Effects of Some Essential Oils on Mycelial Growth of *Penicillium expansum* Link and Blue Mold Severity on Apple

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ABSTRACT— ***In this study, in vitro and in vivo antifungal effects of the essential oils of oregano (Origanum minutiflorum), European pennyroyal (Mentha pulegium) and germander (Teucrium polium) plants, naturally growing in the Mediterranean Region, against Penicillium expansum, storage rot agent of apples, were investigated. In vitro fumigant activities of 1, 2.5, 5 ve 10 µl/petri doses of the essential oils were determined against three virulent isolates of the pathogen and compared with the efficiency of fungicide Thiabendazole which is registered and commonly used against the pathogen. As a result, it was found that antifungal effects of the essential oils increased with the increasing doses and oregano oil at doses over 2,5 µl/petri showed similar effect with the fungicide and totally inhibited the mycelial growth of the P. expansum isolates. This effect was found to be fungistatic at 2,5 µl/petri dose, while it was fungicidal at higher doses. Antifungal effects of the essential oils of other plants were lower. In vivo experiment showed that none of the essential oils were as effective as the fungicide against the pathogen and inhibition rates at 10 µl dose changed between 55-88%. In consequence, promising results on the possible use of oregano oil in the postharvest control of P. expansum were obtained.***

Keywords— *Malus domestica* Borkh., storage rot, plant volatiles, antifungal effect

1. INTRODUCTION

Apple is known as the earliest tree to be cultivated and is one of the most common fruits all over the world. Turkey comes third in apple production, with about 3 million tones apple production, after China and The United States [1,2]. Regarding the year-round demand of apples, long term storage and marketing becomes very important [3]. Post harvest diseases are among the problems decreasing shelf life of apples. It is known that these diseases can cause 20-50% losses depending on the type of crop and storage conditions [4]. *Penicillium expansum* Link is the most important fungal agent causing fruit decay on stored apples. The fungus requires a wound on the fruit to penetrate and initiate infection and can easily spread under suitable conditions [5]. It was reported that this fungus was the most common rot agent in the apple storages in Isparta province supplying one-fifth of Turkey's apple production [6]. The fungus can also produce carcinogenic mycotoxin patulin [7].

Although different methods are developed to control plant diseases, chemical control is the most common way of controlling post harvest diseases of fruits. However, because of the toxicity of pesticides to humans and the environment, use of alternative natural chemicals like plant essential oils gained importance [8]. Essential oils are complex chemical compounds with antimicrobial, allelopathic and antioxidant activity [9,10,11]. Antimicrobial effects are known to be resulted from their phenolic elements [12]. Since plant essential oils natural compounds that are not dangerous for human and environment, they can be thought as alternatives for synthetic pesticides [13,14]. Besides, they can be effective against pathogen strains resistant to fungicides [15].

The aim of this study is to determine the *in vitro* and *in vivo* efficiency and possible use of essential oils of oregano, pennyroyal and germander against *Penicillium expansum*, blue mold agent of apple.

2. MATERIALS AND METHODS

2.1. Preparation of the Essential Oils

Leaves, stem and flowers of oregano (*Origanum minutiflorum* O. Schwarz et. P. H. Davis), European pennyroyal (squaw mint) (*Mentha pulegium* L.) and germander (*Teucrium polium* L.) plants collected from the plateaus of Serik district of Antalya province (Turkey) during June and July 2014 were used to obtain the essential oils. Herbariums of the plant

samples were prepared and they were identified by Prof. Dr. Hüseyin Zengin (Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University). Plant samples were dried at room temperature on a dry, shady place, ground by a grinder and kept in sealed plastic bags under dry and dark conditions.

Essential oils were obtained by Clevenger apparatus and were kept in a fridge until they were used. Component analysis of the oils were performed by GC-MS at the Experimental and Observational Student Research and Application Center of the Süleyman Demirel University.

2.2. Isolation and Selection of the Pathogen Isolates

Most of the *Penicillium* isolates used in the study were obtained from the infested fruit samples collected from the apple storages in Isparta province. Small pieces (1-2 cm) from the fruit samples containing healthy and infected tissue were surface sterilised in 1% NaOCl for 2-3 minutes, transferred to sterile distilled water and blotted dry. Four pieces for each sample were then transferred onto Petri plates with PDA containing streptomycin sulfate (50 mg/l). Pure cultures were obtained after incubation at 24°C for 5 days and isolates were kept on agar slants at 4°C until used [16]. Two additional isolates were also obtained one (4-5-Y-20) from Dr. Evrim ÖZKALE (Department of Biology, Faculty of Science and Arts, Celal Bayar University), and the other (Hö-Pe-1) from Dr. Hülya Özgönen Özkaya (Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University).

In order to determine the virulent isolates used in the rest of the study, pathogenicity tests were performed with Golden Delicious apples. Healthy and chemical free fruits were surface sterilised with 1% NaOCl for 2 minutes, transferred from sterile distilled water and dried on sterile filter papers for 20 minutes at room temperature. Five ml of sterile distilled water with Tween 20 (%0.05) was added to each petri dish with an isolate and spores were forced to transfer into water by gentle agitation with a sterile glass rod. Spore suspension were then filtered through muslin and concentration was adjusted to 10^7 spores/ml by using Thoma counting chamber. Fruits were wounded (3 mm deep and 3 mm diameter) at two opposite sides by a cork borer and inoculated with 20 µl spore suspension. Fruits wounded and inoculated with the same amount of sterile distilled water with Tween 20 served as controls. Apple fruits were then placed in polyethylene bags with three layers of blotters moistened with sterile water at the bottom. Two apples were used for each isolate and lesion diameters were determined 7 days after incubation at 23˚C [17]. Three virulent isolates were selected and identified for the rest of the study.

Isolates were first identified according to their cultural and morphological characteristics [18, 19, 20], then in order to confirm the identifications, ITS regions of the rDNA of the isolates were amplified by using ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) universal primers. A colony from the single spore cultures of the isolates were transferred to potato dextrose broth and incubated for 4-6 days on orbital shaker. Mycelia were filtered through muslin, packed in aluminum foil and kept at -80˚C for an hour, then ground into fine powder using liquid nitrogen with a sterile pestle and mortar. Qiagen DNA easy plant mini kit was used to isolate DNA and DNA samples were visualised after agarose jel electrophoresis. Sequences of the ITS regions were determined by REFGEN gene research center after amplification using a thermocycler. BIOEDIT program was used to compare the sequences by those of the known isolates [20].

2.3. Determination of the Efficiency of the Essential Oils

Spore suspension with 10^7 conidia/ml concentration was prepared from 7 day old cultures of *P. expansum* isolates and 20 µl suspension of each isolate were transferred to 1-2 mm depth wells at the center of the PDA plates with an automatic pipette. Similarly, 1, 2.5, 5 and 10 µl/petri doses of the essential oils were applied at the center of the lids of the petri plates. Same amount of sterile distilled water was applied on the lids of control plates. Fungicide Thiabendazole was used as recommended (0.2%) to compare the efficiency of the essential oils. Petri plates were sealed with parafilm and incubated at 23°C in the dark for 7 days [17]. At the end of the incubation period, area covered by the pathogen colonies were calculated by the help of 1 cm^2 squares drawn on the opposite sides of the plates. Efficiency of the essential oils were determined by Abbott's formula by comparing the area of the colonies on control plates. If there was no mycelial growth on plates with essential oils, the wells with spore suspension of the pathogen were transferred with a 5 mm cork borer to new PDA plates without essential oil and incubated for an additional 7 days. If mycelial growth was observed, the effect of the essential oils were regarded as fungistatic, if not the effect was regarded as fungicidal.

Golden Delicious apple cultivar known as susceptible to the pathogen was used for the *in vivo* experiments. Apple fruits were obtained from an orchard without pesticide application and care was taken to select healthy fruits with similar size. Fruits were kept 2 minutes in 1% NaOCl solution for surface disinfection, washed with sterile distilled water and allowed to dry at room temperature. Wounds with 3mm diameter and 3mm depth were formed on two opposite sides at the ecvatorial regions of the fruits were made with a sterile cork borer [21]. Inoculations were performed by transferring 20 µl spore suspension with 10^7 conidia/ml concentration to the wounds. Apple fruits were then placed in plastic boxes with three layers of blotters moistened with sterile water at the bottom. Above mentioned doses of the essential oils were applied to the small filter papers placed on the lids and boxes were sealed with parafilm. Boxes with filter papers moistened with similar amounts of sterile distilled water were used as controls. Fungicide Thiabendazole was again used to compare the

efficiency of the oils. Inoculated fruits were dipped into fungicide solution for a minute. All fruits were incubated at 24 °C in the dark for 7 days and lesion diameters on the fruits were measured [22]. Lesion diameters were compared with controls and efficiency of the applications were calculated by Abbott's formula [23].

Experiments were conducted in completely randomized design with 3 replicates in petri trials and 4 replicates in fruit trials. Data were subjected to analyses of Variance and means were compared by Tukey test using SPSS program. Arc sin transformation was applied to the data with percentages before statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Chemical components of the essential oils

The yields of the essential oils obtained from the leaves, stem and flowers of oregano, pennyroyal and germander plants collected from Antalya province were 1.8%, 1.4% and 0.225, respectively. Characterization of the oregano oil by GC-MS analyses showed that carvacrol was the main component with 80.25%, followed by γ-terpinene with 5.18% and ρ-cymene with 4.32%. It was previously reported that carvacrol (78.6%), ρ-cymene (7.7%) and γ-terpinene (2.2%) were the major components of oregano oil [24]. Pulegone was the main component of pennyroyal oil with 94.76%, while 3-octanol with 1.84% and isopulegone with 1.36% were the other major components. Similarly, pulegone was reported as the major component of this plant by other researchers [25,26]. In germander essential oil, again carvacrol was the main component with 47.40%. Other major components of the germander oil were β-elemene, β-caryophyllene and sabinene with the ratios 19.92, 9.21 and 5.15, respectively. Components of germander essential oil and their percentages showed differences with the results reported not only in other countries, but also from Turkey. It was mentioned that ecological and genetic differences among plant populations could affect the components and their rates [27,28,29].

3.2. Characteristics of the Penicillium isolates

In the pathogenicity test performed by using a total of 22 isolates two of them obtained from two researchers and the others isolated from the infected fruits in apple storages, lesion diameters were significantly different from each other. As a result of Tukey test, LT-10, 4-5-Y-20 and Hö-Pe-1 isolates caused the largest lesions on the apple fruits and selected for the rest of the study (Table 1).

Table 1: Mean lesion diameters on the apple fruits inoculated with *Penicillium* isolates

Isolate Code	Lesion diameter (mm)
Control	3.00 g *
E- 1	28.00 abc
E-2	26.25 abcd
E-10	26.50 abcd
LT-1	22.00 cdef
LT-2	17.75 ef
LT-3	19.50 def
LT-5	29.50 ab
LT-6	31.00 ab
LT-7	26.75 abcd
LT-8	26.75 abcd
LT-9	16.00 f
LT-10	**32.50 a**
LT-11	16.50 f
LT-16	26.00 abcd
LT-23	29.75 ab
LT-25	24.00 bcde
LT-26	18.00 ef
S-1	26.50 abcd
S-2	24.25 bcde
S-3	19.50 def
Hö-Pe-1	**32.50 a**
4-5-Y-20	**32.75 a**

*Means shown by the same letters were not significantly different from each other according to Tukey test ($P \leq 0.05$)

Three virulent isolates were identified as *P. expansum* according to their cultural and morphological features. Base sequences of their ITS regions of the rDNA also showed 99-100% similarity with those of the *P. expansum* isolates (KP128916.1, KP204876.1, KP204877.1, KP204879.1) in the gene bank.

3.3. Effects of the essential oils on the mycelial growth of P. expansum

In vitro experiments showed that the essential oils supressed the mycelial growth of *P. expansum* isolates in different rates depending on the oils and doses (Table 2). For all isolates in the experiment, oregano oil was the most effective one among three essential oils and 2.5 µl and higher doses of this oil totally inhibited the pathogen growth as fungicide Thiabendazole (Figure 1). It was determined that its effect was fungistatic in 2.5 µl dose, while it was fungicidal in higher doses for all isolates. Although the inhibition rates of pennyroyal and germander oils generally increased with increasing doses, they both could not totally inhibit the growth of the pathogen even in the highest dose and changed between 35-75%. The effect of the fungicide thiabendazole, used in comparison with the oils, showed fungicidal effect for all isolates.

Table 2: Inhibitory effects (%) of different doses of oregano, pennyroyal and germander oils on the mycelial growth of *P. expansum* isolates 7 days after application

Isolates	Doses (µl)	Oregano	Pennyroyal	Germander	Fungicide
LT-10	1.0	74.26[v] b B[w]	21.56 c C	29.40 b C	100.00 A[x]
	2.5	100.00 a A[y]	26.79 c B	41.30 ab B	100.00 A
	5.0	100.00 a A[z]	46.78 b C	60.78 a B	100.00 A
	10.0	100.00 a A[z]	74.50 a B	60.78 a C	100.00 A
Hö-Pe-1	1.0	62.80 b B	12.24 c C	21.34 b C	100.00 A
	2.5	100.00 a A[y]	25.94 bc C	48.26 a B	100.00 A
	5.0	100.00 a A[z]	41.27 b C	56.11 a B	100.00 A
	10.0	100.00 a A[z]	72.25 a B	61.28 a C	100.00 A
4-5-Y-20	1.0	28.20 b B	21.36 c B	28.20 a B	100.00 A
	2.5	100.00 a A[y]	38.46 bc B	35.72 a B	100.00 A
	5.0	100.00 a A[z]	41.17 b B	30.25 a C	100.00 A
	10.0	100.00 a A[z]	65.81 a B	35.04 a C	100.00 A

[v]Arcsin transformation was applied to the data before statistical analyses and real data were shown in the table.
[w]Means in the same column shown by the same lowercase letter and the means in the same row shown by the same uppercase letter were not significantly different from each other according to Tukey test ($P \leq 0.05$).
[x]Fungicide Thiabendazole was used (0.02%) to compare the efficiency of the oils and its effect was found to be fungicidal for all isolates.
[y]fungistatic
[z]fungicidal

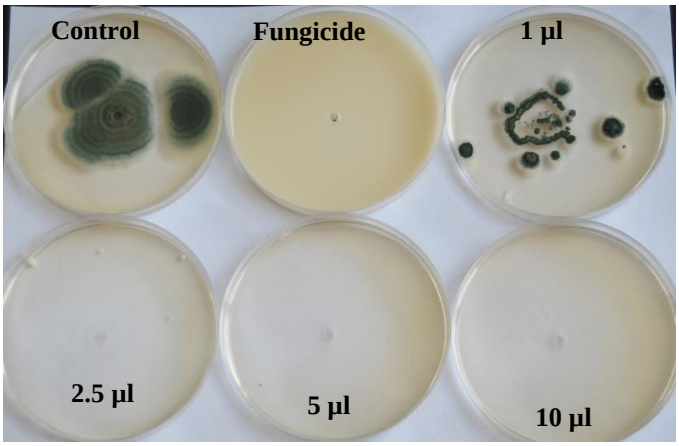

Figure 1: Inhibition of the mycelial growth of *P. expansum* LT-10 isolate 7 days after incubation with different doses of oregano oil and the fungicide Thiabendazole (0.02%).

In the petri trials, there was no statistically significant difference among the isolates in terms of their susceptibilities against the essential oils when all doses were evaluated together (Table 3).

Table 3: Inhibition (%) of the mycelial growth of *P. expansum* isolates 7 days after application of oregano, squaw mint and germander oils

Isolates	Oregano	Pennyroyal	Germander
LT-10	93.57[x] a[y]	42.41 a	48.03 a
Hö-Pe-1	90.70 a	38.13 a	46.78 a
4-5-Y-20	82.05 a	38.80 a	33.16 a

[x]Arcsin transformation was applied to the data before statistical analyses and real data were shown in the table.
[y]Means in the same column shown by the same letter were not significantly different from each other according to Tukey test (P≤ 0.05).

3.4. Effects of the essential oils on blue mold formation on apple fruits

Similar with *in vitro* experiments, oregano, pennyroyal and germander essential oils decreased the blue mold formation on apple fruits in different rates. For all isolates the inhibitory effects of the essential oils increased with the increasing doses (Table 4). This is a common experimental evidence for the effects of various essential oils against different pathogens [30,31]. *In vivo* efficiency of the oregano oil was higher than those of other two oils and reached 87.5% with the 10 µl dose (Figure 2). However, in comparison with the *in vitro* experiment, its effect was lower and could not statistically arrange in the same group with the fungicide Thiabendazole. Inhibitory effects of the oils also showed differences depending on the isolates. For all doses oregano oil showed the highest effect, while the inhibitory effect of germander oil was statistically similar with oregano oil with the 1 µl and 2.5 µl doses in the isolate LT-10, and with the 5 µl and 10 µl doses in the isolate 4-5-Y-20. Similarly, the efficiency of the squaw mint oil was statistically same with oregano oil with the two higher doses in the isolate Hö-Pe-1 and for all doses in the isolate 4-5-Y-20.

Table 4: Inhibitory effects (%) of different doses of oregano, pennyroyal and germander oils on blue mold formation on apple fruits 7 days after application

Isolates	Doses (µl)	Oregano	Pennyroyal	Germander	Fungicide
LT-10	1.0	31.97[x] c B[y]	12.92 c C	28.61 c B	100.00 A[z]
	2.5	41.49 b B	20.40 bc C	36.05 bc B	100.00 A
	5.0	83.67 a B	38.09 b C	45.57 b C	100.00 A
	10.0	83.67 a B	64.62 a C	59.18 a C	100.00 A
Hö-Pe-1	1.0	34.70 d B	22.22 d C	18.01 d C	100.00 A
	2.5	46.52 c B	36.80 c C	38.88 c C	100.00 A
	5.0	57.63 b B	56.24 b B	45.83 b C	100.00 A
	10.0	87.50 a B	87.50 a B	56.24 a C	100.00 A
4-5-Y-20	1.0	42.54 c B	34.32 d BC	28.15 c C	100.00 A
	2.5	48.84 bc B	45.59 c B	36.77 c C	100.00 A
	5.0	54.02 ab B	55.74 b B	53.44 b B	100.00 A
	10.0	66.66 a B	71.26 a B	64.90 a B	100.00 A

[x]Arcsin transformation was applied to the data before statistical analyses and real data were shown in the table.
[y]Means in the same column shown by the same lowercase letter and the means in the same row shown by the same uppercase letter were not significantly different from each other according to Tukey test (P≤ 0.05).
[z]Fungicide Thiabendazole was used (0.02%) to compare the efficiency of the oils.

Like in petri trials, there was no statistically significant difference among *P. expansum* isolates used in the experiments, regarding blue mold severity on apple fruits 7 days after application of the essential oils (Table 5).

Our findings on the highest effect of oregano oil against *P. expansum* mycelial growth was consistent with the results of many other studies. Essential oils obtained from various *Oregano* species showed the highest antifungal effects against different pathogens and carvacrol was mentioned as the cause of its fungicidal effect [32,33,34]. In a similar study, oregano oil was found as the most effective one among 5 plant essential oils against seven fungal pathogens causing storage rot on fruits. Like in the present study, the fungicidal dose of the oregano oil against *P. expansum* was found as 5 µl [35]. Both *in vitro* and *in vivo* experiments showed that squaw mint and germander oils showed similar effects on the pathogen especially with the lower doses, but the efficiency of pennyroyal oil was better than germander oil for higher doses. Antifungal effect of pennyroyal oil was attributed to its high pulegone content. In another study, limonene, menthone, menthol and pulegone were tested against some bacterial

and fungal pathogens and only pulegone showed antimicrobial activity especially against *Salmonella* species [36]. Similarly, it was found that germander oil somewhat inhibited the mycelial growth of *Alternaria solani*, but its efficiency was not satisfactory [27].

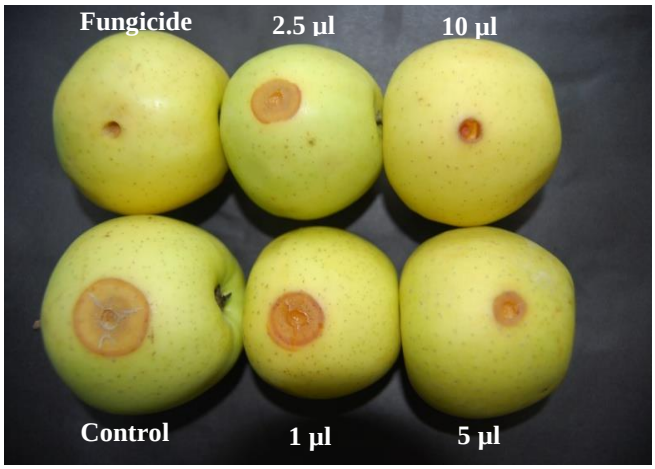

Figure 2: Inhibition of mold formation on apple fruits inoculated with *P. expansum* HÖ-Pe-1 isolate 7 days after incubation with different doses of oregano oil and the fungicide Thiabendazole (0.02%).

Table 5: Inhibition (%) of the blue mold formation by *P. expansum* isolates on apple fruits 7 days after incubation of oregano, pennyroyal and germander oils

Isolates	Oregano	Pennyroyal	Germander
LT-10	52.12[x] a[y]	33.94 b	43.01 a
Hö-Pe-1	54.93 a	51.24 a	44.96 a
4-5-Y-20	57.54 a	58.62 a	47.13 a

[x]Arcsin transformation was applied to the data before statistical analyses and real data were shown in the table.
[y]Means in the same column shown by the same letter were not significantly different from each other according to Tukey test (P≤ 0.05).

In the present study, none of the oils could totally inhibit blue mold formation. In a previous study on apples, similar results were obtained and none of the oils in the experiment could totally inhibit the lesion formation on the fruits [35]. However, with the highest dose, oils showed more than 50% inhibition and the efficiency reached over 85% against some isolates. Therefore, these oils may be used as alternatives to synthetic fungicides which has negative side effects like toxicity to non-target organisms, disruption of the equilibrium of ecosystems, carcinogenic residues in food, and development of resistant pathogen strains. Essential oils and such secondary plant metabolites are known to be biodegradable to nontoxic products and safer for the environment and human health [37]. So, these naturally growing plants having such metabolites should better be prevented from excessive usage and studies on their cultivation and use as natural pesticides should be encouraged.

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