LABORATORY TESTING OF ENTOMOPATHOGENIC FUNGI FOR THE CONTROL OF WIREWORMS (*Agriotes* sp. L.)

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ABSTRACT

The aim of the study was to assess entomopathogenic potential of 7 entomopathogenic fungal species (EPF) isolated from various substrata in Slovenia against larvae of *Agriotes* sp. The fungal isolates tested were *Beauveria bassiana, B. brongniartii, Metarhizium anisopliae, M. brunneum, M. robertsii, Purpureocillium lilacinum* and *Clonostachys solani* f. *nigrovirens*. Conidia of these species were incorporated into the test substrate as a water suspension to reach a final concentration of 3.85 x 10^6 conidia g^{-1} air-dried soil. The larval mortality was observed on a weekly basis for a total of 90 days. Most of the mortality curves observed exhibited a linear trend with slopes ranging from 0.09 to 1.62 for the fungal treatments and 0.16 to 0.74 for the control treatment, in which only 0.1% Tween 80 solution was used. Abbott's corrected mortality at day 90 ranged from 14.3 to 100 %. The most promising candidate biological control agents were *Metarhizium brunneum* isolate 1868, *M. robertsii* isolate 1880 and *B. brongniartii* isolate 1877.

Keywords: biological control, biopesticides, pests, wireworms.

IZVLEČEK

LABORATORIJSKI POSKUSI Z ENTOMOPATOGENIMI GLIVAMI ZA ZATIRANJE STRUN (*Agriotes* sp. L.)

Namen raziskave je bil oceniti entomopatogenost več vrst gliv, izoliranih iz različnih substratov iz Slovenije. Preskušali smo glive *Beauveria brongniartii* (1 izolat), *B. bassiana* (1), *Metharhizium robertsii* (1), *M. anisopliae* (2), *Purpureocillium lilacinum* (1) in *Clonostachys solani* (1). V poskusih smo konidije dodajali testnemu substratu v obliki vodne suspenzije. Substrat smo temeljito premešali, da smo dosegli končno koncentracijo $3{,}85\times10^6$ g^{-1} zračno suhega substrata. Smrtnost ličink smo opazovali tedensko v skupnem trajanju 90 dni. Krivulje smrtnosti so izkazovale linearni trend z nakloni od 0,09 do 1,62 pri glivnih tretmajih in od 0,16 do 0,74 pri kontrolah tretiranih le z 0,1% Tween 80. Smrtnost po Abbottovemu popravku je na 90. dan znašala 14,3 do 100 %. Najbolj obetavni izolati so bili *Metarhizium brunneum* izolat 1868, *M. robertsii* izolat 1880 in *B. brongniartii* izolat 1877.

Ključne besede: biotično varstvo, bioinsekticidi, škodljivec, strune

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1 INTRODUCTION

Wireworms, soil-burrowing larval stages of click beetles (Coleoptera: Elateridae), are major pests of a wide range of crops including potato in many parts of the world (Ansari *et al*., 2009; Furlan *et al*., 2010). Wireworm tunnelling in potato creates an entry point for other plant pathogens, which can cause tuber rot (Ester & Huiting, 2007). In areas highly infested with wireworms, entire batches can become unmarketable (Ansari *et al*., 2009). In Slovenia, *Agriotes ustulatus* Schall., *A. lineatus* L., *A. obscurus* L., and *A. sputator* L. live in grasslands and fields and thus have the potential to be agricultural pests (Gomboc & Milevoj, 2000).
Several attempts have been made to control wireworms and other pests from the click beetle family with biological agents (Tinline & Zacharuk, 1960; Ester & Huiting, 2007; Ansari *et al*., 2009). The experimental methodology in most of these attempts did not follow standardized protocols. Also, the mortality rates and lethal times varied considerably. Therefore, each newly discovered entomopathogenic fungi (EPF) isolate must undergo rigorous testing, in order to determine its potential as a biocontrol agent. The aim of this study was to assess the entomopathogenic potential against wireworms of several newly discovered EPF in Slovenia.

2 MATERIALS AND METHODS

2.1 Entomopathogenic fungi (EPF) isolation and culturing

The EPF were isolated from various substrates in Slovenia. The fungal strains were routinely cultured on potato dextrose agar media at 24°C in darkness. The fungal isolates tested were *Beauveria bassiana* (strain 1174), *B. brongniartii* (1877), *Metarhizium anisopliae* (1154), *M. brunneum* (1868), *M. robertsii* (1880), *Purpureocillium lilacinum* (1797) and *Clonostachys solani* f. *nigrovirens* (1828).

2.2 *Agriotes* sp. larvae collection and maintenance

Agriotes sp. larvae were collected in maize-wheat bait traps according to the description provided by Kirfman *et al*. (1986) and Chabert and Blot (1992). The traps were placed on the 14th of April, 2012 and collected on the 28th of April, 2012. The contents were hand-sorted and all living *Agriotes* sp. larvae transferred to a 15 l plastic container, containing ca. 8 kg damp soil from the original location. The container was placed in a glasshouse on the AIS premises in Ljubljana, Slovenia. Carrot and potato slices were added regularly as food and the container was watered as needed.

2.3 Soil exposure experiment

Conidial suspensions were prepared by transferring conidia to 100 ml of sterile 0.1% Tween 80 solution. A hemocytometer was used to adjust concentrations of conidia. The final concentration of EPF conidia was 3.85 x 10^6 g^{-1} substrate, which was air-dried for 48 h before the conidial suspension was added. The test substrate was a light commercial substrate, rich in organic matter. The spore infected substrate was mixed thoroughly in a large sterile plastic bag to insure homogenous conidial distribution. Thirty ml of substrate containing conidia was transferred into an individual 50 ml centrifuge tube. Into each 50 ml centrifuge tube, a single *Agriotes* sp. larva was placed. Finally, a thin slice (ca. 3 mm thick) of potato tuber was placed on top of the substrate in each tube. The tubes were loosely capped, so air could freely circulate. Fifteen test vessels were used for each treatment. 0.1% Tween was used for negative controls. The positive control was the insecticide 'Marshall' (Marshall 25 CS', based on Carbosulfan, 24.5 % active ingredient, used at a recommended concentration of 0.1 %). The larval mortality was observed on a weekly basis for a total duration of 90 days. Dead or immobile larvae lacking a coat of sporulating mycelium were

removed from the test vessels and placed in sterile 24-well plates to initiate growth of potentially present fungi. The experiment was carried out in an environmental chamber set to 20°C, 80 % relative humidity and total darkness. Potato slices and water was added to the test vessels as needed.

2.4 Data calculations and statistics

From the number of living larvae at each observation point, rate of mortality (M = 100 x living / initial larvae) was calculated. Linear and nonlinear regression was performed on these data. Mortality curve slopes from linear regression were compared against control curves. Data for nonlinear regression were first log-transformed before LT_{50} values were calculated. Additionally, Abbott's corrected mortality (ACM) was calculated for the observed mortalities at day 90. ACM is calculated as follows: ACM = 100 x ((X - Y) / X, where X represents the percent of living larvae in the untreated control sample and Y the percent of living larvae in the treated sample. Calculation using this method eliminates errors due to deaths in the control samples, which were not due to the treatments with our selected EPF (Abbot, 1925). Data presented are mean values. The experiment was performed twice independently. Statistical analysis was performed by computer software GraphPad Prism 5.00 and Microsoft Excel 2007.

3 RESULTS AND DISCUSSION

The mortality curves observed in the soil experiment exhibited a linear trend when *Purpureocillium lilacinum* (1797), *Metarhizium anisopliae* (1154), *Metarhizium brunneum* (1868) and *Metarhizium robertsii* (1880) were tested (Figure 1; Table 1). The 95 % confidence intervals of the EPF treatment slopes for *M. brunneum* (1868) and *M. robertsii* (1880) differed significantly from the slope of the control samples. The second experiment gave similar results with two notable differences: higher mortality was observed in the treatment with *Metarhizium anisopliae* 1154, and lower mortality in the treatment with *Beauveria brongniartii* (1877) (not shown).

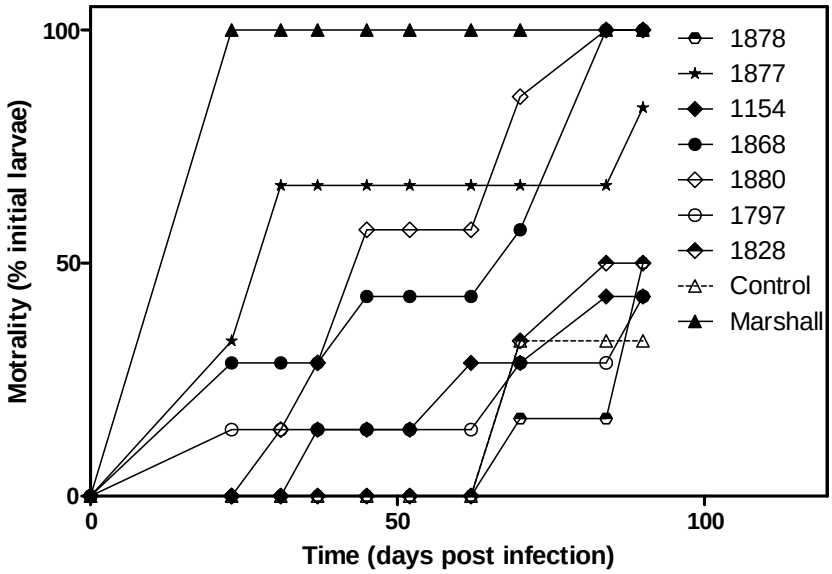

Figure 1: Mortality of *Agriotes* sp. larvae during a typical experiment. The experiments were followed for 90 days. The soil was amended with a concentration of 3.85 x 10^6 conidia g^{-1} air-dried soil at the start of the experiment. *Marshall* - a Carbosulfan-based insecticide, used as a positive control. For the list of EPF strains please see the Materials and Methods section.

The calculated 95 % confidence interval of time needed to reach a 50 % mortality (LT_{50}) based on nonlinear regression was lowest in the treatment with *B. brongniartii* (strain 1877) (14.9 – 43.4 days) and highest in the treatment *Purpureocillium lilacinum* (1797) (85.9 – 244 days), followed by control treatment (82.8 – 121 days). The positive control (treatment with Marshall 25 CS) reached a LT_{50} of less than one day (Table 1). The highest ACM (at day 90) was calculated for the treatment with *M. brunneum* strain 1868 and *M. robertsii* (both 100 %), followed by the treatment with *B. brongniartii* strain 1877 (75.0 %). The lowest ACM was calculated for the treatment with *M. anisopliae* 1154 and *P. lilacinum* 1797 (both 14.3 %) (Table 1).

Table 1: Statistical analysis of the mortality curves and Abbott's corrected mortalities (ACM) calculated for day 90. *Slope* - 95% confidence interval of the mortality slope obtained by linear regression; r^2 – goodness of fit of linear regression; LT_{50} - 95% confidence interval of time needed to reach a mortality of 50 % assessed by nonlinear sigmoid curve fitting; *Marshall* - a Carbosulfan-based insecticide used as a positive control.

Treatment	**1878**	**1877**	**1154**	**1868**	**1880**	**1797**	**1828**	**Control**	**Marshall**
Slope	0.0904-0.742	0.272-1.10	0.408-0.712	0.755-1.37	1.01-1.62	0.221-0.528	0.247-1.01	0.161-0.740	0-1.42
r^2	0.520	0.646	0.900	0.888	0.925	0.799	0.644	0.617	0.383
LT_{50} [days]	87.8-92.5	14.9-43.4	87.1-110	40.6-63.8	42.3-52.2	85.9-244	/	82.8-121	< 1
ACM [%]	25.00	75.00	14.29	100.00	100.00	14.29	25.00	0.00	100.00

The results from the treatments with *M. brunneum* 1868, *M. robertsii* 1880 and *B. brongniartii* 1877 were comparable to the insecticidal activity of a *M. anisopliae* isolate reported by Kölliker *et al*. (2011) for *A. lineatus*. Kölliker *et al*. (2011) obtained lower LT_{50} mortality rates against *A. sputator* and higher for *A. obscurus*. They hypothesized that the pathogenicity of their isolate was species specific. Our study did not allow for differentiation of toxicity assessment against different *Agriotes* sp. species as we performed our experiments with field collected larvae and did not classify them to the species level. This could be overcome by rearing our own *Agriotes* sp. by using the protocol of Kölliker *et al*. (2009) and evaluating insecticidal activity for individual species. Despite these shortcomings the isolates *Metarhizium brunneum* 1868, *M. robertsii* 1880 and *B. brongniartii* 1877 gave promising results. After successful glasshouse and field testing, they could be considered as an environmentally friendly alternative for wireworm management in conventional or organic farming systems.

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