolates

2.4 Gel electrophoresis and staining

PCR products of RAPD analysis were size-separated in 1% agarose gel under 1× TAE buffer (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA) at 70 V for 45 min at room temperature. Gels were stained with ethidium bromide, visualized under UV light and photographed using a gel documentation system (GeneSnap Ver 6.03, Syngene Laboratories, Cambridge, United Kingdom). The sizes of amplified and digested DNA fragments were estimated using GeneTools (Ver 3.00.13, Syngene Laboratories) by comparison with a 2-Log DNA Ladder (0.1–10 kb) marker.

2.5 RAPD data analysis

The bands were considered as binary characters, and were scored as 1 for presence and 0 for absence of DNA bands. The scores were then entered into a matrix for analysis by the numerical taxonomy and multivariate analysis system, NTSYS-pc 1.8 program (Applied Biostatistics Inc., Setauket, NY, USA) (Rohlf 1993). The similarity matrix was calculated among the isolates using Jaccard's similarity coefficient. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) to generate the dendrogram. And the RAPD clustered analyses were compared to geographical origins of the isolates for presence of any correlation coefficients.

2.6 Pathogenicity

Pathogenicity test was conducted in a greenhouse using all 28 isolates of *A. alternata*. The experiment was arranged in a completely randomized design in 10 replications (pots) for infected plants as well as control plants. Each replication consisted of 10 leaves of each plant. Conidial suspension (10^3 spores per ml) was used to inoculate one month old potato leaves (var. *Agria*). Seven days after inoculation, disease rating was scored based on a scale of 0–7 points, where: 0 = no disease symptoms, 1 = lesions as pinpoints and non-measurable, 2 = <10% of the leaves with brown necrotic lesions, 4 = 10 ≤ to 25%, 8 = 25≤ to 50%, 16 = 50≤ to 75% of the leaves with brown necrotic lesions, and 32 = 75≤ to ≥100% of the leaves with brown necrotic lesions or completely brown (NIAB 1985). The experiments were repeated twice. Re-isolation of the inoculated fungi was performed to fulfill Koch's postulate. The following formula was used to calculate percent disease severity (*PDS*) in each replication. In this formula, *T* is the total number of leaves in each category; *R* is the disease severity scale; *N* is the total number of leaves tested; *S* is the highest number in the scale:

$$\textit{Percent disease severity } (PDS) = \frac{\sum RT \cdot 100}{S \cdot N}$$

Data were transformed to arcsine square-root and then subjected to the analysis of variance procedure (ANOVA, $P < 0.05$), and the means were compared by Duncan's multiple range test using SAS software version 9.2.

3 Results and discussion

3.1 RAPD analysis

Application of six RAPD primers generated a total of 47 consistently amplified fragments (100–3000 bp), of which all the 47 fragments were polymorphic (100%). The dendrogram produced from UPGMA analysis based on Jaccard's similarity coefficient indicated that the variability was high among isolates of *A. alternata*, and the similarity value was ranged from 0 to 100% with the mean value of the Jaccard's similarity coefficient 0.50 (Fig. 1). The isolates obtained from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces were clustered into different groups with no correlation to geographical regions of the isolates.

3.2 Pathogenicity

Symptoms of leaf spot were observed in all the inoculated potato plants seven days after inoculation. Foliar lesions initially were as pinpoints, irregular to circular, brown spots on the lower leaves, and then became circular spots spreading over much of the leaves. Control plants remained healthy without showing any symptom of the disease. *Alternaria alternata* isolates was re-isolated from the inoculated plants and was found to be identical to the original isolates based on morphological characteristics. These results revealed that *A. alternata* was the causal agent of leaf spot of potato plants in Iran; however, virulence variability was observed among the isolates (Table 1). The isolate Aa-F4 (*PDS* of 69.17) was the most virulent isolate and located in a single subgroup in comparison to the others three close virulent isolates, Aa-A3, Aa-I2 and Aa-I17 (Fig. 1). While, the three isolates Aa-H6 (*PDS* of 12.50), Aa-A1 (*PDS* of 16.50) and Aa-A4 (*PDS* of 17.67) were the least virulent isolates. Other isolates had the moderate virulence.

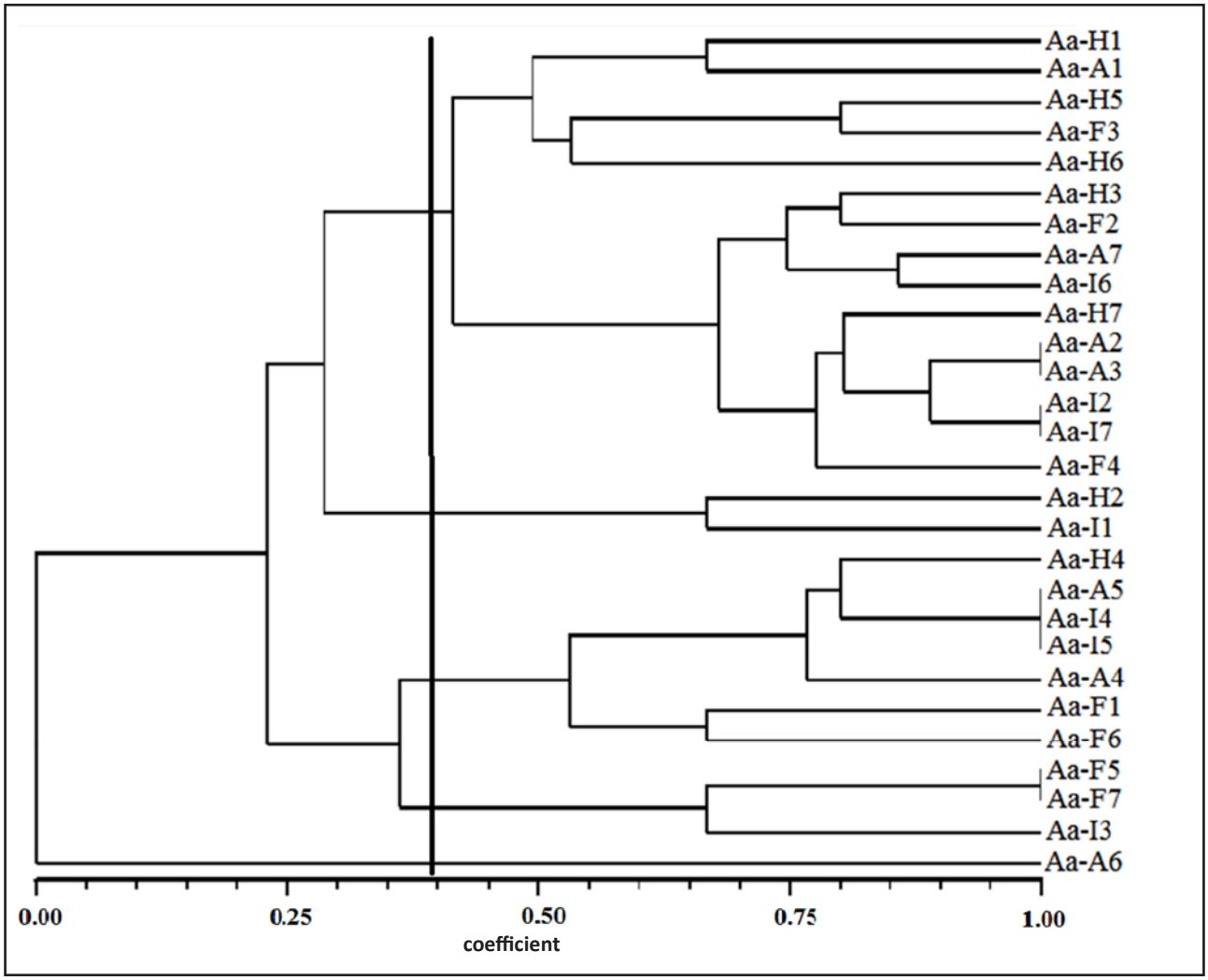

Figure 1 Dendrogram generated by UPGMA showing the genetic variability of 28 *Alternaria alternata* isolates derived from combination of six RAPD primers (OPA-16, OPC-06, OPC-08, OPP-16, OPP-19 and OPX-12). The name of the isolates is based on Table 1

RAPD PCR method is an extremely powerful tool to separate individuals having intraspecific and interspecific variability. This method has been employed to provide comprehensive information regarding the genetic variation in populations of *A. alternaria* (Morris et al., 2000; Pryor and Michailides, 2002; Weir et al., 1998) and other plant pathogenic fungi (Mahmodi et al., 2014; Nasehi et al., 2014). In this study, RAPD analysis indicated that isolates of *A. alternata* have a high diversity in main potato growing regions of Iran with no correlation to geographical origins of the isolates.

The results of pathogenicity indicated that all isolates of *A. alternata* used in this study were pathogenic on potato, but virulence variability was observed among them. Based on the virulence variability, all isolates were clustered into three groups of most virulent (one isolate), least virulent (three isolates) and moderate virulent isolates (25 isolates). These results were in agreement with the previous studies which have confirmed the existence of virulence variability among *A. alternata* isolates obtained from different geographical regions (Kakvan et al., 2012; Meena et al., 2015). In addition, the grouping based on virulence variability was not correlated with the result of RAPD analysis, as well as geographical regions, which is in agreement with previous study on *A. alternata* (Kakvan et al., 2012).

4 Conclusion

In conclusion, this study was conducted to examine the structure of pathogen population by studying of diversity in pathogenicity and genetics of *A. alternata* isolates. The results indicated that because of extant diversity in pathogenicity and genetics of *A. alternata* isolates, a single isolate could not be used for evaluating resistance of potato. This study suggests that the isolate Aa-F4 as the most virulent isolate and a few representative isolates which had moderate virulence could be used for evaluating resistance of potato. The data of RAPD markers could also be expanded for a wider genetic diversity of *A. alternata* on different host plants from different geographical regions.

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