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Research Article

Genetic diversity and pathogenic variability among *Cercospora beticola* Sacc. isolates causing leaf spot of sugar beet in Iran

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Abstract: Cercospora leaf spot caused by *Cercospora beticola* has a great negative impact on yield and quality of sugar beet. In the present study, pathogenic and genotypic variation of 24 *C. beticola* isolates collected from different regions of Iran were studied using RFLP of the Internal Transcribed Spacer (ITS-RFLP), and Random Amplified Polymorphic DNA (RAPD-PCR). Pathogenic variability and genotype × isolate interaction were evaluated in greenhouse experiments on five sugar beet cultivars (FD0018, HM1836, Puma, Eudora and Monatuna). All of the 24 isolates tested were found to be pathogenic on the cultivars with significant variation in disease severity. Results of RAPD analysis showed wide DNA polymorphism among the Iranian *C. beticola* isolates. Restriction pattern of the internal transcribed spacer of rDNA (ITS1-5.8-ITS4) was studied using three restriction endonucleases: *EcoR1*, *Taq1*, and *Busr1*. The length of undigested DNA fragment of all isolates was estimated to be 500bp without rDNA polymorphism after digestion with *EcoR*1 (280, 270 bp), *Taq*1 (330 bp) and *Busr*1 (240, 220, 90 bp). RAPD and ITS-RFLP markers showed the highest level of genetic diversity which confirms the variation in *C. beticola* detection.

Keywords: *Cercospora beticola*, genotype × isolate interaction, Pathogenicity, RAPD fingerprinting

Introduction

Cercospora leaf spot caused by the fungus *Cercospora beticola* Sacc. is the most important, widespread, and destructive foliar disease of sugar beet (*Beta vulgaris* L.) worldwide (Holtschulte, 2000; Trkulja *et al*., 2017). Continued cultivation of the crop spreads the disease far and wide (Panizza, 1998). In Iran, Cercospora leaf spot has been reported from different sugar beet growing areas including Khuzestan, Mazandaran, West Azerbaijan and Fars provinces (Ershad, 1995). High intensity of the disease incidence has been observed annually in the vicinity of the Caspian Sea and Khuzestan province (Holtschulte, 2000). The disease symptoms are necrotic lesions that can expand on leaves and leaf petioles.

The economic losses to recoverable sucrose due to the disease can be as high as 48% (Khan *et al*., 2007). The increased amounts of non-sucrose factors and root

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storage decay are considered as indirect damages of Cercospora leaf spot to sugar beet (Holtschulte, 2000).

The most reliable method to control the disease is to utilize resistant cultivars which is preferred to chemical control due to economic and environmental concerns (Hemayati *et al*., 2017). *C. beticola* isolates resistant to fungicides are identified in European sugar beet fields. For example, in Poland, cercospora leaf spot is mainly controlled with single-site fungicides such as quinone outside inhibitors (QoI) and a gradual increase in QoI tolerance has been observed (Piszczek *et al*., 2017). Nevertheless, application of fungicides is necessary in humid and warm areas (Secor *et al*., 2010; Khare *et al*., 2017).

Before the development and release of a pathogen resistant cultivar, more information such as pathogen-cultivar interaction is needed. Information concerning pathogen × cultivar × environment interaction is of particular relevance because sugar beet cultivars developed in a particular geographic area, may or may not be resistant in other areas (Smith, 1985). Differential interaction of *C. beticola* isolates and sugar beet cultivars was evaluated (Solel and Wahl, 1971; Whitney and Lewellen, 1976; Smith and Martin, 1978; Karaoglanidis and Ioannidis, 2010).

Despite the fact that leaf spot is a destructive disease of sugar beet and genetic resistance is an effective way to control the disease, very little information is available on genetic variability of *C. beticola* isolates (Almeida *et al*., 2005). Cultural variation and the degree of pathogen virulence on cultivars, together with different levels of resistance, are the main criteria used to study the genetic diversity of *C. beticola* (Ruppel, 1972; Solel and Wahl, 1971; Vaghefi *et al*., 2017). A wide range of phenotypic diversity was reported for *C. beticola* isolates (Vaghefi *et al*., 2016). Genetic variability of *C. beticola* isolates collected from different areas throughout the Mediterranean basin was studied using RAPD technology (Chiusa *et al*., 1996). Results showed DNA polymorphism for a large number of loci. In another study, *C. beticola* isolates collected from Western Europe, Iran and New Zealand indicated high genetic variation of the pathogen due to sexual reproduction (Groenewald *et al*., 2008). Authors also reported that the *C. beticola* isolates from Iran and Europe were genetically similar, whereas New Zealand populations were different (Ali, 2012). Previous studies have shown high phenotypic diversity and genotypic variation in populations of *C. beticola* (Moretti *et al*., 2004, Chiusa *et al*., 1996; Groenewald *et al*., 2008). The objective of the present study was to clarify whether there would be any pathogenic variability among diverse isolates on different cultivars. For this reason, genetic diversity of the *C. beticola* isolates in Iran was studied using RAPD and ITS-rDNA techniques and also their interaction with different sugar beet cultivars was evaluated.

Materials and Methods

Fungal isolates

Sugar beet leaves with typical symptoms of *Cercospora* leaf spot were collected from infected sugar beet fields of Mazandaran, Khuzestan, Golestan and Ardabil provinces in Iran. Single spore isolations were made and cultures were established on 1.5% potato dextrose agar (PDA). In order to maintain the virulence level of the isolates, a susceptible sugar beet cultivar was inoculated with selected isolates (Isolates C22, C1, C23, C13 and C6) under greenhouse condition, and used to re-isolate the pathogen from the infected leaves. Isolates were selected based on geographical origin (Table 1).

RAPD-PCR and ITS-rDNA analyses

Total genomic DNA was extracted from 24 isolates of *C. beticola* grown on potato dextrose broth (PDB) and incubated at 25 °C for five days as described by Weiland (2002) with some modification (Mahmoudi *et al*. 2005, Table 1). The RAPD-PCR was performed as described

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by Chiusa *et al*., (1996). PCR reactions were performed in a DNA thermal cycler (Biometra Co. Germany). All PCR products were analyzed on 1.5% agarose gel (Sambroek *et al*., 1989). Initially, 38 random decamer primers were used for amplification of an isolate. Then, based on the results, 14 primers from Advanced Biotechnology (AB1-10, AB4-2, AB4-10, AB6-2, AB6-4, AB6-8, AB6-13, AB-14, AB6-17, AB8-8, and AB-9-19) and University of British Colombia (UBC204, UBC210 and UBC 211) were chosen for *C. beticola* isolates amplification. PCR reactions were performed for twelve isolates collected from Khuzestan (Dezful 1-12), four isolates from Mazandaran (Ghaemshahr 13-16), six isolates from Ardebil (Moghan 17-22), and two isolates from Golestan (23-24).

For ITS-rDNA analysis, genomic DNA of each isolate (Table 1) was amplified using ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') as forward and reverse primers, respectively (Mahmoudi *et al*., 2005). PCR products were digested with *EcoR*1, *Taq*1 or *Busr*1 restriction enzymes under recommended conditions by the manufacturer's protocol. Using agarose gel electrophoresis, the DNA restriction fragments were separated and visualized under UV light (Sambroek *et al*., 1989). The reactions were repeated twice to confirm RAPD and ITS-rDNA reproducibility.

Table 1 List of *Cercospora beticola* isolates and their origin.

No	Province	Location	Year of sampling	Host
1	Khuzestan	Motahhari district	2001	Sugar beet
2	Khuzestan	Shoshtar	2001	Sugar beet
3	Khuzestan	Daylam	2001	Sugar beet
4	Khuzestan	Andimeshk	2001	Sugar beet
5	Khuzestan	Safiabad	2001	*Beta maritima*
6	Khuzestan	Safiabad	2001	Sugar beet
7	Khuzestan	Shoush	2002	Sugar beet
8	Khuzestan	Dezful	2002	Sugar beet
9	Khuzestan	Shoshtar	2002	Sugar beet
10	Khuzestan	Dezful	2002	Sugar beet
11	Khuzestan	Safiabad	2002	Sugar beet
12	Khuzestan	Shoushtar	2002	Sugar beet
13	Mazandaran	Ghaemshahr, Gharakhil	2001	Sugar beet
14	Mazandaran	Ghaemshahr, Gharakhil	2001	Sugar beet
15	Mazandaran	Ghaemshahr, Gharakhil	2001	Sugar beet
16	Mazandaran	Ghaemshahr, Gharakhil	2002	Sugar beet
17	Ardebil	Moghan Pars4	2001	Sugar beet
18	Ardebil	Moghan Pars 5	2001	Sugar beet
19	Ardebil	Moghan Pars1	2001	Sugar beet
20	Ardebil	Parsabad	2001	Sugar beet
21	Ardebil	Moghan, Topraghkandi	2001	Sugar beet
22	Ardebil	Moghan	2001	Sugar beet
23	Golestan	Aliabad Katoul	2002	Sugar beet
24	Golestan	Azadshahr	2002	Sugar beet

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Pathogenicity test

To evaluate the interaction of *C. beticola* isolates with sugar beet cultivars, a greenhouse experiment was conducted by inoculation of five sugar beet cultivars with five selected *C. beticola* isolates. Isolates were selected based on aggressiveness and the cluster analysis of RAPD-PCR. Sugar beet cultivars were selected on the basis of their resistance to *C. beticola* and existence in the Iranian national list of registered varieties for autumn sowing. Five mature leaves were marked on each individual plant and inoculated with different *C. beticola* isolates. Mature sugar beet plants were inoculated with 3×10^4 spores per ml. of spore suspension. About 10 weeks after sowing and shortly before inoculating the plants with *Cercospora* isolates, the temperature was increased from 22 ± 2 ºC to 28 ± 2 ºC and the relative humidity was adjusted to ≥ 90%. This experiment was conducted as factorial arrangement based on completely randomized design with four replications (each replication contained 8 individual plants) in greenhouse condition. Disease severity was scored 30 days after inoculation using a 1-15 standard scale (Shane and Teng, 1992) where the scale 1 was allocated to the plant leaves without any symptom and the scale 15 to the leaves completely covered with the disease symptoms. For the evaluation of isolates × cultivars interaction, a cumulative degree-day model was used to determine the duration of the incubation period between inoculation and appearance of spots in 50 percent of plants.

Results

Collection of isolates and reaction of varieties

In this study, 24 isolates were collected from different regions of Iran: twelve isolates from Khuzestan province where sugar beet was grown as an autumn crop and the rest from other provinces where sugar beet is grown as a spring crop. Only one isolate was collected from weed beet (*B. maritima*) in Khuzestan province (Table 1).

Among the varieties, Puma and Monatuna were considered as tolerant and susceptible varieties to the pathogen, respectively. The results showed that HM1836 was more tolerant than Puma (Table 2), whereas Eudora was found to be susceptible.

Restriction analysis of rDNA-ITS region

Amplification of ITS-rDNA withITS1 and ITS4 resulted in fragments of approximately 500bp. No variation was observed among the 9 isolates. After digestion with *EcoR*1, two restriction fragments of 270 and 280bp were observed, while digestion with *Taq*1 yielded three ITS rDNA-fragments of 240, 220 and 90 p. However, digestion with *Bsur*1 endonuclease resulted in 330 bp fragment size. No rDNA polymorphism among the isolates was observed after digestion with the restriction endonucleases (Figs. 1-3).

Table 2 The disease severity of *Cercospora beticola* isolates on different sugar beet cultivars.

Isolates	Disease severity[1]					
	Puma	Monatuna	HM1836	FD0018	Eudora	Mean
C22	7.69	10.99	6.63	6.06	11.46	8.56 [a**]
C1	7.16	10.10	6.05	5.74	10.86	7.98 [ab]
C23	5.74	9.44	5.71	5.36	9.26	7.10 [b]
C13	7.41	10.53	6.12	5.81	10.58	8.09 [ab]
C6	7.48	10.48	6.57	5.91	11.14	8.31 [ab]
Mean	7.10 [b]	10.31 [a]	6.22 [c]	5.77 [c]	10.66 [a]	

[1] Disease severity: Scale 1 means without any symptoms of the disease and scale 15 with severe symptoms.
Means followed by the same letter(s) are not significantly different at P ≥0.05 by Duncan's multiple range test.

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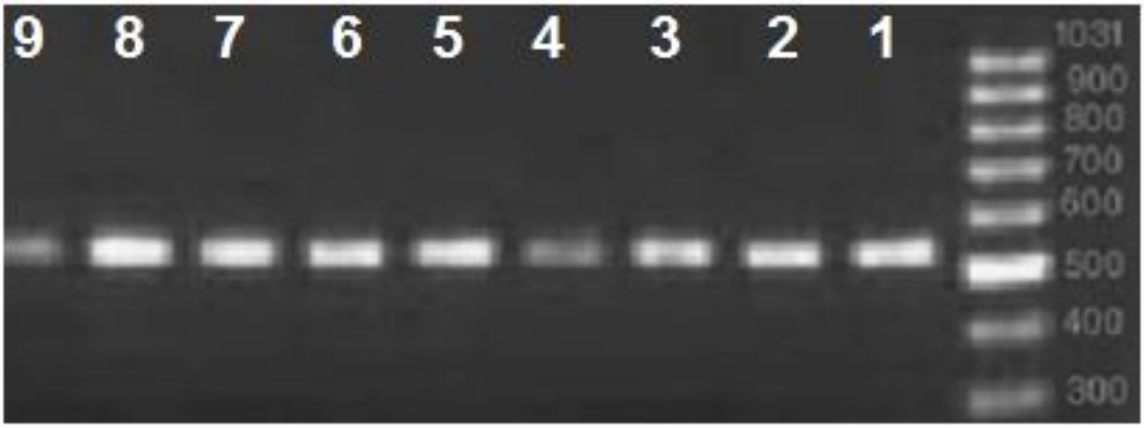

Figure 1 Electrophoretic pattern of PCR-amplified ITS-rDNA for *Cercospora beticola* isolates in 2% agarose gel. The first lane from the right is molecular weight marker and other lanes (1-9) are the *Cercospora* isolates that are described in Table 1.

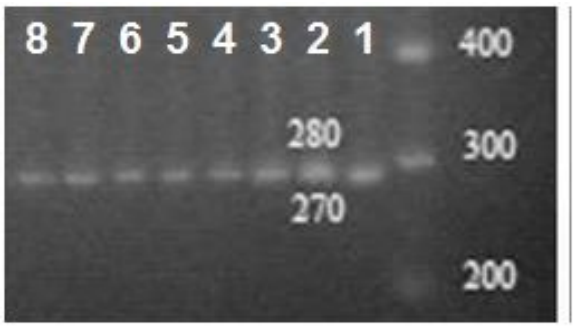

Figure 2 Electrophoretic pattern of PCR-amplified ITS-rDNA for *Cercospora beticola* isolates digested with *Eco*R1in 2% agarose gel. The first lane from the right is molecular weight marker and other lanes (1-8) are the *Cercospora* isolates that are described in Table 1.

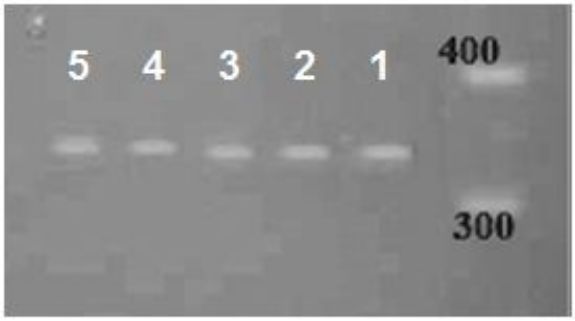

Figure 3 Electrophoretic pattern of PCR-amplified ITS-rDNA for *Cercospora beticola* isolates digested with *Bsur*1 in 2% agarose gel. The first lane from the right is molecular weight marker and other lanes (1-5) are the *Cercospora* isolates that are described in Table 1.

RAPD analysis

Fourteen 10-mer oligonucleotides produced a total of 528 RAPD reproducible (monomorphic and polymorphic) bands (Figure 4). Results of cluster analysis for RAPD is presented in Figure 5. The isolates were clustered in distinct groups, with different rate of similarities among each other. The isolates were roughly grouped according to their geographic origin; for instance, the isolates 13, 14, and 15 collected from Ghaemshahr region during 2001 were grouped together, while the isolate 16 which had been collected from the area in 2002 formed a separate group. In some cases, the isolates collected simultaneously from the same area and year were totally distinct from each other; for example, the isolates 17, 22, 18, 19, 20 and 21 collected from Moghan area in 2000 were categorized in different groups. This clustering method has been used in different studies (Matsumoto *et al.*, 1996; Schneider *et al.*, 1997; Mahmoudi *et al.*, 2005).

Pathogenicity test

Significant differences were found among the isolates for disease severity and incubation period (data not shown). Genotype × isolate interaction was significant for disease severity and as a result, the isolates had different influence on genotypes (Table 3). Among the five isolates (C22, C1, C23, C13 and C6), the isolate C22 collected from Moghan (with disease severity value 8.56) and

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isolate C23 collected from Golestan (with disease severity value 7.1) had the highest and lowest disease severity, respectively (Table 2). On the other hand, the isolates 1 (collected from Dezful) and 6 (collected from Safiabad) had the lowest and Isolate 13 had the highest incubation period, respectively (Table 4). Sugar beet cultivars were categorized into resistant (FD0018 and HM1836), tolerant (Puma), and relatively susceptible (Eudora and Monatuna) groups based on disease severity (Table 2), while they did not show significant difference for incubation period (Table 4).

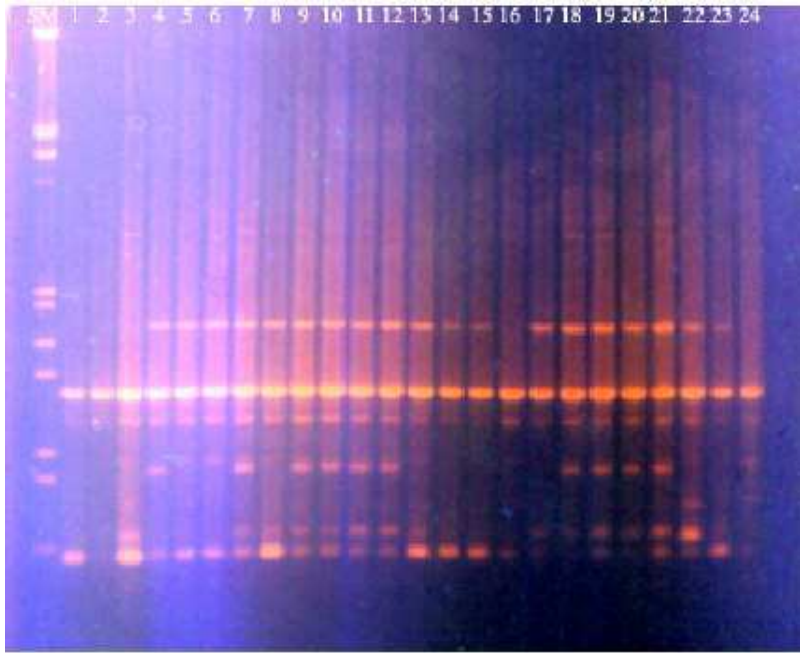

Figure 4 Fingerprint pattern of *Cercospora beticola* isolates generated by RAPD-PCR using RAPD and AB6-14 primers. The first lane from the left is size marker and other lanes (1-24) are the *Cercospora* isolates that are described in Table 1. First line from left is size marker (2kb).

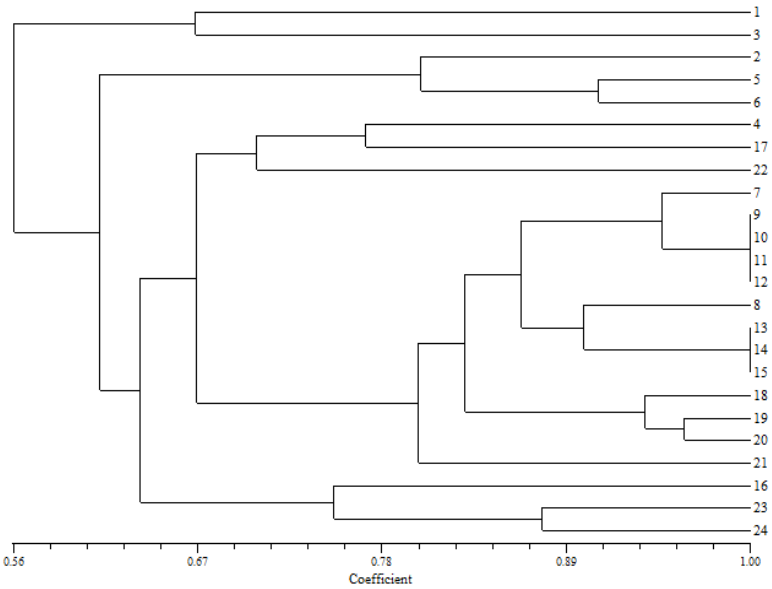

Figure 5 Cluster analysis of *Cercospora beticola* isolates based on fingerprint patterns using RAPD marker.

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Table 3 Results of analysis of variance of isolates × genotype interaction.

Source of variation	df	Mean of squares	
		Disease severity	Incubation period
Genotype	4	106.71**	15951**
Isolate	4	6.18**	111.4*
Genotype × isolate	15	0.336**	60.8^{ns}
Error	75	0.064	41.6

Table 4 Incubation period (cumulative degree-days) of *Cercospora beticola* isolates on different sugar beet cultivars.

Isolates	Incubation period (cumulative degree-days)					
	Puma	Monatuna	HM1836	FD0018	Eudora	Mean
C22	257.50	258.50	264	261	250.50	258.3 c*
C1	234.50	225.25	219.50	232.50	223.50	227.05 d
C23	279.50	276.25	278	282.25	278	278.80 b
C13	289.25	285.25	284.75	286	286	286.25 a
C6	224	231	226.75	226.75	220.25	225.75 d
Mean	256.95 a	255.25 a	254.60 a	257.70 a	251.65 a	

*Means followed by the same letter(s) are not significantly different ($P \geq 0.05$) by Duncan's multiple range test.

Discussion

Cercospora beticola is known as a pathogen of high risk of resistance with vast sporulation, substantial genetic variability and multiple disease cycles during a growing season (Abbasi *et al.,* 2002; Abbasi and Mahmoudi, 2010; Karaoglanidis and Ioannidis, 2010; Trkulja *et al*., 2017). It is also known to infect a wide range of plants from diverse families (Barres *et al*., 2008; Vaghefi *et al.*, 2017). In sugar beet, the disease results in premature death of leaves followed by reduction in assimilation area and finally loss of root yield and sucrose content (Skaracis *et al*., 2010). Amplification of ITS fragments digested by restriction enzymes is one of the methods used for studying genetic diversity at species or sub-species level (Cubeta *et al*. 1996). Different anastomosis groups of *Rhizoctonia solani* were distinguished by rDNA-RFLP polymorphism (Cubeta and Vilgalys 1997; Mahmoudi *et al*. 2005), while ITS-rDNA analysis of *Erysiphe betae* could not differentiate different geographical isolates (Shaykholeslami *et al*. 2005). In our study, ITS-rDNA fragments of different geographical *C. beticola* isolates showed no variation. The banding pattern of PCR products after digestion with *EcoR*1, *Taq*1 and *Bsur*1 enzymes could not differentiate the isolates, while these enzymes provided differentiation among anastomosis groups of *Rhizoctonia solani* isolates of sugar beet (Mahmoudi *et al*. 2005; Kilicoglu and Ozkoc, 2010). Vaghefi *et al*. (2017) used 12 microsatellite markers to characterize the genetic structure of *C. beticola* populations in contrasting table beet production systems in New York. Their results showed high genotypic diversity, detection of admixed genotypes by Bayesian clustering and DAPC analyses which were suggestive of recombination in the *C. beticola* population. Contrary to the results of this study, Meinhardt et al. (2002) showed that the restriction digestion of the ITS1/5.8S/ITS2 ribosomal regions resulted in unique banding patterns specific for an anastomosis group and its subgroups. The ITS restriction digestion (ITS/RFLP), telomere and microsatellite primers identified some genotypes within the isolates of *R. solani*. *Cercospora* leaf spot is a sporadic disease in Iran (Madanian-

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Mohammadi *et al*. 2004), and its occurrence is highly dependent on climatic conditions.

The simplicity, high speed and no need of any prior sequence information has made the RAPD-PCR technique more preferable. In this technique, any oligonucleotide sequence can be used as a primer and it has been successfully used to differentiate diversity among microorganisms species. The RAPD-PCR technique has shown to be an effective marker for determining the diversity among *R. solani* isolates and differentiating the isolates of different anastomosis groups (Mahmoudi *et al*. 2005; Momeni *et al*. 2005). In the present study, DNA polymorphism of Iranian *C. beticola* isolates using RAPD marker classified them into four clusters. In general, cluster analysis differentiated the isolates collected from the same geographical areas.

Although *Cercospora* isolates differed in their aggressiveness, they did not show any interaction with the cultivars. In the present study, the *Cercospora* isolates were distinguished from each other based on sporulation, spore size, pigmentation, and DNA fingerprinting. These differences led to variation in disease severity, but this variation does not seem to be important in a plant breeding program because of absence of isolate × cultivar interaction. On the other hand, the isolate × cultivar interaction cannot explain pathological races of the pathogen. Smith (1985) studied pathological variability among *C. beticola* biotypes under field condition. He evaluated the resistance of 12 sugar beet cultivars in Greece, Italy, Spain, and U.S. for three years. Results of his study showed that the resistance to *C. beticola* was consistent over the four geographical locations.

Although, Solel and Wahl (1971) identified three pathological races of *C. beticola* which were separated from each other using different monogenic resistant cultivars, it was not applied in practice for development of monogenic resistant varieties because of instability of the resistance. However, new commercial sugar beet cultivars have been developed that have a high level of resistance to *C. beticola* with high stability under different climatic conditions (Koch and Jung, 2000). In conclusion, although the genetic diversity was observed among *Cercospora* isolates, the sugar beet cultivars' response to them was similar which illustrates the possibility of the evaluation of resistance to *C. beticola* in any geographical location.

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تنوع ژنتیکی و بیماریزایی جدایه‌های *Cercospora beticola Sacc.* عامل لکه برگی چغندرقند در ایران

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چکیده: بیماری لکه‌برگی سرکوسپورایی چغندرقند که توسط قارچ *Cercospora beticola* ایجاد می‌شود، تأثیر منفی زیادی بر عملکرد و کیفیت چغندرقند دارد. در این مطالعه، تنوع بیماریزایی و ژنوتیپی ۲۴ جدایه *C. beticola* که از مناطق مختلف ایران جمع‌آوری شده بودند با استفاده از نشانگرهای ITS-RFLP و RAPD مورد ارزیابی قرار گرفت. تنوع بیماری‌زایی و اثر متقابل ژنوتیپ و جدایه در گلخانه بر روی پنج رقم چغندرقند شامل FD0018، HM1836، Puma، Eudora و Monatuna مورد مطالعه قرار گرفت. همه ۲۴ جدایه مورد مطالعه، بر روی ارقام بیماریزا بودند و شدت بیماریزایی آنها تفاوت معنی‌داری با یکدیگر داشت. نتایج تجزیه RAPD نشان‌دهنده چندریختی گسترده DNA در بین جدایه‌های ایرانی *C. beticola* بود. الگوی آنزیم‌های برشی EcoR1، Taq1 و Busr1 روی rDNA(ITS1-5.8-ITS4) مورد بررسی قرار گرفت. طول قطعه rDNA تمام جدایه‌ها برابر ۵۰۰bp و بعد از هضم با EcoR1 (280, 270bp)، Taq1(330bp) و Busr1 (240, 220, 90bp) فاقد چندریختی بود. نشانگرهای RAPD و ITS-RFLP بالاترین سطح تنوع ژنتیکی که تأییدکننده تنوع در جدایه‌های *C. beticola* بود را نشان دادند.

واژگان کلیدی: بیماری‌زایی، انگشت‌نگاری به‌کمک RAPD، تعامل جدایه در ژنوتیپ، *Cercospora beticola*

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