TÜBİTAK

Turkish Journal of Zoology

http://journals.tubitak.gov.tr/zoology/

Research Article

Turk J Zool
(2021) 45: 395-407
© TÜBİTAK
doi:10.3906/zoo-2103-47

Susceptibility of *Agriotes* spp. larvae (Coleoptera: Elateridae) to stress-and-kill strategies using spinosad and the entomopathogenic fungus *Metarhizium brunneum*

Pierre-Antoine BOURDON[1,2,*], Ian BAXTER[3], Giselher GRABENWEGER[4], Tariq Mahmud BUTT[1]

[1]Department of Biosciences, College of Science, Swansea University, Swansea, UK
[2]Certis Europe BV, Utrecht, Netherlands
[3]Biobest Group NV, Westerlo, Belgium
[4]Department of Plant Protection, Agroscope, Zurich, Switzerland

Received: 30.03.2021 • **Accepted/Published Online:** 12.06.2021 • **Final Version:** 31.08.2021

Abstract: Wireworms (*Agriotes obscurus, Agriotes lineatus, Agriotes sordidus*) are major pests of arable crops. Damage caused by these subterranean pests has increased following the withdrawal of many traditional synthetic insecticides. Wireworms are susceptible to entomopathogenic fungi belonging to the genus *Metarhizium*. The aim of this study was to determine if exposure of different wireworm species to sublethal doses of the insecticide spinosad altered their susceptibility to different strains of *Metarhizium brunneum*. Of the three wireworm species studied, *A. obscurus* was the most susceptible species to the isolates of *M. brunneum* tested, while *A. lineatus* and *A. sordidus* were not affected by the entomopathogen on its own. However, combination of spinosad and *M. brunneum* could cause significant mortality and, depending on the treatment combination, some interactions were determined as being either antagonistic, additive or synergistic. The efficacy of the stress-and-kill strategy was dependent on the fungal strain, insect species, dose of stressing agent and time. *M. brunneum* strain F52 and ART2825 combined with low doses of spinosad resulted in a synergistic reaction. Scanning electron microscopy shows that without spinosad, poor germination was seen on the wireworm cuticle, indicating that it could be fungistatic. However, the increased susceptibility of the wireworms to the stress-and-kill seem to indicate physiological changes due to spinosad, facilitating germination and penetration of the fungi. Our results show that stress-and-kill can provide wireworm control; yet, the right combination between the fungi, stressing agent and insect species is mandatory.

Key words: *Agriotes*, microbial control, pest management, *Metarhizium*, wireworm, stress-and-kill

1. Introduction

Wireworms are the subterranean larval stage of click beetles (Coleoptera: Elateridae) which include species such as *Agriotes sordidus* (Illiger), *Agriotes lineatus* (L.), *Agriotes sputator* (L.) and *Agriotes obscurus* (L.) (Thibord et al., 2017). The relatively long lived larvae feed on the roots of a wide range of plants causing extensive damage to pasture and food crops (Hicks and Blackshaw, 2008; Barsics et al., 2013; Kergunteuil et al., 2016). Wireworms are responsible for 5% loss of maize production in France with some regions at risk of losing over 36% of their production (Thibord, 2017). In the UK and Canada, wireworms rank among the major pests of potatoes and cereals (Parker and Howard, 2001; Kabaluk and Ericsson, 2007a). The resurgence of wireworms since the 1990's is mostly due to the withdrawal of many insecticides as well as the shift from conventional chemical pest control to integrated pest management (IPM). Many chemical pesticides used for wireworm control are either severely restricted in their use or are slow-acting (Van Herk et al., 2008; Butler, 2018). This is placing considerable pressure on farmers who are trapped between the withdrawal of effective chemical pesticides and the lack of safe, efficient, alternatives. Studies to date show that strains of entomopathogenic fungi (EPF) belonging to the genera *Metarhizium* and *Beauveria* are pathogenic to wireworms, but they are often slow-acting and cause highly variable mortality, ranging between 10% and 83% (Kabaluk et al., 2007; Ansari et al., 2009; Razinger et al., 2013, 2018; Eckard et al., 2014; Rogge et al., 2017).

There is increasing evidence that EPF efficacy can be enhanced when used alongside certain botanicals and insecticides (Shah et al., 2007, 2008; Paula et al., 2011). In some cases the combined use of these agents is synergistic, allowing the application of one or both agents to be reduced and thus offering considerable savings for growers (Purwar and Sachan, 2006; Morales-Rodriguez and Peck, 2009). Synergies or enhanced control has been observed using low dose imidacloprid with *Beauveria bassiana* (Bals.-Criv.)

* Correspondence: 967179@swansea.ac.uk

Vuill. against the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Anderson et al., 1989; Furlong and Groden, 2001) and the root weevil *Diaprepes abbreviates* (L.) (Quintela and McCoy, 1997, 1998a, 1998b). Synergies have also been observed between *Metarhizium anisopliae* (Metch) Sorok. *sensu lato* and imidacloprid against the burrower bug (*Cyrtomenus bergi* Froeschner) (Jaramillo et al., 2005). Not all combinations work synergistically; for example, *B. bassiana* and *Metarhizium brunneum* (Petch) (F52) work synergistically with clothianidin against the white grub (*Popillia japonica* Newman), while *M. anisopliae* NYSAES had an additive or antagonistic activity with clothianidin depending on the dose used (Morales-Rodriguez and Peck, 2009). Altogether, these studies show that the synergy is dependent on the fungal strain, chemical pesticide and target pest.

This study was aimed at understanding the specificity of stress-and-kill strategy against three different species of *Agriotes* larvae. For that purpose, we combined three different *Metarhizium* strains (one highly virulent, one moderately virulent and one weakly virulent) with three different doses of spinosad to understand how the EPF and insecticides interact and influence the mortality of the wireworm species. Cryo scanning electron microscopy analyses were performed to analyze the attachment and germination of the EPF spores on wireworm cuticle.

2. Materials and methods

2.1. Source and maintenance of wireworm

Two populations of *A. lineatus* and *A. obscurus* were collected in the cantons of Zurich and Berne, Switzerland, and provided by Agroscope (Switzerland), while *A. sordidus*, were collected from a field in the South West of France. Another *A. lineatus* population was collected from Brittany (France) (Thibord et al., 2017). Most collections were done in the spring or early summer. Wireworms were maintained in 1-L pots filled with medium compost with clay (Klasmann-Deilmann, Germany) and fed weekly with slices of potatoes. The insects were kept at 23 °C, 65% RH, 16:8 L:D photoperiod. The different species were kept in different insect cages to avoid mixing. The insects used in assays were all late instars, of a similar size, yet, their weight was highly variable: *A. lineatus* (15.9 ± 2.6 mm, 26.38 ± 9.8 mg), *A. obscurus* (17.4 ± 2.7 mm, 28.21 ± 10.23 mg), *A. sordidus* (16.7 ± 3.2 mm, 29.55 ± 13.05 mg). The larvae were kept in pots at least for two weeks prior to trials. Healthy larvae were selected for the trials.

2.2. Fungal strains and preparation of inoculum

Three *M. brunneum* strains ART2825, F52 and 16P were used for these trials. *M. brunneum* 16P was isolated from a dead *Agriotes* larva during previous trials and was moderately virulent against a mixed *A. lineatus*/*A. obscurus* population. The strain ART2825 was isolated by Agroscope from an infected *A. obscurus* in Zurich (Switzerland), and it has shown high virulence towards larvae of *A. obscurus* and *A. lineatus* in a previous study (Eckard et al., 2014). The strain F52 is a commercially available strain and registered as biocontrol organism in Europe and many other countries. F52 is a weakly virulent strain towards wireworms (Ansari et al., 2009; Eckard et al., 2014); however, it is widely used for research (Antwi et al., 2017; Larroudé and Thibord, 2017; Ensafi et al., 2018). The culture used for the experiments was obtained from the Swansea University collection.

Conidia from the three *M. brunneum* strains were harvested from 15-day old cultures grown on Sabouraud dextrose agar (SDA) at 25 ± 1 °C. The conidia were suspended in 0.03% Tween 80 solution, quantified using a Neubauer haemocytometer. These spore suspensions were used to measure conidia viability. An aliquot of 100 µL of the fungal suspension was spread on SDA and three 22-mm square coverslips were placed on top. The rate of germination was assessed under each coverslip 16 h postinoculation at 25 °C (Goettel and Inglis, 1997). Two plates were prepared for each suspension and three counts were performed per plate.

2.3. Compatibility between spinosad and the Metarhizium strains

Sabouraud dextrose agar was prepared and three doses of spinosad (Success 4, Certis Europe) (3.2 ppm, 6.4 ppm or 12.8 ppm) were added to the SDA after autoclaving, without anything being added to control plates. For each fungal strain, 100 µL of a suspension (10^7 conidia/mL) was spread on the agar surface. Spore counts and spore germination tests were performed for each combination as previously described. Conidial viability of 80% or more was obtained for all combinations.

2.4. Stress-and-kill bioassay on different wireworm species

M. brunneum Met52, 16P and ART2825 were used for the stress-and-kill bioassays following a modified version of the Ericsson et al. (2007) protocol. Briefly, 1 mL of the fungal spore suspension was mixed into 30 g of autoclaved sand in a 9-cm Petri dish. The final concentration of the inoculum was 1×10^6 viable conidia per gram of sand (Ericsson et al., 2007). Soon after, 1 mL of different concentrations of spinosad were added to reach a final concentration of 0, 3.2, 6.4 or 12.8 ppm (AI). The fungi and spinosad were also tested on their own, to assess the mortality of the different treatments. For the control treatment, 1 mL of 0.03% Aq. Tween was mixed in the sand. The final sand moisture was adjusted to 10% (wt:wt) with tap water. One wireworm (*A. lineatus, A. obscurus* or *A. sordidus*) was added in each Petri dish. The larvae were fed twice per week with slice of potatoes. There were five replicates per combination and the whole study was repeated twice or thrice depending on

the availability of wireworm larvae. The plates were kept in a Sanyo versatile environmental test chamber (MLR-351H, Sanyo Electric Co., Ltd, Osaka, Japan) at 20 °C, 80% RH. Mortality and fungal sporulation were checked every 3 days for 24 days. *M. brunneum* 16P could not be tested on *A. obscurus* due to a lack of larvae.

2.5. Cryo scanning electron microscopy (cryo-SEM)

Conidia attachment and germination was observed with a cryo scanning electron microscope (Hitachi S-4800). *A. obscurus* larvae were used for this experiment as they were the most susceptible to *Metarhizium*. The larvae were immerged in a 3×10^7 conidia/mL suspension for 10 s, before being placed in a sealed Petri dish with a wet filter paper to keep the humidity. No soil was present in the Petri dish, to avoid residue during the observation. The larvae were incubated at 25 °C for 12 to 72 h. *A. obscurus* larvae with sporulating *M. brunneum* ART2825 (22 days after inoculation) were also observed to look at later infection stages. For the observation, the larvae were glued, using cryogenic glue, to a SEM sample holder, before immersing in a nitrogen slush for rapid freezing. Specimens were transferred to a cryogenic preparation chamber where the temperature was elevated to –90 °C for 10 min to remove surface ice, then returned at –130 °C. Larvae were examined intact or fractured, using a rotating knife to allow imaging of the haemocoel. Specimens were coated with platinum for about 5 min before examination in the cryo SEM (Butt et al., 2013).

2.6. Statistical analysis

The analyses were performed on R version 3.6.2. Kruskal–Wallis tests were performed to compare the germination of the *Metarhizium* strains on SDA with spinosad. Generalized linear model analysis and multiple comparisons of mean (package multcomp, function glht) were done to compare the germination rates of the fungi on SDA with or without spinosad. Quasi Poisson distribution was used due to the overdispersion of the data.

The expected mortality for the stress-and-kill treatment was calculated for each time point using the formula:

$$E = O_{spin} + O_{Met}(1-O_{Spin}),$$

where E is the expected mortality, O_{spin} the observed mortality of spinosad, and O_{Met} the observed mortality of one *Metarhizium* strain. This formula was previously used for stress-and-kill trials to measure the effect of the cotreatment (Trisyono and Whalon, 1999; Hummelbrunner and Isman, 2001; Ericsson et al., 2007). A chi-square test compared the expected mortality and observed mortality of the cotreatment, using the formula:

$$\chi^2 = \{(O_{co} - E)^2\}/E,$$

where O_{co} is the observed mortality of the cotreatment. The chi-square value was compared with the chi-square distribution table using df = 1 and $\alpha = 0.05$; chi-square value higher than 3.84 indicate a significant difference either synergistic or antagonistic. Additive effect was defined as an observed mortality higher than the expected mortality, but with a chi-square <3.84 (Trisyono and Whalon, 1999; Hummelbrunner and Isman, 2001). The expected mortality and chi-square test were calculated using Microsoft Excel.

Survival analysis followed by multiple comparisons using a log-rank test (package: Survminer) were performed on R to compare the mortality of the different treatment with each other.

3. Results

3.1. Compatibility between spinosad and the Metarhizium strains

Spinosad doses had no impact on the germination rate of the *M. brunneum* ART2825 ($\chi^2 = 1.774$, df = 3, p = 0.6206), Met52 ($\chi^2 = 0.90918$, df = 3, p = 0.8232) and *M. brunneum* 16P ($\chi^2 = 0.91879$, df = 3, p = 0.8209).

The lowest and highest doses of spinosad significantly reduced the spore production of *M. brunneum* ART2825 (p = 0.0123 and 0.0310, respectively). Spinosad had no impact on Met52 spore production (p > 0.0968). *M. brunneum* 16P spore production significantly increased with the highest dose of spinosad compared to the two lowest doses (p <0.001). The 6.4 ppm dose of spinosad significantly reduced the number of *M. brunneum* 16P spores produced compared to the control (p = 0.0150).

3.2. Stress-and-kill bioassay with different Metarhizium species

Interactions between *Metarhizium* strains (ART2825, 16P, Met52) and spinosad included neutral (i.e. no interaction), antagonistic, additive and synergistic responses. The type of interaction was dependent on the strain, dose, wireworm species and time of assessment (Tables 1–4).

The mortality caused by spinosad alone was low and not significantly different from the no treatment control for the three wireworm species, confirming that the doses were sublethal (Tables 1–4).

M. brunneum ART2825 on its own was highly virulent to *A. obscurus* and caused 87% mortality 24 days posttreatment (Table 1). Mortality in the presence of ART2825 plus spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 93%, 80% and 73%, respectively (Table 1). ART2825 was avirulent to *A. lineatus* and *A. sordidus* but weak synergy was observed when used with spinosad. The synergies were dependent upon time and dose, with reduced synergy being observed at the highest dose of spinosad (Table 1). The mortality at 24 days posttreatment for *A. lineatus* exposed to ART2825 and spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 40%, 30% and 30%, respectively. The mortality at 24 days posttreatment for *A. sordidus* using the combination of ART2825 and spinosad at 3.2 ppm,

Table 1. Observed and expected mortality of *Agriotes obscurus*, *A. lineatus* and *A. sordidus* exposed to *Metarhizium brunneum* ART2825 and various doses of spinosad, on each sampling date.

Treatment		Time in days	Mortality (%)					
					Combination			
Species	Spinosad		ART2825	Spinosad	Observed	Expected	χ^{2a}	Effect
A. obscurus	3.2 ppm	6	7	0	13	7	6.68*	Synergistic
		12	33	7	47	38	2.09	Additive
		18	60	13	93	65	12.00*	Synergistic
		24	87	13	93	88	0.27	Additive
	6.4 ppm	6	7	0	7	7	0	
		12	33	0	40	33	1.34	Additive
		18	60	7	60	63	0.11	
		24	87	7	80	88	0.65	
	12.8 ppm	6	7	7	0	13	12.88*	Antagonistic
		12	33	7	27	38	3.27	
		18	60	7	60	63	0.11	
		24	87	13	73	88	2.58	
A. lineatus	3.2 ppm	6	0	10	10	10	0	
		12	0	10	10	10	0	
		18	0	20	10	20	5.00*	Antagonistic
		24	0	20	40	20	20.00*	Synergistic
	6.4 ppm	6	0	0	10	0	-	
		12	0	0	10	0	-	
		18	0	0	20	0	-	
		24	0	20	30	20	5.00*	Synergistic
	12.8 ppm	6	0	0	0	0	-	
		12	0	20	10	20	5.00*	Antagonistic
		18	0	20	30	20	5.00*	Synergistic
		24	0	40	30	40	2.50	
A. sordidus	3.2 ppm	6	0	0	0	0	-	
		12	0	0	0	0	-	
		18	0	0	18	0	-	
		24	0	22	36	22	9.00*	Synergistic
	6.4 ppm	6	0	10	0	10	10.00*	Antagonistic
		12	0	10	0	10	10.00*	Antagonistic
		18	0	10	20	10	10.00*	Synergistic
		24	0	20	20	20	0	
	12.8 ppm	6	0	0	0	0	-	
		12	0	10	0	10	10.00*	Antagonistic
		18	0	10	0	10	10.00*	Antagonistic
		24	0	20	20	20	0	

[a] Chi-square comparison that exceeds 3.84, with df = 1 and $\alpha = 0.05$, is considered significantly different and is shown by an asterisk (*).

Table 2. Observed and expected mortality of *Agriotes. lineatus* and *A. sordidus* exposed to *Metarhizium. brunneum* 16P and various doses of spinosad, on each sampling date.

Treatment		Time in days	Mortality (%)					
					Combination			
Species	Spinosad		16P	Spinosad	Observed	Expected	χ^{2a}	Effect
A. lineatus	3.2 ppm	6	0	10	0	10	10.00*	Antagonistic
		12	0	10	0	10	10.00*	Antagonistic
		18	0	20	0	20	20.00*	Antagonistic
		24	0	20	10	20	5.00*	Antagonistic
	6.4 ppm	6	0	0	0	0	-	
		12	0	0	0	0	-	
		18	0	0	0	0	-	
		24	0	20	20	20	0	
	12.8 ppm	6	0	0	0	0	-	
		12	0	20	0	20	20.00*	Antagonistic
		18	0	20	0	20	20.00*	Antagonistic
		24	0	40	40	40	0	
A. sordidus	3.2 ppm	6	0	0	0	0	-	
		12	0	0	0	0	-	
		18	0	0	0	0	-	
		24	0	22	20	22	0.22	
	6.4 ppm	6	0	10	0	10	10.00*	Antagonistic
		12	0	10	0	10	10.00*	Antagonistic
		18	0	10	10	10	0	
		24	0	20	60	20	80.00*	Synergistic
	12.8 ppm	6	0	0	0	0	-	
		12	0	10	10	10	0	
		18	0	10	30	10	40.00*	Synergistic
		24	0	20	50	20	45.00*	Synergistic

[a] Chi-square comparison that exceeds 3.84, with df = 1 and $\alpha = 0.05$, is considered significantly different and is shown by an asterisk (*).

6.4 ppm, 12.8 ppm was 36%, 20% and 20%, respectively (Table 1). *A. obscurus* were significantly more susceptible to ART2825 (with or without spinosad) compare to *A. lineatus* or *A. sordidus* (Table 5).

M. brunneum 16P was avirulent on its own against *A. lineatus* and *A. sordidus* (Table 2). The mortality at 24 days posttreatment for *A. lineatus* using the combination of *M. brunneum* 16P and spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 10%, 20% and 40%, respectively. The interaction was antagonistic with a delayed mortality compared to the mortality of spinosad alone (Table 2). The mortality at 24 days posttreatment for *A. sordidus* using *M. brunneum* 16P plus spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 20%, 60% and 50%, respectively. With the lowest dose of spinosad, no interactions were noted; while, with the highest dose, the interaction was synergistic (Table 2). The interaction of *M. brunneum* 16P with 6.4 ppm of spinosad was synergistic after 24 days; however, compared to spinosad alone, the mortality was delayed (Table 2). *M. brunneum* 16P was not tested against *A. obscurus* due to a limited number of larvae. No significant differences were found between the wireworm species susceptibility to *M. brunneum* 16P (data not shown).

The reference strain, *M. brunneum* F52 on its own was moderately virulent against *A. obscurus* with 40% mortality 24 days posttreatment, but was avirulent against *A. lineatus* and *A. sordidus* (Table 3). Mortality of *A. obscurus* in presence of F52 and spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 20%, 40% and 33% (Table 3). These interactions were antagonistic (Table 3). Mortality at 24

Table 3. Observed and expected mortality of *Agriotes. obscurus*, *A. lineatus* and *A. sordidus* exposed to *Metarhizium. brunneum* F52 and various doses of spinosad, on each sampling date.

Treatment		Time in days	Mortality (%)					
					Combination			
Species	Spinosad		F52	Spinosad	Observed	Expected	χ^{2a}	Effect
A. obscurus	3.2 ppm	6	0	0	0	0	-	
		12	13	7	13	19	1.75	
		18	20	13	20	31	3.71	
		24	40	13	20	48	16.33*	Antagonistic
	6.4 ppm	6	0	0	0	0	-	
		12	13	0	7	13	3.34	
		18	20	7	20	25	1.12	
		24	40	7	40	44	0.36	
	12.8 ppm	6	0	7	0	7	6.66*	Antagonistic
		12	13	7	13	19	1.74	
		18	20	7	27	25	0.07	
		24	40	13	33	48	4.48*	Antagonistic
A. lineatus	3.2 ppm	6	0	10	0	10	10.00*	Antagonistic
		12	0	10	0	10	10.00*	Antagonistic
		18	0	20	40	20	20.00*	Synergistic
		24	0	20	50	20	45.00*	Synergistic
	6.4 ppm	6	0	0	0	0	-	
		12	0	0	20	0	-	
		18	0	0	30	0	-	
		24	0	20	50	20	45.00*	Synergistic
	12.8 ppm	6	0	0	0	0	-	
		12	0	20	10	20	5.00*	Antagonistic
		18	0	20	20	20	0.	
		24	0	40	30	40	2.50	
A. sordidus	3.2 ppm	6	0	0	0	0	-	
		12	0	0	30	0	-	
		18	0	0	50	0	-	
		24	0	22	50	22	34.73*	Synergistic
	6.4 ppm	6	0	10	0	10	10.00*	Antagonistic
		12	0	10	0	10	10.00*	Antagonistic
		18	0	10	10	10	0	
		24	0	20	30	20	5.00*	Synergistic
	12.8 ppm	6	0	0	0	0	-	
		12	0	10	30	10	40.00*	Synergistic
		18	0	10	80	10	490.00*	Synergistic
		24	0	20	90	20	245.00*	Synergistic

[a] Chi-square comparison that exceeds 3.84, with df = 1 and $\alpha = 0.05$, is considered significantly different and is shown by an asterisk (*).

Table 4. Mortality at 24 days after treatment and percentage of dead insect from mycosis with subsequent fungal outgrowth for *A. lineatus*, *A. sordidus* and *A. obscurus* exposed to different stress-and-kill combinations.

		Agriotes lineatus				*Agriotes sordidus*				*Agriotes obscurus*		
		F52	16P	ART2825	No EPF (Control)	F52	16P	ART2825	No EPF (Control)	F52	ART2825	No EPF (Control)
Mortality	No spinosad (control)	0%	0%	0%	0%	0%	0%	0%	0%	40%	87%	13%
	3.2 ppm spinosad	50%	10%	40%	20%	50%	20%	36%	20%	20%	93%	13%
	6.4 ppm spinosad	50%	20%	30%	20%	30%	60%	20%	30%	40%	80%	7%
	12.8 ppm spinosad	30%	40%	30%	40%	90%	50%	20%	20%	33%	73%	13%
Percentage of dead larvae with fungal growth	No spinosad (control)	0%	0%	0%	0%	0%	0%	0%	0%	67%	92%	100%
	3.2 ppm spinosad	60%	0%	0%	0%	0%	50%	0%	0%	33%	86%	50%
	6.4 ppm spinosad	20%	0%	0%	0%	0%	0%	0%	33%	17%	75%	100%
	12.8 ppm spinosad	33%	0%	0%	0%	33%	40%	0%	0%	40%	91%	50%

days posttreatment for *A. lineatus* using F52 plus spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 50%, 50% and 30%, respectively. The mortality was delayed 15 days with the lowest dose of spinosad; however, the mortality increased significantly after, resulting in synergistic interaction (Table 3). With the 6.4 ppm spinosad dose, the interaction was synergistic with an increase in the mortality and the speed of kill; however, with the highest dose, no interaction was noted (Table 3). The mortality 24 days posttreatment for *A. sordidus* using F52 in combination with spinosad at 3.2 ppm, 6.4 ppm, 12.8 ppm was 50%, 30% and 90%, respectively. The highest dose of spinosad significantly increased the susceptibility of *A. sordidus* compared to the medium dose ($p = 0.020$) and the F52 without spinosad ($p = 0.002$). No differences were found between the susceptibility of the *Agriotes* species, except compared to *A. sordidus* exposed to F52 and the highest dose of spinosad (Table 6).

Wireworms had different susceptibilities to *Metarhizium* infection. *A. obscurus* was the most susceptible species (Table 4), while *A. lineatus* and *A. sordidus* were resistant to the three *Metarhizium* strains when used without spinosad (Table 4). No clear relationship was noted between the increased dose of spinosad and the mortality (Tables 5 and 6).

Emergence of *Metarhizium* from dead insects was dependent on the fungal strain, wireworm species and spinosad treatment (Table 4). *A. obscurus* was the most susceptible to *Metarhizium* infection with 67% and 92% of dead larvae with fungal growth when exposed to Met52 and ART2825 without spinosad. However, the addition of spinosad appeared to be antagonistic with the fungal growth. Fungal growth on *A. lineatus* was only seen when the larvae were exposed to Met52 with spinosad (Table 4). While both Met52 and *M. brunneum* 16P grew on *A. sordidus* (Table 4). The fungal growth seen on *A. obscurus* control larvae came from latent field infection.

3.3. Light Microscopy and Cryo scanning electron microscopy

Close examination of the wireworm surface using cryo SEM showed that conidia adhered readily to most parts of the cuticle. The conidia often appeared in clumps and were particularly concentrated on the legs, intersegmental membranes and mouthparts (Figures 1A and 1B). Conidia were also observed in the spiracles and around the setae (Figure 1C). There was a general tendency for the conidia to collect in grooves, or depressions on the insect surface, as well as on the head. Germination was poor with most spores still not having germinated 72 h posttreatment. Of the few germlings observed, these typically produced appressoria at the end of short germ tubes. At 72 hours posttreatment, no sign of colonization of the haemocoel was seen. Even larvae covered with ART2825 fungal growth 22 days posttreatment, had a poor colonization of the hemocoel, with few hyphae found in the fat body, tracheoles, midgut and integument (Figures 2A and 2B), while most of the fungal growth appeared to be external growth. The intersegmental membrane was the prime area of fungal growth; yet, secondary infection was seen on the sclerites (Figure 3).

Melanotic spots were observed on dead larvae in most segments with some patches being quite extensive (Figures 1D and 3B). Only a quarter of the dead larvae had signs of fungal growth (Table 4), while most larvae turned black quickly after death.

Table 5. Comparison of the survival curves of the three wireworm species exposed to M. brunneum ART2825.

Species		A. lineatus				A. obscurus				A. sordidus		
	Treatment	2825	2825 + 3.2ppm	2825 + 6.4ppm	2825 + 12.8ppm	2825	2825 + 3.2ppm	2825 + 6.4ppm	2825 + 12.8ppm	2825	2825 + 3.2ppm	2825 + 6.4ppm
A. lineatus	2825 + 3.2ppm	0.235										
	2825 + 6.4ppm	0.631	0.600									
	2825 + 12.8ppm	0.409	0.827	0.722								
A. obscurus	2825	0.003*	0.022*	0.011*	0.027*							
	2825 + 3.2ppm	0.002*	0.004*	0.003*	0.004*	0.109						
	2825 + 6.4ppm	0.007*	0.068	0.003*	0.044*	0.783	0.149					
	2825 + 12.8ppm	0.013*	0.109	0.004*	0.085	0.657	0.065	0.800				
A. sordidus	2825	0.433	0.060	0.215	0.120	0.001*	0.001*	0.002*	0.004*			
	2825 + 3.2ppm	0.297	0.949	0.631	0.935	0.016*	0.002*	0.041*	0.068	0.075		
	2825 + 6.4ppm	0.684	0.524	0.940	0.631	0.006*	0.001*	0.013*	0.023*	0.231	0.563	
	2825 + 12.8ppm	1.000	0.215	0.611	0.362	0.002*	0.001*	0.004*	0.008*	0.407	0.241	0.634

The asterisks indicate significant differences between the survival curves.

Table 6. Comparison of the survival curves of the three wireworm species exposed to M. brunneum F52.

Species		A. lineatus				A. obscurus				A. sordidus		
	Treatment	F52	F52 + 3.2ppm	F52 + 6.4ppm	F52 + 12.8ppm	F52	F52 + 3.2ppm	F52 + 6.4ppm	F52 + 12.8ppm	F52	F52 + 3.2ppm	F52 + 6.4ppm
A. lineatus	F52 + 3.2ppm	0.055										
	F52 + 6.4ppm	0.055	0.930									
	F52 + 12.8ppm	0.171	0.713	0.620								
A. obscurus	F52	0.093	0.820	0.820	0.810							
	F52 + 3.2ppm	0.320	0.450	0.427	0.820	0.608						
	F52 + 6.4ppm	0.093	0.810	0.805	0.820	0.963	0.620					
	F52 + 12.8ppm	0.146	0.754	0.719	0.886	0.854	0.722	0.886				
A. sordidus	F52	1.000	0.055	0.055	0.171	0.093	0.302	0.09	0.146			
	F52 + 3.2ppm	0.055	0.820	0.854	0.557	0.713	0.303	0.620	0.608	0.055		
	F52 + 6.4ppm	0.171	0.545	0.551	0.928	0.722	0.854	0.722	0.820	0.171	0.450	
	F52 + 12.8ppm	0.002*	0.667	0.128	0.047*	0.471	0.019*	0.027*	0.046*	0.002*	0.303	0.020*

The asterisks indicate significant differences between the survival curves.

4. Discussion

Many wireworms species are considered agricultural pests (Barsics et al., 2013). One or more species or genus can be present in a given area (Thibord et al., 2017). However, the susceptibility of *Agriotes* species is variable depending of the EPF strain used. Our study confirms the susceptibility of *A. obscurus* to the EPF; however, *A. lineatus* susceptibility was lower than what Eckard et al. (2014) found. Our results indicated that stress-and-kill strategies do not requires highly virulent EPF strains, as avirulent and low virulent strains could interact synergistically with spinosad to increase the mortality (Tables 1–3). Yet, the efficacy of the synergies was dependent on the combination, and our results highlighted the importance of measuring the synergy/antagonist effect at multiple dates as the effect was not constant throughout the trials (Ericsson et al., 2007). None of the stress-and-kill strategies tested in this paper resulted in high mortality of the three *Agriotes* species. Yet,

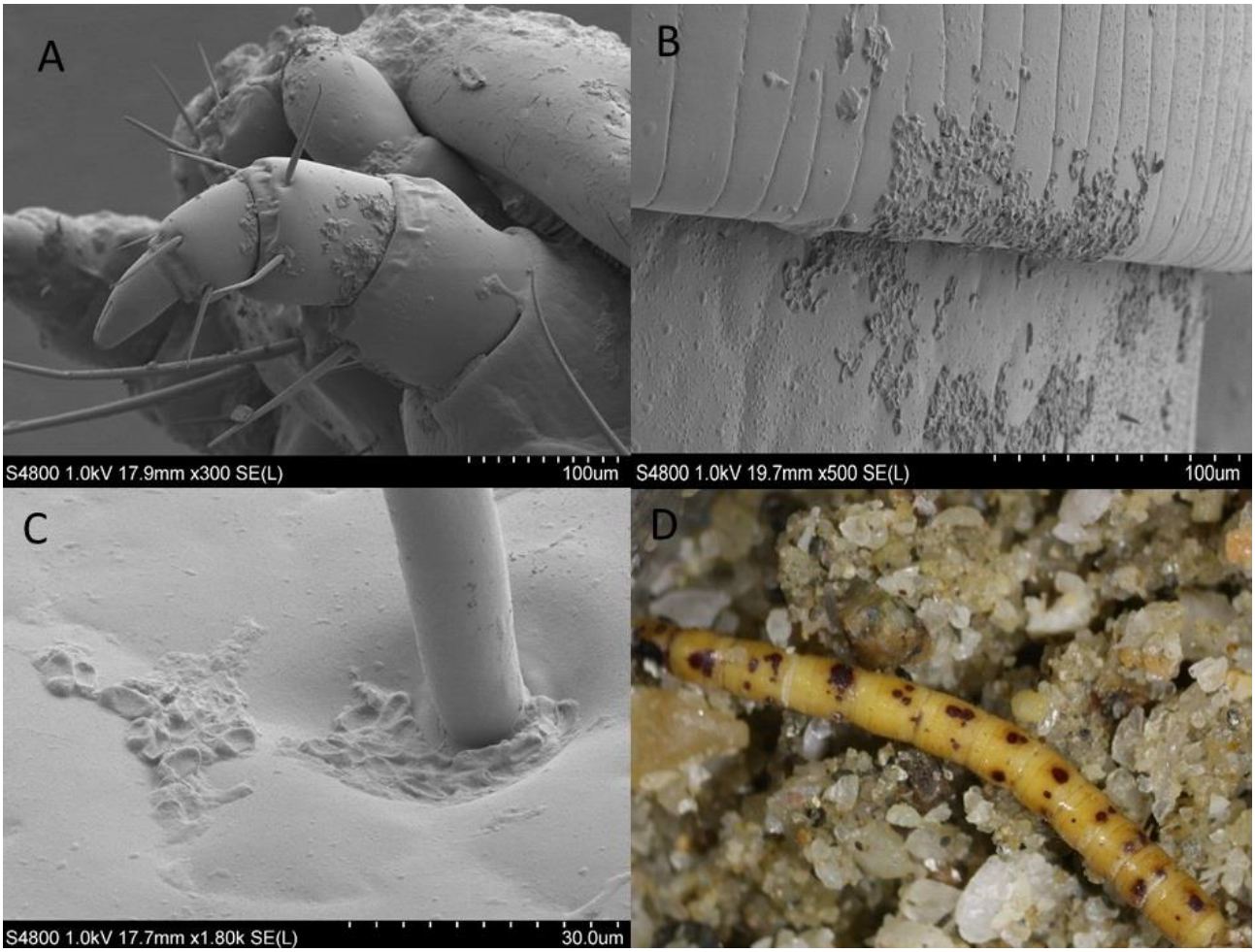

Figure 1. A: Leg of *Agriotes obscurus* exposed to *Metarhizium brunneum* strain ART2825 48 h posttreatment. B: intersegment area of A. obscurus exposed to *M. brunneum* strain F52 36 h posttreatment, C: Depression at the base of a setae of *A. obscurus* with F52 conidia 24 h posttreatment, D: Melanization on *A. sordidus* exposed to *M. brunneum* strain 16P 21 days posttreatment (zoom x 6.7).

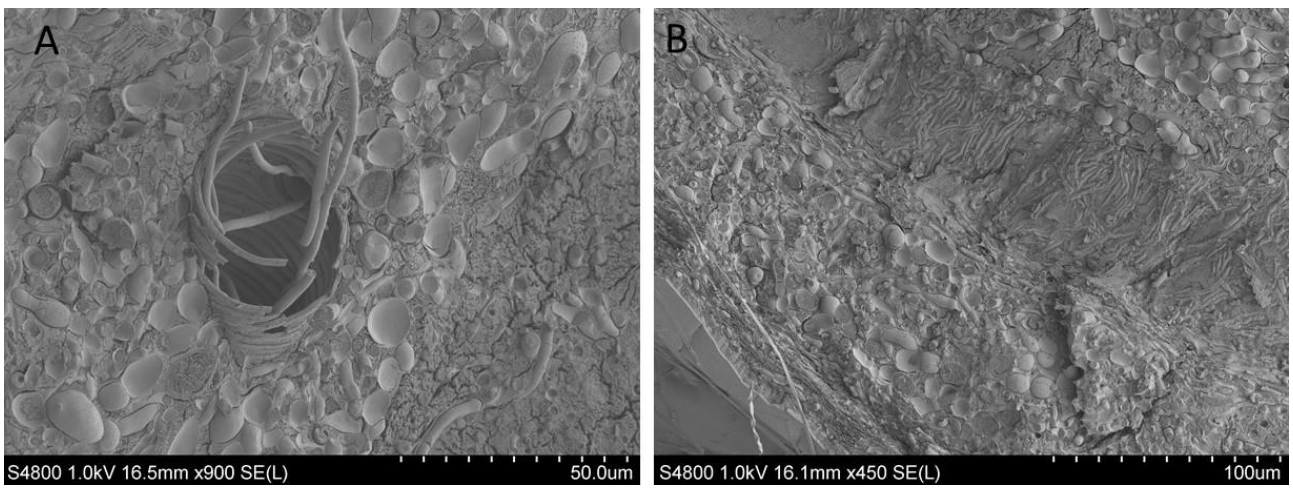

Figure 2. A: *Metarhizium brunneum* strain ART2825 growth in the tracheae of *Agriotes obscurus* 22 days posttreatment. B: ART2825 fungal colonization in the integument of *A. obscurus* 22 days posttreatment.

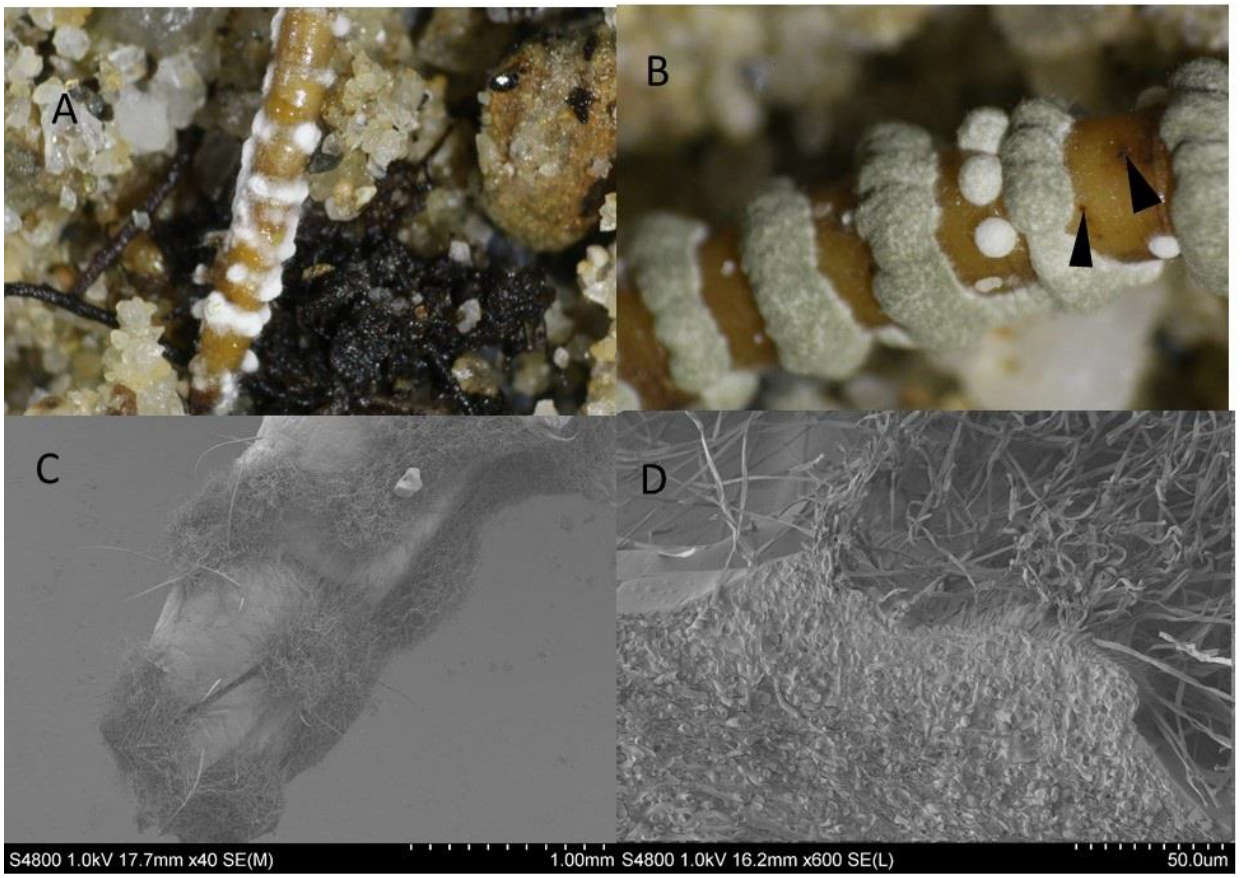

Figure 3. A: Sporulation of *Metarhizium brunneum* strain 16P on *Agriotes sordidus* 21 days posttreatment (zoom ×6.7). B: *M. brunneum* strain 16P primary and secondary fungal growth, with melanotic spots (black arrow) on *A. sordidus* (×6.7). C: ART2825 fungal growth on the cuticle of *A. obscurus* 22 days posttreatment. D: Fungal growth on the cuticle of *A. obscurus*, which could correspond to the secondary growth on the sclerites.

M. brunneum ART2825 and F52 combined with the lowest dose of spinosad mostly resulted in synergistic reactions, killing 36 to 93% and 20 to 50% of the larvae, respectively. Increasing the dose of spinosad did not always result in increased mortality (Table 1). Ericsson et al. (2007) and Morales-Rodriguez and Wanner (2015) also noted that increased doses of insecticides were not always linked with increased susceptibility of the insects.

In coapplication studies, the insecticide is often condidered to be the stressing agent. Spinosad can paralyze the insects; moreover, it caused cellular damage, oxydative stress and cell death (Nguyen et al., 2021). Paralysis could prevent the removal of conidia from the host surface by abrasion with the soil (Quintela and McCoy, 1997, 1998b). Reduced movement could also reduced the number of spores encounter and reduce mycosis. Spinosad had no impact on spores germination rates; yet other insecticides, such as imidacloprid, can increase the germination rate of *B. bassiana* or *M. anisopliae* (Quintela and McCoy, 1997). In this study, the fungal growth observed on *A. obscurus* exposed to spinosad only, came from latent field infection (Kabaluk et al., 2007). The larvae were kept in pots for a couple of weeks prior to the trials, and most larvae who suffered latent infection had died before the beginning of the trials. Even though the fungi emerging from *A. obscurus* were not identified, we do not expect that the latent infection played a major role in the mortality during the coapplication trial. In fact, latent infections are usually weak and coinfection are a rare event. Usually only one of the fungi manage to complete its cycle and is responsible for the death of the insects (Fargues and Bon, 2004). Moreover, in the combination trial, the high rates of conidia present should have overcome any weak latent infection.

Stress-and-kill studies gives interesting results; yet, previous field study have not been able to show consistent synergy between *M. brunneum* F52 and spinosad or clothianidin (Kabaluk and Ericsson, 2007a). However, application of F52 as a seed treatment was enough to increase the stand density and yield of field corn (Kabaluk and Ericsson, 2007b). However, it is not clear from these studies whether this was due to protection provided by

F52 or stimulation of plant growth. *M. brunneum* like many other EPF are known to stimulate plant growth (García et al., 2011; Khan et al., 2012; Dara et al., 2017). Subterranean insects are constantly exposed to a wide range of pathogenic microbes and opportunistic pathogens which lead them to develop powerful defenses, especially at the cuticle surface, which is the first and most important barrier to infection (Butt et al., 2016). Microscopic analysis confirms that conidia could adhere to the cuticle but struggled to germinate. Delaying penetration of the cuticle by the EPF predisposes the fungal inoculum to biotic (e.g., soil microbes) and abiotic factors (e.g., relative humidity, abrasion with soil) that are deleterious to the pathogen and may clear the cuticles from spores. Stress-and-kill strategies could help to weaken *Agriotes* larvae to facilitate fungal penetration and increase mortality.

New strategies are urgently needed to control wireworms. These strategies need to be able to effective against the different wireworm species to be easily use by farmers. The susceptibility of wireworms to EPF varies greatly between *Agriotes* species and EPF strains. However, stress-and-kill can increase the efficacy and the speed of kill of virulent and weakly virulent EPF.

The interaction between spinosad and the EPF strains was weak, further laboratory studies should screen for stressing agents promoting EPF germination and fungal growth, which would increase the efficacy of the stress-and-kill.

Acknowledgments

The authors thank Certis Europe BV and Swansea University for funding this research. We thank P. Larroudé from Arvalis, in field collection of wireworms, B. Clunie for collecting data and L. Hwang for technical support. We thanks our anonymous reviewers for their constructive comments.

References

Anderson TE, Hajek AE, Roberts DW, Preisler HK, Robertson JL (1989). Colorado potato beetle (Coleoptera: Chrysomelidae): effects of combinations of *Beauveria bassiana* with insecticides. Journal of Economic Entomology 82 (1): 83-89. doi:10.1093/jee/82.1.83

Ansari MA, Evans M, Butt TM (2009). Identification of pathogenic strains of entomopathogenic nematodes and fungi for wireworm control. Crop Protection 28: 269-272. doi: 10.1016/j.cropro.2008.11.003

Antwi FB, Shrestha G, Reddy GVP, Jaronski ST (2017). Entomopathogens in conjunction with imidacloprid could be used to manage wireworms (Coleoptera: Elateridae) on spring wheat. The Canadian Entomologist 150 (1): 1-16. doi: 10.4039/tce.2017.58

Barsics F, Haubruge E, Verheggen FJ (2013). Wireworms' management: an overview of the existing methods, with particular regards to A*griotes* spp. Insects 4 (1): 117-152. doi: 10.3390/insects4010117

Butler D (2018). Scientists hail European ban on bee-harming pesticides. Nature. doi: 10.1038/d41586-018-04987-4

Butt TM, Greenfield BPJ, Greig C, Maffeis TGG, Taylor JWD et al. (2013). *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. PLoS ONE 8 (12): 1-11. doi: 10.1371/journal.pone.0081686

Dara SK, Dara SSR, Dara SS (2017). Impact of entomopathogenic fungi on the growth, development, and health of cabbage growing under water stress. American Journal of Plant Sciences 8 (6): 1224-1233. doi: 10.4236/ajps.2017.86081

Eckard S, Ansari MA, Bacher S, Butt TM, Enkerli J et al. (2014). Virulence of in vivo and in vitro produced conidia of *Metarhizium brunneum* strains for control of wireworms. Crop Protection 64: 137+142. doi: 10.1016/j.cropro.2014.06.017

Ensafi P, Crowder DW, Esser AD, Zhao Z, Marshall JM et al. (2018). Soil type mediates the effectiveness of biological control against *Limonius californicus* (Coleoptera: Elateridae). Journal of Economic Entomology 111 (5): 1-6. doi: 10.1093/jee/toy196

Ericsson JD, Kabaluk JT, Goettel MS, Myers JH (2007). Spinosad interacts synergistically with the insect pathogen *Metarhizium anisopliae* against the exotic aireworms *Agriotes lineatus* and *Agriotes obscurus* (Coleoptera : Elateridae). Journal of Economic Entomology 100 (1): 31-38. doi: 10.1093/jee/100.1.31

Fargues J, Bon MC (2004). Influence of temperature preferences of two *Paecilomyces fumosoroseus* lineages on their co-infection pattern. Journal of Invertebrate Pathology 87 (2-3): 94-104. doi: 10.1016/j.jip.2004.07.001

Furlong MJ, Groden E (2001). Evaluation of synergistic interactions between the Colorado potato beetle (Coleoptera: Chrysomelidae) pathogen *Beauveria bassiana* and the insecticides, imidacloprid, and cyromazine. Journal of Economic Entomology 94 (2): 344-356. doi: 10.1603/0022-0493-94.2.344

García JE, Posadas JB, Perticari A, Lecuona RE (2011). *Metarhizium anisopliae* (Metschnikoff) Sorokin promotes growth and has endophytic activity in tomato plants. Advances in Biological Research 5 (1): 22-27.

Goettel MS, Inglis GD (1997). Fungi: Hyphomycetes. In: Lacey L (editor). Manual of Techniques in Insect Pathology. London, UK: Academic Press, pp. 213-249. doi: 10.1016/B978-012432555-5/50013-0

Hicks H, Blackshaw RP (2008). Differential responses of three *Agriotes* click beetle species to pheromone traps. Agricultural and Forest Entomology 10: 443+448. doi: 10.1111/j.1461-9563.2008.00397.x

Hummelbrunner LA, Isman MB (2001). Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lepidoptera, Noctuidae). Journal of Agricultural and Food Chemistry 49 (2): 715-720. doi: 10.1021/jf000749t

Jaramillo J, Borgemeister C, Ebssa L, Gaigl A, Tobón R et al. (2005). Effect of combined applications of *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) strain CIAT 224 and different dosages of imidacloprid on the subterranean burrower bug *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae). Biological Control 34 (1): 12-20. doi: 10.1016/j.biocontrol.2005.03.021

Kabaluk JT, Ericsson JD (2007a). Environmental and behavioral constraints on the infection of wireworms by *Metarhizium anisopliae*. Environmental Entomology 36 (6): 1415-1420. doi: 10.1093/ee/36.6.1415

Kabaluk JT, Ericsson JD (2007b). *Metarhizium anisopliae* seed treatment increases yield of field corn when applied for wireworm control. Agronomy Journal 99 (5): 1377+1381. doi: 10.2134/agronj2007.0017N

Kabaluk JT, Vernon RS, Goettel MS (2007). Mortality and infection of wireworm, *Agriotes obscurus* [Coleoptera: Elateridae], with inundative field applications of *Metarhizium anisopliae*. Phytoprotection 88 (2): 51-56. doi: 10.7202/018055ar

Kergunteuil A, Bakhtiari M, Formenti L, Xiao Z, Defossez E et al. (2016). Biological control beneath the feet: a review of crop protection against insect root herbivores. Insects 7 (70): 1-22. doi: 10.3390/insects7040070

Khan AL, Hamayun M, Khan SA, Kang SM, Shinwari ZK et al. (2012). Pure culture of *Metarhizium anisopliae* LHL07 reprograms soybean to higher growth and mitigates salt stress. World Journal of Microbiology and Biotechnology 28 (4): 1483-1494. doi: 10.1007/s11274-011-0950-9

Larroudé P, Thibord J-B (2017). Interet de l'utilisation de *Metarhizium brunneum* pour lutter contre les taupins en grandes cultures. In: Proceeding of the 11th Association Française de Protection des plantes sur les ravageurs et auxiliaires en agriculture; Montpellier, France. pp. 268-277.

Morales-Rodriguez A, Peck DC (2009). Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. Biological Control 51 (1): 169-180. doi: 10.1016/j.biocontrol.2009.06.008

Morales-Rodriguez A, Wanner KW (2015). Efficacy of thiamethoxam and fipronil, applied alone and in combination, to control *Limonius californicus* and *Hypnoidus bicolor* (Coleoptera: Elateridae). Pest Management Science 71 (4): 584-591. doi: 10.1002/ps.3877

Nguyen J, Ghazali R, Batterham P, Perry T (2021). Inhibiting the proteasome reduces molecular and biological impacts of the natural product insecticide, spinosad. Pest Management Science. doi: 10.1002/ps.6290

Parker WE, Howard JJ (2001). The biology and management of wireworms (*Agriotes* spp.) on potato with particular reference to the U.K. Agricultural and Forest Entomology 3 (2): 85-98. doi: 10.1046/j.1461-9563.2001.00094.x

Paula AR, Carolino AT, Paula CO, Samuels RI (2011). The combination of the entomopathogenic fungus *Metarhizium anisopliae* with the insecticide Imidacloprid increases virulence against the dengue vector *Aedes aegypti* (Diptera: Culicidae). Parasites and Vectors 4 (1): 1-8. doi: 10.1186/1756-3305-4-8

Purwar JP, Sachan GC (2006). Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). Microbiological Research 161 (1): 38-42. doi: 10.1016/j.micres.2005.04.006

Quintela ED, McCoy CW (1997). Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. Environmental Entomology 26 (5): 1173-1182. doi: 10.1093/ee/26.5.1173

Quintela ED, McCoy CW (1998a). Synergistic effect ofimidacloprid on conidial germination and the pathogenicity of two entomopathogenic fungi to larvae of *Diaprepes Abbreviatus* (Coleoptera: Curculionidae). Journal of Economic Entomology 91 (1): 110-122. doi: 10.1093/jee/91.1.110

Quintela ED, McCoy CW (1998b). Conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) treated with imidacloprid. Journal of Invertebrate Pathology 72 (72): 220+230. doi: 10.1006/jipa.1998.4791

Razinger J, Lutz M, Schroers HJ, Urek G, Grunder J (2013). Laboratory testing of insect associated fungi for the control of wireworms (*Agriotes* sp. L.). IOBC-WPRS Bulletin 90: 103-107.

Razinger J, Schroers HJ, Urek G (2018). Virulence of *Metarhizium brunneum* to field collected *Agriotes* spp. Wireworms. Journal of Agricultural Science and Technology 20 (2): 309-320.

Rogge SA, Mayerhofer J, Enkerli J, Bacher S, Grabenweger G (2017). Preventive application of an entomopathogenic fungus in cover crops for wireworm control. BioControl 62 (5): 613-623. doi: 10.1007/s10526-017-9816-x

Shah FA, Ansari MA, Prasad M, Butt TM (2007). Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. Biological Control 40 (2): 246-252. doi: 10.1016/j.biocontrol.2006.10.005

Shah FA, Gaffney M, Ansari MA, Prasad M, Butt TM (2008). Neem seed cake enhances the efficacy of the insect pathogenic fungus *Metarhizium anisopliae* for the control of black vine weevil, *Otiorhynuchs sulcatus* (Coleoptera: Curculionidae). Biological Control 44 (1): 111-115. doi: 10.1016/j.biocontrol.2007.09.009

Thibord J-B (2017). Evaluation de la nuisibilité des ravageurs du maïs en France. In: Proceeding of the 11th Association Française de Protection des plantes sur les ravageurs et auxiliaires en agriculture; Montpellier, France.

Thibord J-B, Larroudé P, Chabert A, Villeneuve F, Quilliot E et al. (2017). Prévision des risques et élaboration de nouvelles techniques de lutte pour la protection des cultures contre les attaques de taupins. Innovations Agronomiques 55: 215-233. doi: 10.15454/1.5137781155320183E12

Trisyono A, Whalon ME (1999). Toxicity of neem applied alone and in combinations with *Bacillus thuringiensis* to Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology 92 (6): 1281-1288. doi: 10.1093/jee/92.6.1281

Van Herk WG, Vernon RS, Tolman JH, Saavedra HO (2008). Mortality of a wireworm, *Agriotes obscurus* (Coleoptera: Elateridae), after topical application of various insecticides. Journal of Economic Entomology 101 (2): 375-383. doi: 10.1093/jee/101.2.375