Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Antifungal activity of *Bunium persicum* essential oil and its constituents on growth and pathogenesis of *Colletotrichum lindemuthianum*

Nima Khaledi[1]*, Farshid Hassani[2]

[1] Department of Crop Protection, Ferdowsi University of Mashhad, Mashhad, Iran

[2] Seed and Plant Certification and Registration Institute, Agricultural Research, Education and Extension Organisation (AREEO), Karaj, Iran

Vol. 58, No. 4: 431–441, 2018

DOI: 10.24425/jppr.2018.124646

Received: May 25, 2018
Accepted: November 9, 2018

*Corresponding address:
khaledi.nima@stu.um.ac.ir

Abstract

Anthracnose disease caused by *Colletotrichum lindemuthianum* (Sacc. and Magnus) Lams-Scrib is one of the most devastating seed-borne diseases of common bean (*Phaseolus vulgaris* L.). In the present study, we evaluated the antifungal activity of *Bunium persicum* essential oil (EO) and its main constituents on mycelial growth, sporulation and spore germination inhibition of *C. lindemuthianum*. The main objective of this study was to investigate the effect of EO and its main constituents on decreasing the activity of cell wall degrading enzymes (CWDEs) produced by *C. lindemuthianum*, which are associated with disease progress. Also, the effects of seed treatment and foliar application of EO and its main constituent, cuminaldehyde, on anthracnose disease severity was investigated. The essential oil of *B. persicum*, was obtained by using a clevenger apparatus and its major constituents were identified by gas chromatography-mass spectrometry (GC-MS). The EO was characterized by the presence of major compounds such as cuminaldehyde (37.7%), γ-terpinene (17.1%) and β-pinene (15.4%), which indicated antifungal effects against *C. lindemuthianum*. This pathogen did not grow in the presence of EO, cuminaldehyde and γ-terpinene, β-pinene at 1,500; 1,010 and 1,835 ppm concentrations, respectively. Also, sporulation and spore germination of *C. lindemuthianum* was completely inhibited by EO and cuminaldehyde. Synergistic effects of the main constituents showed that combing γ-terpinene with cuminaldehyde induced a synergistic activity against *C. lindemuthianum* and in combination with β-pinene caused an additive effect. Activities of pectinase, cellulase and xylanase, as main CWDEs, were decreased by EO and its main constituents at low concentration without affecting mycelial growth. Seed treatment and foliar application of peppermint EO and/or cuminaldehyde significantly reduced the development of bean anthracnose. We introduced *B. persicum* EO and constituents, cuminaldehyde and γ-terpinene, as possible control agents for bean anthracnose.

Keywords: *Bunium persicum*, *Colletotrichum lindemuthianum*, cuminaldehyde, essential oil, *Phaseolus vulgaris*

Introduction

Common bean (*Phaseolus vulgaris* L.) is grown and consumed principally in developing countries in Latin America, Africa, and Asia. It is the most important legume worldwide for direct human consumption. The crop is consumed principally for its dry (mature) beans, shell beans (seeds at physiological maturity), and green pods. It is a major source of dietary protein that complements carbohydrate sources such as rice, maize, and cassava. It is also a rich source of dietary fibers, minerals and certain vitamins (Gepts *et al*. 2008).

Colletotrichum lindemuthianum (Sacc. and Magnus) Lams-Scrib is considered to be a hemibiotrophic

fungus, which causes anthracnose on common bean (*P. vulgaris*) and a few other *Phaseolus* species. It is an important pathogen in almost all bean-growing regions of the world (Ansari *et al.* 2004). The main disease symptoms are discolored leaf veins and sunken brown lesions on stems, petioles and pods. Immature pods shrivel and dry under severe infection conditions (Paulert *et al.* 2009). When seeds are infected, the seed coat often becomes discolored as lesions develop. Such beans have a repulsive appearance and are not popular with consumers. This lowers the marketability and thus the income arising from their sale.

Despite the availability of management practices such as seed and foliar treatment with fungicides, crop rotation, soil solarization, use of certified seed and genetic resistance, bean anthracnose is still of regular occurrence in most areas (Padder *et al.* 2010; Mohammed *et al.* 2013). The best strategy to manage the disease is planting resistant cultivars which are the most effective, the least expensive and the easiest for farmers to adopt. However, high pathogenic variability present in the pathogen population renders their use ineffective due to continuous breakdown of the resistance mainly in recommended cultivars with good agronomic and marketability traits (Pastor-Corrales 2005). Infected seed and crop debris are the two most common sources of primary inoculum for anthracnose outbreaks. The use of pathogen-free seed is one of the key components in an integrated strategy for the control of this disease (Siripornvisal 2010).

The fungicide Bavistin®, being highly effective against anthracnose, is mainly used for both seed treatment and foliar sprays under high rainfall conditions. With time this could create a problem of fungicidal resistance in the pathogen. Therefore, it is important to find an alternative to avoid this risk (Ramos *et al.* 2010). As a result the use of non-chemical ecofriendly means of control i.e. biocontrol agents and secondary metabolites secreted by medicinal plants, have emerged as viable alternatives under such conditions. Plants are potential sources of antimicrobial compounds, which could be used in the management of plant diseases (Balbi-Peña *et al.* 2006). The presence of essential oils (EOs) and plant extracts with antifungal properties has been well recognized and documented, but very few of them have been studied extensively in the case of bean anthracnose. Essential oil bearing plants constitute a rich source of bioactive chemicals, which have been reported to have various antifungal properties. These properties of EOs are a result of the pivotal role of ketones, terpenes and terpenoids, and phenol-derived aromatic and aliphatic components (Kalemba and Kunicka 2003; Bakkali *et al.* 2008).

These chemicals are often active against a limited number of species, including the specific target species. Essential oils and their components are also biodegradable and non-toxic. Although several EOs have been reported to have antifungal properties, few have been developed as commercial formulations for use in plant disease control. General antifungal activity of various EOs is well documented (Khaledi *et al.* 2015; Sharopov *et al.* 2015; Rustaie *et al.* 2016).

Bunium persicum (Boiss.) B. Fedtsch is a member of the Apiaceae family and is an important aromatic perennial plant that naturally grows in Iran (Azizi *et al.* 2009). *Bunium persicum* is an economically important medicinal plant growing wild in arid regions in Iran. In previous studies, antimicrobial (Oroojalian *et al.* 2010) and antifungal (Sekine *et al.* 2007) effects of this plant have been demonstrated. Antifungal activities of volatile compounds on 52 species against four phytopathogenic fungi and their results showed that *B. persicum* had the strongest antifungal activity (Sekine *et al.* 2007).

Although previous studies have identified reduction in the growth of *C. lindemuthianum* via application of various EOs, so far there has been no report on the effects of EO and its main constituents on pathogenesis mechanisms of *C. lindemuthianum*. On the other hand, the geographical region can affect the EO composition and their antibacterial properties.

Therefore, the objectives of this study were (i) to explore the potential role and effect of Iranian cultivated black cumin (*B. persicum*) EO and its main constituents on mycelial growth, sporulation and spore germination inhibition *in vitro*, and (ii) to determine the potential of very low concentrations of EO and its main constituents, without any effects on the fungal growth, in reducing the activity of cell wall degrading enzymes (CWDEs) such as pectinase, cellulase and xylanase, which are involved in the infection process of this fungus on the host plant. In addition, the effects of seed treatment and foliar application of EO and cuminaldehyde, as its main constituent, on decreasing the progress of the diseases caused by *C. lindemuthianum* on bean plant were investigated under greenhouse conditions.

Materials and Methods

Plant pathogenic fungus and inoculum preparation

Colletotrichum lindemuthianum was obtained from the Phytopathology Laboratory in Ferdowsi University of Mashhad, Iran. The fungus isolate was maintained on potato dextrose agar (PDA) medium slants at 4°C, and sub-cultured at monthly intervals. Fungal inoculum was produced in Mathur's agar medium using the methods described by Mathur *et al.* (1950). Conidial suspensions were diluted with autoclaved water to a final concentration of 1×10^5 conidia · ml^{-1} containing 0.05% (v/v) Tween-20.

Plant material and extraction of EO

For the extraction of EO, *B. persicum* leaves were collected in September 2017 from Khorassan-Razavi province, Iran. The leaves 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 EO, 100 g of dried plant 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.

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography analysis of the oil was done by a Shimadzu QP 5000 (FID) chromatograph HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Helium was used as carrier gas at a flow rate of 1 ml · min^{-1} (split ratio 1 : 20) with an injection volume of 0.2 µl. Injector and detector temperatures were set at 220 and 290°C, respectively. Oven temperature was kept at 50°C for 3 min, gradually raised to 160°C at 3°C · min^{-1}, held for 10 min and finally raised to 240°C at 3°C · min^{-1}. The 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 (Shahsavari *et al.* 2008).

Determination of minimum inhibitory concentration (MIC) and inhibitory concentration 50 (IC50)

Minimum inhibitory concentration of EO and its main constituents were determined as described by Plodpai *et al.* (2013) with few modifications. The PDA plates were amended with various concentrations of EO and its main constituents (0–3,000 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 *C. lindemuthianum*. All plates were incubated in triplicate for each concentration at 27 ± 1°C for 168 h. Plates with Tween-20 but without any EO and its main constituents were used as control. Observation of fungal growth was done at a time interval of 12 h up to 168 h after incubation. The MIC values were determined as the lowest concentration of EO and its main constituents that completely prevented visible fungal growth. IC50 (concentration that produces a 50% inhibitory effect) values were graphically calculated from the dose-response curves based on measurements at various concentrations.

Effects of the EO and its main constituents on sporulation and conidial germination of *C. lindemuthianum*

Spore germination inhibition assay was performed using the methods described by Mohammedi and Atik (2013). Conidia of *C. lindemuthianum* cultured on PDA plates were collected using sterile distilled water containing 0.05% Tween-20 and conidial suspensions (1×10^5 conidia · ml^{-1}) were prepared. Various concentrations (1 × MIC; 0.1 × MIC; 1 × IC50; 0.1 × IC50) of EO and its main constituents were mixed on PDA plates. One ml of conidial suspension was spread on each PDA plate containing the EO and its main constituents and they were incubated at 27 ± 1°C for 7 days. Spore germination was investigated with a microscope (Olympus BX51) and inhibition of spore germination was determined using the following formula:

$$\text{Inhibition of spore germination} = \frac{N_c - N_t}{N_c} \times 100\ [\%],$$

where: N_c – number of fungal colonies in control, N_t – number of fungal colonies in treatment.

The effects of various concentrations of EO and its main constituents on the ability of *C. lindemuthianum* for sporulation was evaluated on PDA medium according to the method described by Siripornvisal (2010). The percentage of sporulation inhibition was determined using the previous formula.

Nature of toxicity of EO

The nature of toxicity (fungistatic/fungicide) of the EO against fungus 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.

Comparing the fungitoxicity of EO with some prevalent synthetic fungicides

The efficacy of the EO was compared with some common fungicides, such as Carbendazim (Bavistin®) and Mancozeb (Dithane M-45) by the agar medium assay.

Identification of synergistic effects between EO constituents

The microdilution checkerboard method according to Turgis *et al.* (2012) was carried out on 96-well

plates to evaluate synergistic effects of EO constituents (γ-terpinene, cuminaldehyde and β-pinene). Seventy microliters of each dilution (2 × MIC; 1 × MIC; 0.5 × MIC; 0.25 × MIC; 0.125 × MIC; 0.062 × MIC; 0.0312 × MIC and 0.015 × MIC) were dispensed to each row, and then 80 µl of another constituent added to each row of wells in a direction perpendicular to the previous constituents in different dilutions. Finally, 10 µl of potato dextrose broth (PDB) media containing 1×10^5 conidia · ml^{-1} was added to each well. The plates were incubated at 27 ± 1°C on a rotary shaker at 125 rpm for 24 h. All treatments were triplicated. A fractional inhibitory concentration index (FICI) of the dual combination of EO constituents was calculated by using the following formula:

$$FICI = FIC\ A + FIC\ B =$$

$$= \frac{MIC\ A\ combined}{MIC\ A\ alone} + \frac{MIC\ B\ combined}{MIC\ B\ alone}.$$

Interaction of the combination of two substances was defined as a synergistic effect, if the FIC index was ≤ 0.5, additive if 0.5 < FICI < 1, indifferent if 1 < FICI ≤ 4, and antagonistic if FICI > 4 (Gutierrez *et al.* 2008).

Effects of EO and its main constituents on the activity of pectinase, cellulase and xylanase

The efficacy of EO and its main constituents at 0.01 × IC50 concentration, which in previous experiments had no effect on the fungal growth, in reducing the activity of pectinase, cellulase and xylanase, was determined using the methods described by Khaledi *et al.* (2015). According to previous studies on the activity of CWDEs *in vitro*, most enzyme activities are observed within 10 days after inoculation (Herbert *et al.* 2004). The test for each enzyme had three replicates and the experiment was repeated three times.

Effects of *B. persicum* EO and cuminaldehyde on the progress of diseases caused by *C. lindemuthianum* on bean

Greenhouse experiments were performed using seeds of *Phaseolus vulgaris* cv. Naz (obtained from Seed and Plant Certification and Registration research institute). The seeds were surface-sterilized in 1% sodium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and placed in Petri dishes on sterile wet filter paper. For seed treatment with EO or cuminaldehyde, various concentrations (1 × IC50; 0.1 × IC50; 0.01 × IC50) of the EO or cuminaldehyde were obtained by suspending in distilled water containing 0.05% Tween-20. In the control, the seeds were soaked in sterile distilled water containing 0.05% Tween-20. The seeds were soaked in each treatment for 5 min before sowing in soil. After 3 days at room temperature, germinated seeds were sown in 15-cm-diameter plastic pots containing a combination of clay, sand and leaf compost at a ratio of 1 : 1 : 1 (v/v) which had been autoclaved at 121°C for 30 min on 2 successive days. The plants were grown in a greenhouse (26 ± 2°C; 16L : 8D h photoperiod) and irrigated when needed. After 7 days, hypocotyls and abaxial surfaces of the primary leaves were sprayed with 4 µl of a spore suspension (1×10^5 conidia · ml^{-1}) amended with 0.05% Tween-20. Inoculated plants were subjected to a 12 h photoperiod, 400 µE light intensity and maintained at 26 ± 2°C and 90–100% humidity.

For foliar spray treatment, EO or cuminaldehyde at 1 × IC50; 0.1 × IC50 and 0.01 × IC50 concentrations was sprayed on plants until run-off at 2 days post inoculation (dpi). Inoculated plants were kept for 1 week in the greenhouse. In all cases, when disease symptoms developed, the pathogen was re-isolated from infected plants. Four replicate plants were inoculated in a completely randomized design, and the experiment was repeated three times. Disease severity was estimated at 7 dpi using a 0–10 disease scale (Ansari *et al.* 2004), and the disease index (DI) was calculated (Taheri and Tarighi 2010). The percentage of disease decrease, which is equal to the control efficacy of treatments on the disease caused by the pathogen, was evaluated using the formula described by Plodpai *et al.* (2013):

$$\text{Control efficacy} = \frac{\text{Disease severity of control} - \text{Disease severity of treated group}}{\text{Disease severity of control}} \times 100\ [\%].$$

Statistical analysis

Data were subjected to analysis of variance (ANOVA) with for a completely randomized design with four replicates using SPSS (version 23) software. The means were separated using Duncan's multiple range tests at $p < 0.05$, where the F-value was significant.

Results

Composition of the EO

The chemical composition EO, as determined by GC-MS analysis is shown in Table 1. Thirteen compounds were identified in the oil which constitute about 96.1% of this oil. The main chemical constituents of the *B. persicum* EO were α-thujene (0.5%), α-pinene (1.7%), sabinene (1.3%), β-pinene (15.4%),

Table 1. Chemical composition of *Bunium persicum* essential oil determined by gas chromatography-mass spectrometry (GC-MS)

No.	Compound name and class	RI*	Composition [%]
1	α-Thujene	920	0.5
2	α-Pinene	936	1.7
3	Sabinene	970	1.3
4	β-Pinene	980	15.4
5	Myrcene	990	1.5
6	Limonene	1,025	3.8
7	ρ-Cymene	1,030	6.2
8	γ-Terpinene	1,060	17.1
9	Linalool	1,093	0.1
10	Terpinen-4-ol	1,170	0.5
11	Cuminaldehyde	1,243	37.7
12	Cuminyl alcohol	1,265	9.5
13	Thymol	1,289	0.8
	Total	–	96.1

*Retention Index calculated on the basis of retention time of a mixture of n-alkanes (C8–C30)

myrcene (1.5%), limonene (3.8%), ρ-cymene (6.2%), γ-terpinene (17.1%), linalool (0.1%), terpinen-4-ol (0.5%), cuminaldehyde (37.7%), cuminyl alcohol (9.5%) and thymol (0.8%).

Antifungal activities of EO and its main constituents on mycelial growth, sporulation and spore germination inhibition *in vitro*

Minimum inhibitory concentration (MIC) and inhibitory concentration 50 (IC50) values of EO and its main constituents with antifungal properties were determined and are shown in Table 2. Different values of MIC for treatments against the growth of *C. lindemuthianum* were observed. The MIC values for the EO and its main constituents ranged between 1,010 and 2,539 ppm. The lowest MIC value was related to cuminaldehyde and the EO with 1,010 ppm and 1,500 ppm, respectively. In addition, the lowest and highest IC50 values for cuminaldehyde and β-pinene were 428 ppm and 1,180 ppm (Table 2). The lowest levels of IC50 and MIC were obtained for cuminaldehyde against *C. lindemuthianum* among the EO, γ-terpinene and β-pinene tested.

Investigating fungistatic and/or fungicide activity revealed that the EO had fungistatic effects on *C. lindemuthianum*. The MICs of synthetic fungicides including Carbendazim and Mancozeb against *C. lindemuthianum* were found to be 2,000 and 3,000 ppm, respectively, which were higher than that of the EO tested (Table 2).

Table 2. *In vitro* antifungal activity of the essential oil (EO) and its main constituents compared to synthetic fungicides against mycelial growth of *Colletotrichum lindemuthianum*

Treatments	MIC [ppm]	IC50 [ppm]
Essential oil		
Bunium persicum	1,500 b	650 b
Compound		
γ-Terpinene	1,835 c	905 c
Cuminaldehyde	1,010 a	428 a
β-Pinene	2,539 e	1,180 e
Fungicides		
Carbendazim	2,000 d	1,000 d
Mancozeb	3,000 f	1,500 f

MIC – minimum inhibitory concentration; IC50 – inhibitory concentration 50
Means within a column indicated by the same letter were not significantly different according to Duncan's multiple range test at the level $p < 0.05$

The effect of different concentrations of EO and its main constituents on mycelial growth of *C. lindemuthianum* are shown in Figure 1. The EO and its main constituents inhibited the growth of the pathogen in a dose-dependent manner. The 1 × MIC and 1 × IC50 concentrations of each EO and its main constituents were equally effective against *C. lindemuthianum* without significant differences. A low level of antifungal activity was observed for EO and its main constituents at 0.01 × MIC concentration against *C. lindemuthianum*. At 0.01 × IC50 concentration, the EO and its main constituents did not have any inhibitory effect on the fungal growth (Fig. 1).

The results of the effects of the EO and its main constituents on sporulation and conidial germination of *C. lindemuthianum* are shown in Table 3. The results of counted spores in various concentrations of EO and its main constituents compared to the control revealed that the EO and cuminaldehyde were significantly effective on sporulation of *C. lindemuthianum* at the 1 × MIC concentration (Table 3). Germination of *C. lindemuthianum* spores was completely inhibited by EO and cuminaldehyde at the 1 × MIC concentration compared to the control in which spore generation occurred about 24 h after inoculation (Table 3).

Synergist assay

To investigate *in vitro* synergistic interactions of combinations of γ-terpinene, cuminaldehyde and β-pinene a microdilution checkerboard method was used. According to the obtained results, synergistic effects between γ-terpinene × cuminaldehyde, γ-terpinene × β-pinene, and cuminaldehyde × β-pinene were observed, and no antagonistic effect was found between the tested constituents. The highest level of synergistic

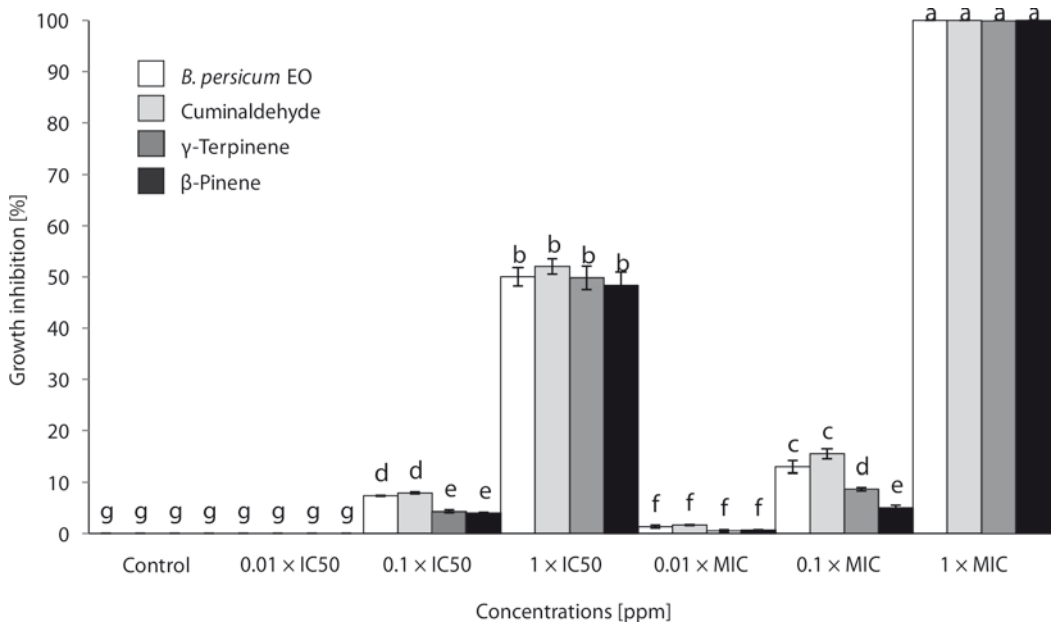

Fig. 1. Effects of different concentrations of *Bunium persicum* essential oil (EO) and its main constituents on the mycelial growth of *Colletotrichum lindemuthianum*. Different letters indicate significant differences according to Duncan's multiple range test at the level $p < 0.05$. The bars indicate standard errors (SE). IC50 – inhibitory concentration 50, MIC – minimum inhibitory concentration

Table 3. Effects of various concentrations of essential oil (EO) and its main constituents on inhibition of sporulation and spore germination of *Colletotrichum lindemuthianum*

Concentration	Inhibition of sporulation [%]				Inhibition of spore germination [%]			
	Bunium persicum EO	γ-terpinene	cuminaldehyde	β-pinene	*Bunium persicum*	γ-terpinene	cuminaldehyde	β-pinene
Control	0.7 ± 0.02 e	0.6 ± 0.04 e	0.6 ± 0.08 e	0.7 ± 0.05 d	0.5 ± 0.03 e	0.7 ± 0.0 e	0.9 ± 0.01 e	0.7 ± 0.03 e
1 × MIC	100.0 ± 0.0 a	32.67 ± 0.52 a	100.0 ± 0.0 a	16.85 ± 0.13 a	100.0 ± 0.0 a	46.1 ± 0.25 a	100.0 ± 0.0 a	32.8 ± 0.32 a
0.1 × MIC	13.5 ± 0.95 c	10.4 ± 0.67 c	16.2 ± 0.55 c	3.1 ± 0.62 c	30.5 ± 0.15 c	26.8 ± 0.01 c	35.7 ± 0.87 c	15.2 ± 0.23 c
0.01 × MIC	3.11 ± 0.01 f	1.29 ± 0.05 f	4.58 ± 0.08 f	0.95 ± 0.02 f	6.49 ± 0.62 f	4.16 ± 0.09 f	8.84 ± 0.03 f	3.37 ± 0.09 f
1 × IC50	37.6 ± 0.54 b	26.1 ± 0.28 b	47.3 ± 0.09 b	12.3 ± 0.89 b	65.8 ± 0.51 b	43.5 ± 0.35 b	76.2 ± 0.48 b	26.1 ± 0.45 b
0.1 × IC50	5.05 ± 0.27 d	3.5 ± 0.30 d	7.5 ± 0.46 d	2.7 ± 0.41 c	9.5 ± 0.33 d	6.5 ± 0.58 d	11.1 ± 0.80 d	5.8 ± 0.11 d
0.01 × IC50	0.07 ± 0.02 g	0.01 ± 0.01 g	0.09 ± 0.01 g	0.0 ± 0.0 g	3.09 ± 0.21 g	1.33 ± 0.42 g	5.39 ± 0.14 g	1.12 ± 0.51 g

MIC – minimum inhibitory concentration; IC50 – inhibitory concentration 50
The results are means ± standard errors of four replications. Means within a column indicated by the same letter were not significantly different according to Duncan's multiple range test at the level $p < 0.05$

effect was related to a combination of γ-terpinene × cuminaldehyde with 0.225 FIC index (Table 4).

Effects of EO and its main constituents on CWDEs activity of *C. lindemuthianum*

The effects of the EO and its main constituents on CWDEs activity of *C. lindemuthianum* are shown in Figure 2. Investigating the effects of EO and its main

Table 4. The fractional inhibitory concentration index (FICI) essential oil (EO) of constituents against *Colletotrichum lindemuthianum*

Compound	FICI	Activity
γ-terpinene × cuminaldehyde	0.225	synergistic
γ-terpinene × β-pinene	0.682	additive
cuminaldehyde × β-pinene	1.269	indifferent

Means within a column indicated by the same letter were not significantly different according to Duncan's multiple range test at the level $p < 0.05$

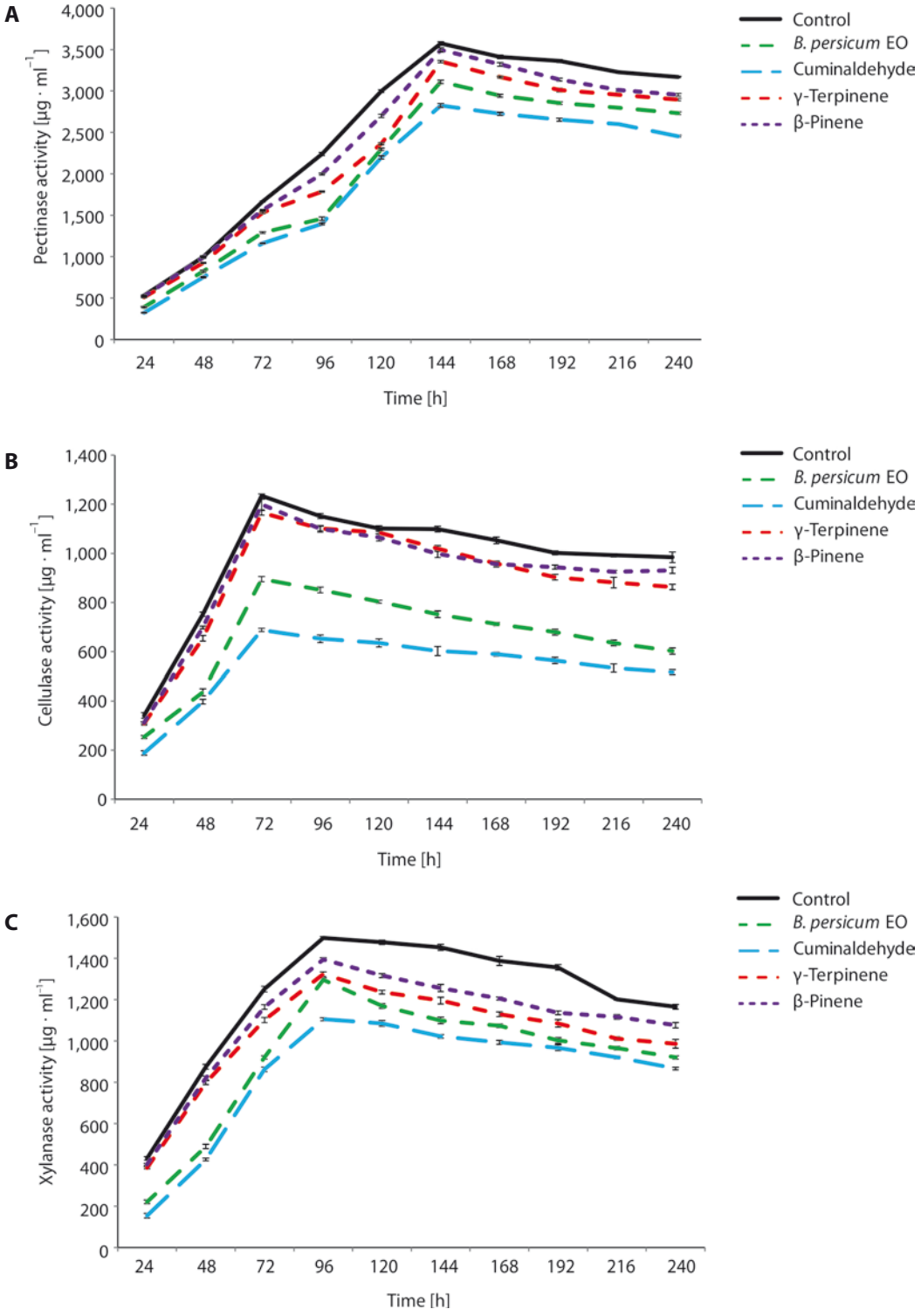

Fig. 2. Effect of *Bunium persicum* essential oil (EO), γ-terpinene, cuminaldehyde and β-pinene on pectinase (A), cellulase (B) and xylanase (C) activity of secreted by *Colletotrichum lindemuthianum*. The bars indicate standard errors (SE)

constituents on the activity of pectinase, cellulase and xylanase secreted by *C. lindemuthianum* revealed that the 0.01 × IC50 concentration of these EO and its main constituents, which did not have any effect on fungal growth, reduced the activity of both enzymes *in vitro*. These results suggested that the EO and its main constituents differed in their ability to reduce the activity of CWDEs secreted by *C. lindemuthianum*, which may be related to their chemical composition. *Colletotrichum lindemuthianum* showed maximum pectinase, cellulase and xylanase activity after 144, 72 and 96 h post culturing on liquid medium (hpc), respectively, and then decreased (Fig. 2). Overall, the highest reduction in CWDEs activity of *C. lindemuthianum*

was observed using cuminaldehyde, followed by EO, cuminaldehyde and β-pinene. Cuminaldehyde had a greater effect than thyme oil on reducing the CWDEs activity of *C. lindemuthianum*. The pectinase, cellulase and xylanase activities of *C. lindemuthianum* were reduced by treatment with EO and its main constituents at various time points investigated (Fig. 2). There was a significant decrease in CWDEs activity of treatments compared to the control. So, the decrease in CWDEs activity observed in this research may reflect an elaborate process of effective compounds of EO to reducing pathogenicity.

Efficiency of *B. persicum* EO and cuminaldehyde on disease severity

The data presented in Table 5 indicated that both seed treatment and foliar application of EO and/or cuminaldehyde markedly reduced the development of bean necrotic lesions and anthracnose caused by *C. lindemuthianum*. Severity of the disease caused by the pathogen on bean significantly decreased with seed treatment using EO or cuminaldehyde at 1 × IC50 concentration followed by 0.1 and 0.01× IC50 concentrations. Similar results for anthracnose disease was obtained in the experiments using foliar spray. No phytotoxicity on the plant leaves at the low concentrations of EO and cuminaldehyde was observed in this study. Overall, higher levels of suppression were obtained for EO than for cuminaldehyde (Table 5). The EO showed the highest antifungal efficacy against *C. lindemuthianum*, which could be associated with cuminaldehyde as its main constituent.

Discussion

Anthracnose is one of the most destructive bean diseases in bean-producing areas. However, disease management strategies including fungicides, resistant cultivars, crop rotation, and soil solarization have not been able to completely eliminate the disease. The limitations in using fungicide resources and probability of the development of resistance to the fungicides prompted us to search for new fungicides with no side-effects on human health. For this purpose, we used EO and its main constituents for bean anthracnose control. In the present study, the EO was obtained from leaves and its constituents were identified using GC-MS analysis. Then, the antifungal ability of the EO and its main constituents against *C. lindemuthianum* was investigated using *in vitro* and *vivo* assays. The effects of the EO and its main constituents on CWDEs activity such as pectinase, cellulase and xylanase, as well as part of the mechanisms involved in the infection process of this fungus, were demonstrated.

The main components were identified in the *B. persicum* EO including γ-terpinene, cuminaldehyde and

Table 5. Efficiency of seed treatment and/or foliar spray using *Bunium persicum* essential oil (EO) and cuminaldehyde to control bean disease caused by *Colletotrichum lindemuthianum* under greenhouse conditions

Treatment	Application type	Disease index	Suppression efficacy [%]
Untreated control	seed treatment	76.2 ± 1.3 a	–
	foliar spray	73.1 ± 1.5 b	–
B. persicum EO (1 × IC50)	seed treatment	31.7 ± 2.0 j	76.3 ± 0.3 a
	foliar spray	38.5 ± 0.5 h	65.5 ± 1.1 c
B. persicum EO (0.1 × IC50)	seed treatment	54.2 ± 1.6 f	43.7 ± 0.6 e
	foliar spray	61.4 ± 0.1 d	34.3 ± 1.2 g
B. persicum EO (0.01 × IC50)	seed treatment	72.1 ± 1.0 b	20.7 ± 1.7 i
	foliar spray	74.5 ± 0.8 a	19.5 ± 0.9 j
Cuminaldehyde (1 × IC50)	seed treatment	36.2 ± 0.2 i	73.8 ± 0.9 b
	foliar spray	42.8 ± 1.1 g	61.7 ± 1.4 d
Cuminaldehyde (0.1 × IC50)	seed treatment	58.3 ± 1.5 e	46.3 ± 1.2 f
	foliar spray	65.1 ± 1.8 c	37.1 ± 2.6 h
Cuminaldehyde (0.01 × IC50)	seed treatment	72.8 ± 0.8 b	22.6 ± 1.5 i
	foliar spray	75.1 ± 0.7 a	18.3 ± 1.2 j

IC50 – inhibitory concentration 50

The results are means ± standard errors of four replications. Means within a column indicated by the same letter were not significantly different according to Duncan's multiple range test at the level $p < 0.05$

β-pinene, which are in accordance with Shahsavari *et al*. (2008) and Sekine *et al*. (2007). Our observations showed a high percentage of cuminaldehyde in the *B. persicum* EO sampled from Iran. This agrees with Foroumadi *et al*. (2002) and Azizi *et al*. (2009).

The *B. persicum* EO and its main constituents indicated antifungal activity. These results were similar to results reported by other investigators. Sekine *et al*. (2007) showed that the *B. persicum* EO and cuminaldehyde as its main constituent were responsible for its antifungal activity against phytopathogenic fungi such as *Fusarium oxysporum*, *Verticillium dahliae*, *Botrytis cinerea* and *Alternaria mali*. Rao *et al*. (2010) reported that γ-terpinenes were ineffective as fungicides against *Saccharomyces cerevisiae*. Koutsoudaki *et al*. (2005) compared the effects of α-pinene, β-pinene, ρ-cymene, β-myrcene, β-caryophyllene, limonene, and γ-terpinene against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, and reported that their antimicrobial activities were low or absent.

This is the first report on the effects of *B. persicum* EO and its main constituents on mycelial growth, sporulation and spore germination inhibition of *C. lindemuthianum*. In our investigations, the EO and the constituent of cuminaldehyde had the best inhibitory effects on the mycelia growth of *C. lindemuthianum* with MIC value of less than 1,500 ppm *in vitro*. This is in accordance with the results obtained by Sekine *et al*. (2007), who demonstrated the best antifungal effect of *B. persicum* among 52 plant species tested.

In comparison to the control our findings showed that sporulation and spore germination of *C. lindemuthianum* were completely inhibited by the MIC concentration of EO and cuminaldehyde. Caraway extract had the strongest inhibitory effect by inhibiting the spore germination of *Emericella nidulans*, *Penicillium commune*, *P. implicatum*, *Aspergillus tamarii*, *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme* (Soliman and Badea 2002; Dimić *et al*. 2009). Therefore, the EO and the constituent of cuminaldehyde were capable of controlling anthracnose infection since they were able to neutralize the fungal pathogen *C. lindemuthianum*.

Investigating fungistatic and/or fungicidal effects of the EO and its main constituents showed that the EO and its main constituents had fungistatic activity against *C. lindemuthianum*. The MIC values obtained for EO and its main constituents used in this assay were considerably lower than the values obtained for synthetic fungicides such as Carbendazim and Mancozeb.

Antifungal activity of EO and its main constituents increased with increasing its concentration. The minimum concentration of the EO and its main constituents required to inhibit mycelial growth of *C. lindemuthianum* differed. The IC50 and MFC values obtained for cuminaldehyde were considerably lower than the values obtained for the synthetic fungicides tested. This is a novel finding, suggesting that the EO and cuminaldehyde might be used as a powerful biological or natural agent instead of synthetic fungicides for reducing or suppressing *C. lindemuthianum* growth and damage caused by this pathogen.

In the present study, synergistic effects among γ-terpinene × cuminaldehyde, γ-terpinene × β-pinene and cuminaldehyde × β-pinene were observed. The combination of γ-terpinene with cuminaldehyde induced synergistic activity against *C. lindemuthianum* and in combination with β-pinene caused an additive effect. Ouedrhiri *et al*. (2017) reported that the synergistic effect could be explained by the interactions between the compounds, (-)-terpinene-4-ol, trans-4-thujanol, p-cymene and γ-terpinene induced a synergistic effect. A combination of γ-terpinene and p-cymene to (-)-terpinene-4-ol led to a significant antagonistic effect against *Escherichia coli* (Cox *et al*. 2001). Using a combination of EO constituents could lead to a reduction in the effective dose of constituents and expand the antifungal spectrum.

Our study revealed that the EO and its main constituents were capable of decreasing pectinase, cellulase and xylanase activity of *C. lindemuthianum*. Degradation of plant cell walls by *C. lindemuthianum* is due to CWDEs such as pectinase, cellulase and xylanase secretion (Herbert *et al*. 2004; Ramos *et al*. 2010). Overall, cuminaldehyde was more effective in reducing pectinase, cellulase and xylanase activity of *C. lindemuthianum* at most of the time points investigated. Similarly, a previous report indicated that EOs of *B. persicum*, *M. piperita* and *Thymus vulgaris* reduced the activity of pectinase and cellulose enzymes secreted by *Macrophomina phaseolina* and *Rhizoctonia solani* compared with the control (Khaledi *et al*. 2015). Abd-El-Khair and El-Gamal Nadia (2011) reported that aqueous extracts of various plants reduced mycelial growth of *R. solani* and *Fusarium solani*, together with significant inhibition of polygalacturonase and cellulose activities of these fungi. The present data revealed a considerable decrease in CWDEs activities of M *C. lindemuthianum*, in all treatments with the EO and its main constituents at 0.01 × IC50 concentration which did not suppress mycelial growth of the fungus *in vitro*. The decrease in the activity of CWDEs may be a part of mechanisms involved in reducing the virulence of *C. lindemuthianum*.

Investigations on mechanisms of disease suppression by plant extracts and EOs have suggested that the active constituents of them may either act on the pathogen directly or induce activation of defense responses in host plants leading to a reduction of disease progress (Abdel-Monaim *et al*. 2011; Kagale *et al*. 2011). In the

present research, first we used soil (data not shown) and seed treatment (Table 5) to evaluate the efficacy of EO and cuminaldehyde in disease control. In most cases, soil treatment was less effective in disease suppression than seed treatment (data not shown). One of reasons for this result might be the possibility that the EO and cuminaldehyde are degraded in soil. It seems that seed treatment leads to higher induction of plant defense mechanisms as previously demonstrated by Abd-El--Khair and El-Gamal Nadia (2011). So, based on these observations, we decided to present only the results of seed treatment and then, we carried out another experiment using foliar spray of different concentrations of EO and cuminaldehyde. Finally, we compared the results of seed treatment with foliar spray to determine their ability to control disease.

Greenhouse experiments indicated that using EO and cuminaldehyde as seed treatment or foliar spray were effective in reducing anthracnose of bean caused by *C. lindemuthianum* in a dose-dependent manner. Foliar application of plant extracts is significantly effective in reducing the disease caused by *C. lindemuthianum* on bean (Paulert *et al.* 2009). Numerous reports indicated that plant extracts or EOs can significantly reduce the severity of plant diseases, which agrees with our results (Plodpai *et al.* 2013; Khaledi *et al.* 2015).

Our study is the first to demonstrate the efficacy of *B. persicum* EO and cuminaldehyde against *C. lindemuthianum* using seed treatment or foliar spray *in vivo*. The EO was more effective in decreasing the DI of *C. lindemuthianum* on bean than cuminaldehyde. A very low concentration (0.01 × IC50) of EO and cuminaldehyde decreased the activity of CWDEs, as the main virulence factors of *C. lindemuthianum*. Essential oil and cuminaldehyde decreased the DI of *C. lindemuthianum* on bean and may represent new alternative disease management strategies.

The current study revealed that EO and its main constituents were capable of decreasing mechanisms of pathogenesis of *C. lindemuthianum*. These results confirm that the high antifungal activity of a broad collection of EO increased activity, can be attributed to the functional moieties. In conclusion, *B. persicum* EO could be applied as an alternative to synthetic fungicides for the control of *C. lindemuthianum*. These results indicate that *B. persicum* EO after suitable formulation could be used for the control of anthracnose of bean caused by *C. lindemuthianum*.

Acknowledgements

The authors wish to thank Ferdowsi University of Mashhad, Iran for financial support of this research.

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