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***Journal of Scientific and Engineering Research*, 2024, 11(7):85-91**

Research Article

ISSN: 2394-2630
CODEN(USA): JSERBR

In Vitro Antifungal Efficacy of Five Plant Essential Oils on Monilinia laxa

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Abstract Monilinia laxa is one of the most important pathogens, causing significant crop losses worldwide. The antifungal effect of mint, thyme, laurel, eucalyptus, and fennel plant essential oils (PEOs) was evaluated under in vitro conditions against M. laxa using the vapor-phase method. Mycelial agar discs (5 mm) from 7-day-old test pathogen were placed in Petri plates with potato dextrose agar. The PEOs were dropped on a steril filter paper adhered to the covers of Petri plates with a micropipette at the doses of 1.0, 2.0, 4.0, 8.0, and 16.0 µL petri-1. The Petri dishes without dripping PEOs were used as a negative control. A licensed fungicide (25.2% Boscalid + 12.8% Pyraclostrobin-BASF company) was used as a positive control at the recommended dosage. Every Petri plate was sealed with parafilm and incubated at 25±1oC for 10 days under darkness. The experiment was carried out in a randomized plot design with three replications. As a result of the study, mint, thyme, laurel, eucalyptus, and fennel PEOs were found to inhibit the growth of M. laxa in a dose-dependent manner, whereas thyme and mint PEOs were determined more inhibitory effects against M. laxa. Thyme and mint PEOs have shown a fungicidal effect against pathogen because of the presence of carvacrol, thymol, and menton compounds, respectively. After thyme and mint PEOs, fennel, laurel, and eucalyptus PEOs showed the highest effect against the pathogen. Results of our study suggest the possibility of using thyme and mint PEOs as an alternative natural fungicide to control M. laxa

Keywords Essential oil, Monilinia laxa, Antifungal activity, Vapor-phase, Biopesticide

Introduction

Monilinia disease caused by Monilinia spp. [Monilinia laxa (Aderh. & Ruhl.), Monilinia fructigena (Aderh. & Ruhl.), Monilinia fructicola (G. Winter) Honey] is one of the most important problems in stone fruits and pome fruits, causing significant yield losses worldwide [1–2]. The incidence and development of Monilinia disease are highly affected by climatic conditions and can cause up to 80% fruit loss under favorable environmental conditions [3]. M. laxa and M. fructigena cause a negative economic impact and cause high losses in Central Europe [4], while M. fructicola is more common in Asia and North and South America [5]. M. laxa was a common disease agent in California (USA) in the early 20th century [6]. In European stone fruit orchards, it was originally found to be the cause of brown rot [7].

Ascomycete fungus M. laxa affects pome fruits like apple, pear, and quince as well as stone fruits including peach, nectarine, apricot, cherry, and plum. It also causes brown rot, blossom blight, and spur infection [8]. The pathogen is spread globally, with variations in distribution according on host plant and geographic region [9]. The next year, M. laxa spores can cause primary infection when they overwinter in fruit mummies and cankers on a tree [4]. The pathogen's spores are transported by wind and rain, enter the flower, further down the branch in the sap, and infect other parts of the host plant. On the attacked branches, necrosis and cankers appear, which hinder the host plant's sap circulation and dry out the branches [10]. The pathogen requires low temperature (optimal 10-12oC) and high air humidity for sporulation and infection of the plant's branches and flowers [11].

Prior to 2000, the only species known to exist in Europe were M. laxa and M. fructigena [12]. M. laxa was one of the Monilinia species that was subject to quarantine in China and several regions of North America [13]. Controlling the infection is critical since M. laxa severely lowers yield and quality both domestically and internationally. Chemical fungicides are not sustainable in the long run since M. fructicola and M. laxa races have grown more resistant to them [14-17]. During the 1970s, benomyl and thiophanate-methyl, two benzimidazole fungicides, were

widely used and effectively managed brown rot during the flowering stage. But after a few years, benomyl was stopped because benomyl-resistant M. laxa isolates were found in the USA [18, 19]. The extensive use of these fungicides has a negative effect on the ecosystem because of the fungicide residues that remain in the soil, spread, and eventually find their way into groundwater [20]. Moreover, the use of these synthetic fungicides to control Monilinia disease presents a serious risk to human health [21]. In this context, safer and more eco-friendly alternative biological treatments to control fruit post-harvest diseases are needed, such as microbial antagonists and natural chemicals [22].

PEOs are one of the first picks for many biopesticides because of their various bioactivities [23], such as antibacterial [24], antioxidant [25], anti-inflammatory [26], cytotoxic [27], and anticancer [28]. About 300 of the 3.000 PEOs known so far have commercial applications, leading to a demand of 370.000 metric tons in 2024 with an estimated market value of US$13.94 billion [29]. The antimicrobial effect of essential oils (EOs) is associated with several mechanisms, including alteration in cell wall and membrane permeability and changes in gene expression patterns [30]. The EO composition of plants is influenced by many factors, such as plant variety [31], geographical area [32], extraction method [33], etc. PEOs may represent a potential management strategy with important and unknown mechanisms [34, 35]. PEOs were fungistatic and inhibited the mycelial growth of fungus, but they did not completely eradicate the fungal colony [36]. Meanwhile, many researchers have reported that PEOs are more effective against fungi in the vapor phase [37, 38]. Studies reported that Thymus vulgaris L. (vapor-phase; at 16.7 µL L-1) was effective in the control of brown rot in peaches caused by M. laxa [39]. PEOs are highly concentrated, volatile, aromatic liquids extracted from plants. PEOs have been reported to control M. laxa in stone fruits [40], postharvest diseases in tomatoes, citrus fruits, molds, foodborne, and various bacteria [41]. PEOs obtained from other plant species have also been reported to be effective against Monilinia spp. [42]. The aim of the study was to evaluate the antifungal effect of five PEOs on M. laxa (MAp 5 isolate) in vitro mycelial growth.

Materials and Methods

A. Fungus culture

The virulent and highly pathogenic MAp 5 isolate of M. laxa (GenBank accesion number ON791287) used in the experiments was obtained from the stock culture collection at the Department of Plant Protection, Mustafa Kemal University, Faculty of Agriculture [43]. Among these stock cultures, 7-day-old cultures developed in Petri plates (90 mm) containing potato dextrose agar (PDA-Difco) at 25±1oC were used.

B. Plant essential oils

A private company (Arpaş Arifoğlu Company, İstanbul-Türkiye) produced five PEOs of mint (Mentha piperita L.), thyme (Thymus vulgaris L.), laurel (Laurus nobilis), eucalyptus (Eucalyptus citriodora Hook.), and fennel (Foeniculum vulgare Mill.) plants by water vapor distillation method. These plants were used as plant material. (Table 1). PEOs were hermetically stored at +4oC in the refrigerator until further use.

Table 1: Basic data of PEOs used in the experiment

Scientific name	Family	English name	Brand name
Mentha piperita L.	Lamiaceae	Mint	Mint Oil
Thymus vulgaris L.	Lamiaceae	Thyme	Thyme Oil
Laurus nobilis L.	Lauraceae	Laurel	Laurel Oil
Eucalyptus citriodora Hook.	Myrtaceae	Eucalyptus	Eucalyptus oil
Foeniculum vulgare Mill.	Umbellifereae	Fennel	Fennel Oil

C. Antifungal effect of five PEOs on mycelial growth of M. laxa in vitro conditions

The antifungal test of five PEOs against M. laxa was examined using the vapor-phase method [44]. For determination of vapor phase effect, Petri plates (90 mm) containing 25 mL of PDA medium were inoculated with 5-mm-diameter plugs of the actively growing test pathogen from the periphery of 7-day-old cultures.Then, sterilised filter papers (70 mm diameter, Whatman No: 1) were placed on the inner surface ofthe Petri dish lid. Thereafter, an aliquot of PEOs (1-16 µL petri-1) were added onto the filter papers in a Petri plate using a micropipette and it allowed only volatile compounds to be the causative agents for mycelial growth. Petri dishes with sterile distilled water were used as a negative control. As a positive control, a fungicide with a license (25.2% Boscalid + 12.8% Pyraclostrobin-BASF company) against pathogen was used at the recommended dosage. The Petri plates were sealed with parafilm immediately after adding PEOs to prevent the loss of vapours and incubated at 25±1oC for 10 days under darkness. The mean radial mycelial growth of the pathogen was determined by measuring the diameter of the colony in two directions at right angels when the plate surface of the untreated control Petri dishes was covered by fungus ten days after inoculation. The antifungal efficacy was calculated as the percentage of mycelial growth inhibition (MGI %) using the following formula (Equation 1) according to the study by Dev et al. [45]. The experiment was carried out in a randomized plot design with three replications.

MGI (%) = [(dc-dt) / dc] x 100 (1)

where dc and dt represent mycelial growth diameter in untreated control and treated Petri plates, respectively. PEOs were scored using Onaran's scale [46] based on their ability to suppress mycelial growth of M. laxa and are presented in Table 2.

Table 2: 0-4 scale of Onaran used in the experiment

Scale value	Definition
0	No inhibition (0%)
1	Weak inhibition (0-25%)
2	Moderate inhibition (26-50%)
3	High inhibition (51-75%)
4	Extremely high inhibition (76-100%)

The fungicidal/fungistatic effect of the minimum inhibition concentration of PEOs on inhibition of mycelial growth in the vapor-phase method was determined according to Thompson [47]. Mycelial discs that did not develop in the Petriplates were transferred to untreated PDA after the tests and incubated again for ten days at 25±1oC. A fungistatic effect occurred when microbial growth in the Petridishes was temporary inhibited, while a fungicidal effect occurred when there was no growth in the Petriplates [48].

D. Statistical analysis

Colony diameters measured at five PEO concentrations were analyzed using one-way ANOVA with the JMP IN package statistical program (SAS Institute, Carry, NC, 13.0 PC version). The differences between the treatments were compared using the LSMeans Differences Student's test ($P \leq 0.01$).

Results & Discussion

The results of the antifungal activity of the PEOs are shown in Table 3. The effect of mint, thyme, laurel, eucalyptus, and fennel PEOs on the pathogen differed depending on the PEOS and treatment doses. Comparing the tested PEOs to the negative control, all concentration levels significantly ($P \leq 0.01$) reduced M. laxa's mycelial growth. Depending on the dose increase, each PEO used in the experiment reduced the mycelial development of M. laxa at different rates. The high-dose (16 µL petri-1) and positive control treatments with mint PEO had a 100% fungicidal effect on M. laxa, whereas an 8 µL petri-1 application had the next-highest effect (89.9%). The percentage of M. laxa mycelial growth inhibition ranged from 40.6% to 69.6% at different dosages of mint PEO. However, the high-dose and positive control treatments (100.0%) had the highest effect on M. laxa in thyme PEO. It was found that these treatments had fungicidal effects. 8 µL of petri-1 (90.3%) was administered after these treatments. On the other hand, different dosages of thyme PEO reduced M. laxa's mycelial growth by 45.9% to 79.2%. The highest inhibitory effect of laurel PEO against M. laxa was shown by the positive control treatment at 100% (fungicidal effect); high-dose application showed the second highest effect at 92.8%. The percentage of M. laxa mycelial growth inhibition ranged from 31.9% to 82.2% at different laurel PEO dosages. The fungicidal effect of applying eucalyptus PEO as a positive control was 100.0% on tested M. laxa; the next highest effect was 86.9% with high-dose treatment. The reduction of M. laxa's mycelial development in four different dosages of eucalyptus PEO ranged from 30.8% to 76.7%. Fennel PEO positive control treatment showed 100% fungicidal effect on tested M. laxa; high-dose application showed the next highest effect, at 95.0%. The percentage of M. laxa mycelial growth suppression ranged from 38.4% to 82.4% with different dosages of fennel PEO. The thyme PEO treatments in 4, 8, 16 µL petri-1, and the positive control were given a scale value of 4. The applications of 8, 16 µL petri-1, and the positive control of the remaining four PEOs were given a scale value of 4. Overall, a low to moderate inhibitory percentage was observed upon dosage reduction (Table 3).

The vapor-phase method demonstrated that the thyme and mint PEOs were the most effective in inhibiting M. laxa's mycelial growth when compared to other PEOs. PEOs containing thyme and mint showed fungicidal effect against M. laxa in both high-dose and positive control applications. The other PEOs' antifungal activity were found to be relatively close to each other, depending on the pathogen and dosage.

Table 3: In vitro antifungal activity of five PEOs on the mycelium development of M. laxa (MAp 5 isolate)

PEO	Concentration (µLpetri^{-1})	M. laxa (MAp 5 isolate)		
		MG (mm)[1]	MGI (%)	Onaran's scale**
M. piperita	0 (Negative control)	42.3 a*	0.0	S0
	1	25.1 b	40.6	S2
	2	20.6 c	51.3	S3
	4	12.8 d	69.6	S3
	8	4.3 e	89.9	S4
	16	0.0 f	100.0^{+}	S4
	Positive control	0.0 f	100.0^{+}	S4
	$CV_{(0.01)}$	1.7		

T. vulgaris	0 (Negative control)	42.3 a	0.0	S0
	1	22.9 b	45.9	S2
	2	16.4 c	61.3	S3
	4	8.8 d	79.2	S4
	8	4.1 e	90.3	S4
	16	0.0 f	100.0+	S4
	Positive control	0.0 f	100.0+	S4
	$CV_{(0.01)}$	2.4		
L. nobilis	0 (Negative control)	42.3 a	0.0	S0
	1	28.8 b	31.9	S2
	2	23.3 c	45.0	S2
	4	17.4 d	58.8	S3
	8	7.5 e	82.2	S4
	16	3.0 f	92.8	S4
	Positive control	0.0 g	100.0+	S4
	$CV_{(0.01)}$	1.5		
E. citriodora	0 (Negative control)	42.3 a	0.0	S0
	1	29.3 b	30.8	S2
	2	24.8 c	41.4	S2
	4	18.3 d	56.8	S3
	8	9.9 e	76.7	S4
	16	5.5 f	86.9	S4
	Positive control	0.0 g	100.0+	S4
	$CV_{(0.01)}$	1.3		
F. vulgare	0 (Negative control)	42.3 a	0.0	S0
	1	26.0 b	38.4	S2
	2	22.0 c	47.9	S2
	4	14.5 d	65.6	S3
	8	7.4 e	82.4	S4
	16	2.1 f	95.0	S4
	Positive control	0.0 g	100.0+	S4
	$CV_{(0.01)}$	1.6		

1The mean mycelial growth of M. laxa was determined at 10 days after inoculation. Based on three replicate plates, each observation. *Mean values followed by different letters within the column are significantly different according to LSD Test (P≤ 0.01). CV: Coefficient of variation. MG: Mycelial growth. MGI: Mycelial growth inhibition. **Onaran's scale:S0: No inhibition (0%), S1: Weak inhibition (0-25%), S2: Moderate inhibition (26-50%), S3: High inhibition (51-75%) and S4: Extremely high inhibition (76-100%). +: fungicidal effect.

The PEOs of T. vulgaris and M. piperita showed the highest inhibitory effect against M. laxa in the present study. The findings of this investigation validated the previously documented potent inhibitory capability of thyme EO against M. fructigena [49]. Oils of the Lamiaceae family have attracted considerable attention because of their strong antifungal effect [50]. For example, thyme and oregano EOs have shown very strong effects against Monilinia spp. in vitro [51]. Thymol and carvacrol, because of their antioxidant activity, have significant anti-fungal plant pathogen properties [52]. Thyme, oregano, and savory EOs have been found to be able to control the growth of M. laxa and Botrytis cinerea on stonefruit [42]. Using a slightly modified agar overlay technique, in vitro investigations demonstrated that both the new formulation and the initially emulsified thyme EO strongly suppressed M. fructigena's mycelial growth [53]. According to study, thymol vapors decrease conidia viability and prevent M. fructicola from growing mycelially. Actually, thymol crystallizes on the fungal cell walls outside, exposing formations that are typified by tangled cell membranes and disjointed cytoplasmic organelles [54]. Benomari et al. [55] found that several Algerian mentha EOs show a strong fumigant antifungal effectiveness against fungi like M. laxa, M. fructigena, and B. cinerea. Their findings that the previously stated mentha EOs might be used as biological antifungal agents to protect apple and pear plants against fungal diseases caused by Monilinia sp. and B. cinerea. Tanović et al. [56] reported that, relative to the control group, the thyme EO formulation showed growth suppression of M. fructicola and M. laxa of 73.9% and 68.4%, respectively. Similar to the results of laurel PEO, using dosages of 200, 400, 600, 800, and 1000 µg mL-1, tests were performed in vitro to determine whether laurel EO affected M. laxa and B. cinerea's ability to produce mycelial growths. Laurel EO showed fungistatic effects in all applications; at the lowest dosage, it totally inhibited M. laxa, while at the highest dosage, it entirely inhibited B. cinerea [57]. Garello et al. [58] reported that all examined EO considerably reduced M. fructicola growth as compared to the untreated control; the most notable reductions in growth were observed in fennel, thyme,

and basil. Neri et al. [59] found that eucalyptus and thyme EOs completely inhibited the mycelial growth of M. fructigena. Thyme and eucalyptus EOs contained 81.25% carvacrol and 24.9% cymene, respectively.

Conclusion

The results showed that PEOs of mint, thyme, laurel, eucalyptus, and fennel were inhibit the mycelial growth of M. laxa (MAp 5 isolate) under in vitro conditions. The inhibitory effect against M. laxa varied according to each PEO's dosage. The highest inhibitory effect against M. laxa was obtained from positive control and high-dose treatments of thyme and mint PEOs. Fungicidal effect has been reported for this effect. The inhibitory effects of thyme and mint PEOs are thought to be due to the chemical compounds carvacrol, thymol, and menton, respectively. The PEOs of fennel, laurel, and eucalyptus showed the second-best effect against the pathogen. As a result, thyme and mint PEOs might be used as an alternative to synthetic fungicides in the control of M. laxa. In addition to the commercial formulation's potential to effectively control Monilinia disease, further research is needed to determine the precise amount and timing of delivering certain PEOs in extensive field testing.

Acknowledgment

The authors thank to Prof. Dr. Şener Kurt (Hatay Mustafa Kemal University, Faculty of Agriculture, Hatay, Türkiye) for kindly providing isolates of M. laxa (MAp 5).

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