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The occurrence of entomopathogenic fungi in the Chojnowski Landscape Park in Poland

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Abstract: *The occurrence of entomopathogenic fungi in the Chojnowski Landscape Park in Poland.* The study was aimed at estimating species composition and abundance of entomopathogenic fungi in the Chojnowski Landscape park. The effect of site, season (spring, summer, autumn) and temperature on the frequency of isolation of entomopathogenic fungi was also analysed. The effect of the first two factors was estimated based on the analysis of soil samples taken from meadow 1, forest 1 and orchard in spring, summer and autumn 2010. Three species of entomopathogenic fungi (*Beauveria bassiana, Metarhizium anisopliae* and *Isaria fumosorosea*) were isolated in the study area. Site and temperature affected the frequency of isolation of particular species. On meadow 1 and in orchard *M. anisopliae* appeared to be the dominating species while forest 1 was dominated by *B. bassiana*. From among meadow and forest sites sampled in the autumn, forest 3 (nature reserve) was characterised by the highest density of entomopathogenic fungi. *M. anisopliae* and *B. bassiana* were most often isolated from meadow sites while *B. bassiana* and *I. fumosorosea* – from forest sites. *B. bassiana* and *I. fumosorosea* infected insects with significantly higher frequency at 20°C than at 25°C.

Key words: entomopathogenic fungi, biology, bioinsecticides, *Beauveria bassiana, Metarhizium anisopliae, Isaria fumosorosea*, the Chojnowski Landscape Park

INTRODUCTION

Entomopathogenic fungi focussed researchers' attention already in the 19th century. Now, they are used in a small scale in organic farming in Western Europe, USA, in the counties of Latin America and in Australia. The fungi proved useful in replacing chemical insecticides, particularly when controlling pests which move vertically in soil according to ground water changes and the range of the root zone. Most promoted are the bio-preparations based on local strains of microorganisms. The use of imported ecotypes developed under different selective factors (e.g. temperature ranges) does not often bring expected results (Mierzejewska 2001).

Entomopathogenic fungi are an environmental-friendly alternative to plant protection chemicals. They are used to control harmful insects and arachnids (mainly mites, Acarina) which make losses in agricultural and forest crops and transfer microorganisms that are pathogenic to humans, animals and plants (Bałazy 2004). Biological control of pests does not result in the acquired resistance in contrast with the use of chemical pesticides (Paruch and

Janowicz 2004). Moreover, fungal bio-preparations do not need obeying the waiting periods or special protective measures (Mierzejewska 2001).

It is extremely important to create appropriate habitat conditions (e.g. refuges) to enable survival of species susceptible to man-made changes in the landscape. Such approach would allow for maintaining possibly richest gene pool of potential entomopathogenic organisms (Bałazy 2004, Quesada et al. 2007).

The Chojnowski Landscape Park has great landscape and natural values. It is part of the so-called green ring surrounding the city agglomeration of Warsaw. The park is localised on light sandy or sandy-loamy soils of relatively high fertility which supports many forest and bog-meadow communities. Apart from rich flora, the park provides also favourable conditions for the development of abundant fauna (Walczak et al. 2001). Practically no chemical control measures are applied to protect tree stands in forest areas of the park. This is favourable for the development of soil mesofauna including insects and their natural enemies like entomopathogenic nematodes and fungi. Forest entomopathogenic fungi find convenient conditions for the growth and development in forest litter and on the soil surface. For the year round they have a chance to contact their hosts permanently dwelling forest bottom or penetrating soil for pupation or wintering (Tkaczuk 2008).

MATERIAL AND METHODS

Entomopathogenic fungi were isolated from soil with the method of trap insects (Zimmerman, 1986 in Górny and Grüm 1993) used also to isolated entomopathogenic nematodes. The method consists in placing in soil some live insects e.g. caterpillars of various butterfly species which are attacked by isolated pathogens. Soil with trap insects is incubated in the lab at appropriate temperature (Górny and Grüm 1993).

Caterpillars of the greater wax moth (*Galleria mellonella* L.) from own culture of the Department of Zoology, Warsaw University of Life Sciences were used as trap insects in this study. Soil samples were collected in 2010 from various meadow and forest habitats and from an orchard situated in the Chojnowski Landscape Park (Masovian Province). Sampling sites were characterised by the following properties (names of sites are further used throughout the text):

- meadow 1 – situated in the village Łoś near the Jeziorka River on peat soils and bordering forest 1,
- meadow 2 – wet meadow growing on alluvial and peat soils situated by the Jeziorka River in the village Gołków,
- meadow 3 – several hundred metres SW of Wólka Pęcherska. Single young pine, birch and oak trees grow on this untypical meadow situated in the close vicinity of forest 3,
- forest 1 – mixed forest (pine, oak, birch, small-leaved linden and other tree species) situated in the village Prace Duże. Soil samples were taken from sites covered by brown soils,
- forest 2 – mixed forest (pine, oak, birch, small-leaved linden and other tree species) situated in the village Bogatki. Soil samples were taken from sites covered by brown and rusty soils,

- forest 3 – nature reserve Biele Chojnowskie, soil samples were taken from sited overgrown by mixed coniferous forest *Querco roboris-Pinetum* (old-growth forest of Scotch pine with a small admixture of the common oak and the common birch). Soil samples were taken from the forest outskirts,
- orchard – a young apple orchard situated on brown soils in a village.

Mixed soil samples were taken from studied habitats with the Egner's cane. Soil from each site was placed in a plastic container (V = 250 cm^3) and 10 larvae of the greater wax moth (*G. mellonella* L.) were added to each container. Caterpillars with soil were incubated at 20 and 25°C. Experiments were made in triplicate. In total, entomopathogenic fungi infected 508 trap larvae out of 1,753 isolated dead insects during the whole experiment.

The first observation of larval mortality was made after 4 days of contact of caterpillars with soil, the next were made every 3 days until the 40^{th} day of experiment. Dead insects were removed and containers with soil were supplemented with live caterpillars. Dead insects when soft suggested infection by nematodes while tough insects with the symptoms of mummification were superficially sterilised with disinfectant 1% sodium hydroside solution and rinsed in distilled water. Individuals with a visible mycelium did not undergo sterilisation. Then, the caterpillars were transferred to Petri dishes lined with filter paper to achieve sporification of fungi necessary for their determination.

The effect of habitat was determined for 6 sites (meadow 1, meadow 2, meadow 3, forest 1, forest 2, forest 3), from which soil samples were taken in autumn 2010. Results obtained from the analysis of soil samples taken in spring and summer (forest 1, meadow 1 and orchard) and in autumn (meadow 1, meadow 2, meadow 3, forest 1, forest 2, forest 3 and orchard) served for testing the effect of temperature on the frequency of isolation of particular species of entomopathogenic fungi. Obtained results were statistically processed with the Statgraphics Plus 4.1 software using simple and multi-parametric ANOVA. Tukey's test was used to compare the means at the significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Polish soils are characterised by a great abundance of entomopathogenic fungi and, as shown in studies by Tkaczuk (2008), by rather diverse species composition. This author found at least two species of entomopathogenic fungi in more than 80% of soil and litter samples from various habitats in Poland (one species in 18.2% samples, two species in almost 40% samples, three species in 34.3% samples and four or more species in 7.9% samples). For comparison, in more than 40% of soil samples from abroad the author found the presence of only one species of entomopathogenic fungi, in more than 30% samples – two species and in 22.5% and 5.6% samples – the presence of three and four or more species, respectively. In each of these examples the respective figures are smaller than those from Polish soils.

Despite the fact that entomopathogenic mitosporic fungi are widespread, especially in the soil habitat, the knowledge of the factors affecting their occurrence, population structure, mechanisms of their persistence and virulence against potential hosts is still scarce. Definitely better studied in this aspect are fungi of the order Entomophthorales (Tkaczuk 2008).

Three species of entomopathogenic fungi: *B. bassiana*, *M. anisopliae* and *I. fumosorosea* were found in studied sites. They were isolated from 96, 77 and 73% of collected soil samples, respectively. *B. bassiana* was the most often isolated species. It was present in every site in a given season. *M. anisopliae* was not found in summer in forest 1 and *I. fumosorosea* was absent from meadow 1 in spring and from meadow 1 and meadow 2 in autumn (Table 1).

Common occurrence of these fungi in soils from various country habitats was confirmed in many studies (Miętkiewski et al. 1991, 1991–1992 and 1998, Miętkiewski and Kloczarek 1995, Bajan and Kmitowa 1997, Marjańska-Cichoń et al. 2005, Sapieha-Waszkiewicz et al. 2006). Clear dominance of mentioned species in Polish soils, without the distinction of particular habitats, was also noted by Tkaczuk (2008). According to this author, the species less frequently recorded in Polish soils are *I. farinosa*, *L. lecanii* and *M. flavoviride* which were not found in the study sites.

Entomopathogenic fungi caused death of 15 to 53% (mean 29%) of trap caterpillars depending on site and season. The lowest mortality caused by these microorganisms was found in soil taken from forest 1 in summer and the highest – in that taken from forest 3 in autumn. A high fungal activity was also observed in soil sampled from

TABLE 1. Mortality of trap insects placed in soil in relation to season and site (%)

Mortality factor	Spring								
	Orchard			Meadow 1			Forest 1		
	20°C	25°C	mean	20°C	25°C	mean	20°C	25°C	mean
B. bassiana	3.03	8.33	5.68	20.69	5.56	13.12	12.90	18.18	15.54
M. anisopliae	3.03	11.11	7.07*	3.45	50.00	26.72	0.00	6.82	3.41*
I. fumosorosea	12.12	0.00	6.06	0.00	0.00	0.00	12.90	9.09	11.00
Total fungi	18.18	19.44	18.81	24.14	55.56	39.85	25.81	34.09	29.95
Other factors	6.06	13.89	9.97	6.90	2.78	4.84	1.61	9.09	5.35
	Summer								
Mortality factor	Orchard			Meadow 1			Forest 1		
	20°C	25°C	mean	20°C	25°C	mean	20°C	25°C	mean

B. bassiana	7.14	2.63	4.89	3.80	3.45	3.62	15.89	6.90	11.39
M. anisopliae	11.22	6.58	8.90	31.65	29.31	30.48*	0.00	0.00	0.00
I. fumosorosea	9.18	2.63	5.91	10.13	1.72	5.93	3.74	3.45	3.59
Total fungi	27.55	11.84	19.70	45.57	34.48	40.03	19.63	10.34	14.99
Other factors	5.10	11.84	8.47	10.13	18.97	14.55	3.74	8.62	6.18

Mortality factor	Autumn								
	Orchard			Meadow 1			Forest 1		
	20°C	25°C	mean	20°C	25°C	mean	20°C	25°C	mean
B. bassiana	4.82	0.00	2.41	18.68	2.94	10.81	7.59	21.74	14.67
M. anisopliae	19.28	14.44	16.86*	13.19	11.76	12.48	0.00	6.52	3.26
I. fumosorosea	13.25	2.22	7.74	0.00	0.00	0.00	12.66	26.09	19.37*
Total fungi	37.35	16.67	27.01	31.87	14.71	23.29	20.25	54.35	37.30
Other factors	3.61	2.22	2.92	2.20	1.47	1.83	2.53	2.17	2.35

Mortality factor	Autumn											
	Meadow 2			Meadow 3			Forest 2			Forest 3		
	20°C	25°C	mean	20°C	25°C	mean	20°C	25°C	mean	20°C	25°C	mean
B. bassiana	47.92	1.59	24.75*	25.00	21.05	23.03	6.36	6.52	6.44	20.00	44.59	32.30*
M. anisopliae	6.25	3.17	4.71	4.69	7.89	6.29	0.00	6.52	3.26	0.00	4.05	2.03
I. fumosorosea	0.00	0.00	0.00	10.94	26.32	18.63*	7.27	6.52	6.90	30.67	6.76	18.71
Total fungi	54.17	4.76	29.46	40.63	55.26	47.94	13.64	19.57	16.60	50.67	55.41	53.04
Other factors	4.17	11.11	7.64	3.13	2.63	2.88	0.00	2.17	1.09	1.33	8.11	4.72

*Tukey's test $\alpha < 0.05$.

meadow 1 in summer and in soil from meadow 3 taken in autumn (Table 1).

Most trap larvae infected by entomopathogenic nematodes, which caused death of from 45 to 79% of the larvae of *G. mellonella*, were found in soil taken from meadow 1, forest 1 and orchard in spring, summer and autumn. In soil taken from meadow 2, meadow 3 and forest 2 entomopathogenic nematodes were also the reason of the highest mortality of trap larvae (from 49 to 82%). Only in soil samples taken from forest 3 the number of *G. mellonella* larvae infected by nematodes (42%) was lower than those infected by entomopathogenic fungi (53% – Table 1). The death of caterpillars was also caused by other factors like saprophytic fungi, mites, non-fruiting mycelium, bacteria or other unidentified factors.

Not all species of entomopathogenic fungi are equally characteristic for various ecosystems – forests, meadows, croplands and other (Bajan and Kmitowa 1997, Miętkiewski et al. 1998, Tkaczuk 2008). As shown by Tkaczuk (2008), *M. anisopliae* is the dominating species in the country meadow and pasture soils. According to other studies (Miętkiewski et al. 1991–1992 and 1998), *M. anisopliae* predominated over other species of fungi in meadow soil. This species appeared also characteristic for such type of soils in the Chojnowski Landscape Park, especially for meadow 1, where it dominated in every season and was the reason of remarkable mortality of trap insects.

B. bassiana definitely dominated in meadow 2, from where soil samples were taken only in autumn. *M. anisopliae* caused higher mortality of trap caterpillars in meadow 2 than in forest habitats but showed much smaller activity there as compared with that in meadow 1. Miętkiewski et al. (1991–1992 and 1998) also reported abundant occurrence of *B. bassiana* in meadow soil but *M. anisopliae* was the dominating species in their studies. *B. bassiana* is known to grow better in habitats rich in organic substances which is probably associated with its ability to develop in the saprophytic phase. Maybe this was the reason of the dominating role of *B. bassiana* in infecting trap insects from soil of meadow 2 since the meadow was situated on river alluvia and peat soils.

M. anisopliae showed relatively high activity in soil sampled from orchard being the dominating species there. The prevalence of *M. anisopliae* compared with other species could result from its resistance to agricultural factors like plant protection chemicals, mineral fertilisation, intensive soil cultivation (Tkaczuk 2008). Some studies (Janowicz et al. 2004, Paruch et al. 2004) indicate, however, quite opposite that *M. anisopliae* is a species particularly sensitive to plant protection chemicals. The species can best stand the absence of potential hosts from among other species of entomopathogenic fungi. Its conidia are able to survive on a primary host for a long period of time (Miętkiewski 1992).

Three species of fungi: *M. anisopliae*, *I. fumosorosea* and *B. bassiana* were isolated from the soil from orchard. The presence of the same species of entomopathogenic fungi in orchard soils was noted by many authors (Marjańska-Cichoń et al. 2003, 2005, Janowicz et al. 2004, Paruch et al. 2004). Paruch et al. (2004) reported higher mortality of caterpillars caused by fungi from orchards without chemical protection measures compared with the soil from a control orchard that was subjected to chemical protection and nursing practices. As seen from their studies, frequent application of plant protection chemicals reduced the populations of beneficial, entomopathogenic soil fungi.

Pesticides were used moderately in the studied orchard. Observed occurrence of entomopathogenic fungi, mainly of *M. anisopliae* and *I. fumosorosea*, differed from that presented by Paruch et al. (2004). In the studied orchard *M. anisopliae* was dominating and its activity was highest in autumn. In the control orchard of Paruch et al. (2004), *M. anisopliae* was not found while in orchards, where pesticides were not used, the species was most active only in spring. *I. fumosorosea* dominated in orchards studied by Paruch et al. (2004).

Statistical analysis showed significant site-specific occurrence of *I. fumorososea*. This fungus was isolated more frequently from forest than from meadow habitats. The least frequent in studied sites was *M. anisopliae*. This species was isolated more often from meadow and orchard than from forest habitats. In autumn it dominated in meadow 1 and in spring and summer – in orchard (Table 1). In the soil from meadow 1, the mean contribution of *M. anisopliae* to all entomopathogenic fungi isolated there was 66% while in the soil from orchard it amounted 50%. The frequency of isolation of *M. anisopliae* in forest 1 was significantly lower than that in meadow and orchard.

Numerous occurrence of *M. anisopliae* in orchard soil as compared with other species of fungi was noted by Miętkiewski et al. (1992). The authors found also an abundant presence of *B. bassiana*. In my study *B. bassiana* was much less frequent but the second numerous species after *M. anisopliae* was *I. fumosorosea*, the species not recorded by Miętkiewski et al. (1992). Studies carried out by Miętkiewski et al. (1992) differed from mine in that they compared the occurrence of entomopathogenic fungi in soils from herbicide fallow and orchard sward. They found that *M. anisopliae* infected larvae more often in soils from herbicide fallow than in soils from orchard sward, *B. bassiana* showed the reverse pattern.

Two species of entomopathogenic fungi: *B. bassiana* and *I. fumosorosea* dominated in studied forest habitats. In each of the three studied forests, *M. anisopliae* was less frequent. According to Tkaczuk (2008), the dominating species in Poland, in both forest soil and litter, is *B. bassiana*. Similarily Głowacka and Świeżyńska (1993) and Bajan et al. (1995) pointed to *B. bassiana* as the most frequent species in forest habitats. The species dominated in studied forest 1 and forest 3, from which soil samples were taken only in autumn. *I. fumosorosea* was also numerous in studied forests being the most frequently isolated species in forest 1 and 2 in autumn. As shown by Tkaczuk (2008) *I. farinosa*, *I. fumosorosea* and *M. anisopliae* are almost equally frequent in soil-litter samples from various forests in Poland and their abundance is nearly equal. In my studies *I. farinosa* was not found at all and *I. fumosorosea* was isolated more often than *M. anisopliae*.

Studied habitats were characterised by different abundance of entomopathogenic fungi. More of these microorganisms were found in meadow 1 than in orchard and forest 1. Various factors like favourable location (close to the river and forest) that provided appropriate soil moisture and enhanced the development of diverse flora and fauna could be the reason of observed pattern. Greatest richness of meadow soil in entomopathogenic fungi compared with orchard soil might be also associated with sporadic application of pesticides and less intensive cultivation.

From among meadow and forest habitats sampled in autumn particularly great abundance of entomopathogenic fungi was noted in the nature reserve – forest 3. This confirms earlier findings (Miętkiewski et al. 1991–1992, Bałazy 2006, Tkaczuk 2008) that more diverse ecosystems with richer flora and fauna (including entomofauna) are more attractive for entomopathogenic fungi, which may find their potential hosts there.

In general, a greater activity of entomopathogenic fungi is observed in autumn due to the translocation of insects to soil for wintering. Insects dying of mycosis substantially enrich the soil in the infection material (Tkaczuk 2008). The number of conidia produced by *B. bassiana* on insects in the forest litter in autumn varies from 109 to 1,010 m^2 while in late spring and summer it is 107–108 m^2 (Bałazy 2006).

Temperature at which trap insects contacted the soil significantly affected the activity of *B. bassiana* and *I. fumosorosea*. Both species infected more larvae at 20°C than at 25°C. No significant differences were noted in the infection of trap larvae by *M. anisopliae* at different values of temperature. The species was slightly more frequent at 25°C. Miętkiewski et al. (1994) obtained different results in this aspect. They confirmed lower thermal requirements of *I. fumosorosea* but found that *B. bassiana* infected most often at 25°C. However, in the study by Sapieha-Waszkiewicz et al. (2006) *B. bassiana* and *I. fumosorosea* infected trap larvae more frequently at 20°C than at 25°C, similarly as they did in my experiments. Both species dominated also at lower (18°C) but not at higher (28°C) temperature in the study of Tkaczuk and Miętkiewski (1996). This phenomenon may be explained by a marked differentiation of features of the strains originating from various habitats.

M. anisopliae infected more trap larvae at 25°C but the difference was not statistically significant. High thermal requirements of this species were also observed by various authors (Miętkiewski et al. 1993 and 1994, Tkaczuk and Miętkiewski 1996, Sapieha-Waszkiewicz et al. 2006).

Entomopathogenic fungi have a great biocoenotic value being a factor controlling population density of insects. Therefore, some species may be used for biological control of plant pests. That is why the understanding of species composition and ecology of this group of fungi and protection of their habitats is so important. Protected forests, nature reserves and national parks should play a role of shelters and refuges for these fungi (Bałazy 1981). Semi-natural habitats play similar role for the maintenance and species diversity of entomopathogenic fungi in agricultural landscapes (Tkaczuk 2008).

CONCLUSIONS

1. The following species of entomopathogenic fungi were isolated in the study area:
   - *Beauveria bassiana*,
   - *Metarhizium anisopliae*,
   - *Isaria fumosorosea.*
2. Habitat and temperature affected the frequency of isolation of particular species of entomopathogenic fungi. Such a relationship was not found for season.
3. *B. bassiana* and *I. fumosorosea* infected insects significantly more often at 20°C than at 25°C.
4. The species most often isolated in autumn were: *B. bassiana* and *M. anisopliae* in meadow habitats and *B. bassiana* and *I. fumosorosea* in forest habitats.

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Streszczenie: *Występowanie grzybów entomopatogenicznych na terenie Chojnowskiego Parku Krajobrazowego.* Celem podjętych badań było określenie składu gatunkowego i nasilenia występowania grzybów entomopatogenicznych na wybranych siedliskach w Chojnowskim Parku Krajobrazowym. Zbadano także, czy na częstość izolowania grzybów entomopatogenicznych wywierają wpływ następujące czynniki: siedlisko, pora roku (wiosna, lato, jesień) oraz temperatura. Na podstawie badań prób glebowych pobranych z łąki 1, lasu 1 i sadu w okresie wiosny, lata i jesieni 2010 roku został oceniony wpływ siedliska i pory roku na występowanie grzybów entomopatogenicznych. Na badanym terenie wyizolowano trzy gatunki grzybów entomopatogenicznych

(*Beauveria bassiana*, *Metarhizium anisopliae* i *Isaria fumosorosea*). Stwierdzono wpływ siedliska i temperatury na częstość izolacji poszczególnych gatunków. Na łące 1 i w sadzie gatunkiem dominującym okazał się *M. anisopliae*, zaś w lesie 1 *B. bassiana*. Z siedlisk łąkowych i leśnych, z których glebę pobrano jesienią, największe nasilenie grzybów entomopatogenicznych obserwowano w lesie 3 (rezerwacie). Na siedliskach łąkowych najczęściej izolowanymi gatunkami były *M. anisopliae* i *B. bassiana*, na siedliskach leśnych *B. bassiana* i *I. fumosorosea*. *B. bassiana* i *I. fumosorosea* istotnie częściej infekowały owady w 20°C niż 25°C.

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