TESTING OF ENTOMOPATHOGENIC FUNGI IN BIOLOGICAL CONTROL AGAINST PINE WEEVIL

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ABSTRACT

Coniferous forests in Slovakia as well as in other European countries in recent decades are attacked by various harmful factors. The most important abiotic factor is the wind, which can in a short time destroy large complexes of spruce stands. After processing such calamities frequently create huge areas of afforestation, making very good conditions for pine weevil presence. It is a beetle causing a lot of damage by eating bark of the trunks of young seedlings, thereby, causing a weakening or dieback of them. Actual research is focused on study of reactions of the pine weevil adult (*Hylobius abietis* L.) (Coleoptera: Curculionidae) to infection caused by various entomopathogenic fungi species (*Beauveria bassiana, Metarhizium anisopliae, Isaria fumusorosea*). *Beauveria bassiana* is one of the longest known insect pathogens. The most significant effect in reducing of food intake has fungus *Beauveria bassiana. Metarhizium anisopliae* had no effect on food intake, and beetles infected with fungus *Isaria fumusorosea* had even increased food intake. Mortality of pine weevil has increased by fungus *Beauveria bassiana*, and good efficacy in this regard has also fungus *Metarhizium anisopliae. Isaria fumusorosea* had almost no effect on their mortality rate.

Keywords: entomopathogenic fungi, *Beauveria bassiana, Hylobius abietis,* biological control

INTRODUCTION

Coniferous forests in recent decades were being attacked by various biotic or abiotic factors. The most important abiotic factor is the wind that is capable to destroy large forest complexes in a short time. An example of a great calamity in Slovakia may be a calamity in Tatras in November 2004, which hit the area of 12,600 hectares of forest. Processing of this calamity created a great area for afforestation while also created very good conditions for some biotic pests, for example pine weevil. The calamity area had enough pine stumps, suitable for pine weevil females to lay eggs, which resulted in outbreak of this pest. Swarming of a new individuals is associated with feeding on the trunks of young seedlings, feeding on the bark leads to a weakening of seedlings; or to their death. The research was aimed at testing different entomopathogenic fungi with regard to their use in biological control of this pest.

Entomopathogenic fungi infect the wide range of hosts of different insect orders. They attack at different life stages from eggs to adults, while larvae or pupae are the most commonly attacked. Some species of fungi can parasitize on different developmental stages of the same host, or only at a particular stage of development of the same kind of insects.

Conidia of the fungi caught on the host's body surface grow into the host body. Conidia start to germinate under appropriate conditions. The tip of the penetrating hyphae, after a short growth, gets into the body using the assistance of mechanical pressure and the intake of nutrients contained in the cuticle. In the form of blastospores it is rapidly spreads to the various tissues and organs. Decrease in food intake is often associated with the ongoing changes in the contested host. The insects die due to the cell collapse caused by depletion of nutrients and poisoning by toxic metabolites produced by the fungus, which occurs within a few days after infection. The host's body is gradually filling the mycelium, which in sufficient humidity overgrows on the surface and subsequently develops a great number of aerial conidia.

Success rate of invasion and killing of the host is highly dependent on the spores concentration. More spores means bigger chance for elimination of host. Entomopathogenic fungi are able to survive in different environmental conditions; however their growth and development is greatly influenced by factors of the abiotic environment, especially by the humidity and temperature of the environment and UV radiation.

Until now we have described at least 90 genera, more than 700 species of fungi which are pathogenic to arthropods, but only few of them are effectively used in biological control. The best known, practically used species of entomopathogenic fungi are: *Beauveria bassiana*, *Entomophaga maimaga*, *Verticillium lecanii*, *Metarhizium anisopliae*, *Nomurea rileya*a

Hirsutella thompsonii [1, 2]. The prior research experience in this area has shown that the *Beauveria bassiana* species appears most suitable for biological control against pine weevil.

***Beauveria bassiana* (Balsamo) Vuillemin**

The first mention of the entomopathogenic fungus *Beauveria bassiana* is from the first half of the seventh century BC. At this time an unknown disease of silkworm occured in ancient China which reduced their numbers. Later, through the increasing trade contagion came to Europe and started to attack the local insects. The losses and damages caused to bee colonies were the most serious. Extinctions of entire honeybee colonies, however, were also accompanied by previously observed unnatural behavior of individuals. At that time, because of the final stage of its symptoms - overgrowth of the host with white woolly mycelium known as “white muscardine disease” [3].

Despite the relatively rapid and large-scale expansion of the fungus, its importance in terms of usefulness to mankind was not fully known. Within the scientific community it was named *Tritirachium shiotae*.In nineteenth century, an Italian entomologist, Agostino Bassi undertook more detailed research. After a number of experiments and gathering a sufficient amount of knowledge, he presented his results to the public in 1936. He concluded that manifesting symptoms are caused by parasitic fungus in the body of adults. The first successful results of applying *Beauveria bassiana* has been recorded by a Ukrainian doctor and the discoverer of phagocytosis - Ilya Mechnikov, who successfully used it against bedbugs *Blissus leucopterus*. The genus was officially described only in the 1912 by Vuillemin, then finished the final taxonomic classification scientists Petch, MacLeod and de Hoog [4].

Entomopathogenic fungus *B. bassiana* is a cosmopolitan widespread species, which a common component of the soil microbial community. It can be found mainly in the soil as a saprophyte on organic substrate, but also as a optional parasite in the form of a thick white mycelia on the surface of dead insects while it produces a number of hydrophobic conidia.

Disease of insects normally starting vital and virulent conidia, which are distributed by random mechanisms in the environment. Of the abiotic factors involved in the wide dissemination is mostly wind, rain or water movement in the soil. Dissemination also by usual mechanisms such as contact with an infected healthy individual or contamination of eggs during their laying.

Attachment of dry, strongly hydrophobic conidia, is ensured by direct interaction between two hydrophobic surfaces or by electrostatic forces, or by the molecular interaction between substances presented on the surface of conidia and substances on the surface of the host´s cuticle. Conidia are sufficiently stocked to the germination, without the need to absorb external nutrients. Their germination on the surface of the host starts in the appropriate temperature and high relative humidity.

From a certain phase of germination further, development of the fungus is dependent on external nutrient intake. The fungus begins to receive substances contained in the cuticle of the insect, then simply absorb the nutrients from the internal tissues of the insect. For this purpose, the fungus penetrates through the direct penetration or through natural openings into the body of the contested individual.

After penetrating of the individual, the fungus begins to produce secondary metabolite beauvericin, which weakens the immune system. Consequently, there is a rapid filling of tissues. This development phase is characterized by transition of filamentous fungus forms on the rapidly dividing and proliferating hyphal bodies, which by means of hemolymph are spreading throughout the body. They are propagated by gemmation, exponentially and very quickly. Individual dies after the destruction of all lymphocytes.

Inside the body fructification fibers are created, on which the blastospores arise. The new hyphae growing up out of them, consume nutrients in the hemolymph needed for their growth, and fructificate. Thus, there is an increase of the mycelial mass and rapid filling of the body of insect.

At sufficiently high relative humidity hyphae grow on the surface of the killed host through the weaker parts of the cuticle, where they form a dense white mycelium to which conidiophores develop which shall progressively become airborne conidia. Conidia in a dormant state are able to sustain life for several weeks to months. The most important environmental factors needed for the development of fungi include temperature and high humidity. Ideal temperatures are between 20°C and 30°C. For mycelial growth on the surface of the body and the formation of conidia requires usually very high humidity (above 90 per cent) but for the release of conidia the sufficient humidity is below 50 per cent [1, 5].

Pine weevil (*Hylobius abietis* L.) (Coleoptera: Curculionidae)

The adult is dark brown with pale little spots on elytra. It is 10 to 14 mm long. The head is elongated into a noticeable nose with antennae on its end. The female lays eggs onto roots of fresh pine and spruce stumps. The larva is whitish, limbless, slightly arched, 12 – 23 mm long. It pupates in the bark. Feeding under the stump bark is not harmful; but, it speeds up their decay. The maturation feeding of sexually immature adults is dangerous on conifer seedlings, especially pines. Feeding of adults on trunks is square. The adult beetle feeds on the bark just above the ground, in deep, funnel-like holes up to sapwood, which causes intense resin production. In case of severe attack, it damages the entire little trunk and the seedling dies [6].

One possible way to control pine weevil is the use of biological methods, which are an important part of the integrated forest protection. Biological control is environmentally friendly way of fighting, which has a growing importance, and in the future is expected to increase its share. This method of protection is often used in areas where the use of chemicals is not possible. There are places with a higher degree of conservation or water resource protection zones. From biological methods, which are used in forestry to protect seedlings, we can use biological control by means of nematodes and biological control by means of entomopathogenic fungi [7, 8].

Entomopathogenic fungi theoretically have the potential to control the pine weevil´s population, but their successful use in field conditions is still just starting. Some laboratory experiments in different model conditions were carried out at the laboratories of the Forest Protection Service Centre in Banska Stiavnica (Slovak Republic) [8].

MATERIALS AND METHODS

Works for the current research was carried out in the laboratories of the National Forestry Centre Zvolen, workplace the Forest Protection Service Centre in Banska Stiavnica. For the experiment we have chosen 150 viable pine weevil's imago, disaggregated by gender. The sex ratio was 1:1 in 75 males and 75 females. The adults were infected by entomopathogenic fungi *Beauveria bassiana* L., *Metarhizium anisopliae* (Metchnik off) Sorokin and *Isaria fumusorosea* Wize, also we created a control, uninfected sample:

Control - 0.05% solution of surfactant Tween 80, 30x, 15 males, 15 females

Bauveria bassiana- dry application of 30 pieces, 15 males, 15 females, spore concentration of 1.58 x 10^7 /ml

Bauveria bassiana - a suspension of 30 pieces, 15 males, 15 females, spore concentration of 1.58 x 10^7/ml

Metarhizium anisoliae - dry application 30 pieces, 15 males, 15 females, spore concentration of 1.58 x 10^7 /ml, *Isaria fumosorosea* - dry application 30 pieces, 15 males, 15 females, spore concentration of 1.58 x 10^7 /ml

Since the spores of the fungus - *Metarhizium anisopliae* are green, the main feature of the disease is the green color of the contested individual. When these asexual spores come into contact with the body of the host insect, begin to germinate and hyphae penetrate into the cuticle. The fungus then develops within the body, and ultimately kills the insects. The killing effect is very effective and helps in the production of insecticidal cyclic peptides. The skin of dead individuals often turns red. If the ambient humidity is sufficiently high, then white mould grows on dead beetles and soon changes to green color. Most insects living near the soil have developed natural defenses against entomopathogenic fungi such as *Metarhizium anisopliae.*

The last test species was *Isaria fumosorosea.* This fungus is a natural enemy of many insects belonging to the order family of Hemiptera and Homoptera, Bemisia and Trialeurodes. It also has activity against Diptera and Lepidoptera. The fungus works in contact with the insect and starts producing bacteria passing through the insect body and excreted toxins which kill the host. The mummified remains of corpses and insects are at high humidity covered with whitish-violet coating. Fungi was originated from the collections of the Arboretum Mlynany, workstation of the Slovak Academy of Sciences.

The adults were infected with dry and wet way. After infection beetles were placed in Petri dishes, where they had food - pine twigs 80 mm long with diameter around 6 mm (for maintaining humidity there were two pieces of moistened cotton wool). Petri dishes were placed into the room in which the temperature varied 18-24 °C and the humidity was about 50%.

Food in Petri dishes was supplemented at regular intervals from 7 to 10 days. This food was registered, we also measured the surface area (in mm^2) and evaluated the size of nibbled area. The results were evaluated according to the type of infection and in the second experiment by gender of pine weevils.

Figure 1. Dry infection of pine weevils (Photo: JurajŠkvarenina)

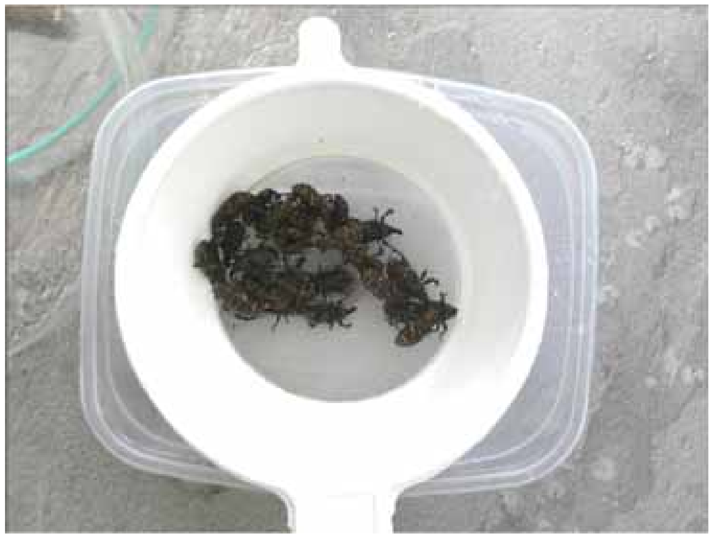


Figure 2. Wet infection of pine weevils in the liquid suspension (Photo: JurajŠkvarenina)

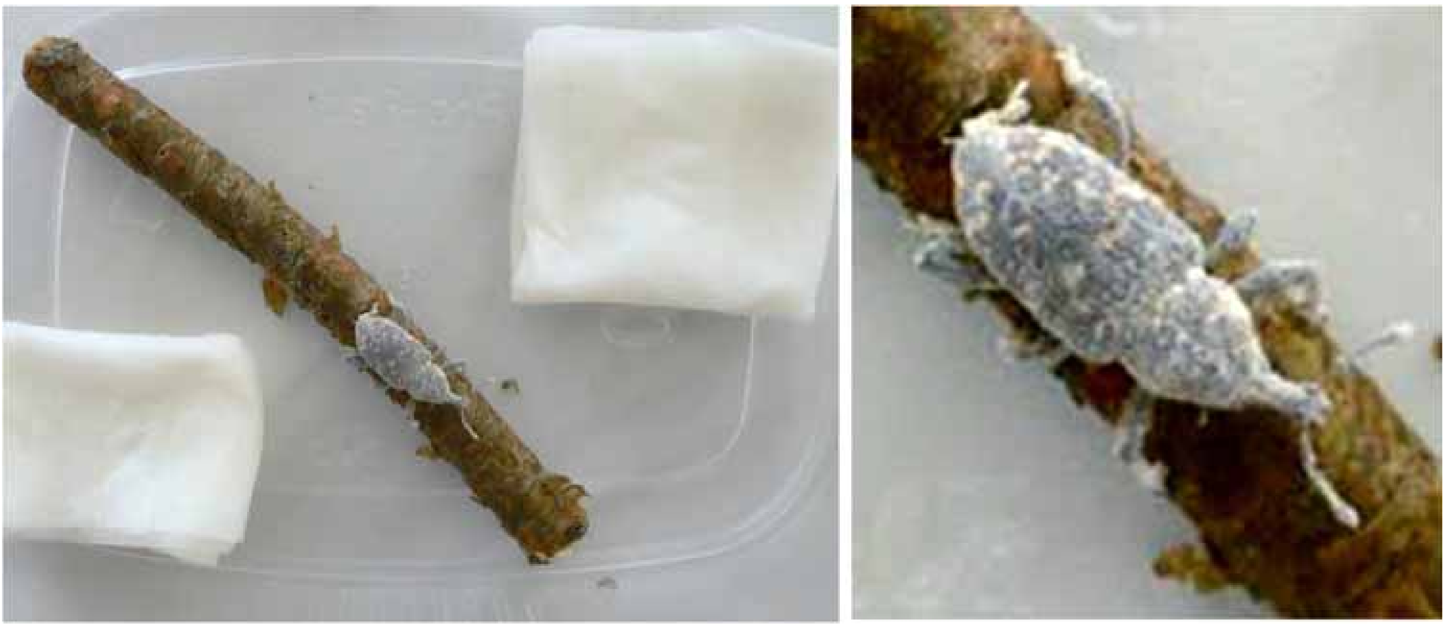


Figure 3. Adult of pine weevil infected by *Beauveria bassiana* (Photo: JurajŠkvarenina)

RESULTS AND DISCUSSION

When evaluating the effectiveness of different species of fungi with regard to achieving pine weevil's mortality under the given circumstances, best results are obtained with *Bauveria bassiana*. Within six days after the "dry application" of spores all male beetles died, females died after ten days. Overgrowth of imago by fungus was observed since the tenth day after infection. The male adults infected by "wet" application died within seventeen days and females within 28 days.

Number of authors dealt with fungus *Beauveria bassiana*, many of them have confirmed its pathogenic effect on various insect species. Markova, Simsiankova (1990) tested its effectiveness to the *Limanria dispar* [9]. Doberski (1981) found that the fungi species *Beauveria bassiana* and *Metarhizium anisopliae* were killing the adults of *Scolytus scolytus* very well [10]. Recently Agulló-Guerra *et al*. (2010) demonstrated that the "dry" spores of *Beauveria bassiana* had stuck to the cuticle of an adult *Rhynchophorus ferrugineus* better than 'wet' spores [11].

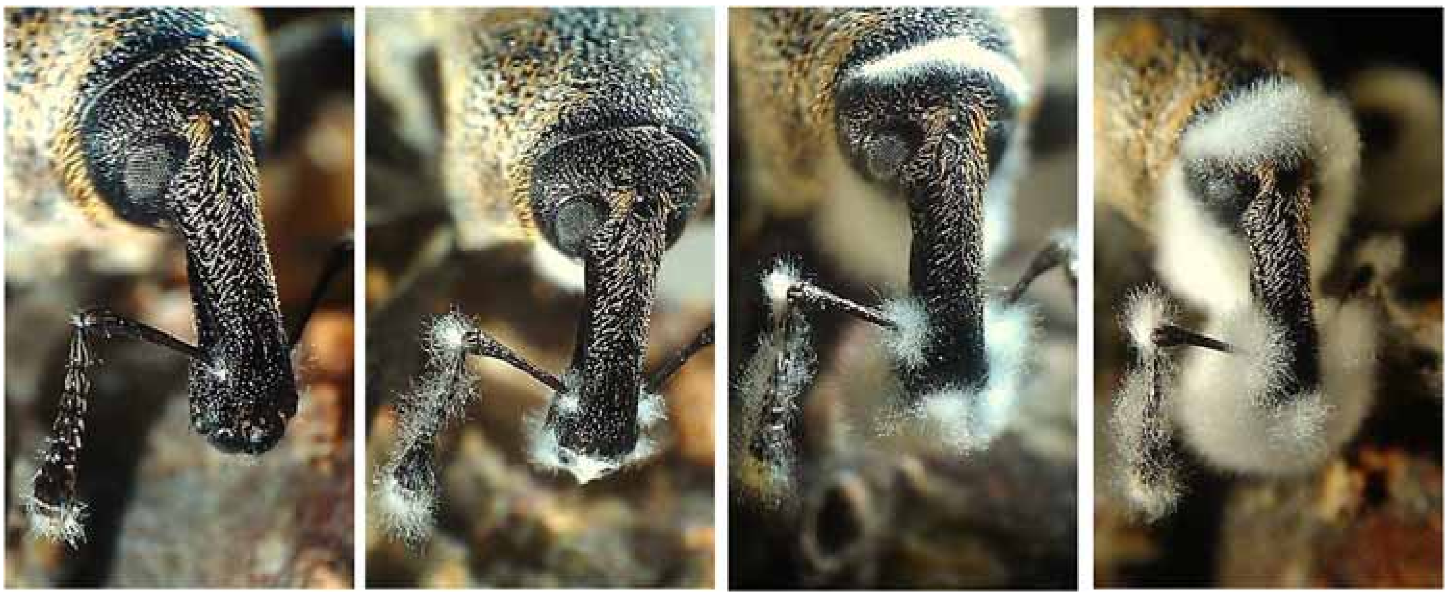
During the experiment, the *Metarhizium anisopliae* species killed all the pine weevils to the 45th day. The result was in agreement with Ansari and Butt (2012) who used *Metarhizium anisopliae* to fight pine weevil and achieved 100% mortality. All imago were killed by fungus within twelve days [12]. *Isaria fumosorosea* was not effective against pine weevil as it killed only one male and only two females after one week. Efficiency of this fungus to other kinds of insects was confirmed by several works such as Pell and Vanderberg (2002) who investigated the efficacy of this fungus on aphid *Diuraphis noxia* [13]. Very encouraging results of testing *B. bassiana* in the biological control against bark beetle (*Ipstypog raphus*) under practical conditions of forestry in the Slovak Republic was obtained by Noge (2013) [14].

One of the first signs of the effect of entomopathogenic fungi was reduction of food intake.Among the different fungi tested, best efficiency to reduce the intake of food was caused by *B. bassiana* fungus. Fungus-infected beetles died after ten days, and food was changed after twenty-two days. It is assumed that if the pine weevils survive to the age fifteen days, the nibbled areas would be smaller than the control.This assumption was confirmed by the results of Lalik (2013), beetles in its experiment had the same mortality rate, but the nibbled areas were smaller compared to the control. Fungus *M. anisopliae* did not affect the reduction of nibbled areas during sexual feeding. The fungus *I. fumosorosea* did not affect feed intake, but on the contrary, it resulted in increase in food consumption[15].

The other results of testing the effectiveness of the biological products suggest that adult pine weevils are susceptible to different strains of entomopathogenic fungi depending upon the concentration used. Very effective preparations had a high concentration of spores. Fastest reaction of pine weevils was recorded by use the entomopathogenic fungus *B. bassiana* from the natural environment applied by dry method in which pine weevils died within 6-8 days of infection. Quick reaction of pine weevils was also noticed by application of wet process, using the suspensions at concentrations of 10^7 and 10^8 spores /ml.

Good mycelial growth depends mainly on very high relative humidity reaching 90% or more. Entomopathogenic fungal hyphae overgrowth was observed mostly under the microscope already on the first day after killing of beetle. Single hyphae of *Beauveria bassiana* started to overgrow among the softer parts of individual body parts of dead beetle mostly joints of the legs, the connections of the head and chest, chest and abdomen, but primarily it was an oral and anal hole.

Growing of mycelium was macroscopically visible 1-4 days after death. Mycelium was visible in the form of initially little groups of tiny fungal fibers, which began to form larger sparse clusters, filling the space between the elements of the insect body. Mycelial growth was relatively quick, and at the third to fourth day after the occurence of the fungus, snout white, thick fluffy formations were created in the limbs and joints area. At this stage its mycelium grew very quickly, fluffy formations were connected together and formed a continuous layer of dense white covering mainly the lower body beetle; fully covered in exceptional cases, in addition to its elytron. Entomopathogenic fungus *Metarhizium anisopliae* over grown essentially the same part of the body as *Beauveria bassiana*. The mycelium was initially very dense and created the white coatings that gradually changed the color to olive green. Its growth was not as intense as the kind *Beauveria bassiana*.



2nd day **3rd day** **4th day** **5th day**

Figure 4. The growth of the mycelium on the head of Beauveriabassiana recorded automatically every 24 hours from the second to the fifth day from the death. (Photo: JurajŠkvarenina)

Table 1. Pine weevil´s mortality depending on time, sex and infection

	Number of male and female pine weevils dead/ overgrown by different entomopathogenic fungi (15 insects in each treatment)									
On date	**Control**		***Beauveria bassiana* - dry**		***Beauveria bassiana* -suspenion**		***Metarhizium-anisopliae***		***Isaria fum-osorosea***	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
19.7	0	0	0	0	0	0	0	0	0	0
23.7	0	0	15//1	12//2	0	0	0	1/0	0	0
26.7	0	0	15/15	15/15	5//2	2//1	1/0	1/0	0	1/0
30.7	0	0	15/15	15/15	14//12	10//7	3//3	6/4	0	1//1
2.8	0	0	15/15	15/15	15/14	13//9	4//4	8/4	0	1//1
6.8	0	0	15/15	15/15	15/14	14//11	4//4	8/4	0	1//1
9.8	0	0	15/15	15/15	15/15	14//12	8//6	9/7	0	5//4
13.8	0	0	15/15	15/15	15/15	15/14	10/9	9/7	0	5//5
16.8	0	0	15/15	15/15	15/15	15/14	11/10	12/11	0	5/5
20.8	0	0	15/15	15/15	15/15	15/14	13/10	12/11	0	5/5
23.8	0	0	15/15	15/15	15/15	15/14	14/13	14/14	0	5/5
27.8	0	0	15/15	15/15	15/15	15/14	14/13	14/14	1//1	6/6
30.8	0	0	15/15	15/15	15/15	15/14	15/15	15/15	1//1	6/6
3.9	0	1/1	15/15	15/15	15/15	15/15	15/15	15/15	1//1	6/6
6.9	1/1	1/1	15/15	15/15	15/15	15/15	15/15	15/15	1//1	6/6

CONCLUSION

Damage caused by the activity of pine weevil in several European, but especially in the Scandinavian countries, is forcing to intensify research on possible methods of protection of seedlings and young plants in forests by control of this pest. The forest stands status after the large-scale, but also the size of the smaller disasters, creates favorable conditions for its expansion and the emergence of significant damage. Due to the limited options for avoid the emergence the appropriate conditions for its reproduction and distribution, they are searching possibilities of its active control. Therefore, the biological control appears as one of the most effective, close to the nature. The aim of our research was to evaluate the biological activity of three species of entomopathogenic fungi on adults of pine weevil and to evaluate survival and food intake by infected imago.

Entomopathogenic fungus had certain influence on feeding of pine weevil only at higher concentrations in applicated biological preparations. The results show that under laboratory conditions fungus work quite well and their research should definitely continue. It is appropriate to focus for the particular application of different entomopathogenic fungi at higher concentrations and of selecting the fungi species that are effective.

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