

Virulence of *in vivo* and *in vitro* produced conidia of *Metarhizium brunneum* strains for control of wireworms

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Wireworms are the soil inhabiting larvae of click beetles and can cause severe damage to arable crops such as potatoes (*Solanum tuberosum*, L.). Several strains of the entomopathogenic fungus *Metarhizium brunneum* (Petch) are pathogenic to wireworms. In this study, three European strains of *M. brunneum* were tested in the laboratory against the most damaging wireworm species in Europe, *Agriotes lineatus* (L.), *Agriotes obscurus* (L.) and *Agriotes sputator* (L.). A Swiss strain, isolated from an *A. obscurus* cadaver, proved to be most effective, killing up to 73% of *A. lineatus* and 83% *A. obscurus* individuals, respectively. The median lethal time (LT₅₀) was 21 days post inoculation (dpi) for *A. lineatus* and 14 dpi for *A. obscurus*. The strain did not lose virulence through subsequent cultivation on artificial medium and thus seems to be suitable for mass production as a biocontrol agent for wireworm control.

1. Introduction

Wireworms, the soil dwelling larvae of click beetles (Coleoptera, Elateridae), cause significant economic damage in agricultural crops, especially cereals, legumes and potatoes (*Solanum tuberosum*, L.). Average yield losses of potatoes typically range between 5 and 25% in the US and UK, even when insecticides for wireworm control are applied (Parker and Howard, 2001). Keiser (2007) observed economically important wireworm damage on 12% of Swiss potato fields with up to 60% of the tubers damaged by the larvae. Feeding damage is also assumed to advance infection of tubers with potato pathogens such as *Rhizoctonia solani* causing "Drycore symptoms" (Keiser, 2007; Keiser et al., 2012).

Preventative control of wireworms is possible with agricultural measures, including an adjusted crop rotation and repeated tillage or by use of chemical insecticides. The efficacy of these methods, however, varies considerably (Parker and Howard, 2001; Ritter and Richter, 2013). In addition, many synthetic soil insecticides with reliable efficacy against wireworms, such as insecticides containing

organochlorine or organophosphate compounds, have been withdrawn due to human health concerns and their adverse effects on the environment (Ritter and Richter, 2013; Vernon et al., 2008). Other less persistent insecticides with fewer side effects on non-target organisms show repellent properties to wireworms, but do not effectively reduce wireworm population densities (van Herk et al., 2008). Furthermore, recent EU legislation, in particular the so called "Sustainable Use Directive" 2009/128/EC, obliges EU Member States to implement the principles of integrated pest management (IPM) with preference to be given to environmentally benign biological control agents (BCA). These alternative control options are, however, lacking for many important pests, especially for root feeding herbivores.

Entomopathogenic fungi (EPF) are considered as promising biocontrol agents in augmentative biocontrol strategies (Butt et al., 2001; Ekesi and Maniania, 2007). *Metarhizium* fungi occur in the soil (Keller et al., 2003) and contribute to the natural control of a wide range of insects (Bidochka and Small, 2005). Selected strains of *Metarhizium* spp. are already in commercial use, e.g. the strain BIPESCO 5 as a biocontrol agent against black vine weevils (*Otiorhynchus sulcatus*, Fabricius) (Ansari et al., 2006; Keller and Schweizer, 2007; Shah et al., 2007b). Based on the findings of Bischoff et al. (2009), the former type species *Metarhizium*

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anisopliae (Metschnikoff) Sorokin, which is widely known and used as BCA, was recently subdivided into four species, namely *Metarhizium pingshaense*, *M. anisopliae*, *Metarhizium robertsii* and *Metarhizium brunneum*. There is evidence that wireworms are also natural hosts of EPFs (Kabaluk et al., 2005; Kleespies et al., 2013). *Beauveria bassiana* as well as *Metarhizium* spp. have already been tested as potential biocontrol agents of wireworms with varying success (Ester and Huiting, 2007; Kabaluk et al., 2005; Kabaluk, 2007; Kölliker et al., 2011; Ladurner et al., 2009).

A key character of a BCA is its efficacy in killing the target pest. This character is usually referred to as “virulence”, when microorganisms like fungal pathogens interact with a host insect (Casadevall and Pirofski, 1999). Moreover, it is important that the fungal BCA retains a stable virulence under commercial mass production conditions. Promising fungal pathogens may fail because they degenerate as a result of successive *in vitro* subcultivation and attenuate in virulence (Butt et al., 2006).

Our aim was to evaluate the potential of three different *M. brunneum* strains as biocontrol agents against three wireworm species which are important pests in European agriculture, *Agriotes lineatus* (L.), *Agriotes obscurus* (L.) and *Agriotes sputator* (L.). We examined the virulence of the fungal strains against the target pests in bioassays. To determine the stability of the best strain, we compared the virulence of inoculum from successive subcultures with that harvested directly from wireworm cadavers. As a second stability criterion, we tested whether virulence of fungal spores deriving from one wireworm host species is similar between wireworms of the same species and wireworms of another species of the same genus.

2. Material and methods

2.1. Fungal strains and host insect species

Three strains of *M. brunneum* were compared in the virulence tests, two of which were isolated from wireworms: (1) V1002 isolated from *Agriotes* spp. in the UK (Ansari et al., 2009) and (2) ART2825 isolated from *A. obscurus* in Switzerland (Kölliker et al., 2011). The third strain used in this study, BIPESCO 5/F52, is the only *Metarhizium* strain registered for commercial use against pests in several European countries (officially deposited as ARSEF1095 or DSM3884). Although it was originally isolated from *Cydia pomonella* (L.) in Austria, it exhibits high efficacy against soil dwelling pests such as black vine weevils (Shah et al., 2007b) and was already used against wireworms in a previous study by Kabaluk (2007). Prior to use in experiments, all fungal strains were passed through Greater Wax Moth larvae (*Galleria mellonella*, L.). Conidia were harvested from the *Galleria* cadavers and single-spore isolates were produced on a selective medium: Sabouraud 2% Glucose Agar (SDA) with antibiotics (Cycloheximide: 0.05 g/l, Streptomycin sulfate: 0.6 g/l, Tetracycline: 0.05 g/l) and the fungicide Dodine (50 mg/l, Strasser et al., 1996).

The wireworm species used were *A. lineatus*, *A. obscurus* and *A. sputator* and originated from a laboratory livestock established with the method of Kölliker et al. (2009): Pots were filled with a mixture of top soil and sand (6:1, v/v) and a grass mixture (*Festuca rubra* (L.), *F. pratensis* (Huds.), *Poa pratensis* (L.), *Lolium perenne* (L.)) was sown into these pots. Adult beetles caught in the field at springtime were sorted to species level and unambiguously determined individuals were placed into these pots for oviposition. Pots were sealed with nets for several weeks to prevent escape of adults. Emerging larvae fed on grass roots and were kept in the pots for approximately half a year. Before being used in experiments, larvae were stored in boxes with peat substrate at 10 °C until they had reached at least the 7th larval stage.

2.2. Virulence of selected *M. brunneum* strains against different *Agriotes* species

Conidia were harvested from agar plates, with 0.03% (v/v) aqueous Tween[®]80 and the concentration adjusted to 10⁸ conidia/ml. Ten late instars per species of *Agriotes* were dipped into the conidia suspension for 5 s and subsequently incubated individually in unsealed small cups (surface area 16 cm²) containing 30 g of non-sterile moist (7% w/w; water content) loamy field soil at 23 °C and 65% relative humidity. A similar method was already successfully used in previous experiments (e.g. Pilz et al., 2007; Ansari et al., 2009). A carrot slice in each cup served as food source. All cups of the same treatment (ten of each species) were incubated together in a sealed plastic box, containing 30 cups. Keeping different treatments in the same boxes was avoided to prevent cross contamination. Control treatments were 0.03% (v/v) aqueous Tween[®]80 and the insecticide Ethoprophos used at the recommended rate of 60 kg/ha (9.6 mg insecticide per cup mixed with the field soil). Mortality was assessed weekly for eight weeks and cadavers were further incubated until mycosis was clearly visible on the insect's surface. Morphological characters were used for determination of the mycosis and only cadavers with clear *Metarhizium* infection were counted as killed by the treatment. The whole experiment was repeated three times and each repetition was performed with a fresh batch of inoculum and wireworms.

2.3. Virulence of strain ART2825 after passage through *Agriotes* species and repeated *in vitro* subcultivation

The stability of virulence after repeated *in vitro* subcultivation was tested with strain ART2825. Further, it was tested if the virulence of the strain is influenced by the host species which it is passed through (see Fig. 1). Four treatments were compared: Conidia for the first treatment originated from a tenth subculture of the fungus on modified SDA. Conidia for the second and third treatment were directly harvested from fresh *Agriotes* cadavers. In the second treatment, each *Agriotes* species was inoculated with spores obtained from cadavers of the same species (homologous host passage, Fig. 1). In the third treatment, *A. sputator* larvae were treated with conidia originating from *A. obscurus* cadavers (heterologous host passage). The fourth treatment with only 0.05% (v/v) aqueous Tween[®]80 solution (pH 6.7) served as control. Eight larvae each of *A. lineatus* and *A. obscurus* and 12 larvae of *A. sputator*, which is less susceptible to ART2825, were inoculated per treatment and replicate. Larvae were dipped for 5 s into suspensions of 10⁶ conidia/ml and incubated in a cup with 10 g moist peat substrate. A hundredfold lower conidia concentration was chosen, because a potential increase of *A. obscurus* mortality after inoculation with host-passed conidia would not be visible due to the high mortality that occurs already after inoculation with *in vitro* produced, non-passed conidia. Incubation and mortality assessment were the same as described in 2.2. Each treatment was replicated four times.

2.4. Statistical analyses

Effects of treatments on wireworm mortality were tested with linear mixed effect models fitted by the Laplace approximation, using the package “lme4” (version 0.999999–0; Bates et al., 2012) of the statistical software R (version 2.14.1; R Development Core Team, 2012). The status of the larvae (alive/killed by treatment) was the dependent variable and assumed to be binomially distributed.

First, the success of the treatments versus the control was analysed. Therefore, the independent variable determined if a pathogen or insecticide was present (all fungal spore treatments

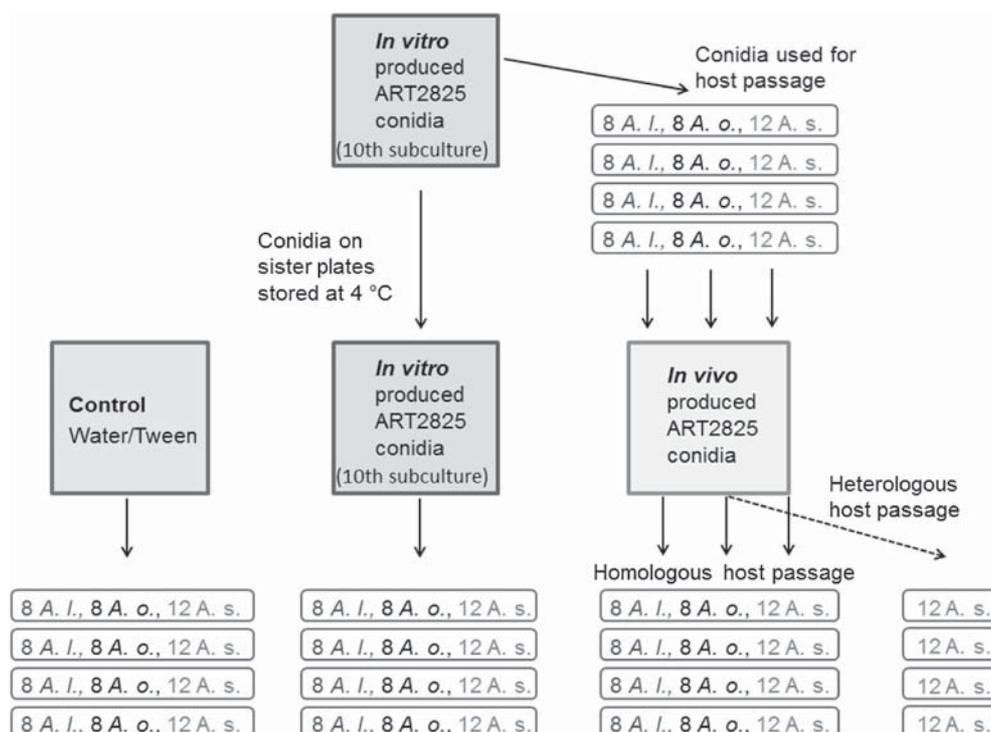


Fig. 1. Experimental set-up of host passages. *A. l.* = *A. lineatus*, *A. o.* = *A. obscurus*, *A. s.* = *A. sputator*; boxes represent one replicate.

and Ethoprophos) or not (control). Because three different *Agriotes* species were infected, the species was also included as independent variable. Cups with larvae of one treatment were incubated in the same box to avoid cross contamination between fungal treatments. Thus, cups with larvae from the same box cannot be regarded as independent and we therefore included the box identity nested within the replicate as random factor in all analyses.

In a second step, every *Agriotes* species was analysed individually to examine the effect of the different treatments on that particular species. In the bioassay comparing the virulence of strains, the independent variables were the treatment (three fungal strains, control and insecticide). In the bioassay with subcultivated and host-passed conidia, the independent variable was the origin of conidia: host-passed or from agar plate. Finally, using the subset of *A. sputator* larvae that were inoculated with host-passed conidia, the independent variable was the origin of host-passed conidia: collected after host passage through the same or a different *Agriotes* species.

The median lethal time (LT_{50}) was only calculated for treatments killing significantly more than 50% of the larvae in all three replicates within eight weeks. Kaplan–Meier survival curves for determination of LT_{50} values were computed with SigmaStat[®]3.5 (Systat Software Inc., USA).

3. Results

3.1. Virulence of selected *M. brunneum* strains against different *Agriotes* species

Strain ART2825 was the most efficient fungus strain against two of the three tested wireworm species in the virulence tests. It caused up to $83 \pm 21\%$ mortality (mean \pm standard deviation) of *A. obscurus* and $73 \pm 15\%$ of *A. lineatus* 56 days post inoculation (dpi, Fig. 2) and killed significantly more wireworms of these two species than the other two *M. brunneum* strains (in *A. lineatus* compared to

V1002: $p = 0.047$, $z = 1.986$; compared to BIPESCO 5: $p = 0.0002$, $z = 3.689$; in *A. obscurus* compared to V1002: $p = 0.0063$, $z = 2.730$; compared to BIPESCO 5: $p = 0.0001$, $z = 3.802$). Strain V1002 caused 77% mortality of *A. sputator* after 56 days, which was not significantly different from the weaker effects of BIPESCO 5 ($p = 0.496$, $z = -0.680$) and ART2825 ($p = 0.899$, $z = 0.127$). The insecticide treatment killed all larvae within 21 dpi whereas mortality in the control treatments was negligible (only one single *A. obscurus* larva killed by *Metarhizium*, no mortality among *A. lineatus* and *A. sputator*).

The LT_{50} value for wireworms treated with Ethoprophos was reached at 7 dpi in all wireworm species (Table 1). The LT_{50} value for *A. lineatus* and *A. obscurus* treated with strain ART2825 was 21 ± 5.4 dpi and 14 ± 1.3 dpi, respectively. *A. sputator* treated with strain V1002 had a LT_{50} value of 42 ± 9.3 dpi. The LT_{50} value for BIPESCO 5 treatments was not calculated, because maximum wireworm mortality was only close to 50%.

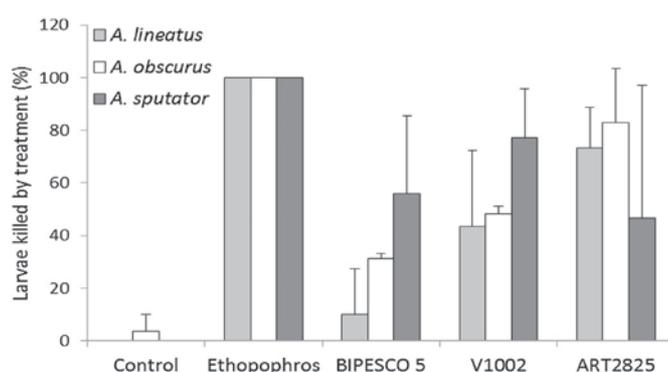


Fig. 2. Mortality of *Agriotes* larvae in % (mean + standard deviation) eight weeks after treatment with three different *M. brunneum* strains, the insecticide Ethoprophos and Tween[®]80 solution (control).

Table 1

LT₅₀ in days post inoculation (dpi, median ± standard error) and 95% upper and lower confidence intervals (CI) of *Agriotes* larvae treated with three different *M. brunneum* strains or an insecticide.

Species	Ethoprophos	BIPESCO 5	ART2825	V1002
<i>A. lineatus</i>	7 ± 0.7	n.a. ^a	21 ± 5.4	n.a. ^a
95% upper/lower CI	5.7/9.2	n.a. ^a	10.5/31.6	n.a. ^a
<i>A. obscurus</i>	7 ± 0.6	n.a. ^a	14 ± 1.3	n.a. ^a
95% upper/lower CI	5.8/8.3	n.a. ^a	11.03/16.7	n.a. ^a
<i>A. sputator</i>	7 ± 1.6	n.a. ^a	n.a. ^a	42 ± 9.3
95% upper/lower CI	3.8/10.2	n.a. ^a	n.a. ^a	23.8/26.0

^a n.a. = not available, because maximum mortality was only close to 50%.

3.2. Virulence of strain ART2825 after passage through *Agriotes* and repeated *in vitro* subcultivation

The mortality of larvae treated with fungal spores of ART2825 directly harvested from host cadavers was similar to that of larvae treated with spores produced on modified SDA after ten subcultivations (Fig. 3) (comparison between host-passed and *in vitro* produced spores in *A. lineatus*: $p = 0.327$, $z = 0.981$; in *A. obscurus*: $p = 0.617$, $z = -0.499$; in *A. sputator*: $p = 0.925$, $z = -0.094$). As in the virulence test, *A. obscurus* was significantly more susceptible towards ART2825 than the other two wireworm species (compared to *A. lineatus*: $p < 0.0001$, $z = -5.727$; compared to *A. sputator*: $p = 0.0126$, $z = 2.496$).

Mortality of *A. sputator* larvae treated with fungal spores harvested from *A. sputator* cadavers (homologous host passage) was similar ($25.3 \pm 9.5\%$) to that of larvae treated with spores from *A. obscurus* cadavers (heterologous host passage, $15.0 \pm 14.0\%$). Thus, the origin of the inoculum had no significant effect on the virulence of ART2825 ($p = 0.271$, $z = 1.101$).

4. Discussion

The wireworm species tested, *A. lineatus*, *A. obscurus* and *A. sputator*, are the most abundant and most destructive ones in Europe (reviewed in Ritter and Richter, 2013). Moreover, these species became established in parts of North America after unintentional introduction (LaGasa et al., 2006; Vernon and Pätz, 1997). Effective antagonists of these three *Agriotes* species are therefore of particular interest for development as a biocontrol agent. All three *M. brunneum* strains tested in the presented experiments were able to kill larvae of these three *Agriotes* species. Mortality rates between different combinations of *M. brunneum* isolates and wireworm species, however, varied considerably.

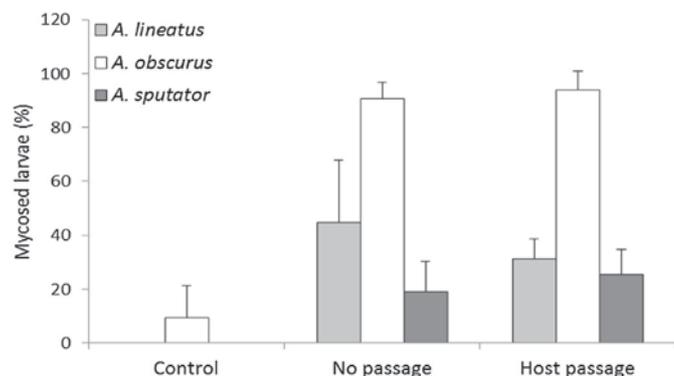


Fig. 3. Number of mycosed *Agriotes* larvae in % (mean + standard deviation) treated with *M. brunneum* strain ART2825 spores of different origin. Conidia originated either from a tenth subculture on modified SDA (no passage) or directly from wireworm cadavers (host passage).

Strain ART2825 was most virulent against *A. obscurus*, causing more than 80% mortality after eight weeks. Indeed, *A. obscurus* seems to be highly susceptible to this fungal strain since the mortality rate in the host passage test was similar to that in the virulence test, although concentration of the inoculum was 100 times lower (10^8 versus 10^6 conidia/ml). Furthermore, strain ART2825 caused 73% mortality in *A. lineatus*, but only when treated with the higher conidia concentration. A hundred times lower dose of conidia used in the host passage test led to only half of the mortality in *A. lineatus*. An analysis of the survival curves points in the same direction. In *A. obscurus*, ART 2825 reached LT₅₀ in two weeks, whereas it was three weeks in *A. lineatus*. Results are comparable to those of Kabaluk et al. (2005), who tested 14 *Metarhizium* isolates against three different wireworm species in Canada. In this study, LT₅₀ values ranged from 11 to 32 dpi, depending on *Metarhizium* strain and wireworm species. They also found *A. lineatus* to be in general more resistant to the fungal treatments than *A. obscurus*. Virulence of ART2825 against the third wireworm species tested, *A. sputator*, is difficult to assess due to high variation of the data in the virulence test. Similar to *A. lineatus*, however, mortality drops significantly when spore concentration is lower.

The *M. brunneum* strains V1002 and BIPESCO 5 were significantly less virulent against *A. lineatus* and *A. obscurus* than ART2825. These results are partially in accordance with those of Ansari et al. (2009), who found 35% mortality of BIPESCO 5 treated *A. lineatus* after three weeks of incubation. However, we were not able to confirm their results with strain V1002 (90% mortality after three weeks), which did not cause high mortality rates of *A. lineatus* larvae in our experiments. One reason may be that relatively young wireworm stages (4th and 5th instars) were used in the study of Ansari et al. (2009), whereas older and presumably less sensitive instars (Butt and Goettel, 2000; Feng et al., 1985) were used in our virulence tests (mainly 7th and 8th instars). Another reason may be that hosts from different eco-geographic regions may not be equally sensitive to a single *Metarhizium* strain (Keller et al., 1999).

The stability of a fungal strain under commercial mass production conditions is a decisive criterion for its success as a BCA. A decline of virulence due to subsequent cultivation on artificial media, also known as attenuation, is a common phenomenon in EPF (reviewed in Butt et al. (2006); Ansari and Butt (2011)). The host passage test was included in this study to estimate the stability of ART 2825 virulence under *in vivo* and *in vitro* cultivation conditions. Results show that inoculum produced on artificial medium for ten subcultivations caused similar mortality rates in wireworms as inoculum directly harvested from host cadavers. In addition, we did not detect any sector formation of the mycelium on agar plates as described in Shah et al., 2007a. Likewise, germination rates of spores varied considerably with medium type or storage conditions, but not with increasing numbers of *in vitro* subcultivations (data not shown).

Attenuated fungal strains may regain virulence by a passage through a natural host (Butt et al., 2006). Besides an effect of the host passage per se, we also expected the virulence of the fungal strain to depend on the type of host passage. Fargues and Robert (1983) demonstrated an increased virulence of *M. anisopliae* against larvae of *Oryctes* sp. and *Cetonia* sp. after one homologous host passage (conidia for experimental treatments harvested from cadavers of the same grub species), while infection after a heterologous host passage (conidia for treatment of *Oryctes* sp. produced on *Cetonia* sp. and vice versa) was not successful. Reason may be that host passages constitute selection events, with the host acting as a genetic bottleneck (Scully and Bidochka, 2006). In the current experiment, it was possible to infect *A. sputator* larvae with conidia obtained from *A. obscurus* cadavers. Moreover, inoculum obtained from a single homologous host passage exhibited similar virulence

to that from a heterologous host passage. One explanation may be that a passage through a congeneric host does not narrow the genetic bottleneck in the same way as is the case with the two scarabaeids from different subfamilies used in the experiment of Fargues and Robert (1983). On the other hand, a similar virulence of the fungal inoculum after homologous and heterologous host passages may also demonstrate the genetic stability of the tested strain. Hence, results of the host passage test corroborate those from the test with *in vivo* and *in vitro* produced conidia shown before.

Besides *Metarhizium* spp., another entomopathogenic fungus, *B. bassiana*, was found to be a natural antagonist of wireworms (Kleespies et al., 2013). Investigations on its efficacy gave contradicting results. Ladurner et al. (2009) and Ester and Huiting (2007) reported positive effects of *B. bassiana* strain ATC74040 in the field in Italy and the UK regarding tuber damage, while Kölliker et al. (2011) was not able to infect wireworms in the lab with the same strain. One explanation could be that Kölliker et al. (2011) worked with species-identified *A. lineatus*, *A. obscurus* and *A. sputator* in the laboratory, while in the other studies naturally occurring field populations of wireworms were used without accurate species determination. Differences between wireworm species' susceptibility was clearly shown in the present experiments and may even exist between populations of the same species from different geographic origins (Keller et al., 1999).

5. Conclusion

M. brunneum strain ART2825 seems to be promising for development as a biocontrol agent against wireworms. We found high virulence and low LT₅₀ values for *A. lineatus* and *A. obscurus*, two major pest species in European arable crops. Beyond that, virulence seems to be stable in this fungal strain, since we did not detect any signs of attenuation after repeated *in vitro* cultivation, and virulence was similar after homologous and heterologous host passage.

The presented laboratory assays are the first steps in the development of a biocontrol strategy against wireworms in Europe, based on the use of an entomopathogenic fungus. Further studies will include assessment of potential synergistic interactions between *M. brunneum* ART2825 and other antagonists like entomopathogenic nematodes or repellents (Ansari et al., 2008, 2004; Shah et al., 2008) as well as testing of innovative formulation technologies to improve the fungus' shelf life and viability in the field.

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