JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Mudrončeková et al. 2013 : 2 (6) 2469-2472

ENTOMOPATHOGENIC FUNGUS SPECIES *BEAUVERIA BASSIANA* (BALS.) AND *METARHIZIUM ANISOPLIAE* (METSCH.) USED AS MYCOINSECTICIDE EFFECTIVE IN BIOLOGICAL CONTROL OF IPS TYPOGRAPHUS (L.)

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ARTICLE INFO

Received 8. 2. 2013
Revised 21. 5. 2013
Accepted 23. 5. 2013
Published 1. 6. 2013

Short communication

OPEN ACCESS

ABSTRACT

The agricultural world is overloaded with chemical substances. Undesirable effects and the resistance of vermin opens a new way for biological control of pathogenic species of animals. *Ips typographus* (L.) is overgrowth across Central Europe. Its natural habitat are forest communities, where chemical spraying is forbidden or restricted. The natural bioregulators of this cosmopolitan pest are entomopathogenic fungi of *Hypocreales* species. In this study, we focused on two types of entomopathogenic fungi – *Beauveria bassiana* (BALS.) and *Metarhizium anisopliae* (METSCH.). They are suitable for biological control because they are attacking a wide range of pathogenic insects in agro-systems. Entomopathogenic fungi were isolated from soil samples and dead infected insects. The samples collected from different sites of the High Tatras. In our experiments, we followed infectivity and mortality of selected isolates of entomopathogenic fungi *Beauveria bassiana* (BALS.) and *Metarhizium anisopliae* (METSCH.). The gDNA of isolates was used for identification and we put them to the analysis of specific DNA segments by amplified PCR method. Results of experiments show the high pathogenicity of entomopathogenic fungi strains. *Beauveria bassiana* (BALS.) caused 99% mortality and *Metarhizium anisopliae* (METSCH.) reached 97%. Infectivity followed by *Beauveria bassiana* (BALS.) reached 92% and *Metarhizium anisopliae* (METSCH.) 90%. For the purpose of limiting the numbers of populations of harmful pest is essential the ability of entomopathogenic fungi to infect, kill the host, and remain in a natural environment without disturbing the biota.

Keywords: *Beauveria bassiana,* biological control, entomopathogenic fungi, High Tatras, *Metarhizium anisopliae, Ips typographus*, spruce bark beetle

INTRODUCTION

Entomopathogenic fungi are an important regulatory factor for bio-control in agriculture and forestry. Their biology and ecology have been studied more than a century. The attention of scientists was focused mainly on fungi genus *Beauveria* and *Metarhizium*.

Entomopathogenic fungi are non-selective and may attack the broad spectrum of insects and these fungi reproduce exclusively asexually. The natural environment is the soil as a reservoir of their resistant spores. They are able to infect their hosts waiting in natural habitat. The first step of infection is capture of fungi conidia on terrestrial insects **(Talaei-Hassanloui *et al.*, 2007)**. It usually snaps the insect cuticle **(Lefebvre, 1934)**, and passes through created tube to the digestive tract, trachea and wounds into the body of the host **(Madelin, 1963)**. Under favorable conditions, there is overgrowth of hyphae and the host finally dies. Hyphae of fungus grow through the cuticulum and produces new spores under suitable environment conditions **(Liang *et al.* 1991)**.

Beauveria bassiana is a filamentous fungus producing white conidia on aerial mycelium. Aerial conidia are formed in the conidiophores **(Holder, 2005)**. Their growth is relatively slow. Conidia appear in the form of powder or hyphae in white, yellow and pink color. Aerial hyphae are divided into smooth septa, 2,0 mm wide, while submerged cultivated are 1,5 to 3,0 mm wide although similar structure **(De Hoog, 1972)**. From swollen branched cells originate conidiogenous cells that occur in dense clusters. They constitute the basic spherical shape. Aerial conidia are smooth, thin-walled, and oval to spherical, depending on the nature and impact of external conditions **(Huang *et al.*, 2002)**. *Beauveria bassiana* occupies especially undisturbed soils in humid forest habitat and is more sensitive to mechanical destruction of the soil, high temperature, drought, and UV radiation.

Metarhizium anisopliae is a fungus known as green Muscardini. Traditionally, this fungus was classified based on phenotypic analysis and characteristics. It was divided into two varieties based on the length of the conidia **(Meyling *et al.*, 2007)**. It contains a number of morphologically distinct species than *M. album*, *M. anisopliae*, *M. cylindrosporae*, *M. flavoviride*, *M. guizhouense*, and *M. pingshaense* **(Driver *et al.*, 2000)**. According to the morphological characters it is really difficult to identify individual species. Conidia *M. anisopliae* have a size of 5,0 to 8,0 µm, and are oval. Fungal colonies are initially white or cream colored and during formation of spores change color to shades of yellow, green to dark green **(Tangthirasunun *et al.*, 2010)**. They germinate at low temperatures **(Meyling *et al.*, 2007)**. **Keller *et al.* (2003)** found that the habitat of M. anisopliae an agricultural environment where mushroom cultivation affected soil. Characterized as an agricultural species, which is most common on exposed sites in regularly disturbed soil environment.

Isolation and characterization of entomopathogenic fungi is the first step to get valuable information on biological control against insect pests in their natural environment.

MATERIAL AND METHODS

Sampling of bark beetles

Dead infected bark beetles, live beetles and larvaes have been collected from bark of the trees and pheromone traps into sterile cryo-tubes with 1 mL of sterile 10% glycerol and 1.5% Triton X-100. The samples collected were stored until further processing at -20 ° C. Mature beetles of *Ips typographus* have been maintained in quarantine laboratory for 7 days to prevent infection that could acquire under natural conditions and only no infected bugs have been used for further laboratory assays. Mature beetles have been multiplied under artificial conditions on spruce cut trunks in Insectarium at 25 °C and 60 % of relative humidity, 14 beetle samples has been used for experimental tests.

Sampling of entomopathogenic fungi

Entomopathogenic fungi have been isolated from infected individuals of *Ips typographus* and samples of soils from locations of the High Tatras - Tatranská Lomnica, Velická Valley, Hrebienok and Otongachi area in Ecuador. On the individual sites were marked 5 trees distant a few meters from each other (**Reay *et al.*, 2008**).

Sampling of soil

Around a tree trunk and at a distance of 20 - 30 cm were taken 3 - 4 soil samples. Collected samples have been taken by sterile shovel. The next samples of soil from another location have been taken with paddle thoroughly cleaned with ethanol 70% to avoid contamination. Consequently these samples are stored and in a sterile box or plastic bag and have been kept at 4° C until used in the laboratory.

Isolation of fungi from soil and bark beetles

For isolation of entomopathogenic fungi from soil samples has been selected method named "Isolation using sensitive insect - *Ips typographus* (L.)". Non infected beetles have been exposed by obtained soil samples. One sample of Petri plate contained 50 g of soil and 5 bark beetles. The soil has been periodically wetted with distilled water dispenser. Petri plates have been stored at room temperature and checked daily after 7 days. Dead beetles were transferred to Petri dishes with moist filter paper and the present entomopathogenic fungi have been let to grow a next couple of days. Subsequently, these fungi have been inoculated at Sabouraund dextrose agar (SDA) (produced by Raymond Sabouraund, 1982) medium in Petri plates and cultured at 30° C in incubator. The same protocol has been used with defrost samples of infected beetles.

Isolation of pure culture

Isolates of entomopathogenic fungi were purified by single colony inoculation method to obtain pure cultures. Individual colonies inoculated on plates with the SDA contained 0.01% Triton X-100. The plates were kept at 30 ° C until single colonies did not grow again. Thus purified cultures are safely stored on silica gel in the freezer at - 20°C. Purified strains have been used for further experiments. The strains were inoculated after cooling and solidification of SDA medium. For removing of bacteria in soil samples, we used antibiotics (50 mg/L tetracycline hydrochloride, 350 mg/L streptomycin sulfate, 125 mg/L cycloheximide) (**Reay *et al*., 2008**) in some SDA mediums. SDA medium contains: Agar – 2g, Glucose – 4g, KH_2PO_4, $MgSO_4.7H_2O$, $NaNO_3$ and peptone 0,1g for 100 ml of water. After adjusting pH on 6.7-6.8 the medium was sterilized and watered into sterile Petri dishes.

Preparation of the spore's suspension

The cultures grown in Petri dishes have been inoculated into liquid medium - Sabouraund agar (SA (produced by Raymond Sabouraund, 1982) and cultivated 2 weeks at 25 °C. Subsequently, the medium was filtered through sterile gauze and spores overflow (supernatant) have been centrifuged 10 minutes, 4500 rpm at 4 ° C. After that, they have been washed 2 times with distilled water and again centrifuged 10 minutes at 4500 RPM at 4 °C. The spores thus obtained have been treated by 0,01 % Triton X-100 (detergent, which prevents agglomeration of spores). The number of spores in 1 ml was counted in Burker's chamber and then diluted on required concentration.

Testing the effect of selected isolates of entomopathogenic fungus on mortality *Ips typographus* (L.)

Mortality of spruce bark beetles caused by entomopathogenic fungi was tested by direct contact. Fungal conidia were collected in sterile distilled water (0.2 ml / L Triton X-100) and filtered through sterile gauze. Collected conidia were lyophilized and lyophilized number of conidia per gram of lyophilized powder was determined by Burker`s cell. We used $1x10^8$ conidia/ml dilution of 10 mg of freeze-dried conidia. In a Petri dish (45 mm in diameter) on the size of the space 1 cm^2 was applied 20 µl of prepared spore suspension. It was induced natural way of infection of bark beetle by entomopathogenic fungal spores. These infected bark beetles were placed in Petri dishes. Petri dish contains one bark beetle and sterile material- daily moisten filter paper (Whatman 3 mm) and 1-1,5 cm^2 of spruce bark. Infected individuals were kept at room temperature of 23 ± 2 °C for a period of 14 days. For the control sample we applied sterile distilled water with 0.2% Triton-X-100 instead of spores. The infected individuals were controlled and moistened with sterile water every 24 hours. It has been tested the mortality and infection.

RESULTS AND DISCUSSION

In our experiments we observed 9 strains *Beauveria bassiana* (BALS.) and 1 strain *Metarhizium anisopliae* (METSCH.). On tab. 1. are shown strains of entomopathogenic fungi, sampling sites, type of samples taken and DNA genotyping. From obtained samples were isolated 4 types of microscophic fungi – *Beauveria bassiana*, *Metarhizium anisopliae*, *Aspergilus flavus/oryzae* and *Peniciulium ochlochloron*.

The strain *Beauveria bassiana* CCM8267 has been obtained from CCM Czech collection of microorganisms stored at Masaryk University, Faculty of Natural Sciences in Brno, Czech Republic and other strains of *Beauveria bassiana* - 1-3-L, 1-5-L, 1-6 -L, X01, X07, X08, 8-A have been marked according to the location of isolation. Isolate marked 9-C was *Metarhizium anisopliae*. Collection of entomopathogenic fungi and spruce bark beetles have been collected from locations in the Tatranská Lomnica, Tichá a Kôprová dolina in the High Tatras, in Brezno and Malá Fatra and strains have been isolated at the Institute of Chemistry, Slovak Academy of Sciences in Bratislava. The strain named E2 - *Beauveria bassiana* has been isolated from obtained samples of infected locusts from Ecuador (province of Santo Domingo de los Colorados) (Institute of Zoology, SAS).

Table 1 List of entomopathogenic fungi isolates and sampling location

Labeled sample	Locality	Source	DNA genotyping
1-L-3	Brezno	spruce bark beetle	*B. bassiana*
1-L-5	Brezno	spruce bark beetle	*B. bassiana*
1-6-L	Brezno	spruce bark beetle	*B. bassiana*
X01	Malá Fatra	spruce bark beetle	*B. bassiana*
X07	Brezno	spruce bark beetle	*B. bassiana*
X08	Vysoké Tatry	spruce bark beetle	*B. bassiana*
8-A	Brezno	soil	*B. bassiana*
9-C	Brezno	soil	*M. anisopliae*
B. bassiana CCM8267	Brno		*B. bassiana*
E2	Ecuador		*B. bassiana*
TP7A	Vysoké Tatry	spruce bark beetle	*Aspergilus flavus /oryzae*
TP7C	Vysoké Tatry	spruce bark beetle	*Aspergilus flavus /oryzae*
TP4BI	Vysoké Tatry	spruce bark beetle	*B. bassiana*
8A-BI	Vysoké Tatry	spruce bark beetle	*Penicillium ochrochloron*

Isolates have been identified by their morphology growth on SDA medium according to shape, size of conidia, type and form of fructification structures. Since the visual identification of the species is not sufficient, the isolates were prepared in submerged culture for isolation of gDNA.

The natural habitat of entomopathogenic fungi is soil. Entomopathogenic fungi of genus Beauveria, Metarhizium and Issaria are most frequently occurring fungi in soils of temperate climate zones, tropical rainforest, but also the arctic zone **(Kram and Kram, 2012)**, (**Meyling and Eilenberg, 2006**), **(Landa et al, 2001)**, **(Reay et al., 2010)**. **Medo (2009)** dealt in his work with the occurrence of entomopathogenic fungi of order Hypocrales on the Slovak territory from different habitat types. From 901 soil samples taken from forest, grassland and farmland habitat, he found out, that the most occurring fungi are *Beauveria bassiana, Metarhizium anisopliae, Isaria farinosa* and *Isaria fumosorose*a. **Barta (2010)** focused in his study on the occurrence of entomopathogenic fungi in forest communities. He collected samples from spruce, deciduous forrests and grassland vegetation. He concluded that the most frequent fungus is *Beauveria bassiana*, subsequently *Metarhizium anisoplie* and finally *Isaria fumosorosea*. These findings confirm our right choice in choosing the appropriate biological "enemy" spruce bark beetle.

Knowing of the optimal concentration reduces the economic costs for the future research and industrial production. Three spore suspensions with a concentration of $1x10^6$ spores per ml, $1x10^7$ spores per ml and $1x10^8$ spores per ml were prepared. Suitable choice of concentration was tested by direct contact by spruce bark beetle mortality. The figure 2 shows the achieved results. The most significant decrease in mortality was observed at a concentration of $1x10^6$ spores/ml. From our results is clear that the most appropriate and most effective concentration is - $1x10^8$ spores/ml. Based on the findings of other authors, we can conclude, that they use the same practice, concentrations of suspensions, which achieved a high percentage success. **Kreutz et al. (2004**) used 4 strain types of *Beauveria bassiana* and a commercial composition Boverol ® (Fytovita, Ostrožská Lhota, Czech Republic) to test the effectiveness of entomopathogenic fungi against *Ips typographus* of three concentrations ($1x10^6$ conidia/ml, $1x10^7$ conidia/ml, $1x10^8$ conidia/ml). The results confirmed us about the correctness of concentration used in our experiments. The concentration $1x10^6$ spores per ml reached the mortality of 93 percent, $1x10^7$ spores per ml and $1x10^8$ spores per ml reached 100% mortality of the *Ips typographus*. Similar results with a high mortality percentage with concentration of $1x10^8$ conidia/ml also achieved authors like **Ahmed *et al* .(2007), Kaeng *et al.* (2009), Bustillo *et al.* (2002), Prasad & Seyd (2010), Steinwender *et al.* (2010).** From this knowledge, we can sumarize: The higher the content of spores in the suspension , the more faster and likely is the onset of infection and death of the host.

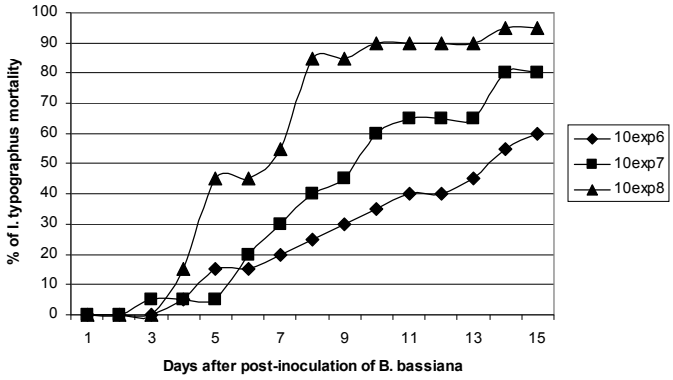


Figure 1 The effect of different concentrations of spores *Beauveria bassiana* on the mortality of spruce bark beetle *Ips typographus*

Entomopathogenic fungi of species *Beauveria bassiana* (BALS.) and *Metarhizium anisopliae* (METSCH.) infect the host through the cuticle or after ingestion, where it kills it with its toxins. Killing the host is manifested with the hyphae ovegrowth on the surface of the body. Insects are dying from mytotoxin exposure, virulent proteins and desiccation. Fungal germination was observed especially in cuticular folds around the mouth and anal, in areas of the body with the continental shelf areas of body and body nesses, consequently in the area of antennae and tarsal metamare. The growth and overgrowth of fungi was made with a light microscope in order to observe even the most insignificant signs of hyphae.

Infection rates are set at three stages – infected, middle infection and strong infection. The sample was classified as infected immediately, as soon as the growth of hyphae could be visible on the surface of the body, (Fig. 2A). The middle infection has been characterized by an average hyphae overgrowth of the body (Fig. 2B). Strong infection occurs, when the body of bark beetle was full overgrowth with fungi (Fig. 2 C-D).

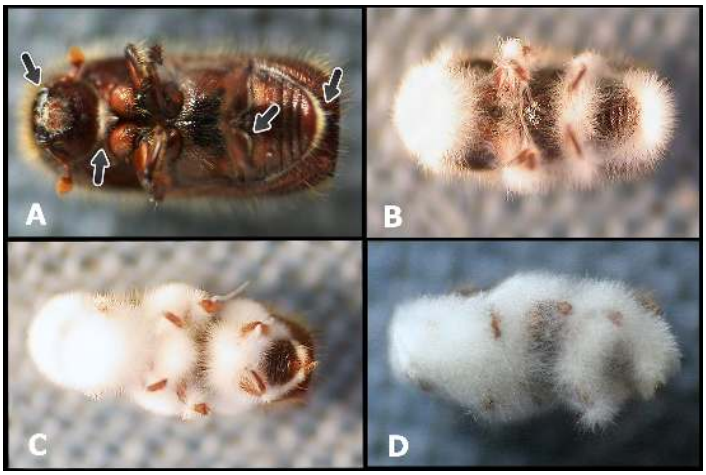


Figure 2 A – first signs of germination of *Beauveria bassiana*, B- Secondary infection caused by *Beauveria bassiana*, C–D – Severe infection caused by *Beauveria bassiana* (Mudrončeková 2012)

The results shown in figure 3, 4 show that kinds of entomopathogenic fungi are highly infectious and cause high mortality of *Ips typographus* (L.). During 14 days fungus *Beauveria bassiana* (BALS.) killed 99% of the spruce bark beetle and *Metarhizium anisopliae* (METSCH.) caused 97% mortality. Infectivity grew to 92% by *Beauveria bassiana* (BALS.) and 90% by *Metarhizium anisopliae* (METSCH.). The collective **of Draganová (2006)** tested the infectivity of isolates *Beauveria bassiana* and *Paecilomycetes farinosus* on *Ips sexdentatus* and *Ips acuminatus*. She used a spore suspension of a concentration $1x10^8$ spores/ml and they realized the experiment 10 days. She obtained the strains of *Beauveria bassiana* from dead *Ips typographus* and *Lepidoptera* larvae. Spruce bark beetle mortality reached 96.67% by *Beauveria bassiana* and by *Paecilomycetes farinosus* 66.67%.

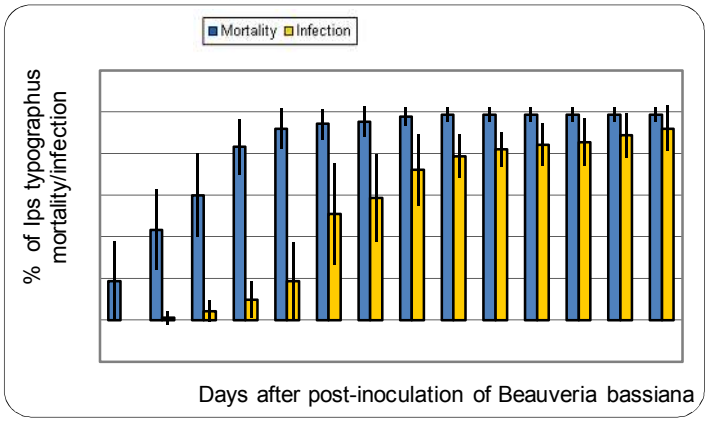
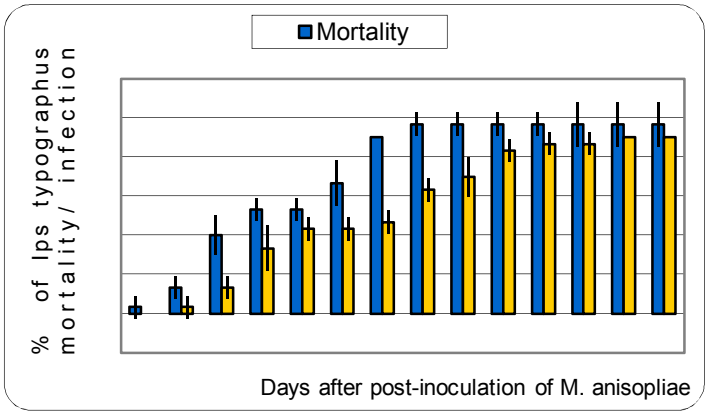


Figure 3 Mortality and infection of spruce bark beetle *Ips typographus L.* caused by induced infection of conidia *Beauveria bassiana* in concentration of $1x10^8$ conidia/ml of water.



Fiure 4 Mortality and infection of spruce bark beetle *Ips typographus L.* caused by induced infection of conidia *Metarhizium anisopliae* concentration of $1x10^8$ conidia/ml of water.Values of infectivity of *P. farinosus* suggest that it is important to choose a suitable biological pest eliminator, because the effects are not adequate to abundant populations of insects. She also found out, that the isolates obtained with from *Ips typographus* achieved a higher mortality than by the isolate obtained from the *Lepidoptera* larvae. This means that an important factor is also the isolation of entomopathogenic fungi from the environment/host of pathogenic pests occurence.

Vakula *et al.* (2010) presented their research using a product Boverol. Its active ingredient is a *Beauveria bassiana*. Tests were made in military forests Kremnica Mountains, where they placed 18 traps of three types (Ecotrap, Multiwal, IT Ecolure Extra). Pheromone traps are adjusted in such a way that the bark beetles trap in airstrike after being infected with entomopathogenic fungi and they are allowed to return to nature. This infected bark beetles spread the infection further. To determine whether the captured bark beetles were infected, they were collected and then bred in the laboratory. Testing was carried out in two ways - on filter paper and fresh crust. On filter paper appear the infection (48.3%), and the infectivity of fresh bark shows 67.5% in both cases after 5 days. These not very pleasing results were affected by the weather, which has been caused by extreme temperatures and humidity in traps, which are the spores of fungi *Beauveria bassiana* sensitive. Another research in Slovakia was conducted by **Jakuš and Blažec (2001)** devoted to testing of Ips typographus mortality in forests Spišská Magura, where the chosen cut trees invaded by bark beetles were treated by aqueous solution containing *Beauveria bassiana*. The concentration of the spore suspension was $1x10^7$ conidia / ml and they applied spray directly on the tree bark. The period of research was carried out from July to October 2007. This type of experiment could infect 28.75% of bark beetles. The authors believe that the anticipated success by entomopathogenic fungi did not reach the expected results due to expected conditions (temperature, relative humidity). And under laboratory conditions the efficiency of entomopathogenic fungi reaches 88 to 100% mortality of bark beetles (**Vaupel et al. 1996, Kreutz et al. 2004, Kunca et al. 2009**). These findings suggest us a strong influence of various factors on the viability of entomopathogenic fungi. Similar experiments were conducted on a wide range of insects with similar results: On termites (**Krutmuanga and Mekchayb, 2005),** na *Spodoptera littoralis* (**Ahmed *et al.* 2007),** *Helicoverpa Armigera* (**Prasad and Seyd 2010**), *Boophilus microplus* (**Kaeng *et al.* 2009**), *Tribolium castaneum* (**Lord, 2008**). Either if were used entomopathogenic fungi strains obtained from land, or affected by insects which were cultivated on SDA, SDAY or PDA plates. The most commonly used

concentration of 1x108 conidia / ml, grown under the same conditions as described in this work. Other possible sources of entomopathogenic fungi were mycoinsecticides. From 2007 is the published work (**Faria and Wraight, 2007**), where the authors completed and updated the complete lists of mykoinsecticides. From these results by different authors it can be concluded that entomopathogenic fungal have high viability, virulence and adaptability in the natural environment of pests, but have suitable conditions for its existence.

CONCLUSION

In the study, the purpose was testing effect of entomopathogenic fungal spores on mortality and infectivity of spruce bark beetle. Study of influence of selected isolates demonstrated positive results for the use of biological protection of forest trees instead of insecticides.

Fungi cultivations from soil samples and from infected and dead spark beetles showed, that in the soil of selected sites mainly occurs the entomopathogenic fungus *Beauveria bassiana*. Our results demonstrate the high pathogenicity of strains *B. bassiana* and *M. anisopliae*, which gives a solid basis their using in biological protection of the forest ecosystems before calamity outbreak of bark beetles. Mortality caused by effects of *B. bassiana* isolate achieved percentage success of 99 % and 97 % by *M. anisopliae*. Virulence climbed to 92 % in *B. bassiana* and *M. anisopliae* to 90 %. These values of entomopathogenic fungal isolates are characterized by high viability and virulence of conidia. Therefore we can confirm about them that they are important regulators of natural spruce bark beetle.

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