BioControl (2019) 64:159–172
https://doi.org/10.1007/s10526-019-09927-3

Natural occurrence of *Beauveria* spp. in outbreak areas of cockchafers (*Melolontha* spp.) in forest soils from Poland

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Received: 9 May 2018 / Accepted: 7 February 2019 / Published online: 14 February 2019

Abstract We investigated the occurrence and pathogenicity of *Beauveria* spp. (Hypocreales: Cordycipitaceae) in forest soils in Poland, in outbreak areas of cockchafers (Coleoptera: Scarabaeidae): *Melolontha melolontha* L. and *M. hippocastani* F. We also examined the occurrence of *Beauveria* in relation to soil pH. *Beauveria* spp. isolates were characterised at species and genotype levels using ITS and microsatellite markers. *Beauveria* spp., which were detected at over 80% of sites, were sensitive to pH, preferring neutral or alkaline soils. This suggests that the acidity of forest soils in Poland can affect their efficacy as biological control agents (BCAs). *B. brongniartii* (Sacc.) Petch as a pathogen of cockchafers occurred at 41% of sites, but often at densities below the threshold values for infection, and it infected only 1.3% of cockchafer grubs. Our results suggest that *B. brongniartii* genotype isolated from cockchafers in forest soils can potentially expand the pool of BCAs in this environment.

Keywords *Melolontha* · *Beauveria brongniartii* · Soil PH · Entomopathogenic fungi · Outbreak area · Forest soil

Handling Editor: Helen Roy

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Introduction

Cockchafers (*Melolontha* spp.) are the most damaging root pests in forest ecosystems in many European countries, including Poland (Blaisinger 1988; Dolci et al. 2006; Fodor et al. 2005; Keller 1988; Malinowski et al. 1996; Niemczyk 2015; Niemczyk et al. 2017; Strasser and Schinner 1996; Švestka 2006, 2010; Wagenhoff et al. 2014). Due to the lack of insecticides registered against *Melolontha* spp. (Directive 2009/128/EC of the European Parliament and of the Council), biological methods are needed.

During the last several decades, biological control agents (BCAs) have been identified as feasible

alternatives to chemical pest treatments (Canfora et al. 2016; Mazid et al. 2011). Although numerous studies have identified and evaluated beneficial bacteria and fungi strains that are pathogenic to insects, the application of BCAs in forestry is still limited by several factors. First, the inoculants are mainly isolated from agricultural soils, which can affect their viability and persistence in different habitats, such as natural forest soil environments. Many studies have shown that the persistence and efficacy of entomopathogenic hyphomycetous fungi in soil depends on complex interactions of intrinsic, edaphic, biotic, and climatic factors (Goble et al. 2012; Kessler et al. 2003; Scheepmaker and Butt 2010). The use of inundative, inoculative, conservative, or classical approaches for fungal BCAs requires an understanding of the biology and ecology of the fungi and different biotic and abiotic factors present (Jackson et al. 2010; Lacey et al. 2015; Meyling and Eilenberg 2007). Soil pH is an abiotic factor that can affect the survival, ecological distribution, and virulence of entomopathogenic fungi (Galani 1988; Inglis et al. 2001; Padmavathi et al. 2003; Sanzhimitupova 1980; Sharma et al. 1992). Due to the influence of soil pH, the actual effects of BCAs may differ from the predicted results. Assessing natural infection rates and the occurrence of entomopathogenic fungi in forest environments in areas where there are mass outbreaks of pests provides a behavioural baseline for these organisms and is thus a key task for improving BCA strain selection and efficacy.

One of the most important entomopathogenic fungal genera distributed worldwide is *Beauveria* (Bals.) Vuill. (Ascomycota: Hypocreales) (Imoulan et al. 2017; Li et al. 2001). In Europe, the most prevalent natural pathogen of *Melolontha* spp. is *Beauveria brongniartii* (Saccardo) Petch, which infects all developmental stages of these pests (Trzebitzky 1996). Because of the ability of *B. brongniartii* to specifically infect and kill insects, several strains have been tested and used commercially as BCAs against cockchafer grubs in various European countries (Enkerli et al. 2001, 2004; Keller et al. 1997; Mayerhofer et al. 2015; Sierpińska 2008; Strasser and Enkerli 2001; Strasser et al. 2000). These BCAs have been tested in agricultural and forest environments, but in the latter no satisfactory results have been achieved (Sierpińska et al. 2015). The identification of edaphic factors in natural forest habitats (soil types, pH ranges, etc.) that influence the occurrence and distribution of *Beauveria* spp. in the soil will help to improve the efficacy of biological control in forests. Simultaneously, the identification of indigenous entomopathogenic fungi from the forest soil environment can provide insight into naturally occurring fungal biodiversity and can expand the pool of potential BCAs for pest control purposes. The aims of the present study were therefore to: (1) investigate the natural occurrence and density of *Beauveria* spp. in forest soils in areas of cockchafer outbreaks in Poland, (2) characterise *Beauveria* species richness and variability, (3) investigate the effects of soil pH ranges and edaphic factors on the occurrence of *Beauveria* spp., and (4) determine the rate of natural infection of cockchafer grubs caused by *B. brongniartii*.

Materials and methods

Study sites

Research plots were selected in areas in Poland that experience outbreaks of cockchafers, and where these insect pests cause the most serious economic losses in forestry. The sites were in three forest districts in central and southeastern Poland: Ostrowiec Świętokrzyski (50°56′00″N 21°24′00″E), Lubaczów (50°09′33″N 23°07′19″E), and Narol (50°21′01″N 23°19′38″E). The mean annual temperature ranged from 7.2 °C in Lubaczów to 8.3 °C in Ostrowiec Św. The annual rainfall exceeded 700 mm at all research sites, and the growing season lasted for approximately 200 days. Detailed information on research sites is given in Table 1.

Study design

Research was carried out at 12 stands (sites) from 2013 to 2014. Sites were chosen in the two most representative (i.e., most common) forest site types for the selected forest districts: fresh broad-leaved forest (six sites) and fresh mixed broad-leaved forest (six sites). Forest site types were classified according to geographical climatic conditions, spatial structure, species composition, site index, physiographical climatic factors, and undergrowth vegetation (Kliczkowska et al. 2003). Preliminary identification of cockchafer grubs was carried out in 2013. At each site, 25

Table 1 Basic characteristic of forest research sites in Poland. Each site was characterised (in accordance with Instrukcja ochrony lasu (2012) as a forested area that was homogeneous in terms of habitat conditions and forest stand elements (dominant tree species, age, spatial structure, site index, forest site type, etc.)

Forest district	Stand	Latitude N (Wgs84)	Longitude E (Wgs84)	Main species	Area covered by main species at site (%)	Age of stand (years)	Forest site type	Soil type
Ostrowiec Św.	1	51,00299	21,48032	*Pinus sylvestris* L.	70	3	Mixed Broad-leaved Forest	Brunic Arenosol (Dystric)
Ostrowiec Św.	2	51,00053	21,46876	*Pinus sylvestris* L.	60	36	Mixed Broad-leaved Forest	Brunic Arenosol (Dystric)
Ostrowiec Św.	3	50,78952	21,5333	*Quercus robur* L.	40	26	Broad-leaved Forest	Haplic Cambisol (Eutric)
Ostrowiec Św.	4	50,94104	21,52045	*Quercus robur* L.	60	7	Broad-leaved Forest	Haplic Cambisol (Eutric)
Ostrowiec Św.	5	51,00591	21,50341	*Pinus sylvestris* L.	50	17	Mixed Broad-leaved Forest	Brunic Arenosol (Dystric)
Ostrowiec Św.	6	51,00824	21,48193	*Quercus robur* L.	20	21	Broad-leaved Forest	Haplic Phaeozem
Lubaczów	7	50,23186	23,38211	*Pinus sylvestris* L.	60	89	Broad-leaved Forest	Haplic Regosol (Calcaric)
Narol	8	50,3356	23,26993	*Pinus sylvestris* L.	70	57	Mixed Broad-leaved Forest	Rendzic Leptosol
Lubaczów	9	50,22318	23,36223	*Pinus sylvestris* L.	80	124	Mixed Broad-leaved Forest	Haplic Cambisol (Dystric)
Narol	10	50,33357	23,2669	*Pinus sylvestris* L.	70	60	Mixed Broad-leaved Forest	Rendzic Leptosol
Ostrowiec Św.	11	51,01408	21,49013	*Quercus robur* L.	40	44	Broad-leaved Forest	Haplic Phaeozem
Ostrowiec Św.	12	51,00086	21,4665	*Pinus sylvestris* L.	50	27	Mixed broad-leaved forest	Brunic Arenosol (Dystric)

The study was carried out in the two most representative forest site types for the selected districts: fresh broad-leaved (six sites) and fresh mixed broad-leaved forests (six sites)

sampling pits measuring 0.5 m^2 (1 × 0.5 m at a depth at least of 0.5 m) were excavated in an overall area of 120 × 200 m to assess grub occurrence. The pits were placed according to a grid superimposed over the sample area, and each pit was permanently marked, both physically and with its GPS position. In 2014, six of the 25 sampling pits were re-excavated at each of the 12 sites. All grubs were collected and identified to genus level (*Melolontha* spp.) in a laboratory using the key presented by Sierpiński (1975). Instars were determined by measuring the width of the head capsule (L_1: 2.6–2.7 mm, L_2: 4.2–4.5 mm, L_3: 6.5–6.9 mm) (Śliwa 1993).

The white grubs collected in 2014 were reared separately for six weeks in 120-ml laboratory vials containing sterilised sand and were fed carrot slices.

Each vial was inspected twice a week for insect mortality. All dead grubs were sterilised in 0.01% $HgCl_2$ in 70% ethanol for 1 min. and washed three times in distilled, sterile water. The dead larvae were then incubated at 23 °C for two weeks in sterile glass Petri dishes, on microscopy glass on wet filter paper. When grub mortality was caused by mycosis, the fungi species responsible were isolated and identified to the genus level using a taxonomy key (Humber 2012) on the basis of morphological characteristics that were determined with a stereoscope (Zeiss, Stemi 2000, Germany). Mortality caused by diseases other than mycosis was not evaluated.

Soil analysis

General information about the soil characteristics for each stand was taken from soil habitat surveys in the particular forest districts. Soil types and texture were classified in accordance with The Polish Soil Classification (SgP 2011), taking into account the World Reference Base for Soil Resources (FAO 2006).

In addition, in 2014 soil samples were taken from each sampling pit, using a cylindrical soil corer (inner diameter: 55 mm), from a depth of 50–150 mm. The samples were placed separately in two sterile 120 ml vials. One vial was used to measure soil pH, and the other to quantify the occurrence of *Beauveria* spp. Prior to the pH analyses, all visible plant materials (roots, stems, and leaves) were removed, and the soil samples were air dried and then ground with a rolling pin. The material was then passed through a 2 mm sieve. In accordance with ISO 10390 (ISO 10390 2015), representative 10 ml samples of the air-dried soil (fraction < 2 mm) were potentiometrically measured using a glass electrode in a 1:5 (volume fraction) suspension of soil in water (to measure pH in H_2O), and in 0.01 mol l^{-1} calcium chloride solution (to measure pH in $CaCl_2$).

The quantification of *Beauveria* spp. in soil was carried out as described by Laengle et al. (2005), with modifications. Prior to the analyses, soil samples were subjected to the same protocols as mentioned above, except that they were passed through a 2.5 mm sieve. Soil samples from each pit were mixed thoroughly and 10 g of soil was added to 90 ml 0.01% (w/v) Tween 80 and shaken at 150 rpm for 60 min. Three *Beauveria*-selective agar plates (Strasser et al. 1996) were inoculated with 100 µl of undiluted soil suspension and incubated at 23 °C for 14 days. Fungal colonies were identified as *Beauveria* spp. if they demonstrated the following two characteristics (Rehner et al. 2011): (1) white, yellowish white, or pale-yellow colour of colonies on Sabouraud agar and (2) conidia aggregated as < 0.1 mm spherical clusters, white in colour, as determined with a stereoscope. Colonies that were identified as *Beauveria* spp. were transferred to Sabouraud agar with the use of a sterile inoculating needle to obtain pure cultures. The number of colonies of *Beauveria* spp. was determined as the number of colony-forming units (CFUs) per gram of dry weight of soil.

DNA isolates and sequencing

We used sequence comparison of the Bloc Intergenic region to determine *Beauveria* species affiliation (Rehner et al. 2006), and we used six variable simple sequence repeat (SSR) markers for genotype identification (Mayerhofer et al. 2015) of *Beauveria* spp. isolates, obtained from the soil samples and from infected cockchafer grubs. DNA of *Beauveria* spp. isolates was extracted using a Syngen Tissue DNA Mini Kit. Using polymerase chain reaction (PCR) analysis, we amplified the internal transcribed spacer (ITS) region marker with the primers B5.1F/B3.1R (Rehner et al. 2006). The PCR thermal profile was as follows: 95 °C for 3 min; 40 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 15 min. Amplifications were carried out in 50 µl with 3 µl of DNA, 25 RedTag Ready Mix (Sigma-Aldrich), 1 µl of each primer (10 µM), and 20 µl of PCR water. After visualization of PCR products on agarose gel and purification with a clean-up kit (A&A Biotechnology), nucleotide sequencing was performed with BigDye Terminator Cycle Sequencing Kit using an ABI 3500 Genetic Analyser (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analysed with Data Collection software ver. 2 (Thermo Fisher Scientific, Inc). Sequences were aligned in BioEdit ver. 7.2.5 (Hall 1999) with reference sequences of two *Beauveria* species haplotypes.

SSR markers were amplified in two multiplex PCRs: (Bb1F4, Bb2A3, Bb2F8) and (Bb4H9, Bb5F4, Bb8D6) in a total reaction volume of 10 µl. The reaction volume contained 1 µl of DNA, 5 µl Multiplex PCR Kit (Qiagen, Germany), 0.2 µl of each

primer (forward and reverse) (10 µM), and 2.8 µl of PCR water. The PCR thermal profile was as follows: 95 °C for 15 min; 35 cycles at 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 90 s; and a final extension at 60 °C for 30 min. Genotyping was performed using an ABI 3500 Genetic Analyser (Applied Biosystems) and allele lengths were scored using GeneMapper® ver. 5 (Thermo Fisher Scientific, Inc.).

Data analysis

Analysis of variance (ANOVA) was performed to test for significant differences between the densities of grubs in 2013 and 2014. Means and SE of *Beauveria* spp. CFU g^{-1} dry weight soil were calculated for each sampling site. Medians were determined for *Beauveria* spp. CFU g^{-1} dry weight soil per soil sample. We used Spearman's rank correlation test to evaluate the relationship between the density of *Beauveria* spp. and soil pH range. Logistic regression was used to assess the effects of soil properties on the occurrence of *Beauveria* spp. and to identify significant variables as predictors of occurrence for given site characteristics. The dependent variable was the absence or presence (0 or 1, respectively) of *Beauveria* spp. For the independent factors, soil pH and density of white grubs were chosen as quantitative variables, while forest site type, soil type, main pedogenic factor, and similar direction of development of the soil were chosen as qualitative variables. A binomial distribution and logit link function were used. The choice of the optimal model (the best subset) was based on the AIC criterion. The derivation of explanatory variables was based on Wald's statistics and their associated probability values. When evaluating model parameters, the odds ratio (OR) was calculated as a measure of the relationship between the variables. The statistical analyses were performed using the statistical package Statistica 10.0 (2011).

Results

Density of *Melolontha* spp. in forest soils

In 2013, the first year of the observation period, the second instar cockchafer larvae (L_2) were the most common stage in the mass outbreak areas. In 2014, the numbers of cockchafers were similar, and ANOVA showed no statistically significant differences between the two years ($F_{1,350} = 0.0002$, $p = 0.9883$). In 2014, 91% of cockchafers were third instar larvae (L_3). *Melolontha* spp. densities varied among the sites from 0 to 16 L_3 per 0.5 m^2. There were only two sites (sites 5 and 6) at which cockchafer grubs were not found. At 50% of the sites, the population density of *Melolontha* spp. was higher than the threshold level for economic losses defined in Instrukcja ochrony lasu (2012) (i.e., 3 L_2 or L_3 per 0.5 m^2 for forest site types where the research was performed) (Fig. 1).

In 2014, the grubs were reared in sterile sand and observed in the laboratory. After six weeks, 76 out of 232 cockchafers had died. Entomopathogenic fungi caused the death of only four of these individuals: three grubs were infected with *Beauveria* spp., and one with *Metarhizium* spp.

Soil analyses

Soil samples revealed the presence of *Beauveria* spp. at ten of the 12 sites. At sites 4 and 8, *Beauveria* spp. were not detected (Fig. 2). At the other sites, *Beauveria* densities reached up to 2.7×10^4 CFU g^{-1} dry weight soil. However, at each site, there were individual samples in which *Beauveria* colonies were not detected. Only 33.3% of all soil samples contained *Beauveria* spp.

According to the classification of soil pH ranges (United States Department of Agriculture Natural Resources Conservation Service), the soil pH ranged from extremely acidic (4.3) to moderately alkaline (7.4). In general, very strongly acidic soils predominated (pH of 4.5 to 5.0) (Table 2). We found a positive correlation between pH ranges both in H_2O and in $CaCl_2$ and *Beauveria* densities from the same sampling pits, with $\alpha = 0.05$ level of significance. The Spearman's rank correlation coefficients were as follows: $r_s = 0.1908$ ($p = 0.0049$) and $r_s = 0.2291$ ($p = 0.0007$).

Logistic regression

On the basis of Akaike criteria, the best subset among six candidate predictors were soil type and soil pH (Table 3). The results of Hosmer–Lemeshow goodness of fit test of the final model, choosing nine groups ($g = 9$), were as follows: $\chi^2 = 8.4217$ ($df = 7$, $p = 0.2968$ for the model with pH in H_2O as an

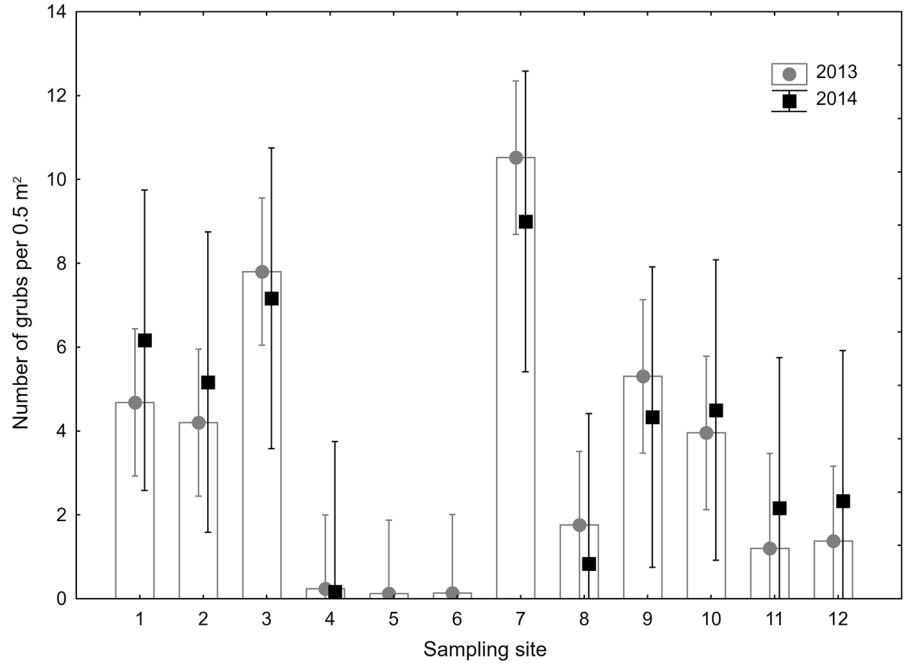

Fig. 1 Number of cockchafer white grubs (mean ± SE) (*Melolontha* spp.) per sampling pit (1 × 0.5 m at a depth of at least 0.5 m) at forest sites in two consecutive study years. Means were determined from 25 pits excavated in 2013 and six pits re-excavated in 2014 per site. Pits were placed using a grid superimposed over the sample area. Site numbers correspond to site numbers in Table 1

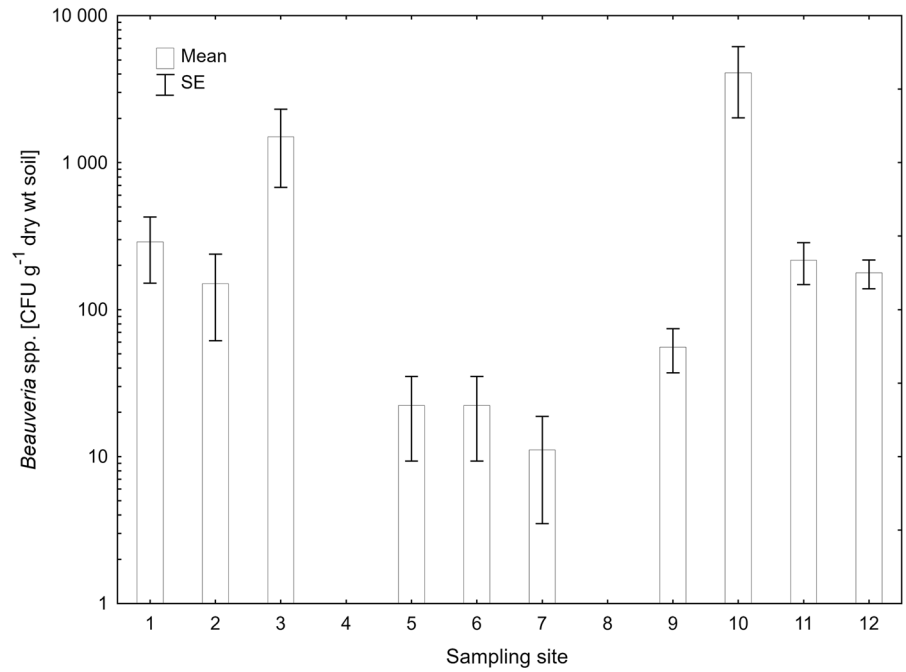

Fig. 2 Density of *Beauveria* spp. (CFU g^{-1} dry weight soil) in forest stands (mean ± SE). *Beauveria* spp. densities were determined at six sampling pits per site and for three replicates per soil sample. Y-axis values are shown in a logarithmic scale. Site numbers correspond to site numbers in Table 1

explanatory variable) and $\chi^2 = 7.5499$ ($df = 7$, $p = 0.3739$ for the model with pH in $CaCl_2$ as an explanatory variable), which indicates that there is no evidence of poor fit (there are no differences between the observed and predicted values of the dependent variable). The model correctly predicts the presence of *Beauveria* spp. in 81% of cases and their absence in 69% of cases for the model with pH in H_2O, and 78% and 71% respectively, for the model with pH in $CaCl_2$.

The evaluation of the model parameters showed that the presence of *Beauveria* spp. in soil is most affected by soil pH (Table 3), and that an increase in

Table 2 Soil pH values, numbers of cockchafer grubs (*Melolontha* spp.), and density of *Beauveria* spp. at forest sites, as determined at six sampling pits per site. Values of *Beauveria* spp. colony-forming units (CFUs) were determined as three replicates per soil sample

Site[a]	pH in H_2O			pH in $CaCl_2$			Number of grubs per 0.5 m^2 sampled soil			Density of *Beauveria* spp. (CFU g^{-1} dry wt soil)		
	Min	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max
1	4.8	5.1	5.4	3.9	4	4.2	0	7	12	0	50	2,300
2	4.7	4.75	5.3	3.6	3.8	4.3	0	4.5	7	0	0	1,500
3	5	5.3	5.9	4.1	4.4	4.6	1	5	15	0	0	10,300
4	4.6	4.7	5	3.7	3.85	4.1	0	0	1	0	0	0
5	4.5	4.7	4.9	3.7	3.85	4.1	0	0	0	0	0	200
6	4.4	4.9	5.1	3.4	4	4.3	0	0	0	0	0	200
7	4.4	4.5	4.8	3.7	3.75	3.9	2	8	15	0	0	100
8	4.4	4.95	6.1	3.6	3.95	5.4	0	1	3	0	0	0
9	4.3	4.55	4.8	3.6	3.8	4.1	0	3	9	0	0	200
10	6.8	8	8.1	6.3	7.3	7.4	0	4.5	8	0	300	27,000
11	4.4	4.7	4.9	3.7	3.85	4.1	0	1	8	0	100	800
12	4.4	4.7	4.9	3.7	3.85	4	0	1	3	0	100	600

[a]Site numbers correspond to site numbers in Table 1

pH of one unit was associated with an increased chance of *Beauveria* spp. occurrence by 14.6 (OR) (for the model with pH in H_2O). The logistic regression model also showed that *Beauveria* spp. occurrence varied with soil type. The probability of *Beauveria* occurrence was highest in Albic Luvisol soil and lowest in Rendzic Leptosols and Haplic Cambisols (Eutric) (Fig. 3).

DNA sequence alignment

The number of *Beauveria* spp. isolates collected from different sites varied, ranging from 0 (at sites 4, 7, and 8) to six isolates per site (at site 10). The sequence of ITS markers of *Beauveria* spp. was 1385 bp long. From 30 samples of *Beauveria* spp., we obtained three haplotypes: two from *B. pseudobassiana* (Bals.) Vuill. and one from *B. brongniartii*. We detected length differences between haplotypes from these two species (three INDELs in total). Two haplotypes of internal transcribed spacer B *locus* were identical to sequences deposited in the GeneBank database, from Rehner et al. (2011): HQ880728 (*B. pseudobassiana*) and HQ880713 (*B. brongniartii*). One of the sequences for *B. pseudobassiana* was recorded for the first time in the present study (MG029116) and differed by two substitutions from HQ880728. The nucleotide differences on the analysed fragment between these two species were 117 point mutations, giving a genetic distance of 8.6%, discounting deletions.

Among mass outbreak areas, only one site (site 10) contained all three haplotypes of *Beauveria* spp. Both *B. pseudobassiana* and *B. brongniartii* were represented in five other sites. At the rest of the sites, only one of the two above-mentioned haplotypes was identified (Table 4). Isolates obtained from infected cockchafer grubs came from the 2nd (two isolates) and 10th (one isolate) sites and were identified as *B. brongniartii* (HQ880713).

SSR analysis showed the presence of 21 *Beauveria* genotypes out of 30 isolates. PCR amplification of the SSR markers Bb4H9, Bb5F4, and Bb8D6 yielded products from all the isolates. The SSR marker Bb8D6 was monomorphic for *B. brongniartii* (166 bp), and the markers Bb1F4, Bb4H9, and Bb5F4 were monomorphic for *B. pseudobassiana* (190, 198, and 148 bp respectively). The markers Bb1F4, Bb2A3, and Bb2F8 were partially amplified for *B. brongniartii*, and the latter was also partially amplified for *B. pseudobassiana.* There were 18 genotypes that were represented as single isolates only, and three

Table 3 Optimal model of logistic regression predicting the occurrence of *Beauveria* spp. as a function of soil characteristics at study sites (two independent analyses for pH in H_2O and pH in $CaCl_2$)

Independent variable	df	Wald's stat.	*p*	Level	Coef.	SE	Wald's stat.	*p*	OR	− 95% CI	+95% CI
Intercept	1	7.660	**0.006**	–	− 13.692	4.947	7.660	**0.006**	–	–	–
soil type	5	12.103	**0.033**	Brunic Arenosol (Dystric)	1.479	0.577	6.567	**0.010**	4.389	1.416	13.599
				Haplic Cambisol (Eutric)	− 1.783	0.808	4.865	**0.027**	0.168	0.034	0.819
				Albic Luvisol	1.690	0.712	5.628	**0.018**	5.418	1.342	21.873
				Haplic Regosol (Calcaric)	0.785	0.938	0.700	0.403	2.191	0.348	13.775
				Rendzic Leptosol	− 3.628	1.767	4.219	**0.040**	0.027	0.001	0.862
pH H_2O	1	7.556	**0.006**	–	2.679	0.975	7.556	**0.006**	14.571	2.156	98.498
Intercept	1	6.523	**0.011**	–	− 17.010	6.660	6.523	**0.011**	–	–	–
soil type	5	11.388	**0.044**	Brunic Arenosol (Dystric)	2.266	0.836	7.348	**0.007**	9.643	1.873	49.64
				Haplic Cambisol (Eutric)	− 1.455	0.817	3.169	0.075	0.233	0.047	1.156
				Albic Luvisol	2.235	0.912	6.003	**0.014**	9.346	1.564	55.839
				Haplic Regosol (Calcaric)	1.305	1.121	1.356	0.244	3.689	0.410	33.199
				Rendzic Leptosol	− 6.241	3.048	4.193	**0.041**	0.002	< 0.001	0.786
pH $CaCl_2$	1	6.491	**0.011**	–	3.982	1.563	6.491	**0.011**	53.612	2.505	1147.399

The output provides the coefficients for explanatory variables and their levels. The Haplic Cambisol (Dystric) was chosen as a reference level for soil types (SE = standard error, CI = confidence intervals, boldface text indicates statistical significance at $\alpha = 0.05$, OR = odds ratio)

genotypes were represented by a larger number (two to five). The highest number of different genotypes (five genotypes) was found at site 10. The most common genotypes, C and I, were found at two (sites 1 and 12) and three (sites 3, 11, and 12) different sites, respectively (Table 4).

Discussion

Numerous studies have demonstrated the close relationship between *B. brongniartii* and *Melolontha* spp. (Keller et al. 2003; Kessler et al. 2004). *B. brongniartii* has been reported to be a highly host-specific fungus that exclusively infects *Melolontha* spp. under natural conditions in Central Europe (Kessler et al. 2004; Neuvéglise et al. 1994). In Switzerland, Keller et al. (2003) demonstrated the natural occurrence of *B. brongniartii* and *M. melolontha* together in meadow soils. During a forest cockchafer outbreak in southwest Germany, Trzebitzky (1996) found that more than 50% of the natural infections of *M. hippocastani* grubs in forest soils were caused by *B. brongniartii*. In contrast, during an outbreak of the common cockchafer (with a grub population density of 0 to 72 per m^2) in Valle D'Aosta, Italy, only two larvae were affected by mycosis in one year (Cravanzola et al. 1996). Similarly, in the present study, only 1.3% of cockchafer grubs were infected by *B. brongniartii*. The low levels of infection of cockchafer grubs and maintenance of stable populations of larval cockchafers in the present study can be partly explained by the high resistance of older (L_2 or L_3) instar larvae, which were dominant during the study period. Sukovata et al. (2015) tested the efficacy of a biocide product against *Melolontha* grubs and observed a higher resistance rate and lower mortality rate among L_3 grubs compared with the L_1 and L_2 instar larvae at the same biocide concentration. Kessler (2004) found that the age and origin of

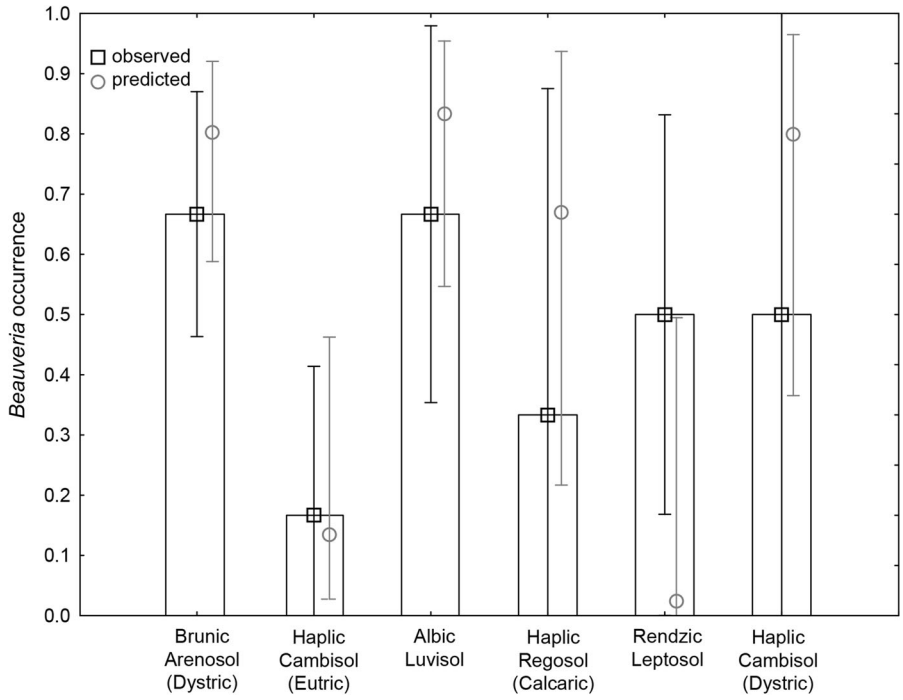

Fig. 3 Predicted and observed probability [± confidence intervals (CI)] of *Beauveria* spp. occurrence depending on forest soil types, using the optimal model of logistic regression

Melolontha larvae influence the efficacy of BCA as much as does the virulence of the spore types.

The low level of infection caused by *B. brongniartii* in forest soils can be further explained by the pH conditions, which were suboptimal for the growth of *Beauveria* spp. According to Enkerli et al. (2001), sustainable cockchafer control can be achieved when fungal density reaches 1×10^3–1×10^4 CFU g^{-1}. Densities at this level were found at sites 1, 2, 3, and 10, but the fungus was parasitic only at sites 2 and 10. Notably, site 10 was characterised by the highest soil pH ranges. The importance of pH in *Beauveria* development and pathogenicity in the present study was reflected by the positive relationship between the pH ranges and fungal densities (Spearman's rank correlation). Logistic regression analyses confirmed that pH ranges, supported by soil type, were significant predictive variables for the occurrence of *Beauveria* spp. Strong soil acidity was responsible for the absence of these hyphomycetous fungi. An increase in the pH by one unit resulted in a 14.6-fold higher probability of *Beauveria* occurrence within the studied pH ranges.

Our findings confirm the results of previous studies. According to Padmavathi et al. (2003), a pH of 3 was toxic to all tested isolates of *B. bassiana* (closely related to *B. brongniartii*). Conidia germinated at this pH, but growth was completely inhibited. Qazi (2008) noted that differences in the germination capability of *B. bassiana* conidia under differing substrate pH conditions were explained by the specific optimal pH values required for the expression of proteases produced by the fungus. Overly acidic (or overly alkaline) reactions can adversely affect conidia germination in *B. brongniartii*, which may explain our observation of hindered mycosis in cockchafer grub populations.

Considering the isolation of entomopathogenic fungi (including *Beauveria*) from natural and cultivated areas, Quesada-Moraga et al. (2007) detected a narrow pH that was optimum for *B. bassiana*, with 52.9% of samples falling within 8.0–8.5. Karthikeyan et al. (2008) confirmed that the optimal soil pH for *Beauveria* spp. development ranges from 6 to 8. Moreover, these fungi typically occur in lowland soils with neutral or alkaline pH (Medo and Cagáň 2011) and are detected more frequently in natural forest soils than in cultivated ones (Shin et al. 2013). Taking into account that our soil samples were representative of most Polish forest pH ranges (acidic and very acidic soils make up 50% of Poland's area), our results demonstrate that strong soil acidity in forests provides

Table 4 Characteristics of *Beauveria* spp. genotypes for six simple sequence repeat (SSR) markers (allele size), species affiliation, and GenBank (ITS) accession number identification with references for isolates obtained at study sites

Stand	Isolate no.	Isolated from	SSR marker[c]						Genotype[d]	GenBank similarity	GenBank accession number
			Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6			
1	415	Soil	241	112	229	180	196	166	A	*B. brongniartii*	HQ880713[a]
1	416	Soil	**	**	**	177	196	166	B	*B. brongniartii*	HQ880713[a]
1	419	Soil	190	115	196	198	148	182	C	*B. pseudobassiana*	HQ880728[a]
1	459	Soil	190	115	196	198	148	182	C	*B. pseudobassiana*	HQ880728[a]
1	460	Soil	190	115	196	198	148	182	C	*B. pseudobassiana*	HQ880728[a]
1	427	Soil	190	115	196	198	148	182	C	*B. pseudobassiana*	HQ880728[a]
2	464	Grub	262	115	226	165	166	166	D	*B. brongniartii*	HQ880713[a]
2	465	Grub	238	112	220	165	217	166	E	*B. brongniartii*	HQ880713[a]
2	442	Soil	190	115	**	198	148	186	F	*B. pseudobassiana*	HQ880728[a]
2	443	Soil	190	115	196	198	148	186	G	*B. pseudobassiana*	HQ880728[a]
3	417	Soil	244	112	166	168	211	166	H	*B. brongniartii*	HQ880713[a]
3	425	Soil	190	115	172	198	148	182	I	*B. pseudobassiana*	HQ880728[a]
3	426	Soil	190	112	199	198	148	182	J	*B. pseudobassiana*	HQ880728[a]
3	435	Soil	190	115	172	198	148	182	I	*B. pseudobassiana*	HQ880728[a]
5	447	Soil	**	**	**	204	220	166	K	*B. brongniartii*	HQ880713[a]
5	448	Soil	190	112	**	198	148	182	L	*B. pseudobassiana*	HQ880728[a]
9	453a	Soil	202	115	211	159	160	166	M	*B. brongniartii*	HQ880713[a]
9	455	Soil	190	115	211	198	148	182	N	*B. pseudobassiana*	HQ880728[a]
10	429	Soil	241	115	199	177	202	166	O	*B. brongniartii*	HQ880713[a]
10	431	Soil	205	115	178	159	160	166	P	*B. brongniartii*	HQ880713[a]
10	433	Soil	**	**	**	165	199	166	Q	*B. brongniartii*	HQ880713[a]
10	466	Grub	205	115	178	159	160	166	P	*B. brongniartii*	HQ880713[a]
10	428	Soil	190	115	178	198	148	182	R	*B. pseudobassiana*	MG029116[b]
10	432	Soil	190	115	**	198	148	182	S	*B. pseudobassiana*	HQ880728[a]
11	449	Soil	190	112	223	198	148	182	T	*B. pseudobassiana*	HQ880728[a]
11	451	SOIL	190	115	172	198	148	182	I	*B. pseudobassiana*	HQ880728[a]
12	437	Soil	190	115	172	198	148	186	U	*B. pseudobassiana*	HQ880728[a]
12	439	Soil	190	115	172	198	148	182	I	*B. pseudobassiana*	HQ880728[a]
12	440	Soil	190	115	172	198	148	182	I	*B. pseudobassiana*	HQ880728[a]

Table 4 continued

Stand	Isolate no.	Isolated from	SSR marker[c]						Genotype[d]	GenBank similarity	GenBank accession number
			Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6			
12	441	Soil	190	115	196	198	148	182	C	*B. pseudobassiana*	HQ880728[a]

**lack of PCR product

[a]Rehner et al. (2011)

[b]Present study

[c]Allele size is given as number of base pairs

[d]The capital letters are used for the identification of genotypes obtained from isolates in our study. The same capital letters obtained from different isolates indicate identical genotypes

a suboptimal environment for the development of *Beauveria* spp.

Our study area encompassed several types of soil, including extremely gravelly and/or stony Leptosols, sandy Arenosols, soils of increasing clay content such as Cambisols, and high-activity clays throughout the argic horizon in the Luvisols. The probability of *Beauveria* occurrence was highest in the latter types of soil. Many previous studies (Mietkiewski et al. 1997; Milner 1989; Quesada-Moraga et al. 2007) reported that the occurrence of entomopathogenic fungi is associated with soils with high clay content. This may be because leaching of the inoculum is correlated with the water infiltration value of soils, which is higher in sandy soils than in finer-textured soils (Storey and Gardner 1988). Some studies also suggested that a high clay content in soil enhances the abundance and persistence of many insect pathogenic fungi because conidia are adsorbed onto clay particles (Inglis et al. 2001; Studdert et al. 1990). Therefore, the soil type is another source of information (in addition to pH ranges) that indicates the potential occurrence and persistence of entomopathogenic fungi in the forest environment.

Beauveria spp. isolates were detected at more than 80% of sites and in 33.3% of soil samples, comparable to recovery rates from other countries with cold/humid temperate climates. According to Vänninen (1996), *Beauveria* were detected in 19.8% of soil samples from Finish soils. Typical recovery rates were 18% in the Pacific Northwest (Bruck 2004). Based on sequence alignment in the present study, three different haplotypes of *Beauveria* spp. were identified in forest sites: two haplotypes belonging to *B. pseudobassiana* species and one to *B. brongniartii*. *B. brongniartii*, an important species of entomopathogenic fungus that is indigenous to the study area, was present in 11 of 30 isolates, and ten different genotypes were detected in the samples. By comparison, 41 different *B. brongniartii* genotypes were detected among 63 isolates from two sites in Switzerland (Enkerli et al. 2001) and 13 *B. brongniartii* genotypes were detected from 92 isolates from the Tyrol region (Mayerhofer et al. 2015). In the present study, *B. brongniartii* was identified in soils in 41% of forest sites (five sites) and was also isolated from cockchafer grubs in two sites.

In summary, only two species of *Beauveria* were found in the forest soils we sampled: *B.*

pseudobassiana and *B. brongniartii*. *B. brongniartii*, an important natural pathogen of cockchafers, did not occur frequently and its density was often below the threshold value for the effective infection of cockchafer grubs. We determined that *Beauveria* genotypes are sensitive to soil pH and soil types in forest environments. Our results suggest that the *B. brongniartii* genotype isolated from cockchafers from forest soils can expand the pool of potential BCAs in the forest environment. However, additional studies are needed to explore the genotypes of virulence and optimal pH conditions for *Beauveria* spp. for use as BCAs.

Acknowledgements This research was financially supported by the Forest Research Institute (Project No. 240226). We thank our colleague from the Forest Research Institute, Szymon Krajewski for assistance during field data collection.

Author contributions All authors have read and approved the final version of the manuscript. All authors have agreed to authorship and the order of authorship for this manuscript; and all authors have the appropriate permissions and rights to the reported data.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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