Fungicidal properties of *Pinus sylvestris* L. for improvement of air quality

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Key words: *microorganisms, pine, essential oils, indoor air.*

Summary. *Sick building syndrome is a term commonly used to describe the consequences of poor indoor air quality. It is well documented that first of all air quality depends on the chemical composition, and until now negligible attention has been paid to air pollution by microorganisms. Some species of fungi (Aspergillus flavus, A. fumigatus, A. niger, A. parasiticus, A. oryzea and other) and their toxins cause difficulty in breathing, allergic rhinitis, watery eyes, headaches, and flu-like symptoms. Over recent years considerable interest has been developed for plant extracts that would be of great use for the improvement of air quality. The biological activity of Pinus sylvestris L. has been investigated in order to find out its fungicidal activity against airborne microorganisms. It was determined, that fungi from Aspergillus and Penicillium genera dominated indoors. Antimicrobial activity of pine oil was evaluated by technique of oil diffusion to Czapek agar (for fungi), malt extract agar (for yeast and yeast-like fungi) and nutrient agar (for bacteria). Minimum inhibitory concentrations of pine oil to 13 species (8 fungi, 2 yeast-like fungi, yeast and 2 bacteria,) were determined: 1.0–2.5, 1.0–1.2, 0.5–0.75, and 0.75–1.2% (v/v), respectively. According to resistance to pine oil action, microorganisms grouped themselves as following: fungi, spore bacteria, yeast-like fungi, yeast, and bacteria (fungi being the most resistive and bacteria being the least resistive). The most active concentration of pine oil against all tested microorganisms was 2.5%, and the most sensitive fungus to volatiles was Ulocladium oudemansii.*

Introduction

Last two decades have been characterized by a significant increase of the research data on bioaerosols in indoor environment. Development of new techniques and analytical methods allowed more precise identification of the sources of microbial contamination, evaluation of the quality of indoor air and the assessment of potential hazards (1–3). Control of microorganisms in the indoor environments has traditionally focused on source control, ventilation, and air cleaning. Disinfecting compounds often are toxic. Harmful substances interfere with vital body processes by destroying enzymes, blocking oxidation, restricting the functions of various organs and initiate cellular changes and mutations. This concern has encouraged researchers to look for other solutions to new disinfectants. Recently considerable interest in plant extracts has been expressed. Plant extracts are generally assumed to be more acceptable and less hazardous than the synthetic compounds. Essential oils are chemically very diverse in their effect and cause different actions, unlike synthetic chemicals, which basically have one action. In screening for new less toxic compounds, family Pinaceae is one of the important objects for investigation due to its medical properties. Pine was first investigated by Hippocrates, the father of Western medicine, due to its benefits to the respiratory system. During the 1800's, tar from the pine was used in many medicines, especially for treatment of skin diseases like eczema and psoriasis. The needles were pressed into a stuffing called "pine wool" that was thought to repel lice, fleas, and other pests. Several different species of pine are distilled for the essential oil, often labeled simply as "pine oil". The oil is distilled from different species depending on country of origin. In Scandinavia, pine oil is distilled from *P. sylvestris*, while in France *P. palustris* is often used as well. In the Alps, Siberia and the Carpathian Mountains the oil is distilled from *P. cabrea, P. lambertiana, P. palustris, P. taeda, P. ponderosa* or *P. sabiniana*.

According to the composition of essential oils the genus *Pinus* is divided into two groups (4). One group comprises the species rich in monoterpene hydrocarbons (α-β-pinene, limonene, β-caryophylene, germacrene D, Δ-3-carene) and other is rich in the oxygenate monoterpenes (borneol, bornyl acetate).

During the last years most of the phytochemical studies were related to aromatherapy, a branch of her-

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bology. Historically essential oils have been used for thousands of years to promote well being and health (5, 6). Essential oils have been used in the form of massage or bath oils or inhalations, however, lately their introduction into the other fields of practice increases. One sphere of their new employment is the improvement of the indoor air quality. The air and indoor environment is colonized by propagules of the various microorganisms. Their distribution in the air depends on the physiological properties of individual species, as well as on the type of activities of occupants (7). Fungi produce mycotoxins, which are capable of producing illness and death in humans. Based on the microbiological analysis of air samples from occupied spaces, aflatoxins, produced by *Aspergillus spp.*, are common contaminants related with health problems (7–9). Fungal propagules and bacterial cells are important contagious agents causing infectious diseases such as allergic rhinitis, asthma and hypersensitivity pneumonitis (2, 10–12). Asthma is one of the most common childhood respiratory illnesses, and its worldwide prevalence continues to increase (13, 14). The other problem is that nurseries are crowded and the presence of fungi on children's hair or nails creates an opportunity for them to invade the skin (15). Many studies have documented fungi from the *Aspergillus, Penicillium genera* and yeast *Candida* as the main causal factors for human illnesses (15, 16). However, list of the dangerous to human fungal species (belonging to *Alternaria, Acremonium, Fusarium, Absidia, Phoma genera*) increases every year (13, 15).

The antimicrobial activity of essential oils from family *Pinaceae* was investigated by many authors (17–20). It was reported that essential oil of pine was very active only against bacteria but not the fungi (20). Later Krauze-Baranowska et al (2002) determined that essential oil (at 2% concentration) from North American pine inhibited the growth of two fungi: *Fusarium culmorum* and *F. solani.* Fungicidal activity against *F. poae* was observed at 5% concentration of pine oil.

The system of protection against fungi and their mycotoxins and public health promotion are divided into three parts: risk assessment, decontamination of indoor environment and education on health promotion (21). In many countries Environmental Health Monitoring systems are functioning, however, better attention should be paid to the air contamination by microorganisms and to the prevention of the diseases caused by them.

During current investigation we evaluated the quality of the indoor air in order to specify potential agents responsible for human health problems, and continued search for the new preventive measures against them. The aim of the present investigation was to determine the antimicrobial activity of *Pinus sylvestris* L. against the microorganisms most frequently isolated from the human environment.

Materials and methods

Microorganisms tested. Microorganisms were isolated from the indoor air by sedimentation method as described in our previous work (22).

Effect of biomass of the pine needles on the fungal development was studied by cup method (23). Fungal conidia suspension was mixed in Czapek's agar and poured into Petri dishes 20 ml each. After agar was cooled, the cups (7 mm in diameter) were made and were filled with squash of the pine needles (1 g biomass/cup). Control ones were Petri dishes without pine biomass. Zones (mm) of fungal growth stimulation or inhibition by pine biomass were determined after 7-day incubation at 25°C in the dark.

Agar diffusion method was used to evaluate minimum inhibition concentration (MIC) of pine oil to the investigated microorganisms. The sterile Czapek's agar (for fungi), malt extract agar (Difco) (for yeast and yeast-like fungi), and nutrient agar (Difco) (for bacteria), at 1% oil concentration was poured into Petri dishes. Microorganisms were cultivated for 24 h (for bacteria), 48 h (for yeast and yeast-like fungi) and 72 h (for fungi). The essential pine oil of investigated concentration was placed in the wells (9 mm in diameter) cut in the media, and the dishes were incubated at 32°C for bacteria (24 h) and 25°C for yeast, yeast-like fungi and fungi (3 and 7 days for yeast and fungi, respectively). The MIC for pine oil was defined as the lowest concentration that inhibited the visible growth by 100%. The data are reported as the concentration of pine oil necessary to inhibit the visible growth of the evaluated isolates by 90% (MIC_{90}). All assays were done in duplicate to verify the results.

Effect of the volatile fraction of the commercial pine oil was studied by the invert Petri dishes method (23). Czapek's agar was poured into one Petri dish, and pine oil (at different concentrations) tested was poured into the other dish of the pair. After agar was cooled, dishes containing agar medium were inoculated with fungus conidia. The lids of the dishes were sealed with Para film. Dishes were incubated for 7 days at 25°C. Diameter of the fungal colony for various concentrations was measured. The antifungal action of pine oil and its vapor was determined on the 6–9th day by breaking the fungus colony growth, using Ebbot (Golyshin, 1970) formula:

$$T=(D_K-D_O)/D_K\times100\%,$$

D_K – diameter of mycelium control colonies, cm; D_O – diameter of mycelium colonies in the experiment; T – percent of mycelium growth inhibition.

Results and discussion

Results of our *in vitro* studies revealed new data related to biological activity of biomass of the pine needles, oil and their volatile compounds against common indoor environment microorganisms. The indoor environment of dwellings is colonized by a number of various microorganism species. Their quantity in the air depends on the physiological properties of individual species, as well as on the type of activities of occupant's. Fungi, which were observed in the investigated indoor environments, *Aspergilus spp., Chaetomium spp, Penicillium spp., Stachybotrys chartarum, Rhizopus stolonifer, Aureobasidium pullulams, Phoma glomerata* possess allergenic and toxic properties and are known risk factors for occupational respiratory diseases (1, 10, 16, 21). Relative frequency of the prevalent genera in the indoor environment (mean of all studied flats) is presented in Table 1. Isolated fungi belong to the following genera: *Alternaria* (was observed in 34.2% of collections by sedimentation method), *Aureobasidium* (in 56–84.6%), *Aspergillus* (96.8–100%), *Chaetomium* (26.8–33.4%), *Cladosporium* (53.4–69.9%), *Paecilomyces* (84.1–100%), *Penicillium* (67.3–82.5%), *Phoma* (23.6–45.7%), *Rhizopus* (34.2–41.7%), *Sporotrichum* (12–15.6%), *Stachybotrys* (36.7–49.8%), *Trichoderma* (23.4–35.4%) and *Ulocladium* (in 25.2–36.9% of collects) (Fig. 1). Among the isolated fungi, *Absidia corymbifera, Aspergillus niger, A. versicolor, Aureobasidium pullulans, Paecilomyces variotii, Penicillium chrysogenum, Penicillium spp. and Rhizopus stolonifer* were predominant, but *Cladosporium spp., Mortierella spp., P. carneus, Geotrichum sp.* and *Trichoderma spp.* were also isolated. Bacteria, *Micrococcus spp.* and *Rhodococcus spp.*, and yeast *Candida lipolytica* (= *Mycotorula lypolytica*) were also predominant in microflora of indoor air. Few strains of *Foma, Cladosporium, Chaetomium* and *Trichoderma genera* were included in this investigation as were found frequently (41.7%, 69.9%, 33.4% and 35.4%, respectively) on

***Table 1.* List of species and strains investigated and mean relative frequencies (%) of the microorganisms genus in the indoor environment**

Microorganisms (species)	Strains	Relative frequency of genera (%) in the indoor air
Chaetomium globosum Kunze	No BG-23	26.8–33.4
Cladosporium cladosporioides (Fresen.) G.A. de Vries	No Wg-14	53.4–69.9
Aspergillus niger Tiegh.	No BN-3	96.8–100
	No OG-25	96.8–100
Aspergillus versicolor (Vuill.) Tirab.	No BG-12	36.5–64.2
	No BG-3	36.5–64.2
Aureobasidium pullulans (de Bary) G. Arnaud	No OG-8	56.0–84.6
	No OG-53	56.0–84.6
Paecilomyces variotii Bainier	No OG-7	84.1–100
Penicillium chrysogenum Thom	No BG-31	67.3–82.5
Phoma glomerata (Corda) Wollenw. et Hochapfel	No VG-2	23.6–45.7
Phoma sp.	No BG-17	23.6–45.7
Rhizopus stolonifer (Ehrenb. ex Fr.) Vuill.	No OG-24	34.2–41.7
	No DG-16	34.2–41.7
Stachybotrys chartarum (Ehrenb. ex Link) Hughes	No WG-5	36.7–49.8
Trichoderma viride Pers.	No BG-19	23.4–35.4
Ulocladium atrum Preus	No BG-55	25.2–36.9
Rodococcus sp.	No BB-2	6.5–7.8
Bacillus sp.	No BB-6	14.3–22.4
Candida lipolytica (F.C.Harrison) Diddens et Lodder (= *Mycotorula lypolytica* Harrison)	No BM-8	3.6–5.4
Geotricum candidum *Link: Fr.*	No BM-11	16.8–19.4

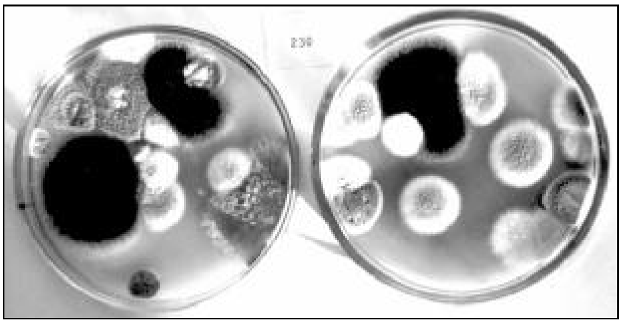

***Fig. 1.* Colony formed by viable airborne fungal propagules on Czapek's medium**

the walls of mould-affected dwellings, especially on the bath-walls. Pieckovį and Kunovį (2002) reported that in the most cases walls were colonized by the "first colonizers" (*Aspergillus Fr.: Fr. sp.* and *Penicillium sp.*) and afterwards by the "second" ones (namely *Cladosporium Link sp.*); the third group of colonizers includes *Alternaria sp., Phoma sp. and some Aspergillus and Penicillium spp.* These filamentous fungi are known to be able to produce compounds with very high ciliostatic toxicity (21, 24).

Recent work has shown that biomass of the pine needles, when added into the cups in the agar, stimulate growth of the fungi (Table 2). Fungi grew on biomass of the pine needles, and fungal growth stimulation near this biomass was also observed. The highest stimulation of fungal growth by addition of pine biomass was during the first days of incubation.

Penicillium funiculosum was the most sensitive to volatile fraction of pine needles biomass (45.5 and 32.6% inhibition after 3 and 7 days, respectively). After 3-day incubation, vapor of pine needles inhibited

***Table 2.* Stimulation of the fungus growth on Czapek's agar by biomass of *Pinus sylvestris* needles (1 g)**

Days of cultivation	Zone of stimulation, mm		
	Aspergillus niger	*Penicillium funiculosum*	*Trichoderma viride*
3	10.0±0.5	9.2±0.6	10.0±0.2
4	9.3±0.3	8.8±0.9	8.0±0.5
5	8.1±0.5	8.2±0.4	8.0±0.5
6	8.0±0.4	8.2±0.5	6.0±0.3
7	5.0±0.3	4.3±0.3	2.0±0.2

***Table 3.* Antifungal activity of volatile fraction of pine oil, expressed as fungus growth inhibition (%)**

Fungi (species)	Days	Quantity of pine oil, volatile fraction of which caused visible growth inhibition		
		0.05 ml	0.1 ml	0.15 ml
Penicillium funiculosum	3	9.0	24.6	28.7
	4	20.5	35.5	38.0
	5	21.8	31.5	37.1
	6	25.8	31.8	35.1
	7	22.3	28.7	30.0
	8	18.5	28.6	29.7
Ulocladium oidemansii	3	81.3	83.3	93.2
	4	75.9	72.8	81.7
	5	68.7	70.2	77.8
	6	64.4	67.8	70.9
	7	61.1	63.8	69.6
	8	59.1	60.2	65.9
	9	63.1	66.4	70.1
	10	60.4	65.3	73.2
	11	58.0	65.6	76.7
	12	58.0	65.6	76.5

growth of *Trichoderma viride* (growth inhibition by 22.2%) and *Aspergillus niger* (21.3% growth inhibition). After 7-day incubation effect of vapor of pine needles on *T. viride* growth decreased to 6.4%. Growth of *A. niger* was inhibited (22.2–20.3% inhibition) during all period of that investigation.

Essential oils often are fungistatic rather than fungicidal. Our results suggested that tested fungi are able to use biomass of the pine needles as carbon or energy source. On the other hand, extract obtained from pine needles was fungistatic. Recent work has shown that essentials of the commercial pine oil were antagonistic against the tested indoor fungi. Inhibition level depended on the fungus species (Table 3). Obtained data suggested that fungistatic activity of vapor of pine oil depended on the oil concentration and fungus species. At 0.05 ml/plate pine oil concentration, growth inhibition of fungus P. funiculosum by vapor remained stable during the whole incubation period. When oil concentration was 0.15 ml/plate, the highest growth inhibition was evaluated during the 4th and 6th days of cultivation. Fungistatic activity of volatile fraction of pine oil against fungus Ulocladium oudemansii was the highest at the beginning of incubation (1–2 days) and later slightly decreased (Fig. 2, 3)

Antibacterial and antifungal activity tests of pine oil in this investigation were made by use of paper disc-agar diffusion and broth dilution techniques. Commercially available pine oil gave a high degree of antibacterial activity against *Rhodococcus sp.* (No BB-2) and *Bacillus sp.* (No BB-6); significant activity against yeast *Candida lipolytica* (No BM-6) and yeast-like fungi *Aureobasidium pullulans* (No OG-8) and *Geotrichum candidum* (No BM-11); and no antifungal activity at 0.25–0.5% pine oil concentration (Table 4). MICs against fungi determined by cup-agar diffusion were 2–3 times higher that those against yeast and bacteria. Fungal isolates *Penicillium chrysogenum*

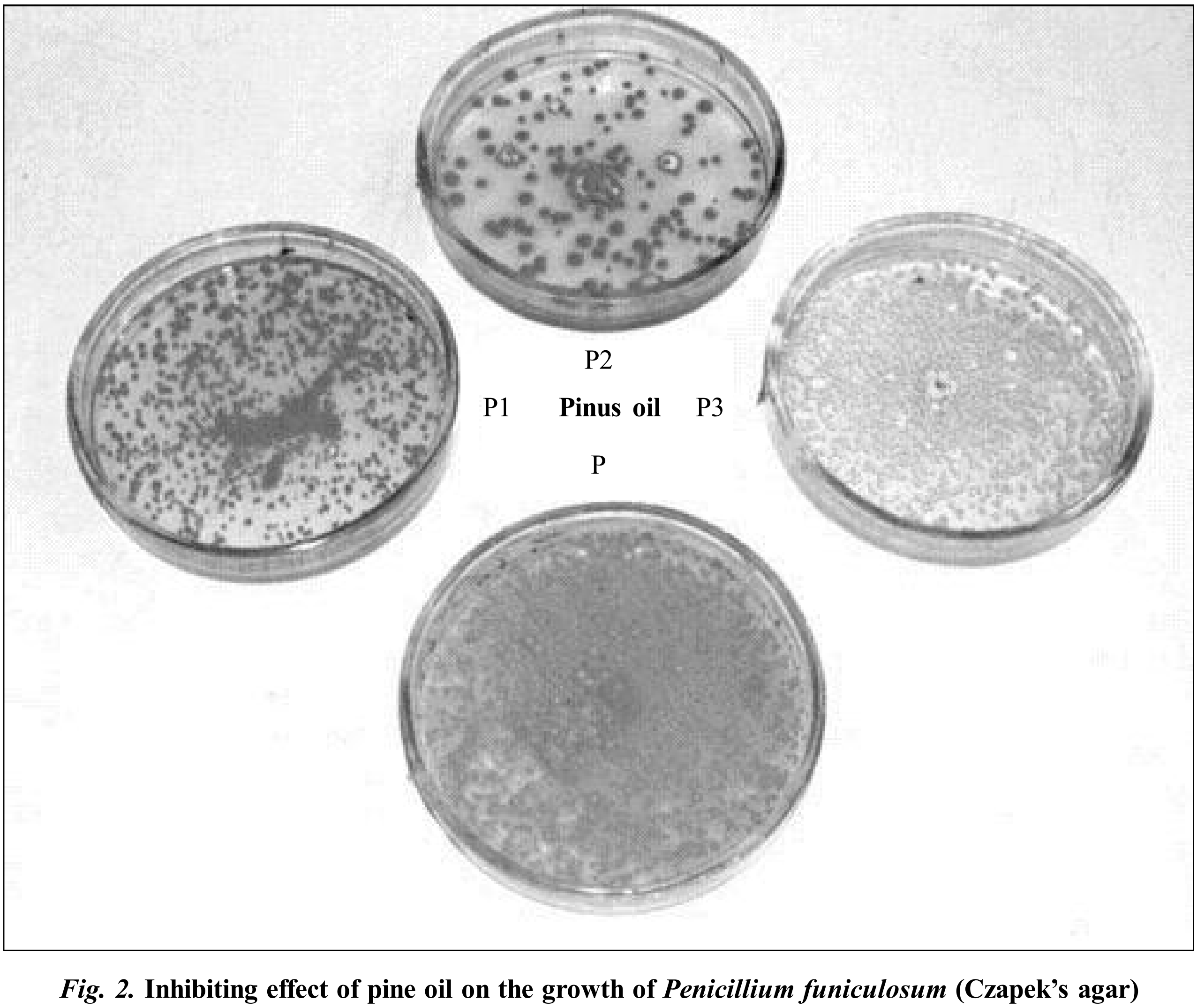

***Fig. 2.* Inhibiting effect of pine oil on the growth of *Penicillium funiculosum* (Czapek's agar)**
P – control; pine oil concentrations: P1 – 0.05 ml/dish, P2 – 0.1 ml/dish, P3 – 0.15 ml/dish. At P3 pine oil concentration inhibition of fungus sporulation was noticeable.

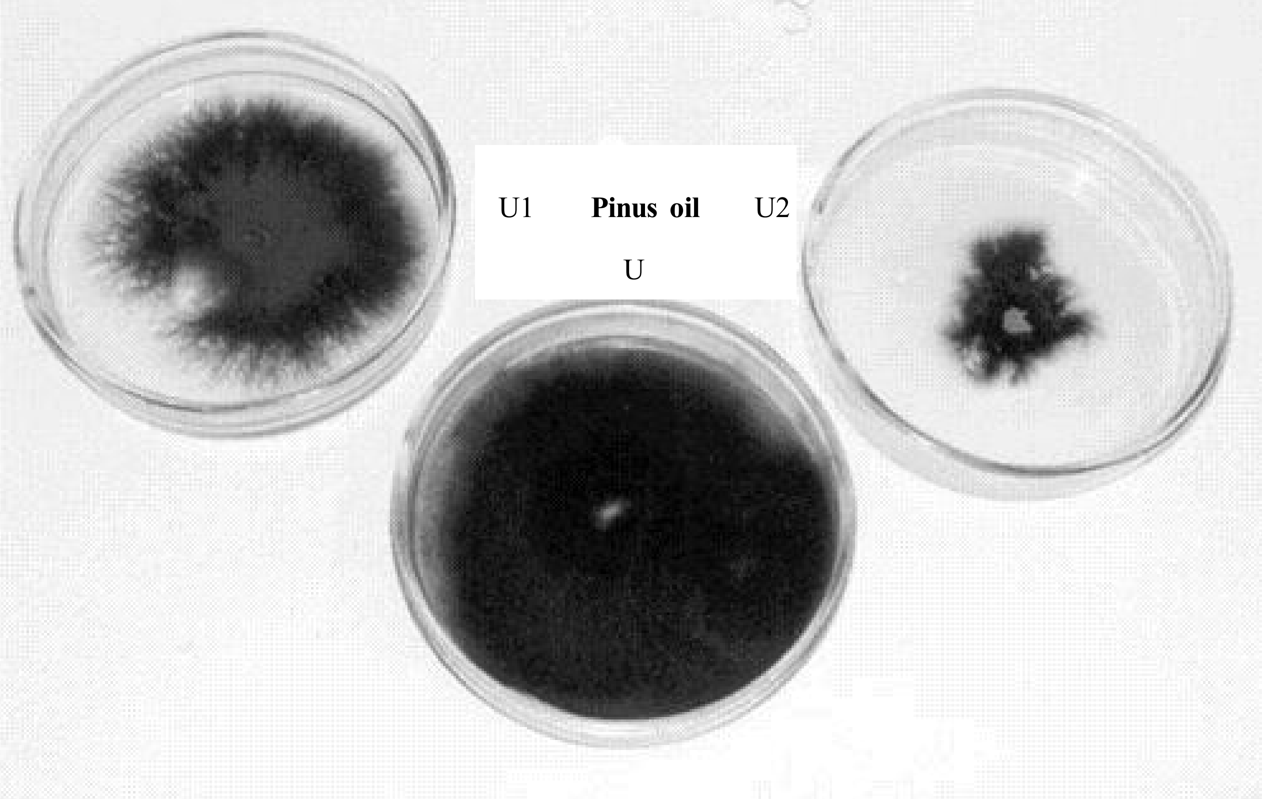

Fig. 3. **Inhibiting effect of pine oil on fungus on the growth of *Ulocladium oidemansii* (12 days incubation on Czapek's agar)**

U – control; pine oil concentrations: U1 – 0.05 ml/dish, U2 – 0.15 ml/dish.

Table 4. **MIC_{90} values (% v/v) to microorganisms determined by cup-agar diffusion technique (means from two investigated pine oil samples and three replicates of each experiment)**

Microorganisms	Strains	MIC_{90}
Chaetomium globosum	No BG-23	0.5
Cladosporium cladosporioides	No Wg-14	0.5
Aspergillus niger	No BN-3	1.0–1.5
	No OG-25	0.75
Aspergillus versicolor	No BG-12	1.0–1.5
	No BG-3	0.75–1.0
Aureobasidium pullulans	No OG-8	0.55–0.75
	No OG-53	0.5
Paecilomyces variotii	No OG-7	1.2
Penicillium chrysogenum	No BG-31	1.0–2.5
Phoma glomerata	No VG-2	1.5–2.5
Phoma sp.	No BG-17	0.75
Rhizopus stolonifer	No OG-24	0.5
	No DG-16	0.75
Stachybotrys chartarum	No WG-5	1.0–1.5
Trichoderma viride	No BG-19	0.55
Ulocladium atrum	No BG-55	0.75-1.0
Rodococcus sp	No BB-2	0.5
Bacillus sp.	No BB-6	0.5
Candida lipolytica	No BM-8	0.5
Geotricum candida	No BM-11	0.35–0.5

(No BG-31), *Paecilomyces variotii* (No OG-7), *Phoma glomerata* (No VG-2) were the least susceptible to pine oil. MICs against those fungi were in the rage 1.5–2.5%. Slight antifungal activity of pine oil also was shown against *Aspergillus niger* (No BN-3 and No OG-25), *Stachybotrys chartarum* (No WG-5) and *Aspergillus versicolor* (No BG-3). Only 1.0–1.5% pine oil concentrations induced inhibition zone in cultures of these fungi.

The results of this study confirm the excellent in vitro efficacy of the pine oil against the more common microorganisms in the indoor environment. *Pinus sylvestris* oil demonstrated the lowest MICs and was the most active against yeast and bacteria, with similar MIC and narrow MIC ranges. Pine oil also has similar activity against *Chaetomium globosum* and *Aureobasidium pullulans*. On the other hand, pine oil demonstrated less activity against *Geotrichum candidum* although still within the efficacy range, and not much higher than the MICs of the very susceptible strain of *Candida lipolytica*. Moreover, the MIC results indicate that *Pinus sylvestris* is fungicidal for all of the fungal species evaluated. In addition to the broad antimicrobial activity of pine oils, the most exciting observation was remarkably good activity of volatiles demonstrated against the strains of bacteria and yeast. Essential pine oil also exhibits antifungal activity against a wide range of common post harvest pathogens. Pine oil has antibacterial and antifungal properties that have secured it a place in the commercial pharmaceutical market.

This work highlights the potential for using pine oil for post harvest disease control of fresh fruits and vegetables. Application of pine oil via the vapor phase should also make its use more cost-effective than dipping. Application of the oil as vapor at continuous, low concentrations should prevent tainting of the products. Increased concentrations should serve as fungistatic mean for indoor air improvement. Research has shown that with their immune-stimulating properties, essential oils enhance and support the building of the immune system, whether they are inhaled or rubbed on the body topically. Even those who contract a cold or the flu recover 70 percent faster, if using essential oils. Oils increase ozone and negative ions in the home, which inhibit bacteria growth. They prevent and destroy existing odors from mold, cigarettes, animals, etc. Essential oils have the electrical magnetic attraction to fracture the molecular chain of chemicals and take them out of the air, rendering them non-toxic to the body. Scientists in European countries have found that essential oils will bond to metallic and chemicals and carry them out of the body, working as natural chelators, inhibiting these toxic substances from staying in the tissues. Essential oils remove dust particles out of the air and, when diffused in the home, can be the greatest air filtration system.

We are continuing our work aiming to determine the optimum concentrations of pine oil for maximum control of the airborne fungi with acceptable levels of air quality. Additional work is required to determine the extent of these effects, suitable parameters of stability and appropriate formulation and cultivation procedures.

Conclusions

The results indicate that the essential oil of *Pinus sylvestris*, used in the study, inhibited development of bacteria, yeast and fungi to a variable extend. The most active concentration of pine oil against tested microorganisms was 2.5% of oil in culture medium.

On the other hand, it is determined that the pine essential oil was more active against bacteria and yeast than fungi and the antimicrobial activity of oil increased with increase of oil concentration in the medium.

Needles of *Pinus sylvestris* are well nutrient substrate for fungal growth and development. Volatile compounds of pine needles inhibit growth of fungi. Fungistatic activity of volatile fraction of pine oil depends on fungus species: strong inhibition effect on *Penicillium funiculosum* and *Trichoderma viride* growth was determined.

Effect of pine oil against fungi depended on its concentration and fungus species. Growth inhibition of investigated fungi increased with increased concentration of oil. *Fungus Ulocladium* oudemansii was the most sensitive to pine oil action of the fungi tested in current investigation.

Pinus sylvestris L. fungicidai – patalpų oro kokybei gerinti

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Raktažodžiai: mikroorganizmai, pušis, eteriniai aliejai, patalpų oras.

Santrauka. Patalpų ekologija glaudžiai susijusi su žmogaus sveikata. Norint užtikrinti saugią ir sveiką aplinką, ypač svarbus rodiklis – oro kokybė, kuri dažniausiai vertinama pagal jo cheminę sudėtį. Deja, dar nepakankamai skiriama dėmesio oro užterštumui mikroorganizmais. Kai kurių rūšių (*Aspergillus flavus, A. fumigatus, A. niger, A. parasiticus, A. oryzae* ir kitų) padermės ir jų toksinai sukelia kvėpavimo takų, akių ligas, alergiją. Dabar ypač susidomėta augalų ekstraktų panaudojimo galimybėmis oro kokybei gerinti. Šio tyrimo tikslas – *Pinus sylvestris* L. fungicidų savybių tyrimai siekiant įvertinti jos ekstraktų poveikį oru plintančių mikroorganizmų vystymuisi. Nustatyta, kad tirtose patalpose vyravo *Aspergillus* ir *Penicillium* mikromicetų genčių padermės. Antimikrobinis aktyvumas vertintas naudojant pušies aliejaus difuzijos agarizuotose terpėse metodą. Mikromicetai buvo auginami ant Čapeko, mielės ir mieliagrybiai – ant alaus misos, bakterijos – ant mitybinio agaro terpių. Nustatytos minimalios 13 mikroorganizmų (8 mikromicetų, 2 bakterijų, 2 mielių ir mieliagrybio) vystymąsi slopinančios pušies aliejaus koncentracijos: 1,0–2,5; 1,0–1,2; 0,5–0,75; 0,75–1,2 proc. (v/v), atitinkamai mikromicetams, mielėms, mieliagrybiams ir bakterijoms. Pagal atsparumą pušies aliejui mikroorganizmai pasiskirstė taip: mikromicetai > mieliagrybis > mielės > bakterijos. Bakterijos jautriausiai iš visų tiriamų mikroorganizmų reagavo į pušies išskiriamų lakiųjų medžiagų poveikį. Mažiausia pušies aliejaus koncentracija, slopinusi visų tirtų mikroorganizmų vystymąsi, buvo 2,5 proc. aliejaus terpėje. Pušies aliejus stipriausiai slopino mikromiceto *Ulocladium oudemansii* augimą.

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