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THE POTENCIAL OF USE OF ENTOMOPATHOGENIC FUNGI AS BIOLOGIC REGULATORS OF POPULATIONS OF BARK BEETLES *I.TYPOGRAPHUS*

Abstract. In this study, we observed several points of České Švycarsko National Park (Czech Republic) and collected more than 50 adults of bark beetles which were covered with white mycelium. After partial sequence of secretory lipase partial mRNA isolates, most of them were identified as *B. bassiana.* Testing of isolates of the entomopathogenic fungi *Beauveriabassiana (Bals.) Vuill.* and *Isariafarinosa (Holm.)* against adults of *Ipstypographus* (Coleoptera: Scolytidae) was during 11 days.

As a result, more than 20 isolates had a high virulence. Most of them had highest lethal effect after 9th day of inoculation. The isolates Inc1 and Inc22 of *Paecilomyces* caused low mortality to bark beetles.

Key words: Entomopathogenic fungi, *Beauveriabassiana, Isariafarinosa, Ipstypographus*.

Introduction. Three phloeoxylophagous insects are indexed in the law of the Czech Republic as pests that might cause calamities in forests – *Ipstypographus*, *Pityogeneschalcographus* and *Hylobiu-sabietis* [1].

Attacks by spruce bark beetle (*Ipstyphographus*) on Norway spruce (*Piceaabies*) forest stands can cause huge damage in Europe. This bark beetle is the most important pest beetle in Europe. Its population densities have increased during the recent years. Except of mountainous and boreal parts of its distribution (i.e. native distribution of Norway spruce), has this species more generations per season [2].

There are records of outbreaks dating from the eighteenth century. The losses that occurred during some of these outbreaks, in millions of cubic meters of wood, were as follows (Wellenstein, 1954; Schwerdtfeger, 1955; Worrell, 1983; Christiansen & Bakke, 1988): outbreaks have also occurred in Italy (Lozzia, 1993), Poland, Czech Republic (Pfeffer&Skuhravy, 1995) and on Hokkaido Island, Japan [3, 4, 13].

Synthetic formulations of entomopathogenic microorganisms, such as fungi, bacteria, and viruses, may also be useful for managing bark beetle populations. Efforts have focused largely on the fungus *Beauveriabassiana (bals.) Vuill.* (*Ascomycota: Hypocreales*), which has been demonstrated to cause high levels of mortality in several species of bark beetles, including *I. typographus* (Wegensteiner, 1992, 1996; Kreutz et al., 2000, 2004) [5-7].

Material and methods. Bioassays were conducted under laboratory conditions in 2016. 33 isolates of entomopathogenic fungi were from the collection of the biotechnology laboratory of the Kazakh Institute of Plant Protection and Quarantine. Bioassays to evaluate the efficacy of entomopathogenic fungi isolates against of *I.typographus* were conducted under laboratory conditions in the Czech University of Life Science.

The incidence of entomopathogenic fungi directly associated with the adults of spruce bark beetles and with soil closely related to sites with a presence of *I.typographus* has been monitored in the 2016. The new 50 entomopathogenic isolates used in bioassays was isolated into the pure culture on Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) from dead adult bark beetles found in spruce in the České Švycarsko National Park (Czech Republic). The infected bark beetles were taken to the laboratory.

Two solid media Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were used for the study. Twenty ml of autoclaved solid media were poured into a sterilized Petri plates. Potato dextrose agar was prepared and sterilized with streptomycin as an antibiotic to avoid bacterial contamination. A 10 mm actively grown culture of selected entomopathogenic fungi was placed individually in the center of the respective medium. The inoculated plates were incubated at 25^0 C for 10 days. Three replications were maintained. The diameter of the fungal colony was measured following Daggupati (1988).

The fungal pathogen was identified according to morphological characteristics as on the host as on a culture [8].

Conidia of the isolates used as infectious units in bioassays were obtained after twenty-days cultivation on SDAY in tubes at 25^0 C following by washing down with sterilized water. The concentrations of conidia were determined by counting in hemocytometer. Aqueous suspensions applied in bioassays were prepared by dilution at concentration of 1×107 conidia/ml.

Adults of *I. typographus* used in bioassays were from natural populations of the pests collected from spruce bark in Českе `Švycarsko National Park (Czech Republic). They were treated by a surface contact with 1 ml of conidial suspensions for 24 h placed on filter paper discs (100 mm in diameter) in Petri dishes (Draganova and Staneva, 1988). Experiments were carried out in three replicates with 10 adults per replicate at temperature $25±2^0$ C and 60% RH. Insects were fed on spruce bark 24 h after the treatment. Adults in control variants were treated with water instead of conidial suspension [7].

Molecular identification of strains. After cultivation, DNA from the isolates on PDA media for 7 days at 25±1°c, DNA was extracted by the sigma's gene lute Tm plant genomic DNA miniprep kit provides a simple and convenient way to isolate pure DNA from a variety of fungi. The Gene lute kit combines the advantages of a silica-based system with a micro spin format and eliminates the need for expensive resins, RNASE treatment, and hazardous organic compounds such as phenol and chloroform.

Several micrograms of DNA can be obtained from up to 100 mg of fresh tissue or 10 mg of freeze-dried material in less than an hour. The pure DNA is greater than 20 kb in length and can be used in sensitive downstream applications such as restriction endonuclease digests and PCR amplification.

The polymerase chain reaction (PCR) for amplification of fragment secreted lipase (slip) region was performed by a pair of universal primers slip generating estimated size of 600 bp product.

The amplification reaction. Conditions consisted of 5 min at 94°c followed by 40 cycles of 1 min at 94°c, 45 s at 54°c and 1 min at 72°c with a final extension of 4 min at 72°c. PCR conditions were adapted essentially as described by Rehner & Buckley (2005). PCR products were separated on 1% agarose gel and visualized under UV light. Amplification products were extracted from agarose gels with the Gel Extraction Kit (50) and sent to sequencing. Obtained sequences were used to carry out BLAST searches by using the NCBI GenBank database to confirm isolate identification. Additionally, the sequences were used to compare the representative sequences from the study that were included for comparison of *Beauveria* strains of Rehner & Buckley (2005) [9, 10].

Data analysis. Sequences were assembled and edited with BioEdit and aligned (Hall, 1999). Cluster analyses of the sequences was performed using BioEdit (version 7.09) with Clustal W followed by Kimura-2 parameter analysis with neighbor joining analysis on aligned sequences was performed with MEGA 7.0 software. Alignment gaps were treated as missing data. Reliability of phylograms was tested by bootstrap analysis with 1000 replicates using MEGA 7.0[11].

Mortality values were corrected according to Abbott's formula (Abbott, 1925) [12]. For statistical evaluation all experiments were used one-way ANOVA and Fisher LSD in SPSS program. For all studies, controls were always statistically different from treatments ($p<0.05$).

Results and discussion. This study was conducted in the ČeskeŠvycarsko National Park (Czech Republic) between May and September 2015 to found the fungal infection of *I. typographus*. After collecting dead adults with mycosis from several points, we found more than 50 adults bark beetles which were covered with white mycelia (figure 1). The fungus was isolated and cultivated on SDAY medium to determine some of the morphological features. The conidia were like globose and diameter of the conidia was measured as 2.93 ± 0.24 μm. The conidial chains were long and conidial heads diffuse. The colony color was white on SDAY medium after two weeks. Based on its morphological features, it was identified as *Beauveriabassiana* sensulato.

a *b*

Figure 1 – Collection of live and dead bark beetles with mycosis

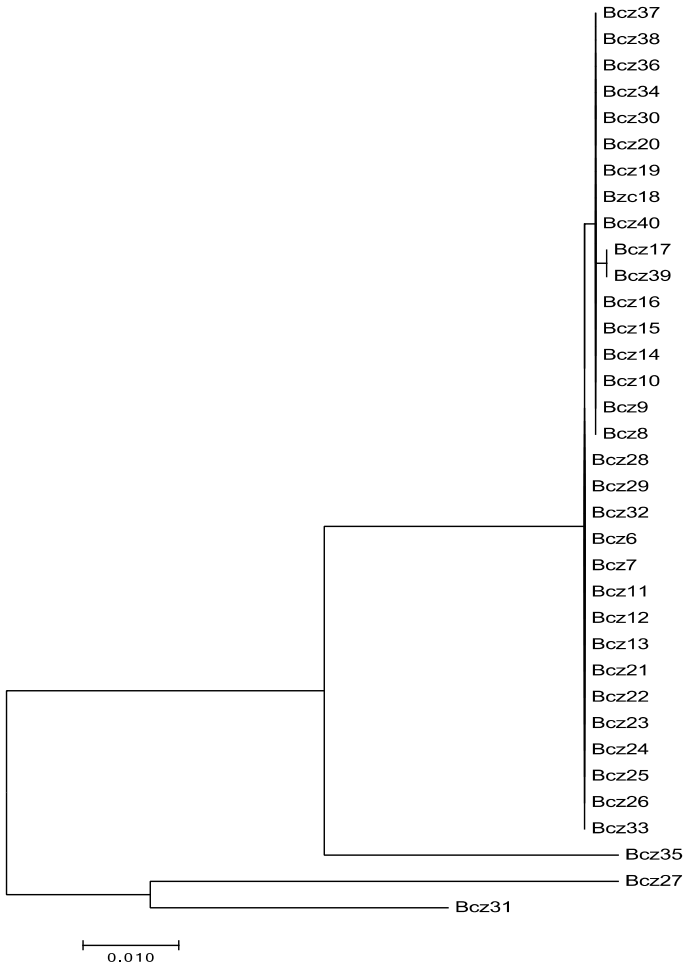

Figure 2 –
Phylogenetic position of the isolates
from bark beetles within Beauveria genus based
on SLIP sequence.
The phylogenetic tree construction
was conducted with neighbour –
joining method packaged
in software MEGA 7.0

A large body of information and data has been acquired over the past years on various ecological aspects of fungal entomopathogens. The use of molecular techniques has increasingly influenced ecological research on fungal entomopathogens and in combination with other disciplines has contributed to the progress made during the past decade. After partial sequence of secretory lipase partial mRNA isolates, most of them were identified as *B. bassiana* (figure 2).

In the next stage of the study, we are testing this isolates against *I. typographus.* Adults *I. typographus* used in bioassays were from natural populations of the pests collected from ČeskeŠvycarsko National Park (Czech Republic). They were treated by a surface contact with 1 ml of conidial suspensions for 24 h placed on filter paper discs (100 mm in diameter) in Petri dishes. Experiments were carried out in three replicates with 10 adults per replicate at temperature 25 ± 2^0 C and 60% RH. As a result, more than 20 isolates had a high virulence. Most of them had highest lethal effect after 9th day after inoculation. The isolates Inc1 and Inc22 of *Paecilomyces* caused low mortality to adults bark beetles.

Efficacy of entomopathogenic fungi isolates from ČeskeŠvycarsko National Park (Czech Republic) in bioassays with adults *I.Typographus*

Isolates	% Bark beetles mortality, days				Beetles with signs of mycosis
	5	7	9	11	
1	2	3	4	5	6
BCz1	47.5±11.0	70±4.0	90±7.0	100	68
BCz2	35±8.6	50±4.0	100	100	89
BCz3	45±8.6	55±5	80±4.0	100	93
BCz4	27.5±6.2	50±4.0	85±2.8	100	74
BCz5	45.5±2.8	60±4.0	80±7.0	100	59
BCz6	22.5±4.7	50±4.0	75±2.8	100	67
BCz7	67.5±10.3	75±10.4	92.5±4.7	100	50
BCz8	22.5±4.7	50±4.0	100	100	80
BCz9	22.5±4.7	50±5.7	100	100	89
BCz10	15±6.4	85±3.7	92.5±4.7	100	54
BCz11	40±12.2	55±14.4	85±2.8	100	90
BCz12	20.0±4.4	50±3.1	100	100	66
BCz13	60±19.5	80±10.8	100	100	70
BCz15	50±7.0	82.5±4.7	100	100	64
BCz16	60±10.8	72.5±5.7	100	100	76
BCz17	50±12.5	80±16.8	100	100	89
BCz18	75±18.9	77.5±14.3	100	100	90
BCz19	40±12.9	57.5±12.5	100	100	71
BCz20	40±13.5	77.5±13.1	100	100	68
BCz21	57.5±16.5	62.5±13.1	100	100	67
BCz22	72.5±2.5	80±12.2	100	100	78
BCz23	65±6.4	72.5±8.5	100	100	77
BCz24	70±10.8	90±5.7	90±5.7	100	65
BCz25	32.5±8.5	42.5±12.5	100	100	100
BCz26	42.5±8.5	62.5±15	90±10.0	100	56
BCz27	42.5±8.5	57.5±15.0	90±5.7	100	92
BCz28	30±4.0	55±12.9	100	100	72
BCz29	50±10.8	67.5±10.3	100	100	67
BCz30	45±17.5	72.5±8.5	100	100	90

1	2	3	4	5	6
BCz31	35±8.6	72.5±22.1	100	100	100
BCz32	50±4.0	62.5±14.3	100	100	82
BCz33	45±17.5	57.5±8.5	100	100	70
BCz34	22.5±4.7	42.5±8.5	75±2.8	100	93
BCz35	35±8.6	57.5±8.5	100	100	70
BCz36	37.5±13.1	50±8.1	75±2.8	100	93
BCz37	25±2.8	55±5.0	72.5±2.5	100	60
BCz38	42.5±16.5	50±20.8	67.5±10.3	100	100
BCz39	24±4.0	45±8.5	100	100	65
BCz40	65±5.0	72.5±2.5	80±7.0	100	91
BCz41	50±20.0	60±19.5	75±25.1	100	57
BCz42	57.5±14.3	75±18.9	87.5±7.5	100	70
BCz43	62±8.5	75±14.7	74±2.4	100	94
BCz44	55±17.0	65±6.5	75±12.5	80±11.5	63
BCz45	45±2.8	50±4.0	80±8.1	100	88
BCz46	65±12.5	75±10.4	85±12.9	100	63
BCz47	50±12.9	60±4.0	70±11.2	100	69
IsCz1	30±10.0	58±1.3	75±15.0	100	78
IsCz2	30±12.9	50±10.0	70±20.8	85±2.8	51
Inc1	25±9.5	30±5.7	60±14.1	70±17.3	43
Inc2	40±14.1	50±3.2	60±8.3	65±12.5	61
Control	0.00	0.00	0.00	10±2.3	
$LSD_{.05}$	11.3	10.7	5.4	2.3	

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

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ПОТЕНЦИАЛ ИСПОЛЬЗОВАНИЯ ЭНТОМОПАТОГЕННЫХ ГРИБОВ КАК БИОЛОГИЧЕСКИЙ РЕГУЛЯТОР ЧИСЛЕННОСТИ КОРОЕДА *I.TYPOGRAPHUS*

Аннотация. В этом исследовании мы обследовали разные точки Национального парка Чешская Швейцария (Чехия) и собрали более 50 имаго- короедов, покрытых белым мицелиям. После секвенирования секреторной части липазы mRNA изолятов, многих из них мы идентифицировали как *B. bassiana.* Тестирование изолятов энтомопатогенных грибов*Beauveriabassiana (Bals.) Vuill.* И *Isariafarinosa (Holm.)* против имаго *Ipstypographus* (Coleoptera: Scolytidae) проводилось в течение 11 дней. В результате больше чем 20 изолятов имеет высокую вирулентность. Большая часть показала наивысший летальный эффект после 9 дней инокуляции. Изоляты Inc1 и Inc22 рода *Paecilomyces* показали относительно низкую смертность.

Ключевые слова: энтомопатогенные грибы, *Beauveriabassiana, Isariafarinosa, Ipstypographus.*

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*I.TYPOGRAPHUS*АҒАШ ҚАБЫҚ ЖЕГІШ ҚОҢЫЗЫНЫҢ САНЫН РЕТТЕУ ҮШІН ЭНТОМАПОТЕГЕН САҢЫРАУҚҰЛАҚТАРДЫҢ ҚОЛДАНУ ПОТЕНЦИАЛЫ

Аннотация. Бұл зерттеу жұмысында Чешская Швейцария Ұлттық паркінің әртүрлі нүктерлі зерттеліп, 50 ден астам ақ мицелиймен қапталған ағаш қабық жегішінің имагосы табылды. Изоляттардың липазаның секреторлық бөлігіне секвенирования жасап, көбісін *B. Bassiana* туысына жататынын анықтадық.11 күннің ішінде энтомопатоген саңырауқұлақтар *Beauveriabassiana (Bals.) Vuill.* және *Isariafarinosa (Holm.)* ағаш қабық жегіш *Ipstypographus* қоңызына қарсы тестілеу жүргізілді. Нәтижесінде, 20 астам изолят жоғары вируленттік көрсетті. *Paecilomyces* туысына жататын Inc1 және Inc22 изоляттары төмен көрсеткіштер көрсетті.

Түйін сөздер: энтомопатоген саңырауқұлақтар, *Beauveriabassiana, Isariafarinosa, Ipstypographus*.

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