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Virulence of *Metarhizium brunneum* to Field Collected *Agriotes* spp. Wireworms

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ABSTRACT

The number of active substances registered for wireworm control is decreasing. Consequently, inventorization programs were launched to find and identify potential wireworm biological control agents. *Metarhizium brunneum* Petch was isolated from an adult *Agriotes* sp. (Coleoptera: Elateridae) in Slovenia. The strain belongs to a phylogenetic lineage of *M. brunneum* accommodating isolates from Asia and North America while it was reported from Europe (Denmark and Switzerland) only recently, and for the first time in Slovenia. Its pathogenicity to field-collected *Agriotes* spp. was tested in feeding and soil experiments. The latter lasted either 15 or 90 days and adopted different concentrations of fungal conidia. Coating potato slices with conidia had no effect on mortality. However, *M. brunneum* in soil significantly increased wireworm mortality in short- and long-term bioassays. The average LT_{50} based on Probit analysis was 44.6 days for the *M. brunneum* treated wireworms and 741 days for the negative controls.

Keywords: Biological control, Entomopathogenic fungi, Integrated pest management, Potato, Soil pest.

INTRODUCTION

Wireworms are soil-burrowing larval stages of click beetles (Coleoptera: Elateridae), and major pests of crops including potatoes, wheat, and maize (Ansari *et al*., 2009; Gomboc and Milevoj, 2001; Johnson *et al*., 2008; Reddy *et al*., 2014) in many parts of the world. There are at least 180 genera of click beetles in Europe. Species of the genus *Agriotes*, including *A. ustulatus* Schall., *A. lineatus* L., *A. obscurus* L., *A. sordidus* Illiger and *A. sputator* L. are economically most important pests in Central and Eastern Europe. These species live in grasslands and fields and have the potential to be agricultural pests (Gomboc and Milevoj, 2001).

Wireworms damage seed potatoes shortly after planting in April–May only weakly, typically not causing plant losses. Their impact on developing tubers in summer and early autumn can be very serious. Damaged potato tubers show characteristic round boring holes and tunnels and have a reduced market quality (Johnson *et al*., 2008). More importantly, this leads to secondary microbial infections (Ester and Huiting, 2007). In high pest pressure areas, the entire potato crops can become unmarketable (Ansari *et al*., 2009). Significant economic losses were reported from North America (Jansson and Seal, 1994), Germany (Schepl and Paffrath, 2007) and Slovenia (Zidarič *et al*., 2013). In Slovenia, potatoes and maize are the most affected crops by wireworms (Bohinc and Trdan, 2013), causing direct or indirect economic losses amounting up to 64 million EUR, annually (Groznik *et al*., 2009).

Granular organophosphates can effectively control wireworms (Chaton *et al*., 2008), but high application rates are required and they have been banned or restricted in many countries (Ester and Huiting, 2007). The number of active

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substances registered for wireworm control in Slovenia has decreased from 13 in 1995 to only three in 2017: Synthetic pyrethroid tefluthrin (Force 1,5 G), thiacloprid based insecticide Sonido and the bioinsecticide Naturalis (*Beauveria bassiana*, strain ATCC 74040) (Bohinc and Trdan, 2013; Zidarič *et al*., 2013; List of authorised plant protection products in Slovenia, http://spletni2.furs.gov.si/FFS/REGSR/index.htm , March 5, 2017). However, no chemical substance is available at all in organic farming systems (Ester and Huiting, 2007).

Several authors reported that members of the species complexes that centre on *Metarhizium anisopliae* (Metschn.) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill. or their close relatives can be highly pathogenic to wireworms or click beetles (Ansari *et al*., 2009; Eckard *et al*., 2014; Reddy *et al*., 2014; Tinline and Zacharuk, 1960; Zacharuk and Tinline, 1968). Zacharuk (1973) suggested that hyphae of *M. anisopliae* penetrate cuticular layers of wireworms and prevent ecdysis. Although different kinds of experimental designs were used in these reports, the consensus emerged that different wireworm species are differently susceptible to entomopathogenic fungi (Eckard *et al*., 2014; Kabaluk *et al*., 2005; Kölliker *et al*., 2011) and that different fungal agents can vary in their pathogenicity and virulence as their mean lethal time (*LT50*) values cover a range from six to 120 days.

In May 2011, an *Agriotes* sp. imago strongly infected with *Metarhizium* sp. was gathered in Jablje, Slovenia. The aim of this study was to identify this strain and assess its virulence against field-collected *Agriotes* wireworms.

MATERIALS AND METHODS

Entomopathogenic Fungus, Isolation and Identification

The *Metarhizium* strain was isolated from a male adult beetle trapped in a YATLORf pheromone trap (Csalomon, Hungary) equipped with pheromones known to attract either *Agriotes sputator* or *A. ustulatus*. The trap was placed on 14 April 2011 in experimental fields of the Agricultural Institute of Slovenia (AIS) at Jablje near Ljubljana, Slovenia (14.578° E, 46.141° N). When the dead beetle was collected on 10 May 2011, it was covered by numerous *Metarhizium* sporodochia.

Metarhizium conidia were suspended into a drop of sterile water on a glass microscope slide. Conidia were then sucked into a fine glass capillary tube and the tube was emptied by moving the tip over the surface of 1/3 strength potato dextrose agar enriched with antibiotics (14 g potato dextrose, Biolife, Italy; 10 g technical agar, Biolife, Italy; 12 mg penicillin G potassium salt, Fluka, Buchs, Switzerland; 54 mg streptomycin sulfate salt, Sigma-Aldrich). Conidia that were well separated from each other were identified microscopically at low magnification and transferred to fresh agar plates once they germinated. Isolated fungal strains were kept and sub-cultured on 1/3 strength PDA in darkness.

The obtained *Metarhizium* strain was identified on the basis of morphological characters and a molecular barcode sequence of the intron-rich part of the elongation factor 1-alpha (*tef*) by adopting the strategies described by Bischoff *et al*. (2007) but using the EF2 primer of O'Donnell *et al*. (1998). The 50 µL reaction mixture for PCR consisted of 5 µL of Taq PCR buffer with $(NH_4)_2SO_4$ (Fermentas, USA), 2 mM $MgCl_2$, 0.2 mM dNTP (Promega, USA), 0.5 mM of each of the primers, 1 unit of native Taq polymerase (Fermentas, USA) and 1 µL of genomic DNA. In PCR, we used an initial denaturation step at 94°C for 3 minutes, 5 cycles of 94°C for 60 seconds (denaturation), 56°C for 45 seconds (annealing), 72°C for 60 seconds (elongation) and 35 cycles as described before but with an annealing temperature of 53°C, and a final extension at 72°C for 8 minutes. Sequencing reactions were performed at the Macrogen Europe sequencing facility (Amsterdam, The Netherlands) in both directions by using the same primers as used in PCR. The data were inspected and edited with the aid of the

software program BioEdit v7.2.0 (Hall, 1999). The generated sequence, deposited at GenBank (accession number KF233885) was compared with other *Metarhizium TEF1* sequences published by Bischoff *et al*. (2009, 2007), Nishi *et al*. (2011), Reineke *et al*. (2014), Steinwender *et al*. (2014), Wyrebek *et al*. (2011) in November 2015. Relevant sequences selected from Genbank and the newly generated sequence KF233885 were aligned using the software MEGA6. Maximum Likelihood analyses (ML) of aligned sequences were performed with PhyML 3.0 (Guindon *et al*., 2010) at the ATGC website (http://www.atgc-montpellier.fr/phyml/; November 2015) by adopting a GTR model of nucleotide substitutions and a gamma distributed rate variation among sites. ML bootstrap inferences were based on 1000 re-sampled datasets. Clade credibility was also tested using MrBayes 3.2.2 (Ronquist *et al*., 2012) implementing six substitution types, gamma-shaped rate variation across sites and four rate categories as implemented in the general time reversible model with gamma-distributed rate heterogeneity. MrBayes ran two simultaneous analyses for five million generations; the number of chains was set to four and trees were sampled every 100 generations, starting from a randomly selected tree. A 50% majority rule consensus tree and posterior probabilities (pp) for each split was calculated after excluding the first 12,500 sampled trees. The analyses used *M. robertsii* as out- and *M. pingshaense* and *M. anisopliae* as sister-groups.

Wireworm Collection and Rearing

Wireworms were collected in maize-wheat bait traps (Kirfman *et al*., 1986) that were placed approximately 8.8 km SW of the location where the *Metarhizium*-infected beetle was found. The traps were laid out on 14th April 2011 and kept in the field for 14 days. Their content was hand-sorted and all live, undamaged and intermediately-aged wireworms were transferred to a 15 L plastic container, containing 8 kg of damp soil from the original location. Lettuce leaves and potato slices were added regularly as food and the container was watered as needed.

Short-Term Soil Exposure Experiments

In the short term soil exposure experiments the wireworms were exposed to *Metarhizium* conidia incorporated into soil. The conidia were sampled from 14 day-old PDA cultures and suspended in 100 mL sterile water with 0.01% Tween 80 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The soil substrate was a light commercial planting soil, rich in organic matter (Potgrond H, Klasmann-Deilmann GmbH, Geeste, Germany). The non-sterile soil substrate was inoculated with conidia at a concentration of either 3.75×10^5 conidia g^{-1} air-dried soil (designated 'low soil treatment') or 2.5×10^6 conidia g^{-1} air-dried soil (designated 'high soil treatment') by adding 5 mL of conidial suspension to 20 g of air-dried soil substrate. Concentration of conidia was adjusted with the aid of a haemocytometer (Faust Laborbedarf AG, Schaffhausen, Switzerland). To allow for uniform inoculum incorporation, conidial suspensions were mixed into the soil in a large tray with a spatula. The bioassay included a control treatment of 5 mL of a 0.01% Tween 80 solution mixed equally into soil. Twenty grams of the inoculated soil substrate was placed into a 150 mL plastic cup and loosely capped to allow aeration. Three wireworms, 1.5–2.5 cm long, were added to each cup. For each of the two conidial concentrations a total of five cups were prepared. The cups were placed into sealed zip-lock bags containing moist paper towels and maintained in darkness at 21°C and 75% relative humidity. A potato slice (2 cm^2, 3 mm thick) was added after 24 hours to each cup as food for the wireworms. The experiment was performed twice independently, each time with five replicates. Larval mortality was evaluated after 15 days after the start of the experiment

by counting dead larvae covered with *Metarhizium* sporodochia. Additionally, the extent of potato consumption was determined by a visual scale with levels 1 to 3: (1) No feeding; (2) Intermediate feeding; and (3) Most of the potato drilled and eaten.

Long-Term Soil Exposure Experiments

The methodology of the long-term soil exposure experiments was similar to the short-term soil exposure experiments. The concentration used was 3.85×10^6 living conidia g^{-1} non-sterile air-dried soil substrate. Aliquots of 30 mL were transferred into 50 mL centrifuge tubes (Vitaris AG, Baar, Switzerland). Into each tube a single wireworm was placed together with a potato piece on top of the substrate. Fifteen test tubes each were used for the negative controls that were treated with Tween 80 (0.01 %) and for treatments with conidia. The experiment was repeated three times independently and lasted 90 days. Larval mortality was observed 9 times in experiment 1, and 10 times in experiments 2 and 3, making 29 observations in total. All experiments were carried out in an environmental chamber at 20±1°C, 80±5% relative humidity in darkness.

Feeding Experiments

The experimental setup in feeding experiments was similar to the short-term soil exposure experiments, with an important difference; namely the conidia were added to the test system via infected potato slices. The potato slices (4 cm^2, 3 mm thick) used as food source for the wireworms were incubated for 10 min under gentle agitation in a 0.01 % Tween 80 suspension with 1.5×10^6 conidia mL^{-1} or 1.0×10^7 conidia mL^{-1}. For the negative control experiment, the potato slices were drenched in sterile 0.01% Tween 80 solution. To all cups, 5 mL of deionised water was added. Potato slices drenched with conidia were added to the cups at the beginning of the experiment. The cups were placed into sealed zip-lock bags containing moist paper towels and maintained in complete darkness at 21°C and 75% relative humidity. The experiment was performed twice independently, each time with five replicates. Larval mortality was determined after 15 days.

Statistical Evaluation of Data

Statistical analyses were performed using the computer software GraphPad Prism 5.00. The resulting data were corrected by Abbott's formula (Abbott, 1925) and examined for normality of distribution according to the D'Agostino-Pearson test. The significance of difference between treated and non-treated samples in short term experiments was analyzed using one-way Analysis Of Variance (ANOVA) and Tukey's multiple comparison post-tests for each experiment separately. Significant differences between fungi-treated and control groups were observed in both experiments, subsequently, the data were pooled. The virulence assay mortality curves from the long-term soil experiment were examined by linear regression, one-way ANOVA with Tukey's multiple comparison post-tests, Kaplan-Meier and Probit analysis (Finney and Stevens 1948; Motulsky 1995).

RESULTS

Olivaceous colony pigments observed on PDA after 14d and conidia measuring 5–7× 2–3 µm suggested that the fungus on the *Agriotes* beetle could be a member of the *M. anisopliae* species complex (Bischoff *et al*., 2009). Phylogenetic inferences based on the five end of *tef* sequences identified the strain as a member of one of, at least, three phylogenetic *M. brunneum* subgroups. Standard deviation of split frequencies in MrBayes analyses were below 0.01 after about 350.000 generations and a log

likelihood of -1421.51795 for the best tree was found in ML tests. Sequence data of H.J.S. 1868 was near identical with *M. brunneum* strains from Japan, Canada, Denmark, and Switzerland that formed a lineage within *M. brunneum* subclade three (Figure 1). Representatives of the other lineages in subgroup three were from Australia, USA, Israel, Philippines, and Mexico. The sequence of the strain from Slovenia differed by approximately five nucleotide substitutions from the sequence EU248855 that derived from the ex-epitype strain ARSEF 2107 of *M. brunneum* (Figure 1, subclade one) and by six from members of another subclade that centred on *M. brunneum* isolates known also from Europe (Figure 1, subclade two).

The *M. brunneum* strain H.J.S. 1868 was pathogenic against wireworms in laboratory

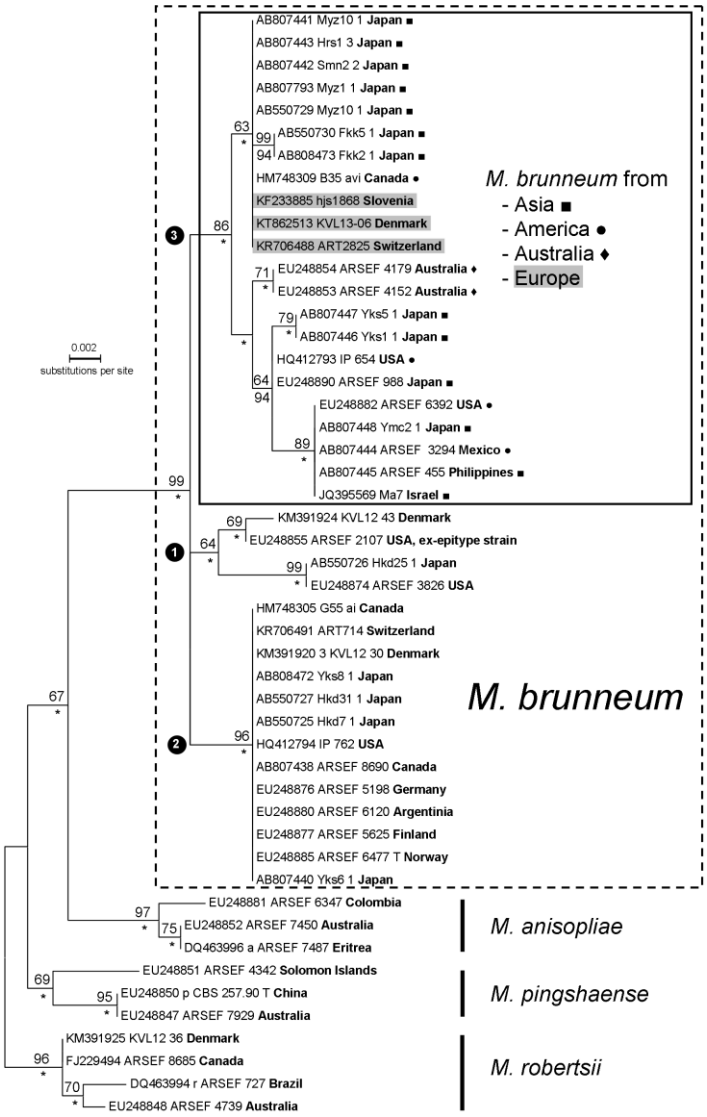

Figure 1. Maximum likelihood tree from PhyML analysis of TEF1 sequences for representatives of the three *Metarhizium brunneum* subgroups. Node support is provided as ML bootstrap proportions (Numbers> 60% are shown) or posterior probabilities from MrBayes analyses (asterisks for PP of 0.95–1.00).

bioassays. Exposing the wireworms to high concentrations of *M. brunneum* conidia (2.5×10^6 conidia g^{-1} soil; 'high soil treatment') resulted in a mortality of approximately 42% in 15 days (Table 1) in the short-term soil tests. Fifty percent mortality was achieved in 35 to 55 days (Figure 2) in the long-term soil experiments. The Analysis of variance has shown a significant effect of different conidial concentrations in the short-term soil experiments ($F_{2, 27}$=3.84, P= 0.034). Tukey's post-tests have shown that the number of living wireworms was significantly lower in the high soil treatment after 15 days of exposure as compared to the control (Table 1). No significant effect of *Metarhizium* exposure on potato consumption was observed (P= 1.000 in the 'low' and P= 0.195 in the 'high' treatment). No fungal infections were observed in the control group.

The mortalities observed in the long-term soil experiment exhibited a linear trend. Morality curves of the groups treated with *M. brunneum* had slopes of 1.15±0.07, 1.06±0.13 and 0.63±0.06 in the three independently performed experiments. The control groups' mortality curves had slopes of 0.42±0.05, 0.23±0.06, or 0.13±0.02. The ANOVA test suggested a $F_{5, 52}$ of 8.28 (P< 0.001). All Tukey's post-tests within three control repetitions and within three *Metarhizium*-treatment repetitions had *P*> 0.05; those between *Metarhizium*-treatments and their respective controls had *P*< 0.05.

Table 1. Effect of conidia concentration on mortality of wireworms in 15-day soil exposure experiment. [a]

Treatment	Control	Low	High
Conidia concentration (Viable conidia g^{-1} soil)	0	3.75×10^5	2.5×10^6
Number of living wireworms	2.6±0.2	1.9±0.4	1.5±0.3*
Percent of living wireworms (%)	86.7±5.4	63.3±11.6	50.0±10.2*
Mortality after Abbott's correction	0.0±6.2	26.9±13.4	42.3±11.8*
Potato consumption (Scale 1–3)	1.8±0.2	1.8±0.2	2.2±0.2

[a] Data were pooled from two experiments performed in 5 replicates (N= 10). Data shown are means±SE. Results significantly differing from controls are marked by an asterisk (P< 0.05).

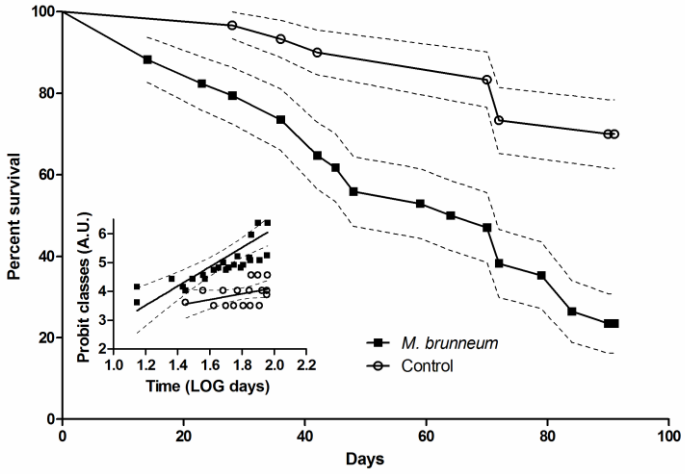

Figure 2. Kaplan-Meier wireworm survival curves with 95% confidence intervals (dashed lines). The survival curves are based on data pooled from three independent experiments. Inset: A graphical representation of Probit analysis, where the results are presented as raw mortality data and linear regression models (full lines) with 95% confidence intervals (dashed lines).

The individual *Metarhizium* mortality curves were significantly different from their respective controls, but not from each other. Accordingly, the Kaplan-Meier and Probit analysis was performed on pooled data (Figure 2). The individual Kaplan-Meier survival curves of *Metarhizium*-treatments differed significantly from their respective controls, resulting in a median survival time of 67 days for *Metarhizium*-treatments and > 90 days for the control. LT_{50} based on Probit analysis was 45 days (95% confidence interval(CI) 35 to 55 days) for treated wireworms and 741 days for the negative controls (95% CI 178 to > 900 days; Figure 2). No fungal infections were observed in the control group.

In the feeding experiment, where *M. brunneum* conidia were added to potato slices, the number of living wireworms did not differ significantly in the high and low conidia exposure treatments ($F_{2, 27}$= 1.5, P= 0.241; Table 2). No significant effect of *Metarhizium* exposure on potato consumption was observed (P= 1.000 in the 'low' and P= 0.608 in the 'high' treatment).

DISCUSSION

Metarhizium brunneum, originally described from the Philippines (Petch, 1935), is closely related to *M. anisopliae*, *M. pingshaense* and *M. robertsii* (Bischoff *et al*., 2009). Due to morphological similarities, strains of *M. brunneum* may have been misidentified in the past as *M. anisopliae*. *Metarhizium brunneum* became a frequently cited species only after its epitypification (Bischoff *et al*., 2009) (Web of Science™ database, http://apps.webofknowledge.com, accessed on 17 November 2015). *Metarhizium brunneum* has so far been reported from Argentina, Australia, Canada, Europe, Japan, and the USA (Bischoff *et al*., 2009; Steinwender *et al*., 2014, 2011; Wyrebek *et al*., 2011), but only since 2015 subclade three representatives were reported for Europe (Mayerhofer *et al*., 2015; Steinwender *et al*., 2015; this study). The *M. brunneum* biological control strain F52, cited earlier as *M. anisopliae* (e.g., Behle *et al*., 2013) and also originating from Europe, belongs to subclade two (Stephen Rehner, USDA-ARS, personal communication to H.J.S.). On the basis of analyses of *TEF1* sequences disseminated through GenBank, we conclude that *M. brunneum* consists of numerous phylogenetic lineages that group into three major subclades and that H.J.S. 1868 is the first report of *M. brunneum* (subclade three) for Slovenia.

The virulence of strain H.J.S. 1868 encountered in the short-term soil experiments appears to be higher than *Metarhizium anisopliae* strain V 275 (Ansari *et al*., 2009). The latter caused ca. 35% mortality against *Agriotes lineatus*, however, the fungal inoculum tested was higher and exposure different (the larvae were dipped into 1×10^8 conidia ml^{-1}), and the experiment lasted 21 days. Additionally, the same strain of *M. anisopliae* (in this report referred to as BIPECSO 5) caused less than 50% mortality of *A. lineatus*, *A. obscurus* and *A. sputator*

Table 2. Effect of conidia concentration on mortality of wireworms in 15-day feeding experiment.[a]

Treatment	Control	Low	High
Concentration of conidial suspension[b] (Viable conidia ml^{-1})	0	1.5×10^6	1.0×10^7
Number of living wireworms	2.3±0.2	2.6±0.2	2.1±0.3
Percent of living wireworms (%)	76.7±5.1	86.7±5.4	70.0±9.2
Mortality after Abbott's correction	0.0±6.6	-13.0±7.1	8.7±12.0
Potato consumption (Scale 1-3)	1.8±0.2	1.8±0.2	2.0±0.3

[a] Wireworms were exposed to conidia by incubating potato slices in the conidia suspensions. Data was pooled from two experiments performed in 5 replicates (N=10). Data shown are means±SE. [b] Potato slices, used as food, were incubated for 10 seconds in the conidia suspensions.

after eight weeks of exposure to 1×10^8 conidia mL^{-1} (Eckard *et al*., 2014). Ericsson *et al*. (2007) reported 13.3% mortality at a concentration of 1.0×10^5 conidia g^{-1} soil after 20 days in their experiments with *M. anisopliae* and the Spinosad insecticide at a concentration of 1.5 or 3 ppm.

The results from the long-term soil experiments were comparable to the virulence for *M. brunneum* strain ART 2825 reported by Kölliker *et al*. (2011) against *A. lineatus* (LT_{50} 56 days). The authors obtained longer LT_{50} values against *A. sputator* (LT_{50} ca. 84 days) but shorter LT_{50} values for *A. obscurus* (LT_{50} 21 days). Kabaluk and Ericsson (2007) reported 100% mortality for *A. obscurus* within 16–25 days and 80–95% mortality for *A. lineatus* in 28–30 days when exposing the wireworms to 10^6 conidia of *M. brunneum* strain F52 g^{-1} wet soil. Eckard *et al*. (2014) reported higher virulence for strain ART 2825 (LT_{50} 21 and 14 days for *A. lineatus* and *A. obscurus*, respectively) and comparable virulence of strain V1002 to *A. sputator* (LT_{50} 42 days) also belonging to the subclade 3 of *M. brunneum*.

Some authors report that predation and cannibalism among wireworms can occur when several are kept in small test vessels (Chaton *et al*., 2003; Zacharuk and Tinline, 1968). It is unlikely that cannibalism influenced the results in our study because aggressive behaviour between two wireworms was only observed once in the short-term experiment and the involved animals were alive at the time of inspection. Although the long-term experiments showed a somewhat lower mortality rate, we believe that these results are more reliable because we excluded potential transfer of conidia and cannibalism between experimental subjects (Kabaluk *et al*., 2005).

The incubation of potato slices with low or high conidial concentrations (1.5×10^6 conidia mL^{-1} versus 1.0×10^7 conidia mL^{-1}) had no effect on the mortality of the wireworms in the feeding experiments (Table 2). Some reports suggest that *Metarhizium* may infect insect larvae after ingestion (Lacey *et al*., 1988) while other reports suggest that *Metarhizium* is inhibited in the insect gut (Chouvenc *et al*., 2009). Our results support the conclusion by Thomas and Read (2007) and Beys-da-silva *et al*. (2014) that *Metarhizium* may exploit its insect pathogenicity through conidial adhesion, exoskeleton colonization and penetration and that ingestion by the insects is not required.

The *Metarhizium* strain was isolated from a male adult beetle trapped in a YATLORf pheromone trap (CSALOMON, Hungary) equipped with pheromones known to attract either *Agriotes sputator* or *A. ustulatus*. Because it was severely damaged, no further identification of the host beetle was possible on the basis of morphological characters, however, both species mentioned are known to inhabit the wider Ljubljana area (Gomboc and Milevoj, 2001).

Due to our interest in the virulence of strain 1868 against actual field population of *Agriotes* spp. wireworms, and partly due to their difficult classification (Furlan, 2004), they were not identified to species level. However, we presume that the *Agriotes* species composition in central Slovenia at the time of the experiment was: *A. lineatus* 41.4%, *A. ustulatus* 29.6%, *A. sputator* 20.7%, and *A. obscurus* 11.2% (Gomboc and Milevoj, 2001). It is possible that we observed a linear increase in mortality in the soil experiment because different species of *Agriotes* often have different LT_{50} values (Eckard *et al*., 2014; Kabaluk *et al*., 2005; Kölliker *et al*., 2011).

To conclude, *M. brunneum* is reported for the first time in Slovenia. Ingestion of *M. brunneum* H.J.S. 1868 conidia did not have a significant effect on wireworm mortality. However, spiking the soil with high conidial doses significantly increased wireworm mortality. The locally isolated strain of *M. brunneum* H.J.S. 1868 exhibited similar virulence as in some previously reported studies, and may form a base of an environmentally friendly strategy for wireworm management in conventional or

organic potato farming systems after successful on-farm evaluation.

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شدت بیماری زایی *Metarhizium brunneum* در کرم مفتولی گونه *Agriotes* spp. جمع آوری شده در مزرعه

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چکیده

از آنجا که شمار مواد ثبت شده موثر برای کنترل کرم مفتولی رو به کاهش است، برنامه های تهیه فهرست موجودی عامل های کنترل بیولوژیکی این آفت و یافتن و شناسایی آنها اجرا شده است. در این زمینه، *Metarhizium brunneum* Petch از موجودی بالغ از گونه *Agriotes sp* (سخت بالپوش:Elateridae) در اسلوونی جدا سازی شد. این ریسه متعلق به تبار زایی (phylogenetic) خاندان *M. brunneum* بود که جدایه هایی از آسیا و آمریکای شمالی را در بر می گیرد، در حالیکه حضور آن در اروپا (دانمارک و سوئیس) اخیرا و در اسلوونی برای نخستین بار گزارش شده بود. در این آزمایش، بیمارگری (pathogenicity) آن در مورد گونه *Agriotes spp.* جمع آوری شده از مزرعه، در یک آزمون تغذیه وآزمون تلقیح خاک بررسی شد. ازمون خاکی به مدت ۱۵ یا ۹۰ روز ادامه داشت و در آن غلظت های مختلفی از کنیدیوم های قارچ به کار رفت. پوشش دادن برش

های سیب زمینی با کنیدیوم هیج اثری روی مرگ ومیر کرم مفتولی نداشت.اما، افزودن *M. brunneum* به خاک در زیست-آزمونهای های کوتاه مدت و دراز مدت، مرگ ومیر کرم مفتولی را به گونه ای معنادار افزایش داد. میانگین LT_{50} بر مبنای تجزیه تحلیل Probit برای کرم مفتولی تیمار شده با *M. brunneum* در حد ۴۴/۶ روز وبرای تیمارنشده ها برابر ۷۴۱ روز بود.