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Full Length Article

Intraspecific Variation within *Fusarium oxysporum* f. sp. *cumini* from *Cuminum cyminum* in Turkey

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Abstract

Thirty-nine isolates of *Fusarium oxysporum* f. sp. *cumini* were isolated from symptomatic tissues of cumin plants from Ankara and Konya provinces of Turkey during 2011 and 2012 years. Species-specific primers produced a 340-bp DNA fragment from *F. oxysporum* isolates. All isolates tested for pathogenicity on local cultivar under controlled conditions were highly pathogenic to cumin seedlings, showing variation in their aggressiveness. Also, the restriction digestions of PCR-amplified nuclear ribosomal DNA intergenic spacer (IGS) region were used for determining genetic variability within *F. oxysporum* f. sp. *cumini*. Five IGS haplotypes were defined within *F. oxysporum* f. sp. *cumini* based on restriction analysis of IGS region with the four restriction enzymes: *Bsu*RI, *Tru*1I, *Eco*RI, and *Msp*I. UPGMA analysis revealed four major groups at an arbitrary level of 85% similarity. These findings indicate that isolates of *F. oxysporum* f. sp. *cumini* are genetically distinct from each other and the presence of different subgroups of the formae speciales cumini in the important cumin production areas of Turkey.

Keywords: Cumin; Fusarium wilt; Intraspecific variation; Pathogenicity; PCR-RFLP

Introduction

Cumin (*Cuminum cyminum*) is cultivated on 200 117 decares and production of 13 293 t yr^{-1} in Turkey, accounting for 6% of world cumin production (TUIK, 2013). Among the major diseases of cumin, Fusarium wilt of cumin, caused by a soil- and seed-borne vascular wilt pathogen *Fusarium oxysporum* f. sp. *cumini* is a devastating disease that occurs in major cumin growing areas of the world (Pappas and Elena, 1997; Tawfik and Allam, 2004; Mehta *et al*., 2012). The pathogen usually increases in warm areas and under dried conditions and causes yield losses up to 80% (Divakara Sastry and Anandaraj, 2013). Disease symptoms include damping off, wilting, shriveling of the foliage, discoloration within the vascular tissue, growth retardation and plant death according to the stage of plant growth (Pappas and Elena, 1997).

Pathogenic forms within *F. oxysporum* have been further assigned to formae speciales that infects a range of host plants. Also, the pathogen populations belonging to some formae speciales are classified into subgroups, named races based on the reactions of differential sets. The pathogenic variability within *F. oxysporum* f. sp. *cumini* has not been characterized into a race structure, although the classification of different races are reported into many formae speciales (Tawfik and Allam, 2004; Mehta *et al*., 2012).

The most efficient and viable alternative for the control of soil-borne disease is the breeding of resistant cultivars. However, low genetic diversity among different cumin accessions makes it difficult for breeding of resistant varieties (Champawat and Pathak, 1990). Some lines have been identified as tolerant to Fusarium wilt of cumin (Mandavia *et al*., 2000). Knowledge of population structure of plant-pathogenic fungi can offer to the development of an effective breeding approach for resistance. However, a few studies have been conducted to evaluate intraspecific variation within *F. oxysporum* f. sp. *cumini* in the world (Deshwal and Kumari, 2012; Mehta *et al*., 2012).

Molecular techniques have widely been used to evaluate the extent of genetic variability within fungal populations. Genetic diversity within *F. oxysporum* from cumin was characterized by RAPD (Random amplified polymorphic DNA) and VCG (Vegetative compatibility grouping) tests (Mofrad *et al*., 2005; Deshwal and Kumari, 2012; Mehta *et al*., 2012). The conserved regions of nuclear rDNA provide informative inter- and intra-specific polymorphisms in plant-pathogenic fungi. Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified IGS region of ribosomal DNA has been frequently applied to examine intraspecific variations in the genus Fusarium (Appel and Gordon, 1995; Llorens *et al*., 2006; Bayraktar *et al*., 2010).

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This study evaluated the population differences of Fusarium wilt isolates from cumin fields based on pathogenic variations and restriction banding patterns of IGS region.

Materials and Methods

Fungal Materials

Isolates of *Fusarium oxysporum* were recovered from diseased cumin roots obtained from cumin fields at least 5 km apart in Ankara and Konya provinces located in Central Anatolia region of Turkey during 2011 and 2012 years. Infected samples were cut into small pieces and surface sterilized in 1% NaOCl for 3 min. Then, the samples were washed two times with sterilized H_2O and cultured on potato dextrose agar (PDA) medium at 24°C under 12 h photoperiod. Every isolates were purified by single-spore culture and preserved on filter papers at 8°C. The isolates were identified based on morphological characteristics according to Leslie and Summerell (2006).

Pathogenicity Tests

For the pathogenicity test, thirty one isolates of *F. oxysporum* were cultured on PDA medium for two weeks at 24°C under 12 h photoperiod. Spore concentration of each isolates was adjusted to a final concentration of 1×10^5 conidia/mL by diluting in sterile distilled water. Also, five cumin seeds were planted in 10 cm in diameter plastic pots and grown at 24°C for 30 days. The pathogen inoculation was performed by pouring 10 mL of the prepared inoculum onto the surface of each pot. The inoculated seedlings were grown at 24°C for 40 days (Pappas and Elena, 1997). Three replicate pots were used with five plants per the isolate. Disease severity (%) was evaluated using 0-4 scale (0=plants healthy; 1=0-25% wilt; 2=26-50% wilt; 3=51-75% wilt and 4=severe wilt) of Gour and Agrawal (1988). The data were analyzed using analysis of variance (MSTAT, Michigan State Univ., USA) and compared by least significant differences (LSD, P= 0.05).

DNA Isolation

DNA isolation was conducted by the minipreparation method of Edel *et al.* (2000) with some modifications. Mycelia of each isolates were gently scraped from the surface of PDA medium and placed in 1.5 mL eppendorf tube, containing 500 µL of lysis buffer (50 mM Tris-HCl pH: 7.5, 3% SDS, 50 mM EDTA). The mixture was subjected to twice phenol-chloroform extractions, and DNA was precipitated by addition of 0.5 vol. of 7.5 M NH_4OAc and 1.5 vol. of isopropanol. The resultant DNA pellet was washed in ethanol, dissolved in ddH_2O, stored at -20°C until use.

Species-specific PCR Assays of *F. oxysporum* Isolates

The pathogen identification was also confirmed by using the primer pairs FOF1 (5'-ACA TAC CAC TTG TTG CCT CG-3') and FOR1 (5'-CGC CAA TCA ATT TGA GGA ACG-3'), specific to *F. oxysporum* described by Mishra *et al.* (2003) and according to the protocol of Bayraktar and Dolar (2011).

PCR-RFLP Analysis of IGS Region

The IGS region of the ribosomal DNA was amplified with the flanking primer pairs CNL12/CNS1 described by Appel and Gordon (1995). PCR reaction was carried out in 50 µL mixture containing 5 µL reaction buffer (10 X), 0.5 µM primer forward and reverse, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, and 2 U *Taq* DNA polymerase (MBI Fermantase, Germany) and remaining deionized water.

The thermocycling profile was initiated by a 2 min incubation at 94°C, followed by 35 cycles of at 95°C for 35 s, at 58°C for 55 s, and at 72°C for 2 min, and ending with a single 10-min extension step at 72°C. After completion of amplifications, 5 µL PCR product was visualized on 1% agarose gel by ethidium bromide staining (Sambrook *et al*., 1989). The size of amplified fragments was estimated with 100 bp DNA ladders (MBI Fermantase, Germany).

The amplified PCR products, 10 µL aliquots, were cut with the enzymes: *Bsu*RI, *Tru*1I, *Eco*RI, and *Msp*I according to the manufacturer's recommendations (MBI Fermantase, Germany). The separation of restriction fragments were performed by 3% agarose gel electrophoresis in 1xTAE buffer. Band profiles were visualized under UV light after staining with ethidium bromide. All isolates were evaluated for restriction profiles obtained from four enzymes, and each four-letter code was assumed as a distinct IGS haplotype. The similarity coefficient was calculated for each pair of isolates by the Dice coefficient. A dendrogram was constructed by the unweighted pairgroup method using the arithmetic average (UPGMA). All data analysis was performed using the NTSYS-pc numerical taxonomy package, version 2.0 (Rohlf, 1998).

Results

Fusarium wilt of cumin, caused by *F. oxysporum* f. sp. *cumini* was detected widely in cumin fields located in Ankara and Konya provinces, the major producing areas of Turkey during 2011 and 2012. The pathogen was observed to cause typical wilt symptoms including a drooping and yellowing of the leaves, browning of underground plant parts, and eventual death of the plant in infected cumin fields (Fig. 1).

A total of thirty-nine isolates were isolated from symptomatic plant tissues from different fields (Table 1). Based on morphological characteristics, including the

Table 1: Disease severity (%) and IGS types of Fusarium wilt isolates of cumin

Isolate name	Location	Disease Severity (%)[a]	Restriction Enzymes[b]				IGS haplotypes
			*Bsu*RI	*Tru*1I	*Eco*RI	*Msp*I	
FC-1	Ankara/Polatlı	75	B	B	B	B	1
FC-2	Ankara/Polatlı	85.42	B	B	B	B	1
FC-3	Ankara/Polatlı	91.67	B	B	B	B	1
FC-4	Ankara/Polatlı	79.17	B	B	B	B	1
FC-5	Ankara/Polatlı	-	B	B	B	B	1
FC-6	Ankara/Polatlı	77.08	B	B	B	B	1
FC-7	Ankara/Polatlı	-	A	A	A	A	2
FC-8	Ankara/Polatlı	77.08	C	B	B	A	4
FC-9	Ankara/Polatlı	-	B	B	B	B	1
FC-10	Ankara/Polatlı	97.92	B	B	B	B	1
FC-11	Ankara/Haymana	79.17	A	A	A	A	2
FC-12	Ankara/Haymana	91.67	A	A	A	A	2
FC-13	Ankara/Haymana	83.33	B	B	B	B	1
FC-14	Ankara/Haymana	100	B	B	B	A	5
FC-15	Ankara/Haymana	72.92	B	B	B	B	1
FC-16	Ankara/Haymana	-	B	B	B	B	1
FC-17	Ankara/Haymana	87.5	B	B	B	B	1
FC-18	Ankara/Haymana	-	B	B	B	B	1
FC-19	Ankara/Haymana	95.83	A	A	A	A	2
FC-20	Ankara/Haymana	89.58	B	B	B	B	1
FC-21	Ankara/Haymana	83.33	A	A	A	A	2
FC-22	Ankara/Haymana	95.83	A	A	A	A	2
FC-23	Ankara/Gölbaşı	79.17	B	B	B	B	1
FC-24	Ankara/Gölbaşı	79.17	A	A	A	A	2
FC-25	Ankara/Gölbaşı	91.67	B	B	B	B	1
FC-26	Konya/Kulu	93.75	A	A	A	A	2
FC-27	Konya/Kulu	54.17	B	B	B	B	1
FC-28	Konya/Cihanbeyli	-	B	B	B	B	1
FC-29	Konya/Kulu	-	D	C	B	C	3
FC-30	Konya/Kulu	97.92	B	B	B	B	1
FC-31	Konya/Kulu	91.67	A	A	A	A	2
FC-32	Konya/Kulu	81.25	B	B	B	B	1
FC-33	Konya/Cihanbeyli	97.92	D	C	B	C	3
FC-34	Konya/Kulu	-	A	A	A	A	2
FC-35	Konya/Kulu	95.83	A	A	A	A	2
FC-36	Konya/Cihanbeyli	89.58	B	B	B	B	1
FC-37	Konya/Kulu	95.83	A	A	A	A	2
FC-38	Konya/Cihanbeyli	85.42	B	B	B	B	1
FC-39	Konya/Kulu	85.42	B	B	B	B	1
LSD(P=0.05)		10.08					

-No tested. [a]Disease severity (%) was calculated by 0-4 scale (0=plants healthy; 1=0-25% wilt; 2=26-50% wilt; 3=51-75% wilt and 4=severe wilt) described by Gour and Agrawal (1988). [b]IGS haplotypes detected within of *F. oxysporum* f. sp. *cumini* with restriction digestions

shapes of macroconidia and microconidia, the morphology of conidiogenous cells, the production of chlamydospore, and the growth of culture, all isolates were identified as *F. oxysporum*. Species-specific PCR assay was also performed to confirm morphological identification of the isolates identified as *F. oxysporum*. The primer pairs FOF1⁄R1 resulted in the amplification of a 340 bp target fragment from *F. oxysporum* isolates.

In the pathogenicity test, thirty-one isolates of *F. oxysporum* were tested for their virulence on local cumin cultivar under controlled conditions. All tested *F. oxysporum* isolates were highly pathogenic to cumin seedlings, causing typical wilt symptoms. Pathogenic variability among the isolates was statistically significant (P= 0.05) and ranged from 54.2% to 100% (Table 1). Isolate FC-14 was the most aggressive, while isolate FC-27 was the least aggressive. No disease symptoms were observed on control plants.

The PCR-RFLP analysis of IGS region was used to evaluate genetic differences among Fusarium wilt isolates of cumin. Amplification of IGS region with primer CNL12 and CNS1 produced a PCR product of approximately 2.6 kb from the pathogen isolates. The purified PCR product of each isolate was cut with restriction enzymes *Bsu*RI, *Tru*1I, *Eco*RI, and *Msp*I. Digestion with *Bsu*RI gave four different patterns among the isolates. Representative restriction patterns from *Bsu*RI digestion are shown in Fig. 2. Restriction digestion of IGS region with *Eco*RI showed two different patterns, while digestions of *Tru*1I and *Msp*I revealed three distinct banding patterns (Fig. 3). However, some smeared and weak bands were not possible to determine their correct size on agarose gel and were excluded from the analysis. The results of PCR-RFLP analysis revealed five distinct

Fig. 1: Fusarium wilt symptoms observed in cumin fields infected by *Fusarium oxysporum* f. sp. *cumini*

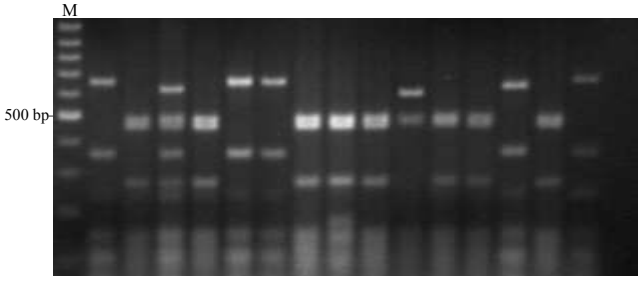

Fig. 2: Restriction band patterns of *Fusarium oxysporum* f. sp. *cumini* isolates digested with the enzyme *Bsu*RI. M: 100 bp DNA Ladder (MBI Fermantase, Germany)

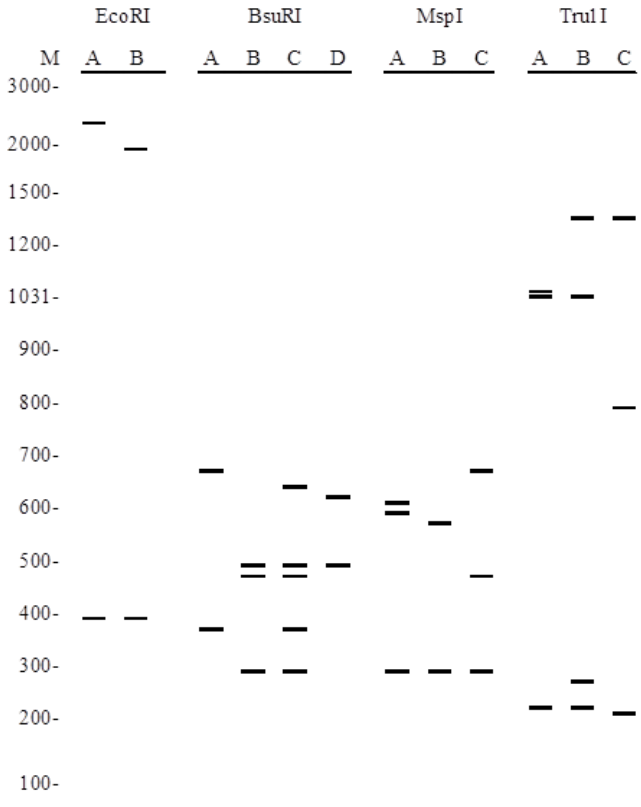

Fig. 3: Restriction patterns of IGS-PCR products of *Fusarium oxysporum* isolates digested with four enzymes. M: 100 bp DNA ladder (MBI Fermantase, Germany)

IGS haplotypes among 39 *F. oxysporum* isolates (Table 1). Enzyme pattern representing IGS haplotype 1 was the most frequent pattern and observed in 23 isolates. IGS haplotypes 2 and 3 were formed on 12 and 2 isolates, respectively whereas IGS haplotypes 4 and 5 were observed in one isolate each. Also, IGS haplotypes 1 and 2 were prevalent in different districts of both Ankara and Konya provinces. IGS haplotypes 4 and 5 were only detected in Ankara province, while IGS haplotypes 3 was found in Konya province. The cluster analysis of restriction patterns classified all isolates into four major groups at an arbitrary level of 85% similarity (Fig. 4). Isolates of IGS haplotypes 1, 2 and 3 clustered into different groups, while IGS haplotypes 4 and 5 clustered into the same group. However, the results did not show a significant relationship between restriction enzyme profiles of IGS region and pathogenic variability of Fusarium wilt isolates.

Discussion

Fusarium wilt of cumin is a major risk factor for cumin production of Turkey. Determination of intraspecific variations within *F. oxysporum* f. sp. *cumini* is essential for developing the efficient disease management programs to Fusarium wilt. To determine the pathogenic and genetic structure of *F. oxysporum* f. sp. *cumini* and to get an insight into the population structure of this pathogen, this study revealed some useful scientific information.

The occurrence of Fusarium wilt disease was surveyed at cumin cultivation areas in Central Anatolia region, providing approximately 85% of Turkey's cumin production during 2011 and 2012 (TUIK, 2013). Isolation from collected disease samples resulted in 39 *F. oxysporum* isolates based on cultural characteristics and specific PCR assay. All isolates tested for the pathogenicity were pathogenic to local cumin cultivar and caused disease symptoms that were similar to those described by Pappas and Elena (1997). Also, the pathogen isolates showed significant variability in their aggressiveness, ranging from 54.2% to 100%. Pathogenicity tests using Fusarium wilt isolates of cumin in different countries revealed aggressiveness variation among the pathogen isolates, but these studies were conducted by using a limited number of the isolates. Mehta *et al.* (2012) observed high degree of pathogenic variability among ten pathogen isolates from the major growing areas of India and detected disease incidence ranging 23.31 to 95.00 and 18.46 to 80.00 in sterilized and unsterilized soil, respectively. Similarly, five *F. oxysporum* f. sp. *cumini* isolates from cumin growing areas in Egypt

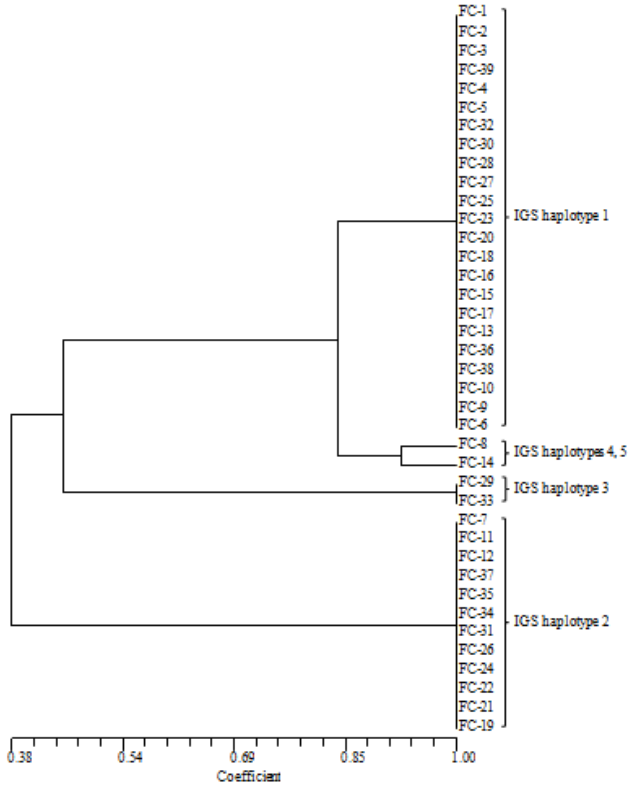

Fig. 4: Dendrogram generated by UPGMA of Dice's coefficients based on PCR-RFLP data of *F. oxysporum* f. sp. *cumini* isolates

showed pathogenic variability, ranging from 45% to 87.3% (Tawfik and Allam, 2004).

F. oxysporum f. sp. *cumini* is considered as a devastating disease, limiting cumin production in many countries (Pappas and Elena, 1997; Tawfik and Allam 2004; Mehta *et al.,* 2012), although the studies for determining of genetic diversity within the pathogen populations are quite limited. Ten Indian isolates of *F. oxysporum* f. sp. *cumini* were grouped into different clusters with the genetic similarity values, ranging from 0.21 to 0.68 based on RAPD data (Mehta *et al.,* 2012). Similarly, six VCG groups and two major RAPD clusters were detected among ten Fusarium wilt isolates from cumin plant at the level of 66.25% genetic similarity (Deshwal and Kumari, 2012). However, there have been no reports based on genetic diversity within conserved regions of ribosomal DNA.

Restriction analysis of IGS region has been used successfully to examine intraspecific polymorphisms within *F. oxysporum* (Appel and Gordon, 1995; Edel *et al*., 1997; Nagarajan *et al*., 2004) and other *Fusarium* species (Hinojo *et al*., 2004). Cai *et al*. (2003) detected a close relationship between IGS haplotypes and VCG groups of *F. oxysporum* f. sp. *lycopersici*. Similarly, genetic diversity within IGS region allowed the discrimination of lettuce isolates of *F. oxysporum* and other formae speciales and the identification of race 1 isolates with specific restriction enzymes (Rimondi *et al*., 2010). In this study, restriction patterns of IGS region were used for determining genetic variability within *F. oxysporum* isolates from cumin. Isolates from different cumin fields showed high level of genetic diversity and clustered into five IGS haplotypes based on the restriction banding profiles of IGS region with four enzymes. UPGMA analysis clustered all isolates into four major groups. In previous studies, the other formae speciales of *F. oxysporum* were classified into different number of IGS haplotypes and genetic groups related to restriction polymorphisms (Appel and Gordon, 1995; Edel *et al*., 1997; Cai *et al*., 2003). However, genetic variability within IGS region of Fusarium wilt isolates did not correlated with pathogenic variation and geographical locations of the isolates. Intraspecific variation among the pathogen isolates may be correlated to mutations, gene flow or recombination within IGS region. These results were consistent with the findings of different researchers reported that there was no relationship between restriction analysis of IGS region and pathogenicity, geographic location and VCG groups of *F. oxysporum* in different hosts (Alves-Santos *et al*., 1999; Bayraktar *et al*., 2010; Hafizi *et al*., 2013).

Conclusion

These results indicated that the pathogen isolates from cumin were genetically distinct from each other and different subgroups of the formae speciales *cumini* were found in Turkey. These results contributed to our understanding of the extent of genetic variation among *F. oxysporum* f. sp. *cumini* isolates in Turkey. Thus, the prevailing genotypic groups should be taken into consideration in the developing processes of resistance cumin cultivars to this destructive pathogen.

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