

Investigations

Comparison Analysis of Species Composition of Entomopathogenic Fungi Isolated from Bark Beetles (Coleoptera: Scolytidae)

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Abstract: The monitoring outbreaks of bark beetles, flight activity of imago and development of larvae were conducted with pheromone traps and trap logs. We found more than 50 dead adults with symptoms white muscardine from several points under the bark. Healthy adults of *Ips typographus* without any pathogenic symptoms collected from pheromone traps and trap logs under bark in the forest Czech Bohemian Switzerland (České Švýcarsko). For adaptation bark beetles *Ips typographus* in the laboratory conditions and excluded any other abiotic factors as a potential reason for their mortality. They were placed in bark in the Petri plates with filter paper and treated by 1 ml of entomopathogenic fungi conidial suspensions. The isolates Bcz38 and Inc2 showed low level of mortality of *Ips typographus*. But the isolate Bcz2, Bcz8, Bcz9, Bcz12-Bcz25 of *B. bassiana* and Bcz31 *B. pseudobassiana* had high virulence against of *Ips typographus* 100%.

Keywords: Entomopathogenic Fungi, *Beauveria bassiana*, *Ips typographus*

Introduction

One of the most dangerous groups of pest xylophages in coniferous forests is bark beetles (Coleoptera, Curculionidae, Scolytinae). Bark beetles have been often serious pests in spruce monocultures but also in close-to-nature forests in the last decades (Juha and Turčáni, 2008; Holuša *et al.*, 2012). *Ips typographus* found throughout the natural range of its main host *Picea abies*. The spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae, Scolytinae), is one of the most destructive insects infesting spruce forests in Europe (Faccoli and Bernardinelli, 2014).

The outbreaks of *Ips typographus* (L.) in Central Europe after severe storms in the 1990s triggered extensive research (Wermelinger, 2004).

The aim of forest managing against bark beetles is to minimize attacks on living trees. The measures most commonly applied for this purpose are clearing windthrows (trees uprooted or broken by wind), sanitation felling of infested trees and the installation of trapping devices. The felling and removal of infested trees from forests are one of the most effective control methods (Wegensteiner and Weiser, 1996). There is a long-term discussion about possibility of sanitary cutting

of trees infested by bark beetles particularly in strictly protected areas. Strictly protected zones of natural parks become often the areas with large-scale bark beetles outbreaks. On the places where removing of cut trees is difficult, logs are left on site. No chemical treatment is used within the national park (Kníílek and Trýzna, 2014).

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 *Beauveria bassiana* (bals.) Vuill. (Ascomycota: Hypocreales), which has been demonstrated to cause high levels of mortality in several species of bark beetles, including *I. typographus* (Prishepa and Kanapackaya, 2005; Wegensteiner, 2000).

Materials and Methods

The monitoring of flight activity and development *Ips typographus* was studied in the several points of Czech Bohemian Switzerland (České Švýcarsko) with pheromone traps and logs, at an altitude of 340-471 m above sea level (N 50.927371°W 14.447837°; N 50.813300°W 14.388281°; N 50.866989°W 14.319150°) in 2016 (Fig. 1).

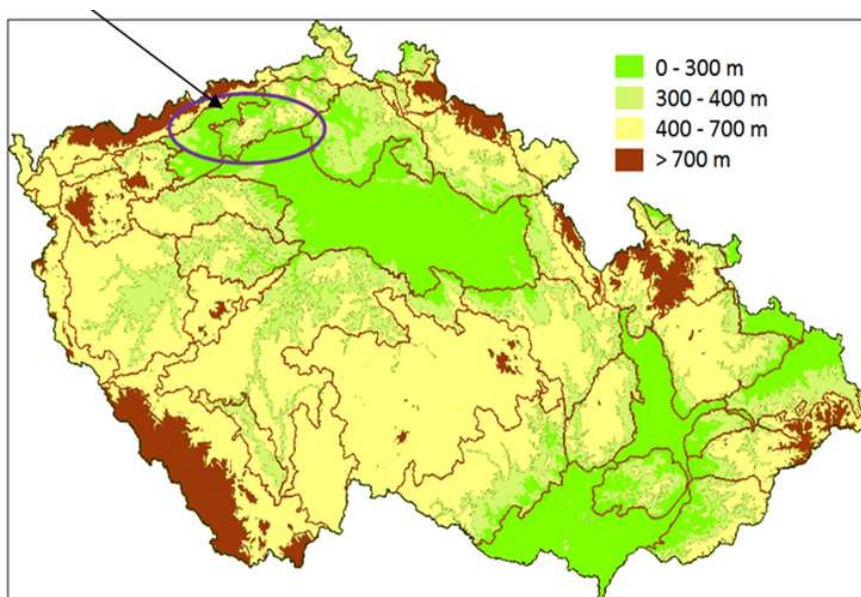


Fig. 1: Map of Czech Republic with marked research location

Traps were controlling weekly from April through September. The number of bark beetles caught was determined or by direct counting or measured by volume. Naturally infected adults bark beetles were collected under the bark. We found more than 70 adults with symptoms mycosis white muscardine. From this dead bark beetles were isolated fresh 49 isolates entomopathogenic fungi on the medium Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA). PDA and SDA were prepared and sterilized by autoclave, after that added antibiotic to avoid bacterial contamination.

In the process of isolating a pure culture of new isolates entomopathogenic fungi, they was identified respectively to morphological characteristics when growing on the medium PDA and SDA: Size, shape, structure, edge of colonies (Lednev *et al.*, 2002). They are growing very well on nutrient medium PDA and SDA. On SDA main mass of culture has morphology characteristic of *Beauveria* sp.: Rounded colonies of white or beige color, often with darker color in the center, dense, mealy, mealy-fluffy or powdery.

Bio assays to evaluate the efficacy of entomopathogenic fungi isolates against adults of *I. typographus* were conducted under laboratory conditions in the Czech University Of Life Science. Healthy insects were placed in Petri plates with filter paper disks in the optimal conditions. They were treated by 1 ml of entomopathogenic fungi conidial suspensions in concentration 1×10^7 .

Molecular Identification of Strains

After cultivation, DNA from the isolates on PDA media for 14 days at $25 \pm 1^\circ\text{C}$, was extracted by the GenElutePlant Genomic DNA Miniprep Kita.

Determine the concentration and purity of the DNA by 1.5% agarose gel electrophoresis.

The fragment of DNA of EF1- α was also amplified by using primers EF1T (5'-ATGGGTAAGGARGACAAGAC-3') and 1567R (5'-ACHGTRCCRATACCACCSATCTT-3'). PCR conditions consisted of 5 min at 95°C followed by 35 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 (Atkins *et al.*, 2005).

Data Analysis

Results of DNA sequences were collected and edited with software Mega7. Alignment of sequences ClustaW. For phylogenetic analysis was use programs jModelTest, Paup*v4., MrBayes 3.1.2., TreeView.

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

Results

The field study was conducted in the Czech Bohemian Switzerland (České Švýcarsko) between April and September 2016. The flight activity, outbreaks of bark beetles and development were controlling *Ips typographus* with pheromone traps and trap logs. We found more than 50 dead adults with symptoms mycosis white muscardine from several points under the bark. The fungus was isolated and cultivated on PDA medium to determine some of the morphological features (Fig. 2). The colony color of entomopathogenic fungi was white on PDA medium.



Fig. 2: Entomopathogenic fungi were isolated from bark beetles

Table 1: Characterization of haplotypes EF1- α isolates from bark beetles were found Czech bohemian in comparison with the records in genbank

Strains	Haplotypes	Strain in Genbank	Access number in Genbank	Isolates	Level of similarity with a typical strain from Genbank, %
<i>B. bassiana</i>	A (09/16)	EABb 09/16	KX911197.1	Bcz1-Bcz30, Bcz32-Bcz34, Bcz36-Bcz47	100
B (492)	L492BA	JQ043236.1	Bcz35	100	
<i>Beauveria caledonica</i>	A (4302)	ARSEF 4302	HQ881014.1	Inc2	100
<i>B. pseudobassiana</i>	A (09/05)	EABb 09/05	KJ536059.1	Bcz31	100
<i>I. farinosa</i>	A (1031)	YHIFXGL-I1031	KP743154.1	Isc1, Isc2	100

They was identified as *Beauveria* sensu lato based on morphological specificity. Additionally, we sequenced partial sequence of EF1- α to confirm 45 isolates *B. bassiana*, 1 isolates *Beauveria caledonica*, 1 isolates *B. pseudobassiana* and two isolates *I. farinose* (Table 1). Results showed in all new isolates from bark beetles dominated entomopathogenic fungi *B. bassiana* 92%,

respectively *Beauveria caledonica* 2% and *B. pseudobassiana* 2%, *I. farinosa* 4% (Fig. 3).

Healthy adults of *Ips typographus* without any pathogenic symptoms collected from pheromone traps and trap logs under bark in the forest Czech Bohemian Switzerland (České Švýcarsko). For adaptation bark beetles *Ips typographus* in the laboratory conditions and

excluded any other abiotic factors as a potential reason for their mortality. They were placed in bark in the Petri plates with filter paper and treated by 1 ml of entomopathogenic fungi conidial suspensions. The isolates Bcz38 and Inc2 showed low level of mortality of *Ips typographus*. But the isolate Bcz2, Bcz8, Bcz9, Bcz12-Bcz25 of *B. bassiana* and Bcz31

B. pseudobassiana had high virulence against of *Ips typographus* 100% (Table 2).

After 5 days inoculation, entomopathogenic fungi killed bark beetles. Dead beetles transfer in the humidity Petri dishes to determine the reason of mortality abiotic factors or influence of entomopathogenic fungi (Fig. 4).

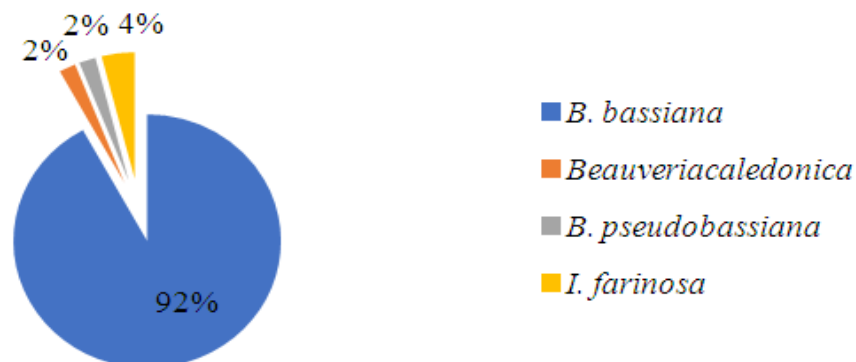


Fig. 3: Structure of species composition of entomopathogenic anamorphic ascomycetes isolated from adults dead bark beetles



Fig. 4: Mycosis on adult bark beetles caused by isolate of *B. bassiana*

Table 2: Efficacy of entomopathogenic fungi strains in bioassays with adults *Ips typographus*

Isolates	% Bark beetles mortality, days				Beetles with signs of mycosis
	5	7	9	11	
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
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
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	

Discussion

In the study 49 new isolates from bark beetles were observed. In the process of isolating a pure culture of new isolates entomopathogenic fungi, they were identified respectively to morphological characteristics when growing on the medium PDA and SDA: Size, shape, structure, edge of colonies (Lednev *et al.*, 2002). They are growing very well on nutrient medium PDA and SDA. On SDA main mass of culture has morphology characteristic of *Beauveria* sp.: Rounded colonies of white or beige color, often with darker color in the center, dense, mealy, mealy-fluffy or powdery. Additionally, we sequenced partial sequence of EF1- α to confirm 45 isolates *B. bassiana*, 1 isolates *Beauveriacaledonica*, 1 isolates *B. pseudobassiana* and two isolates *I. farinosa*. Results showed in all new isolates from bark beetles dominated entomopathogenic fungi *B. bassiana* 92%, respectively *Beauveriacaledonica* 2% and *B. pseudobassiana* 2%, *I. farinosa* 4%. A similar group of entomopathogenic anamorphic ascomycetes on bark beetles is also characteristic for some European countries (Takov *et al.*, 2007). After analysis of the location largest number of samples at an altitude of 340 m above sea level (more than 90%) was collected among only *B. bassiana*. An assessment of the intraspecies of the identified taxa has been shown that both species of the genus *Beauveria* represent two haplotypes, and the rest-one by one.

After 11th days, efficacy adjusted for control (according to Abbott's formula) varied from 60 to 100%. The strains Bcz2, Bcz8, Bcz9, Bcz12- Bcz25 and Bcz31 showed high virulence effect to adults of *Ips typographus* 100% after 9 days. In our study, we focused to find isolates from bark beetles and definitions of virulence against this pest.

The virulence of *B. bassiana*, *I. farinosa*, *M. anisopliae* and *V. lecanii* was tested on *I. typographus* with significant mortality effect, indicating their suitability for biological control against this forest pest (Popa *et al.*, 2012).

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. In the result of the data is convincing about this, entomopathogenic fungi isolates from the Czech Republic could use to produce mycoinsecticides against bark beetles.

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Author's Contributions

Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Ethics

Authors declared no conflicts of interests.

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