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CURRENT KNOWLEDGE ABOUT MICROORGANISMS USED IN BIOLOGICAL CONTROL OF PESTS AND THEIR RELATION TO BEES: A REVIEW

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ABSTRACT

The aim of the review is to describe a control of pests by microorganisms and its possible effect on bees, other pollinators and beekeeping. Biological control seems to be a natural way to solve the problem with pests in agriculture as an alternative to the use of pesticides. However, the proposed solution must be closely associated with the safety to pollinators, which are an important part of plant production as well as forest ecosystems. Entomopathogenic bacteria (mainly bacilli) and entomopathogenic fungi (e.g., *Beauveria bassiana* and *Metarhizium anisopliae*) are often used to suppress the pests in agriculture. The application of entomopathogenic microsporidia is controversial because their frequent representatives are pollinators' pests. Moreover, biocontrol can be applied in the form of pollinator strips near the fields with monocultures resulting in plant and pollinator protection. In some countries, bees are also used as biovectors of control agents for the plant protection in the fields. On the other hand, specific pests pose a threat to bees themselves in the hives. Varoosis is a problem in beekeeping all over the world. The suppression of bee pests using microorganisms was tested. An activity of *Beauveria bassiana* against *Varroa destructor* shows promising results. Surprisingly, *Beauveria bassiana* can be isolated from cadavers of *Galleria mellonella* larvae, another bee pest, which destroy wax combs. Therefore, understanding of various links between the organisms could be helpful for sustainable beekeeping. Overall, humans are more conscious that everything is connected to each other. Protecting agents designed on natural basis often possess excellent results in practice. Therefore, testing them is more than desirable.

Key words: entomopathogenic bacteria; *Galleria mellonella*; microscopic fungi; *Varroa destructor*

INTRODUCTION

Since the half of the 20th century, agriculture had used more intensively fertilizers and pesticides during the "green revolution". Desired results were an increase of harvest and more efficient use of land. Intensive agriculture has both advantages and disadvantages. A negative impact on food security,

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environment and human health has been also observed (Chaikasem and Na Roi-et, 2020; Ramadan *et al*., 2020; Shalaby *et al*., 2021; Zúñiga-Venegas *et al*., 2021). Some new strategies have been developed as a biological alternative aimed at the effectiveness against pests, but without harmful effects on the environment. They are described as IPM (Integrated Pest Management) (Ondráčková, 2015; Mandal, 2019; Vondruška *et al*., 2019; Francis *et al*., 2020; Singh *et al*., 2020).

In beekeeping, various stressors in terms of nutrition, environment and management affect the health and productivity of bees during the different stages of their life (Sperandio *et al*., 2019); There are two main connections of biocontrol of pests associated with beekeeping: i) bees as a part of IPM in plant production and ii) biological control of bee pests.

The review is aimed at the current research of biocontrol of pests using microorganisms, especially entomopathogenic bacteria, fungi and microsporidia, with the description of important representatives, mode of action, isolation as well as case studies in general and their effect on bees and/or other pollinators.

BEE AS A PART OF PLANT PRODUCTION AND FORESTRY

Beekeeping is a special sector of animal production, which is very closely linked to plant production because of pollination. Pollinators are also an important part of forest ecosystems. Each activity, including chemical substances used in agriculture, influences pollinators. On the other hand, farmers try to obtain optimal harvest of the crops by using plant protection preparations. Current efforts in EU agriculture are centred on sustainable technologies, including the protection of pollinators. Pollinating insects are crucial for the functioning of ecosystems, our food security as well as for medicines and our wellbeing (European Commission, 2021).

Integrated Pest Management (IPM)

Insects, pathogens, weeds and vertebrates are considered pests in plant production. Biological control is commonly based on natural enemies against pests. Natural enemies are predatory insects, parasitoids and microbial pathogens. Microbial biological control agents − MBCAs, ("biopesticides") suppress the pest and allow the adequate development of the crop. They are derived from a wide range of microorganisms such as bacteria, viruses and fungi including their metabolites, entomopathogenic nematodes and protozoa. (Ehler, 2006; Köhl *et al*., 2019a, b; Singh *et al*., 2019; Francis *et al*., 2020).

IPM is applied to a larger extent in countries with tropical climate in comparison to countries with temperate climate. However, there also exists a possibility of its greater use in temperate climate conditions in the future due to climate changes associated with the arrival of new pathogens and pests.

In general, biological agents are safer for wildlife than chemical pesticides (Dutka *et al*., 2015). Long-term effects, persistence in the environment and side effects on the entomofauna, including beneficials, such as entomophagous predators/parasitoids and pollinators, must be carefully evaluated for all products that will be developed (Zibaee and Malagoli, 2020; Francis *et al*., 2020). Risks in IPM should be a potential dominance of applied organisms in the environment resulting in negative effects of them or their metabolites on other organisms in natural environment (Köhl *et al*., 2019a).

Pollinator strips

Non-crop margins (called nectar margins, field margins, or buffer strips) or lines of bee plants in monoculture (called pollinator strips) can be beneficial in terms of bee forage diversity as well as protection of monoculture against pests. These important habitats for pollinators provide food for bees and living space for other beneficial insects and contribute to the reduction in use of chemical agents. Moreover, the insects also support other important wildlife species in agroecosystems, such as wild birds, and help the farm ecosystem to thrive (Henderson, 2020).

Volatile compounds and extrafloral nectar are common defence mechanisms of wild plants. However, they bear in crops an as-yet underused potential for biological control of pests. Natural plant volatiles with antifungal or repellent properties can serve as direct resistant agents (Stenberg *et al*., 2015).

Pollinator strips can also act as a natural way for the presence of beneficial microorganisms. Francis *et al*. (2020) divided microorganisms associated with an indirect biological pest control into the following five groups according to their origin:

i) rhizosphere microorganisms (soil microbiome),
ii) phyllosphere microorganisms (from surface of plants),
iii) endophytes (from inside of plant tissues),
iv) insect microbes, v) nectar and honeydew microbes.

Bee vectoring technology

Bees can also act as a biovector to control pests in crops. They can transport biological control agents to control weeds, plants' pathogens or insect pests in the fields as they are able to carry microscopic particles (Kevan *et al*., 2008). Honey bee (or also bumble bee) hives can be fitted with special dispensers of biological control agents (bacteria, fungi, viruses) that are antagonistic to microbial pathogens and pest insects (Kevan *et al*., 2003). Maebe *et al*. (2021) tested a commercial preparation on the basis of *Bacillus amyloliquefaciens* (ex Fukumoto) Priest *et al*.[a] in apple and pear trees using bumble bees *Bombus terrestris* Linnaeus[c] and mason bees *Osmia bicornis* Linnaneus[c], *O. cornuta* Latreille[c] as entomovectors delivering successfully the active substance to untreated trees. Joshi *et al*. (2020) also confirmed a good ability of Japanese orchard bee *Osmia cornifrons* Radoszkowski[c] to deliver the drug. In both researches, the aim of the treatment was to reduce the bacterium *Erwinia amylovora* (Burrill) Winslow *et al*. (Approved Lists)[a], which causes the fire blight, a serious disease in apple and pear orchards.

PESTS IN BEEKEEPING

Shimanuki and Knox (2000) indicated the wax month (*Galleria mellonella* Linnaeus[c], *Achroia grisella* Fabricius[c], *Ephestia kuehniella* Zeller[c]), small hive beetle (*Aethina tumida* Murray[c]) and the bee-louse (*Braula coeca* Nitzsch[c]) as the honey bee pests. Abou-Shaara and Staroň (2019) also included *Varroa destructor* Anderson and Trueman[c] mites (Figure 1), *Vespa* Linnaeus[c] hornets and parasitic flies into the group of honey bee pests. They stated that while pests are controlled by using chemical methods, which cause some negative effects on honey bees and contaminate their products, the use of biological control agents is promising and has no serious hazards. Currently, the ectoparasitic honey bee mite *Varroa destructor* is considered to be the major threat for apiculture (Rosenkranz *et al*., 2010; Giacobino *et al*., 2017; Steinhauer *et al*., 2018), including Slovakia (Chlebo, 2017).

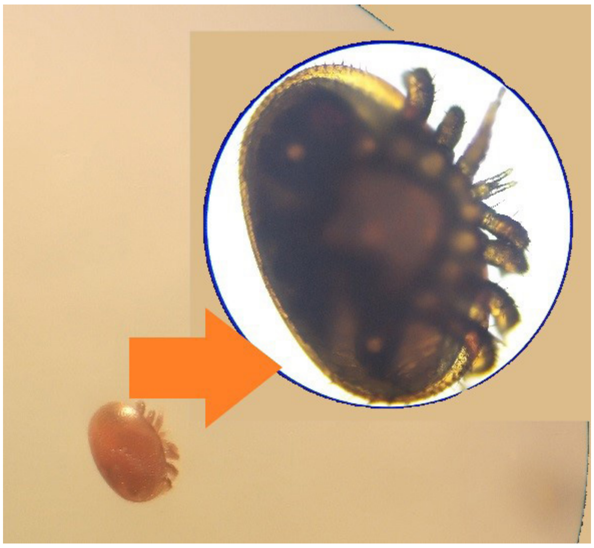

Figure 1. *Varroa destructor* under the macro-lens (left down) **and microscope** (right up) (photo: V. Kňazovická, 2020)

ENTOMOPATHOGENIC BACTERIA (EPBs)

Characterization and important representatives

The term "entomopathogenic" refers to those microorganisms that can attack insects or use them as hosts to develop a part of their life cycle (Singh *et al*., 2020). Bacteria pathogenic to insects can be found in a variety of habitats worldwide, including water, soil, plants and animals (Fisher and Garczynski, 2012). In biocontrol, bacilli are often used. They originated from soil, where they dominate and most of them are pathogenic for plants.

According to Ruiu (2015), entomopathogenic bacteria belong to the following families and classes: families *Bacillaceae*: *Bacillus thuringiensis* Berliner[a], *Lysinibacillus sphaericus* (Mayer and Neide) Ahmed *et al*.[a], Paenibacillus Ash *et al*.[a] spp., *Brevibacillus laterosporus* (Laubach) Shida *et al*.[a]; *Clostridiaceae: Clostridium bifermentans* (Weinberg and Séguin) Bergey *et al.*[a]; classes *Gammaproteobacteria: Photorhabdus* Boemare *et al*.[a] spp., *Xenorhabdus* Thomas and Poinar[a] spp., *Serratia* Bizio[a] spp., *Yersinia entomophaga* Hurst *et al*.[a], *Pseudomonas entomophila* Mulet et al.[a]; *Betaproteobacteria: Burkholderia* Yabuuchi *et al*.[a] spp., *Chromobacterium* Bergonzini[a] spp.; and phylum *Actinobacteria: Streptomyces* Waksman and Henrici[a] spp. and *Saccharopolyspora spinosa* Mertz and Yao[a].

The most well-known example of entomopathogenic bacteria used in the fields is *Bacillus thuringiensis* (*Bt*), a Gram-positive spore-forming bacterium (Singh *et al*., 2019). *Lysinibacillus* spp. and *Brevibacillus* spp. have similar properties as a *Bt* (O'Callaghan *et al*., 2012).

Mode of action

The insecticidal activity of *Bt* is based on *Cry* toxins and acts against the species of Lepidoptera, Coleoptera and Diptera (Lacey and Siegel, 2000; Chakroun *et al*., 2016). The pathogenic action of this bacterium normally occurs after the ingestion of spores and crystalline inclusions containing insecticidal δ-endotoxins that specifically interact with receptors in the insect midgut epithelial cells (Pigott and Ellar, 2007; Ruiu, 2015). *Cry* toxins of *Bt* possess the possible health impact on vertebrates, particularly because they might be associated with immune-activating or allergic responses (Rubio-Infante and Moreno-Fierros, 2016).

Except of *Cry* or *Cyt* proteins, Chakroun *et al*. (2016) also described another proteins – *Vip* (vegetative insecticidal proteins), which are divided into four families according to their amino acid identity:

- *Vip 1* component binds to receptors in the membrane of the insect midgut;
- *Vip 2* component enters the cell, where it displays its ADP-ribosyltransferase activity against actin preventing microfilament formation, and *Vip 1* and *Vip 2* proteins act as a binary toxin against Coleoptera;
- *Vip 3* is similar to *Cry* proteins in terms of proteolytic activation binding to the midgut epithelial membrane and pore formation, and *Vip 3* is toxic to Lepidoptera;
- *Vip 4* has been recently reported and its activity against target insect is still unknown.

Regaiolo *et al*. (2020) studied the bacterium *Photorhabdus luminescens* (Thomas and Poinar) Boemare *et al.*[a], which lives in symbiosis with entomopathogenic nematodes. They are highly pathogenic towards insects. The study showed that *P. luminescens* is adapted in the rhizosphere. The analysis highlighted genes involved in chitin degradation, biofilm regulation, formation of flagella and secretion system. Furthermore, they provided evidence that *P. luminescens* can inhibit the growth of phytopathogenic fungi.

Isolation

Fisher and Garczynski (2012) described the isolation, cultivation and preservation of entomopathogenic bacilli. They stated that entomopathogenic bacteria must be isolated from the soil, insect or water as fast as possible. The first processing is different depending on the sort of material. The soil (2-4 g) is homogenized with 10 mL of sterile distilled water. The insect (0.2-0.4 g of abdominal part) is homogenized with 1 mL of sterile distilled water and 0.5 % Tween 80. The water is filtered (through 0.22 µm filter) to concentrate the bacteria and the filter is placed in a tube with 10 mL of sterile distilled water. Next, the heat treatment is performed at 80 °C for 10 min followed by chilling on ice. The processed material is inoculated onto agar (e.g. MBS, Nutrient agar, UG or no. 17) plates and cultivated at 30 °C for 24 h. Then, the culture is transferred by a sterile loop to tubes with a growth broth and cultivated on a shaker (at 250 rpm) at 30 °C for 48 h. Bacterial cultures can be cryopreserved by freezing with glycerol (830 µL of cultured bacteria + 170 µL of sterile glycerol).

Case studies in general

Entomopathogenic spore-forming bacteria, mainly *Bt*, are widely used against several insect pests in crops, forest and aquatic habitats because of the broad spectrum of susceptible hosts, production on artificial media and the ease of application using conventional equipment (Lacey and Siegel, 2000). *Bt* pesticides are available as formulated spray-able products of bacterial spores and endotoxin crystals. They are used on broad planted crops. A high level of selectivity and safety is required when they are sprayed on fruits and vegetables (Singh *et al*., 2019). Fuentealba *et al*. (2015) found that *Bt* is effective against the budworm in spruce trees. As indicated by Tancik and Cagáň (1998), the preparation based on *Bt* could be very efficient in controlling the European corn borer (*Ostrinia nubilalis* Hűbner[c]). However, its effect usually varied. The effectiveness of preparation based on *Bt* depended on outside factors as well as on the method of application and application time. In the same experiments in South-Western Slovakia, *Bt'* formulations were as efficient as pyrethroids in 1993 and 1994, but not enough efficient in 1995. *Bt* has also been tested in terms of bioremediation and was effective in degradation of ibuprofen, whose intake has increased in recent years (Marckhlewicz *et al*., 2017). L*ysinibacillus sphaericus* or *Clostridium bifermentas* are effective against mosquitoes or blackflies (Ruiu, 2015).

Influence on bees

Some organisms used in IPM can have negative effects on pollinators. Bacteria *Photorhabdus luminescens* live in symbiosis with entomopathogenic nematodes in the rhizosphere (Regaiolo *et al*., 2020). Dutka *et al*. (2015) observed a high mortality in bumble bee *Bombus terrestris* Linnaeus[c] colonies caused by entomopathogenic nematodes (*Heterorhabditis* Poinar[c] spp. and *Steinernema* Travassos[c] spp.).

Concerning bacterial bee pathogens, the most serious is *Paenibacillus larvae*, a causative agent of American foulbrood (AFB) (Shimanuki and Knox, 2000). Interestingly, *Paenibacillus* spp. are common in bee environment. However, under the certain conditions, *P. larvae* convert from the form of spore to the vegetative form, live an active life and can cause the most devastating disease of bee brood. Adult honey bees are resistant to AFB. *P. larvae* spores do not germinate in their digestive tract but the spores maintain their viability and germinate in the midgut of bee larvae (Dingman and Stahly, 1983). Other bacterial bee pathogens are *Melissococcus pluton* (ex White) Bailey and Collins[a] with associated microbiota, *Pseudomonas aeruginosa* (Schroeter) Migula (Approved Lists)[a] and *Spiroplasma* Saglio *et al*.[a] spp. (Shimanuki and Knox, 2000).

Bt, EPB extended in practice, has a practical application in beekeeping as well. Beekeepers try to prevent the stored combs from damage caused by wax moths. For this purpose, they sort the combs and/or use a chemical way, e.g. sulphur wicks. However, it is possible to use *Bt* for biocontrol of wax moths (Ahmad *et al*., 1994). Commercial protective agents based on *Bt* are designed for spraying stored combs (McKillup and Brown, 1991).

ENTOMOPATHOGENIC FUNGI (EPFs)

Characterization and important representatives

Entomopathogenic fungi (EPFs) are a group of phylogenetically diverse, heterotrophic, eukaryotic, unicellular or multicellular (filamentous) microorganisms that reproduce via sexual or asexual spores or both (Singh *et al*., 2020). They are used in microbial control to prevent arthropod species in cultivated lands (Altinok *et al*., 2019).

EPFs have evolved from the "calcinaccio" described from the *Beauveria bassiana*-covered spore caterpillars, by the "Father of Insect Pathology" Agostino Bassi (Davidson 2012, cit. Barra-Bucarei *et al*., 2019). Important EPFs species are *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin[b], *Metarhizium anisopliae* (Metschn.) Sorokin[b], *Lecanicillium lecanii* (Zimm.) Zare and Gams[b], *Isaria fumosorosea* Wize[b] and *Purpureocillium lilacinum* (Thom) Luangsa-ard *et al*.[b] (Altinok *et al*., 2019). *Beauveria bassiana* and *Metarhizium anisopliae* are well-known in biocontrol.

Mode of action

EPFs are typically applied as contact insecticides (Francis *et al*., 2020). They use chitin, the main component of insects' exoskeleton, as a source of carbon (Barra-Bucarei *et al*., 2019) and act as plant growth promoters (Mantzoukas and Eliopoulos, 2020). The life cycle is divided into a parasitic phase, which starts with the infection and lasts until the host dies, and a saprophytic phase, which takes place after the death of the insect (Barra-Bucarei *et al*., 2019). The mode of action of EPFs was described by Zimmermann (2007) in six steps:

1. Attachment – conidia adheres to the cuticle using a hydrophobic interaction and specialized adhesion proteins;
2. Germination and appressoria formation;
3. Penetration through the cuticle – mechanical, aided by the production of enzymes (including proteases, chitinases and lipases);
4. Overcoming host defences and the production of novel destruxins;
5. Proliferation within the host – generally via the production of blastospores or hyphae;
6. Outgrowth and production of new infective conidia.

Hyphae development and multiplying in insect body and blood cells cause the death of the insect (Altinok *et al*., 2019), which can occur from the second day after infection and initiating the second phase of the cycle (Barra-Bucarei *et al*., 2019).

EPFs can be used by foliar, stem, root or seed applications. Soil characteristics can enhance or inhibit the endophytic action of EPFs (Mantzoukas and Eliopoulos, 2020). EPFs represent potential risks to immune-depressive people (Singh *et al*., 2020).

Isolation

EPFs can be isolated from the soil, plant tissue (e.g. leaf, stem) or insect cadavers. If the isolation is from the soil, the top layer is removed and the soil from approximately 10-15 cm depth is obtained. Then the larvae of *Galleria mellonella* are placed into the soil and cultivated under specific conditions for 14 days. When the larvae are dead, the cadavers are transferred to Petri dishes on filtration paper and fungi will grow from them. The larvae of a greater wax moth *Galleria mellonella* or a large flour beetle *Tribolium destructor* Uyttenboogaart[c] (Coleoptera: Tenebrionidae) are the most commonly used for the cultivation of EPFs. Firstly, the surface of plant tissue/cadavers is sterilized by 70 % ethanol or 1-5 % sodium hypochlorite. Then it is rinsed 3 times with distilled water. The water from the third rinse is inoculated into Petri dishes. If the water is free of microorganisms, the fungi from the material are cultivated on dishes at 20-25 °C for 3-7 days (Inglis *et al*., 2012).

Case studies in general

Vondruška *et al*. (2019) stated that preparations of EPFs are used in EU agriculture only marginally, mainly in horticulture. They indicated that practical application was tested, for example, in orchards, grassland (Switzerland) and greenhouse cultures (Netherlands), where the main part of available preparations is used.

On the other hand, there are various studies of EPFs applied e.g. on bean or maize fields in countries of tropical climate. Mutune *et al*. (2016) observed a bean stem maggot (BSM) *Ophiomyia phaseoli* Tryon[c], which attacks seedlings of *Phaseolus vulgaris* in Africa with serious effects on its production. They found out that using the fungi from genera *Metarhizium*, *Beauveria*, *Hypocrea* Fr.[b] and *Trichoderma* Pers.[b] resulted in the reduction of BSM feeding, oviposition, pupation and emergence. Ondráčková (2015) observed that some strains of genera *Lecanicillium*, *Isaria* and *Beauveria* showed the efficacy against adults of bean weevil, but did not prevent the reproduction of adults. Ramos *et al*. (2020) confirmed the effectiveness of EPFs (*Beauveria bassiana* with *Metarhizium anisopliae*) against the fall armyworm *Spodoptera frugiperda* (J. E. Smith)[c] in maize production in Cuba. Moreover, Ramos *et al*. (2017) evaluated *Beauveria bassiana* on bean fields and came to the conclusion that these endophytes occur naturally in higher amounts in organic fields compared to conventional fields. Consequently, organic fields are natural reservoirs of enemies against the pests of beans.

The mortality of the European corn borer (*Ostrinia nubilalis*) larvae was very low when the preparation containing *Beauveria bassiana* was used in an experiment in South-Western Slovakia in 1993-1995 (Cagáň *et al*., 1995; Tancik and Cagáň, 1997). Concerning the experiment carried out in South-Western Slovakia in 2004, the effectivity of native isolates of *B. bassiana* in the control of *O. nubilalis* was 45.9-60.1 %, and it was 0-33 % in the control of the adults of the Western corn rootworm, *Diabrotica virgifera virgifera* LeConte[c] (Cagáň *et al*., 2005). The conidial suspension *Beauveria bassiana* with the concentration of 10^7 conidia per ml was used for the virulence test against the fourth instar larvae of *O. nubilalis* at the temperature of 25 °C. After 14 days, the mortality of larvae was in the range 34-96 %. The median lethal time LT50 was estimated on 5.5-21.3 days (Medo *et al*., 2021).

In terms of forestry, Hricáková and Hleba (2019) mentioned that fungal genus *Beauveria* showed the most effective virulence in the fight with the spruce bark beetle, *Ips typographus* (C. Linnaeus)[c]. Jakuš and Blaženec (2011) tested the biopreparation based on *B. bassiana* against the spruce bark beetle in Slovak spruce stands and found out that the results of biopreparation were comparable to the variant using the insecticide. Mudrončeková *et al*. (2013) tested *B. bassiana* and *M. anisopliae* isolates from various places of the High Tatras in Petri dishes and determined high mortality (97-99 %) of spruce bark beetle.

Influence on bees

Using EPFs is expected for a short-term pest control. However, a long-term persistence is also observed depending on habitat conditions (Francis *et al*., 2020). Residues of EPFs have no known adverse effects on the environment (Singh *et al*., 2020). The advantage of EPFs is little likelihood of insect resistance development to them and their disadvantage is that the efficiency of fungi against pests is dependent on environmental conditions, particularly on temperature and humidity (Ondráčková, 2015). In general, *Beauveria bassiana* has no harmful effects on non-target organisms (Jakuš and Blaženec, 2011).

On the other hand, Foote *et al*. (2020) recorded that wild bee communities may benefit from changes in the forest structure following bark beetle outbreaks. Maybe, they thought that the activity of bark beetle results in the formation of trees' cavities, which serve as a suitable living space for bees in forests. A similar example, when the process is not so good for one group (fruit or fruiterers) but is beneficial for the second group (drosophilae, wasps and then bees), is collecting the fruit juice by bees (Staroň, 2020, Figure 2). Interestingly, this process is managed by microorganisms − yeasts. Bellutti *et al*. (2018) evaluated the egg-laying behaviour of fruit flies *Drosophila suzukii* Matsumura[c], an invasive pest species that damages unwounded, healthy fruit. They observed that the number of eggs laid by flies increased on cherry fruits artificially colonised with *Candida* Berkhout[b] spp. and *Saccharomyces cerevisiae* (Desm.) Meyen[b,c], and concluded that these findings can be useful for improving both attract-and-kill technologies and mass rearing of *D. suzukii*. De Medeiros and Da Silva (2019) mentioned

Figure 2. Bees on raspberries (photo: M. Nábělková, 2020)

that some agents consume free nutrients on the plant and scavenge them from the pathogen; for instance, certain yeasts protect fruits from postharvest infection caused by *Botrytis cinerea* Pers.[b, c].

Concerning filamentous fungi, there are two main bee pathogens. The fungus *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive and Spiltoir[b] is a causative agent of chalkbrood resulting in chalklike mummy of bee larvae and *Aspergillus flavus* Link[b] (associated with *A. fumigatus* Fresenius[b] or *A. niger* van Tieghem[b]), which causes stonebrood (Shimanuki and Knox, 2000). Yaremenko *et al*. (2020) stated that *Ascosphaera apis* is a dangerous EPF and investigated a fungicide activity of peroxidases from *Bombus terrestris* against this fungal bee pathogen.

Another EPFs can probably be applied in the control of bee pests successfully. In terms of biological control of varoosis, it seems that the use of entomopathogenic fungi is more effective and practical than predators, e.g., pseudoscorpions (Abou-Shaara and Staroň, 2019). Ditrich (2021) considered that in practice, a suitable candidate from EPFs will not be able to kill the varroa alone. However, it could be a useful helper together with other actions.

Poidatz *et al*. (2019) discovered *Beauveria bassiana* as naturally parasitizing fungi in hornet *Vespa velutina* Buysson[c], a predator of bees that was accidentally introduced in Europe from China in 2004 and suggested to consider its use in biocontrol. In Russia, wax moth larvae became more susceptible to fungal infections after envenomation by the ectoparasitoid *Habrobracon hebetor* Say[c] – a wasp (Polenogova *et al*., 2019).

ENTOMOPATHOGENIC MICROSPORIDIA (EPMs)

Characterization and important representatives

Microsporidia, microscopic organisms closely related to the Fungi, are primary pathogens of many aquatic and terrestrial insect species and have important roles in insect population dynamics and managed insect disease. Moreover, entomopathogenic Microsporidia (EPMs) are important regulatory factors in the population dynamics of insect pests (Solter *et al*., 2012; Bjornson and Oi, 2014).

Representatives of the relatively controversial Microsporidia group are also used in pest control (Solter *et al*., 2012). Only for the last fifteen years, microsporidian genes have been analysed to the extent that the placement of this eukaryotic pathogen group in the Protozoa by Balbiani (1882) was seriously questioned. A recent molecular research has placed the Microsporidia within the kingdom of Fungi (Hirt *et al*., 1999; James *et al*. 2006). Another controversy of the group lies in the fact that Microsporidia are important primary pathogens of both pest and beneficial insects. To date, this significant but lesser-known group includes only a few hundred of the species described, with each species within insects or other arthropods being assumed to have its own specific species of parasite from the Microsporidia. There are known several microsporidian species that have been globally introduced as biological control agents, e.g. *Amblyospora connecticus* Andreadis[c], *Paranosema locustae* (Canning) Sokolova *et al.*[c], *Kneallhazia solenopsae* (Knell *et al*.) Sokolova and Fuxa[c], *Vairimorpha invictae* Jouvenaz and Ellis[c], *Vairimorpha disparis* (Timofeja) Vavra *et al*.[c] (Solter *et al*., 2012; Bjornson and Oi, 2014).

Mode of action

Microsporidian species do not reproduce as free-living organisms. The life cycle varies for individual groups and for species. However, the basic mechanism is the same or very similar for the majority of microsporidia representatives. They have only a simple asexual phase or a complex life cycle that consists of both asexual and sexual phases (Ironside, 2007). Host organisms are infected through the ingestion of spores, e.g., on consumed vegetation and cannibalism or necrophagy of infected hosts (Canning, 1962; Ewen and Mukerji, 1980). Later, spores germinate in a specific way. A polar tube that is coiled within the spore punctures the host midgut cells, injecting the spore contents into the cell cytoplasm. Mitochondria and Golgi apparatus are lacking in these pathogens, and the energy is evidently extracted from host cells via the direct uptake of ATP. Different types of spores may be produced at different stages, probably with different functions including autoinfection. This pathogen mainly infects the fat bodies, disrupting metabolism and energy storage. In several cases of infection, the fat body is greatly hypertrophied with spores. The process is also associated with a change in the colour of the fat body. Effects on the host are typically chronic. This is one of the main reasons why these microorganisms are also used in pest biocontrol (Canning, 1953; Solter *et al*., 2012).

Isolation

Species identification is based on a combination of host identification, electron microscopy and molecular analyses. In laboratory conditions, a routine diagnosis of microsporidia relies mostly on special staining and microscopic techniques. In addition, microsporidian organisms cannot be cultivated axenically because of their intracellular development, but they have been successfully cultivated in their specific type of host (Joseph and Sharma, 2009). For example, several species of microsporidia were isolated from various species of grasshopper and locust, of which *Paranosema locustae* was the most extensively evaluated for grasshopper control (Johnson, 1997). The isolation of individual microsporidian species was performed mostly from typical hosts, e.g., *Vairimorpha disparis* is often isolated from *Lymantria dispar* Linnaeus[c] (Solter and Maddox, 1998).

Case studies in general

Most representatives of the Microsporidia group are still unknown. However, the species used in pest biocontrol are examined in some details. For instance, studies by Lockwood *et al*. (1999) and Lange and Cigliano (2005) deal with the elimination of economic pests such as rangeland grasshoppers using *Paranosema locustae*. The main aim of the studies of Solter and Hajek (2009) and Solter *et al*. (2012) is the prospective use of microsporidian species, e.g. *Vairimorpha disparis* in gypsy moth pest control.

When the combination of *Beauveria bassiana* and *Nosema pyrausta* (Paillot) Weiser[c] was used in laboratory, the mortality of the European corn borer larvae, *Ostrinia nubilalis*, increased significantly in all instars. Relative to the *B. bassiana* treatment alone, the *B. bassiana + N. pyrausta* treatment decreased the LC50s by 42.16 %, 37.63 %, 21.60 %, 27.11 % and 33.95 % for the first to the fifth instars, respectively (Rahman *et al*., 2010).

Influence on bees

Many species are used in biocontrol of pests (eliminate grasshoppers, mosquitoes, ants etc.). On the other hand, many species in this group also cause the mass deaths of bees, fish and crabs (Tirjaková, 2010). *Nosema apis* causes nosema disease in adult honey bees, mainly when they are confined, so the heaviest infections are present in winter bees, package bees and bees from hives used for pollination in greenhouses (Shimanuki and Knox, 2000). Nosema spores cast into the epithelial layer of the ventriculus and the midgut (Sharma *et al*., 2019) and pass to the rectum (Hornitzky and Anderson, 2010). This causes digestive disorders (Sharma *et al*., 2019). Bee colonies with a strong infection weakened considerably (Čavojský *et al*., 1981). *N. apis* and *N. cerana* are considered quite devastating for apiculture (Sharma *et al*., 2019).

OTHER ORGANISMS RELATED TO BEES

Predatory insects

Predatory insects were observed and described in the last century as an important agent in the hive responsible for bee health. The action mode of predatory insects is the elimination of some small bee pests by consuming them (Čavojský *et al*., 1981). In the hive environment, pseudoscorpions can defuse the first larval stages of the wax moth *Galleria mellonella*, lesser wax moth *Achroia grisella* and a small hive beetle *Aethina tumida* (Schiffer, 2017).

Vitali-di Castri (1973) determined the greatest number of pseudoscorpions in Mediterranean climate regions (Mediterranean Basin, a part of North-Western America, central Chile and south of South Africa and Australia) while expecting a lower number of them in tropical rain forests compared to Mediterranean zones. Čavojský *et al*. (1981) stated that in Slovakia, there are also several species of *Pseudoscorpionidea* in hives.

In organic beekeeping, mites *Stratiolaelaps scimitus* Womersley[c] found in compost or pseudoscorpion *Chelifer cancroides* Linnaeus[c] seem to be available agents to biocontrol of varroosis. This way of biocontrol requires the hives to be adapted to the natural habitat of these animals (Bajko, 2020).

Wax moth

The wax moth *Galleria mellonella* is well known for beekeepers who try to eliminate it. *G. mellonella* is not known only as a honeycomb pest. Bombelli *et al*. (2017) reported the fast biodegradation of polyethylene (PE) by larvae of the wax moth *G. mellonella* producing ethylene glycol. They also highlighted its potential for biotechnological applications because plastics are largely resistant to biodegradation.

Smith (1938) stated that *G. mellonella* is found wherever bees are found. It is a typical holometabolous insect, which develops within four distinct life stages, namely egg, larva, pupa (Figure 3) and adult (Kwadha *et al*., 2017). The wax moth larvae (called wax worms) are fed on the combs and their contents. They prefer older dark combs that contain high levels of impurities (Conrad, 2013). These moths attack wax combs to feed on cast larval skin and stored food (Abou-Shaara and Staroň, 2019). They obtain nutrients from honey, castoff pupal skins, pollen and other impurities found in the beeswax, but not from the beeswax itself. Consequently, older combs are more likely to be damaged than new combs or foundations (Shimanuki and Knox, 2000).

Conrad (2013) provided a different viewpoint that the wax moth is an opportunist, a scavenger, to whom nature has delegated the task of cleaning up the abandoned bee wax, thereby acting as a natural form of disease prevention to help keep other colonies in the neighbourhood healthy. Relationships between parasites and hosts can be mediated by endosymbiotic microorganisms from both the parasite and host sides (Polenogova *et al*., 2019). Cadavers of *Galleria mellonella* larvae are used as "cultivating media" for *Beauveria bassiana*, which is potentially effective against varroosis. In modern beekeeping, we suppress the *G. mellonella*. However, the relationship between *Varroa destructor* and *Galleria mellonella* seems to be interesting in terms of maintaining bee health in a natural way. Rosenkranz *et al*. (2010) used the term "natural pest control" for *Varroa* (pest) management using antagonistic, parasitic or pathogenic organisms without the contamination of bee products.

CONCLUSION

In scientific literature, there are many case studies about the application of biocontrol in pest management using entomopathogenic micro-organisms. Currently, biocontrol has not been commonly used in Slovakia. However, there is a potential increase in its future use exists, mainly because of its advantages connected to sustainable agriculture. Entomopathogenic organisms must not be harmful for bees and other pollinators.

Figure 3. Pupae of *Galleria mellonella* inside the hive's box
(photo: I. M. Nábělek, 2018)

Concerning EPBs, *Bt* is often applied. Moreover, it is also used in beekeeping to suppress the wax moth. EPFs, particularly *Beauveria bassiana* and *Metarhizium anisopliae*, are frequent biological control agents in IPM with unknown adverse effects to pollinators. Furthermore, *Beauveria bassiana* seems to be a promising agent also against varroosis. The application of EPMs in IPM could be hazardous because many representatives are pests for pollinators, e.g., *Nosema* spp. Overall, the harmony between the organisms in the surrounding is very gentle. We can observe that in above presented studies, pests are generally scavenged by microscopic fungi and they are scavenged by bacteria and so on. Therefore, the protection against pests in plant production, forestry or beekeeping must be comprehensive.

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INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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