Entomopathogenic Fungi in the Soils of Forest Plantations: Towards the Control of Large Pine Weevil, *Hylobius abietis*

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Abstract

The study was aimed at estimating species structure and density of entomopathogenic fungi in soils of forest plantations that are a natural habitat of the large pine weevil, *Hylobius abietis* L. Soil samples were collected in spring and autumn 2010 and 2011 in forest districts of northern, central and southern Poland. Fungi were isolated from soils with the trap insect method and on selective growing media.

As a result, 5 species of entomopathogenic fungi were isolated: *Beauveria bassiana*, *Isaria farinosa*, *Isaria fumosorosea*, *Metarhizium anisopliae* and *Verticillium lecanii*, of which *I. fumosorosea* and *M. anisopliae* were isolated most frequently. The number of infected trap insects and the number of fungal colonies isolated on growing medium differed depending on the term and place of soil sampling. It seems that type of forest habitat might be the factor affecting species structure and the number of entomopathogenic fungi in forest plantations established on clear cuttings, where stumps of coniferous trees provide the place for development of the large pine weevil.

Key words: entomopathogenic fungi, forest plantation, *Hylobius abietis*, soil

Introduction

The large pine weevil *Hylobius abietis* L. is a species, whose beetles feed on seedlings of all coniferous tree species causing damage to bark, phloem and, to a less extent, needles. They may also damage deciduous trees, especially in mixed plantations (Wallertz et al. 2006, Thorpe and Day 2008, Gradinariu et al. 2012). Larvae of the insect are not harmful since they develop in roots of stumps that remain after falling the trees mainly the Scots pine *Pinus sylvestris* L. and Norway spruce *Picea abies* (L.) H. Karst. The large pine weevil is a vector responsible for transmitting of tree pathogens, e.g. spores of the fungus *Heterobasidium annosum* (Fr.) Bref. causing root rot - one of the most important economic disease of forest trees (Kadlec et al. 1992). *Hylobius abietis* may also contribute to the distribution of other phytopathogenic fungi like *Leptographium lundberii*, *Ophiostoma floccosum*, *O. piliferum*, *Sporothrix inflate* (Jankowiak and Bilanski 2013).

Protection of forest plantations against the large pine weevil is still one of more important problems in forest management. To protect young forests against this species different methods are used in practice. To directly reduce the number of pine weevils, freshly cut and debarked conifer billets or various kinds of traps baited with alpha-pinen and ethanol are used. Chemical treatments consist in dipping the above-ground parts of seedlings before planting or spraying them with contact insecticides after planting have been most often form of forest protection until recently (Glowacka et al. 1991). However, in Sweden for many years, the studies have been conducting on using of various kinds of physical barriers assumed to the stems of seedlings (Lindström et al. 1986, Eidmann et al. 1996, Petersson et al. 2004). As a result, a new method

was developed, in which the stems of conifer seedlings are covered with flexible sand coating (Conniflex) (Nordlander et al. 2009). The use of Conniflex leads to a significant increase (more than 85 %) of young tree survival.

An alternative to mechanical and chemical methods would be to use pathogenic microorganisms and parasitoids, predatory insects and vertebrates as control factors reducing the number of harmful entomofauna are carried out in many scientific institutions. In the case of the large pine weevil, the studies attempt to use entomopathogenic nematodes and fungi. In Europe such studies focussed mainly on nematodes of the genera *Steinernema* and *Heterorhabditis* (Brixey et al. 2006, Dillon et al. 2007, Skrzecz et al. 2011, Tumialis et al. 2013).

Studies on the use of entomopathogenic fungi in limiting the number of the large pine weevil were carried out by Wegensteiner and Führer (1988), who analysed mortality of beetles infected by *Beauveria bassiana* (Bals.) Vuill. in laboratory conditions. Similar tests were made by Ansari and Butt (2012), who infected all growth stages of *H. abietis* with fungi: *B. bassiana* and two species of the genus *Metarhizium*: *M. robertsii* (Metschn.) Sorokin and *M. brumneum* Petch. Field tests of combined effect of nematodes *S. carpocapsae* (Weiser) and *Heterorhabditis downesi* (Stock, Griffin and Burnell), and fungi *B. bassiana* with *Metarhizium anisopliae* (Metschn.) Sorokin on various growth stages of *H. abietis* were made in Ireland (Wiliams et al. 2013).

The most frequent entomopathogenic fungi in field and forest soils of Poland are: *B. bassiana*, *M. anisopliae*, *Isaria farinosa* (syn. *Paecilomyces farinosus*), *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*) and *Verticillium lecanii* (Bajan and Kmitowa 1997). They belong to the fungi imperfecti of the class Hyphomycetes and are potentially most effective in the control of pest insects in Poland (Balazy 2000).

So far, there is little information on a possible use of entomopathogenic fungi in controlling *H. abietis*. Therefore, species composition of these pathogens in forest plantations, where stumps provide a basis for growth and young trees are food for the large pine weevil has not been analysed. Such studies may help isolate a strain of entomopathogenic fungus, whose pathogenic properties would be great enough to use it in biological preparations potentially applicable in the insect control.

The aim of studies performed in 2010-2011 was to estimate species structure and density of entomopathogenic fungi in the soils of forest plantations at various time of growing season in different sites that are natural habitats of the large pine weevil.

Materials and methods

Study sites were localised on 1 to 3-year-old clear cuttings. In spring (the first half of May) and autumn (the second half of September) 3 soil samples were taken every year with the Egner's sampler (2.5 cm in diameter) to the depth of 20 cm. Each soil sample consisted of 5 contents of the sampler (1 kg in total), which were mixed to obtain a homogenous sample from each site. Soil was then placed in foil bags and taken to the laboratory.

Studies were carried out in forest districts in northern, central and southern Poland. Each site was given a symbol denoting its location in the country (N – north, C – centre, S – south) followed by a number (Figure 1). Characteristics of study sites and their symbols are shown in Table 1. Regardless of the samples, the air temperature was similar, but spring rainfall was more than 2-fold higher (Table 2). The weather data came from meteorological stations of the State Forests located up to 30 km from experimental plots. Acidic podzolic soils were mainly present in the study areas and their pH ranged from 5.0 to 5.5.

In the laboratory, entomopathogenic fungi were isolated from soils with the method of trap insects using larvae of the greater wax moth *Galleria mellonella* L. (Zimmerman 1986). The larvae of this species are commonly used in microbiology to isolate the entomopathogenic microorganisms and nematodes from the soils due to high sensitivity to infections caused by pathogens. By this, the larvae die as a result of infections caused by the fungal spores that are present in the soil sample. Larvae of *G. mellonella* came from continuous laboratory rearing on bee wax in temperature 25 °C and the darkness. To isolate entomopathogenic fungi, each soil sample was placed in 10 plastic containers of 250 cm^{-3}, filled with tested soil to ¾ height. Then, 10 of *G. mellonella* larvae

Figure 1. Location (geographical coordinates) of forest plantations, from which soil was sampled: N-1 (53°53'57.3"N, 20°47'37.5"E); N-2 (53°51'18.9"N, 20°49'06.2"E); C-1 (52°03'34.3"N, 21°23'09.7"E); C-2 (52°05'19.3"N, 21°17'15.2"E); C-3 (52°10'28.3"N, 21°46'29.9"E); C-4 (52°10'21.5"N, 21°32'48.3"E); S-1 (49°39'02.1"N, 19°17'33.2"E); S-2 (49°40'08.5"N, 19°04'11.9"E); S-3 (49°39'47.4"N, 19°04'28.8"E)

Table 1. Characteristics of forest plantations, from which the soil samples were taken

Symbol of area	Forest habitat	Area of clear cut (ha)	Harvested stand: main species/age (years)	Date of clear cut establishment	Date of reforestation
N-1	Fresh coniferous forest	3.20	*P. sylvestris*/105	February 2010	April 2010
N-2	Fresh mixed coniferous forest	1.80	*P. sylvestris*/100	February 2006	April 2008
C-1	Fresh coniferous forest	1.36	*P. sylvestris*/95	September 2007	April 2009
C-2	Fresh coniferous forest	1.01	*P. sylvestris*/100	February 2008	April 2010
C-3	Fresh coniferous forest	2.50	*P. sylvestris*/107	February 2008	April 2010
C-4	Fresh coniferous forest	2.22	*P. sylvestris*/102	February 2009	April 2010
S-1	Mixed mountain coniferous forest	1.50	*P. sylvestris*/90	September 2007	April 2008
S-2	Mixed mountain forest	2.50	*P. abies*/85	February 2008	April 2008
S-3	Mixed mountain forest	2.15	*P. abies*/90	September 2007	April 2008

Table 2. The average monthly air temperature and total rainfall in the months of sampling

Year	Months	Localization of experimental plots	Air temperature (^{0}C)	Rainfall (mm)
2010	May	North	12.72	56
		Center	12.61	48
		South	10.94	93
	September	North	10.11	22
		Center	11.39	18
		South	9.28	44
2011	May	North	11.78	58
		Center	11.28	49
		South	10.62	118
	September	North	11.50	9
		Center	11.56	2
		South	10.48	17

in L3 stage were placed in each container. The tested soil was not moistening in the containers. After 21 days of rearing (temp. 25 °C, humidity 60%), dead insects were transferred to Petri dishes lined with wet filter paper and individuals covered with mycelium were counted. Then, mycelia from insects were placed in Petri dishes filled with Sabouraud Dextrose Agar (medium based on peptone, glucose and agar). Fungi from grown colonies were determined to species using keys by Samson et al. (1988) and Humber (2012).

To determine the number of entomopathogenic fungi in analysed samples, soil was distributed on selective media with the surface methods (from 10^{-2} dilution of soil solution) and developed colony forming units (cfu) of fungi were counted (Strasser et al. 1996). Fungi were determined to species as above.

The results were statistically processed with the IBM SPSS Statistics 21 software. Non-parametric Kruskal-Wallis test was used to compare the degree of infection of test insects by particular fungi species and the number of isolated colonies in study sites. In case of finding differences, Mann-Whitney U test was applied at $P < 0.05$.

Results

Five species of entomopathogenic fungi were isolated in this study: *B. bassiana, Isaria farinosa* (Holmsk.) Fr., *Isaria fumosorosea* Wize, *M. anisopliae* (Metsch.) Sorok and *Verticillium lecanii* (Zimm.) (Figures 2, 3).

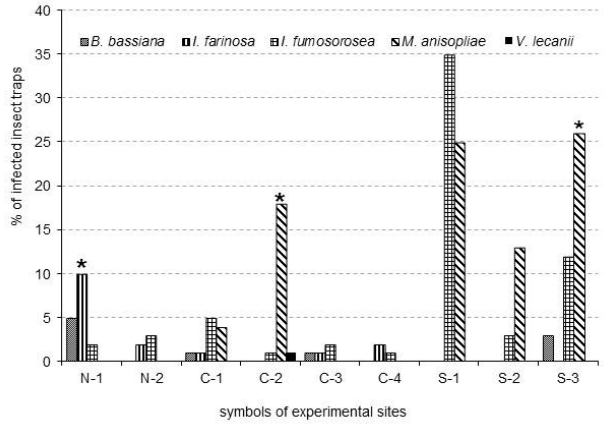

Figure 2. Percentage of insects infected by entomopathogenic fungi isolated from soil in spring. *Statistically significant differences between the number of trap insects infected by fungi from sites N-1 ($P = 0.048$), C-2 ($P < 0.001$) and S-3 ($P = 0.006$)

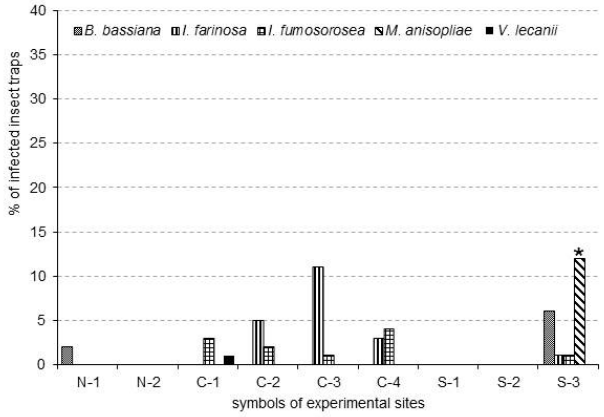

Figure 3. Percentage of insects infected by entomopathogenic fungi isolated from soil in autumn. *Statistically significant differences between the number of trap insects infected by fungi from site S-3 ($P = 0.006$)

In soil samples collected in spring, most trap insects were infected by *I. fumosorosea* and *M. anisopliae* (Figure 2). *Isaria fumosorosea* was isolated from soils of all analysed forest plantations. Most insects (35 %) were infected by isolate of this species obtained from soil of a single site in southern Poland (S-1). The second most infecting species was *M. anisopliae*, which occurred in all sites of southern Poland (13-26 % of infected insects) and with less intensity (4 and 18 % infected insects) in two sites of central Poland (C-1 and C-2, respectively). Other three species infected fewer insects. *Isaria farinosa* was mainly isolated from soils of central and northern Poland. Most pathogenic isolate of this species, which infected up to 10 % of insects, was from northern Poland (N-1). *Beauveria bassiana* was isolated from only a few sites in northern (N-1), central (C-1 and C-4) and southern Poland and infected no more than 5 % of test insects. *Verticillium lecanii* was isolated from only one soil sample from central Poland (C-2) and infected only 1% of insects.

In samples collected in autumn, the presence of above mentioned species was found in all sites of central Poland and only in single sites in the north and south of the country (Figure 3). Moreover, the fungi infected much fewer (up to 12 %) trap insects. Most insects (12 %) were infected by *M. anisopliae* isolated from soil sample taken exclusively in one site in southern Poland (S-3). Most frequently isolated were fungi from the genus *Isaria* (soils from central Poland and one site in the south of the country – S-3) recorded in 11 % (*I. farinosa*) and 4% (*I. fumosorosea*) of insects. *Beauveria bassiana* was isolated from only one site in northern Poland (N-1) and one in southern Poland (S-3) and infected up to 5 % of insects. The least frequent was *V. Lecanii,* which infected 1 % of insects. As in spring samples (Figure 2), it was isolated in autumn from soil of only one site in central Poland (C-1) (Figure 3).

Three species of entomopathogenic fungi (*I. farinosa*, *I. fumosorosea* and *M. anisopliae*) from soils taken in spring and 4 species (all above plus *B. bassiana*) from soils obtained in autumn (Figures 4, 5, respectively) were isolated on selective medium. *Verticillium lecanii* was not isolated on selective medium. None of the fungal species from soils taken in spring in site N-2 and in two sites in central Poland was isolated on selective medium (Figure 4) despite the fact that infection of trap insects by several species of fungi had been observed in these sites earlier.

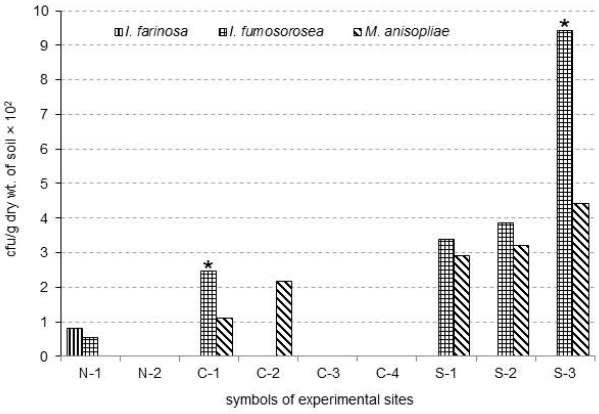

Figure 4. The number of colony forming units (cfu) of entomopathogenic fungi isolated from soil collected in spring. *Statistically significant differences between the number of cfu of fungi isolated from soil of site C-1 ($P = 0.048$) and S-3 ($P = 0.021$)

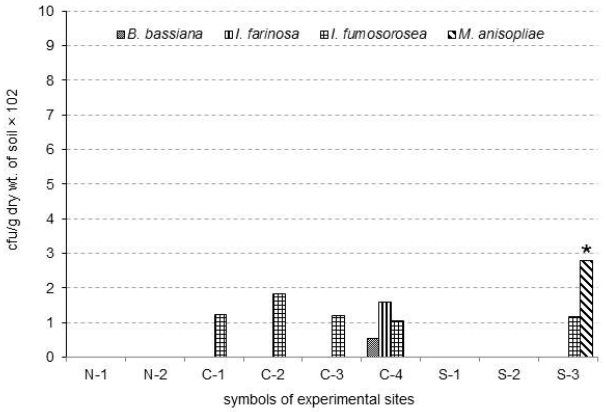

Figure 5. The number of colony forming units (cfu) of entomopathogenic fungi isolated from soil collected in autumn. *Statistically significant differences between the number of cfu of fungi isolated from soil of site S-3 ($P = 0.007$)

Similarly, in autumn samples entomopathogenic fungi were isolated from soils in central Poland and from only one site in southern Poland (S-3) (Figure 5).

Isaria fumosorosea was the species most frequently isolated from both spring and autumn samples on selective medium (Figures 4, 5). The highest number of cfu of this species was isolated from samples collected in spring in the south of Poland (S-3: over 9×10^2 cfu/g dry wt. of soil) (Figure 4), about 4 times less from soils of central Poland (C-1: about 2.5×10^2 cfu/g dry wt. of soil) and the least from soils in the north of the country (N-1: about 0.5×10^2 cfu/g dry wt. of soil). In autumn, *I. fumosorosea* was isolated on growing medium from soils of all sites in central Poland and from one site in the south (S-3) (Figure 5). However, in autumn the number of isolated cfu of this species was lower, up to 1.8×10^2 cfu/g dry wt. of soil.

Metarhizium anisopliae was more often isolated on growing medium from soils taken in spring than in autumn (Figures 3, 4). In spring the species was isolated from soils of central and southern Poland: from about 1.0×10^2 (C-1) to nearly 4.5×10^2 (S-3) cfu/g dry wt. of soil (Figure 4). In autumn, the species was isolated only from southern sites (S-3: nearly 3×10^2cfu/g dry wt. of soil) (Figure 5).

The remaining two species: *I. farinosa* and *B. bassiana* were isolated on growing medium from soils of single sites: *I. farinosa* obtained in spring only from one site in the north (N-1: less than1×10^2cfu/g dry wt. of soil) (Figure 4) and in autumn from soil in central Poland (C-3: over 1.0×10^2cfu/g dry wt. of soil) (Figure 5). *Beauveria bassiana* was isolated only from soil sampled in autumn in one site of central Poland (C-4: less than 1.0×10^2cfu/g dry wt. of soil) though the species was not found in this site with the use of trap insects.

Discussion

It was found that soils of 1-3-year-old forest plantations are the habitats of 5 species of entomopathogenic fungi (*M. anisopliae*, *I. fumosorosea*, *B. bassiana*, *I. farinosa* and *V. lecanii*), which are commonly found in soil habitats (Bajan and Kmitowa 1997, Bałazy 2000). These species are classified as facultative pathogens. They can develop in the soils as saprotrophic fungi, which is very important for their survival after application in the form of biopreparations.

Definitely more isolates of fungi were found in soils sampled in spring than in those taken in autumn. The differences resulted certainly from higher soil moistures in spring time, which is commonly known to facilitate the development of fungi in soil habitat. In spring, southern soils (mountain sites) were most frequented by entomopathogenic fungi and in autumn – those in the centre of the country. Such result could be an effect of the higher moisture of mountain sites in spring. The effect of geographic location and habitat type on the presence of entomopathogenic fungi was studied by Vanninen (1995). Based on her analyses of soils in Finland, the author found that geographic location was important for *M. Anisopliae,* while *B. bassiana* and *P. farinosus* (syn. *I. farinosa*) were more affected by habitat type. According to the study by Leger et al. (1992), the occurrence of fungi in the environment can also be affected by host range (wide or narrow) and their availability. These authors concluded that the most of *M. anisopliae* pathotypes has potentially broad host ranges, and minority had a narrow specialisation towards a particular group of hosts. The isolates genetically adapted to a narrow host range have a lower degree of physiological adaptation compared with isolates adapted to a wide host range.

The results demonstrated that *M. anisopliae* and *I. fumosorosea* were most frequent species of entomopathogenic fungi in soils of forest plantations, especially in spring. However, a high number of their spores in sampled soil (i.e. cfu on the growing medium) not always corresponded with the number of infected insects. This was observed in *I. fumosorosea* isolated from soil in southern Poland (S-3). In spite of relatively high number of spores of this fungus, infection of trap insects was rather low, which might evidence low biological activity of the pathogen. A reverse situation was observed in *M. anisopliae* isolated from soil of the same site, which indicated a high activity of its spores in the habitat. It seems that if in the soil sample there is a high number of spores and parasitism of insect is low, it may be related to reduced activity of this fungus in these environments. Such correlation may affected by the life strategy of a fungus (Chandler 2009). According to the strategy "sit and wait",

the fungus can produce a large number of spores, which greatly increases the possibility of insect infection and survival of spores in the soil, even in the absence of the host. Miętkiewski et al. (1991) found the domination of *Paecilomyces fumosoroseus* (syn. *I. fumosorosea*) among entomopathogenic fungi inhabiting 50 to 60-year-old pine tree stands. However, these authors did not find the presence of *M. anisopliae*, which was often found in soils of forest plantations studied by us. Such results may suggest that *M. anisopliae* prefers soils of open areas such as clear cuts with up to 5-year old seedlings, compared to soils covered with older trees.

Beauveria bassiana also found in analysed soils, in spite of its presence in many sites had no high biological activity. Miętkiewski et al. (1991) noted that the species is infrequent in forest soils. So far, this fungus can kill the large pine weevil only in laboratory conditions. Such a conclusion may be supported by results obtained by Ansari and Butt (2012), who observed 100 % mortality of all growth stages of *H. abietis* infected in the laboratory by *B. bassiana* and by two fungi of the genus *Metarhizium*: *M. robertsii* and *M. brumneum*. Wegensteiner and Führer (1988) found 86-100 % mortality of the large pine weevil beetles infected with a dose of 4×10^7 conidia of *B. bassiana*/insect at 20 and 33 ^{0}C in the laboratory conditions. However, no fungal infections were noted in beetles feeding on bark treated with the fungus in the field conditions. This means that *B. bassiana* present in natural soils probably exerts an insignificant effect on the density of the large pine weevil in forest plantations. Field attempts to control populations of the large pine weevil with *B. bassiana* and *M. anisopliae* applied together with entomopathogenic nematodes: *Sterneinema carpocapsae* and *H. downesi* made by Wiliams et al. (2013) showed a higher effectiveness of nematodes, which were responsible for 50 % mortality of the larvae of the lesser pine weevil. Fungi infected 20 % of larvae and pupae of the pest. No synergy between the effect of applied species of nematodes and fungi was found. Perhaps a poor ability to reproduce within the forest environment is the reason for low biological activity of *B. bassiana* applied against *H. abietis* in field conditions.

The next species isolated from forest soils was *V. lecanii* present exclusively in one site of central Poland. The number of trap insects infected by this species was low (1 %). This allows for supposition that *V. lecanii* is present sporadically in studied forest habitats. *Isaria farinosa* was isolated from one site in the north and one in the centre of the country and may be thus classified to infrequent fungi in soils of forest plantations. Miętkiewski et al. (1991), however, did not find *I. farinosa* in forest soils. On the contrary, Chandler et al. (1997) and Medo and Cagáň (2011) found that *B. bassiana* and *I. farinosa* were more frequent than *M. anisopliae* in forest habitats.

So variable results of presented studies, sometimes different from those obtained by other authors, may results from the fact that the presence and activity of entomopathogenic fungi in soils are affected by many factors like organic matter content, pH, type of soil and the way of its cultivation (Walstad et al. 1970, Quesada-Moraga et al. 2007, Bouamama et al. 2010). Also microclimatic conditions (temperature, moisture) and host density strongly influence on trophic interaction between entomopathogenic fungi and insect populations (Walstad et al. 1970, Karg and Balazy 2009). Dispersion of fungi in soils is facilitated by various arthropod species of the order *Collembola* (Dromph 2001, 2003). A decrease of biomass and taxonomic differentiation of entomofauna was found to limit pathogens due to a lack of appropriate host species (Karg and Bałazy 2009). It was noted, for example, that *M. anisopliae* finds favourable conditions for development as a saprophyte in the rhizosphere and its occurrence depends also on plant community overgrowing soil (Hu and Leger 2002, Wang et al. 2005). Moreover, Leger (2008) is of the opinion that rhizosphere is a place of mutual interactions between insects and their pathogens and the effectiveness and survival of the pathogen is affected by interactions among plant, insect and pathogen. Literature data also reveal that fungi in soil may interact with each other. Some authors even suggest that competition between soil fungi may sometimes be stronger than effects of habitat factors such as soil moisture (Widden and Abitbol 1980).

Obtained results are the first in the country contribution on species composition of entomopathogenic fungi in soils of forest plantations established on clear cuttings. Continuation of these studies will allow for selecting native isolates of fungi with optimum activity against beetles of the large pine weevil. Application of such isolates in practice may supplement other biological methods as an element of integrated protection of crops from the large pine weevil. This is evidenced by many studies aimed at the selection of entomopathogenic fungi characterized by high biological activity as the promising factors in the protection of forest plantations against the large pine weevil (Williams et al. 2013).

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