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MICROBIOTA OF *PINUS* POLLEN AS ADJUVANT FACTOR OF ALLERGY

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ABSTRACT

Bacteria, their endotoxin and mold found on pollen can be a reason of respiratory symptoms in sensitized individuals. This question concerns an anemophilous pollen more acute. In this work quantitative by dilution plating method and qualitative microbial analysis by MALDI-TOF MS Biotyper of pollen and other plants organs of *Pinus sylvestris* L., *P. nigra* Arnold, *P. mugo* Turra, *P. armandii* Franch., *P. wallichiana* A.B. Jacks from Nitra, Slovakia are performed which shows quantitative and species differences in mesophilic aerobic (0.00-6.27 log cfu/g) and anaerobic bacteria (0.00-3.70 log cfu/g), enterococci (0.00 log cfu/g), coliform bacteria (0.00-5.29 log cfu/g), lactobacilli (0.00-4.20 log cfu/g), microscopic fungi and yeasts (2.60-5.29 log cfu/g) content. Representatives of *Pseudomonas* (14), *Bacillus* (2), *Acinetobacter* (1), *Arthrobacter* (1), *Pantoea* (1), *Klebsiella* (1), *Penicillium* (6), *Aspergillus* (4), *Cladosporium* (1), *Debaryomyces* (1) genera were revealed on pine trees. The allergenic potential of the identified association of microorganisms on pollen has been evaluated based on published data. The results may be useful for aerobiologists, allergists and microbiologists, at least at the local level.

Keywords: *Pinus* species, microbiota, pollen contamination, microbiological quality, flowering, allergy

INTRODUCTION

Pinus pollen has natural properties to be allergenic: 1. it's anemophilous; 2. released in large quantities; 3. the genus is widespread in nature (**Noks, 1985; Behrendt and Becker, 2001; D'Amato *et al.*, 2007; Maňka *et al.*, 2013; Turos *et al.*, 2013**). At the same time, a *Pinus* pollen grain, for example, *Pinus sylvestris* L. is heavier (30.08 mμg) in comparison with a pollen grain of *Betula verrucosa* Ehrh. (9.48 mμg) – known potent allergen. They also differ slightly both settling velocity of grains in still air: for *P. sylvestris* L. is 3.69 cm/sec, for *B. verrucosa* Ehrh. – 2.94 cm/sec and the probable range drift of pollen by wind – 1700-1775 and 600 km, respectively (**Dyakowska and Zurzycki, 1959**). It is known more widely about the beneficial properties of pine pollen (**Lee *et al.*, 2009; Solntseva and Glazunova, 2010**). Pine pollen has long been considered a non-allergenic pollen (**Gastaminza *et al.*, 2009; Vinhas *et al.*, 2011**). The main reasons of this are the large size of a grain, its low levels of proteins and the presence of a waxy hydrophobic layer that averts protein release. Thus, the length of the polar axis of the *P. sylvestris* L. pollen grain is 67.4 (60-74) μm, *P. nigra* – 75.5 (67-87) μm. For comparison, the size of *Betula pendula* pollen grain is only 22.6 (21-25) μm (**polleninfo**). As **Přidal (2003)** determined the amount of proteins in the pollen of *Betula* is 23.02%, in *Pinus* pollen – 14.14% (**Nôžková *et al.*, 2014**). **Özler *et al.* (2009)** defined pollen of *Pinus nigra* subsp. *nigra* var. *caramanica* as important allergenic tree pollen because of content of amino acids and total protein. **Gastaminza *et al.* (2009)** detected high degree of cross reactivity among the pollen of the distinctive pine species. Cases of allergic sensitization by pine pollen are known and enough spread (**Green *et al.*, 2003; Gastaminza *et al.*, 2009**).

The genus *Pinus* includes 116 species. This genus is subdivided into subgenus *Strobus* with 43 species and subgenus *Pinus* with 73 species (**Maňka *et al.*, 2013**). *Pinus* pollen is predominant in the atmosphere of most environments: from the Arctic Circle to Guatemala, the West Indies, North Africa and Malayan Archipelago (**Green *et al.*, 2003; Maňka *et al.*, 2013**). Slovakia, as a mountainous and forested country (40.6% forest cover) in central Europe, has a large variety of vegetation zones, forest types, and a rich variety of forest tree species. *Pinus sylvestris* L. is one of the most important tree species (**Oszlányi, 1997**). Pines are frequently planted in city parks or near streets (**Ivanová and Bernadovičová, 2010; Dušička *et al.*, 2013**). Plant wealth, closeness to living environments and regional geography are additional factors that can prompt to local allergic sensitization by pine pollen (**Shah and Grammer, 2012**).

Gram-negative and Gram-positive bacteria, mould are the source of allergens. They were revealed on the surface of the pollen grains of anemophilous plants as well as endotoxin (**Śpiewak *et al.*, 1996; Heydenreich *et al.*, 2011; Mittag *et al.*, 2013**). These microorganisms are attached to the pollen exine or located within the pollen cells likely before the opening of the catkins. On the other hand they may be airborne contaminants that alight on the pollen during collection and handling (**Madmony *et al.*, 2005**). Airborne bacteria are more likely use pollen grain as a transport or source of nutrients (**González *et al.*, 2005; Madmony *et al.*, 2005; Després *et al.*, 2011**). Such pollen-associated bacteria have a privilege: ability to thrive within plant tissues gives them, in turn, with several advantages, e.g., an environment with little competition, protection from environmental stresses and a reliable food source (**Madmony *et al.*, 2005**). **Heydenreich *et al.* (2011)** showed that Gram-positive and Gram-negative bacteria on the surface of grass pollen grains may serve as adjuvants by augmenting dendritic cells maturation and inflammatory Th1, Th2 and Th17 responses helping to initiate allergic immune responses.

Based on the aforesaid the aim of this study is to analyze the surface of *Pinus* pollen grains on the presence of microorganisms, identify them and conclude about allergenic potential of *Pinus* pollen known as pollen with low allergenicity.

MATERIALS AND METHODS

Selected *Pinus* species

Five *Pinus* species were selected for microbiological analysis. They are: Scots pine (*P. sylvestris* L. (PS)), Austrian pine also called European black pine (*P. nigra* Arnold (PN)), Dwarf pine (*P. mugo* Turra (PM)), Chinese white pine (*P. armandii* Franch. (PA)), Blue pine or Himalayan pine (*P. wallichiana* A.B. Jacks (PW)). *P. sylvestris*, *P. nigra*, *P. mugo* belong to the subgenus *Pinus*, *P. armandii* and *P. wallichiana* – to the subgenus *Strobus* (**Maňka *et al.*, 2013**).

Scots pine and dwarf pine represent the two principal species of pines indigenous to Slovakia. They distribute mainly allopatric in the country (**Kormuťák *et al.*, 2013**). *P. sylvestris* L. has been chosen as the main object of this study as it is the widespread species.

All trees are growing in the same natural conditions at the Botanical garden of the Slovak Agricultural University in Nitra.

Plant and pollen materials description

Unopened male cones were collected before they were ready to shed pollen aseptically in April-May 2015. Then they were dried at room temperature overnight. Pollen was collected the next day, keeping sterile conditions. Samples were placed in a cooler and conserved for a few days until analysis at +4°C. Since some *Pinus* species flowered with a difference of one month, the microbiological analysis of pollen samples was carried out when the plant material was collected.
Samples of the other organs of *Pinus sylvestris* L. trees were collected for a more detailed analysis. They are: male cones before, during and after flowering, young and mature female cones, needles. Also male cones during and after flowering were prepared for the other *Pinus* species. Altogether thirteen pine samples were collected.
All the trees are without visible damage.

Air samples

Air samples around the trees were studied for comparison the qualitative composition of the microbiota of trees and air. Air is not a medium in which microorganisms can develop, but has a carrier particulate matter, dust and droplet which can be weighed down with microbes (**Latha and Mohan, 2013**). "Koch-type" sedimentation method was used for sampling the air (**Tóth *et al.*, 2013**). Open Petri dishes with meat peptone agar medium (Imuna, Slovak Republic) and malt extract agar base (Biomark Laboratories, India) were placed under the studied tree's crown and exposed for 10 and 15 minutes. Air sampling was performed three times on different days. Altogether fourteen air samples were investigated.

Quantitative microbial analysis

One gram of each sample of pollen were diluted in 99 mL of sterile saline solution and mixed for 30 min by vigorous shaking in Rotamax 120 Orbital shaker (Heidolph, Germany) (175 rpm) at room temperature. Ten-fold serial dilutions of suspension were made up to 10^{-3}. The serial dilutions were inoculated on appropriate nutrient media. In whole every sample was cultivated on Petri dishes quadruplicate on six kinds of nutrient media. The formed colonies on the plates were counted and expressed as log colony forming units/g (log cfu/g).
Total mesophilic aerobic and anaerobic microbiota was determined by spreading 1 mL from the appropriate dilution into sterile Petri dishes to which meat peptone agar (Imuna, Slovak Republic) was poured. Plates were incubated aerobically at 25°C and counted after 48-72 h.
Total *Enterococcus* was determined in the same way but only Slanetz-Bartley medium was used (HiMedia, India). Plates were incubated at 37°C for 48 h.
Total coliforms: A 1 mL aliquot of the appropriate dilution were transferred into Petri plates and poured with violet red bile with lactose agar (Pronadisa, Spain). Plates were incubated at 37°C and colonies were counted after 24 h.
Lactobacilli were determined by spreading of 1 mL from the appropriate dilutions on MRS Agar Modified (HiMedia, India) and incubated at 37 °C for 48-72 h.
Total microscopic fungi and yeasts were determined by spreading 0.1 mL from appropriate dilutions on Sabouraud medium (Biomark Laboratories, India). Plates were incubated at 28°C and counted after 7 days.

Qualitative microbial analysis

Qualitative microbial analysis of plant materials was performed using MALDI-TOF Mass Spectrometry (Bruker Daltonics, Germany). MALDI-TOF MS is a rapid, reliable diagnostic tool for the identification of most microorganisms (Barberis et al. 2014). For identification, the Biotyper software compares the sample spectrum to its database of spectra generated using characterized isolates (DeMarco and Burnham 2014). After counting of the colonies best of them were selected for the identification. All isolates were pick out from the Petri dishes and transferred into 300 µL of distilled water in Eppendorf tubes. Then 900 µL of ethanol was added and the tubes were centrifuged for 2 minutes at 14,000 npm. The supernatant was carefully pipetted off and discarded. The same spin was repeated on the pellet. All remaining ethanol was removed, and the pellet was allowed to dry. Ten microliters of 70% formic acid was mixed with the pellet by pipetting and vortexing. Then 10 µL of acetonitrile was added. Tubes were centrifuged for 2 minutes at 14,000 npm and 1 µL of the supernatant was applied to the MALDI target. Once dry, every spot was overlaid with 1 µL of HCCA matrix and left to dry at room temperature before analysis. Spectra were generated and MALDI-TOF analyzed on the Microflex LT (Bruker Daltonics) instrument using Flex Control 3.4 software and Biotyper Realtime Classification 3.1 with BC specific software. Criteria for successful identification as proposed by the manufacturer were a confidence score of ≥2.0 for species level and ≥1.7 for genus level (**DeMarco and Burnham, 2014**).

Statistical analysis

Results were evaluated by standard techniques using MS Excel and Statistica 10. Mann-Whitney U-test was used for pairwise comparisons of the data.

RESULTS

Microbial content of *Pinus* trees samples is presented in Tables 1 and 2.

Table 1 Microbial enumeration of *Pinus* trees samples collected in Nitra, Slovak republic in 2015, log cfu/g

Plant part	Group of microorganisms	
	Total mesophilic aerobic counts	**Total mesophilic anaerobic counts**
Male cones PS BF[a]	2.70	0.00
Male cones PS DF	3.85	0.00
Male cones PN DF	6.02	0.00
Male cones PM DF	5.16	2.30
Male cones PS AF	3.48	0.00
Needles PS AF	3.00	0.00
Female cones mature PS DF	0.00	3.00
Female cones young PS AF	0.00	0.00
Pollen PS	5.66	0.00
Pollen PN	6.27	3.70
Pollen PM	4.23	0.00
Pollen PA	5.57	2.00
Pollen PW	6.02	0.00
	***Enterococcus* counts**	**Total coliforms**
Male cones PS BF	0.00	2.00
Male cones PS DF	0.00	2.00
Male cones PN DF	0.00	2.60
Male cones PM DF	0.00	2.70
Male cones PS AF	0.00	2.00
Needles PS AF	0.00	0.00
Female cones mature PS DF	0.00	0.00
Female cones young PS AF	0.00	0.00
Pollen PS	0.00	0.00
Pollen PN	0.00	5.29
Pollen PM	0.00	5.17
Pollen PA	0.00	3.48
Pollen PW	0.00	5.01
	Total lactobacilli counts	**Microscopic fungi and yeast**
Male cones PS BF	4.08	4.54
Male cones PS DF	3.30	4.63
Male cones PN DF	0.00	4.63
Male cones PM DF	3.60	4.99
Male cones PS AF	3.48	4.18
Needles PS AF	3.30	2.60
Female cones mature PS DF	3.85	3.95
Female cones young PS AF	4.20	3.00
Pollen PS	3.90	4.32
Pollen PN	0.00	3.69
Pollen PM	3.00	4.79
Pollen PA	0.00	5.03
Pollen PW	3.48	5.29

BF – before flowering; DF – during flowering; AF – after flowering

As can be seen from Table 1 the total content of mesophilic aerobic bacteria of the investigated samples is 0.00 (young and mature female cones of *P. sylvestris*) – 6.27 (pollen of *P. nigra*) log cfu/g. A higher level of the presence of aerobic bacteria on pollen and male cones compared with female cones and needles clearly expressed. Within this group of microorganisms *Pseudomonas asplenii, P. chlororaphis, P. corrugate, P. koreensis, P. orientalis, P. tolaasii, P. rhodesiae, P. fluorescens, P. libanensis, P. veronii, P. extremorientalis, P. trivialis, P. synxantha, P. grimontii, Bacillus flexus, B. licheniformis, Acinetobacter lwoffii, Arthrobacter* sp. were identified using MALDI-TOF (see Table 2). All these bacteria were determined only on pollen grains.
The level of contamination by mesophilic anaerobic bacteria in comparison with the aerobic bacteria is much lower: 0.00 (all samples, except male cones of *P. mugo*, mature female cones of *P. sylvestris*, pollen of *P. armandii*) – 3.70 (pollen of *P. nigra*) log cfu/g. Samples of *P. nigra* and also *P. armandii* are the most contaminated by mesophilic bacteria among all the samples of pollen. In this group of microorganisms was not possible to identify bacteria using MALDI-TOF on a reliable level.
Enterococci were not detected on investigated *Pinus* samples. It's a good result from the sanitary point of view.
Unfortunately, total coliforms were found out on samples at the level from 0.00 (young and mature female cones, needles and pollen of *P. sylvestris*) to enough

high – 5.29 (pollen of *P. nigra*) log cfu/g. Pollen is more contaminated by coliforms than male cones. *Pantoea agglomerans* was identified using MALDI-TOF within this group of microorganisms. This is the most common species on investigated *Pinus* samples. It was revealed on all pollen samples and male cones. Also *Klebsiella pneumonia* was identified on male cones of *P. sylvestris* before and during flowering.

The amount of lactobacilli varies from 0.00 (male cones of *P. nigra*, pollen of *P. nigra* and *P. armandii*) to 4.20 (young female cones of *P. sylvestris*) log cfu/g. In general, lactobacilli are much observed on other parts of the tree rather than on pollen. During the analysis it was found that this group of microorganisms to identify the hardest. It was possible to identify only unusual to this group of microorganisms.

Colonies of microscopic fungi and yeasts are most numerical. They were detected in all the samples, without exception. The fewest of microscopic fungi and yeasts were defined on the needles of *P. sylvestris* (2.60 log cfu/g), the greatest number – on the pollen *P. wallichiana* (5.29 log cfu/g). During flowering the level of microscopic fungi and yeasts is higher on parts of the tree involved in flowering, meaning male cones and pollen. A number of yeast colonies in comparison with the colonies of microscopic fungi is more numerical. Representatives of the genera *Penicillium, Aspergillus, Alternaria, Cladosporium, Rhodotorula* and unidentified genus *Mycelium sterillium* without creation fruiting bodies were determined microscopically. Then presence of *Penicillium chrysogenum, P. digitatum, P. italicum, P. roqueforti, P. expansum, P. commune, Cladosporium herbarum, Aspergillus oryzae, A. flavus, A. versicolor, A. parasiticus*, among yeast – *Debaryomyces hansenii* was confirmed by MALDI-TOF. Also, the presence of such yeasts like *Candida sorbosa, C. guilliermondii, C. dubliniensis, Lodderomyces elongisporus* have been revealed, but at the level of less than 1.7.

Table 2 The microbial species composition isolated from the parts of *Pinus* trees

№	Identified microorganism	Part of *Pinus* trees
1	*Pseudomonas asplenii*	Pollen PS
2	*Pseudomonas chlororaphis*	Pollen PS
3	*Pseudomonas corrugata*	Pollen PS
4	*Pseudomonas koreensis*	Pollen PS
5	*Pseudomonas orientalis*	Pollen PN
6	*Pseudomonas tolaasii*	Pollen PN, PS
7	*Pseudomonas rhodesiae*	Pollen PN
8	*Pseudomonas fluorescens*	Pollen PN
9	*Pseudomonas libanensis*	Pollen PN
10	*Pseudomonas veronii*	Pollen PN
11	*Pseudomonas extremorientalis*	Pollen PN
12	*Pseudomonas trivialis*	Pollen PN
13	*Pseudomonas synxantha*	Pollen PN
14	*Pseudomonas grimontii*	Pollen PN
15	*Bacillus flexus*	Pollen PW
16	*Bacillus licheniformis*	Pollen PS, PM, PW
17	*Acinetobacter lwoffii*	Pollen PM, PS, PA
18	*Arthrobacter* sp.	Pollen PA
19	*Pantoea agglomerans*	Pollen PS, PM, PW, PA, PN, male cones PS, PM, PN DF, male cones PS AF
20	*Klebsiella pneumonia*	Male cones PS BF and DF
21	*Penicillium chrysogenum*	Pollen PS, PM
22	*Penicillium digitatum*	Pollen PS, PM, PA
23	*Penicillium italicum*	Pollen PS, PM
24	*Penicillium roqueforti*	Pollen PM, PA, PS
25	*Penicillium expansum*	Pollen PA, PS
26	*Penicillium commune*	Pollen PA
27	*Cladosporium herbarum*	Pollen PS, PN, PM, PA, PW
28	*Aspergillus oryzae*	Pollen PA, PN, PW, PS
29	*Aspergillus flavus*	Pollen PN, PW, PA, PS
30	*Aspergillus versicolor*	Pollen PS
31	*Aspergillus parasiticus*	Pollen PS, PN, PA
32	*Debaryomyces hansenii*	Pollen PS, PN, PM, PA, PW, male cones PS DF, AF, mature female cones PS

BF – before flowering; DF – during flowering; AF – after flowering

As shown the microbiological and statistical analysis, samples of pine pollen are more contaminated by microorganisms than other parts of trees (see Table 2). There are no significant differences between the number of microorganism colonies of pollen and male cones during flowering period after paired comparison (see Figure 1). However, there was found a strong tendency to the predominance of anaerobic bacteria in the pollen, in comparison with the cones at the stage of flowering ($p=0.07$). It is also proved that the number of fungi and yeasts colonies on the male cones after flowering less than on pollen ($U=4$, $p=0.015$).

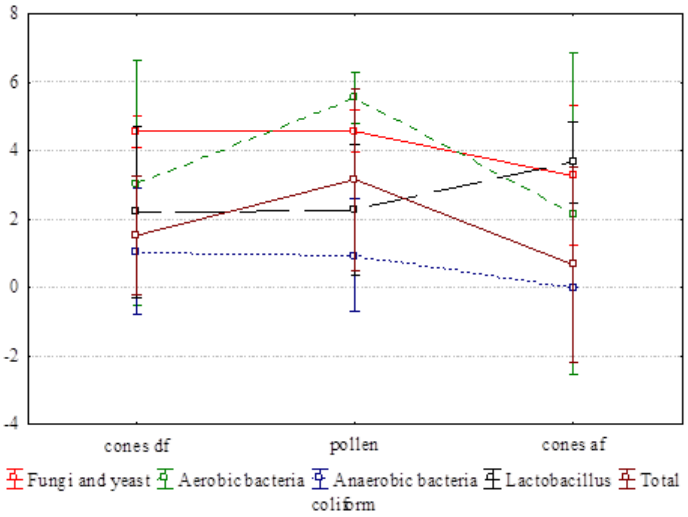

Figure 1 Comparison of the number of colonies of microorganisms groups on pollen and male cones of *Pinus* during and after flowering using Mann-Whitney U-test

Pollen of *P. sylvestris* has the most diverse composition of the microbiota. Of the 32 identified microorganisms 20 species of them belong to *P. sylvestris* samples, 15 to *P. nigra*, whereas 11 to *P. armandii*, 8 to *P. mugo*, only 6 to *P. wallichiana*. *Pantoea agglomerans, Debaryomyces hansenii* and *Cladosporium herbarum* are microorganisms the most commonly found on *Pinus* samples.

Bacillus licheniformis, Pantoea agglomerans, Penicillium commune, P. verrucosum, P. roqueforti, P. italicum, P. digitatum, P. expansum, P. lanosum, P. corylophilum, Mucor ramosissimus, M. circinelloides, Chaetomium funicola, Rhizopus stolonifer, Absidia coerulea, Alternaria alternata, Aureobasidium pullulans, Paecilomyces lilacinus, Cladosporium sp., unidentified genus *Mycelium sterillium* without creation fruiting bodies were found in the air samples around the pines at the Botanical garden. The composition of the microbiota of the air is quite diverse. According to data of the aerobiological station of Nitra region of the Slovak Republic concentrations of fungal spores were at level "very high" in the days of sampling the air (more than 150 particles per 1 m^3 of air). During the flowering season of *Pinus* only daily concentration of *Cladosporium* spores were at significant level in the Nitra region (values of average weekly concentrations of spores per 1 m^3 of air were in the range 27-141). The *Cladosporium* spores generally exceed all other airborne biologic particles in outdoor air (**Ozdemir, 2015**). Concentration levels of *Alternaria* (1-8/m^3), *Epicoccum* (0-3/m^3), *Stemphylium* (1-3/m^3), *Helminthosporium* (0/m^3) spores in the air of Nitra region were low or very low. Allergic relevant concentrations of *Pinus* pollen in the air was not observed (**www.alergia.sk**).

DISCUSSION

Thus, it has been detected and partially identified representatives of mesophilic aerobic and anaerobic bacteria, lactobacilli, coliform bacteria, microscopic fungi and yeasts on samples of pollen, male and female cones, needles of five representatives of *Pinus* species, that grow at the Botanical garden in Nitra, Slovakia. Microscopic fungi and yeasts are the largest group of microorganisms for the *Pinus* trees (mean=4.28 log cfu/g) and aerobic bacteria (4.00 log cfu/g), the least – anaerobes (0.85 log cfu/g). Young female cones are the least contaminated by microorganisms among all investigated parts of the tree (identified only representatives of lactobacilli, microscopic fungi and yeast). It is quite logical: young female cones are just beginning to take shape after pollination. There are enough nutrients on other organs of the tree for the bacteria. The presence of microscopic fungi and spores can be explained by the fact that fungi are the most common organisms in nature (**Latha and Mohan, 2013**). Moreover, pollen is better partner in the air for the airborne microorganisms (**Després *et al.*, 2012; Sapiňa *et al.*, 2013**). Mature last year's female cones and needles are also less attractive to microorganisms in comparison with pollen and male cones. Representatives of only three groups of microorganisms from the six examined were identified on it. Perhaps, this is because of the flowering period. Pollen abundantly releases, pour out on all parts of the plant, it is in the air and bacteria cannot not dealing with it.

There are differences among *Pinus* species in the quantitative composition of the microbiota of the male cones in the flowering period. These differences are most appreciable in quantitative composition of aerobes, anaerobes and lactobacilli. There are also species differences in the level of pollen microbial contamination. Pollen of *P. sylvestris* contaminated least by all groups of microorganisms (on average 2.78 log cfu/g). Pollen of *P. wallichiana* contaminated most (3.96 log cfu/g). The result is interesting because although pollen of *P. sylvestris* less contaminated quantitatively, the qualitative composition of it microbiota is most diverse. In the case of *P. wallichiana* pollen the result is opposite (see Table 2).

The level of contamination by all groups of microorganisms of *P. nigra* pollen is 3.79 log cfu/g, *P. mugo* – 3.44 log cfu/g, *P. armandii* – 3.22 log cfu/g. It was found the absence of representatives of microorganisms, at least, for two groups for all species of pollen.

With regard to the microbiota of *P. sylvestris* – species, for which the microbiological composition was done the most complete, quantitatively differences were revealed between the trees organs of this species. Thus, male cones most contaminated by coliform bacteria, fungi and yeast at different stages concerning the period of flowering, pollen – by aerobic bacteria, mature female cones – by anaerobic bacteria, young – by lactobacilli.

Quantitative microbiological analysis of *Pinus* trees and air samples near the trees showed that microscopic fungi, yeast, aerobic bacteria are the most widespread microorganisms during the flowering period of this genus. Their colonies are most often on the pollen and the male cones in the flowering period. And after this period there is a tendency to reduce them on the male cones. This indicates that the reason for the presence of microorganisms is pollen. Since pollen samples were investigated before their release into the environment, it is possible to assume that pollen contamination by these representatives occurs before the mass flowering. **Madmony *et al.* (2005)** also consider that different fungi and bacteria are located inside the pollen cells probably before the opening of the catkins. Likely the pollen contamination is caused by airborne microbiota encountered during pollen collection and handling. Content of air microbiota may be the proof. With regard to species differences in composition of the microbiota of pollen, the clear tendency for representatives of taxonomically related species subgenus *Pinus* and subgenus *Strobus* have not been identified. The representatives of these subgenera flowered with a difference of one month. Weather conditions were optimal. **Maňka *et al.* (2013)** presented results that show closeness of the pollen viability characteristics in taxonomically related species of pines growing on the same territory and shedding their pollen in the same period of time as compared with the comparing characteristics of pollen in taxonomically distant species shedding their pollen in different periods of the flowering time.

Based on the analysis of the quantitative composition of the microbiota of *Pinus* representatives can assume about an allergenic potential of pollen. Based on the analysis of qualitative composition the assumptions will be more thorough.

In general, 32 species of microorganisms belonging to the aerobic and coliform bacteria, fungi and yeasts were identified with MALDI-TOF. Their number is maybe even more diverse, but to confirm this on confidence level was not possible. Representatives of *Pseudomonas* (14 species), *Penicillium* (6 species) and *Aspergillus* (4 species) appeared the most various. In recent years, *Pseudomonas* strains have been studied with increasing interest due to their importance in medical, food and environmental microbiology and phytopathology (**Baïda *et al.*, 2002**). Genus *Pseudomonas* has environmental interest. Plant growth promoters, plant pathogens and xenobiotic degraders are among them. It is also one of the most important and best-studied bacterial taxa in soil (**Ivanova *et al.*, 2002; Bultreys *et al.*, 2003; Garbeva *et al.*, 2004; Moore *et al.*, 2006**). Really, 9 from 14 representatives of *Pseudomonas* genus can be found in the soil (**Brodey *et al.*, 1991; Bodelier and Laanbroek, 1997; Behrendt *et al.*, 2003; Kwon *et al.*, 2003; Haas and Keel, 2003**), other five were isolated from the aquatic ecosystems (**Elomari *et al.*, 1996; Dabboussi *et al.*, 1999; Baïda *et al.*, 2002; Ivanova *et al.*, 2002**). *Pseudomonas*, as different epiphytes, may influence plant productivity negatively, e.g., through induction of frost injury, or positively, e.g., by production of phytohormones that improve development. Phytopathogenic *Pseudomonas* species are distributed worldwide, causing diseases of most major groups of higher plants. They generally can be found only on diseased plants, in which they appear as relatively homogeneous populations when the pathological lesions are young (**Moore *et al.*, 2006**).

From the natural airborne allergens, pollen of grass and trees are the most important outdoor allergenic sources. Other important allergenic sources are airborne mould (**Benndorf *et al.*, 2008**). The genera of molds causing allergy and allergy-related problems most often are *Alternaria alternata, Cladosporium herbarium, Aspergillus fumigates* and *Penicillium* (**Ozdemir, 2015**). *Penicillium* species are among the most common fungi present in the environment. Observably, *Penicillium* is one of the most abundant fungal floras with the intention that there are 10^6–10^8 spores in one gram of normal soil and 10^4 spores in one milliliter of unpolluted groundwater. They are usually considered non-pathogenic to humans. *Penicillium* species can cause opportunistic infections (**Oshikata *et al.*, 2013**). *Aspergillus* infections have grown in importance in the recent years (**Hedayati *et al.*, 2007**). Several species of *Aspergillus* have been shown to be allergenic, including *A. fumigatus, A. niger, A. flavus* and *A. oryzae. A. oryzae* and *A. flavus* are the two species are so closely related. *A. flavus* is the second leading cause of invasive aspergillosis and it is the most common cause of superficial infection (**Hedayati *et al.*, 2007**). Aflatoxins, produced predominantly by fungi such as *A. flavus* and *A. parasiticus*, are among the most potent natural carcinogens known (**Yu *et al.*, 2003**). In humans there is cross-reactivity to *Aspergillus* and *Penicillium*: most sera from patients with precipitins against *Penicillium* have precipitins against *Aspergillus* (**Oshikata *et al.*, 2013**).

Also interesting representatives of the microbiota of anemophilous pine pollen is *Pantoea agglomerans*, *Pseudomonas libanensis*, *P. veronii*, *P. extremorientalis*, *P. grimontii* (**Špiewak *et al.*, 1996; Baïda *et al.*, 2002; Leclerc, 2003; Nam *et al.*, 2003; Egamberdieva, 2011**). *P. agglomerans* (formerly *Enterobacter agglomerans*) is phytopathogen causing human disease. **Špiewak *et al.* (1996)** showed that *P. agglomerans* is present on pollen grains as well as endotoxin – the bacterial product having strong immunomodulating properties. From the literature it is known that the abovementioned *Pseudomonas* were isolated from natural springs in Lebanon, Russia, France. *Pseudomonas extremorientalis*, *P. chlororaphis* and *P. veronii* have the ability to survive in ecologically stressed conditions, such as saline soils, contaminated soils with simple aromatic organic compounds (**Nam *et al.*, 2003; Egamberdieva, 2011**).

We classified representatives of identified microorganisms as to their pathogenic properties based on published data. Thus, eight microorganisms belong to phytopathogens (*Pseudomonas asplenii, P. corrugata, P. tolaasii, P. digitatum, P. italicum, P. expansum, C. herbarum, P. agglomerans*), 4 species to human pathogen (*P. fluorescens, K. pneumonia, P. chrysogenum, P. commune*), with possible pathogenic properties 6 microorganisms (*P. trivialis, A. lwoffii, A. oryzae, A. flavus, A. versicolor, A. parasiticus*), not pathogen – 5 (*P. chlororaphis, B. licheniformis, Arthrobacter* sp., *P. roqueforti, D. hansenii*), unknown pathogenicity – 9 representatives of identified species (*P. orientalis, P. koreensis, P. rhodesiae, P. libanensis, P. veronii, P. extremorientalis, P. synxantha, P. grimontii, B. flexus*,). So, according to literature data, 56% of the identified microorganisms in samples of *Pinus* representatives have pathogenic nature, 16% – not pathogenic and 28% – unknown. According to the «List of airborne pathogens, including allergenic, toxigenic, and suspected respiratory and non-respiratory pathogens» eight identified microorganisms (*K. pneumonia, Acinetobacter, P. chrysogenum, P. commune, P. expansum, A. flavus, A. versicolor, C. herbarum*) correspond to the list (**Breitenbach and Simon-Nobbe, 2002; Schwab *et al.*, 2004; Fomicheva *et al.*, 2006; Hedayati *et al.*, 2007; Debarry *et al.*, 2010**). Cases of allergic sensitization are known also for *A. oryzae* and *D. hansenii* (**Barbesgaard *et al.*, 1992; Yamamoto *et al.*, 2002**). Frequency of occurrence of allergenic species on pollen of *P. sylvestris* is 9 from 10, *P. armandii* – 8 from 10, *P. nigra*, *P. mugo* and *P. wallichiana* – 5 from 10. That is, theoretically, all the investigated pollen samples of *Pinus* carry allergenic stimuli on themselves.

Also among the identified microorganisms are those that have useful properties. For example, *Pseudomonas chlororaphis* (acting against various fungal plant pathogens by creating phenazine), *Pseudomonas synxantha* – a fluorescent rhizosphere bacterium with nematicidal properties, *Bacillus licheniformis*, that demonstrates antifungal activity by producing an antibiotic that acts against fungi, *Penicillium roqueforti* are used to produce compounds that can be employed as antibiotics, flavours and fragrances (**Wechter *et al.*, 2002; Rij *et al.*, 2004; Pringle, 2005; Ropars *et al.*, 2012**). Among «bad» microorganisms there are representatives also with useful properties. For example, *Aspergillus versicolor* is very effective at removing lead ions, *Aspergillus oryzae* is used in rice saccharification for sake brewing (**Kitamoto, 2002; Fomicheva *et al.*, 2006**).

On the basis of microbiological analysis, and analysis of literature data, making a conclusion about the potential allergenicity of *Pinus* pollen it can be assumed that pollen of *P. sylvestris* would most allergenic. Most of microorganisms were identified on these samples (20 of 32), the frequency of occurrence of allergenic species is maximal – 9 out of 10, but in general, the pollen of *P. sylvestris* least contaminated by microorganisms (2.78 log cfu/g), the third highest level of contamination by aerobic bacteria (5.66 log cfu/g), and the fourth – of contamination by fungi and yeasts (4.32 log cfu/g). The percentage of occurrence of allergenic species of microorganisms among identified should be consider as more important factor. It is 45% for *P. sylvestris* pollen. According to the same criteria pollen of *P. nigra* possess the minimum allergenic potential – 33.3%. This pollen sample is characterized by a variety of the presence of *Pseudomonas. P. mugo* (62.5%) and *P. armandii* (72.7%) have the average potential of allergenicity. Pollen of *P. wallichiana* has a minimum number of the identified microorganisms – only 6 out of 32, but it is more contaminated with microorganisms (3.96 log cfu/g), has the maximum values of the presence of fungi and yeast (5.29 log cfu/g), a second indicator of contamination by aerobic bacteria (6.02 log cfu/g), and 83% of the identified species of microorganisms among the presented on the pollen grains of *P. wallichiana* have the allergenic properties.

As a conclusion, available microbiota on pine pollen or associated with pines can be an addition factor of allergic sensibilization of sensitive persons. The gained results may be useful for aerobiologists, allergists and microbiologists, at least at the local level.

CONCLUSION

Microbiota of anemophilous *Pinus* pollen has been analyzed to evaluate it allergenic potential. Microbiota of other plant organs of *Pinus* trees has been examined to compare it with pollen. The content of revealed microbiota is multifarious: absence of representatives of *Enterococcus*, minimum mesophilic anaerobic bacteria and maximum microscopic fungi, yeast and mesophilic aerobic bacteria. Among them *Pseudomonas, Penicillium* and *Aspergillus* are most widespread on pine pollen grains. Among 32 identified microorganisms 56% of them have pathogenic nature and 16% – not pathogenic. All the investigated *Pinus* pollen contact with potentially allergenic species of

microorganisms, *P. wallichiana* mostly, *P. nigra* least of all. Based on literature data airborne *Pinus* pollen can be a contributing factor in development of respiratory allergy. For the development of the allergenic properties of pine pollen for sensitive person match a range of other environmental factors is necessary. For example, the mass distribution of the species, weather conditions, the negative impact of anthropogenic factors, etc.

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