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EFFECTS OF ESSENTIAL OILS ON IN VITRO GROWTH OF FUNGI *Cladobotryum dendroides* AND *Mycogone perniciosa* INFECTING BUTTON MUSHROOM

Abstract: The aim of the studies was to investigate the effect of camel grass, lavender, patchouli, peppermint and tea tree essential oils, and their mixtures on the *in vitro* growth of pathogenic fungi *Cladobotryum dendroides* and *Mycogone perniciosa*, occurring in the cultivation of button mushroom (*Agaricus bisporus*). The mycelial growth of the tested pathogens was evaluated on PDA medium. Essential oils were added in three doses: 0.25; 0.5 and 1 mg·cm^{-3} of PDA medium. Camel grass and peppermint essential oils applied at the highest dose inhibited completely the *in vitro* growth of *C. dendroides* mycelium. Lavender oil used at the amount of 1 mg·cm^{-3} reduced the growth of the pathogen by 90 %. In the case of *M. perniciosa* the complete inhibition of the pathogen's growth was observed after the addition of camel grass oil to PDA medium, irrespective of a dose, and lavender oil at the doses of 0.5 and 1 mg·cm^{-3}. The efficacy of the tested mixtures against *M. perniciosa* was high. Generally, all mixtures of essential oils, irrespective of a dose, completely controlled the growth of the pathogen. The complete inhibition of the growth of *C. dendroides* was observed only on the medium with the addition of the mixture of camel grass and peppermint oils at the highest dose. The conducted research showed that natural essential oils due to their antifungal properties could be useful in the Integrated Disease Management for the protection of button mushroom against diseases. They could be an effective alternative to synthetic chemical fungicides.

Keywords: *Agaricus bisporus*, antifungal activity, natural essential oils, *Cladobotryum dendroides*, *Mycogone perniciosa*

Introduction

A number of diseases cause problems in the white button mushroom (*Agaricus bisporus* (Lange) Imbach) production, because they significantly reduce both the quality and quantity of mushrooms. Due to the short growing period, the special cultivation technology and the lack of available synthetic substances, the scope of applying any chemical against pathogens is very limited. The most serious diseases of cultivated mushrooms are caused, among others, by *Cladobotryum dendroides* (Bull.) and *Mycogone*

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perniciosa (Magn.) [1, 2]. The latter one is the causal agent of wet bubble, whereas *Cladobotryum* spp. cause cobweb disease [3]. *Agaricus bisporus* fruit bodies infected with *M. perniciosa* become large and irregular, and tumorous fungal masses are formed. The exudation of the accumulated extracellular fluid is observed on the surface of diseased mushrooms [4-6]. The wet bubble disease occurs quite frequently and may induce from 10 to 46 % loss in yield, especially if infection occurs in the first flush [4]. Cobweb disease, caused by *Cladobotryum* spp., is characterized by the growth of a coarse mycelium covering affected mushrooms. As it ages, the white mycelium becomes pink [6-9]. Occurrence of cobweb in commercial crops results in reductions in yield and quality, mainly due to cap spotting, a lesser surface area that can be used for cultivation and to the need for early crop termination when the disease becomes epidemic [10].

Few fungicides are officially recommended for mushroom production: prochloraz in the EU countries, and chlorothalonil, thiabendazol and tiophanate-methyl in North America. Resistance against registered products can be observed in more and more cases. A decreased sensitivity of the pathogenic fungi infecting white button mushroom to prochloraz has already been reported [11]. The use of synthetic chemical fungicides contaminates the environment (land, air, and water), increases the potential human exposure to fungicide, and the deposition of residue on or in the plants (crops, fruits). Moreover, they are the main cause of fungicide resistance of plant pathogenic fungi [12, 13]. To improve the resistance evolution control of plant pathogenic fungi, FRAC (Fungicide Resistance Action Committee) has given recommendations to use fungicide mixtures to broaden the spectrum of disease control due to synergistic interactions leading to more potent fungal diseases control and higher flexibility. This recommendation is appropriate for the natural fungicide action. Plants produce a great variety of secondary metabolites related to the mechanisms of adaptation and important in mediating interactions between plants and the biotic environment. Among secondary metabolites, essential oils are very important natural products, which have different therapeutic and biological activities. Volatile oils are complex mixtures of odorous principles stored in special plant cells and glands in different parts of plants [14]. They contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic and aliphatic components [15]. Plant metabolites, including essential oils, contain many antimicrobial compounds which can reduce the development of pathogen resistance due to the variation of the metabolic processes [16, 17]. Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural and food industries [15]. Essential oils and their components have demonstrated strong fungistatic effects against pathogenic fungi and bacteria on the cultivated mushrooms [18].

The mechanism of interaction of essential oils with fungi has not been sufficiently explained. Voda et al. [19] found that the action of secondary metabolites is associated with the inhibition of sulfhydryl-containing active sites of fungal enzymes. Essential oils belonging to hydrocarbon monoterpens have the lowest antifungal activity, while oxygenated terpens or oils with phenolic structures have a considerable antifungal potential [20]. Essential oils from the group of hydrocarbons tend to be relatively inactive regardless of their structural type, and this inactivity is closely related to their limited hydrogen binding capacity and water solubility. Ketones, aldehydes and alcohols show different levels of activity, which is connected with the functional groups present, but also associated with hydrogen-binding parameters in all cases [21]. Thymol is anaromatic *p*-menthane type

monoterpene phenol and one of the plant's most active secondary metabolites. This compound is thought to interact with both outer and inner cytoplasmic cell membranes, by integrating at the polar head group region of the lipid bilayer. This alternates the cell membrane and leads to its increased permeability/disintegration [22]. Moreover, thymol can also take part in the up- or down regulation of genes involved in outer membrane protein synthesis, inhibition of enzymes involved in protection against thermal stress, synthesis of ATP, citric metabolic pathways, etc. [23]. Carvacrol, a compound isomeric to thymol, interacts with the cytoplasmic membrane by inserting between acyl chains of phospholipids [24]. The mentioned process leads to disturbance (increase) of the membrane fluidity and its higher permeability as a consequence. Increased permeability results with an efflux of ions and ATP, and with a disturbed membrane potential and pH gradient. By measuring the release of lipopolysaccharides (present in the outer membrane), it has been proved that it also affects the outer membrane, a structure responsible for higher resistance of Gram-negative bacteria [25]. Moreover, although it has been found that carvacrol affects both the outer and inner membrane, further studies have showed that its main site of action is the cytoplasmic membrane, where the effect of this compound is significantly enhanced by its hydroxyl group which functions as a transmembrane carrier of monovalent cations, leading to the disturbance of the membrane potential [26]. Research carried out by Veldhuizen et al. [27] showed that this group is not essential for the antimicrobial activity of carvacrol, but significantly enhances its action. Eugenol, a phenylpropanoid found in many plant species, shows a lytic effect on cells and its mode of action is a non-specific membrane permeabilization [28], demonstrated in several studies by the efflux of potassium and ATP [29]. It is thought that eugenol binds to membrane proteins, inhibiting and changing their functions. Evident from the change in the fatty acid composition of the eugenol-treated cells, the membrane fluidity seems to be also affected. It is a well known adaptive mechanism of microorganisms, developed in order to maintain optimal membrane features [30]. Permeabilization of the membrane and subsequent processes are induced by a number of other plant metabolites frequently present in antimicrobial plant extracts: linalool [31], linalyl acetate, menthol, and citral [32]. On the other hand, limonene changes cell morphology and the membrane fluidity [33]. Numerous studies have shown that many essential oils, extracted from different plant species, induce changes in the cell membrane permeability/integrity. Examples of such species from a number of plant families include: *Origanum vulgare* L. [34], *Rosmarinus officinalis* L. [35], *Thymus vulgaris* L. [36], *Coriandrum sativum* L. [37, 38] and *Mentha longifolia* (L.) Huds. [39]. *Melaleuca alternifolia* (Maiden & Betche) Cheel essential oil has an exceptional antimicrobial activity, owing to its mainly monoterpene composition, with terpinene-4-ol as the major constituent [40]. This oil possesses a remarkable activity against fungal cells. In studies carried out by Hammer et al. [41] its mode of action was investigated against yeasts *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. The mentioned studies showed that tea tree oil changed the fluidity and, thus, permeability of the fungal membrane [41] and damaged mitochondria, resulting in matrix loss and increased mitochondrial irregularity. The next consequence of mitochondrial damage was the disruption of the tricarboxylic acid cycle and the accumulation of reactive oxygen species [42, 43].

According to Inouye et al. [44] antifungal effects of the vapourising essential oils seemed to be correlated with their respiration-inhibitory activity in fungi cells.

The main target of the present work was to find natural products (essential oils) which could be tested under the production conditions against the major fungal diseases of *Agaricus bisporus* (Lange) Imbach, with an effect of prevention and/or as a curative method.

Material and methods

Material

Isolates D25B *Cladobotryum dendroides* (Bull.) and M15 *Mycogone perniciosa* (Magn.), obtained from the gene bank of cultivated and medicinal mushrooms of the Department of Vegetable Crops, Poznan University of Life Sciences, were used in the studies on the effect of natural essential oils on *in vitro* growth of pathogens.

In the first part of the experiment the efficacy of camel grass (*Cymbopogon schoenanthus* Ethkher), lavender (*Lavandula officinalis* L.), patchouli (*Pogostemon patchouli* Pellet., syn. *Pogostemon cablin* Benth.), peppermint (*Mentha x piperita* L.), and tea tree (*Melaleuca alternifolia* (Maiden & Betche) Cheel) essential oils was tested. They were produced by „Pollena Aroma" company from Warszawa. The chemical composition of the applied essential oils, according to ISO standards, is given in Table 1.

Methods

The selection of essential oils, used in the further research (evaluation of the efficacy of oils' mixtures), was based on the results of statistical analysis. Only essential oils, which significantly inhibited *in vitro* growth of the mycelium of the tested pathogens, were chosen for preparing mixtures. They were mixed in the ratio 1:1 (v/v). The aim of the application of oils' mixtures was to increase their efficacy against the examined pathogens. Moreover, the use of several essential oils together, and hence combinations of numerous active ingredients included in them, can control the mechanism of fungicide resistance in plant pathogenic fungi. It is very important in plant protection.

The growth of *C. dendroides* and *M. perniciosa* was evaluated on PDA medium (potato dextrose agar). The medium was poured into conical flasks of volume 0.2 dm^3, which subsequently were plugged with cotton wool bungs and aluminium foil and sterilized in an autoclave at 121 °C for 30 min. After cooling down the liquid medium to 40 °C, essential oils, dissolved in 0.2 % Tween 20, were added in three doses i.e. 0.25; 0.5 and 1 $mg \cdot cm^{-3}$ of PDA medium. The medium supplemented with essential oils was poured into 85 mm diameter sterile Petri dishes. Afterwards, mycelia plugs (7 mm in diameter) excised from the periphery of 6-day-old PDA cultures of the examined fungi were placed on the solid medium in the centre of Petri dishes. Incubation was carried out in a thermostatic chamber at 25 °C and 85-90 % relative humidity. Measurements of the growth of fungal colonies were taken every four days in two perpendicular directions and subsequently mean diameters of the colonies in respective treatments were calculated. Observations were conducted until the entire Petri dishes in control were overgrown by the mycelium of the examined pathogens. Fungistatic efficacy of each essential oil and their mixtures was assessed on the basis of observations of the fungal growth in relation to the control (the medium supplemented only with 0.2 % Tween 20, without the addition of oils). For the last observation, the percentage reduction in the diameter of mycelial colony was calculated for individual treatments in relation to the control treatment. Each treatment was performed in six replicates. The data were statistically analysed. Variance analysis was performed and

the significance of differences between means was determined on the basis Newman-Keuls test at a significance level $\alpha = 0.05$.

Results

The chemical composition of the tested essential oils is shown in Table 1. Camel grass oil included mainly citronellal (30.0-40.0 %), geraniol (20.0-25.0 %) and citronellol (8.5-14.0 %). The most abundant compound in lavender oil was linalyl acetate (25.0-47.0 %), followed by linalool (20.0-43.0 %). The major components of patchouli oil were patchouol (27.0-35.0 %), bulnezen (13.0-21.0 %) and α-guaiene (11.0-16.0 %), whereas in peppermint oil dominated menthol (36.0-46.0 %) and menthone (15.0-25.0 %). The main components of tea tree oil was 4-terpinenol (30.0-48.0 %), followed by γ-terpinene (10.0-28.0 %) and α-terpinene (5.0-13.0 %).

Table 1

The chemical composition of natural essential oils according to ISO standards [%]

Component	**Essential oils**				
	Camel grass (ISO 3848:2001)	**Lavender (ISO 3515:2002)**	**Patchouli (ISO 3757:2002)**	**Peppermint (ISO 856:2006)**	**Tea tree (ISO 4730:2004)**
1	2	3	4	5	6
Aromadendrene					trace* - 3.0
Bulnezen			13.0-21.0		
1,8-Cineol		0-3.0		4.0-6.0	trace -15.0
δ-Cadinene	1.5-2.5				trace -3.0
Camphor		0-1.5			
β-Caryophyllene			2.0-5.0	1.0-2.5	
Citronellal	31.0-40.0				
Citronellol	8.5-14.0				
Citronellyl acetate	2.0-4.0				
Copaene			trace -1.0		
ρ-Cymene					0.5-8.0
Isopulegol	0.5-0.7				
β-Elemene	0.5-2.5				
Elemol	1.3-4.0				
Eugenol	0.5-1.0				
β-Phellandrene		0-1.0			
Geranial	0.3-1.0				
Geraniol	20.0-25.0				
Geranyl acetate	2.5-5.5				
Germacrene-D	1.5-3.0				
α-Guaiene			11.0-16.0		
Globulol					trace -1.0
Isomenthone				2.0-4.5	
Lavandulol		0-3.0			
Lavandulyl acetate		0-8.0			
Limonene	2.0-5.0	0-1.0		1.0-2.5	0.5-1.5
Linalool	0.5-1.5	20.0-43.0			
Linalyl acetate		25.0-47.0			
Menthol				36.0-46.0	
Menthone				15.0-25.0	
Menthofuran				1.5-6.0	
Menthyl acetate				3.0-6.5	

1	2	3	4	5	6
Neomenthol				2.5-4.5	
Norpatchoulenol			0.35-1.0		
cis-β-Ocimene		1.0-10.0			
trans-β-Ocimene		0.5-6.0			
3-Octanol				0.1-0.4	
3-Octanon		0-3.0			
β-patchoulene			1.8-3.5		
Patchoulol			27.0-35.0		
α-Pinene					1.0-6.0
Pogostol			1.0-2.5		
Pulegone				0.5-2.5	
Sabinene					trace -3.5
trans-Sabinene hydrate				0.5-2.3	
4-Terpinenol		0-8.0			30.0-48.0
Terpinolene					1.5-5.0
α-Terpinene					5.0-13.0
γ-Terpinene					10.0-28.0
α-Terpineol		0-2.0			0.5-8.0
Viridiflorene					trace -3.0
Virdiflorol					trace -1.0

*trace - less than 0.1 %

Table 2

Effects of essential oils on the *in vitro* growth of *Cladobotryum dendroides* (Bull.)

Essential oils and their dose ([mg·cm^{-3}] of PDA medium)	**Duration of incubation [days] Diameter of mycelial colony [mm]**								**Mycelial growth inhibition after 16 days of incubation [%]**
	4		**8**		**12**		**16**		
Camel grass (1)	0	a	0	a	0	a	0	a	100
Camel grass (0.5)	0	a	0	a	18.0	b	32.0	b	62.4
Camel grass (0.25)	0	a	0	a	43.0	cd	63.0	de	25.9
Lavender (1)	0	a	0	a	5.2	ab	8.5	a	90.0
Lavender (0.5)	0	a	3.5	a	44.8	cd	59.5	cd	30.0
Lavender (0.25)	0	a	12.0	b	76.0	e	85.0	e	0
Patchouli (1)	0	a	12.8	b	31.8	c	40.0	a	52.9
Patchouli (0.5)	0	a	14.3	b	48.5	cd	56.8	b	33.2
Patchouli (0.25)	0	a	16.3	bc	51.7	cd	63.0	de	25.9
Peppermint (1)	0	a	0	a	0	a	0	a	100
Peppermint (0.5)	0	a	10.8	b	43.5	cd	56.3	cd	33.10
Peppermint (0.25)	0	a	12.2	b	46.2	cd	54.7	cd	35.7
Tea tree (1)	0	a	18.0	bc	55.0	d	64.5	de	24.1
Tea tree (0.5)	0	a	22.2	c	59.5	d	71.5	de	15.9
Tea tree (0.25)	0	a	37.3	d	75.8	e	82.5	e	2.9
Control	40.3	b	60.5	e	78.3	e	85.0	e	-

Means in columns marked with the same letter are not significantly different at the level $\alpha = 0.05$, according to the Newman-Keuls test

It has been found that natural camel grass and peppermint essential oils, applied at the highest dose i.e. 1 mg·cm^{-3} PDA completely inhibited the *in vitro* growth of *Cladobotryum dendroides* mycelium (Fig. 1). Lavender oil used at the amount of 1 mg·cm^{-3} and camel grass oil at the dose of 0.5 mg·cm^{-3} controlled also the growth of the pathogen to a great

extent. In the last day of observations (16th day) they reduced the growth of *C. dendroides* compared to the control by 90 and 62.4 %, respectively. Significant differences in the growth of the pathogen after 16 days from inoculation were found in the case of the use of lavender oil at the dose of 0.5 mg·cm^{-3} (30 %), peppermint oil at the amount of 0.25 and 0.5 mg·cm^{-3} (35.7 and 33.1 %) and patchouli oil at the doses of 0.5 and 1 mg·cm^{-3} (33.2 and 52.9 %). In other treatments significant differences were not noted (Table 2).



Fig. 1. The effect of camel grass essential oil on the *in vitro* growth of *C. dendroides* after 16 days of incubation

A complete inhibition of the growth of *Mycogone perniciosa* was observed after the application of camel grass oil, irrespective of a dose, and lavender oil at the doses of 0.5 and 1 mg·cm^{-3}. Peppermint oil used at the highest dose i.e. 1 mg·cm^{-3} and lavender oil at the dose of 0.25 mg·cm^{-3} controlled also the growth of the pathogen to a large extent (Fig. 2). After 28 days of incubation they reduced the growth of *M. perniciosa* compared to the control by 90 and 78.8 %, respectively. Strong inhibition of the growth of the fungal mycelium was found after the application of patchouli oil at the doses of 0.5 and 1 mg·cm^{-3} (54.4 and 64.3 %). The decrease of the *in vitro* growth of the pathogen was observed also after the use of patchouli oil at the amount of 0.25 mg·cm^{-3} (28.4 %), peppermint oil at the doses of 0.25 mg·cm^{-3} (35.1 %) and 0.5 mg·cm^{-3} (32.7 %) and tea tree oil at the amount of 1 mg·cm^{-3} (40 %). In other treatments significant differences were not noted (Table 3).

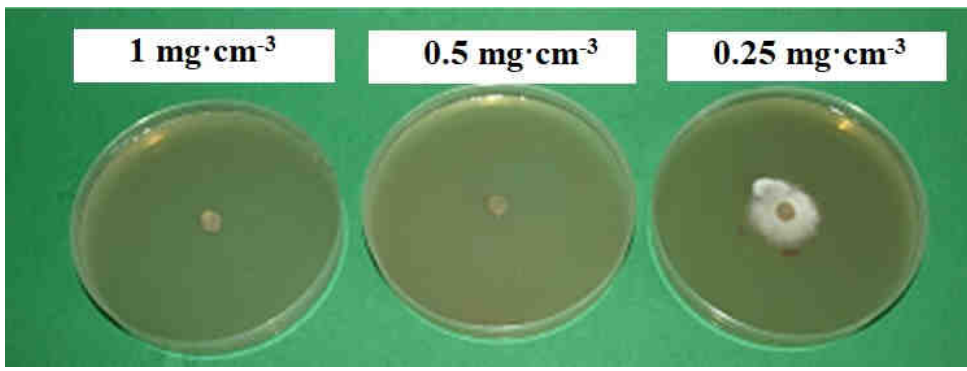


Fig. 2. The effect of lavender essential oil on the *in vitro* growth of *M. pernicosa* after 28 days of incubation

Table 3

Effects of essential oils on the *in vitro* growth of *Mycogone perniciosa* (Magn.)

Essential oils and their dose ([mg·cm^{-3}] of PDA medium)	Duration of incubation [days] Diameter of mycelial colony [mm]												Mycelial growth inhibition after 28 days of incubation [%]
	4		8		12		16		20		24		28		
Camel grass (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Camel grass (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Camel grass (0.25)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender (0.25)	0	a	0	a	0	a	0	a	0	a	0	a	18.0	ab	78.8
Patchouli (1)	0	a	0	a	8.2	bc	8.2	b	8.8	b	23.3	b	30.3	bc	64.3
Patchouli (0.5)	0	a	0	a	12.8	cd	12.8	b	14.3	b	20	b	38.7	cd	54.5
Patchouli (0.25)	0	a	0	a	13.5	cd	16.3	bc	18.2	b	40	c	60.8	ef	28.4
Peppermint (1)	0	a	0	a	0	a	0	a	0	a	0	a	8.5	a	90
Peppermint (0.5)	0	a	0	a	0	a	0	a	0	a	37.3	c	57.2	d-f	32.7
Peppermint (0.25)	0	a	0	a	0	a	0	a	0	a	35.5	c	55.2	d-f	35.1
Tea tree (1)	0	a	0	a	5.5	ab	8.2	b	10.2	b	36.0	c	51.0	de	40
Tea tree (0.5)	0	a	0	a	16.0	d	23.2	c	31.2	c	56.5	d	76.0	fg	10.6
Tea tree (0.25)	0	a	0	a	16.5	d	22.5	c	29.8	c	59.0	d	74.2	fg	12.7
Control	12.5	b	18.8	b	32.5	e	40.0	d	44.0	d	56.8	d	85.0	g	-

Means in columns marked with the same letter are not significantly different at the level $\alpha = 0.05$, according to the Newman-Keuls test

In the conducted studies, the effect of mixtures of essential oils on *in vitro* growth of the examined pathogens was determined, too. A complete inhibition of the growth of *C. dendroides* was observed on the medium with the addition of the mixture of camel grass and peppermint oils at the dose of 1 mg·cm^{-3}. Strong inhibiting activity showed the mixtures of peppermint and lavender oils, as well as peppermint and patchouli oils. In both cases after 16 days of incubation the mycelial growth inhibition was 62.4 % (Fig. 3). The mixture of lavender and patchouli oils reduced the growth of the pathogen by 50.6 %. All mentioned mixtures of natural essential oils were the most effective at the highest dose i.e. 1 mg·cm^{-3}. The decrease in the growth of the pathogen was observed also when the medium was supplemented with the mixtures of lavender and patchouli oils at the amounts of 0.25 and 0.5 mg·cm^{-3} (respectively 43.6 and 35.8 % of inhibition), peppermint and patchouli oils at the dose of 0.5 mg·cm^{-3} (35.7 %) as well as peppermint and lavender oils at the amount of 0.25 mg·cm^{-3} (33.2 %) (Table 4).

Table 4

Effects of mixtures of essential oils on the *in vitro* growth of *Cladobotryum dendroides* (Bull.)

Essential oils and their dose ([mg·cm^{-3}] of PDA medium)	**Duration of incubation [days] Diameter of mycelial colony [mm]**								**Mycelial growth inhibition after 16 days of incubation [%]**
	4		**8**		**12**		**16**		
Camel grass + peppermint (1)	0	a	0	a	0	a	0	a	100
Camel grass + peppermint (0.5)	0	a	0	a	0	a	63.0	de	25.9
Camel grass + peppermint (0.25)	0	a	19.2	b	49.0	c	71.5	de	15.9
Lavender + patchouli (1)	0	a	16.5	b	25.5	b	42.0	b	50.6
Lavender + patchouli (0.5)	10.2	b	21.5	b	36.7	bc	54.6	cd	35.8
Lavender + patchouli (0.25)	12.2	b	22.2	b	33.5	bc	48.0	b	43.6
Peppermint + lavender (1)	0	a	0	a	0	a	32.0	b	62.4
Peppermint + lavender (0.5)	0	a	13.0	b	25.2	b	72.5	de	14.7
Peppermint + lavender (0.25)	9.3	b	17.7	b	41.2	bc	56.8	cd	33.2
Peppermint + patchouli (1)	0	a	12.7	b	21.2	b	32.0	b	62.4
Peppermint + patchouli (0.5)	10.5	b	19.0	b	29.5	b	54.7	cd	35.7
Peppermint + patchouli (0.25)	12.7	b	23.2	b	34.5	bc	64.5	de	24.2
Control	25.8	c	55.8	c	70.0	d	85.0	e	-

Means in columns marked with the same letter are not significantly different at the level $\alpha = 0.05$, according to the Newman-Keuls test

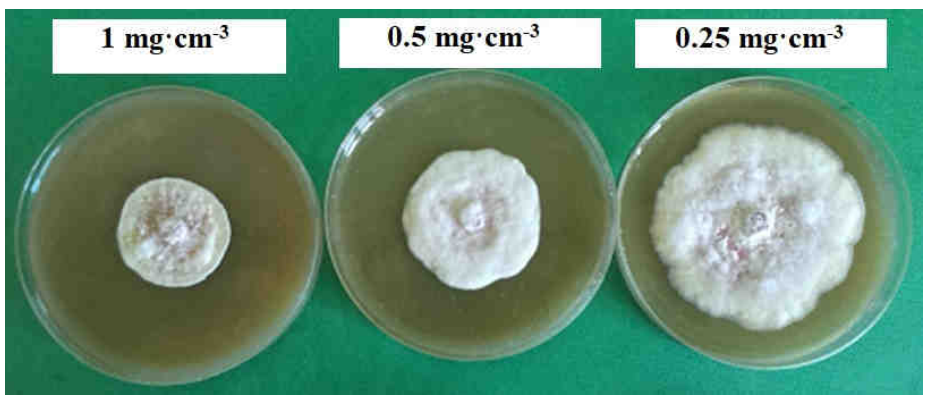


Fig. 3. The effect of the mixture of peppermint and patchouli essential oils on the *in vitro* growth of *C. dendroides* after 16 days of incubation

In the case of *M. perniciosa* the activity of the tested mixtures of natural essential oils was very strong. Generally, all mixtures of essential oils completely controlled the growth of the pathogen (100% inhibition). Only the mixture of peppermint and patchouli oils applied at the dose of 0.25 mg·cm^{-3} inhibited the mycelial growth to a lesser extent. After 28 days of incubation the mixture reduced the growth of *M. perniciosa* by 67.6 % compared to the control (Table 5, Fig. 4).

Table 5

Effects of mixture of essential oils on the *in vitro* growth of *Mycogone perniciosa* (Magn.)

Essential oils and their dose ([mg·cm^{-3}] of PDA medium)	Duration of incubation [days] Diameter of mycelial colony [mm]												Mycelial growth inhibition after 28 days of incubation [%]
	4		8		12		16		20		24		28		
Camel grass + peppermint (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Camel grass + peppermint (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Camel grass + peppermint (0.25)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender + patchouli (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender + patchouli (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Lavender + patchouli (0.25)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + lavender (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + lavender (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + lavender (0.25)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + patchouli (1)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + patchouli (0.5)	0	a	0	a	0	a	0	a	0	a	0	a	0	a	100
Peppermint + patchouli (0.25)	0	a	0	a	0	a	0	a	0	a	18.5	b	27.5	b	67.6
Control	12.0	b	20.5	b	31.7	b	47.7	b	57.0	b	70.0	c	85.0	c	-

Means in columns marked with the same letter are not significantly different at the level $\alpha = 0.05$, according to the Newman-Keuls test

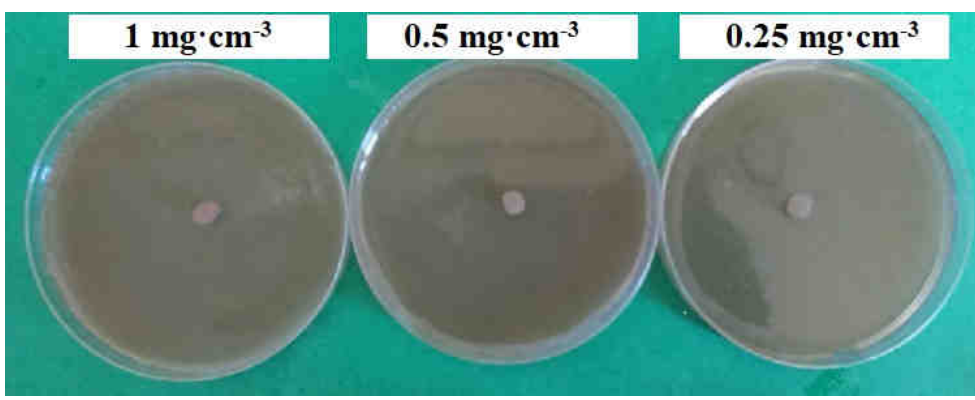


Fig. 4. The effect of the mixture of peppermint and lavender essential oils on the *in vitro* growth of *M. perniciosa* after 28 days of incubation

Discussion

It has been found that peppermint (*Mentha piperita*) oil (1 mg·cm^{-3}) completely inhibited the *in vitro* growth of *Clabobotryum dendroides*. The results of the *in vitro* tests carried out by Geosel et al. [45] showed that spearmint (*M. spicata*) oil had also a strong inhibiting effect on the growth of this pathogen and additionally controlled the growth of *Mycogone perniciosa*, *Lecanicillium fungicola* and *Trichoderma aggressivum* f. *aggressivum*. It has been proven that essential oils not only have a contact effect, but also by vaporizing, the active agents can block the vegetative growth of pathogens. In other studies, Dianez et al. [46] showed the antifungal activity of peppermint oil against *Cladobotryum mycophilum* and *Trichoderma aggressivum* f.sp. *europaeum*. Sokovic et al. [21] investigated various *Mentha* species (Lamiaceae) and they found that menthol (37.4 %), menthyl acetate (17.4 %) and menthone (12.7 %) were the main components of *M. piperita* oil, whereas carvone (69.5 %) and menthone (21.9 %) were dominant in *M. spicata*. Their results suggested that carvone and menthol had greater antifungal activities than the other compounds. The essential oils of peppermint (*M. piperita*), and spearmint (*M. spicata*) have great antifungal potentials, inhibiting the growth of *T. viride* and other pathogens of the button mushroom such as *L. fungicola*, *T. harzianum* and *P. tolaasii* [21]. Todorovic et al. [47] detected a remarkable activity of peppermint oil against *T. aggressivum* f. *europaeum*, showing both an inhibitory and fungicidal effect.

In the conducted studies, 100 % inhibition of *C. dendroides* growth was observed on PDA medium with the addition of camel grass (*Cymbopogon schoenanthus*) oil at the dose of 1 mg·cm^{-3}. The strong fungicidal activity of citronella grass oil, extracted from the species *Cymbopogon winterianus* Jowitt ex Bor, was confirmed in *in vitro* tests by Andila et al. [48] against *Aspergillus niger*, *Cladosporium* spp. and *Fusarium solani* and by Cavalcanti et al. [49] in studies concerning *Candida albicans*. As demonstrated by Amornvit et al. [50], the growth of the *C. albicans* was completely inhibited after being exposed to lemongrass oil, obtained from the species *Cymbopogon citratus* (DC.) Stapf, used at the concentration of 2.5 µL·mL^{-1} [0.25 % (v/v)].

It has been found that lavender and patchouli oils, applied at adequate concentrations, significantly reduced the growth of *C. dendroides*. Tanovic et al. [51] also reported that lavender (*Lavandula officinalis* (L.)) oil exhibited a fungicidal effect at 0.32 µg·mL^{-1}, suppressing the growth of *Cladobotryum* spp. and additionally *L. fungicola*. Dianez et al. [46] showed a strong inhibiting effect of patchouli oil on *C. mycophilum.*

In vitro and *in vivo* bioassays conducted by Gea et al. [52] showed that essential oils obtained from *Thymus vulgaris* and *Satureja montana*, were the most effective for inhibiting the mycelial growth of *Cladobotryum mycophilum*, and were also the most selective to *A. bisporus*. According to the authors, these essential oils may be useful products to manage the mushroom cobweb disease if used as a part of an integrated pest management (IPM) program.

In the case of *Mycogone perniciosa* a complete inhibition of the growth was observed after the application of camel grass oil, irrespective of a dose, and lavender oil at the doses of 0.5 and 1 mg·cm^{-3}. In studies conducted by Regnier and Combrinck [18], lemongrass oil (*Cymbopogon citratus*) was found to substantially inhibit the *in vitro* growth of *M. perniciosa*, while the low toxicity it demonstrated towards *A. bisporus*. Similarly to our results, Tanovic et al. [51] showed, that lavender oil controlled the *in vitro* growth of the *M. perniciosa*. In our studies the significant inhibition of the growth of this pathogen was

observed also in treatments with the use of peppermint, patchouli and tea tree oils at adequate doses. The results of *in vitro* tests carried out by Geosel et al. [45] showed that spearmint oil (*M. spicata*) completely inhibited the mycelial growth of *M. perniciosa*, whereas Tanovic et al. [53] found that tea tree oil had the high antifungal activity against this pathogen.

The world literature reports a range of information concerning antibacterial properties of patchouli oil e.g. against *Bacillus cereus, Staphylococcus aureus* and the antifungal activity e.g. against *Aspergillus* spp., *Candida albicans*, *Rhizopus oligosporus*, *Saccharomyces cerevisiae* [54-56]. However, there is no information about effects of this oil on pathogens, which infest cultivated mushrooms.

A high degree of phytopharmacological synergism should be expected in mixtures of different essential oils obtained from various plant species. The results of our studies with the use of various mixtures of essential oils confirm this finding. In the case of *M. perniciosa* the activity of the examined mixtures was very strong. Generally, all mixtures of essential oils completely controlled the growth of the pathogen (100 % inhibition), irrespective of the applied dose. A similar effect of synergism was not observed in the case of *C. dendroides.* The use of the mixtures of selected essential oils did not increase their efficacy compared to the same oils applied individually. At the same time, the significant decrease in the efficacy was not found. A complete inhibition of the growth of this pathogen was observed only on the medium with the addition of the mixture of camel grass and peppermint oils at the highest dose. Nonetheless, it should be pointed out that the application of several essential oils together can have a great importance in plant protection against pathogens, even if the synergism of their action has been not observed. The use of the plant protection agents containing many active ingredients, often with different mechanisms of action, can prevent the formation of fungal strains resistant to fungicides or slow down this process.

There are studies showing that the pharmacokinetics of certain bioactive natural products can be improved by applying them as mixtures rather than as single compounds. Ultee et al. [57] proposed an explanation for the mode of synergistic activity of *p*-cymene and carvacrol. According to them, *p*-cymene has a high affinity towards the cytoplasmic membrane, and its bonding to the membrane causes its expansion altering its potential and resulting in its higher sensitivity to the action of carvacrol. It has been demonstrated that the biological effects observed for the major compounds of *Ocimum gratissimum* (L.) oil do not sum up to the overall effect of the essential oil, suggesting a possible synergy between the constituting plant secondary metabolites [58]. Study *in vivo* on pear fruits conducted by Nikkhah et al. [59] showed that double combination of thyme/cinnamon oils had more inhibitory effect on *Botrytis cinerea* and *Penicillium expansum* than single essential oil treatments. Hossain at al. [60] studied the antifungal activities of eight essential oils, namely basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme. A combined formulation of oregano and thyme oils resulted in a synergistic effect, showing enhanced efficiency against *Aspergillus flavus, Aspergillus parasiticus* and *Penicillium chrysogenum*. Mixtures of peppermint and tea tree oils produced the synergistic effect against *Aspergillus niger.* On the other hand, in the research conducted by Purkait et al. [61] it was found that the cinnamon/clove oil combination showed the synergistic antifungal activity against the above mentioned pathogen.

In other studies, it has been confirmed that the combination of oregano and thyme essential oils had the synergistic antimicrobial activity against *Leuconostoc citreum*,

a spoilage bacterium in vegetable and fruit juices. These findings will be useful for developing non-thermal food preservation technologies to increase the shelf-life of juices and other foods not containing synthetic preservatives [62]. Ayari et al. [63] indicated the synergistic effect of different mixtures of essential oils against *Bacillus cereus* and *Paenibacillus amylolyticus*. They found that the combination of oregano/thyme essential oils displayed the best antibacterial activity and could be used in food preservation to reduce the contamination by pathogens.

Based on the results of our studies, it can be concluded that examined essential oils and their mixtures due to their antifungal properties may be useful in the integrated protection of button mushroom *Agaricus bisporus* (Lange) Imbach against diseases, especially against those caused by common pathogens of edible mushrooms *Cladobotryum dendroides* and *Mycogone perniciosa*. However, it should be noted that promising results of the studies on the efficacy of natural essential oils, which we obtained under *in vitro* conditions may differ considerably from the observations done *in vivo* i.e. under production conditions where we are dealing with the influence of the environment and cultivated mushrooms. Hence, the studies should be continued.

Conclusion

1. Natural essential oils due to their antifungal properties may be useful in the Integrated Disease Management (IDM) for the protection of button mushroom *Agaricus bisporus* (Lange) Imbach against diseases. They could be an effective alternative to synthetic chemical fungicides, especially that only few of them can be used in the cultivation of edible mushrooms. The complete inhibition of the *in vitro* growth of *Cladobotryum dendroides* (Bull.) mycelium has been found after the application of camel grass and peppermint essential oils at the highest dose i.e. 1 mg · cm^{-3} PDA. In the case of *Mycogone perniciosa* (Magn.) the complete inhibition of the pathogen's growth was observed after the addition of camel grass oil to PDA medium, irrespective of a dose, and lavender oil at the doses of 0.5 and 1 mg·cm^{-3}.
2. The application of the mixtures of essential oils can increase their fungicidal activity. In the case of *M. perniciosa* the activity of tested mixtures was very strong. Generally, all mixtures of essential oils, irrespective of a dose, completely controlled the growth of the pathogen (100 % inhibition). There was no a clear effect of synergism when the mixtures of natural essential oils were used against *C. dendroides.*
3. The studies on the efficacy of natural essential oils and their mixtures should be continued under *in vivo* i.e. production conditions taking into account the influence of the environmental factors and the effects on cultivated mushrooms.

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