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Antifungal Effect of Some Plant Essential Oils against Verticillium Wilt (*Verticillium dahliae* Kleb.) in Cotton

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Abstract In this study, it was aimed to determine the antifungal effect of essential oils of black cumin (*Nigella sativa* L.), cumin (*Cuminum cyminum* L.), chamomile (*Matricaria chamomilla* L.), cedarwood (*Cedrus atlantica*) and ginger (*Zingiber officinale*) plants against non-defoliating and defoliating pathotypes of Verticillium wilt (*Verticillium dahliae* Kleb.) disease *in vitro*. Essential oils were added to petri dishes (90 mm diameter) containing PDA medium at doses of 0.25, 0.5, 1, 2 and 4 µl mL^{-1}, and fungal discs with a diameter of 5 mm were planted in the middle of the petri dishes. Petri dishes with PDA were incubated for 12 days at 24±1°C. At the end of the incubation period, the colony diameters of the fungi were measured and the percent inhibition rates of plant essential oils were calculated compared to the controls. Chemical composition analysis of essential oils was determined by GC-MS method. As a result of the study, the antifungal effects of black cumin, cumin, chamomile, cedarwood and ginger essential oils were found to be very close to each other, although they varied depending on the pathotype and dose. The highest antifungal effect against non-defoliating and defoliating pathotype was found in the high dose (4 µl mL^{-1}) application of cumin essential oil, at a rate of 88.7% and 81.5%, respectively. The high antifungal effect of cumin essential oil against the pathogen can be attributed to the cuminaldehyde (31.44%) compound in its chemical composition. As a result, it was concluded that cumin essential oil can be used as an antifungal agent.

Keywords *Verticillium dahliae*, essential oils, pathotype, antifungal effect, cotton

1. Introduction

Cotton is an economically important plant cultivated in hot regions with tropical and subtropical climates in the world [1]. Cotton, which is an industrial plant, has an important place with the value it adds to the country's economy and employment opportunities. With the increase in the world population, the needs of the increasing population are also increasing. At the beginning of these needs are nutrition and clothing. It is used as a raw material in the textile industry with its cotton plant fiber, which has a wide range of uses, in the oil industry with its oil obtained from the seed, in the feed industry with its high protein content in the pulp, and its linters as a raw material in paper and gunpowder production [2, 3].

There are biotic and abiotic stress factors that limit cotton production [4]. One of the biotic stress factors is wilt disease caused by *Verticillium dahliae* Kleb., which causes economic loss all over the world [5]. The causative agent of the disease is a soil-borne fungus, which infects the plants from the capillary roots, enters and settles in

the transmission bundles. By preventing the transport of water and other mineral substances taken from the root to the leaves and tissues, it causes wilting, drying and decreased photosynthesis in the leaves, and changes in yield and fiber quality characteristics due to shedding in small boll [6, 7]. There are two pathotypes of the pathogen, defoliatng and non-defoliating [8]. The defoliating pathotype causes complete shedding and death of the leaves in the plant, while the non-defoliating pathotype causes wilting and a small amount of leaf shedding [9]. It is reported that the annual loss of product in cotton due to Verticillium in the world is 1.5 million bales [10, 11].

The fact that the pathogen is a soil-borne fungus and there is no economical chemical control makes it difficult to control the disease [12]. In this context, alternative control methods are needed in the fight against *V. dahliae*. Today, it has been reported in studies that essential oils and active substances obtained from plants may have antifungal properties due to their solubility in the phospholipid bilayer of the cell membranes of fungi and their ability to penetrate the cell walls of fungi [13, 14]. These essential oils are broad spectrum, safe, economical and effective. There are biologically effective components such as monoterpenes, diterpenes, sesquiterpenes, phenolic compounds in plant essential oils [15, 16]. Antifungal effects are due to the effective compounds in essential oils [17]. The active substance components may vary from plant to plant, as well as depending on the regions where the plants are grown, climatic conditions, and the type of microorganism [18]. The antifugal effects of essential oils of *Nigella sativa* L., *Cuminum cyminum* L., *Matricaria chamomilla* L., *Cedrus atlantica* and *Zingiber officinale* have been reported by conducted studies [19-23]. In a study conducted to determine the antifungal effect of *C. cyminum* essential oil against *V. dahliae* Kleb., it was reported that *C. cyminum* essential oil inhibited mycelial growth of *V. dahliae* 100% at all concentrations [24]. However, no study was found on the antifungal effect of *N. sativa* L., *M. chamomilla* L., *C. atlantica* and *Z. officinale* essential oils against *V. dahliae* in the literature reviews.

In this study, it was aimed to determine the antifungal effect of essential oils of black cumin, cumin, chamomile, cedarwood and ginger plants against non-defoliating and defoliating pathotypes of Verticillium wilt disease *in vitro*.

2. Materials and Methods

Test microorganisms

V. dahliae Kleb. isolates (PHCVd3-non-defoliating pathotype; PHCVd47-defoliating pathotype) isolated from cotton with known virulence were obtained from the fungal culture collection of Hatay Mustafa Kemal University. Fungal isolates were grown on potato dextrose agar medium (PDA-Difco) in a refrigerated incubator (24±1°C temperature; 12 hours dark/12 hours light) and kept in the refrigerator at +4ºC.

Plant essential oils

Essential oils of plants belonging to different families such as black cumin (*N. sativa* L.), cumin (*C. cyminum* L.), chamomile (*M. chamomilla* L.), cedarwood (*C. atlantica*) and ginger (*Z. officinale*). It was produced by a private company by steam distillation method and used as plant material (Table 1). Essential oils were stored in dark colored and tightly closed bottles at +4ºC in the refrigerator until use.

Table 1: Common name, scientific name and family of the plants species used in this study

Common name	Scientific name	Family
Black cumin	*Nigella sativa* L.	Ranunculaceae
Cumin	*Cuminum cyminum* L.	Apiaceae
Chamomile	*Matricaria chamomilla* L.	Asteraceae
Cedarwood	*Cedrus atlantica*	Pinaceae
Ginger	*Zingiber officinale*	Zingiberaceae

Detection of essential oil components by gas chromatography/mass spectrometry (GC-MS)

Suleyman Demirel University Innovative Technologies Application and Research Center (YETEM) were analyzed chemical compositions of essential oils using gas chromatography-mass spectrometry (GC-MS) (Shimadzu 2010 SE, Kyoto Japan). The individual peaks were identified by comparing linear retention indices (LRI) as well as comparing mass spectra with the Wiley library (Wiley, New York, NY, USA) and the NIST mass spectral database (Gaithersburg, MD, USA) [25].

Efficacy of plant essential oils on mycelial growth *in-vitro* conditions

The antifungal effect of black cumin, cumin, chamomile, cedarwood and ginger plant essential oils belonging to different families against non-defoliating and defoliating pathotypes of *V. dahliae* was investigated by *in vitro* contact application test at petri dishes. The essential oils of black cumin, cumin, chamomile, cedarwood and ginger plants are dissolved in ethanol solution at a ratio of 1:2 and in different concentrations (0.25, 0.5, 1,2, 4 µl mL^{-1}) was poured at petri dishes. After keeping the petri dishes (90 mm diameter) at room conditions overnight, discs with a 5 mm diameter fungal isolates were taken from the ends of the 7-day-old fungal isolates, which were previously grown in PDA medium, and placed in the middle of petri dishes containing essential oil + PDA medium. After inoculation, the petri dishes were covered with parafilm and incubated at 24±1°C for 12 days. As a control, PDA medium free from essential oils was used. At the end of this period, fungal colony diameters were measured and recorded. The measurement of the colony diameter was made by measuring the diameter of the fungus colony in separate directions perpendicular to each other [26]. The percent inhibition of different doses of essential oil was calculated by comparing the mycelial growth in the essential oil-containing petri with that of the negative control petri [27]. All experiments were conducted twice, with three replicates per treatment in a completely randomized plot design.

$$MGI\ (\%) = [(dc-dt)/dc] \times 100$$

MGI: Mycelial growth inhibition rate (%); dc: Represents the mycelial growth diameter of the control Petri dish (mm); dt: Represents the mycelial growth diameter of the amended Petri dish (mm).

Statistical analysis

Statistical analyses of the data were performed with the JMP IN packet statistic program (SAS Institute, Carry, NC, 13.0 PC version). Analysis of variance (one-way ANOVA) was carried out to determine the effects of the treatments. When the treatment effects were statistically significantly ($p \leq 0.01$), the Duncan's multiple range tests was used for means separations.

3. Results and Discussion

In the study, 69 active substances were determined in plant essential oils by GC/MS analysis (Table 2). Among them, Eucalyptol (1,8-Cineole) was the highest in black cumin essential oil component with a rate of 48.28%, followed by chamomile essential oil (46.78%). The biological effects of essential oils depend on their chemical composition, which is determined by plant genotype and is greatly influenced by various factors such as geographical origin, environmental and agronomic conditions. [28, 29]. In general, the antimicrobial effects of essential oils are determined by their composition, structure and also by these compounds in their functional groups [30]. In the current study, although 69 active substances were determined in essential oils in GC/MS analyzes, it was determined that Eucalyptol (1,8-Cineole) (48.28%) and cuminaldehyde (31.44%) came to the fore.

Table 2: GC-MS analysis results of black cumin, cumin, chamomile, cedarwood and ginger essential oils

No.	Compound name[a]	LRI[b]	*N. sativa*	*C. cyminum*	*M. chamomilla*	*C. atlantica*	*Z. officinale*
			% of the oil				
1	Tricyclene	924	0.77	0.04	0.46	0.41	1.44
2	alpha- Thujene	927	2.40	0.32	0.57	0.55	0.16

3	alpha - Pinene	933	14.78	1.68	10.17	9.33	7.01
4	beta- Fenchene	942	-	-	-	-	0.12
5	Camphene	953	4.77	0.24	3.66	3.07	12.33
6	Sabinene	972	2.23	0.32	1.69	1.28	0.51
7	beta- Pinene	978	9.07	16.03	6.34	5.26	2.11
8	4-Methyl-1-hepten-5-one	986	-		-	-	0.54
9	beta- Myrcene	991	-	0.69	0.75	-	1.55
10	Octanal	1006	-		-	-	0.14
11	Phellandrene	1007	-	0.44	0.18	-	0.39
12	DELTA.3-Carene	1009	-	0.05	0.27	-	0.04
13	alpha- Terpinene	1018	-	0.18	0.45	0.27	0.13
14	Cymol	1025	8.41	14.73	3.72	2.84	1.17
15	Limonene	1030	3.10	1.19	7.53	1.72	12.72
16	Eucalyptol (1,8-Cineole)	1052	48.28	1.93	46.78	27.88	16.27
17	gamma-Terpinene	1058	3.36	17.79	2.59	2.21	0.70
18	trans-Sabinene hydrate	1088	0.52	0.03	1.42	0.52	0.20
19	alpha- Terpinolen	1096	-	0.13	-	-	0.28
20	Dimethylstyrene (alpha-para)	1104	-	0.52	-	-	0.65
21	Linalool	1114	-	-	2.91	-	-
22	Chrysanthenone	1133	-	-	0.38	-	-
23	Carveol	1152	-	0.10	4.09	-	-
24	Camphor	1157	2.32	0.09	0.99	1.60	0.87
25	4-Terpineol	1193	-	0.28	-	-	0.18
26	Dimethylbenzylcarbinyl acetate (DMBCA)	1200	-	0.35	-	-	0.62
27	alpha- Terpineol	1207	-	-	0.34	-	-
28	Perilla alcohol	1208	-	0.85	-	-	-
29	Dihydrocarvone	1210	-	0.11	-	-	-
30	p-Allylanisole	1210	-	-	0.85	-	-
31	Z-Citral	1238	-	-	-	-	1.88
32	Cuminaldehyde	1247	-	31.44	-	-	-
33	Carvotanacetone	1260	-	0.33	-	-	-
34	E-Citral	1268	-	-	-	-	2.25
35	Phellandral	1277	-	0.34	-	-	-
36	2-Undecanone	1294	-	-	-	-	0.17
37	2-Caren-10-al	1298	-	6.84	-	-	-
38	1-Phenylpropane-1,3-diol	1302	-	0.89	-	-	-
39	Thymol	1307	-	0.10	-	-	-
40	Carvacrol	1317	-	0.05	-	-	-
41	Citronellyl acetate	1363	-	-	-	-	0.37
42	Eugenol	1372	-	-	3.24	-	-
43	alpha- Copaene	1375	-	-	-	-	0.25
44	gamma- Cadinene	1388	-	0.09	-	-	-

45	Linalyl acetate	1392	-	-	-	-	0.93
46	beta- Elemene	1400	-	-	-	-	0.33
47	alpha- Zingiberene	1414	-	-	-	-	0.09
48	alpha- Cedrene	1414	-	-	-	10.08	-
49	beta- Cedrene	1423	-	-	-	1.47	-
50	Caryophyllene	1428	-	0.12	0.62	-	-
51	Thujopsene	1433	-	-	-	22.59	-
52	Germacrene B	1439	-	-	-	-	0.15
53	Farnesene ((E)-, beta)	1466	-	0.05	-	-	-
54	alpha- Cedrene	1483	-	1.32	-	-	-
55	Germacrene D	1490	-	-	-	-	0.45
56	Curcumene	1491	-	-	-	-	4.30
57	Alloaromadendrene	1503	-	-	-	-	0.52
58	Sesquithujene (7-epi)	1506	-	-	-	-	17.72
59	Cuparene	1515	-	-	-	0.68	-
60	alpha- Farnesene	1517	-	-	-	-	1.40
61	beta- Bisabolene	1519	-	-	-	-	3.80
62	beta- Sesquiphellandrene	1534	-	-	-	-	3.80
63	Carotol	1601	-	0.05	-	-	-
64	alpha- Cedrol	1614	-	-	-	8.23	-
65	Tetracosane	2400	-	0.07	-	-	-
66	Pentacosane	2500	-	0.08	-	-	-
67	Hexacosane	2600	-	0.07	-	-	-
68	Heptacosane	2700	-	0.06	-	-	-
69	Nonacosane	2900	-	0.02	-	-	-
			100	100	100	100	100

[a] Compounds listed in order their elution, [b] LRI: Linear retention index

In this study carried out under *in vitro* conditions, the effects of 0.25, 0.5, 1, 2, 4 µl mL^{-1} doses of black cumin, cumin, chamomile, cedarwood and ginger herbal essential oils of mycelial growth and inhibition rates of PHCVd3 (non-defoliating pathotype) and PHCVd47 (defoliating pathotype) isolates and are given in Table 3. The inhibitory effect of black cumin, chamomile, cedarwood and ginger essential oils on mycelial growth of PHCVd3 and PHCVd47 isolates was found to be statistically significant ($P \leq 0.01$) compared to the control. It was determined that the essential oils used in the study inhibited mycelial growth of both pathotypes of the pathogen at different levels depending on the dose. 4 µl mL^{-1} application of black cumin essential oil had an effect of 66.9% and 67.1% on PHCVd3 and PHCVd47 isolates, respectively. In other doses of black cumin essential oil, inhibition of mycelial growth of PHCVd3 isolate was between 15.3% and 45.3%, while inhibition of mycelial growth of PHCVd47 isolate was between 14.3% and 46.4%. 4 µl/mL application of cumin essential oil was found to be 88.7% and 81.5% effective against PHCVd3 and PHCVd47 isolates, respectively. In other doses of cumin essential oil, inhibition of mycelial growth of PHCVd3 was between 13.2% and 55.5%, while inhibition of mycelial growth of PHCVd47 was between 12.5% and 51.1%. The highest antifungal effect in chamomile essential oil was determined at the rates of 70.0% and 69.4%, respectively, against PHCVd3 and PHCVd47 isolates in 4 µl mL^{-1} application. In other doses of chamomile essential oil, inhibition of mycelial growth of PHCVd3 was between 29.1% and 54.2%, while inhibition of mycelial growth of PHCVd47 was between 12.1% and 51.0%. Application of 4 µl mL^{-1} in cedarwood essential oil had an effect of 76.7% and 73.1% against PHCVd3 and PHCVd47 isolates, respectively. While other doses of cedarwood essential oil

inhibited mycelial growth of PHCVd3 between 22.0% and 59.3%, it inhibited mycelial growth of PHCVd47 between 10.0% and 60.3%. While the 4 µl/mL application of ginger essential oil showed the highest antifungal effect against PHCVd3 isolate (69.0%), this effect was found to be 67.8% in PHCVd47. At other doses of ginger essential oil, mycelial growth of PHCVd3 was inhibited between 16.2% and 40.1%, while mycelial growth of PHCVd47 was inhibited between 10.2% and 39.5% (Table 3).

Black cumin from the Ranunculaceae family is one of the most useful essential oils and is an industrial plant used in the food, medicine and cosmetics industries [31, 32]. GC-MS analysis of *N. sativa* essential oil resulted in the detection of 12 components representing 100% of the essential oil. As a result of the analysis, it was determined that black cumin essential oil contains high amounts of eucalyptol (1,8-cineole) (48.28%) and α-pinene (14.78%). (Table 2). The literature study shows that although the ratios of the compounds in the content are different, the composition is generally the same [33]. Some differences in the quality of oil composition can explained by environmental factors known to strongly influence the chemical composition of essential oils. In a conducted study, it was reported that *N. sativa* has an antifungal effect against *C. albicans* [34]. It has been stated that eucalyptol, the main component of the essential oil of *N. sativa*, completely inhibits the growth of various fungal species [35].

Belonging to the Apiaceae family, cumin is a well-known spice plant with important medicinal properties. *C. cyminum* essential oil contains cuminaldehyde (31.44%), γ-terpipene (17.79%) and cymol (14.73%) as main components showing antibacterial, antifungal and antioxidant activities (Table 2) [36-38]. Our findings, in parallel with other research results, show that cumin essential oil contains the highest level of cuminaldehyde compound [39]. In the study, *C. cyminum* has the highest inhibiting effect on fungal growth among essential oils. Similar to our findings, Ustuner et al. [24] reported that *C. cyminum* essential oil completely (100%) inhibited mycelial growth of *V. dahliae* at all concentrations [24]. Chamomile is a medicinal plant known from the Asteraceae family [40]. According to the results of GC-MS analysis, the main components of chamomile essential oil were determined as eucalyptol (1,8-cineole) (46.78%), α-pinene (10.17%) and limonene (7.53%) (Table 2). In the study, chamomile essential oil shows parallelism with the work of El Mihyaoui et al. in terms of the compounds in our results [41]. It has been reported that MCh-AMP1 peptide found in *M. chamomilla* L. plant has broad-spectrum antifungal activity by changing the cell membrane permeability of fungi. [42]. However, another study conducted indicated that essential oils and extracts from *M. chamomilla* had moderate to weak effects against the growth of fungi [43]. The main components found in cedar essential oil were eucalyptol (1.8-Cineole) (27.88%), thujopsene (22.59%) and α-cedrene (10.08%) (Table 2). As a result of the studies carried out, it has been reported that the essential oils of cedar species have significant antifungal effects [44-46]. However, no study was found on the effect of *C. atlantica* on *V. dahliae*. Our results showed that cedarwood essential oil inhibited defoliatings and non-defoliating pathotypes of *V. dahliae* at varying rates. It is thought that the antifungal effect of *C. atlantica* is due to the high eucalyptol and thujopsene compounds it contains. In this context, researchers reported that eucalyptol and thujopsene have antifungal effects as a result of their studies [47, 48]. Ginger is an important spice plant from the Zingiberaceae family. Sesquithujene (17.72), eucalyptol (16.27%) and limonene (12.72%) have been noted as the main components of ginger essential oil, which has fungicidal effects [49, 50]. In a study in which ginger essential oil was applied against *Fusarium verticillioides*, it was determined that ginger essential oil had a high antifungal effect against *F. vertticillioides* [23].

Table 3: Antifungal activity of plant essential oils on mycelial growth of *V. dahliae*

Essential oils	Doses (µL mL^{-1})	PHCVd3 isolate		PHCVd47 isolate	
		Mycelial growth (mm)[1]	MGI (%)	Mycelial growth (mm)[1]	MGI (%)
N. sativa	0.25	27.4 b*	15.3	35.4 b*	14.3
	0.5	23.2 bc	29.3	28.6 c	30.8
	1	22.4 cd	31.6	28.2 c	31.9

	2	17.9 d	45.3	22.2 d	46.4
	4	10.8 e	66.9	13.6 e	67.1
	Control	32.8 a	0.0	41.3 a	0.0
	$CV_{(0.01)}$	11.5		6.8	
C. cyminum	0.25	27.0 b	13.2	31.5 b	12.5
	0.5	24.2 bc	22.3	25.2 c	30.1
	1	20.4 c	34.4	24.0 c	33.3
	2	13.8 d	55.5	17.6 d	51.1
	4	3.5 e	88.7	6.7 e	81.5
	Control	31.1 a	0.0	36.0 a	0.0
	$CV_{(0.01)}$	10.5		6.8	
M. chamomilla	0.25	27.6 b	29.1	36.2 b	12.1
	0.5	25.0 b	33.2	29.3 c	28.9
	1	19.6 c	49.6	25.8 c	37.2
	2	17.8 c	54.2	20.2 d	51.0
	4	11.7 d	70.0	12.6 e	69.4
	Control	38.9 a	0.0	41.7 a	0.0
	$CV_{(0.01)}$	10.5		8.3	
C. atlantica	0.25	24.0 b	22.0	35.2 a	10.0
	0.5	17.7 c	42.6	25.5 b	34.7
	1	16.4 c	46.6	22.5 b	42.4
	2	12.5 d	59.3	15.5 c	60.3
	4	7.2 e	76.7	10.5 d	73.1
	Control	30.8 a	0.0	39.1 a	0.0
	$CV_{(0.01)}$	7.2		10.5	
Z. officinale	0.25	25.4 b	16.2	36.5 b	10.2
	0.5	21.9 c	27.8	30.6 c	24.8
	1	20.3 cd	33.0	27.5 d	28.7
	2	18.2 d	40.1	24.6 e	39.5
	4	9.4 e	69.0	13.1 f	67.8
	Control	30.3 a	0.0	40.7a	0.0
	$CV_{(0.01)}$	7.6		4.5	

[1] The mean radial mycelial growth of *V. dahliae* was determined at 12 days after inoculation. Each observation is based on 3 replicate plates. Arcsine transformation was performed prior to statistical analysis. *Mean values followed by different letters within the column are significantly different according to Duncan Test (P≤0.01). MGI: Mycelial growth inhibition rate (%)

4. Conclusion

The control of *V. dahliae* is very difficult due to the fact that they have resistant builds that can maintain their vitality for many years in the soil, there is resistance problem and there is no economical chemical control. Today, as a result of the increase in the awareness of both producers and consumers, the demand for synthetic fungicides used in the control against plant pathogens is decreasing day by day and the interest in alternative control methods is gradually increasing. It was determined that high-dose (4 µL mL^{-1}) application of cumin essential oil from black cumin, chamomile, cedarwood and ginger essential oils in the study was more effective against defoliating and non-defoliating pathotypes of *V. dahliae* in *in vitro*. The highest antifungal effect of cumin essential oil against the pathogen can be attributed to the high level of cuminaldehyde content of this essential oil. In particular, the effectiveness of cumin and other plant essential oils, which can be used as an

alternative to synthetic fungicides and are considered safe for human and environmental health, against soil-borne pathogens that are difficult to control should be investigated in more detail.

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