

Determination of Fungal Pathogens Associated with *Cuminum cyminum* in Turkey

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Abstract

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The occurrence of fungal pathogens, associated with cumin production of Turkey, was determined during 2011 and 2012. A total of 379 isolates were classified as *Fusarium oxysporum* f.sp. *cumini*, *F. solani*, *F. acuminatum*, *F. equiseti*, *F. sambucinum*, *F. avenaceum*, *Macrophomina phaseolina*, *Alternaria burnsii*, *A. alternata*, *A. infectoria*, *Embellisia* sp., and *Rhizoctonia solani*. Pathogenicity tests showed that *F. oxysporum* f.sp. *cumini*, *F. solani*, *F. equiseti*, *M. phaseolina*, *A. burnsii*, and *A. alternata* were highly pathogenic. *A. burnsii* and *F. oxysporum* f.sp. *cumini* were the major risk factors for cumin cultivation in Turkey. To our knowledge, this is the first report on *F. oxysporum* f.sp. *cumini*, *F. solani*, *F. equiseti*, *M. phaseolina*, and *A. alternata* in Turkey.

Keywords: fungal diseases; cumin; wilt; blight; root rot; *Alternaria* spp.; *Fusarium* spp.; *Macrophomina phaseolina*

Cumin (*Cuminum cyminum*) is an important seed spice crop that is native to the region from the eastern Mediterranean to India. Turkey is an important producer of cumin, accounting for about 6% of world production with cultivated area of 20 011 ha and production of 13 293 t/year (ANONYMOUS 2013). Cumin seeds are used for adding condiments and flavours to food, and for medicinal and cosmetic properties. Also, essential oils from cumin seeds are used in controlling plant pathogens due to their broad spectrum of antimicrobial activity (DIVAKARA SASTRY & ANANDARAJ 2013).

Cumin production is seriously affected by different fungal pathogens such as *Alternaria* spp., *Fusarium* spp., *Erysiphe polygoni* (KISHOR & JAIN 1999; DIVAKARA SASTRY & ANANDARAJ 2013). Cumin wilt caused by *F. oxysporum* f.sp. *cumini* is a destructive disease of this crop and results in yield losses of up to 80% (LODHA *et al.* 1986; DIVAKARA SASTRY & ANANDARAJ 2013). *A. burnsii* affects cumin plant only after flowering stage and causes complete failure of the crop in some years depending on climatic conditions. Powdery mildew caused by *E. polygoni* is a less important disease that occurs under warm,

humid conditions (DIVAKARA SASTRY & ANANDARAJ 2013). Also, other fungal pathogens such as *A. alternata*, *M. phaseolina*, *F. equiseti*, and *F. solani* have been reported to cause vascular wilt, charcoal rot and blight symptoms on cumin plant (REUVENI 1982; MOHAMMADI & MOFRAD 2009; MAHDIZADEH *et al.* 2011; RAMCHANDRA & BHATT 2012). However, little information is known about the pathogens, causing yield losses in cumin production areas of Turkey. *A. burnsii*, *A. raphani*, and *Fusarium* spp. were found to be associated with cumin plants in Turkey (KOCATÜRK 1988; KOCATÜRK *et al.* 1988).

The aim of this study was to identify fungal pathogens, causing economic losses on cumin production of Turkey. The results will contribute to the development of disease management methods to control these pathogens.

MATERIAL AND METHODS

Fungal materials. Cumin growing areas in Ankara and Konya provinces located in Central Anatolia

region, which provides approximately 85% of Turkey's cumin production, were surveyed during 2011 and 2012. Twenty plant samples that showed symptoms of wilting, damping off, root rot, blight, and leaf spots were collected from different areas of each field. Infected tissues were surface-sterilised in 1% sodium hypochlorite solution for 3 min, rinsed thoroughly with sterile distilled water. The diseased tissues were placed on potato dextrose agar (PDA) medium (Merck, Darmstadt, Germany) and the Petri dishes were incubated for 7 days at 23°C under a 12 h dark/light cycle. Each of the isolates were purified and preserved on filter papers at 8°C. The isolates were identified based on morphological characteristics described by DOMSCH *et al.* (1980), LESLIE and SUMMERELL (2006), and SIMMONS (2007). All isolates were deposited in the culture collection of the Department of Plant Pathology at the Agricultural Faculty, Ankara University, Turkey.

Molecular identification. DNA isolation was conducted by the minipreparation method of EDEL *et al.* (2000). Fungal mycelia of each isolate were gently scraped from the surface of PDA medium and suspended in 500 µl of extraction buffer (50mM Tris-HCl pH 7.5, 50mM EDTA, 3% SDS). After double extraction with phenol/chloroform/isoamylalcohol (24:1:1 v/v/v), DNA was precipitated by addition of 0.5 volume of 7.5M ammonium acetate and 1.5 volume of isopropanol. The resultant DNA pellet was rinsed with ethanol, suspended in ddH₂O, and stored at –20°C.

The identification of the isolates representing different fungal species was confirmed by DNA sequence analysis with the primer pairs ITS1/4 and Alt for/rev described by WHITE *et al.* (1990) and HONG *et al.* (2005), respectively. PCR reaction was carried out in 50 ml mixture containing 5 µl reaction buffer (10×), 1.5mM of MgCl₂, 0.4µM of each primer, 0.2mM of dNTPs, 1.5 unit of *Taq* DNA polymerase (MBI; Fermentas, Leon-Rot, Germany) and remaining deionised water. PCR amplification was performed in a thermal cycler programmed as follows: one cycle of 94°C for 1 min, 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and during 10 min at 72°C.

The amplified DNA products were sequenced in both directions using the same primers at Refgen Biotechnology Laboratory (Ankara, Turkey). The resulting sequences were examined by BLAST analysis using the NCBI website. Sequence alignment was performed using MEGA 5.1 software (TAMURA *et al.* 2011). Also, the identification of *Fusarium* spp., for which DNA sequencing assays were not avail-

able, was confirmed by species-specific PCR assays according to BAYRAKTAR and DOLAR (2011).

Pathogenicity tests of fungal species. To confirm Koch's postulates, 30 representative isolates of *F. oxysporum* f.sp. *cumini* (5 isolates), *F. solani* (4 isolates), *F. acuminatum* (3 isolates), *F. equiseti* (3 isolates), *F. sambucinum* (3 isolates), *F. avenaceum* (1 isolate), *A. burnsii* (3 isolates), *A. alternata* (2 isolates), *A. infectoria* (1 isolate), *Embellisia* sp. (1 isolate), *M. phaseolina* (3 isolates), and *R. solani* (1 isolate) were subjected to pathogenicity test on local cumin variety under controlled conditions.

Five cumin seeds were sown into plastic pots 10 cm in diameter and grown at 23°C for 30 days. For the pathogenicity test of *Fusarium* spp., spore concentration was adjusted to a final concentration of 1 × 10⁵ spores/ml by diluting in sterile distilled water. Inoculation was performed by pouring 10 ml of the final inoculum suspension onto the surface of each pot (PAPPAS & ELENA 1997). Inoculum concentration of *Alternaria* spp. and *Embellisia* sp. isolates was adjusted to a final concentration of 1 × 10⁴ spores/ml. Inoculation was carried out by spraying the spore suspension onto the leaves of cumin seedlings (PRYOR & GILBERTSON 2002). Pathogenicity assays of *M. phaseolina* and *R. solani* isolates were performed by soil-inoculation method of NENE and HAWARE (1980). Thirty-day-old cumin seedlings were transplanted into the soil inoculated with these pathogens. Cumin seedlings inoculated with the isolates of *Fusarium* spp., *M. phaseolina*, and *R. solani* were grown at 23°C for 40 days under controlled conditions. The plants inoculated with *Alternaria* spp. and *Embellisia* sp. were grown at 23°C for 15 days. Three pots were used for each isolate. Experiments were repeated twice at different times. Diseased severity was evaluated with 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) for *Fusarium* spp., *M. phaseolina*, and *R. solani*. The criteria of evaluation were leaf wilt, stunted growth, brown discoloration on stems, and death of all plant. The plants inoculated with *Alternaria* spp. and *Embellisia* sp. were evaluated using 0–5 scale (0 = no disease, 1 = 1% leaf necrosis, 2 = 5% leaf necrosis, 3 = 10% leaf necrosis, 4 = 20% leaf necrosis, 5 ≥ 40% leaf necrosis) of PRYOR and GILBERTSON (2002). Disease index of the isolates belonging to each fungal species was compared by the Least Significant Difference test (*LSD*, *P* = 0.05) using MSTAT (Michigan State University, East Lansing, USA) package program.

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Table 1. Relative frequencies (%) of fungal species obtained from different cumin growing areas in Ankara and Konya provinces

Fungal	Provincec					Percentage compared to all species
	Ankara			Konya		
	Polath	Haymana	Gölbaşı	Cihanbeyli	Kulu	
<i>F. acuminatum</i>	16.67	12.28	15.00	23.02	27.05	21.37
<i>F. avenaceum</i>	0	1.75	0	0	0	0.26
<i>F. equiseti</i>	11.11	3.51	20.00	11.11	5.74	8.71
<i>F. oxysporum</i>	18.52	22.81	15.00	6.35	13.11	13.19
<i>F. sambucinum</i>	5.56	5.56	10.00	2.38	8.20	5.54
<i>F. solani</i>	12.96	15.79	5.00	7.94	7.38	9.50
<i>A. burnsii</i>	20.37	10.53	10.00	47.62	36.89	32.72
<i>A. alternata</i>	3.70	7.02	5.00	1.59	0.82	2.64
<i>A. infectoria</i>	3.70	5.26	10.00	0	0	1.85
<i>Embellisia</i> sp.	0	3.51	5.00	0	0.82	1.06
<i>M. phaseolina</i>	5.56	12.28	5.00	0	0	2.90
<i>R. solani</i>	1.85	0	0	0	0	0.26
Total number of isolates	54	57	20	126	122	379

RESULTS

The occurrence of fungal pathogens associated with cumin plant was surveyed at cultivation areas in Central Anatolia region, the major growing areas of Turkey. Diseased plant samples that showed wilting, blight, and root rot symptoms were collected from cumin fields. Isolation from collected disease samples resulted in a total of 379 fungal isolates. Based on morphological characteristics, the isolates were identified as *F. oxysporum* f.sp. *cumini*, *F. solani*, *F. acuminatum*, *F. equiseti*, *F. sambucinum*, *F. avenaceum*, *M. phaseolina*, *A. burnsii*, *A. alternata*, *A. infectoria*, *Embellisia* sp., and *R. solani* (Table 1).

Morphological identifications were also confirmed by DNA sequence analysis of ribosomal DNA-ITS region for the isolates of *Fusarium* spp., *R. solani*, and *M. phaseolina*, and ITS and major allergen *Alt a1* regions for the isolates of *Alternaria* spp. and *Embellisia* sp. PCR amplification with primers ITS1 and ITS4 yielded a single DNA fragment approximately ranging in length from 510-bp to 540-bp from all isolates. Amplification of the *Alt a1* gene resulted in a PCR product of approximately 425–485-bp in length. BLAST analysis of all isolates except for the five isolates of *A. burnsii* showed a high level of genetic similarity with DNA sequences of fungal species available in GenBank. All *A. burnsii* isolates had the same sequence homology on

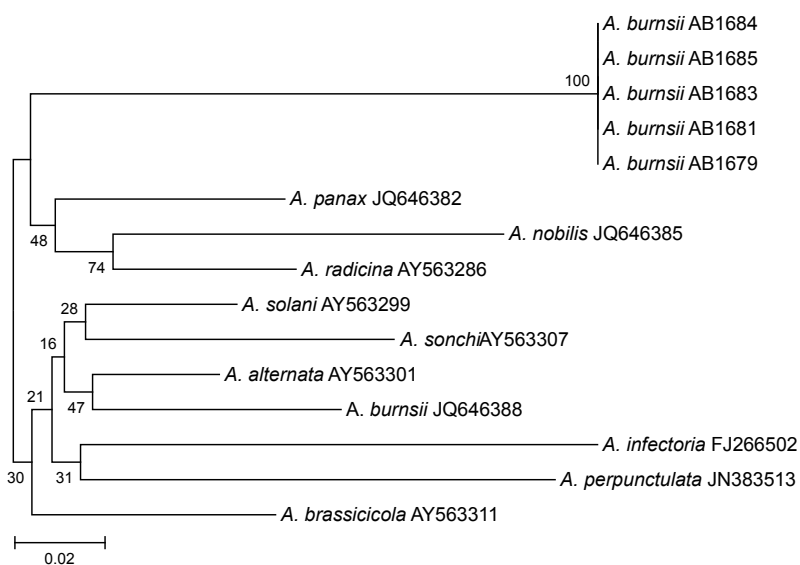


Figure 1. Neighbor-joining tree constructed using *Alt a1* gene sequences of *Alternaria burnsii* isolates from cumin and the reference isolates belonging to different *Alternaria* section described by LAWRENCE *et al.* (2013). The numbers at the branch points indicate bootstrap values based on 1000 replicates

the regions of ITS and *Alt a1* gene. However, the identification of *A. burnsii* isolates could not be confirmed by DNA sequence analysis because ITS sequence of *A. burnsii* was not available in GenBank, and BLAST analysis of *Alt a1* gene did not show similarity with only one sequence (accession No. JQ646388), available for *Alt a1* gene of *A. burnsii* in GenBank. Also, DNA sequencing analysis of *Alt a1* gene revealed that *A. burnsii* was phylogenetically distant to other species-groups of *Alternaria* described by LAWRENCE *et al.* (2013) (Figure 1). Thus, the identification of *A. burnsii* isolates was only confirmed based on morphological characteristics described by SIMMONS (2007). The resulting sequences were deposited in GenBank under accession numbers KF453968, KJ526171–KJ526181. The isolate of *R. solani* was further characterised to AG 3 by sequencing analysis of ITS region. Species-specific PCR assays confirmed the identity of other *F. oxysporum*, *F. equiseti*, and *F. acuminatum* isolates, not analysed by DNA sequencing assay. All *F. oxysporum* isolates gave the specific fragment of 340-bp with the primer set FOF1/R1. Specific primer pair FEF1/FER1 amplified the expected 400-bp DNA fragment from *F. equiseti* isolates. The isolates of *F. acuminatum* produced the expected 600-bp fragment with the primer set FAF1/R.

In pathogenicity tests, the isolates of *F. acuminatum*, *F. sambucinum*, *F. avenaceum*, *A. infectoria*, *Embellisia* sp., and *R. solani* did not cause visible symptoms on cumin plants. These species might be weakly pathogenic or non-pathogenic on this cumin cultivar. The other fungal species caused mean disease severity ratings ranging from 75% to 100% (Table 2). Also, there was no statistically significant difference in disease severity ratings among the isolates belonging to each pathogenic species except for isolate FC-6 of *F. oxysporum* f.sp. *cumini*. *A. burnsii*, and *A. alternata* led to typical blight symptoms including brownish necrotic spots and death of plant tissue. Isolates of *F. oxysporum* f.sp. *cumini* were highly pathogenic and caused red-brown shriveling of the foliage, wilting, and finally death of all plants. *F. solani*, *F. equiseti*, and *M. phaseolina* resulted in different levels of wilting, root rot, and death symptoms on infected plants. The pathogenic fungi were consistently re-isolated from diseased plant tissues. In contrast, the control plants did not show any symptoms.

A. burnsii was determined as the most common pathogen, associated with cumin production in this region (Table 1). The pathogen comprised 32.72% of all isolates recovered from diseased cumin plants. *F. oxysporum* and *F. acuminatum* represented 13.19 and 21.37% of all isolates, respectively. *F. solani*

Table 2. Disease severity (%) of selected pathogens on cumin plants

Isolate	Fungal species	Disease severity (%)
FC-26	<i>F. oxysporum</i> f.sp. <i>cumini</i>	93.75 ^a
FC-19	<i>F. oxysporum</i> f.sp. <i>cumini</i>	95.83 ^a
FC-14	<i>F. oxysporum</i> f.sp. <i>cumini</i>	100.00 ^a
FC-30	<i>F. oxysporum</i> f.sp. <i>cumini</i>	97.92 ^a
FC-6	<i>F. oxysporum</i> f.sp. <i>cumini</i>	77.08 ^b
FC-3	<i>F. equiseti</i>	87.50 ^a
FC-4	<i>F. equiseti</i>	77.08 ^a
FC-5	<i>F. equiseti</i>	87.50 ^a
FC-10	<i>F. solani</i>	89.58 ^a
FC-11	<i>F. solani</i>	91.67 ^a
FC-12	<i>F. solani</i>	75.00 ^a
FC-13	<i>F. solani</i>	85.42 ^a
Mp-1350	<i>M. phaseolina</i>	97.92 ^a
Mp-1351	<i>M. phaseolina</i>	100.00 ^a
Mp-1352	<i>M. phaseolina</i>	97.92 ^a
AC-19	<i>A. burnsii</i>	98.30 ^a
AC-20	<i>A. burnsii</i>	98.30 ^a
AC-21	<i>A. burnsii</i>	96.70 ^a
AC-10	<i>A. alternata</i>	91.70 ^a
AC-11	<i>A. alternata</i>	90.00 ^a

Disease severity (%) was calculated according to GOUR & AGRAWAL (1988) for *Fusarium* spp., *M. phaseolina* and according to PRYOR and GILBERTSON (2002) for *Alternaria* spp., and compared with the Least Significant Difference test (*LSD*, $P = 0.05$) for each fungal species. Within each fungal species, values followed by a common letter were not significantly different from each other followed by a common letter were not significantly different from each other

and *F. equiseti* comprised 9.5 and 8.71%, respectively while *M. phaseolina* and *A. alternata* comprised 2.9 and 2.64% of all isolates. The other fungal species represented 8.97% of all isolates. Compared Ankara and Konya provinces to each other, *F. oxysporum* was more common in Ankara province while *A. burnsii* was found to be more common in Konya province. *M. phaseolina* was only detected in cumin growing areas located in Ankara province.

DISCUSSION

In the present study, the fungal pathogens associated with cumin production in Turkey were characterised in detail for the first time. In previous studies, *Alter-*

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naria burnsii, *A. raphani*, and *Fusarium* spp. were determined to cause wilt, blight, and wither symptoms in the flowering period (KOCATÜRK 1988; KOCATÜRK *et al.* 1988). However, there was no detailed information about these pathogens in previous studies. Isolates of *Fusarium* spp. were not identified at the level of species. In this study, the isolates from the major cumin growing areas of Turkey were identified based on morphological characteristics, DNA sequence analysis of two regions, and pathogenicity test. Among these fungal species identified, *F. oxysporum* f.sp. *cumini*, *F. solani*, *F. equiseti*, *M. phaseolina*, and *A. alternata* have not been reported in cumin plants in Turkey.

Comprising to 45.9% of all isolates recovered from diseased plants, *A. burnsii* and *F. oxysporum* f.sp. *cumini* were determined as the most important limiting factors for cumin production of Turkey. Especially, *A. burnsii* was found in most of the investigated fields and observed to cause the destruction of all products in some cumin fields during the surveys in 2011. *A. alternata* was not as common as *A. burnsii* in cumin fields, although the pathogen was detected in all locations and observed to cause browning, drying, and then death of all plant tissues. Similarly, Alternaria blight of cumin was reported to cause heavy losses in India, the largest producer of cumin (CHAND & JAIN 1999; LODHA & MAWAR 2007).

Cumin root diseases have been reported as a problem limiting cumin production in warm areas and under dried conditions (HASHEM *et al.* 2010; DIVAKARA SASTRY & ANANDARAJ 2013). Several *Fusarium* spp. were related to wilt and root rot diseases of cumin, although *F. oxysporum* f.sp. *cumini* was referred as the main limiting factor that affected cumin cultivation worldwide (PAPPAS & ELENA 1997; HASHEM *et al.* 2010; DIVAKARA SASTRY & ANANDARAJ 2013). In this study, *F. oxysporum* f.sp. *cumini* was widely detected in cumin fields in Turkey and caused typical wilt symptoms that were similar to those described by PAPPAS and ELENA (1997). The isolates identified as *F. solani* and *F. equiseti* were highly pathogenic and caused disease symptoms including stunted growth, root rot, and death of the plant. In previous studies, *F. solani* and *F. equiseti* were reported to be pathogenic to cumin plant (REUVENI 1982; MOHAMMADI & MOFRAD 2009; HASHEM *et al.* 2010; RAMCHANDRA & BHATT 2012). Also, *M. phaseolina* was recovered from some diseased samples, showing blackening of the stem and root rot symptoms, and these isolates were confirmed to be pathogenic to cumin seedlings.

Similarly, this pathogen was determined on cumin plant by MAHDIZADEH *et al.* (2011).

In conclusion, this study is the first known extensive research for characterization of the fungal pathogens, associated with cumin production in Turkey. To our knowledge, isolates of *F. oxysporum* f.sp. *cumini*, *F. solani*, *F. equiseti*, *M. phaseolina*, and *A. alternata* were identified for the first time in cumin fields of Turkey. Also, *A. burnsii* and *F. oxysporum* f.sp. *cumini* were found as the main limiting factor on cumin cultivation. The results obtained may assist in developing an integrated control program for these fungal diseases. More detailed investigations should be carried out on population structure, genetic and pathogenic variability of these pathogens.

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