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ORIGINAL ARTICLE

Effect of seed-borne *Fusarium* species on constituents of essential oils from seeds of black cumin populations

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Abstract

The seed is one of the most important inputs of agricultural products and its quality and health can be affected by seed-borne fungi. Seed-borne fungal pathogens are a major threat to black cumin production and cause considerable yield losses every year worldwide. The aim of this study was to identify seed-borne fungi, the effects of natural fungal infected seeds on some seed quality indicators, and also to investigate cell wall degrading enzymes (CWDEs), pathogenicity and aggressiveness of the isolates obtained from seeds. The constituents of essential oils (EOs) from seeds of Iranian and Syrian black cumin populations were identified and their effect on [isolated] seed-borne *Fusarium* isolates. A total of 17 isolates were identified based on morphological and molecular characteristics of *Fusarium oxysporum* and *F. solani* species. The results of the standard germination test showed that there was a significant difference between the studied seed populations in the germination and vigor indices. Our results indicated that most of the identified isolates were in the seed coat, while a few isolates of *F. oxysporum* were located in embryos. The results of the pathogenicity test showed that about 42% of the isolates were pathogenic and 58% of the isolates were non-pathogenic. Different levels of pathogenicity and aggressiveness were observed for various isolates of *Fusarium* species. All *Fusarium* isolates were not capable of producing CWDEs as pathogenicity factors. Analyzing the activity of CWDEs, including cellulase, pectinase, xylanase and lipase produced by the *Fusarium* isolates, revealed that activity levels of CWDEs are positive and are correlated with variations in pathogenicity and aggressiveness of seed-borne fungal isolates on seeds. The EOs were identified by gas chromatography-mass spectrometry and the major constituents were identified as ρ-cymene, trans-anethole, thymoquinone, limonene, carvacrol and α-thujene. The results showed that the compounds ρ-cymene, limonene, carvacrol, thymoquinone and trans-anethole had antifungal effects against *F. oxysporum* isolate. It seems that the percentage of carvacrol and limonene composition in the EOs components can affect the presence of the seed-borne *Fusarium*. This is the first report on the effect of EO compositions of black cumin seed populations on seed-borne *Fusarium* isolated from the same seeds. The findings of this research showed that the amounts and types of constituents of EOs of black cumin seed populations are different and they can affect the abundance of seed-borne fungi and their level of pathogenicity and aggressiveness.

Keywords: black cumin, carvacrol, cell wall degrading enzymes, limonene, pathogenicity, seed-borne

Introduction

Black cumin (*Nigella sativa* L.) belonging to the Ranunculaceae family, is an important aromatic plant with medicinal properties (Ahmad *et al*. 2013). Black cumin is cultivated on about 24.2 ha with a production of 30,700 t in Iran (Anonymous 2019). There are various reports about how abiotic and biotic stress affect the germination and seedling growth of black cumin (Elwakil and Ghoneem 1999; Ahma-

dian *et al*. 2015; Papastylianou *et al*. 2018). Black cumin seeds are economically important in herbal medicines commonly employed in Iran. In previous studies, different biological activities, including antibacterial, antifungal and antioxidant, antiviral and ant parasitic potentials of this plant, have been demonstrated (Chaieb *et al*. 2011; Ozdemir *et al*. 2018).

Seed health plays an important role in increasing the quantity and quality of yield. Seed health refers specifically to the severity and incidence of pathogens in seeds which play a vital role in carrying pathogens (Mahapatra *et al*. 2019). Fungal pathogens are some of the most important living factors affecting seed health that may be internally associated with seeds or externally seed-borne (Singh *et al*. 2011). Seed-borne fungal pathogens have some deleterious effects on seeds such as reducing seed viability, seed necrosis, seed rot, rotting, reduction or elimination of vigor and germination capacity, variation in plant morphology, wilting and blight of seedlings (Browne and Cooke 2005).

Black cumin production is seriously affected by various diseases like fusarium root rot and wilt which are devastating diseases that occur in major black cumin growing areas of the world. *Fusarium* root rot and wilt of black cumin are caused by several species of *Fusarium*, but primarily they are caused by *F. oxysporum* and *F. solani*, and results in yield losses. So far, the presence of different species of *Fusarium* associated with black cumin have been identified throughout the world, including *F. oxysporum*, *F. solani*, *F. camptoceras*, *F. lateritium* and *F. moniliforme* (Elwakil and Ghoneem 1999; Karakaya and Erzurum 2002; Mohamed *et al*. 2017; Al-Sman *et al*. 2019). These fungi are considered to be soil and seed-borne pathogens. Several management strategies have been used to control diseases of black cumin such as planting uninfected and healthy seeds, and chemical and biological control (Al-Sman *et al*. 2019).

Fungal pathogens belonging to the *Fusarium* have no specialized structures for penetration into plant cells and enter the host via natural openings, e.g., through wounds, and penetrate the epidermal cell walls directly with short infection-hyphae (Pritsch *et al*. 2000; Wanyoike *et al*. 2002). The plant cell wall is a dynamic physical barrier, the first layer of plant defense (Underwood 2012), and fungal pathogens have evolved for overcoming physical defense barriers in the host plants. The ability to produce various cell wall degrading enzymes (CWDEs), as the major type of pathogenicity factors, is necessary for fungal pathogens to penetrate into cell walls and plasma membranes (Kikot *et al*. 2009). Aggressiveness is an important factor of pathogenic adaptability since it indicates the potential ability of pathogens to cause epidemics and induce the disease on a smaller scale of time to the host plants (Sacristan and García-Arenal 2008; Lannou 2012). Pathogenicity and aggressiveness of *Fusarium* species are associated with various mechanisms such as production of extracellular enzymes and mycotoxin, which enable them to penetrate and invade host plant seedlings and to cause wilt disease symptoms (Cho *et al*. 2009). The secretion of CWDEs, such as polygalacturonases, pectate lyases, xylanases and proteases are important in the penetration of *F. oxysporum* hyphae into the host (Michielse and Rep 2009). The increased production of CWDEs by phytopathogens is directly associated with their increased invasion in the plant host (Kikot *et al*. 2009; Noda *et al*. 2010).

Despite the economic and commercial importance of diseases in black cumin production, understanding the percentage of infected seeds in black cumin populations is limited. Thus, the objectives of this study were to (i) identify isolated seed-borne *Fusarium* species isolates, (ii) determine the effect of seed-borne *Fusarium* on germination and vigor indices, (iii) evaluate pathogenicity and some factors affecting it, and also (iv) identify the constituents of EOs from seeds of black cumin populations and evaluate their effect on seed-borne *Fusarium* isolates.

Materials and Methods

Sample collection

Eight samples were collected from the main black cumin-growing areas of two important black cumin-producing countries, Iran and Syria. Samples from each of the black cumin-growing regions were collected according to the International Seed Testing Association guidelines after harvest in 2018–2019 (ISTA 1986). A total of seven samples from Iran including Gorgan (36°52' 3.1"N, 54°28'57.4"E), Bushehr (28°55'00.7"N, 50°58'15.4"E), Shahreza (31°58'28.0"N, 51°52'49.4"E), Fereidan (33°01'10.3"N, 50°14'27.1"E) and Semirom (31°24'57.5"N, 51°32'30.9"E), Sarvabad (35°18'39.6"N, 46°22'21.6"E), Nazarabad (35°59'25.9"N, 50°35'56.8"E) and also one sample from Syria's export population were used in this study. The seed samples were packed in a paper envelope and stored in a freezer until used for subsequent studies.

Isolation and morphological identification of *Fusarium*

The agar plate method was used to detect seed-borne *Fusarium* associated with black cumin seeds. One hundred seeds of each seed lot were randomly selected, surface-sterilized in 1% sodium hypochlorite solution for 1 min, rinsed in sterile distilled water and

left to air dry. Seeds were plated in Petri dishes containing potato dextrose agar (PDA) amended with 25 mg · l^{-1} streptomycin sulphate. Petri dishes were incubated at 25 ± 1°C for 14 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness. The different fungal colonies on the PDA were retrieved from the seeds, isolated and purified using the single spore technique and/or the hyphal tip method. Morphological identification was based on the key described by Leslie and Summerell (2006).

Molecular identification of isolated *Fusarium*

To confirm the morphological identification of the isolated *Fusarium* at the species level, conventional polymerase chain reaction (PCR) was performed using specific primers (Table 1). DNA extraction was performed with a DNA extraction kit (Genomic DNA isolation kit; Pishgam Biotech, Iran) according to the manufacturer's instructions. The reaction mixture and the PCR program were performed as described by Mishra *et al*. (2003). Amplification products were separated by electrophoresis (80–90 V, 60 mA, 100 W, 30 min) in 1.5% agarose gels in 1 Tris-acetate-EDTA (TAE) buffer and visualized by SYBR Green staining on a UV gel documentation system. Positive controls containing the DNA of *F. oxysporum* and *F. solani* in PCR reaction mixture and negative control with molecular grade water were included (Rezaee *et al*. 2018).

Standard germination test

Germination and vigor tests were performed (on Whatman filter paper in 9-cm Petri dishes) using a standard germination test (with 2 mm radicle growth) (ISTA 2013). Four replicates of 25 seeds from each genotype were sown in Petri dishes. The Petri dishes were placed in a germinator at 25 ± 1°C with an alternating 16/8 h light/dark photoperiod. After a 14-day incubation period, we analyzed the germination percentage, normal and abnormal (deformed and diseased) seedlings, shoot and root lengths, and fresh and dry weights were determined (by placing them in the oven for 24 h at 75°C). Also, seedling length vigor index (SLVI) and seedling weight vigor index (SWVI) relations were calculated (Nautiyal 2009).

Location of isolates in black cumin seed

The location of identified isolates in black cumin seed was studied by employing the component plating technique (Maden *et al*. 1975). Naturally infected black cumin seed samples were used for the study. Twenty seeds were washed four times with tap water, surface sterilized in 1% sodium hypochlorite for 3 min. These seeds were again washed with sterile water and soaked in water for 60 min and then the seeds were dissected aseptically using a sterile needle and forceps. The separated seed parts viz., seed coat and embryo were plated immediately before drying on PDA plates. The plates were incubated at 25 ± 1°C for 7 days. Then, they were examined under a stereo-binocular microscope for the presence of *Fusarium* spp. in different seed parts. The infection level of each part was evaluated according to the following formula (Müller *et al*. 2012):

$$\text{Infection level [\%]} = \frac{\text{Total number of infected seed parts}}{\text{Total number of infected parts}} \times 100.$$

Plant materials and extraction of EOs

An Iranian black cumin native population obtained from the Seed and Plant Certification and Registration Institute of Karaj, Iran, was used for pathogenicity tests. The seeds were surface sterilized with 1% sodium hypochlorite for 30 s, rinsed three times with sterile distilled water. Five black cumin seeds were sown in each of the 15 cm-diameter plastic pots filled with sterile potting soil, which had been autoclaved at 121°C for a minimum of 30 min at 100 kPa (15 psi) on 2 successive days and grown in a greenhouse (25 ± 3°C; 16/8 h light/dark photoperiod). The soil used in this experiment was a combination of peat moss, vermiculite and perlite at a ratio of 2 : 1 : 1 (v/v/v).

The extraction of essential oils (EOs) was performed in the Horticulture Laboratory at Ferdowsi University of Mashhad, Iran. The seeds were washed with distilled water and dried at room temperature in the shade and away from direct sunlight. Then, the dried leaves were crushed and plant tissues were passed through a sieve (10 mesh). For isolation of the EOs, 50 g of dried plant

Table 1. Primer sequences, product sizes and reference used for PCR identification of *Fusarium* species

Species	Primer	Sequences (5′-3′)	Product size (bp)	Reference
Fusarium oxysporum	FOF1	ACATACCACTTGTTGCCTCG	340	Mishra *et al*. (2003)
	FOR1	CGCCAATCAATTTGAGGAACG		
F. solani	Fs4F	ATCGGCCACGTCGACTCT	658	Delgado-Ortiz *et al*. (2016)
	Fs4R	GGCGTCTGTTGATTGTTAGC		

materials were subjected to hydro-distillation for about 3 h, using a clevenger apparatus. The oil was dried over anhydrous Na_2SO_4, preserved in sealed glass bottles and protected from the light by wrapping in aluminum foil. It was stored at 4°C until used. Essential oils percentage was determined using the following formula:

$$\text{Essential oil [\%]} = \\ = \frac{\text{Extracted essential oil [g]}}{\text{50 g sample of each seed}} \times 100.$$

Inoculum preparation

Fungal inocula were produced on saltwater nutrient agar (SNA) medium as described by Müller *et al.* (2012). Conidial suspensions were diluted with sterile distilled water to a final concentration of 1×10^5 conidia · ml^{-1} containing 0.05% (v/v) Tween 20.

Pathogenicity test and assessment of aggressiveness

To confirm Koch's postulates, isolates were subjected to the pathogenicity test on black cumin under controlled conditions. Inoculation was carried out with 10 µl of spore suspension (1×10^5 conidia · ml^{-1}) amended with Tween 20, and sprayed onto the leaves of black cumin seedlings. Disease severity was estimated at 10 days postinoculation using a 0–3 disease scale (Al-Sman *et al.* 2019), and the disease index (DI) was calculated (Khaledi *et al.* 2017). Aggressiveness, as the quantitative component of pathogenicity, was investigated for each fungal isolate on seedlings using the methods described by Hassani *et al.* (2019) and Khaledi *et al.* (2017) based on determining hours post inoculation (hpi) for disease symptom appearance.

Enzymatic analyses of pathogenicity factors

Activity of several CWDEs (including cellulase, lipase, pectinase and xylanase) was evaluated in this study within 10 days *in vitro* (37–38). The test for each enzyme had three replicates for each isolate and the experiment was repeated two times. Fungal cultures were grown for each fungal isolate in basic culture media of cellulase, xylanase, lipase and pectinase activities as described by Miller (1959), MacMillan and Voughin (1964), Abdel-Razik (1970), Ortega *et al.* (2013), respectively. Then, cellulase, xylanase, lipase and pectinase activities were investigated using the methods of Wood and Bhat (1988), Khanna and Gauri (1993), Colowich (1995), and Ortega *et al.* (2013), respectively.

The absorbance of cellulase and xylanase activities were spectrophotometrically measured at 550 nm and 540 nm, respectively, and also the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity was defined as the amount of the enzyme that catalyzed 1.0 µmol of glucose per minute during the hydrolysis reaction. One unit of xylanase activity was defined as the amount of enzyme that liberates 1.0 µ mol of reducing sugars equivalent to xylose per minute under the assay conditions described by Colowich (1995) and Wood and Bhat (1988). The absorbance of pectinase activity was measured spectrophotometrically at 540 nm and a standard curve was drawn based on the absorbance in different concentrations (µg · ml^{-1}) of D-galacturonic acid. Then, a unit of pectinase activity was defined as the amount of enzyme that released 1 µmol of galacturonic acid per minute according to the standard curve. Lipase hydrolytic activity was measured spectrophotometrically at 440 nm with pnitrophenyl palmitate and one unit of lipase activity was defined as the amount of enzyme that releases 1 µmol of p-NPP per minute under the above-mentioned reaction conditions (Ortega *et al.* 2013).

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS was performed in the Oil, Cereal and Food Analysis Laboratory at Ferdowsi University of Mashhad, Iran. Gas chromatography was performed in a Shimadzu GC-17A using a DB-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Helium was used as a carrier gas at a flow rate of 1 ml · min^{-1} (split ratio 1 : 30) with an injection volume of 1 µl. Mass spectra were obtained in the electron impact (EI) mode at 70 eV in a full scan of range from m/z 50 to 650. GC-MS analysis was carried out using a Shimadzu QP 5050 operator. Retention indices were determined by using retention times of n-alkanes that had been injected after the oil under the same chromatographic conditions. The components of the EOs were identified by comparison of their retention indices with those published in the literature (Adams 2017).

Determination of antifungal activities of EOs and their main constituents

Minimum inhibitory concentrations of EOs and their main constituents were determined as described by Plodpai *et al.* (2013) with a few modifications. The PDA plates were amended with various concentrations of EOs and their main constituents (0–4,000 ppm). For enhancing the solubility, Tween-20, 0.05% (v/v) was added. Each plate was inoculated with a mycelial

plug (10 mm diameter) of *F. oxysporum* FO7. All plates were incubated in triplicate for each concentration at 25 ± 1°C for 120 h. Plates with Tween-20 but without any EOs and their main constituents were used as control. Observation of fungal growth was done at time intervals of 12 h up to 120 h after incubation. The minimum inhibitory concentration (MIC) values were determined as the lowest concentrations of EOs and their main constituents that completely prevented visible fungal growth. IC_{50} (the concentration that produces a 50% inhibitory effect) values were graphically calculated from the dose-response curves based on measurements at various concentrations.

Nature of toxicity of EOs and their constituents

The nature of toxicity (fungistatic/fungicide) of the EOs and their constituents against *F. oxysporum* FO7 was determined as described by Thompson (1989). The inhibited fungal mycelia plugs of the oil treated sets were reinoculated into fresh medium and the revival of their growth was observed.

Statistical analysis

All experiments were set up in a completely randomized design with four replicates and conducted three times. The data were analyzed by one-way analysis of variance (ANOVA) and comparison of means was carried out using the least significant difference (LSD) at the level of $p < 0.05$. Statistical analysis was performed using SAS software (version 9.2; SAS Institute, Cary, NC, USA).

Results

Morphological and molecular identification of isolated *Fusarium*

In this study, a total of 17 fungal isolates were recovered from different samples of black cumin fields. Morphological observations showed that all isolates were classified into only *Fusarium* genre (Fig. 1). Finally, two fungal species belonging to *F. oxysporum* and *F. solani* species were isolated and identified based on morphological and molecular characteristics. The results of PCR confirmed the morphological identification of fungal isolates and showed that the isolates were properly identified. Among the isolates, 16 isolates of *F. oxysporum* and one isolate of *F . solani* were identified. *Fusarium*-infected seeds were detected in all seed sampling regions except Bushehr and Syria's export populations. Results of the present study indicated that six, four, three, two, one and one isolates were recovered from Gorgan, Semirom, Sarvabad, Fereidan, Shahreza and Nazarabad seed samples, respectively. Among seed samples of populations in the investigated regions, the Gorgan sample showed the highest distribution of fungal isolates. *Fusarium oxysporum* associated with infected seeds was detected in all sampling regions except Bushehr samples. *Fusarium solani* was detected only from the Gorgan region. Among the identified species, *F. oxysporum* was the most frequent, followed by *F. solani* (Table 2).

Germination and vigor indices of black cumin seeds

The results of seed germination tests as affected by natural fungal infection are presented in Table 2. The seed germination percentage ranged from 100 to 91.75% in all samples. Standard germination results showed that the percentage of diseased seedlings was less than 2.75%; moreover, the percent of deformed seedlings was less than 4.25% for all samples. The SLVI and SWVI of the seedlings varied from 5,150.75 to 7,225 and from 1.77 to 2.55, respectively. Samples from the Gorgan region showed the lowest vigor indices. The average shoot and root lengths of the seedlings varied from 34.7 to 27.7 mm and from 37.5 to 29.0 mm, respectively. The fresh and dry weights of the seedlings varied from 0.275 to 0.253 g and from 0.025 to 0.019 g, respectively. The lowest seed germination, shoot and root lengths, fresh and dry weights were observed in samples from Gorgan, Iran (Table 2).

Location of *Fusarium* pathogens in seeds

The incubation of seeds without coats allowed the internal development of *Fusarium* pathogens. The location of *Fusarium* spp. in the seed was studied by employing the component plating technique and the results are presented in Table 3. A high percentage of *F. oxysporum* was noticed in seed coats (28.3%) and a lower degree of infection in embryos. *F. solani* was located in seed coats. Only *F. oxysporum* was recorded in embryos of seeds at low frequency (Table 3).

Pathogenicity and aggressiveness tests

A comparison of the data obtained from the pathogenicity of fungal isolates isolated in black cumin seeds revealed significant differences in the pathogenicity of the different isolates (Table 4). The pathogenicity test revealed that seven isolates of *F. oxysporum* were found to be pathogenic or weakly pathogenic and the others were non-pathogenic. The identified isolate of *F. solani* was found to be non-pathogenic. Significant differences in disease index were observed between isolates (any species) tested. The FO7 isolate (belonging to

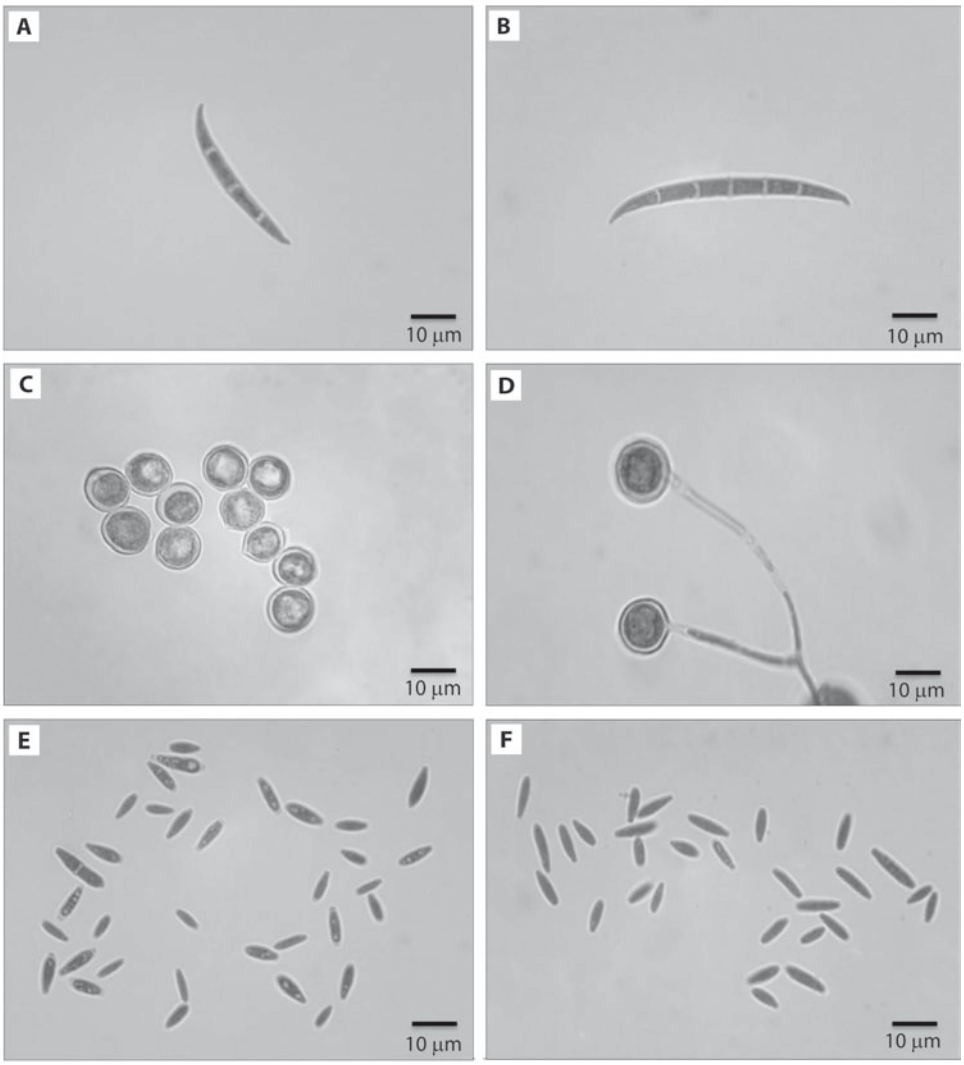

Fig. 1. Morphological characteristics of conidia of *Fusarium oxysporum* and *Fusarium solani* isolated from black cumin seed populations. Macroconidium (A), chlamydospores (B) and microconidia (C) isolate FO7 of *F. oxysporum*; macroconidium (D), chlamydospores (E) and microconidia (F) isolate FS1 of *F. solani*; staining with lactophenol cotton blue. Photographs were taken under a microscope (Olympus BX51) at 400× magnification

Table 2. Frequency of *Fusarium* isolates identified and means comparison of germination and vigor indices as affected by natural fungal infection in black cumin seeds

Sample site	NFI	G	SD	DS	SL	RL	FW	DW	SLVI	SWVI
Gorgan	6	91.75 e	2.75 a	4.25 a	27.7 e	29.0 f	0.253 e	0.019 e	5,150.75 f	1.77 f
Semirom	4	94.00 d	1.50 b	2.75 b	29.5 d	31.0 e	0.261 d	0.021 ed	5,687.25 e	1.90 e
Fereidan	2	96.75 c	1.00 b	1.25 cd	31.2 c	34.0 c	0.266 c	0.022 c	6,313 c	2.18 c
Shahreza	1	98.75 b	0.25 c	0.50 ef	33.2 b	35.7 b	0.270 b	0.024 b	6,814 b	2.37 b
Bushehr	0	100 a	0 c	0 f	34.7 a	37.5 a	0.275 a	0.025 a	7,225 a	2.55 a
Sarvabad	3	96.25 c	1.25 b	1.75 c	29.5 d	32.5 d	0.261 d	0.021 d	5,967.50 d	2.02 d
Nazarabad	1	98.50 b	0.25 c	0.75 de	33.0 b	35.5 b	0.270 b	0.024 b	6,747.50 b	2.34 b
Syria's export population	0	100 a	0 c	0 f	34.5 a	37.2 a	0.274 a	0.025 a	7,175 a	2.52 a
LSD (0.05)	–	0.84	0.59	0.65	0.84	0.87	0.003	0.0008	–	–

NFI = number of *Fusarium* isolates, SD = seedling disease (%), DS = deformed seedling (%), G = Germination (%), SL = shoot length (mm), RL = root length (mm), FW = fresh weight (g), DW = dry weight (g), SLVI = seedling length vigor index and SWVI = seedling weight vigor index. Each experiment was repeated three times with similar results. Different letters indicate significant differences according to LSD analysis using SAS software ($p = 0.05$). Each experiment was repeated three times with similar results

Table 3. Location of *Fusarium* spp. isolates in different seed parts of natural infected black cumin

Fusarium species	Seed component	
	seed coat*	embryo*
Fusarium oxysporum	28.3 a	3.3 a
F. solani	4.2 b	0 b
LSD (0.05)	17.16	3.84

*infection level
Different letters indicate significant difference according to LSD analysis using SAS software ($p = 0.05$). Each experiment was repeated three times with similar results

F. oxysporum) caused the highest level of disease progress on seedlings. The pathogenicity test showed that the disease index of pathogenic and weakly pathogenic isolates for *F. oxysporum* ranged from 67.54 ± 0.45 to 7.48 ± 0.03 (Table 4). The results of aggressiveness of *Fusarium* isolates on seedlings showed that the highest and the lowest aggressiveness belonged to the FO7 (138 hpi) and FO4 (264 hpi) isolates, respectively. In addition, symptoms of other isolates appeared on the seedlings between these time points (Table 4).

Analysis of CWDEs activity

Analysis of CWDEs showed that not all isolates tested were capable of producing cellulase, xylanase, pectinase and lipase, as the main hydrolytic enzymes involved in pathogenicity of pathogenic or weakly pathogenic isolates. These results suggested that all isolates, pathogenic or weakly pathogenic, were capable of producing CWDEs as the main hydrolytic enzymes involved in pathogenicity of isolates. Investigating the activities of CWDEs showed that different isolates tested had various CWDEs activity. The level of CWDEs activity among the tested isolates varied from 684.75 to 312.50 $\mu g \cdot ml^{-1}$ for cellulase, 2345.75 to 1103.25 $\mu g \cdot ml^{-1}$ for pectinase, 950.25 to 516.25 $\mu g \cdot ml^{-1}$ for xylanase, and 25.37 to 17.00 $\mu g \cdot ml^{-1}$ for lipase (Table 4). The FO7 isolate had the maximum CWDEs activity *in vitro* compared to other isolates tested. The lowest levels of CWDEs activities were observed for the FO4 isolate. Among all CWDEs tested, the highest activity levels were observed for pectinase, followed by xylanase, cellulase and lipase (Table 4). The FO7 and FO4 isolates, which showed the highest and lowest levels of CWDEs activity had the highest and lowest levels of pathoge-

Table 4. Pathogenicity and aggressiveness of seed-borne *Fusarium* isolates on black cumin seedling and quantitative analysis of cell wall degrading enzymes produced by them *in vitro*

Isolate code	Sample site	Pathogenicity (DI)	Aggressiveness (hpi)	Enzyme activity [$\mu g \cdot ml^{-1}$]			
				cellulase	pectinase	xylanase	lipase
FO1	Gorgan	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO2	Fereidan	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO3	Shahreza	14.54 ± 0.11 e	234	370.00 ± 0.91 e	1415.00 ± 2.48 e	591.75 ± 0.85 e	18.40 ± 0.01 e
FO4	Fereidan	7.48 ± 0.03 g	264	312.50 ± 1.32 g	1103.25 ± 1.55 g	516.25 ± 1.38 g	17.00 ± 0.02 g
FO5	Gorgan	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO6	Semirom	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO7	Gorgan	67.54 ± 0.45 a	138	684.75 ± 2.01 a	2345.75 ± 4.03 a	950.25 ± 2.29 a	25.37 ± 0.10 a
FO8	Sarvabad	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO9	Nazarabad	10.91 ± 0.31 f	246	349.50 ± 1.19 f	1328.00 ± 1.29 f	572.75 ± 1.25 f	18.05 ± 0.01 f
FO10	Gorgan	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO11	Semirom	35.82 ± 0.28 c	180	545.50 ± 1.32 c	1843.25 ± 1.38 c	721.25 ± 0.63 c	19.81 ± 0.02 c
FO12	Gorgan	28.49 ± 0.26 d	198	512.25 ± 1.25 d	1768.25 ± 2.78 d	696.00 ± 2.04 d	19.35 ± 0.00 d
FO13	Sarvabad	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO14	Semirom	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FO15	Sarvabad	53.27 ± 0.45 b	156	638.50 ± 1.44 b	2160.25 ± 1.11 b	836.25 ± 1.89 b	22.21 ± 0.05 b
FO16	Semirom	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
FS1	Gorgan	0.00 ± 0.00 h	–	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 h
LSD (0.05)		0.75	–	3.37	5.61	3.84	0.11

DI = disease index, hpi = hours post inoculation; average ± standard error
Different letters indicate significant differences according to LSD analysis using SAS software (p = 0.05). Each experiment was repeated three times with similar results

nicity and aggressiveness in bioassays on seedlings, respectively (Table 4). Analysis of CWDEs showed that cellulase and lipase play an important role in pathogenicity by degrading the plant cell wall.

Composition of the EOs

The chemical composition EOs, as determined by GC-MS analysis is shown in Table 5. A maximum of 19 compounds were identified in the EOs, accounting for 91.6–96.5% of the total compositions. The chemical constituents of the EOs were trans-anethole, thymoquinone, ρ-cymene, limonene, carvacrol, α-thujene, sabinene, terpinen-4-ol, anisaldehyde, α-pinene, β-pinene, α-phellandrene, 1-methyl-3-propyl benzene, 3-methyl nonane, myrcene, n-decane, γ-rerpinene and ρ-cymene-8-ol (Table 5). The ρ-cymene, trans-anethole, α-thujene and thymoquinone were the highest components in Gorgan, Semirom and Sarvabad oil (Table 5). The ρ-cymene, trans-anethole, limonene, carvacrol, thymoquinone and α-thujene were the highest components in Bushehr, Syria's export, Shahreza, Nazarabad and Fereidan oil (Table 5). The EO percentage of samples ranged from 0.885 to 0.579%. The EO content in the sample of Bushehr was significantly higher than in other samples (Table 5).

Antifungal activities of EOs and their constituents *in vitro*

Minimum inhibitory concentration (MIC) and inhibitory concentration 50 (IC_{50}) values of EOs and their constituents with antifungal properties were determined and are shown in Table 6. Different values of MIC for treatments against the growth of FO7 isolate of *F. oxysporum* were observed. The MIC values for the EOs and their constituents ranged between 906 and 3875 ppm. The lowest MIC value was related to carvacrol and limonene with 906 ppm and 952 ppm, respectively. In addition, the lowest and highest IC_{50} values for carvacrol and Gorgan were 428 ppm and 2101 ppm (Table 6). The α-thujene, 3-methyl nonane, α-pinene, sabinene, β-pinene, myrcene, n-decane, α-phellandrene, 1-methyl-3-pro-

Table 5. Chemical composition of *Nigella sativa* essential oil determined by gas chromatography-mass spectrometry (GC-MS) in populations

No.	Compound name	RI*	Composition in each sample [%]							
			Bushehr	Gorgan	Semirom	Fereidan	Shahreza	Sarvabad	Nazarabad	Syria's export population
1	α-thujene	928	7.1	7.4	8.3	9.8	9.1	8.7	6.5	6.3
2	3-methyl nonane	931	0.5	0.5	tr**	0.4	tr	tr	tr	tr
3	α-pinene	935	1.6	1.5	1.3	1.4	1.6	1.6	1.6	1.6
4	sabinene	974	2.1	1.9	2.3	1.9	2.2	2.2	2.2	2.1
5	β-pinene	979	1.9	0.7	0.8	1.2	1.5	1.5	1.5	1.9
6	myrcene	992	1.1	0.3	0.6	0.8	0.9	0.9	0.9	1.1
7	n-decane	1001	tr	0.9	0.6	0.3	tr	tr	tr	tr
8	α-phellandrene	1007	tr	tr	1.1	0.7	0.8	0.8	0.8	tr
9	ρ-cymene	1026	26.9	32.3	28.8	25.9	25.5	26.7	25.5	26.6
10	limonene	1030	13.5	2.6	2.9	10.4	11.4	2.4	11.4	11.5
11	carvacrol	1032	10.1	3.5	3.9	8.9	9.3	2.3	9.2	9.7
12	1-methyl-3-propyl benzene	1052	0.8	0.7	1.1	0.8	0.7	0.8	0.7	0.8
13	γ-terpinene	1059	0.4	0.3	tr	0.3	0.4	0.4	0.4	0.4
14	terpinen-4-ol	1179	2.1	1.9	1.8	1.9	2	1.8	2	2.1
15	terpinen-8-ol	1186	0.3	0.6	0.5	0.4	0.3	0.3	0.3	0.3
16	carvone	1245	tr	tr	2	1.9	2	2.1	2	tr
17	thymoquinone	1255	8.1	5.2	5.3	7.2	7.5	5.5	7.5	7.3
18	anisaldehyde	1255	1.5	1.7	1.9	1.8	1.8	1.8	1.8	1.5
19	trans-anethole	1289	18.5	29.6	28.9	16.5	16.4	28.4	16.4	17.2
	Total	–	96.5	91.6	92.1	92.5	93.4	90.2	90.7	91.9
	Essential oil content [%]		0.885 a	0.579 e	0.674 d	0.774 c	0.851 b	0.544 f	0.568 e	0.669 d

*retention index calculated on the basis of retention time of a mixture of n-alkanes (C8-C30); **trace

Table 6. *In vitro* antifungal activity of the essential oils and their main constituents against mycelial growth of the FO7 isolate of *Fusarium oxysporum*

Treatments	*Fusarium oxysporum* FO7	
	MIC [ppm]	IC_{50} [ppm]
Essential oil of black cumin in each sample		
Bushehr	2784 f	1405 f
Gorgan	3875 a	2101 a
Semirom	3658 b	1935 b
Fereidan	3289 c	1723 c
Shahreza	2891 d	1455 d
Sarvabad	3643 b	1911 b
Nazarabad	2879 d	1448 d
Syria's export	2905 e	1424 e
Compound		
α-thujene	ND	ND
3-methyl nonane	ND	ND
α-pinene	ND	ND
sabinene	ND	ND
β-pinene	ND	ND
myrcene	ND	ND
n-decane	ND	ND
α-phellandrene	ND	ND
ρ-cymene	3640 b	1928 b
limonene	952 i	485 i
carvacrol	906 j	459 j
1-methyl-3-propyl benzene	ND	ND
γ-terpinene	ND	ND
terpinen-4-ol	ND	ND
terpinen-8-ol	ND	ND
carvone	ND	ND
thymoquinone	1951 h	983 h
anisaldehyde	ND	ND
trans-anethole	2249 g	1240 g

MIC = minimum inhibitory concentration; IC_{50} = inhibitory concentration 50; ND = not detected
Means within a column indicated by the same letter were not significantly different according to LSD analysis using SAS software ($p = 0.05$)
Each experiment was repeated three times with similar results

pyl benzene, γ-terpinene, terpinen-4-ol, terpinen-8-ol, carvone and anisaldehyde did not have antifungal activities. The lowest levels of IC_{50} and MIC were obtained for carvacrol against *F. oxysporum* among the EOs and their constituents tested. Investigating fungistatic and/or fungicide activity revealed that the EOs, ρ-cymene, trans-anethole and thymoquinone had fungistatic effects on *F. oxysporum*. In addition, carvacrol and limonene had fungicide effects on *F. oxysporum*.

Discussion

Based on morphological observations and molecular analyses, a total of 17 fungal isolates were isolated from black cumin seed samples. Sixteen isolates were identified as isolates of *F. oxysporum* and one isolate as *F. solani*. Similar results of morphological and molecular identification of fungal species were in accordance with the reports of Elwakil and Ghoneem (1999), Karakaya and Erzurum (2002), Mohamed *et al.* (2017), Al-Sman *et al.* (2019). This is the first report on isolation and identification of seed-borne *Fusarium* infection on native cumin populations in Iran. The main *Fusarium* species associated with black cumin seeds in Iran are *F. oxysporum*. Results similar to our findings were obtained in Turkey (Karakaya and Erzurum 2002) and Egypt (Mohamed *et al.* 2017). *Fusarium oxysporum* was found as the main limiting factor in black cumin cultivation of Iran, especially in the Gorgan region.

The *Fusarium* species causing root rot and wilt disease were observed in different sampling regions. Our results showed that the highest incidence of natural fungal infection in Iranian seed samples was observed in the Gorgan region, followed by the Semirom, Sarvabad, Fereidan, Shahreza, Nazarabad and Bushehr regions. The occurrence and diversity of fungal pathogens of various crops were very diverse in the Golestan province, especially in the Gorgan, Aghqla and Gonbad Kavous regions as suggested by previous studies (Khaledi *et al.* 2017; Hassani *et al.* 2019). The Gorgan sample had the highest percentage of *Fusarium* infected seed samples. It is possible that cultures of the sensitive Iranian black cumin population were affected by varying climate and environmental conditions, crop rotation with vegetables, and the lack of disinfection of seeds. These factors may have caused the high incidence of fusarium-infected seeds in the Gorgan region of Iran.

The results showed that the growth indicators used in this research (shoot and root lengths, fresh and dry weights) decreased with increased natural fungal infection in black cumin seeds. Seed-borne *Fusarium* are of considerable importance due to their influence on plant health indicators such as germination and vigor indices (Pareek and Varma 2015). The results showed that the number of normal seedlings decreased with increasing numbers and levels of pathogenicity and aggression of isolates obtained from seeds. Infected seeds may fail to germinate, vigor can be greatly reduced, and disease transmitted from seed to seedling (Islam and Borthakur 2012). Similar results were obtained by Fatima and Khot (2015), who observed that the fungi associated with black cumin seeds significantly affected seed germination and seedling growth. Papastylianou *et al.* (2018) reported inhibitory effects of stress on seed germination parameters of black

cumin. Similarly, Ghiyasi *et al.* (2019) reported that germination and vigor indices were decreased under the influence of stress.

Some studies revealed location activities of some fungal pathogens in seed coats, endosperms, cotyledons and embryonic axes. The results showed that a higher infection level of *F. oxysporum* most commonly occurred in the seed coat (28.3%), followed by the embryo (3.3%). *Fusarium solani* was observed in the seed coat (4.2%). Similar results were obtained by Hassani *et al.* (2019), who reported that *Fusarium* sp. were more active in the seed coat than in the embryo. Among the species of *Fusarium* detected in black cumin seeds, only *F. oxysporum* was observed in the embryos of seeds. Previous studies have shown the location of fungi pathogens in naturally infected black cumin (Elwakil and Ghoneem 1999), guar (Pareek and Varma 2015) and wheat (Pathak and Zaidi 2013). This is the first time the location of *Fusarium* spp. in seeds has been identified.

Our results suggest that these pathogens can cause fusarium root rot and wilt of black cumin. A total of seven (about 42%) out of 17 isolates were pathogenic or weakly pathogenic on black cumin seedlings. The pathogenicity test revealed that seven isolates of *F. oxysporum* were found to be pathogenic or weakly pathogenic and the others were non-pathogenic. The pathogenicity test on seedlings showed that two isolates isolated from Gorgan seed samples were pathogenic or weakly pathogenic, while in other samples only one isolate was pathogenic or weakly pathogenic. The fungal isolates recovered from seeds revealed significant differences in pathogenicity and aggressiveness between *Fusarium* isolates. This finding is in agreement with the observations of other researchers (Purahong *et al.* 2012; Khaledi *et al.* 2017; Hassani *et al.* 2019). Aggressiveness is an important factor for determining the potential ability of isolates to cause pathogenicity and yield losses. More detailed knowledge about the extensive variability of aggressiveness is essential for understanding the interaction between plant and pathogen. Elwakil and Ghoneem (1999) reported that there was variation in severity and incidence of disease symptoms caused by different seed-borne *F. oxysporum* and *F. solani* isolates, which is in accordance with our observations. The results showed that there was a difference in levels of pathogenicity and aggressiveness between fungal species and also between isolates of a species. Similar results were obtained by Amatulli *et al.* (2010), who observed a high variation in aggressiveness among *Fusarium* spp. isolates on rice plants.

Pathogenicity and aggressiveness of these pathogenic fungi involve different mechanisms such as production of extracellular enzymes and mycotoxins (Ortega *et al.* 2013). *Fusarium oxysporum* and *F. solani,* as necrotrophic pathogens, utilize a variety of pathogenicity factors especially production of extracellular enzymes throughout the infection process (Gibson *et al.* 2011; Khaledi *et al.* 2017). The secreted CWDEs produced by *Fusarium* species are important pathogenicity factors. Production of CWDEs such as cellulase, pectinase, xylanase and lipase are important and vital for *Fusarium* spp. establishment in plants (Upasani *et al.* 2017). The activities of CWDEs, as the main pathogenicity factors involved in the infection process of *Fusarium* isolates on black cumin seedlings, were evaluated in this study. Pathogenic isolates produced considerably higher levels of CWDEs than weakly pathogenic isolates. Fungal isolates without the ability of producing CWDEs were unable to cause severe disease in black cumin seedlings. Similar results were reported by Hubballi *et al.* (2011), who reported that non-pathogenic isolates do not produce or produce less of the pectinolytic enzymes.

Maximum activities of CWDEs studied were observed for the FO7 isolate, which showed the highest levels of pathogenicity and aggressiveness. This is in agreement with other reports (Khaledi *et al.* 2017; Hassani *et al.* 2019). Determining the role of individual, secreted CWDEs in pathogenicity is difficult to establish because of functional redundancy, while the collective action of CWDEs such as carbon catabolite repression and nitrogen regulation is important for the infection process (Michielse and Rep 2009), as observed in this study. We found a positive relationship between both pathogenicity and aggressiveness on seedlings and CWDEs produced by fungal isolates *in vitro*. These findings were in accordance with the results obtained by Hassani *et al.* (2019) and Khaledi *et al.* (2017). This is the first report about a strong association between the amount of extracellular CWDEs activities and the pathogenicity of *Fusarium* isolates on black cumin seedlings. Similar to our observations, in chickpea – *F. oxysporum* (Upasani *et al.* 2017), in wheat – *Fusarium* spp. (Paccanaro *et al.* 2017), and also in palm oil – *Fusarium* spp. (Suwandi *et al.* 2018), secreted CWDEs increased pathogenicity and aggressiveness of phytopathogens.

The main components identified in the EOs included ρ-cymene, trans-anethole, thymoquinone, limonene, carvacrol and α-thujene which is in accordance with Ahamad Bustamam *et al.* (2017), Gerige *et al.* (2009) and Wajs *et al.* (2008). Our observations showed a high percentage of ρ-cymene and trans-anethole in the EOs sampled from Iran (Minooeian Haghighi and Khosravi (2013) and Mojab *et al.* (2003). The *N. sativa* EO and its main constituents indicated antifungal activity. These results were similar to those of other investigators. Sitara *et al.* (2008) showed that the *N. sativa* EO was responsible for its antifungal activity against phytopathogenic fungi such as *F. oxysporum*, *F. moniliforme*, *F. nivali*, *F. semitectum*,

A. alternate, *Drechslera hawiinesis*, *Aspergillus niger* and *A. flavus*. Huang *et al*. (2010) reported that trans-anethole has antifungal activity against *F. oxysporum* and *F. graminearum*. Carvacrol antifungal activity also has been reported against phytopathogenic fungi such as *F. oxysporum*, *F. solani*, *F. semitectum*, *F. sambucinum*, *F. nivale*, *F. equiseti*, *F. culmorum* and *F. acuminatum* (Kordali *et al*. 2008). Thymoquinone has antifungal activity against pathogenic dermatophyte fungi such as *F. oxysporum* (Mahmoudvand *et al*. 2014). Also, limonene has antifungal activity against *F. oxysporum*, *F. proliferatum* and *F. verticillioides* (Dambolena *et al*. 2008; Chutia *et al*. 2009; Singh *et al*. 2010; Van Hung *et al*. 2013). However, p-cymene and α-thujene have weak or no antifungal activities (Kordali *et al*. 2008; Rammanee and Hongpattarakere 2011).

This is the first report on the effects of EOs and their constituents on mycelial growth inhibition of *F. oxysporum*. In our investigations, the constituents of carvacrol and limonene had the best inhibitory effects on the mycelia growth of *F. oxysporum* with a MIC value of less than 1000 ppm *in vitro*. This is in accordance with the results obtained by Rahmouni *et al*. (2019), who reported that carvacrol is an effective fungicide on the growth of *F. oxysporum* at a very low concentration. Marei *et al*. (2012) reported that reducing pectin methyl esterase, cellulase and polyphenol oxidase activity has a limonene effect on *F. oxysporum*. The minimum concentration of the EOs and their constituents required to inhibit mycelial growth of *F. oxysporum* differed. The IC50 and MFC values obtained for carvacrol and limonene were considerably lower than the values obtained for the EOs tested.

It seems that the percentage of antifungal composition of the EOs components can affect the presence of the seed-borne fusarium and/or the pathogenicity of them. The results showed that although ρ-cymene was the main component of the EOs of all Iranian populations, relative amounts of carvacrol, limonene and thymoquinone were more in oil from Bushehr, Fereidan, Nazarabad and Shahreza than in the oil from Gorgan, Semirom and Sarvabad. Furthermore, relative amounts of carvacrol, limonene and thymoquinone were higher than other constituents in the oil from Syria's export population. These observations confirm that these compounds are effective on the presence of the seed-borne fusarium. The seed samples of Bushehr and Syria's export did not have any seed-borne fusarium infection. The results of the GC-MS analysis showed that the total percentage of the antifungal composition of the EOs components of these samples was less than that in other samples.

Biotic and abiotic stresses may have an effect on EO content and production. We observed that the EO content was decreased due to increased *Fusarium*-infected seeds. However, the effect of genotype on the EO content should not be ignored. Therefore, the quantity and quality of EO are controlled by genetic, biotic and abiotic factors (Mohammadnejad Ganji *et al*. 2017; Isah 2019). On the other hand, the geographical region can also affect the EO composition and their antifungal and antibacterial properties (Khaledi and Hassani 2018).

In summary, this study indicated that seed-borne fusarium caused a reduction in seed quality indicators such as germination and vigor indices. Knowledge about phytopathogens and the diseases caused by them which affect the production and quality of black cumin seeds, especially seed-borne pathogens that can spread the disease to a new location and also pass it to the next-generation, can be helpful in selecting effective strategies for controlling diseases. Identifying pathogenicity factors of *Fusarium* species especially CWDEs, and their association with pathogenicity and aggressiveness of these *Fusarium* can further our understanding of how phytopathogens cause diseases. In addition, it seems that the composition of the EO in the seed of black cumin, especially carvacrol and limonene, can affect the presence of the seed-borne fusarium and/or the pathogenicity of them. It is suggested that carvacrol and limonene, after suitable formulation, could be used for the control of *Fusarium* sp. Moreover, they could be useful for plant breeders in selecting resistant native populations and other management strategies to reduce destructive effects of the pathogens on the host plants.

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