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ANTIFUNGAL ACTIVITY OF LEMON, EUCALYPTUS, THYME, OREGANO, SAGE AND LAVENDER ESSENTIAL OILS AGAINST *ASPERGILLUS NIGER* AND *ASPERGILLUS TUBINGENSIS* ISOLATED FROM GRAPES

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ABSTRACT

Today, it is very important to find out the protection of products of natural origin as an alternative to synthetic fungicides. The promising alternative is the use of the essential oils (EOs). Essential oils from plants have great potential as a new source of fungicide to control the pathogenic fungi.The main objective of this study was evaluation of the antifungal activity of lemon (*Citrus lemon* L.), eucalyptus (*Eucalyptus globulus* LABILL.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) sage (*Salvia officinalis* L.) and lavender (*Lavandula angustifolia* MILLER.) EOs against *Aspergillus niger* and *Aspergillus tubingensis* isolated from grapes and their ability to affect the growth. It was tested by using the vapor contact with them. At first both tested isolates were identified by using PCR method. Sequence data of 18S rRNA supported the assignment of these isolates to the genus *Aspergillus* and species *A. niger* (ITS region: KT824061; RPB2: KT824060) and *A. tubingensis* (ITS region: KT824062; RPB2: KT824059). Second, EO antifungal activity was evaluated. The effect of the EO volatile phase was confirmed to inhibit growth of *A. niger and A tubingensis*. EOs were diluted in DMSO (dimethyl sulfoxide) final volume of 100 µL. Only 50 µL this solution was distributed on a round sterile filter paper (1 x 1 cm) by micropipette, and the paper was placed in the center of the lid of Petri dishes. Dishes were kept in an inverted position. The essential oils with the most significant activity were determined by method of graded concentration of oils - minimum inhibitory doses (MIDs). The most effective tested EOs were oregano and thyme oils, which totally inhibited growth of tested isolates for all days of incubation at 0.625 $\mu L.cm^{-3}$ (in air) with MFDs 0.125 $\mu L.cm^{-3}$ (in air). Lavender EO was less active aginst tested strains (MIDs 0.313 $\mu L.cm^{-3}$). The results showed that the tested EOs had antifungal activity, except lemon and eucalyptus. Sage EO was the only one which decelerated the radial growth of colony of both tested strains after all days of cultivation in comparison with a control sets. Our study provides the support that essential oils can be used to control plant pathogens such as *A. niger* and *A. tubingensis*.

Keywords: *Aspergillus*; essential oils; antifungal activity; vapor

INTRODUCTION

Fruit deterioration is a key postharvest problem because fungal spoilage can cause great economic losses. Grape, as a perishable fruit, is susceptible to fungal infection, especially from *Aspergillus niger* which causes a disease called black mold, one of the major causes of rapid and extensive deterioration of table grapes during the harvest and the major obstacle for storage **(dos Santos et al., 2012; de Sousa et al., 2013)**. *Aspergillus niger*, the most important member of *Aspergillus* subgenus *Circumdati* section *Nigri*, is primarily a plant pathogenic fungi responsible for deterioration of stored food material, as well as *Aspergillus tubingensis*, which includes species that morphologically resemble *Aspergillus niger* **(Samson et al., 2000)**. In addition, the genus *Aspergillus* and its species are producers of several mycotoxins. *A. flavus* and *A. parasiticus* are the main aflatoxins-producing species, while production of ochratoxin A is mainly associated with *Aspergillus carbonarius* and *A. niger* or *Nigri* section species, which has also been reported to produce fumunosin, sterigmatocystin, cyclopiazonic acid and patulin **(Plascencia-Jatomea et al., 2014)**. Spoilage and poisoning of food by fungi are the major problem for food industry and consumers. Decay may increase post harvest losses up to 50% without fungicide treatment. However, the use of synthetic fungicides is becoming more restrictive and thus alternative treatments need to be developed to reduce environmental risk and satisfy the demands of consumer groups **(Phillips et al., 2012)**. This negative consumer perception of chemical preservatives drives attention towards natural alternatives **(Sharma and Tripathi, 2008)**. Due to an increasing risk of chemical contamination upon the application of synthetic fungicides to preserve fresh fruits and vegetables, essential oils are gaining increasing attention **(Farzaneh et al., 2015)**.

Essential oils are aromatic and volatile liquids extracted from plants. The chemicals in essential oils are secondary metabolites, which play an important role in plant defense as they often possess antimicrobial properties **(Hyldgaard et al., 2012)**. Some of EOs have been reported to be active *in vitro* against *A. niger* such as lemongrass **(Tzortzakis and Economakis, 2007)** and *Matricaria chamomilla* flower **(Tolouee et al., 2010)**. A number of EO components have been registered by the European Commission for use as flavourings in food stuffs **(Commission Decision of 23 January, 2002)**. Some EO formulations are currently used

as food preservatives and are kept in the category "GRAS" in view of their favourable safety profile. Being volatile in nature, such EOs may be used as plant-based fumigants for the stored food commodities. Hence, EOs may play a significant role in overcoming storage losses and in enhancing food shelf life **(Prakash et al., 2015)**.

The objective of this study was evaluation of the antifungal activity of 6 EOs by using vapor contact against the fungal species of the genus *Aspergillus* section *Nigri* isolated from grapes in Slovakia.

MATERIAL AND METHODOLOGY

Fungal isolation and identification

Two isolates of black aspergilly, *Aspergillus niger KMi-116-LR* and *Aspergillus tubingensis KMi-144-LR* isolated from grapes, were used. These isolates belong to the collection of microorganisms at the Department of Microbiology of the Slovak Agricultural University in Nitra. They were inoculated on Czapek Yeast Autolysate Agar (CYA) **(Samson et al., 2002)** dishes.

Culturing conditions and DNA extraction

Single spore fungal isolates grown on SDA (Pancreatic Digest of Casein 5 $g.L^{-1}$, Peptic Digest of Meat 5 $g.L^{-1}$, Glucose 40 $g.L^{-1}$, Agar 15 $g.L^{-1}$, BioLife, Italy, Srl) plates (2 weeks, 26 °C, 16/8 light regime) were used for DNA extraction. DNA was extracted using a ZR fungal/bacterial DNA extraction kit (Zymo Research Corp. USA, CA). Identification of isolates was based on 18S rDNA-ITS1-5.8S rDNA-ITS2-28S rDNA region (ITS). We used also partial sequences of second largest subunit of DNA dependent RNA polymerase II (RBP2) because ITS region has low discrimination power among species in *Aspergillus* sect. *nigri*. Amplification reactions were carried out in 25 μL volumes containing: 200 mM dNTPs, 1x dreamTaq buffer, 0.5 unit DreamTaq DNA polymerase (Life technologies, USA), 0.5 mM of corresponding primer, and 0.5 μL DNA. Conditions of PCR reactions were following: initial denaturation at 95 °C for 3 min, 35 cycles were performed consisting of denaturation at 95 °C for 30 s, annealing at corresponding temperature for each primer set for 45 s, and extension at 72 °C for 90 s, final step was 10 min incubation at 72 °C. PCR reactions were carried out in a Biorad MJ mini thermal cycler (BioRad Corp., USA, CA). Primers used for PCR and sequencing of ITS region were ITS1 and ITS4 **(White et al., 1990)**. Primer pair for PCR amplifdication was partial RPB2 where RPB2-5F and RPB2-7cR and sequencing primer was RPB-6F **(Liu et al.,**

Table 1 The major constituents of essential oils analyzed by Calendula company a.s.

Essential oils	Compound	Amount (%)
Lemon	β-pinene	7.0 – 17
	sabinene	1.0 – 3.0
	limonene	56 – 78
	γ-terpinene	6.0 – 12
	β-caryophyllene	max. 0.5
	neral	0.3 – 1.5
	α-terpineol	max. 0.6
	neryl acetate	0.2 – 0.9
	geranial	0.5 – 2.3
	geranyl acetate	0.1 – 0.8
Oregano	carvacrol	min. 50
Lavender	limonene	<1.0
	cineole	<2.5
	3-octanone	0.1 – 2.5
	camphor	<1.2
	linalool	20 – 45
	linalyl acetate	25 – 46
	terpinen-4-ol	0.1 – 6.0
	lavandulyl acetate	>0.2
	lavandulol	>0.1
	α-terpineol	<2.0
Thyme	ρ-cimene	40 ±3
	thymol	32 ±2
Eucalyptus	α-pinene	9.0
	β-pinene	max. 1.5
	sabinene	max 0.3
	α-phellandrene	max. 1.5
	limonene	12
	1,8-cineole	min. 70
	camphor	max. 0.1
Sage	1,8-cineole	min. 5.0
	thujone	min. 15.0
	borneole	min. 5.0

Note: max. (maximum), min. (minimum).

1999). PCR products were cleaned-up by ExoI/FastAP (Life technologies, USA) and sent to Macrogen (Korea) for Sanger sequencing. Acquired sequences were assembled and processed using the Seaview software **(Gouy et al., 2010)**. Isolates were identified by comparison with records in genbank database using genbank BlastN tool. (http://blast.ncbi.nlm.nih.gov/). Sequences of both used isolates was deposited in genbank database under folowing accesion numbers: *A. niger* isolate KMi-116-LR, ITS: KT824061; RPB2: KT824060. *A. tubigensis* isolate KMi-144-LR, ITS: KT824062; RPB2: KT824059.

Essential plant oils

The essential oils used in this study were extracts of lemon (*Citrus lemon* L.), eucalyptus (*Eucalyptus globulus* LABILL.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) sage (*Salvia officinalis* L.) and lavender (*Lavandula angustifolia* MILLER.), they all were supplied by Calendula company a.s. (Nová Ľubovňa, 238 A, Slovakia). The gas chromatography analysis of the main components of each essential oils were determined by Calendula company a.s. (Table 1). Essential oils were extracted by hydro distillation and its quality and stability were certified by suppliers.

Antifungal activity of essential oils

The antifungal activity of selected EOs was investigated by microatmosphere method. The test was performed in sterile Petri dishes (Ø 90 mm) containing 15 mL of CYA. Evaluation by filter paper was made by the method adapted from **Guynot et al., (2003)**. First, all EOs were tested in highest concentration (0,625 µL.cm^{-3} of air). EOs were diluted in DMSO (dimethyl sulfoxide) final volume of 100 µL. Only 50 µL of this solution was distributed on a round sterile filter paper (1 x 1 cm) by micropipette, and the paper was placed in the center of the lid of Petri dishes. Dishes were kept in inverted position. Filter paper discs impregnated with dimethyl sulfoxide (DMSO) were only used as a control to confirm no solvent effect of bioactivity. Each fungus was inoculated in the center of Petri dishes with needle – inoculated. Dishes were tightly sealed with parafilm and incubated for fourteen days at 25 ±1 °C (three replicates were used for each treatment). Diameters (Ø mm) of the growing colonies were measured at the 3rd, 7th, 11th and 14th day with a ruler. Essential oils able to inhibit each fungus (visible inhibition- non growth of fungus) were used in the following test.

Minimum inhibitory doses (MIDs)

After incubation, the minimum inhibitory doses (MIDs) of EOs with the most significant activity were recorded by the method adapted from **Kloucek et al., (2012)**. The essential oils with the most significant activity were determined by method of graded concentration of oils. EOs dissolved in DMSO were prepared at different concentrations (0.500, 0.313, 0.188, 0.125, 0.063 µL.cm^{-3} of air). Cultivation was carried out at the 25 ±1 °C and measured after 14 days. The MID (expressed as microlitres of EOs per volume unit of atmosphere above the organism growing on the agar surface) was defined as the lowest concentration of the oil which did not permit any visible growth after 14 days in comparison with control sets.

Statistical analysis

All analyses were performed in triplicate and the results were expressed as the mean of the data obtained in each replicate. Statistical analyses were performed with descriptive statistics (mean and standard deviation) and inferential tests (ANOVA followed by 95.0% Tukey HSD test) to determine statistically significant differences ($p < 0.05$) between treatments.

RESULTS AND DISCUSSION

Contamination of grapes and grape products by *Aspergillus* section *Nigri* is known to occur widely. The fungal species *Aspergillus niger*, *Aspergillus tubingensis*, and *Aspergillus carbonarius* are included within this section and during their growth are able to produce mycotoxins **(Somma et al., 2012)**.

The objective of this study was to find the activity of the volatile phase of lemon, oregano, lavender, eucalyptus, thyme and sage essential oils against the fungal growth of *Aspergillus niger* and *Aspergillus tubingensis*. First, all EOs were tested at the higherst concentration (0,625 µL.cm^{-3}). Both tested strains, *Aspergillus niger* (Figure 1) and *A. tubingensis* (Figure 2) were sensitive in treatment with oregano, lavender and thyme EOs, which completely inhibited their growth after all days of cultivation (14 days). Strain *A. niger* was not sensitive in treatment with lemon EO, as same as *A. tubingensis*. Eucalyptus EO had very similar antifungal activity against both tested strains. *A. niger* showed the most significant sensibility to the sage EO at the highest concentration (0,625 µL.cm^{-3}) after 7 days of cultivation. *A. tubingensis* seems to be more resistant in treatment with sage EO. It was inhibited by sage EO only after 3 days of cultivation in a comparison with control sets and *A. niger* strain.

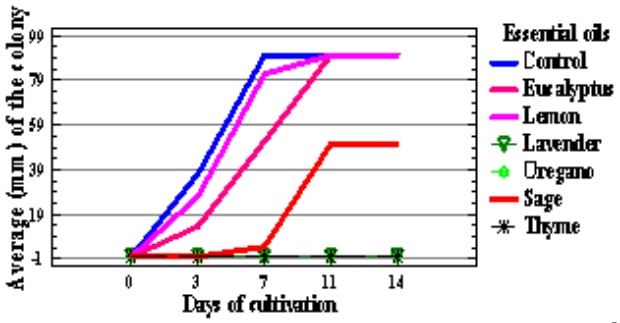

Figure 1 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *Aspergillus niger* KMi-116-LR.

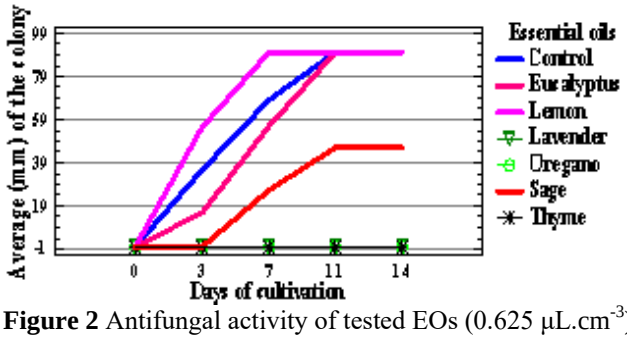

Figure 2 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *Aspergillus tubingensis* KMi-144-LR.

Table 2 Effect of different concentrations of lavender, oregano and thyme essential oils on radial growth inhibition (after 14 days) of *A. niger* and *A. tubingensis*.

Conc. µL.cm^{-3}	***Aspergillus niger*** KMi-116-LR			***Aspergillus tubingensis*** KMi-144-LR		
Essential oils	**Lavender**	**Oregano**	**Thyme**	**Lavender**	**Oregano**	**Thyme**
0.500	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0
0.313	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0	0^{a} ±0
0.188	24.50^{b} ±2.29	0^{a} ±0	0^{a} ±0	7.67^{b} ±2.08	0^{a} ±0	0^{a} ±0
0.125	34.67^{c} ±8.39	0^{a} ±0	0^{a} ±0	22.67^{c} ±6.43	0^{a} ±0	0^{a} ±0
0.063	66.67^{e} ±20.82	7.33^{a} ±0.58	45.67^{d} ±16.01	36.00^{d} ±8.54	6.50^{ab} ±1.80	44.67^{e} ±0.76
Control	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0	90^{f} ±0

* Data in the column followed by different letters are significantly different in 95% Tukey HSD test.

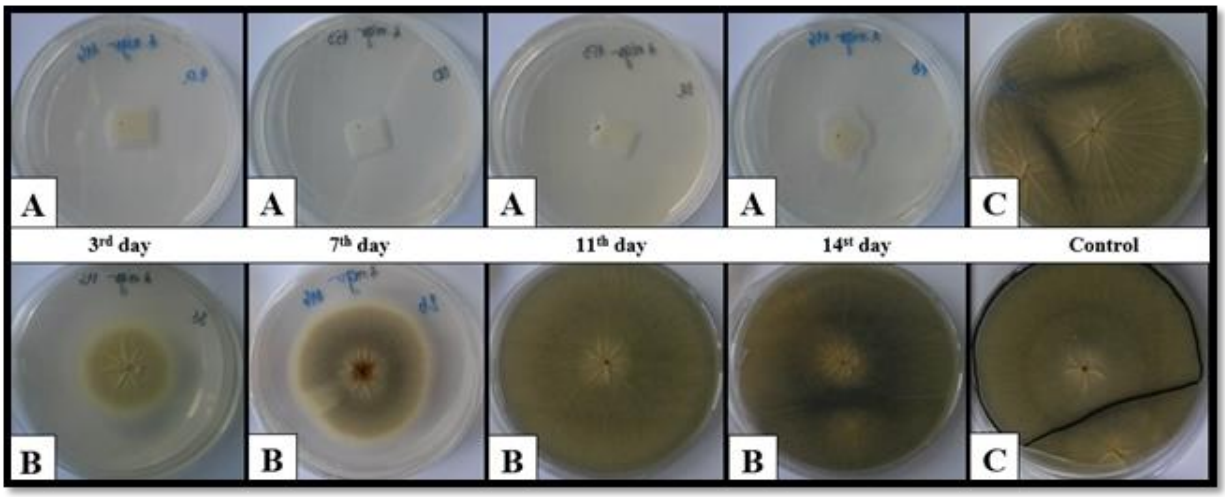

Figure 3 Antifungal activity of oregano (A) and lemon essential oils (B) against *Aspergillus niger*; (C) control.

Pinto et al., (2007) in their study also demonstrated similar results of antifungal activity of sage EO against fungi, but different results were found by **Suhr and Nielsen (2003)** where sage EO showed very poor inhibitor effects. Our results showed that all tested EOs have antifungal activity, except lemon and eucalyptus EOs, and demonstrated significant differences between each other (p <0.001). **Velázquez-Nuñez et al., (2013)** studied antifungal activity of citrus essential oils. They reported the minimum inhibitory concentration for the growth of *A. flavus* by direct addition 16.000 mg.L^{-1}, while for the vapor contact 8000 mg of EO mg.L^{-1} in air. For the both studied methods, growth of *A. flavus* decreased with increasing EO concentration. Further, studies have also documented that eucalyptus and lemon essential oils are effective even against fungal strains in vapor contact, e.g.: *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum and P. verrucosum* **(Viuda-Martos et al., 2008)**, *A. clavatus*, *A. niger*, etc. **(Su et al., 2006)**. Regarding to previous studies, this study demonstrated that lemon and eucalyptus EOs were not effective against tested strains in comparison with other tested EOs (sage, oregano, lavender and thyme). Also **Vilela et al., (2009)** reported that eucalyptus EOs and its major compound 1,8-cineole demonstrated very poor fungicidal activity against *A. flavus* and *A. parasiticus* in both contact and headspace volatile exposure assays.

In this study the most effective EOs were able to inhibit growth of tested strains all days of cultivation at the highest concentration (0.625 µL.cm^{-3}) and were used for determination of MIDs. Among all oils tested, thyme, oregano and lavender oils proved to be the best inhibitor of the black aspergilly. Results are showed in Table 2. The best results (MIDs 0.125 µL.cm^{-3}) (p <0.05) for both, *A. niger* and *A. tubingensis* showed oregano and thyme EOs.

In study of **Combrinck et al., (2011)** thyme EO proved to be the most effective inhibitor, totally inhibiting all of the pathogens tested at concentrations of 1000 µL.L^{-1} and lower, with the exception of a resistant *Penicillium* strain. Several researchers **(Stević et al., 2014; Kocić-Tanackov et al., 2012; Zabka et al., 2014)** found hight inhibitory effect of oregano EOs against fungi, too.

In our study, *A. niger* showed visible growth after 14 days only in treatment with lavender EO with a higher MIDs value 0.313 µL.cm^{-3}, as same as *A. tubingensis* (MIDs 0.313 µL.cm^{-3}) (p <0.05). **Soylu et al., (2010)** tested

rosemary and lavender EOs against *Botrytis cinerea*, and they also found that rosemary and lavender EOs were inhibitory at relatively higher concentrations (25.6 $\mu g.mL^{-1}$). Also **Daferera et al., (2003)** demonstrated that lavender, rosemary, sage, and pennyroyal essential oils have less inhibitory activity against tested fungal species. Although the concentrations of oils tested in this work were not the same. But antifungal activity of tested EOs depends on concentration of EOs, cultivation time and used method. In a previous study conducted by **Goñi et al., (2009)** behavior of clove EO was not the same in direct contact and vapor phase. **Bluma et al., (2009)** demonstrated that the vapor generated by 5000 $\mu L.L^{-1}$ of poleo oil significantly reduced growth of *Aspergillus* section *Flavi* in the order of 78.0%, whereas the dose of 3000 $\mu L.L^{-1}$ completely inhibited fungal development in the direct contact assay **(Bluma and Etcheverry, 2008)**. In study of **Velázquez-Nuñez et al., (2013)** direct addition of orange peel EO had a rapid effect on *A. flavus* growth, but exposure to orange peel EO vapors was more effective, requiring lower concentrations of EO to inhibit mold growth. They concluded that vapor contact is an alternative when essential oils (EO's) and microorganisms are placed separately in some sealed environment.

CONCLUSION

As a conclusion, volatile substances from oregano, thyme (MIDs 0.125 $\mu L.cm^{-3}$) and lavender (MID 0.313 $\mu L.cm^{-3}$) essential oils had a potential antifungal activity against tested strains of black aspergilly. Results showed that the tested EOs had antifungal activity, except lemon and eucalyptus EOs in comparison with control sets. In spite of the fact that sage EO showed only weak antifungal activity, and was able only to delayed growth of *A. niger* (after 7 days of cultivation) and *A. tubingensis* (after 3 days) could be used in food preservation, but further research is needed. Our study gives support that essential oils can be used to control plant pathogens such as *A. niger* and *A. tubingensis*.

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