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Survey for Etomopathogenic Nematodes and Entomopathogenic Fungi in Alfalfa Snout Beetle, *Otiorhynchus Ligustici* (L.) (Coleoptera: Curculionidae), Infested Fields in Hungary and in New York State

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SURVEY FOR ETOMOPATHOGENIC NEMATODES AND ENTOMOPATHOGENIC FUNGI IN ALFALFA SNOUT BEETLE, *OTIORHYNCHUS LIGUSTICI* (L.) (COLEOPTERA: CURCULIONIDAE), INFESTED FIELDS IN HUNGARY AND IN NEW YORK STATE

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ABSTRACT

A survey for etomopathogenic nematodes and fungi in alfalfa snout beetle *Otiorhynchus ligustici* (L.) infested fields was conducted in Hungary, where this beetle is native, and in New York State, where the alfalfa snout beetle is an invasive species. Soil samples were collected in Hungary and in New York in alfalfa snout beetle infested alfalfa fields in spring 2002. *Galleria mellonella* (L.) larvae were used as bait insects. The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* were found in Hungary and New York. The frequency of plots with entomopathogenic fungi was not significantly different between locations in Hungary and New York. The entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis* sp. were found in Hungary and New York and *S. feltiae* was only found in Hungary. The frequency of plots with entomopathogenic nematodes was not significantly different between locations in Hungary and New York. *S. carpocapsae* and *S. feltiae* were found in coexistence at one location in Hungary.

INTRODUCTION

The alfalfa snout beetle, *Otiorhynchus ligustici* (L)., is the single most serious insect pest of alfalfa and clover in North America. This insect was introduced from Europe by trading ships from Europe that carried soil as ballast (York 1974). This insect was first recorded in New York state in 1896 near Oswego, New York (York et al. 1971, Ferguson et al. 1995). Alfalfa snout beetle has a two-year life cycle with the larvae feeding on the tap root of alfalfa plants ultimately resulting in the death of the plants (Lincoln and Palm 1941, Ferguson et al. 1995). Frequently, entire fields are destroyed in a single growing season. Within the infested area, alfalfa snout beetle cannot be contained or managed with currently available insecticides and the infested area is increasing in size every year. To date, the infested area remains relatively small, covering only nine counties in New York State and extends across the St. Lawrence River into Canada (Loan et al. 1986).

The alfalfa snout beetle is not considered a serious enough alfalfa pest in Hungary to use chemical or biological control methods to reduce population numbers. Mass movements of the adults have been observed several times in Hungary (Manninger and Csehi 1963, Jermy and Balázs 1990), but yearly, range-wide mass movements, when the emerged adults walk in great numbers, as observed in northern New York state, have not been recorded in Hungary in the last decade. There was no empirical data available on the population densities of alfalfa snout beetle in Hungary in recent years but the fact that farmers were unaware of this pest in Hungary (with the exception of the area near to

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Mezõhegyes in southern Hungary) suggests that the populations of the alfalfa snout beetle may be lower in Hungary than in New York.

A survey was carried out in Hungary and in New York State for the parasitic microsporidium *Nosema otiorhynchi* (Weiser) (Neumann 2003) that was reported to have affected alfalfa snout beetle populations significantly in the Czech Republic (Weiser 1961). This survey yielded results of limited value; however, infections by entomopathogenic nematodes and fungi were found in some of the adult beetles. Our objective was to conduct a broader survey of entomopathogenic nematodes and fungi in Hungary and in New York State in 2002 during the beetle's spring emergence.

MATERIALS AND METHODS

Soil sampling and isolation. Soil samples were taken in alfalfa fields at four different locations in central Hungary (Szada, Valkó), southern Hungary (Hódmezõvásárhely), and south-eastern Hungary (Mezõhegyes,) and at four different locations in New York State (sites in Oswego County, Wayne County, and two sites in Jefferson County). All sites had sandy type soils. The sampling was conducted during the spring emergence of adult alfalfa snout beetles (22-27 March 2002 in Hungary and 4-21 May 2002 in New York State). Ten random 1-m^2 plots were chosen in each field and, within each sample plot, 10 random soil samples were taken. GPS readings were taken at each plot. A 2.5 cm diameter soil core sampler was used to collect 10 cm deep soil samples. The soil cores were divided into 0-5 cm and 5-10 cm depths, so each soil core yielded two samples (same soil core, different depths). The sampling depth targeted the upper regions of the soil where adult alfalfa snout beetle is found during the spring emergence and oviposition. The soil samples were then placed into 180 ml plastic cups. The soil cores were broken up with a fork and lightly moistened with water mist if necessary. Two healthy wax moth, *Galleria mellonella* (L.), last instar larvae were placed on top of each soil sample. The cup was closed tightly and was incubated at room temperature in an inverted position. The larvae were monitored every 24 hours for 72 hours and dead larvae were removed and placed individually into small plastic tubes. The *G. mellonella* larvae used in Hungary were from a wild population and these larvae often formed cocoons which had to be removed to force the larvae to remain in contact with the soil. Larvae that survived the three-day exposure time were also transferred individually into plastic tubes and transported back to the US for further observation. This protocol ensured the detection of the infections by entomopathogenic fungi. All dead larvae that showed no externally detectable signs of fungal infections were placed onto White traps (White 1927). Fungal infections were recorded and the fungi were isolated on SDAY (Sabouraud dextrose agar supplemented with 1% yeast extract) media. Dead larvae on the White traps that did not show any signs of fungal infection or emergence of infective juveniles (IJ) from cadavers were dissected 18 days after death.

If IJ emergence occurred, healthy *G. mellonella* larvae were infected by transferring the recently emerged IJs onto autoclaved soil in a 180 ml plastic cup, placing 10 *G. mellonella* larvae on top of the soil and then incubating the cups at room temperature in an inverted position. Dead larvae were transferred onto White traps immediately after death. A few dead larvae were dissected three to five days after death in order to isolate adult nematodes for identification. Entomopathogenic fungi on the SDAY media were identified after sporulation and deposited with the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) at Cornell University.

The frequency of infections by entomopathogenic fungi and nematodes were based on the number of plots with positive soil cores because the plots were chosen randomly within a field and the soil cores were randomly chosen within

the plots. Frequencies were evaluated by comparing the actual frequencies of positive plots between Hungary and New York. The chi-squared test was used to analyze the data.

The focus of this soil bioassay was to survey and identify entomopathogenic nematodes and fungi. Saprophytic fungal and bacterial infections were not investigated.

RESULTS

Entomopathogenic fungi in Hungary. *Metarhizium anisopliae* (Metsch.) was found in one plot in Szada and in one plot in Hódmezõvásárhely. *Beauveria bassiana* (Vuill.) was found in three plots in Szada, six plots in Valkó, and one plot in Mezõhegyes. *Cladosporium* sp. and *Fusarium* sp. were also recovered in Szada, Valkó, and Hódmezõvásárhely but these fungi were considered to be weak pathogens. Entomopathogenic fungi were found in 60% of the plots (6 positive plots out of 10 in total) in Valkó, 50% of the plots (3 positive plots out of 6 in total) in Szada, and 10% of the plots (1 positive plot out of 10 in total at each location) in Hódmezõvásárhely and Mezõhegyes.

Entomopathogenic fungi in New York State. *M. anisopliae* was found in eight plots at the first location in northern Jefferson County, two plots at the second location in southern Jefferson County, four plots in Oswego County, and three plots in Wayne County. *B. bassiana* was found in one plot at the first location in northern Jefferson County, one plot at the second location in southern Jefferson County, two plots in Oswego County, and three plots in Wayne County. Entomopathogenic fungi were found 80% of the plots (8 positive plots out of 10 total) in northern Jefferson County, 30% of the plots (3 positive plots out of 10) in southern Jefferson County, and 50% of the plots (5 positive plots out of 10 in total at each location) in Oswego and Wayne Counties.

The frequencies of entomopathogenic fungi in Hungary and in New York State based on the number of plots with entomopathogenic fungi (species pooled) are summarized in Table 1.

There was no significant difference between the frequencies of positive plots (plots with entomopathogenic fungi, species pooled) in Hungary and in New York ($\chi^2 = 3.743$, df = 1, $P = 0.053$).

Table 1. The frequencies of entomopathogenic fungi in Hungary and in New York State.

Location	# of Plots	% of Positive Plots
Hungary	36	30.5
Hódmezõvásárhely	10	10.0
Valkó	10	60.0
Mezõhegyes	10	10.0
Szada	6	50.0
New York State	40	52.5
Oswego County	10	50.0
Wayne County	10	50.0
Jefferson County (north)	10	80.0
Jefferson County (south)	10	30.0

Entomopathogenic nematodes in Hungary. *Heterorhabditis* sp. was recorded in three plots in Szada. *Steinernema carpocapsae* (Weiser) was recorded in two plots in Valkó. *S. feltiae* (Filipjev) was recorded in four plots in Valkó, and one plot in Hódmezõvásárhely. Entomopathogenic nematodes were found in 50% of the plots (3 positive plots out of 6 in total) in Szada, 50% of the plots (5 positive plots out of 10 in total) in Valkó, 10% of the plots (1 positive plot out of 10 in total) in Hódmezõvásárhely, and 0% of the plots (0 positive plots out of 10 in total) in Mezõhegyes. All attempts failed to recover one of the nematode infections from Hódmezõvásárhely (another sample from the same plot contained *S. feltiae*).

Entomopathogenic nematodes in New York State. *S. carpocapsae* was found in four plots in northern Jefferson County, one plot in southern Jefferson County, and one plot in Wayne County. *S. feltiae* was not recorded at any of the locations in New York State. One nematode-infected *G. mellonella* larva from Oswego County failed to produce IJs. This nematode was identified as *Heterorhabditis* sp. based on the brick red coloration of the infected wax moth larva. Entomopathogenic nematodes were found in 40% of the plots (4 positive plots out of 10 in total) in northern Jefferson County and 10% of the plots (1 positive plot out of 10 in total at each location) in southern Jefferson, Oswego (considering the lost nematode), and Wayne Counties.

The frequencies of entomopathogenic nematodes in Hungary and in New York State based on the number of plots with entomopathogenic nematodes (species pooled) are summarized in Table 2.

There was no significant difference between the frequencies of positive plots (plots with entomopathogenic nematodes, species pooled) in Hungary and in New York ($\chi^2 = 0.641$, df = 1, $P = 0.423$).

Vertical distribution of nematodes. When entomopathogenic nematode occurrences were pooled by species from all locations, *S. carpocapsae* was found in the top 5 cm of soil in 90% of cases, *S. feltiae* occurred in the top 5 cm in 60% of cases while *Heterorhabditis* sp. was found 50% of cases in the top layer of soil.

Table 2. The frequencies of entomopathogenic nematodes in Hungary and in New York State.

Location	# of Plots	% of Positive Plots
Hungary	36	25.0
Hódmezõvásárhely	10	10.0
Valkó	10	50.0
Mezõhegyes	10	0.0
Szada	6	50.0
New York State	40	17.5
Oswego County	10	10.0
Wayne County	10	10.0
Jefferson County (north)	10	40.0
Jefferson County (south)	10	10.0

DISCUSSION

Entomopathogenic nematodes and fungi. The occurrence of entomopathogenic fungi and nematodes may have been underestimated in Hungary due to the wild *G. mellonella* larvae used as trap insects. These larvae formed cocoons in the soil cups and/or often climbed to the top of the container before forming the cocoon, shortening the exposure time. It was not feasible to continuously remove the cocoons so, in some cases, the larvae may have escaped infection. The limited number of available host larvae in Hungary also reduced the sample size.

All species of entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) found in Hungary were also found in New York State. The frequency of entomopathogenic fungi tended to be higher in New York State (52.5%) than in Hungary (30.5%) when species were pooled (Table 1), but this difference was not statistically different. The occurrence of entomopathogenic nematodes tended to be higher in Hungary (25%) than in New York State (17.5%) but this did not prove to be statistically different (Table 2). A larger sample size may show a significant difference with sites sampled in New York having a higher frequency of entomopathogenic fungi.

The generally high frequency of entomopathogenic fungi compared with entomopathogenic nematodes suggests that the conditions may be more favorable to fungi than nematodes. There are no data on nematophagous fungi that may affect entomopathogenic nematode frequencies at the sites surveyed or on the interactions of microbial agents. Also, it must be noted that the results presented here are based on one-season data with limited number of replications.

Preliminary testing with all recovered fungi showed that they are capable of infecting and killing alfalfa snout beetle adults and larvae but no quantitative testing was done.

All nematodes found in Hungary and New York State were found to infect and kill adult alfalfa snout beetle in a different research project (Neumann 2003) and ongoing experiments are investigating the penetration rates of IJs of these nematodes into adult and larval alfalfa snout beetle.

One important finding was that *S. feltiae* occurred in Hungary at two sites infested by the alfalfa snout beetle while this nematode species was not recovered anywhere in New York State during the survey. Alfalfa snout beetle populations at these sites in Hungary were at lower levels than at the sites in New York State. Koulova et al. (1998) reported that *S. feltiae* was able to infect 5-43% of adult alfalfa snout beetles under laboratory conditions at temperatures as low as 10 oC and *S. feltiae* was mentioned to attack adult beetles when they enter the soil for oviposition. Generally, steinernematids remain active at lower temperatures than heterorhabditids (Molyneux 1986). Therefore, it seems possible that *S. feltiae* has an advantage over *Heterorhabditis* sp. attacking adult beetles in the soil during the spring emergence.

Although the main focus of this survey was to identify potential natural enemies that may affect the alfalfa snout beetle adults early in the spring, the importance of *Heterorhabditis* sp. as a biological control agent should not be underestimated (Schroeder et al. 1994). Ferguson et al. (1995) found that *H. bacteriophora* (Poinar) was more persistent than the two steinernematid species mentioned. Schroeder et al. (1994) also found that *H. bacteriophora* dispersed deeper into the soil than *S. carpocapsae* and *S. feltiae*. The ability of *H. bacteriophora* to persist over the long term and to reach greater depths in the soil probably makes this nematode the best candidate for decreasing the number of alfalfa snout beetle larvae in later instars that move further down into the soil. *H. bacteriophora* could also attack ovipositing adults when they enter the soil and the soil temperatures are higher later in the spring.

S. carpocapsae and *S. feltiae* were found in coexistence at the site in Valkó where the alfalfa snout beetle was present but not in great numbers suggesting acceptable natural control. Whether the presence of these two species of nematodes can provide better control than a single species cannot be determined by this study. Alatorre-Rosas and Kaya (1990) discussed whether there was an advantage to using two or more species of nematodes for the control of soil-inhabiting insect pests. They suggested that depending on the location of the host, the dispersal ability of the nematode will determine its effectiveness. For example, if the pest is deeper in the soil, a well dispersing, cruiser type nematode would be most effective. Therefore, no advantage is gained by the use of more than one nematode species. The alfalfa snout beetle, however, during its two-year life cycle, can be a target of nematodes with different foraging strategies. *S. carpocapsae* is an ambusher nematode while *S. feltiae* adopts both ambushing and cruising strategies (Lewis et al. 1995). *S. carpocapsae* therefore, may be more effective infecting the adult beetles moving on the soil surface during the spring emergence and oviposition while *S. feltiae* may be more effective infecting the larvae as they move deeper down into the soil. The alfalfa snout beetle in alfalfa may be a suitable system for the study of the effectiveness of using more than one species of nematodes with different foraging behaviors against a soil-inhabiting pest.

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