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FROM STUDIES ON POSSIBILITY OF PROTECTING BLUE SPRUCE (*Picea pungens* Engelm.) AGAINST FUNGI. PART II. LABORATORY ASSESSMENT OF ANTIFUNGAL ACTIVITY OF SELECTED ESSENTIAL OILS

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Abstract. The antifungal properties of selected essential oils, i.e. lavender, mint, orange, spruce and thyme oils, were checked *in vitro* to fungi isolated from blue spruce (*Picea pungens* Engelm.) and its cultivar 'Glauca'. The degree and extent of antifungal activity for essential oils listed above were tested on the following fungi: *Acremonium tubakii, Anthostomella conorum, Arthrinium* state of *Apiospora montagnei, Aureobasidium pullulans, Botryodiplodia rubi, Fusarium camptoceras, F. moniliforme* var. *lactis, Penicillium canescens, Phoma pomorum, Rhizosphaera kalkhoffii, Ulocladium consortiale* and *Zythiostroma pinastri*. Among essential oils tested the thyme oil showed the highest antifungal activity to all fungi under examination even at the lowest concentration used. The mint, lavender and spruce oils demonstrated a medium activity. The lowest activity was found for orange oil.

Key words: *Picea pungens*, fungi, control, essential oils

INTRODUCTION

The biological methods play more and more important role in plant protection against diseases. In these methods both living organisms and various herbal extracts are used to control harmful pathogens [Orlikowski i in. 2002]. The herbal extracts include often essential oils distinguishable for its high antibacterial and antifungal activity [Góra 1997]. This depends on their chemical composition, origin, application method and use form [Reddy i in. 1998]. Among a number of essential oils those extracted from Labiatae are of special interest. They show a very high activity in inhibiting growth of various fungal species [Klimach i in. 1996, Zambonelli i in. 1996, D'Aulerio i Zambonelli

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1997, Bartyńska 1998, 1999, Reddy i in. 1998, Bartyńska i Budzikur-Ramza 2001, Budzikur-Ramza 2003, Mirowska 2003, Snieškienė i in. 2003].

The aim of this paper is to find a protection of *Picea pungens* against fungi by assessing antifungal properties of the selected essential oils.

MATERIAL AND METHODS

The aim of *in vitro* tests was to evaluate the effectiveness and the extent of antifungal activity of selected essential oils to fungi isolated from diseased plants. The following commercial oils supplied by Avicenna-Oil were tested: lavender (*oleum lavandula officinalis*), mint (*oleum mentha piperite*), orange (*oleum citrus aurantium*), spruce (*oleum piceae*) and thyme (*oleum thymus vulgaris*).

The laboratory tests were carried out by using the method described by Kowalik and Krechniak [Kowalik i Krechniak 1961]. The essential oils were used at the following concentrations 2.5 $ml \cdot l^{-1}$; 5 $ml \cdot l^{-1}$ and 7.5 $ml \cdot l^{-1}$. When selecting oil concentrations the available data related to the degree and extent of antifungal activity to various fungal species were taken into account [Singh i in. 1993, Klimach i in. 1996, Budzikur-Ramza 2003, Mirowska 2003]. The most frequently isolated fungi were selected for testing, namely *Acremonium tubakii, Anthostomella conorum, Arthrinium* state of *Apiospora montagnei, Aureobasidium pullulans, Botryodiplodia rubi, Fusarium camptoceras, F. moniliforme* var. *lactis, Penicillium canescens, Phoma pomorum, Rhizosphaera kalkhoffii, Ulocladium consortiale* and *Zythiostroma pinastri* [Bartyńska i Mirski 2005].

During the test essential oils were introduced directly into liquid and slightly cooled PDA agar. After thorough mixing with the agar the obtained suspension was poured into Petrie dishes of 70 mm in diameter. Afterwards, an agar disk with mycelium of fungi under examination was placed centrally into each dish for each combination. The medium without amendments with a disk of appropriate fungus was used as the control for each combination. The test was carried out in three repetitions for each combination. The antifungal activity of essential oils under consideration was then calculated form Abbot's formula [Kowalik i Krechniak 1961]:

$$I = \frac{C - T}{C} \cdot 100$$

where: I – fungus linear growth inhibition index (percentage), C – fungus colony diameter in the control combination, T – fungus colony diameter in combination containing a specified essential oil concentration in the agar.

Finally, an effect of essential oils under examination on fungal biology (mycelium morphology, presence of spores, sporification intensity, presence and number of chlamydospores) was determined.

RESULTS AND DISCUSSION

The tested essential oils showed different effect on the mycelium growth of the fungi species under examination depending on oil type and its concentration in the medium (tab. 1).

Table 1. An effect of essential oils on inhibiting mycelium linear growth (%)
Tabela 1. Wpływ badanych olejków eterycznych na zahamowanie rozrostu liniowego (%) grzybni testowanych gatunków grzybów

Essential oil Olejek eteryczny	Concentration Stężenie $ml \cdot l^{-1}$	Fungus / I = Inhibition index – Grzyb / I = współczynnik zahamowania, %											
		Acremonium tubakii W. Gams	*Anthostomella conorum* (Fuckel) Sacc	*Arthrinium* state of *Apiospora montagnei* Sacc.	*Aureobasidium pullulans* (de Bary) Arnaud	*Botryodiplodia rubi* Syd.	*Fusarium camptoceras* Wollenw. et Reinking	*Fusarium moniliforme* Sheld var. *lactis* (Pir. et Rib.) Bilai	*Penicillium canescens* Sopp	*Phoma pomorum* Thüm	*Rhizosphaera kalkhoffii* Bubák	*Ulocladium consortiale* (Thüm.) Simmons	*Zythiostroma pinastri* (Karst.) Höhn.
Lavender Lawendowy	2.5	17.14 g*	100 a	44.29 h	100 a	52.63 d	44.06 f	95.71 b	19.22 f	75.36 b	85.98 c	45.96 c	28.81 e
	5	100 a	100 a	73.10 c	100 a	100 a	95.96 b	100 a	83.51 b	100 a	100 a	100 a	25.24 f
	7.5	100 a	100 a	100 a	100 a	100 a	32.63 g	93.10 c	100 a	100 a	100 a	100 a	100 a
Mint Miętowy	2.5	100 a	100 a	48.10 fg	100 a	58.10 c	100 a	100 a	20.34 e	63.10 c	100 a	100 a	87.39 b
	5	100 a	100 a	87.63 b	100 a	100 a	94.53 c	100 a	75.83 c	100 a	100 a	100 a	100 a
	7.5	66.67 c	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
Orange Pomarańczowy	2.5	16.91 h	56.43 e	53.57 e	18.61 d	25.71 h	5.96 j	1.43 i	9.89 i	20.71 g	-8.46 i	6.43 h	8.93 i
	5	17.63 g	61.67 d	47.63 g	15.12 e	43.81 f	16.43 i	37.86 g	12.10 h	40.96 e	-5.29 h	20.27 g	7.63 i
	7.5	22.86 f	87.86 b	69.77 d	13.01 f	48.34 e	23.10 h	38.34 f	1.64 k	37.14 f	-3.97 g	44.77 d	11.67 g
Spruce Świerkowy	2.5	31.67 e	86.20 c	48.57 f	26.32 c	48.34 e	0 k	46.43 e	8.80 j	0 h	41.54 d	41.67 f	10.96 h
	5	42.20 d	100 a	100 a	88.80 b	82.39 b	80.24 e	31.34 h	18.69 g	59.29 d	15.08 e	78.93 b	34.53 d
	7.5	81.20 b	100 a	100 a	13.01f	29.29 c	86.20 d	78.10 d	35.71 d	100 a	88.10 b	43.81 e	35.71 c
Thyme Tymiankowy	2.5	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
	5	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
	7.5	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
Control Kontrola		0 i	0 f	0 i	0 g	0 i	0 k	0 j	0 l	0 h	0 f	0 i	0 j

* Values marked with the same letter in columns have no significant differences at P = 0.05 (Duncan test)
* Wartości oznaczone takimi samymi literami w kolumnach nie różna się istotnie przy p = 0,05 testu Duncana

It was found that thyme oil is the most effective in inhibiting mycelium growth at each concentration used (photos 1, 2, 3).

For mint oil, that was of lower activity than that of thyme oil, it was recorded that its activity increased with concentration in the medium (photos 1, 2, 3). Only in combination with *Acremonium tubakii* in the medium of the highest concentration, the activity of this oil was reduced considerably compared to those of lower concentrations.

Lavender oil at the highest concentration was the most effective in inhibiting mycelium growth in most of fungal species under examination (photos 1, 2, 3).

The effectiveness of spruce oil compared to those of thyme, mint and lavender oils was considerably lower (photos 1, 2, 3). This oil was effective at the highest concentration tested in inhibiting the growth of some fungal species only, namely *Anthostomella conorum, Arthrinium* state of *Apiospora montagnei* and *Phoma pomorum*. For *Aureobasidium pullulans* and *Botryodiplodia rubi* its effectiveness was reduced even at the highest concentration in the agar.

Orange oil showed the lowest antifungal activity (photos 1, 3). In combination with *Rhizosphaera kalkhoffii* this oil even stimulated the mycelium growth regardless of concentration (table 1, photo 2).

The tests indicated very high antifungal activity of selected essential oils to all fungal species under examination. The highest antifungal activity (100%) and very wide range of application was found for thyme oil. Similar properties of this oil were found previously by a number of other authors [Klimach i in. 1996, Zambonelli i in. 1996, Bartyńska 1998, 1999, Bartyńska i Budzikur-Ramza 2001, Mirowska 2003, Motiejūnaitė i Kalėdienė 2003, Sajdak 2004].

Slightly lower activity was recorded for mint oil compared to earlier information on its both antimicrobiological and antibacterial and antifungal properties [8], and antifungal activity to the genus *Fusarium* [Paran i in. 1996, Sajdak 2004].

The relationship between the mycelium growth of selected fungi and lavender oil origin and concentration was demonstrated previously [Bartyńska 1998, 1999, Bartyńska i Budzikur-Ramaz 2001, Budzikur-Ramza 2003, Mirowska 2003, Motiejūnaitė i Kalėdienė 2003, Snieškienė i in. 2003, Sajdak 2004]. Similarly, the relationships between antifungal activity of lavender and mint oils and its concentration in the medium have been also found [D'Aulerio i Zambonelli 1997, Bartyńska 1998, 1999, Bartyńska i Budzikur-Ramaz 2001, Budzikur-Ramza 2003, Sajdak 2004,].

In turn, the low antifungal activity of orange oil found in this paper is inconsistent with the results reported by Snigh et al. [1993], where they reported both high activity and a wide range of application for this oil. The cause of such discrepancy can be assigned among other things to plant material and oil chemical composition [Reddy i in. 1998].

Within the scope of the effect of selected essential oils on biology of selected fungal species it was found that oils changed morphology of hyphae and reduced or stimulated the sporulation and chlamydospore production in some fungi (table 2) – compared to the control cultures, presented in the Mirski research work [2007].

In the medium with thyme oil no mycelium was observed in any combination due to 100% efficiency of antifungal activity and very wide spectrum of activity for this oil.

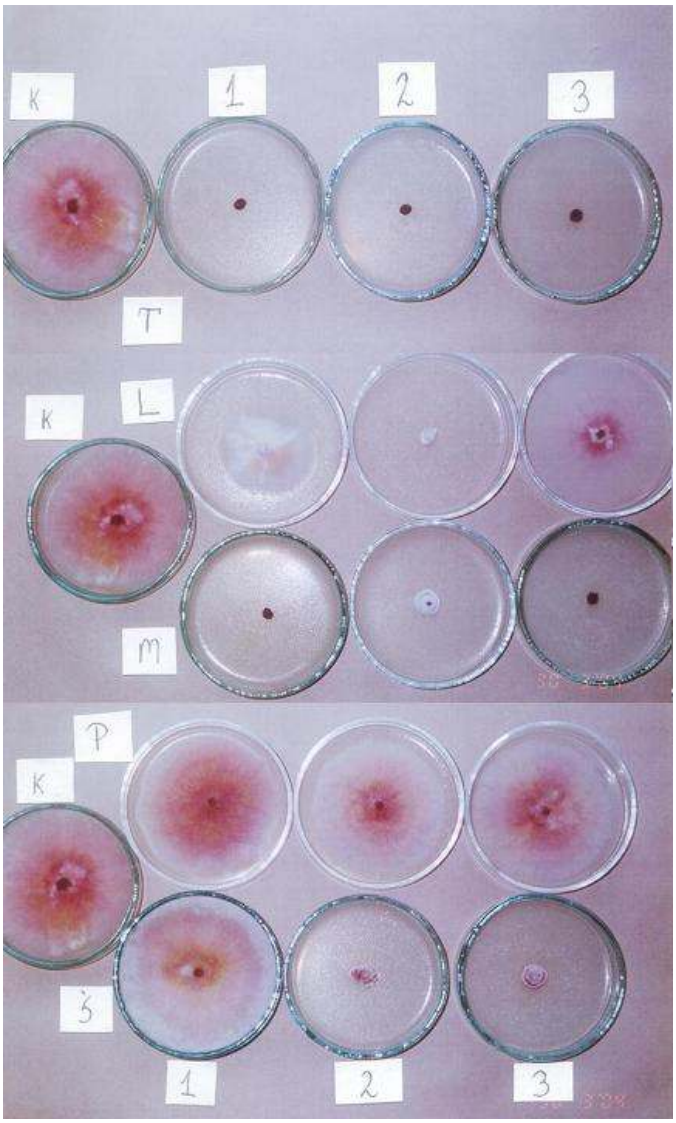

Photo 1. An effect of thyme, lavender, mint, orange and spruce oil on linear growth of *Fusarium camptoceras* (K – control; T – thyme oil, L, – lavender oil, M – mint oils, P – orange oil and Ś – spruce oils; 1 – concentration 2.5 ml/l; 2 – concentration 5 ml/l; 3 – concentration 7.5 ml/l).

Fot. 1. Wpływ olejku tymiankowego, lawendowego, miętowego, pomarańczowego i świerkowego na rozrost liniowy *Fusarium camptoceras* (K – kontrola; T – olejek tymiankowy, L – olejek lawendowy, M – olejek miętowy, P – olejek pomarańczowy, Ś – olejek świerkowy; 1 – stężenie 2,5 ml/l; 2 – stężenie 5 ml/l; 3 – stężenie 7,5 ml/l)

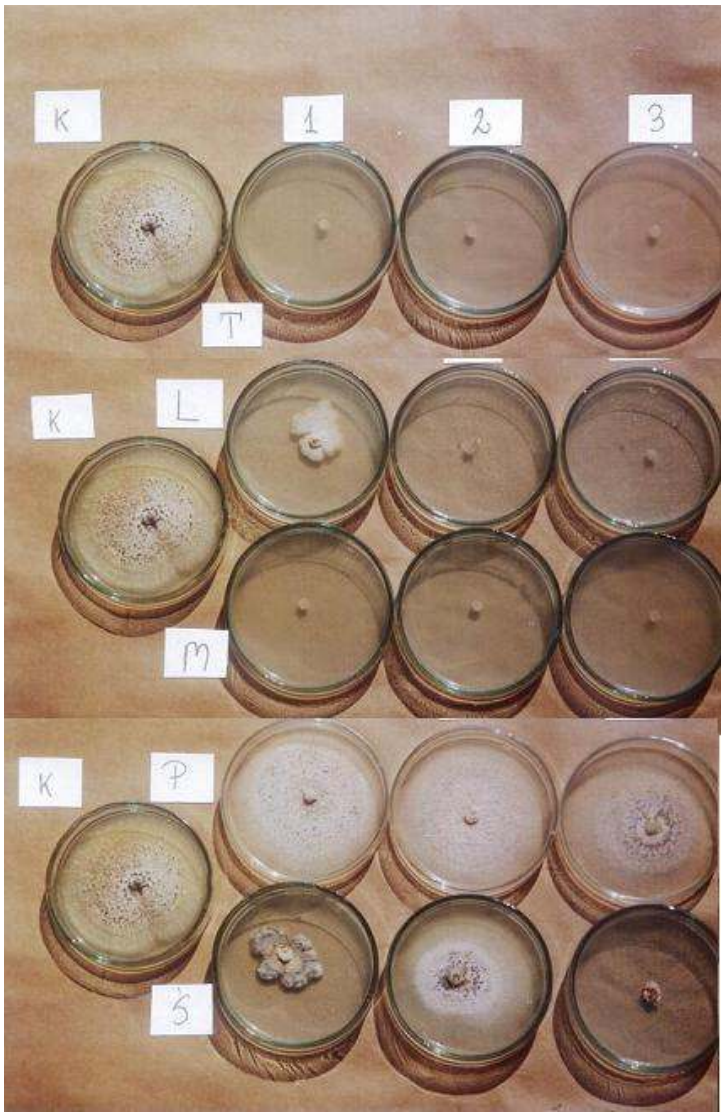

Photo 2. An effect of thyme, lavender, mint, orange and spruce oil on linear growth of *Rhizosphaera kalkhoffii* (K – control; T – thyme oil, L, – lavender oil, M – mint oils, P – orange oil and Ś – spruce oils; 1 – concentration 2.5 ml/l; 2 – concentration 5 ml/l; 3 – concentration 7.5 ml/l)

Fot. 2. Wpływ olejku tymiankowego, lawendowego, miętowego, pomarańczowego i świerkowego na rozrost liniowy *Rhizosphaera kalkhoffii* (K – kontrola; T – olejek tymiankowy, L – olejek lawendowy, M – olejek miętowy, P – olejek pomarańczowy, Ś – olejek świerkowy; 1 – stężenie 2,5 ml/l; 2 – stężenie 5 ml/l; 3 – stężenie 7,5 ml/l)

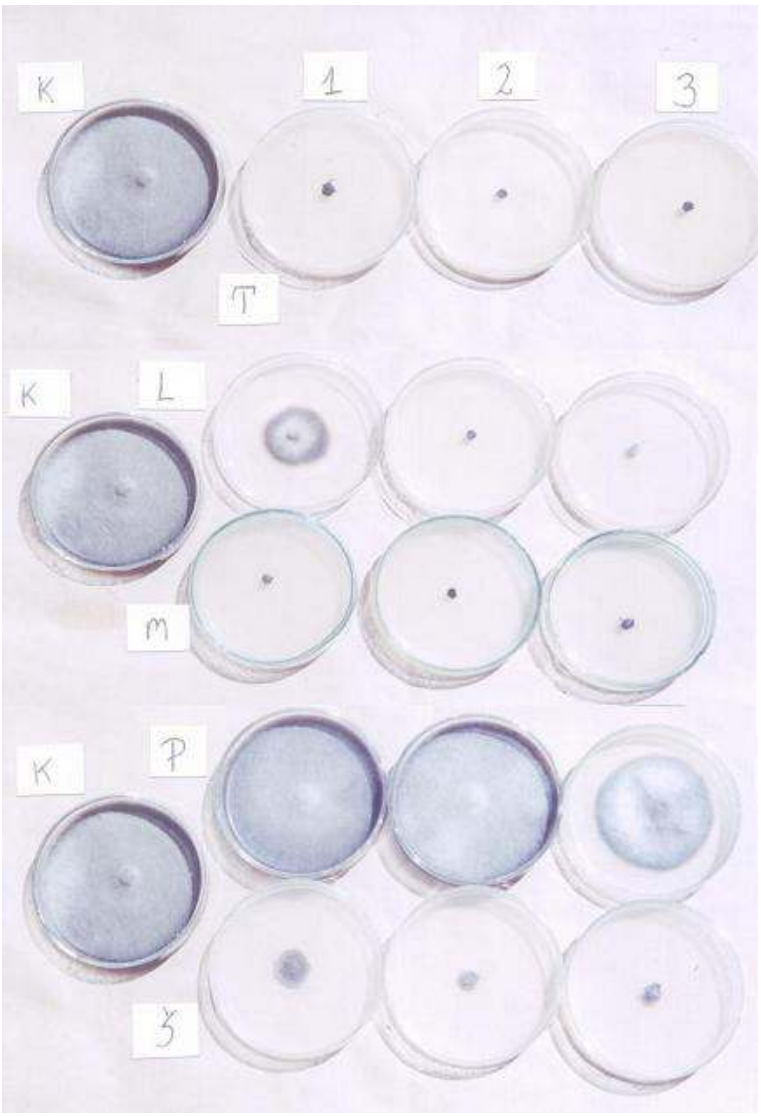

Photo 3. An effect of thyme, lavender, mint, orange and spruce oil on linear growth of *Ulocladium consortiale* (K – control; T – thyme oil, L, – lavender oil, M — mint oils, P – orange oil and Ś – spruce oils; 1 – concentration 2.5 ml/l; 2 – concentration 5 ml/l; 3 – concentration 7.5 ml/l)

Fot. 3. Wpływ olejku tymiankowego, lawendowego, miętowego, pomarańczowego i świerkowego na rozrost liniowy *Ulocladium consortiale* (K – kontrola; T – olejek tymiankowy, L – olejek lawendowy, M - olejek miętowy, P – olejek pomarańczowy, Ś – olejek świerkowy; 1 – stężenie 2,5 ml/l; 2 – stężenie 5 ml/l; 3 – stężenie 7,5 ml/l)

Table 2. An effect of essential oils on some features (spore and hyphae appearance, sporification intensity, presence of endosporous forms) of the tested fungal species compared to those of the control cultures.

Tabela 2. Wpływ badanych olejków eterycznych na niektóre cechy testowanych gatunków grzybów (wygląd zarodników i strzępek grzybni, obfitość zarodnikowania, obecność utworów przetrwalnikowych) w porównaniu do kultur kontrolnych.

Fungus Grzyb		Essential oil and its concentration – Olejek eteryczny i jego stężenie, in $ml \cdot l^{-1}$ / fungus features														
		lavender – lawendowy			mint – miętowy			orange – pomarańczowy			spruce – świerkowy			thyme– tymiarkowy		
		2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5
Acremonium tubakii	1*	no sporification	no mycelium growth in the medium with amendments				less chlamydospores	no sporification						no mycelium growth in the medium with amendments		
	2*	less chlamydospores						no differences			less chlamydospores					
Athostomella conorum	1	no mycelium growth in the medium with amendments						no perithecium, imperfect stage formation			no perithecium	no mycelium growth in the medium with amendments				
	2							no differences								
Arthrinium state of *Apiospora montagnei*	1	stimulated sporification					no mycelium growth in the medium with amendments	stimulated sporification				no mycelium growth in the medium with amendments				
	2	no differences						thinner hyphae	thickened hyphae		thinner hyphae	thickened hyphae				
Aureobasidium pullulans	1	no mycelium growth in the medium with amendments						no differences						no mycelium growth in the medium with amendments		
	2															
Botryodiplodia rubi	1	no pycnidum	no mycelium growth in the medium with amendments		no pycnidum		no pycnidum	less pycnidum	no pycnidum		less pycnidum	no pycnidum		no mycelium growth in the medium with amendments medium		
	2	thickened hyphae			thinner hyphae			no differences	thickened hyphae		no differences					
Fusarium camptocerans	1	no differences	chlamydospore within macroconidia	no differences	no mycelium growth in the medium with amendments			no differences	reduced sporification		stimulated sporification	no differences		no mycelium growth in the medium with amendments		
	2	no differences							no differences	more chlamydospores	no differences					

Fungus Grzyb		Essential oil and its concentration – Olejek eteryczny i jego stężenie, in $ml \cdot l^{-1}$ / fungus features															
		lavender – lawendowy			mint – miętowy			orange – pomarańczowy			spruce – świerkowy			thyme– tymiarkowy		
		2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5	2.5	5.0	7.5
Fusarium moniliforme var. *lactis*	1	reduced sporification	no mycelium growth in the medium with amendments	reduced sporification	no mycelium growth in the medium with amendments			no sporification		few spores		no sporification		no mycelium growth in the medium with amendments		
	2	colorless hyphae		colorless hyphae				no differences		slightly colored hyphae		colorless hyphae				
Penicillium canescens	1	no differences		no mycelium growth in the medium with amendments	no differences	no mycelium growth in the medium with amendments		no differences						no mycelium growth in the medium with amendments		
	2															
Phoma pomorum	1	no differences	no mycelium growth in the medium with amendments		no differences	no mycelium growth in the medium with amendments		no differences						no mycelium growth in the medium with amendments		
	2	thinner hypha, more chlamydospores			more chlamydospores			thinner hyphae, no chlamydospore	more chlamydospores	thinner hyphae, more chlamydospores						
Rhizosphaera kalkhoffii	1	no differences	no mycelium growth in the medium with amendments					no differences						no mycelium growth in the medium with amendments		
	2	thinner hyphae														
Ulocladium consortiale	1	no sporification	no mycelium growth in the medium with amendments					stimulated sporification	no differences					no mycelium growth in the medium with amendments		
	2	thin hyphae						no differences								
Zythiostroma pinastri	1	no pycnidum	more pycknidums	no mycelium growth in the medium with amendments	no pycnidum	no mycelium growth in the medium with amendments. Thickened hyphae		no pycnidum						no mycelium growth in the medium with amendments		
	2	thickened or thin hyphae			thickened hyphae			thickened hyphae		thin hyphae		thickened hyphae				

* 1 – sporification; 2 – hyphae appearance
* 1 – zarodnikowanie; 2 – wygląd strzępek

In turn, in the medium treated with lavender oil various biology reactions were noted for fungi under examination. In combinations with the following species: *Ac. tubakii, B. rubi, Ph. pomorum, U. consortiale* and *Z. pinastri* – there were no sporification; for *F. moniliforme* var. *lactis* – sporification was highly reduced; for *Arthrinium* state of *Apiospora montagnei* – sporification was stimulated. In addition, it seemed that lavender oil stimulated the chlamydospore production in *Ph. pomorum* and chlamydospore production within macroconidia in *F. camptoceras*. In combinations with *Ph. pomorum* and *Rh. kalkhoffii* this oil caused that hyphae were significantly thinner that those of the control, while thickened hyphae in *B. rubi* and *U. consortiale*.

In the medium with amendments with mint oil no sporification was found in fungi *Ac. tubakii, Ph. pomorum* and *Z. pinastri*, while sporification stimulation was recorded for *Arthrinium* state of *Apiospora montagnei*. In addition, an increased number of chlamydospores was recorded in *Ph. pomorum*, and reduced number for *Ac. tubakii*.

It was found in this paper that orange oil shown a low mycelium growth inhibiting activity for fungi under consideration and had only an effect on sporification intensity in some fungal species, namely. *Ac. tubakii, F. moniliforme* var. *lactis, Ph. pomorum* and *Z. pinastri.* In addition, this oil eliminated perithecium formation in *An. conorum* thus causing that this fungus was in imperfect stage only. Orange oil led also to thickening hyphae in *B. rubi* and *Z. pinastri*, while hyphae in *Arthrinium* state of *Apiospora montagnei* were considerably thinner than those of the control.

Spruce oil eliminated completely the sporulation in *Ac. tubakii, An. conorum, F. moniliforme* var. *lactis* and *Z. pinastri*. In combination with *B. rubi* and *F. camptoceras* this oil reduced sporification, while stimulated sporification in *Arthrinium* state of *Apiospora montagnei* (2.5 $ml \cdot l^{-1}$) and *F. camptoceras* (5 $ml \cdot l^{-1}$). For *Ac. tubaki* this oil reduced chlamydospore production, while increased the number of chlamydosphores in *F. camptoceras* and *Ph. pomorum*. In *Arthrinium* state of *Apiospora montagnei* and *Z. pinastri* (5 $ml \cdot l^{-1}$) spruce oil thickened hyphae, while in *Ph. pomorum* and *Z. pinastri* (2.5 $ml \cdot l^{-1}$) hyphae were significantly thinner.

The results presented in this paper have indicated that selected essential oils can be used in practical protection of blue spruce against fungi. In particular, this would be highly advantageous in urban green areas, where contrary to forest environment, the mycobiose of these trees is more diversified. Thus, the trees may require an intensive protection against diseases caused by fungi.

These oils showed however an adverse effect on some tested fungi considered to be saprobionts of advantageous impact on plants, e.g. *Au. pullulans* [Kowalski i Sadłowski 1993, Patkowska 2003] and fungi belonging to genus *Penicillium* [Pięta i in. 2002, Patkowska 2003] and *P. canescens* described in this paper.

CONCLUSIONS

1. It would be justified to implement essential oils into practical protection of blue spruce against fungal pathogens, especially in urban green areas.

2. Among essential oils tested *in vitro*, only thyme oil shows 100% efficiency of antifungal activity to all fungi under examination. Lavender and mint oils shows slightly

lower activity. Spruce oil is of significantly lower antifungal activity and narrower range of application, while orange oil shows the poorest properties.

3. It seems that reaction of tested fungi to essential oils used is selective and variable. This indicates a destructive effect of these substances on fungi as demonstrated by stimulating or reducing some life-processes in fungi.

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Z BADAŃ NAD MOŻLIWOŚCIĄ OCHRONY ŚWIERKA KŁUJĄCEGO (*Picea pungens* Engelm.) PRZED GRZYBAMI. CZEŚĆ II. LABORATORYJNA OCENA SKUTECZNOŚCI GRZYBOBÓJCZEJ WYBRANYCH OLEJKÓW ETERYCZNYCH

Streszczenie: W warunkach *in vitro* badano właściwości grzybobójcze wybranych olejków eterycznych, tj. lawendowego, miętowego, pomarańczowego, świerkowego i tymiankowego, w stosunku do grzybów wyizolowanych z roślin świerka kłującego (*Picea pungens* Engelm.) i jego odmiany 'Glauca'. Stopień i zakres działania grzybobójczego wyżej wymienionych olejków przetestowano na grzybach: *Acremonium tubakii, Anthostomella conorum, Arthrinium* state of *Apiospora montagnei, Aureobasidium pullulans, Botryodiplodia rubi, Fusarium camptoceras, F. moniliforme* var. *lactis, Penicillium canescens, Phoma pomorum, Rhizosphaera kalkhoffii, Ulocladium consortiale* i *Zythiostroma pinastri*. Wśród przebadanych olejków eterycznych bardzo wysoką aktywnością odznaczał się olejek tymiankowy, bo już przy najniższym zastosowanym stężeniu i w stosunku do wszystkich testowanych gatunków grzybów. Średnią aktywność grzybobójczą wykazały olejki: miętowy, lawendowy i świerkowy. Najmniej skuteczny okazał się olejek pomarańczowy.

Słowa kluczowe: *Picea pungens*, grzyby, zwalczanie, olejki eteryczne

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