Acta Chimica Slovaca, Vol.3, No.1, 2010, 95 - 102

Pollen microbial colonization and food safety

Ján Brindza[1], Ján Gróf[2] , Kamila Bacigálová[3], Peter Ferianc[4]. Dezider Tóth[1]

[1]Institute of Biodiversity Conservation and Biosafety, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Tr. A.Hlinku 2, 949 76 Nitra, Slovakia.
[2]Vega Konti Ltd., Hlohovecká 850, 925 53 Pata, Slovakia.
[3]Botanical Institute of the Slovak Academy of Sciences, Dúbravská cesta 14, 842 24 Bratislava, Slovakia.
[4]Institute of Molecular Biology of the Slovak Academy of Sciences, Dúbravská cesta 21, 845 51 Bratislava, Slovakia.

dezider.toth@uniag.sk

Abstract

Pollen samples collected in the spring of 2002 in 8 south-western Slovakia localities and 40 live individuals of bumblebee were analyzed for the presence of bacteria and microscopic fungi. Microorganisms occurring on pollen and bumblebees were identified using cultivation and microscopic methods supplemented with biochemical tests. In the pollen were found fermenting and non-fermenting Gram-negative rods, Gram-positive sporulating cocci and non-haemolytic Gram-positive cocci. Analyses of microscopic fungi on bumblebee bodies showed the presence of only four species - *Acremonium murorum, Aspergillus penicilloides, Fusarium oxysporum, Harpografium fasciculatum* representing Fungi imperfecti. The highest amount of microscopic fungi occurred on drone, lower numbers on queen and worker bees. In the pollen samples 21 fungal species forming 13 genera of microscopic fungi were detected. The highest number of mould species was classified in the genera of *Mucor, Rhizopus, Aspergillus, Alternaria* and *Paecilomyces*, the species of other genera occurred in lower frequency. As the majority of the identified micromycetes represent the mitosporic fungal group of saprophytic microorganisms inhabiting soil or the organic residues of plants lacking pathogenic effects, it could be concluded, the tested pollen samples may be declared for safe resource of food and/or feed.

Keywords: Bacteria, bumblebee, microscopic fungi, pollen microorganisms

Introduction

Pollen has a considerable potential for being used in increasing quantities as food and feed. Pollen contains proteins, carbohydrates, lipids, vitamins and minerals, moreover it is a rich source of free amino acids and, therefore, is appreciated even in human nutrition (Block et al. 1994). In some countries the bee pollen has been recognized as a medicine, e.g. by the German Federal Board of Health (Linskens and Jorde 1997). Pollen as food and medicine is traditionally used in Far East region – especially in China.

Taking into account the food safety aspects, there are coming into question many possible interactions of different importance between microorganisms - plant pollen – bee nutrition - pollination – plant production for food and feed. It is generally accepted, that the human survival is tightly bound with the global cycles of elements enabling the restoration of substrates for synthesis of essential compounds, thus keeping vital processes in progress. Concerning the nutritional aspects, there are several components fulfilling the well-being of mankind including the interaction microorganisms- plants- animals – man as well. In the pollination stage are currently often used bumblebee colonies. These are fed by bee collected pollen and grown for managed pollination in green-houses and open fields as well.

There are several estimations of UNO that the world population of 9-10 billion could be reached around 2050. It is evident that the Earth vegetation is one of the most decisive factors for mankind sustainable development. Coming to needs of vegetation itself – there is necessary to preserve globally the required amount of vegetation and carry for their healthy state and productivity. These requirements are closely connected with an efficient global management, cooperation of plant pathologists and support of the pollination processes.

The biotic pollinators include wasps, flies, butterflies, moths, beetles and other insects and birds, but the pollination dominance in many parts of the world is connected with honey bees and bumble bees (Proctor et al 1996). Around with the pollination the bees are collecting pollen as nutritive substrates what creates connection with the cross-interactions between the bees, pollen and the microorganisms occurring in the micro-environment. Bacteria and fungal spores could be deposited on flowers and on bees. As reported by Huang and Kokko (1985) pollen of snail-clover may carry the fungus *Verticillium alboatrum*. On bees, some fungal spores, such as *Ascosphaera apis* and other species of *Ascosphaera* genus could be found. These have been recorded by Anderson et al. (1998). Of course, different bacterial and fungal

species or their spores are incidentally transported during normal foraging, as documented in this contribution.

Materials and Methods

Corbicular pollen samples were collected in 8 localities of the south-western Slovakia during the spring of 2002, the bumble bees were supplied by the firm Vega Konti Ltd., Pata, Slovakia. The mixture of pollen samples was applied to bumble-bee colonies for their feeding. These samples contained prevailingly cereal and rape seed pollen with smaller fractions of oily squash, opium poppy and cornelian cherry pollen (Brindza et al. 2007a, 2007b, Tóth et al. 2007).

The bacterial strains were grown in 2% Peptone broth, Nutrient agar and/or on MacConkey, Slanetz-Bartley and Cetrimid agar selective media (Biomark Laboratories, Puna, India). Fractions of pollen sample (3 g) were extracted overnight in 5 ml of physiological mineral medium under continual mixing on shaker at 30^{o}C. The obtained suspension was inoculated (2 aliquots per 0.5 ml) on the surface of Nutrient agar – the grown colony forming units indicated the total number of bacteria. The sporulating bacteria were determined after inoculation of the suspension formerly heated for 20 minutes at 80^{o}C. Microorganisms occurring on pollen were identified using cultivation and microscopic observation supplemented with biochemical tests. To differentiate the fermenting and non-fermenting bacteria the NEFER and ENTERO tests (Lachema Brno, Czech Republic) were applied.

Isolation of micromycetes and their spores or hyphae from the bumblebee individuals based on extraction in 10 ml of 0.01 % Tween 80 on shaker (30 min). After sedimentation of solid particles 200 µl of extract was inoculated on agarized cultivation medium in Petri dish with 100 mm diameter. The micromycetes were cultivated on 2% Czapek-Dox, malt and/or sucrose malt agars at 25^{o}C for 8-20 days in the dark, then slide cultures and preparations were made in lactic acid solution. The samples were evaluated on Zeiss Amplival microscope (Carl Zeiss Jena, Germany) with microphotographic equipment. All assays were performed in triplicate. The fungal species were determined on the base of mycelium and spores morphology using the diagnostic literature (Domsch et al. 1980, Ellis 1993, Fassatiova 1979, Samson et al. 1981).

Results and Discussion

Bacteria and spores of microscopic fungi (conidia) could adhere to the bee's body surface as documented by Turner (1974) and Shaw (1993), therefore, the first tests were oriented on the microorganisms occurrence on bumblebee individuals. Results shown in Tables. 1 and 2 proved that the mean values for bacteria per bumblebee individual are 1630 and the mean for fungi only 345, respectively. Bumblebees could be taken as vectors transferring bacteria, yeasts and spores or hyphae of moulds between the environment and their colonies/hives, brood and products (Snowdon and Cliver 1996).

Numbers of microscopical fungi producing colony forming units on Czapek-Dox and malt agars showed for all tested bumblebee individuals except the queen bee significant differences, which apparently resulted from the presence of yeasts as indicated by CFU grown on malt agar (Tab. 2). The highest amount of microscopic fungi occurred on drone, lower numbers on queen bee and worker bee. The total number of bacteria estimated as colony forming units grown on nutrient agar medium related to 1 g of pollen sample ($CFU.g^{-1}$) ranged from 100 to 1250, the only exception being the elevated bacterial colonization (13 125) of pollen sample No. 3 (Tab. 3).

Tab. 1. Total number of bacteria detected on bumble bee individuals

Bumble bees	**CFU/bumble bee**
Queen bee	2250
Worker bee	1200
Drone	1500
Mean/bumble bee	1630

Tab. 2 Total number of microscopic fungi detected on bumble bees

Bumble bees	**CFU on Czapek-Dox Agar**	**CFU on Malt Agar**
Queen bee	231	225
Worker bee	110	267
Drone	694	159
Mean/bumble bee	345	217

Tab. 3. Number of bacteria and microscopic fungi detected on pollen samples

Pollen sample No.	Bacteria: CFU/g of pollen	Microscopic fungi: Ranges of CFU/g of pollen
1	300	630 - 1089
2	1100	566 - 1100
3	13125	2761 - 4688
4	1125	112 - 664
5	1250	606 – 911
6	1000	3733 - 3978
7	100	165 - 690
8	1200	107 - 343

Analyses of the micromycetes occurrence on pollen were more complex than in the case of bacteria, as the samples were subdivided into several fractions and these sub-samples were monitored individually. The highest pollen colonization by fungal spores has been found in samples Nos. 3 and 6. There is clear relevance with bacterial highest level in the pollen sample No. 3, although different levels were obtained with sample No. 6. Numbers of micromycetes found in four fractions of every pollen sample were mostly below 1 100, except of samples 3 and 6, where the maximum values were around 4 thousand. Evidently, the pollen sample No. 3 with increased microbial contamination could be collected from more polluted fields or in environment supporting growth of bacteria and/or fungi (e.g. higher values of water activity; availability of utilizable substrates).On bumblebee bodies were detected two bacterial groups – non-fermenting Gram-negative rods identified as *Agrobacterium radiobacter, Burkholderia cepacia,* Gram-positive sporulating bacteria (mostly of genus *Bacillus*) and non-haemolytic Gram-positive cocci (Tab. 4). Pathogenic bacteria were fully absent.

Exploration of bumblebee´s colonization by microscopic fungi showed the presence of only four species - *Acremonium murorum, Aspergillus penicilloides, Fusarium oxysporum, Harpografium fasciculatum* (Tab. 4) representing the group of mitosporic fungi (Fungi imperfecti). The bacterial isolates were further analysed exploiting several commercial biochemical tests. In the pollen were found fermenting and non-fermenting Gram-negative (G^-) rods, Gram-positive (G^+) sporulating cocci, non-haemolytic G^+ cocci and fermenting G^- rods were qualitatively differentiated on the base of their growth characteristics and biochemical traits as physiological groups.

Tab. 4 Bacterial and fungal species and groups detected on pollen samples and on bumble bee individuals.

Samples of	Bacteria	Microscopic fungi
Pollen no. 1	*Serratia marcescens* *Agrobacterium radiobacter* Fermenting and non-fermenting Gram-negative rods	*Alternaria chartarum* *Aspergillus flavipes* *Aureobasidium pullulans* (De Bary) Arnaud *Cladosporium sphaerospermum* Penz. *Humicola grisea* Traaen *Monodictys castanae* (Wallr) Hughes *Mucor racemosus* Fres. *Penicillium* sp.
Pollen no. 2	*Serratia marcescens* *Agrobacterium radiobacter* Fermenting and non-fermenting Gram-negative rods	*Aspergillus repens* (Corda) De Bary *Cladosporium sphaerospermum* Penz. *Mucor spinosus* Tiegh. *Paecilomyces varioti* Bainier. *Penicillium* sp. *Rhizopus arrhizus* A. Fisher *Rhizopus nigricans* Ehrenb.
Pollen no. 3	Gram-positive sporulating cocci Non-haemolytic Gram-positive cocci Fermenting and non-fermenting Gram-negative rods *Agrobacterium radiobacter*	*Alternaria alternata* (Fr.) Keissler *Aspergillus clavatus* Desm. *Mortierella* sp. *Mucor hiemalis* Wehmer *Mucor circinelloides* Tiegh. *Rhizopus nigricans* Ehrenb. *Trichosporiella hyalina* Kamyschko
Pollen no. 4	Fermenting and non-fermenting Gram-negative rods *Agrobacterium radiobacter*	*Harpografium fasciculatum* Sacc. *Mucor racemosus* Fres.
Pollen no. 5	Fermenting and non-fermenting Gram-negative rods *Agrobacterium radiobacter*	*Mucor racemosus* Fres. *Paecilomyces niveus* Stolk et Samson *Rhizopus arrhizus* A. Fisher
Pollen no. 6	Fermenting Gram-negative rods	*Aureobasidium pullulans* (De Bary) Arnaud
Pollen no. 7	Fermenting Gram-negative rods	*Mucor racemosus* Fres. *Rhizopus nigricans* Ehrenb. *Paecilomyces niveus* Stolk et Samson
Pollen no. 8	Fermenting Gram-negative rods	not identified
Bumble-bees	Non-fermenting Gram-negative rods *Agrobacterium radiobacter* *Burkholderia cepacia* Gram-positive sporulating bacteria *Bacillus sp.* Non-haemolytic Gram-positive cocci	*Acremonium murorum* (Corda) Gams *Aspergillus penicilloides* Speg. *Fusarium oxysporum* Schlecht. *Harpografium fasciculatum* Sacc.

In pollen samples nos. 1 and 2 several species of *Serratia* genus were identified including *Serratia marcescens*, which is an opportunistic pathogen and occasionally could be dangerous

for immunodepressive individuals. This bacterial species is often occurring on plants, anyway on the most pollen samples (nos. 3 – 8) it was absent. Among the bacterial isolates occupying the tested pollen samples species of *Agrobacterium radiobacter* and *Serratia marcescens* have been taxonomically identified.

In the pollen samples were identified 21 species of 13 genera of microscopic fungi totally. Most often there occurred species of *Mucor, Fusarium* (*Fusarium* sp.), *Rhizopus* (*Rhizopus arrhizus* A. Fischer, *Rhizopus nigricans* Ehrenb.) and *Aspergillus* (*Aspergillus flavipes, Aspergillus repens* (Corda) De Bary, *Aspergillus clavatus* Desm.). Over 62 % of the isolates were identified as species of *Mucor*, *Aspergillus*, *Alternaria*, *Rhizopus* and *Paecilomyces* genera. Similarly to results reported by Gillian et al. (1989), the highest share belonged to two genera – *Mucor* and *Aspergillus*, while differently from the above cited authors on lower lever was represented the genus *Penicillium.* Moulds detected on the pollen samples represent relative low number of species compared to other reports cited in the following sentence. Gilliam et al. (1989) isolated 148 different moulds from the almond pollen, Gonzales et al.(2005) found 116 fungal isolates in mixed pollen sample. Great majority of moulds isolated from pollen represented the mitosporic fungal group of saprophytic microorganisms inhabiting soil and organic residues of plants indicating the provenience of these microorganisms from the microenvironment. This statement is supported by the fact reported by Burri (1947), that pollen is germ-free in blossoms that have not opened or in opened blossoms if uncontaminated by insects or wind. So it would be advisable to collect pollen directly from the plant but that task is impossible to fulfil when taking into account any possible mass consumption of pollen. The second best possibility is to withdrawn the corbicular pollen from beehives collectors as often as possible – minimally on daily base. It was shown that pollen left at ambient temperature for longer time could be overgrown by microorganisms when the air is humid Lacey and Magan (1991). The pollen should be stored at low temperature in conditions excluding the possibility of microorganisms reproduction - in such case the bee collected pollen may keep an acceptable level of hygienic standards, allowing to exploit such product as food or feed under controlled conditions. In summary - bacteria and moulds as common microflora of pollen and pollinator insects have received little attention. Therefore this paper tried to stress some aspects of possible mutual effects between the environment, pollinators and pollen quality. Our results could help with

introduction of some new thoughts in the branch of pollen hygienic status, e.g. by elaboration of good manufacturing practices in the process of collection and processing of pollen.

Acknowledgement

This work was supported by the Ministry of Education of Slovak Republic under the contracts No. AV/1121/2004 and 1/0887/102010-2011 of VEGA project.

References

Anderson DL, Gibbs AJ, Gibson NL (1998) Mycological Research, 102:541–547

Block G, Sinha R, Gridley G (1994) American Journal of Clinical Nutrition 59: Suppl. 232-239

Brindza J, Miklošíková Z, Tóth D, Gróf J (2007a) In: Abstracts Book of Apimondia, No.378 (p.216) Apimondia 40th International Apicultural Congress, Melbourne

Brindza J, Tóth D, Gróf J (2007b) In: Abstracts Book of Apimondia, No.344 (p.196). Apimondia 40th International Apicultural Congress, Melbourne

Burri R (1947) Schweizerisches Bienen-Zeitung 1947:273-276

Domsch KH, Gam, W, Anderson TH (1980) Compendium of soil fungi. Academic Press, London-Toronto

Ellis MB (1993) Dematiaceous hyphomycetes. CAB International, London

Fassatiova O (1979) Plísně a vláknité houby v technické mikrobiologii, SNTL, Praha

Gilliam M, Prest DB, Lorenz BJ (1989) Apidology 20: 53-68

Gonzales G, Hinojo MJ, Mateo R, Medina A, Jimenez M (2005) International Journal of Food Microbiology 105: 1-9

Huang HC, Kokko EG (1985) Phytopathology 75: 859–865

Lacey J, Magan N (1991) In: Chelkowski J (Ed.) Cereal grain – mycotoxins, fungi and quality in drying and storage, (p.77-118). Elsevier Science, Amsterdam

Linskens HF, Jorde W (1997) Economic Botany 51: 77-78

Proctor M, Yeo, P, Lack A (1996) The Natural History of Pollination. Harper Collins Publishers, London

Samson RA, Hoekstra ES, Van Oorschot CAN (1981) Introduction to food-borne fungi. Centraalbureau voor Schimmelcultures, Baarn

Shaw DE (1993) Mycologist 7: 182–185

Snowdon JA, Cliver DO (1996) International Journal of Food Microbiology 31: 1-26

Tóth D, Brindza J, Grygorieva O, Klimenko S (2007) In: Abstracts Book of Apimondia, No.343, (p.196). Apimondia 40th International Apicultural Congress, Melbourne

Turner GJ (1974) Transaction of the British Mycological Society 62: 205–206