9th International Scientific Conference
RURAL DEVELOPMENT 2019
RESEARCH AND INNOVATION FOR BIOECONOMY
26-28 September 2019, Vytautas Magnus University Agriculture Academy, Lithuania

VYTAUTAS MAGNUS UNIVERSITY AGRICULTURE ACADEMY

Proceedings of the 9th International Scientific Conference Rural Development 2019

Edited by prof. Asta Raupelienė

ISSN 1822-3230 (Print)
ISSN 2345-0916 (Online)

Article DOI: http://doi.org/10.15544/RD.2019.004

FUNGI AND MYCOTOXINS IN FRESH BEE POLLEN

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The aim of the present study was to determine the concentration of microscopic fungi and selected mycotoxins in fresh bee pollen, stored for different periods. In the study, 12 pollen samples collected from the same apiary families were investigated. Bees have collected the pollen from different plants. The moisture content of fresh pollen varied between 14.2 and 22.7%. During studies, the most prevalent fungal genera of *Fusarium*, *Penicillium*, *Alternaria*, *Mucor* and yeast were found in fresh bee pollen. The amounts of microscopic fungi increased from 2.9×10^3 to 4.4×10^3 cfu g^{-1} as the pollen storage time increased. The significantly higest amounts of fungal colonies was determined after 3 days storage of undried pollen. The most significant *Fusarium* spp. increase (14.9%) was determined after 2 days of storage. The highest levels of mycotoxins ZEN and DON were determined after 3 days of pollen storage.

Keywords: *bee pollen, contamination, fungi, mycotoxins.*

INTRODUCTION

One of the most important criteria for a bee pollen quality standard is microbiological contamination, which is higly influenced by environmental factors that influence the development and reproduction of microscopic fungi and bacteria (Xue et al., 2014). The fresh bee pollen is very hygroscopic and therefore very moist. Therefore pollen is a rich medium for microorganisms to grow and multiply (DeGrandi-Hoffman et al., 2013). Microscopic fungi are very difficult to control under natural conditions (Filola et al., 2007). They are widespread in nature, have spores that are resistant to various environmental factors, wich not only enter in the bee pollen but can also enter in the honey (Popa et al., 2009).

Mycotoxicoses caused by fungal mycotoxins can be caused by the harmful effects of mycotoxins, which are secondary metabolites of fungi (Hani, 2012). Mycotoxin contaminated food may result in cytotoxic, neurotoxic, immunosuppressive, teratogenic, mutagenic and carcinogenic effects (Krnjaja et al., 2012). Fungi of *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium* and *Mucor* genera were identified as the etiologic factors of the above-mentioned diseases (Krysinska-Traczyk et al., 2001; Smith et al., 1994). Special attention should be given to toxins produced by *Fusarium* spp.(fusariotoxins). Deoxynivalenol (DON) and zearalenone (ZEA) produced by *F. culmorum and F. graminearum* are mycotoxins that cause a variety of health problems (Smith et al., 1994). The recovery of storage fungi (*Aspergillus* and *Penicillium*) in frech pollen presents a potential risk for human health, and must look resposibly of the post-harvest process (Nardoni et al., 2016).

Some of the factors that can influence bee pollen storage is water activity (a_w). He plays an important role, it is the amount of water needed for growth and development of microorganisms. In order to grow microorganisms, require a minimum a_w value. Yeasts and fungi are growing to a_w value over 0.61 (Jay et al., 2007). In the fresh collected bee pollen the water content is about 25% (Bogdanov, 2012), which represent a suitable substrate for the growth of microorganisms especially yeasts and fungi (Brinza et al., 2010). Bee pollen schould be dried after harvest to avoid adverse effects on microorganisms. The moisture content of dried bee pollen should not exceed 8% (Melo et al., 2011).

The quality of the bee pollen may result non-compliance with proper handling of the bee pollen after removal from the hives (Deveza et al., 2015). A very important moment is pollen collection from the traps. The quality of the bee pollen also depends on the time of storage (Gonzales et al., 2005). Improper storage of the pollen (increases humidity) can cause the development of mold and bacteria, which leads to the production of mycotoxins, causing poisoning in humans (Estevinho et al., 2011; de Arruda et al., 2013).

Bee pollen is used for human consumption as fresh, freezing and drying. However little is known on its safety related to microbiological hazards, not enough attention was given to the fungal contamination of bee pollen and to mycotoxins risks related to its human consumption (Mauriello, 2017; Nardoni, 2016).

The aim of the present study was to determine the concentration of microscopic fungi and selected mycotoxins in fresh bee pollen, stored for different periods.

RESEARCH METHODS

In the study, 12 pollen samples collected from the same apiary families were investigated. Bees have collected the pollen from different plants. Having removed the pollen on the same day, moisture content (%) of fresh pollen was determined and after 12 hours microbiological studies have been carried out. The collected fresh pollen samples (50 g) were stored at room temperature (20-23°C) in sterile paper bags for 1, 2 and 3 days.

The moisture content was determined by drying 5 g of samples in an oven at 105°C, until a constant weight.

The dilution method (CFU/g) was used to determine the number and amount of fungi at the surface and inside the sample (LST ISO 6611: 2004). Microscopic fungi were isolated by adding 10 g of ground pollen to 90 ml of physiological saline (NaCl, 8.5g/l) and shaking in a shaker for 15 min. (revolutions at 400 rpm). A series of dilutions were prepared from the resulting suspension. 1 ml of 10^{-2}, 10^{-3} and 10^{-4} suspensions were dispensed in Petri dishes (8 cm diameter) on which the Potato Dextrose Agar (PDA) medium was infused. For extracting the yeast, 0.1 ml of 10^{-1}, 10^{-2} and 10^{-3} suspensions were spread in Petri dishes over a suspended Saboraud Dextrose Agar (SDA) medium and dispersed with a Drigalski lens.

All cultures were performed in triplicate. Petri dishes with cultures for 5-7 days were maintained in a thermostat at 26 ± 2°C, and for the isolation of yeast the plates with cultures were maintained for 2-4 days, at 22°C. Grown fungi were counted and evaluated as colony-forming units per gram of bee pollen (CFU/g). Morphological features of the colonies of the raised fungi were evaluated macroscopically (colony occurrence) and examined by light microscopy. Separate fungal colonies were purified to monocultures and identified by cultural and morphological features based on various descriptors (Domsch et al., 1980; Ellis, 1971; 1976; Gerlach et al., 1982; Nelson et al., 1983).

The mycotoxins deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin (T-2) were analysed by ELISA (enzyme-linked immunosorbent assay) method (Wilkinson et al., 1992). The RIDASCREEN® quantitative test kits (R-Biopharm AG, Darmstadt, Germany), were used for the analysis. Mycotoxin extraction and tests were performed according to manufacturer's instructions. The optical densities of samples and controls from standard curve were estimated by a photometer Neogen Stat Fax®303 Plus, using filter of 450 nm. Measured absorbances were converted to the mycotoxin concentration units - $\mu g \times kg^{-1}$. The results were estimated taking into account the lowest calibration curve's mycotoxin concentration value (LOD-limit of detection), which is for: DON – 18.5 $\mu g \times kg^{-1}$ (ppb); ZEN – 17.0 $\mu g \times kg^{-1}$ (ppb); T-2 – 5.0 $\mu g \times kg^{-1}$ (ppb).

The significance of the differences between the treatments was evaluated applying *F* criteria and the least significant difference (LSD) test (Raudonius, 2017). The statistical analysis of the experimental data was performed using the software *ANOVA* from the package *SELEKCIJA*. Confidence limits were added at $P < 0.05$.

RESULTS AND DISSCUSIONS

The moisture content of fresh bee pollen ranged from 14.2 to 22.7% (Table 1). The significantly highest moisture content of pollen was found in the control after 5 hours after collection and after 1 day of storage, compared with the other treatments. After 3 days, the pollen humidity significantly decreased by 8.5%. The high water content in bee pollen is an ideal cultural medium for microorganisms.

The analysis of the microbiological state of fresh pollen maintained at different time periods showed that the amount of microscopic fungi starting increased with the duration of pollen storage from 2.9×10^3 to 4.4×10^3 cfu g^{-1} (Table 1). The significantly highest total amount of microscopic fungi was found after 3 days of retention of undried pollen and the fungal colonies amounted 4.4×10^3 cfu g^{-1}, the fungal colonies increased to 1.5×10^3 cfu g^{-1}, compared to the control.

Table 1. The total number of fungi and moisture content in fresh bee pollen samples

Storage duration	The moisture content %	Total number of fungi cfu g^{-1}
Control (5 hours)	22.7a	2.9×10^3b
After 1 day	22.6a	2.9×10^3b
After 2 days	18.5b	3.1×10^3b
After 3 days	14.2c	4.4×10^3a

Note. The differences between the averages of treatments, marked by different letter (a, b, c), are significant ($P < 0.05$).

During studies, the most prevalent fungal genera of *Fusarium*, *Penicillium*, *Alternaria*, *Mucor* and yeast were found in fresh bee pollen (Table 2). In addition to the dominant genera of fungi, the genera with a detection rate of 6% were isolated, namely the fungi of the *Acremonium, Cladosporium, Chaetomium, Paecilomyces, Aspergillus, Epiccocum* and *Trichoderma* genera. Great majority of identified fungi are classified as saprotrophic, living in soil and in plant

residues. *Fusarium*, *Alternaria*, *Cladosporium*, *Penicillium* and *Aspergillus* are often considered as the most prominent feed-borne filamentous fungi (Galik, 2007).

One of the major producers of mycotoxins *Fusarium* spp. the amount of fungi varied during the study period. The most significant increase (14.9%) was observed after 2 days of pollen retention and was 4.6% and 1.4% higher compared to 1 and 3 days, respectively. However mycotoxins content are not always related to the number of fungi presented (Tarr, 2006). *Fusarium* species were identified during the studies: *F. graminearum*, F. *sporotrichioides*, *F. poae*, *F. oxysporum. F. graminearum*. They was the most dominant species isoliated from all tested samples.

The highest amount of *Penicillium* spp. and *Alternaria* spp. fungi was in the pollen of control samples, 63.0% ir 38.2% respectively. After 1 day of storing amount of *Penicillium* spp. fungi increased 1.5% and this was the highest amount (64.5%) of fungi during study period. After storing pollen for 2 and 3 days, it was found that fungi had spread much less in comparison to the pollen removed after 5 hours. *Penicillium* and *Mucor* are the microorganisms which usually exist in honey (Kacainova et al., 2009). The *Alternaria* genus was detected in 38.2% of the pollen. Compared to pollen stored in traps for 5 hours, contamination after 3 days declined from 38.2 to 33.7%.

Other fungi of low interest regarding mycotoxins production were isolated. The amount of *Mucor* spp. increased depending with duration of storage. Compared to pollen stored for 5 hours, contamination after 3 days increased 8.6%. Yeast contaminated all samples of pollen. The significantly highest number of yeast was found in the control with the highest moisture content (22.7%). With the decrease of humidity in pollen, yeast significantly decreased accordingly.

Table 2. The prevalence of fungal genera in fresh bee pollen samples

Storage duration	Prevalence, %				
	Fusarium spp	*Penicillium* spp.	*Alternaria* spp.	*Mucor* spp.	Yeast
Control (5 hours)	11.2c	63.0a	38.2a	4.00c	10.4a
After 1 day	10.3c	64.5a	38.1a	3.90c	9.00b
After 2 days	14.9a	57.8b	34.3b	6.70b	8.20bc
After 3 days	13.5b	58.0b	33.7b	12.6a	7.50c

Note. The differences between the averages of treatments, marked by different letter (a, b, c), are significant ($P < 0.05$).

Results of mycotoxins (Table 3) indicate that samples of bee pollen were contaminated with mycotoxins. Mycotoxicological analysis by ELISA revealed the presence of zearalenone (ZEN) and deoxynivalenol (DON), with an average concentration of 70 µg kg^{-1} and 50 µg kg^{-1}, respectively. Highest levels of mycotoxins ZEN and DON were determined after 3 days of pollen storage. Zearalenone (ZEN) is mycoestrogen with limited toxicity that is produced by several *Fusarium* species: *F. graminearum*, *F. culmorum*, *F. crookwellense*, and *F. equiseti*. It is regularly present in crops and crop products. T-2 and deoxynivalenol belong to trichothecenes compounds, the sesquiterpenoid metabolites obtained after microbiological activity of several fungi from the following genera: *Fusarium* (primary source), *Trichoderma*, *Myrothecium*, *Phomopsis*. Together with ZEN, they were the most dominant mycotoxins in the bee pollen samples (Bennet, 2003).

Table 3. The concentration of mycotoxins in fresh bee pollen samples

Storage duration	The concentration of mycotoxins, µg kg^{-1}		
	Zearalenon (ZEN)	Deoxivalenol (DON)	T-2
Control (5 hours)	70	50	*<5
After 1 day	70	*<10	*<5
After 2 days	70	50	*<5
After 3 days	300	125	*<5

*Below detection limits

CONCLUSIONS

The moisture content of fresh pollen varied between 14.2 and 22.7%, fresh bee pollen chould be dried to reduce the levels of microbial contamination.

The total count of microorganisms in the pollen during the study period varied from 2.9×10^3 to 4.4×10^3 cfu g^{-1}. The significantly higest amount of fungal colonies was determined after 3 days retention of undried pollen.

The most significant *Fusarium* spp. increase (14.9%) was determined after 2 days of storage. The highest levels of mycotoxins ZEN and DON were determined after 3 days of pollen storage.

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