Attractants and entomopathogenic fungi to control wireworms: field efficacy and impact on non-target species

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LIÈGE université
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Preface

This master thesis was carried out in collaboration between Gembloux Agro-Bio Tech, University of Liège (Sustainable Pest Management Division), and the TERRA research unit (Chemical and Behavioural Ecology). The field tests were carried out thanks to the Royal Belgian Institute for Beet Improvement (IRBAB). This master thesis is presented in article format. The formatting rules applied follow those of the journal Pest Management Science.

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After that, I would like to thank all the friends I met at Gembloux. Being surrounded by people as wonderful as you made these 5 (and a half) years of study even more beautiful and enjoyable. Thank you to all my travelling companions, with whom I shared unforgettable moments, whether it was during a weekend in the Fagnes or a trek in Norway. Special thanks to Hugo for all the sporting hours we spent together, to Emilie for all the confidences we shared and to Maëlle who was an attentive listener and with whom I can't wait to discover Asia.

And finally I would like to mention my parents, my sister and my grandparents who have supported me throughout my studies. I'm grateful to have a family like you and I couldn't have done it without you, you've given me endless emotional support throughout all those years and all those stressful moments. You've always encouraged me to make my own choices and I can't thank you enough for that. A very special thank you to Victor, who I'm lucky to have by my side and who is a permanent source of inspiration and encouragement.

Résumé

Les larves de taupins sont des ravageurs majeurs des cultures, et le retrait des insecticides tels que les néonicotinoïdes complique leur contrôle. Une stratégie attractante et tueuse, qui combine un signal chimique attractif et un champignon entomopathogène, constitue une alternative prometteuse pour réduire les populations de taupins et minimiser les dommages causés aux cultures. Cette étude visait à évaluer l'efficacité biocide de divers champignons, à étudier le comportement d'orientation des taupins vers ces champignons en l'absence d'attractifs supplémentaires et à caractériser leurs profils de composés organiques volatils (COV). Nous avons émis l'hypothèse que des champignons spécifiques efficaces pour tuer les taupins pouvaient également les attirer grâce à leurs COV. Un deuxième objectif consistait à évaluer l'efficacité de la formulation attract and kill dans la protection des cultures de betteraves sucrières dans des conditions semi-contrôlées et sur le terrain, en émettant l'hypothèse d'une réduction des dommages causés aux cultures. En outre, la sécurité de la formulation a été testée sur l'espèce de carabes non-cible *Pterostichus melanarius*, en supposant qu'elle n'augmenterait pas la morbidité ou la mortalité. Les résultats ont permis d'identifier *Metarhizium anisopliae* NEW comme le champignon le plus efficace pour tuer les taupins. De plus, *M. anisopliae* NEW et *Trichoderma harzianum* produisent des COV spécifiques qui attirent les larves de taupins. Les essais en champ ont démontré que la formulation attract and kill améliorait significativement la croissance foliaire des cultures de betteraves sucrières sans avoir d'impact négatif sur la morbidité ou la mortalité des carabes. En conclusion, *Metarhizium anisopliae* NEW combiné à un attractant ciblant les larves de taupins est un candidat très prometteur pour un biopesticide ciblant les taupins tout en étant non nocif pour les espèces non-cibles, ce qui constitue une solution efficace pour la culture durable de la betterave sucrière.

Mots-clés : *Agriotes* spp., biopesticides, Attract and Kill, *Beta vulgaris*, écologie chimique

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Acronyms

EPF	Entomopathogenic Fungi
A&K	Attract and kill
IPM	Integrated Pest Management
VOC	Volatile Organic Compound
OSA	*Ophiocordyceps sporangifera* A
MAN	*Metarhizium anisopliae* NEW

Attractants and entomopathogenic fungi to control wireworms: field efficacy and impact on non-target species.

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Abstract

Wireworms are major crop pests, and the withdrawal of insecticides such as neonicotinoids has made their control increasingly challenging. An attract and kill strategy, which combines an attractive chemical signal with an entomopathogenic fungus, presents a promising alternative for reducing wireworm populations and minimizing crop damage. This study aimed to evaluate the biocidal efficacy of various fungi, investigate the orientation behaviour of wireworms towards these fungi in the absence of additional attractants, and characterize their volatile organic compound (VOC) profiles. We hypothesised that specific fungi efficient at killing wireworms, could also attract them, through their VOCs. A second objective evaluated the effectiveness of the attract and kill formulation in protecting sugar beet crops under semi-controlled and field conditions, hypothesising a reduction in crop damage. Additionally, the safety of the formulation was tested on the non-target carabid beetle species *Pterostichus melanarius*, with the assumption that it would not increase morbidity or mortality. The results identified *Metarhizium anisopliae* NEW as the most effective fungus for killing wireworms. Moreover, *M. anisopliae* NEW and *Trichoderma harzianum* produced specific VOCs that attract wireworms. Results showed that the attract and kill formulation significantly enhanced foliar growth in sugar beet crops without negatively impacting the morbidity or mortality of carabid beetles. In conclusion, *Metarhizium anisopliae* NEW is a highly promising candidate for a biopesticide targeting wireworms while being non-harmful to non-target species, providing an effective solution for sustainable sugar beet cultivation.

Key words: *Agriotes* spp., biopesticides, Attract and Kill, *Beta vulgaris*, chemical ecology

Introduction

Wireworms (Coleoptera: Elateridae) are click beetles' larvae and represent serious crop pests worldwide. Species such as *Agriotes lineatus* L., *A. obscurus* L. and *A. sputator* L. are polyphagous, causing significant losses in sugar beets, potatoes, maize and wheat (Hill, 1987; Furlan, 1996). Recently, significant increases in their damage have been recorded, partly due to the withdrawal of non-specific, long lasting insecticides such as neonicotinoids and carbamates (Parker et al., 2001; Vernon et al., 2009, 2022; "Phytoweb," June-4-2024).

Thus, alternative pest control methods are being developed (Gupta et al., 2010) and include for instance: trapping of adults with sex pheromones (Tóth et al., 2003); biofumigation using Brassicaceae plants (Parker, 2007); introduction of natural antagonists such as predators, parasitoids, and micro-organisms (Kleespies et al., 2013). Agricultural strategies can also help preventing wireworms damage: crop rotation favours plant species unfavourable to wireworm oviposition and survival, such as barley or oats or clover-grass mixtures (Keiser et al., 2012; Milosavljević et al., 2019; Poggi et al., 2021). Planting cultivars that are more tolerant of soil-borne pests can also help reducing their damage (Poggi et al., 2021).

Understanding the behaviour of these larvae can also lead to the development of innovative strategies of control. Wireworms use biotic and abiotic signals to orient themselves in the soil. First, they follow a gradient of CO_2, which signals the presence of food (Doane et al., 1975; Johnson et al., 2012). Then, they perceive the volatile organic compounds (VOCs) emitted by the plant rhizosphere (Johnson et al., 2012) thanks to sensory receptors located on their under-developed antennae and labial or maxillary palps (Barsics et al., 2014). Their searching behaviour is influenced by the nature and concentration gradients of these semiochemical mixtures (Crombie et al., 1947; Thorpe et al., 1947). But their preference for certain chemical cues is also highly influenced by their development status and feeding history. For instance, orientation sensitivity increases in the case of prolonged starvation (Thorpe et al., 1947). Previous studies identified several plant-released chemicals as attractants for wireworms, including 2-pentylfuran and maize aldehydes (Fiers et al., 2013; Gfeller et al., 2013; la Forgia et al., 2023). These volatile cues could be used to attract and trap wireworms.

Biopesticides based on entomopathogenic micro-organisms such as bacteria, viruses, nematodes or fungi, represent promising alternatives (Ansari et al., 2009; Essiedu et al., 2020). They offer several advantages: their low persistence in the environment reduces the risk of long-term pollution, making them more environmentally friendly. Also, they leave no residues, making it possible to harvest the crop shortly after application (Gupta et al., 2010). However, their adoption remains limited due to several challenges, including their short shelf life, high production costs, and inconsistent effectiveness under field conditions (Gupta et al., 2010; Dar et al., 2011). Entomopathogenic fungi (EPF) are naturally present in the soil and are already widely used as biopesticides (Cook et al., 2007). EPF infect their hosts during a parasitic phase through a multi-step process: (i) spores' adhesion to the insect cuticle, (ii) penetration inside the insect body via an appressorium, (iii) colonization of haemolymph, and (iv) death of the insect (Gao et al., 2011). Then, a saprophytic phase begins with the hyphal growth and the production of new spores outside the insect body, facilitating the dispersion and infection of a new host (Sharma et al., 2023). The most commonly used entomopathogenic fungi as inundative control agents include *Beauvaria bassiana*, *B. brongniartii*, *Metarhizium anisopliae* and *M. brunneum* (Zimmermann, 2007; Sharma et al., 2023). *Ophiocordyceps sporangifera* (Hypocreales : Ophiocordycipitaceae) is also an entomopathogenic fungus that has shown good performance on wireworms in biocide tests under direct exposure (Regnier, 2022). Entomopathogenic fungi effectiveness may be influenced by the method of application, whether foliar spraying, endophyte application, seed

treatment or soil irrigation (Sharma et al., 2023). The success of fungal infection also depends on environmental conditions at the time of application, such as humidity, light and temperature, which must be optimal to ensure pathogenicity (Sharma et al., 2023). Given that entomopathogenic fungi are not highly specific and can infect a broad range of arthropods, it is crucial to assess the specificity of any new formulation to ensure it does not negatively impact non-target species or beneficial organisms in the ecosystem (Zimmermann, 2007).

The aim of this study is to develop an attract and kill formulation, combining the attractive action of a chemical cue with the biocide action of an entomopathogenic fungi. This technology is based on a recent development by Brandl et al (2017) who combined *Metarhizium brunneum* conidia (to infect wireworms) with baker's yeast (*Saccharomyces cerevisiae*) that produces carbon dioxide, and consequently attract wireworms (*Agriotes* spp.) to the killing agent. Their encapsulation in biodegradable alginate beads, provides a protective environment for the survival and growth of the micro-organisms, while facilitating their distribution in the soil (Brandl et al., 2017).

The first objective of this research is to evaluate the biocidal capacities of several entomopathogenic and non-entomopathogenic fungi against wireworms, explore the orientation behaviour of wireworms towards these fungi, and identify their odour profile (Figure 1). The second objective aims to confirm the effectiveness of the attract and kill formulation in protecting sugar-beets under semi-controlled and field conditions. Lastly, the third objective assesses the safety of the formulation on a non-target species which can be in contact with the formulation: the carabid beetle *Pterostichus melanarius*.

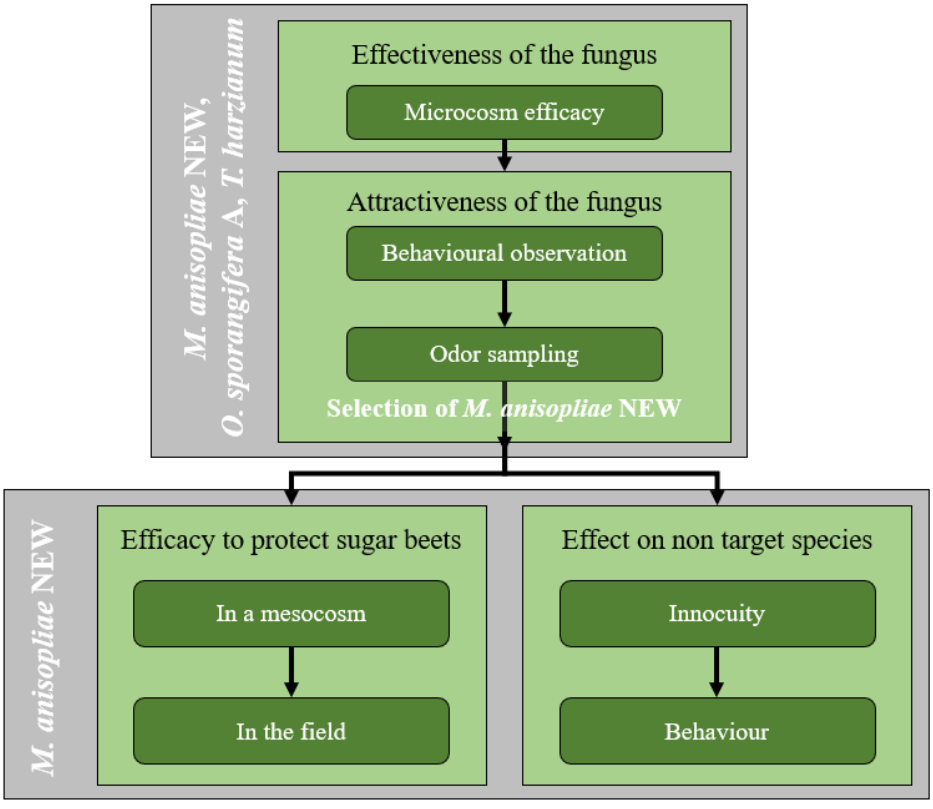


Figure 1: Study workflow

Materials and Methods

Biological material

Wireworms (*Agriotes lineatus* and *A. obscurus*) were reared from adults collected in spring 2023 in Gembloux (45.725833, 4.666111). Larvae were separated by their developmental stage and reared in plastic containers (11×11×6 cm) in a soil-vermiculite mixture (RAJA, France) (1:1, v:v) at 65% water holding capacity (WHC) at room temperature (21±1°C). Larvae were fed germinating organic wheat (Walagri, Belgium). Before each experiment, wireworms were selected based on their developmental stage (L10-L11), isolated and starved for one week.

Adult carabid beetles (*Pterostichus melanarius*) were collected in the field (Wallonia, Belgium) on July 25th, August 19th and 22nd, 2024. They were placed in large plastic containers (50×32×15 cm) at 21±1°C, along with soil, water and pieces of aluminium used as shelters (7×7cm). They were fed either wet dog food (Everyday®, Belgium) or dead *Tenebrio molitor* larvae. Only adult beetles with physical integrity were selected for experimental use. *Tenebrio molitor* larvae were reared in large, aerated boxes (24×13×8 cm) at 21±1°C and fed on flour, bran and carrots.

The fungal strains used in this study (*Metarhizium anisopliae*, *Ophiocordyceps sporangifera* and *Trichoderma harzianum*) were obtained from the Mycothèque of Louvain-la-Neuve (MUCL, Belgium). They were preserved by cryopreservation at -130°C, then reactivated and grown on V8+agar medium at 24°C for multiplication. The strains of entomopathogenic fungi, *M. anisopliae New* (MAN) and *O. sporangifera* A (OSA), were isolated from field-collected wireworms.

Preparation of the Attract and Kill formulation

Attract beads: The beads were made in the Chemical and Behavioural Ecology Laboratory (Gembloux, Belgium) and the protocol has been adapted from la Forgia et al. (2023). A natural organic potato extract (50% v/v; Allians variety, Belgium) was used as an attractant for wireworms. This extract was encapsulated in humid, spherical beads composed of sodium alginate (2% w/v) and alpha-tocopherol (0.7% w/v). The beads were polymerised in a 0.1M solution of calcium chloride ($CaCl_2$) for 30 min. After polymerisation, the beads were rinsed with distilled water, dried under chemical hood for 20 min and stored at 4°C before being used for experimentation the following day. The attract control beads were prepared with water instead of potato juice.

Fungi beads: The beads were made in the laboratory of UCLouvain (Louvain la Neuve, Belgium). Conidia of *Metarhizium anisopliae* NEW were collected in Petri dishes containing a culture medium composed of V8 and agar (Agar agar for microbiology, ROCC S.A.). Conidia were suspended in sterile pure water and quantified using a Thoma cell to achieve a final concentration of 6×10^8 conidia/ml alginate (i.e. 25,000 conidia/ml). A solution based on 2% alginate and 30% corn starch (MP Biomedicals) was mixed with the conidia to formulate the beads. For *Ophiocordyceps sporangifera* A, fragments of mycelium (25,000 fragments/ml) and perithecia were collected from Petri dishes containing a culture medium composed of V8 and agar (ROCC S.A.), as the strain had difficulty sporulating. These elements were incorporated into a solution of 2% alginate and 30% corn starch (MP Biomedicals). Perithecia were quantified after depolymerisation of the formulated beads.

Solutions containing *M. anisopliae* NEW or *O. sporangifera* A were polymerised in a 0.1 M calcium chloride solution for 30 minutes. The beads obtained were then stored at 4°C for 24 hours before being introduced into a sterile microcosm. Sixty beads per treatment were placed in each microcosm. The fungi control beads were manufactured using the same protocol as that described for *M. anisopliae NEW* and *O. sporangifera*, but without the addition of fungi.

EPF selection

The first objective of the present work was to assess the most effective fungus strain to kill wireworms. Additionally, the wireworms' orientation bioassay, supplemented by volatolomic analyses, aimed to determine whether wireworms showed an olfactory preference for these fungi in the absence of an additional attractant, and, if so, to identify the volatile compounds associated with the fungi's odours.

Biocidal tests on wireworms

To determine the sublethal and/or lethal effect of the fungi beads, wireworms were individually placed in a microcosm containing 20 grams of moist soil (La Plaine Chassart, Belgium) at 65% WHC, one gram of fungi beads and 1 cm^3 of potato (Allians variety). Weekly monitoring was carried out to assess the mortality. These tests were carried out over a period of eight weeks.

Wireworms' orientation bioassay

The attractiveness of encapsulated EPF to wireworm was tested using a double-choice bioassay. A two-branch olfactometer (20 cm long, 3.2 cm internal diameter) consisting of a glass tube filled with lightly moistened vermiculite (65% WHC; RAJA, French) was used (Chacon Hurtado et al., 2023). Wireworms' attractiveness of two entomopathogenic fungi (*M. anisopliae* and *O. sporangifera*) and one non-entomopathogenic fungus (*T. harzianum*) were assessed in dual choice bioassays (Figure 2). The olfactometer had a central inlet (GL14 neck) to introduce one wireworm in the system, and its ends were connected to two 500 ml borosilicate jars (Duran, Belgium) attached to the side necks (GL45). In one of the two jars, 1 g of beads of entomopathogenic or non-entomopathogenic fungi were placed, the other jar was filled with fungi control beads. The jars were then filled with moistened vermiculite (65% WHC) and connected to the olfactometer, a fine mesh (8×8 cm) preventing the wireworms from reaching the beads. The system was left for 20 minutes in the dark at 21±1°C to diffuse the odours into the olfactometer, before the introduction of one wireworm. The device was kept in the dark at 21±1°C, giving the wireworm one hour to choose a direction in the olfactometer. At the end of the experiment, the wireworm position was recorded. Wireworm found within the first 3cm of either side of the entry point were considered not responding (neutral zone). Wireworms could also be found close to the fungi control beads or fungi beads (zone A or B). The experiment involved 21 olfactometers running simultaneously, was repeated over a period of two days, with 4 sessions per day. The behaviour of 56 wireworms was recorded per fungus strain.

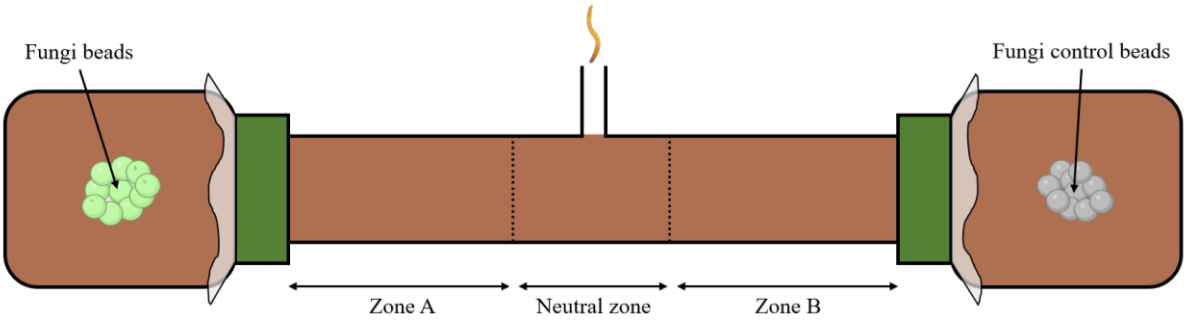


Figure 2: Two-branched olfactometer used for the behavioural test on wireworms.

Volatolomic analysis

The aim of the volatolomic analyses was to identify the volatile organic compounds (VOCs) emitted by encapsulated fungi. The fungi tested included two entomopathogenic (*M. anisopliae and O. sporangifera*), one non-entomopathogenic (*T. harzianum*) and one control, with five replicates per modality. 40 grams of soil and two grams of fungi beads were placed in an airtight glass chamber (20 cm long, 3.2 cm internal diameter) (Figure 3). After three hours of equilibration, the headspace air present in the glass chamber was sampled dynamically in the dark at ambient temperature (21±1°C). A flow of activated carbon filtered air (150 ml/min) was generated through a GilAir^{TM} Plus suction pump and VOCs were trapped on a hydrophobic TenaxTa/Carbograph tube (Marked International, UK) for a 4-hours period. Wadding was placed between the activated charcoal and the soil-containing olfactometer to avoid soiling of the activated charcoal cartridges. After sampling, butylbenzene (25 ng, Sigma-Aldrich, USA) as internal standard was injected in each tube that were then hermetically sealed and stored at +4°C. At least, one sample of each modality was taken for every sampling day. VOCs samples were then analysed using a thermal desorption gas chromatograph coupled with a mass spectrometer and a flame ionisation detector (TD-GC-MS/FID, Shimadzu, Kyoto, Japan). The volatile compounds were first desorbed at 280°C for 8 minutes using a thermodesorber (TD30R, Shimadzu, Kyoto, Japan). A cryofocus was then carried out at -20°C for 12 minutes using the Peltier effect, before the samples were heated to 280°C and injected at the head of the column using a split method. Compounds were separated using a capillary column (30 m × 0.25 mm × 0.50 µm, Agilent Technology, Santa Clara, California, USA) with helium as carrier gas set at a flow rate of 0.94 mL/min. The temperature program was 40°C for 3min; 5°C/min to 200°C; 20°C/min to 300°C; and 300°C for 6 min to purge the column. Compounds were identified by comparing their mass spectra with reference mass spectrum databases (NIST17 and FFNSC3). The injection of a C7-C30 saturated alkanes standard solution (25ng/µL, Sigma-Aldrich, USA) under the same chromatographic conditions allowed to calculate Kovats retention indices (RI). These RI were compared to those available in the literature to confirm identification.

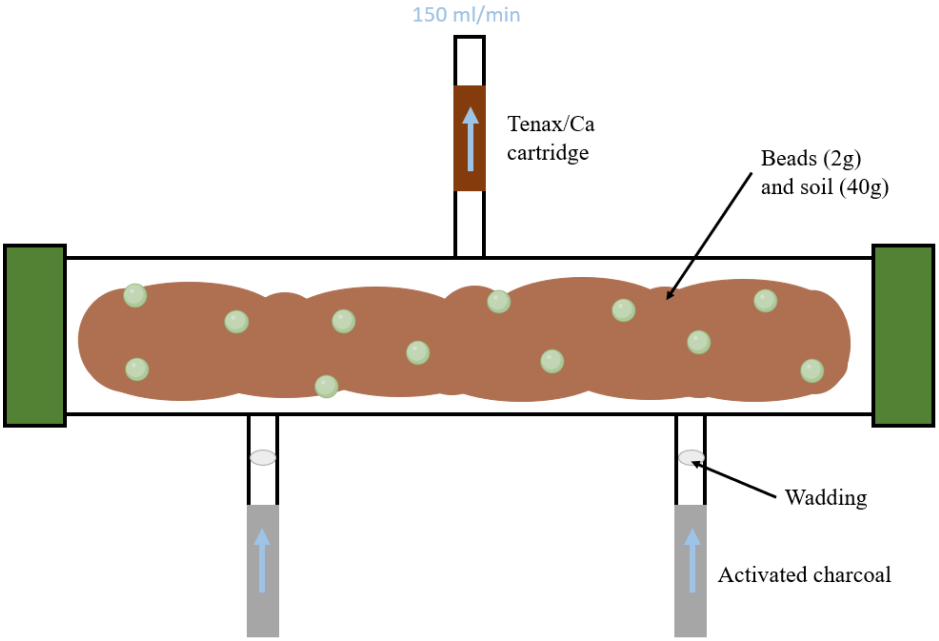


Figure 3: Air-tight glass chamber for VOCs sampling

Effectiveness of *M. anisopliae*

Following the results of previous tests, *M.anisopliae* was selected as the EPF of interest in the formulation for all further tests. The second objective was to assess the effectiveness of the attract and kill formulation in protecting sugar-beets under semi-controlled and field conditions.

Mesocosm trial

The aim of the mesocosm tests was to evaluate the effectiveness of the formulation on an intermediate scale, in a semi-controlled environment. The mesocosms, located in Gembloux (50.5635178N, 4.6979551E), were 50×50 cm aluminium cubes, 80% filled with soil from an organic arable crop (Gembloux, Belgium) free from weeds. The trial consisted of three randomly distributed mesocosms: (1) sugar beets were cultivated in presence of fungi and attract control beads and in absence of wireworms (n=5) (F-W-); (2) sugar beets were cultivated in presence of wireworms and the attract and kill (A&K) formulation (n=12) (F+W+); and (3) sugar beets were cultivated in presence of wireworms and fungi and attract control beads (n=12) (F-W+). In the mesocosms receiving wireworms, 16 wireworms were introduced per mesocosm in groups of four at the location where the sugar beets had been sown (Figure 4). The following day, alginate beads were buried in plots at a depth of 2 cm in nine locations within each mesocosm: for F+W+, 2g of attract beads and 1g of fungi beads per plot; for F-W+ and F-W-, 2g of attract control beads and 1g of fungi control beads per plot. Seven days after formulation application, four sugar beet seeds (without fungicide and insecticide coating) were sown at a depth of two cm. Plant emergence and leaf count were recorded weekly until 75% of the emerged plants reached the BBCH 20 growth stage. Subsequently, all plants were carefully removed from the mesocosms, rinsed with water, and oven-dried at 60°C for 72 hours to ensure their dry mass was stable. Data collected included the percentage of beet emergence, as well as the fresh and dry mass of roots and leaves at the 9-10 leaf stage (BBCH 19-20).

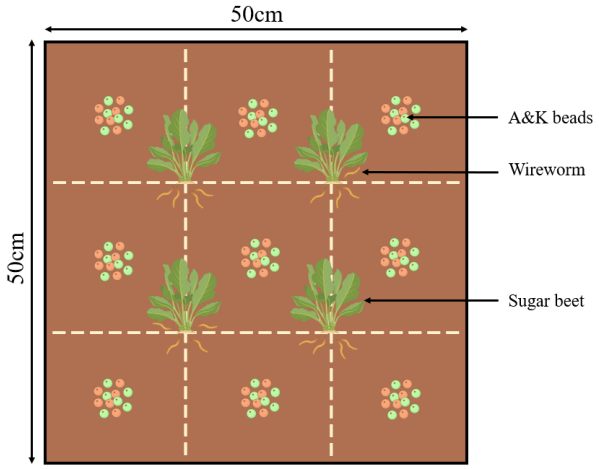


Figure 4: A mesocosm containing 4 sugar beet plants, 9 attract and kill beads plots and 16 wireworms (F+W+).

Field trial

The aim of this test was to evaluate the effectiveness of the formulation under sugar beet field conditions. The trials were conducted in a field infested with wireworms, selected by IRBAB and located in Beloeil (50.551789134578534, 3.6368066174999965). The experiment took place from 11/05/2024 with the sowing of sugar beet seeds, without fungicide or insecticide and ended on 27/06/2024 with field sampling. The field was subdivided into 48 plots, all 2 metres apart. Of these, 12 plots were allocated to the study: six were used to test the formulation and the other six served as control plots. Each plot contained four rows of sugar beet (row length 40 cm) with an inter-plant distance of 18.5 cm. Four days after sowing, 40 dots of attract and fungi beads (2 grams of attract beads and 1 gram of fungi beads) were placed in two of the three rows, so that each row was in direct contact with the formulation (Figure 5). Plant emergence and leaf count were recorded weekly until 75% of the emerged plants reached the BBCH 20 growth stage. Subsequently, eight plants per plot were randomly and carefully removed from the soil, rinsed with water, and oven-dried at 60°C for 72 hours to ensure their dry mass was stable. Data collected included the percentage of beet emergence, as well as the fresh and dry mass of roots and leaves at the 9-10 leaf stage (BBCH 19-20)

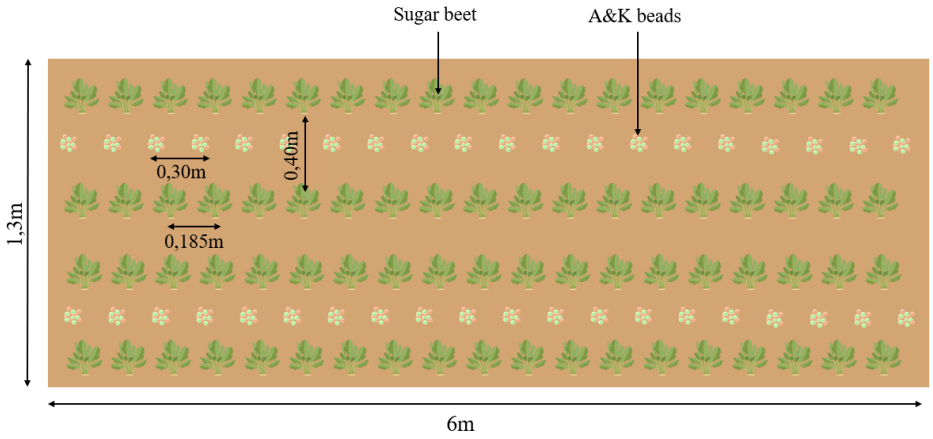


Figure 5: Experimental plan of one field plot

Impact of the formulation on a non-target species (*Pterostichus melanarius*)

The aim of this section was to assess the effect of the attract and kill formulation on the mortality and morbidity of the non-target species *Pterostichus melanarius*.

Innocuity

The experiment to assess the innocuity of the A&K formulation on the carabid beetles *Pterostichus melanarius*, was carried out in plastic boxes (13×18×16 cm, Eurobox). Each box contained 70 grams of potting soil (60% WHC; La Plaine Chassart, Belgium) 10 grams of attract beads and 5 grams of fungi beads well homogenised. Four conditions were tested: (1) attract control beads with fungi control beads (A-K-), (2) attract control beads with fungi beads (A-K+), (3) attract beads with fungi control beads (A+K-), (4) attract beads with fungi beads (A+K+). Each modality was repeated 7 times. Then, 7 adult carabid beetles collected on the 25th of July were placed in each box and received 6.5 grams of food each week during the experiment. The mortality of *P. melanarius* was monitored twice a week. Morbidity was measured once a week by recording the time needed by each individual to exit a circle (Ø = 9 cm) drawn at the bottom of a glass crystalliser (Ø = 20 cm). After 21 days of continuous exposure, the carabid beetles were removed, and the pathogenicity of each microcosm was assessed by introducing mealworms (*T. molitor*). Fifteen mealworms were placed in each microcosm for a continuous exposure period of 21 days, at the end of which mortality was recorded.

Behaviour

The aim of the behavioural tests was to assess whether the formulation was attractive or repulsive to a non-target species. The same four bead modalities were used. A carabid was placed in the centre of a crystalliser (Ø = 20 cm) and maintain in an isolation chamber for 15 seconds, to ensure that it was in a stable position on its legs and to reduce stress. After this period, the insect was given the opportunity to choose between an area (Ø = 5,5 cm) containing A-K- beads and an area (Ø = 5,5 cm) corresponding to one of the four modalities tested (Figure 6). Each zone contained 2 grams of attract beads and 1 gram of fungi beads. For 3 minutes, the carabid beetles' behaviour was recorded using a webcam (Joyaccess, China). After each observation, the crystallizer was cleaned with bleach 5% (La Croix, French) and hexane (Thermo Scientific, Germany). The parameters observed were the time spent in each zone, the number of physical contacts between the carabid beetle and beads (with at least one part of the body), the number of bites on beads (with the mandibles depressed), the number of times the carabid groomed its antennae, the number of attempts to bury the head under the beads.

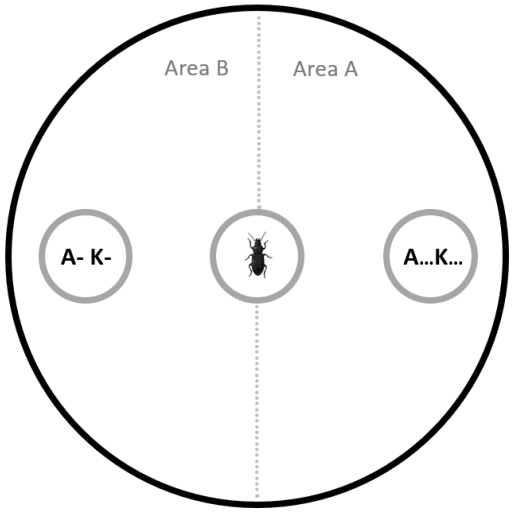


Figure 6: Behavioural test setup

Videos were analysed using BORIS® (Behavioral Observation Research Interactive Software).

Statistical analysis

All statistical analyses were collected in Excel (version 2019, package "readxl") and carried out using R software (version 4.3.2). All graphics were produced using the "ggplot2" package.

Analysis of mortality of wireworms during continuous exposure to beads of different fungi was carried out using Cox proportional hazards regression (package "survival") using kill control beads as reference. Kaplan-Meier survival curves were then generated (package "survminer"), and a Log-Rank Mantel-Cox test was used to determine whether the probability of survival increased. A generalised linear model (GLM; package "lme4", family "binomial") was fitted to assess whether wireworms preferentially chose the side containing the fungus or the control side in the dual choice olfactometer. The data were filtered beforehand (package "dplyr") to exclude the wireworms that remained in the neutral zone. The chromatograms were processed using the GCAligner 1.0 software. To evaluate potential differences in the complete VOC profiles emitted by fungi beads (*Metarhizium*, *Ophiocordyceps*, *Trichoderma*) in the presence of soil, we employed permutative multivariate analysis of variance (perMANOVA) based on a Euclidean distance matrix with 999 permutations (using the "adonis" function from the vegan R package, Oksanen et al., 2022). Bonferroni correction was applied to adjust p-values and control for type I error due to multiple comparisons. The assumption of homoscedasticity was verified using the betadisper function. Additionally, Partial Least Squares Discriminant Analysis (PLS-DA) was used to develop models capable of differentiating the fungal groups based on their volatile profiles. This approach was chosen due to the presence of correlations among certain peaks. PLS-DA constructs a model that separates observations into categories by utilizing the X matrix (formed from linear combinations of volatile components, known as factors) and the Y matrix (containing categorical variables indicating class membership). This analysis defines a discriminant space where observations, projected onto the components, are distinctly grouped according to their respective classes. From the PLS-DA model, confidence scores were extracted for each VOC to evaluate their significance in predicting the volatile profiles of entomopathogenic fungi.

The effect of formulation on sugar beet root dry mass in mesocosms was assessed using generalised linear models (GLM; package "lme4"). The effect of formulation on leaf dry mass was assessed using analysis of covariance (ANCOVA), including the number of leaves as a covariate, as all the ANCOVA assumptions were validated (normality of residuals, homogeneity of variances, linear relationship between covariates and dependent variable, homogeneity of slopes, and absence of influential observations). Two models were calculated, one with leaf dry mass and the other with root dry mass as response variables. The treatment was included as a fixed factor, while the number of leaves was included as a covariate. Multiple comparisons between treatments were performed using the "emmeans" function. The effect of formulation on sugar beet leaves and roots dry mass in the field was evaluated using general linear mixed models (GLMM; packages "lme4" and "emmeans"). Two models were calculated with leaves dry mass and roots dry mass (both with logarithmic transformation) as response variables. Each model was calculated with the treatment as fixed factor and number of leaves as covariate. Plots

were included as random factors to account for plot variabilities.

The analysis of carabid beetle (*Pterostichus melanarius*) mortality in continuous exposure with attract and fungi beads was carried out using a Cox proportional hazard regression (package “survival”) using the control A-K- as reference. Subsequently, Kaplan-Meier survival curves were generated (package “survminer”), and a Log-Rank Mantel-Cox test was employed to determine whether the progression of survival probability, with the modality factor, exhibited similar trends. The evolution of beetle weight during continuous exposure was analysed using a generalised linear mixed model (GLMM; package “lme4”). The model was calculated with the interaction of modalities and day as fixed factor. The box number was included as random factor. Multiple comparisons were performed to estimate the differences in slopes between modalities (package “lmertest”). The effect of formulation on the latency for a beetle to leave a circle was analysed using a generalised linear model (GLM; package “lme4”). A threshold of 3 minutes was set, thus any time exceeding this value was replaced with this maximum to avoid bias from extreme values. The interaction between modalities and day was included in the model as fixed factor. Multiple comparisons were performed to estimate the differences in slopes between modalities (package “lmertest”). The effect of the formulation on the time spent in each zone and the time spent touching the beads was evaluated using generalised linear mixed models (GLMM; family “gamma” package “dplyr”, “lme4”) with the date of the experiment as a random factor. For the A-K+ modality, for the time spent in each zone and the time spent touching the beads, a generalised linear model (GLM; “gamma” family, “dplyr” package, “lme4”) was carried out because the effect of the date was not significant. The effect of the formulation on the number of bites on the beads, grooming and burying under the beads was evaluated using generalised linear mixed models (GLMM; family “poisson” package “dplyr”, “lme4”) with the date of the experiment as a random factor.

Results

EPF selection

Biocidal tests on wireworms

Mortality of wireworms continuously exposed to fungi beads ranged from 45.1% to 70.4% (Figure 7). Survival curves of wireworms exposed to different fungi showed no significant differences compared with the control (log-rank test $\chi 2=10.14$, df=3, p=0.02). *Metarhizium* tended to have a greater impact on wireworm survival, although statistical significance was almost reached ($Z = 1.759$, $p = 0.079$).

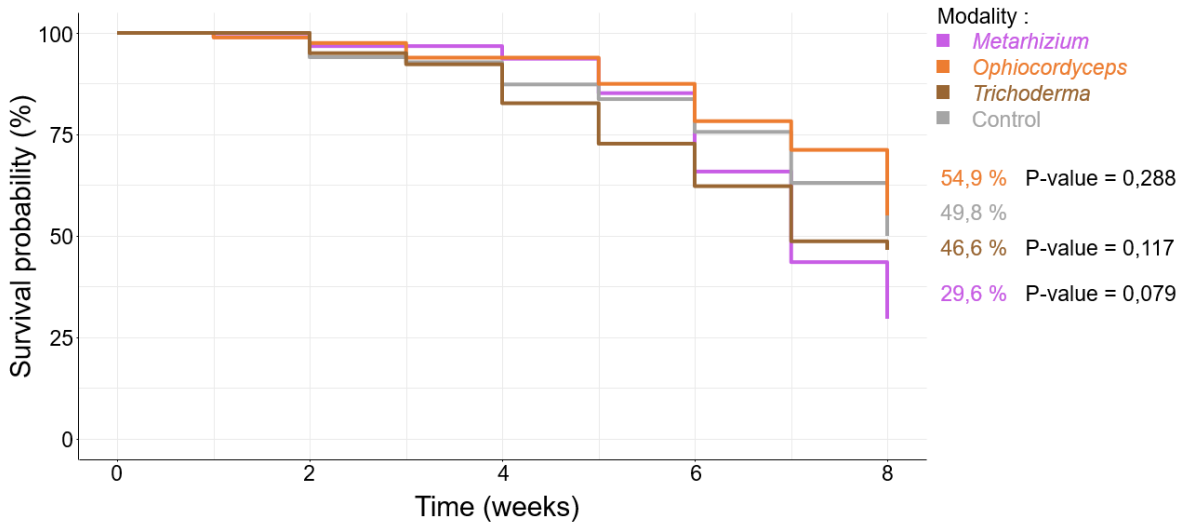


Figure 7: Probability of survival of wireworms in microcosms during continuous exposure to different fungi.

Wireworms' orientation bioassay

Over 60% of the tested wireworms made a choice, leaving the neutral zone and moving towards either the fungus or the control (Figure 8). Wireworms preferentially moved towards the *Trichoderma* fungus rather than the control (Z = 2.683, df = 36, p = 0.007). They were also attracted toward *Metarhizium* (Z = 2.331, df = 105, p = 0.021). Finally, wireworms were not significantly attracted or repelled by the odour of *Ophiocordyceps* (Z = -0.665, df = 35, p = 0.506).

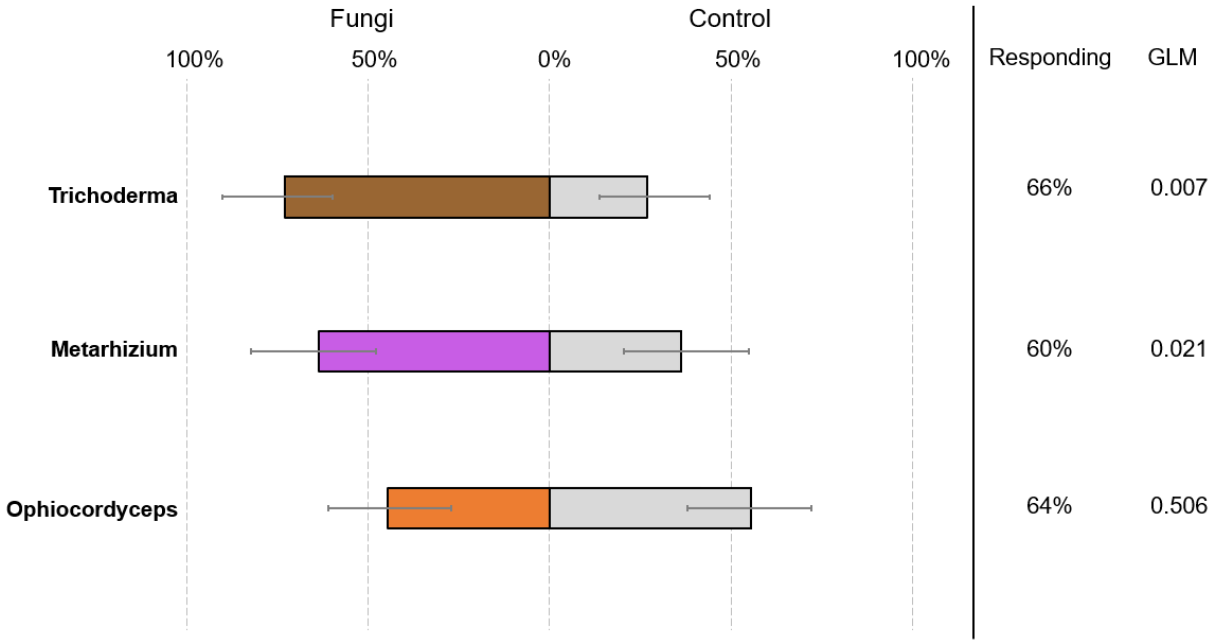


Figure 8: Percentage of wireworms reaching one side of the olfactometer during the dual-choice test. P values were determined by general linear models. The confidence intervals were estimated following a binomial calculation.

Volatolomic analysis

A total of 80 compounds were identified from the fungi analysed: *Trichoderma* (n=58), *Metarhizium* (n=64) and *Ophiocordyceps* (n=70). The volatile composition varies considerably between the studied fungi (Figure 9). Only few compounds account for more than a half of the relative amount of identified VOCs, belonging to the terpenes, aldehydes, alkanes and ketones.

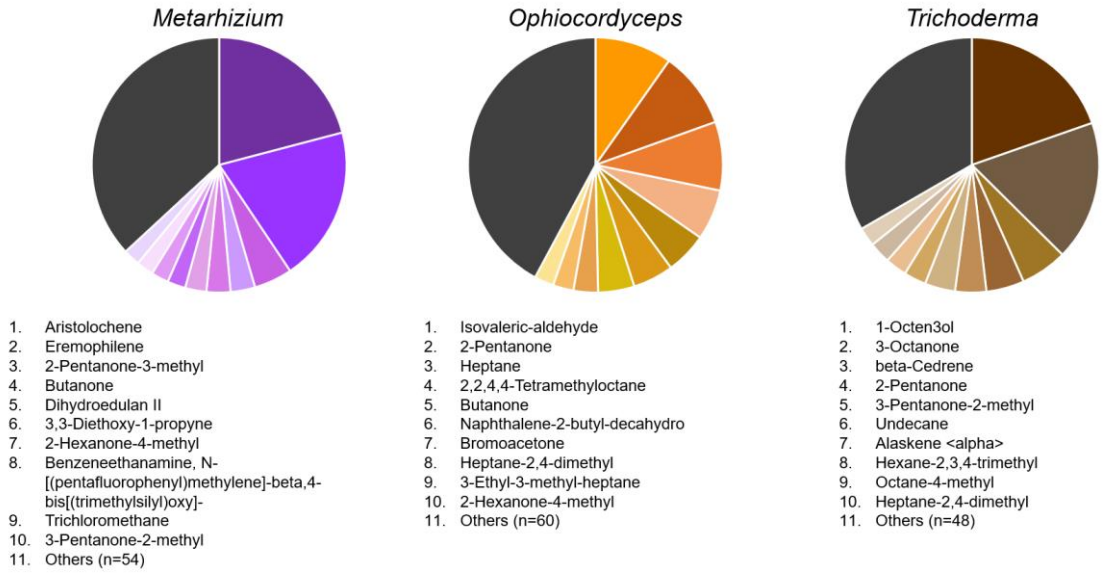


Figure 9: Distribution of the 10 most abundant volatile compounds emitted by each fungi, expressed as relative proportions.

PerMANOVA revealed highly significant differences in VOC profiles between the three groups of fungi ($F_{3,12}$ = 13.01, p = 0.001), indicating that fungi vary on the basis of their odour profiles (pairwise comparisons, all $p < 0.05$).

Discriminant analysis using PLS-DA was used to classify the fungus samples on the basis of their chemical characteristics. The model achieved an excellent accuracy of 100% (Kappa = 1), showed a high sensitivity (100% for all fungi) and specificity (100% for all fungi) with all fungi being correctly classified (Figure 10). The model explained 62% of the total variance in the data. This suggest that their chemical signatures are sufficiently distinct to be accurately discriminated.

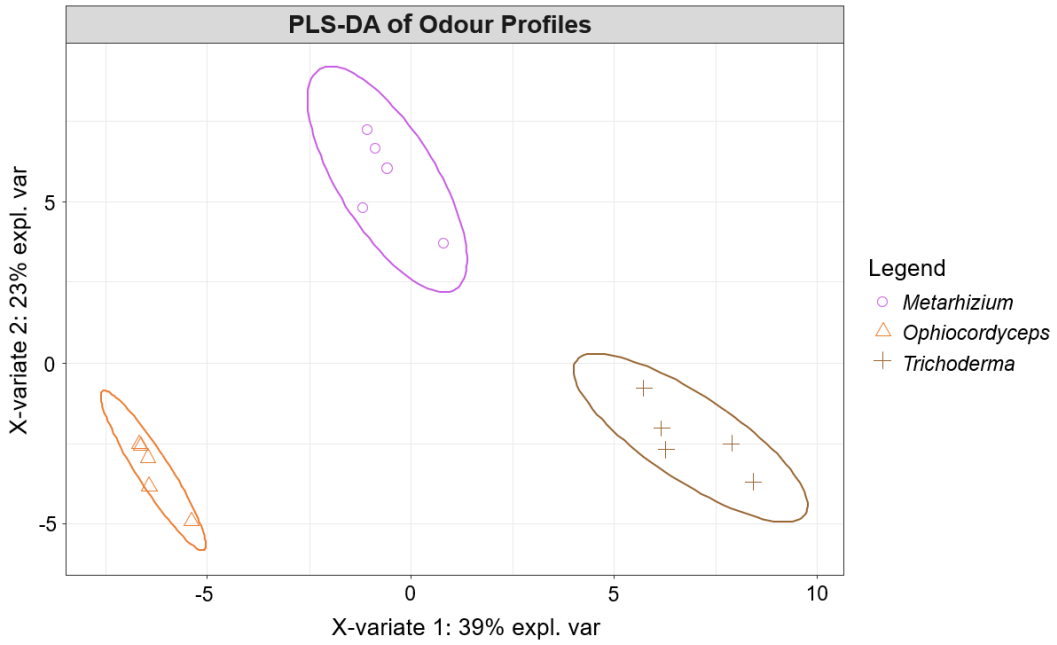


Figure 10: Discriminant analysis by PLS-DA of the three fungi based on their olfactory profiles.

A confidence coefficient is assigned to each VOC identified, indicating its degree of influence in the profile of each fungus. A higher coefficient indicates greater participation of the compound in discriminating the fungi from the other ones. The spider chart (Figure 11) provides insight into the number of compounds necessary for efficient fungi classification. *Metharizium* exhibits distinct compounds essential for their classification. The compounds Selinene alpha and Selina-5,11-diene have high coefficients (100% and 99.47%) in predicting *Metarhizium* profile. On the other hand, *Ophiocordyceps* and *Trichoderma* show no key compound, but their identification is linked by the simultaneous presence of multiple compounds.

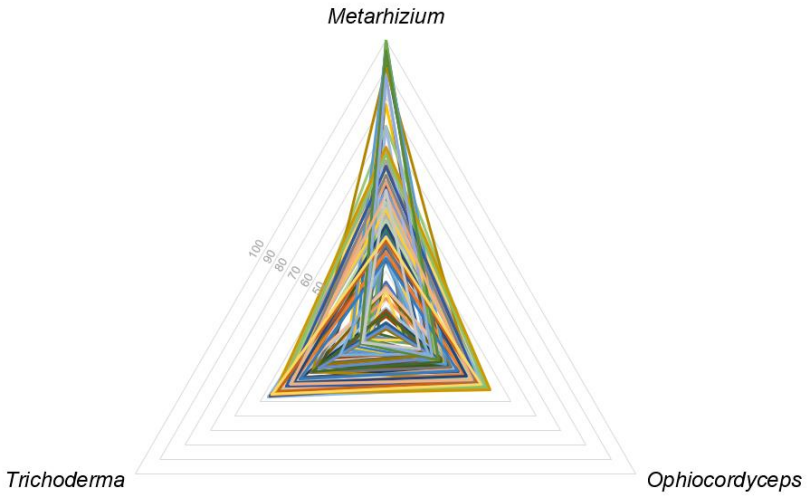
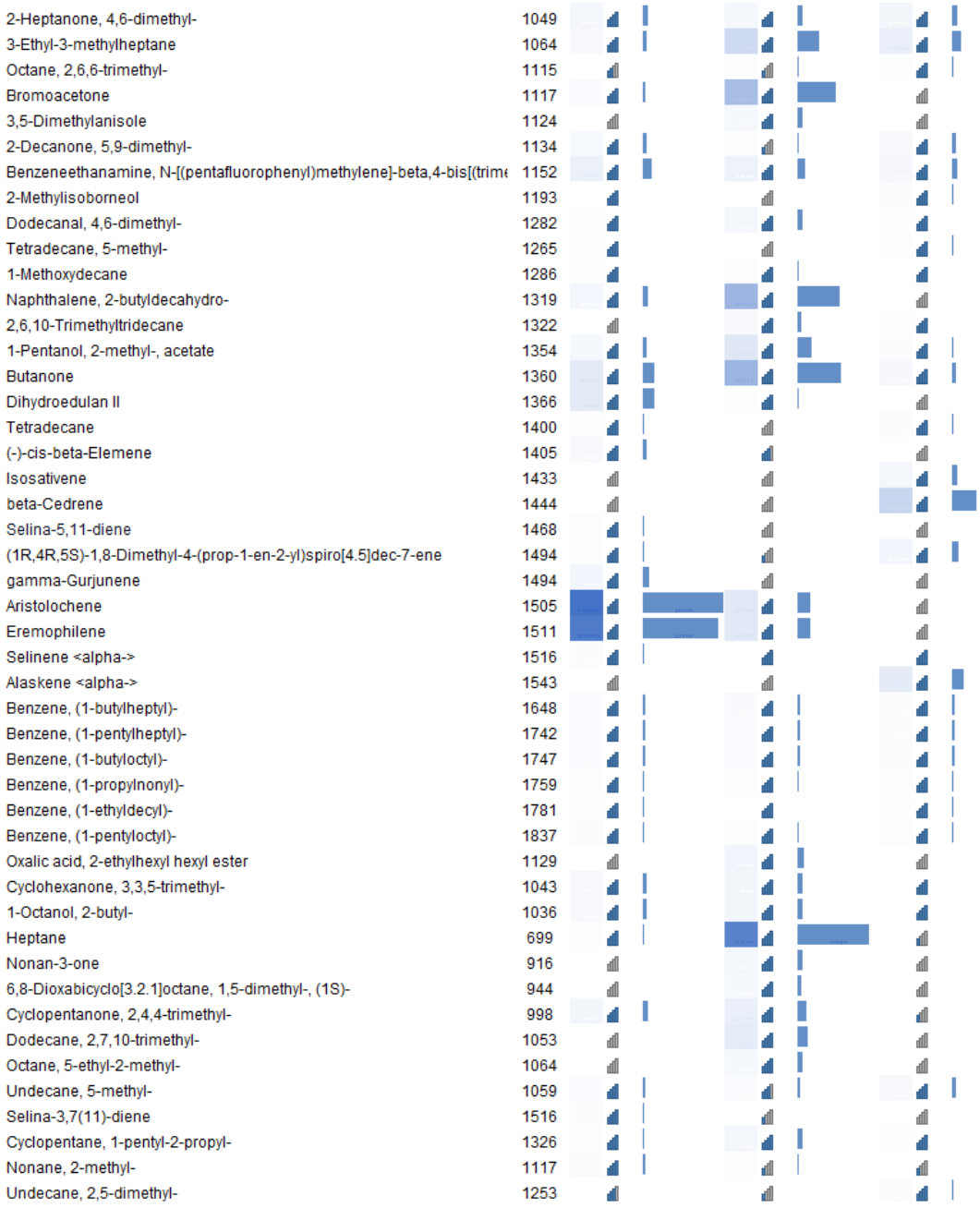


Figure 11: Detected compounds and their respective importance in the predicted models generated from the PLS-DA analyses. One colour correspond to one compound. The percentages on the axes represent the relative importance of each compound in the model prediction.

Metharizium fungus can be identified by the presence of aristolochene and eremophilene, which have a confidence coefficient of 95.93% and 96.93% and a high relative abundance of 20% and 19% respectively (Table 1). Similarly, two compounds allow to identify the odour profile of *Trichoderma*, the two main compounds 1-Octen-3-ol (20%) and 3-Octanone (18%) (Figure 9), which also have a high specificity. In contrast, three compounds characterize *Ophiocordyceps* odour profile from the other: Isovaleric aldehyde, the major compound representing 9% of the total profile, 2-Pentanone (9%) and Heptane (8%).

Various compounds have been identified as major compounds in several fungi, such as butanone, 2-hexanone-4-methyl, which are found in the profile of *Metarhizium* and *Ophiocordyceps*; 2-pentanone and heptane-2,4-dimethyl, which are found in *Ophiocordyceps* and *Trichoderma*; and 3-pentanone-2-methyl, which is found in the major compounds of *Metarhizium* and *Trichoderma*.

Table 1: Volatile organic compounds (VOC) profile of each modality. The specificity highlights the importance of the 80 compounds in the predicted profile provide by the partial least square analyses (dark blue: high specificity; light blue: low specificity). The occurrence represented by four rectangles highlight the number of times the VOC was identified in the replicates.



Effectiveness of *M. anisopliae*

Mesocosm trial

There is no impact of the tested formulation on the emergence of sugar beet plants (54% for F+W+, 58% for F-W+ and 60% for F-W-). There was no effect of the treatments on the sugar beet root dry mass (F+W+ *vs* F-W+: t.ratio = -1.123, df = 22.4, p = 0.510, F+W+ *vs* F-W-: t.ratio = -1.538, df = 19.9, p = 0.295, F-W+ *vs* F-W-: t.ratio = -0.646, df = 17.4, p = 0.797). Similarly, we found no effect of the treatments on the sugar beet leaf dry mass (F+W+ *vs* F-W+ : t.ratio = -1.392, df = 23.9, p = 0.361, F+W+ *vs* F-W- : t.ratio = -0.937, df = 22.7, p = 0.623, F-W+ *vs* F-W- : t.ratio = 0.169, df = 21.3, p = 0.984) (Figure 12).

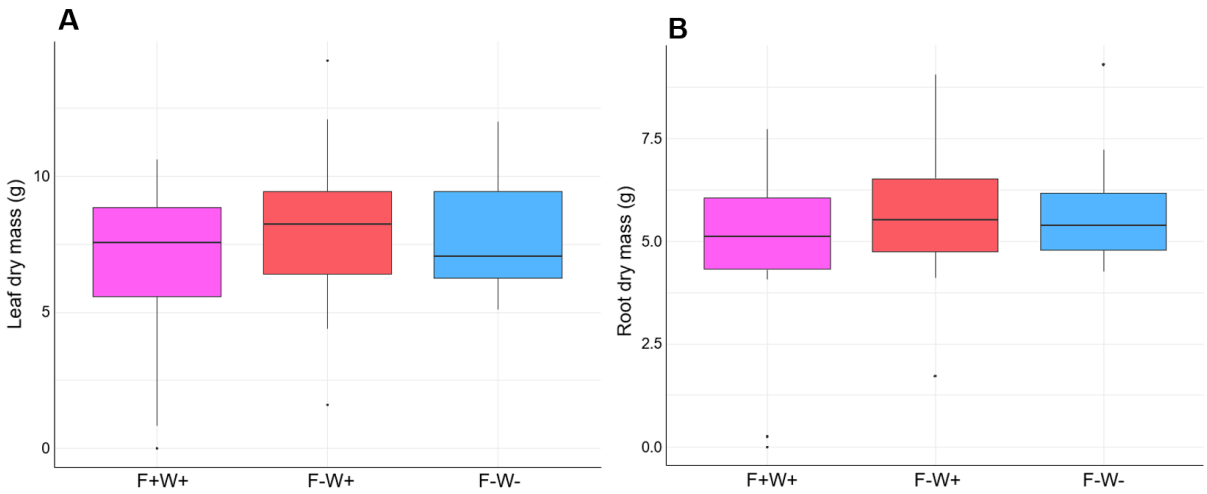


Figure 12: Leaf (A) and root (B) dry mass of sugar beet plants harvested in mesocosms for each treatment.

Field trial

The results showed a higher percentage of plants emerged in the control plots (73%) compared with the A&K formulation plots (68%; t = 3.088, p = 0.011). Among the emerged plants, the number of healthy plants was not significantly different between the control (95%) and formulation (90%) plots (Z = 0.823, p = 0.41). Comparisons between treatments show that the presence of the treatment results in a nearly significant difference in root dry weight (t= -1.804, df = 88.029, p = 0.074). However, in the presence of the A&K formulation, plant leaf dry weight was significantly higher than in the absence of the A&K formulation (t= -2.041, df = 88.119, p = 0.044; Figure 13).

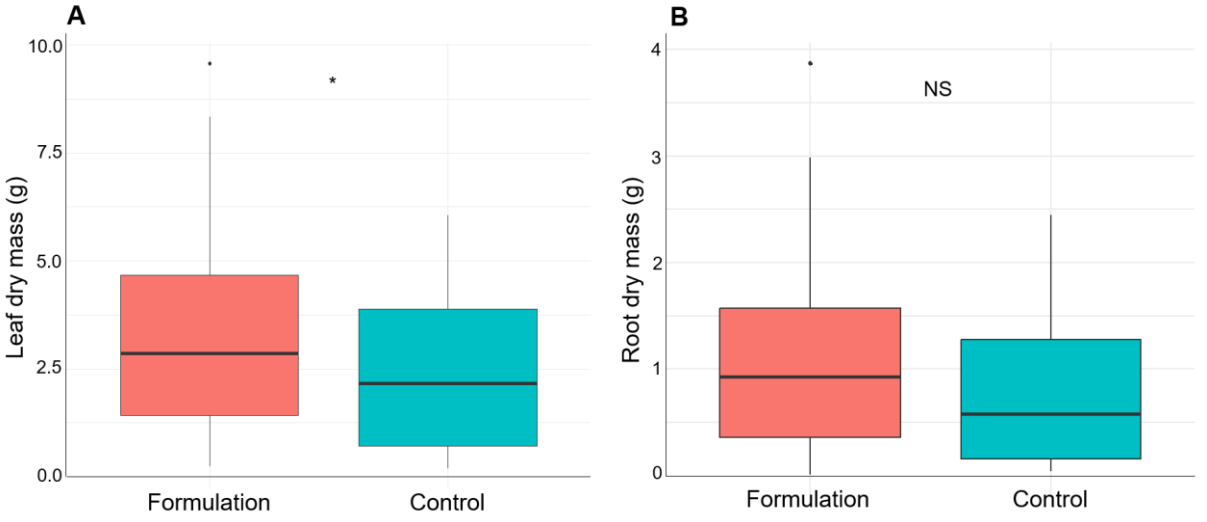


Figure 13: Leaf (A) and root (B) dry mass of sugar beet plants harvested in the field for each treatment.

Impact of the formulation on a non-target species (*Pterostichus melanarius*)

Innocuity

Observed mortality across all modalities ranged from 17% to 29%, and survival curves of carabid beetles exposed to the attract and kill formulation showed no significant differences compared with the control (log-rank test $\chi^2 = 0.23$, df = 3, p = 1; Figure 14). The mortality rate observed in the control condition (A-K-) was not significantly different from that observed in the presence of any formulation (A+K-, z = -1.546, p = 0.122; A-K+, z = -1.713, p = 0.087; A+K+, z = -1.221, p = 0.222). However, of the dead individuals, only 1.5% were found intact, due to a high rate of cannibalism observed in the plates (A-K- = 24%; A+K- = 16%; A-K+ = 26%; A+K+ = 16%). Sporulation was only observed on 3 wireworms in conditions A-K+ (n = 1) and A+K+ (n = 2). Carabid beetle eggs and larvae were found in the boxes even though they could not be sexed (Table 2). Statistical analyses could not be carried out, but the results showed twice as many eggs in the modalities with the fungus. The good sporulation of the fungus and its entomopathogenic nature were confirmed by continuous exposure of *T. molitor* for 21 days. The mortality rate of *T. molitor* after 21 days of continuous exposure was 47% for A-K-, 56% for A+K-, 100% for A-K+ and 98% for A+K+.

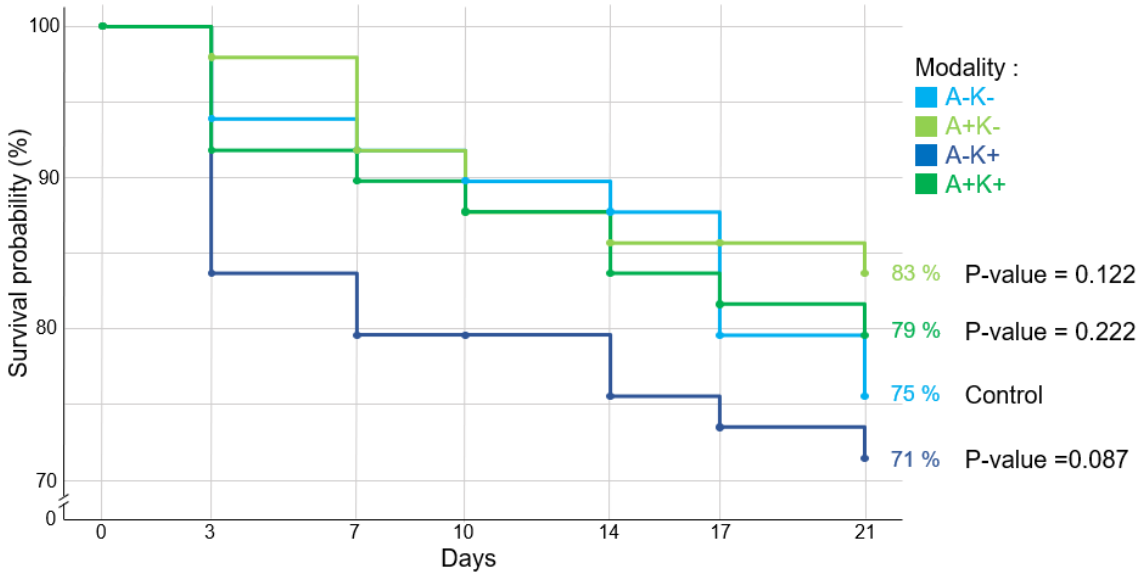


Figure 14: Survival probability of carabid beetles during the continuous exposure to different formulations.

Table 2: Average number of carabid beetle eggs and larvae found in the boxes by modality during continuous exposure.

	Day 14		Day 21	
	Eggs	Larvae	Eggs	Larvae
	mean±SD	mean±SD	mean±SD	mean±SD
A-K-	1.25±0.5	1.667±1.155	2.5±1.225	1±0
A+K-	0	1±0	2.6±1.140	1.333±0.577
A-K+	2.143±1.069	1±0	7.143±4.598	2.25±1.892
A+K+	1±0	1.5±0.707	7.571±5.798	1.75±0.957

During the 21-day continuous exposure, there was a variation in the weight of carabid beetles in all modalities (Figure 15). Results showed a significant effect of exposure duration on weight variation with an increase in weight on day 14 ($\beta = 0.031$, $p = 3.53 \times 10^{-6}$). There was a significant interaction effect between modalities and exposure duration at 14 days, with a significant increase in weight at this time point for all modalities compared to the control ("A+K- : Day 14", $\beta = 0.029$, $p = 0.002$; "A-K+ : Day 14", $\beta = 0.055$, $p = 1.60 \times 10^{-8}$; "A+K+ : Day 14", $\beta = 0.048$, $p = 5.93 \times 10^{-7}$). In contrast, no significant interaction was observed for days 7 and 21 (all $p > 0.05$). Multiple comparisons showed a significant effect of the treatment on the average weight of carabid beetle at 14 days of exposure. Carabid beetles' weight was significantly higher when exposed to both A-K+ and A+K+ compared to A-K- ("A-K+": t ratio = -7.616, $p < 0.001$; "A+K+": t ratio = -6.773, $p < 0.001$). Additional differences were detected with carabid beetles showing significantly higher weight when exposed to A-K+ and A+K+ compared to A+K- ("A-K+": t ratio = -4.693, $p < 0.001$; "A+K+": t ratio = -3.771, $p = 0.016$). There was no significant difference between A+K- and A-K- at day 14 (t ratio = -2.996, $p = 0.173$).

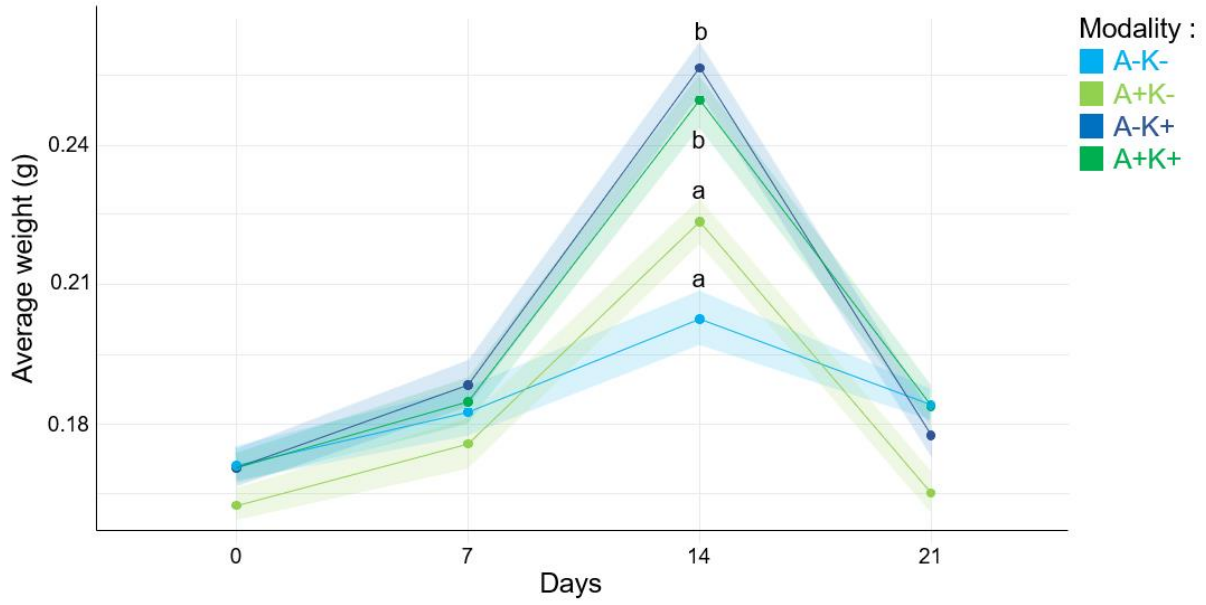


Figure 15: Weight evolution of carabid beetles during the continuous exposure to different formulations. The variance is represented by the transparent areas behind the curves. Letters assigned denote significant differences between treatments for day 14, determined by emmeans pairwise post hoc multiple comparisons.

Overall analysis of the time taken for a carabid to leave a circle 7 cm in diameter, used as an indicator of morbidity, revealed no significant effect of modality on carabid speed (F-statistic = 1.452, df = 667, p = 0.1175) (Figure 16).

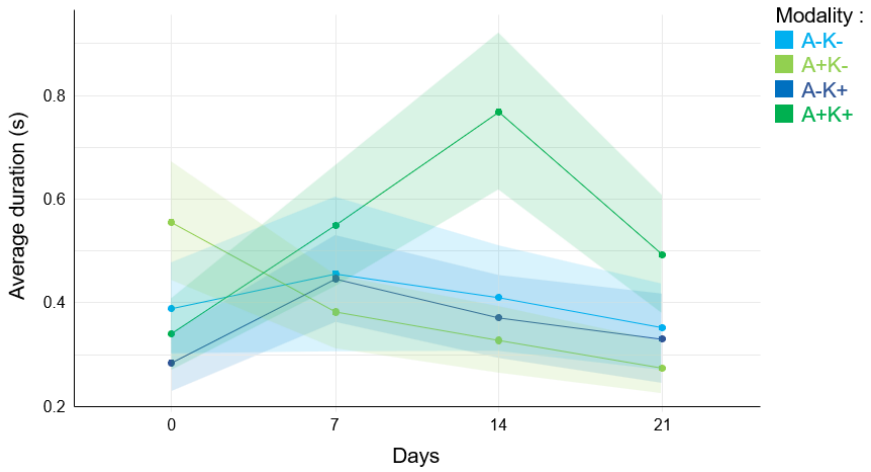


Figure 16: Average duration for a carabid beetle to emerge from a circle during the continuous exposure to different formulations. The variance is represented by the transparent areas behind the curves.

Behaviour

Carabid beetles spent significantly more time in the A+K- zone than in the A-K- zone (p = 0.003 (Table 3). In contrast, this duration was not significantly different for all the other modalities tested (all p > 0.05, table 3). Regarding the 'bead-biting' behaviour, carabid beetles bite significantly more A+K- and A-K+ beads than A-K- beads (A+K- *vs* A-K- : p = 0.049 ; A-K+ *vs* A-K- : p = 0.043). They also did more antennal grooming when they were close to A+K+ beads compared to A-K- beads (p < 0.005). Regarding the time spent touching the beads and the burying of carabid beetles in the beads, there was no significant difference among the three modalities tested (all p > 0.05).

Table 3: Results of carabid beetle behaviour analysis. Values in bold represent the experimental modality (A+K+, A-K+, A+K-), while values in normal type indicate the control (A-K-). For durations, the left-hand column represents the mean ± standard error (SE) and for occurrences it represents the mean. The right-hand column shows the associated p-value obtained by GLMM or GLM.

	Time per zone (duration in seconds)		**Touching (duration in seconds)**		**Biting**		**Grooming**		**Burying**	
A+K+	**16.9±2.6**	0.168	**22.8±6.15**	0.975	**6.57**	0.187	**7.33**	< 0.005	**1.5**	0.707
A-K-	14.4±2.5		23.2±8.35		6.25		2.67		1	
A+K-	**16.5±2.4**	0.003	**18.6±5**	0.872	**6.57**	0.049	**3**	0.058	**3**	0.378
A-K-	11.9±1.9		23.0±11.4		5.7		3.67		1.5	
A-K+	**15.0±2.6**	0.655	**18.1±6.7**	0.892	**6.33**	0.043	**4.75**	0.173	**3**	0.333
A-K-	16.7±2.6		19.5±7.3		6.5		2.5		3.5	

Discussion

The primary objective of this section was to select a fungus effective against wireworm. The results showed that the mortality of wireworms varied according to the fungus tested, and that *Metarhizium anisopliae* NEW (MAN) proved to be the most effective in killing wireworms, justifying its selection for future studies. These results are consistent with Kölliker et al. (2011) who showed that the efficacy of *M. anisopliae* reached 97% against *A. obscurus*, but was considerably less virulent against *A. lineatus* and *A. sputator*. Ansari et al. (2009) also demonstrated that *M. anisopliae* can cause mortality in *A. lineatus*; however, the mortality rate varied depending on the EPF isolates. In our experiment, we observed a mortality rate of 70.4% for wireworms exposed to MAN. This *M. anisopliae* strain was isolated from a dead wireworm, which may reflect a close and specific relationship between this fungus and its natural host, as supported by previous results showing that entomopathogenic fungi tend to exhibit high virulence against the hosts from which they were originally isolated (Chandler, 1992; Altre et al., 1999; Pilz et al., 2007). As well as having good pathogenicity, MAN was attractive to wireworms, which can be explained by the volatolomics. *M. anisopliae* NEW is characterized by the predominance of the sesquiterpenes aristolochene and eremophilene, standing out for their role as toxins and bioregulators, as key metabolites produced by filamentous fungi (Barton et al., 1999; Jeleń, 2002). Bojke et al. (2018) compared the volatile compounds released by *Metarhizium anisopliae* and reported the presence of several alcohols, with 1-octen-3-ol being the most abundant and other notable compounds such as 2-octen-1-ol, octanol and hexanol. Among the ketones, 3-octanone and 1-octenone were the most abundant in the mycelium of *Metarhizium anisopliae.* However, no studies have explored their possible role in attracting wireworms.

Regarding *O. sporangifera* A (OSA), Seib et al. (2024) and Ansari et al. (2009) reported that Cordyceps strains had no pathogenic effect against wireworm larvae. Our results partially fit into this observation, with no influence of OSA on short-term mortality was observed. However, we hypothesise that this fungus could alter wireworm behaviour in the longer term. Indeed, certain species of Cordyceps are known for their ability to manipulate the behaviour of their hosts (Hughes et al., 2016; de Bekker, 2019). It is conceivable that *O. sporangifera* could induce behavioural changes in wireworms that were not measured in this study, but which could be relevant to crop protection, such as a change in foraging behaviour. However, the absence of orientation behaviour of wireworms exposed to OSA may be attributed to the absence of dominant compounds in its volatolomic profile. However, the presence of isovaleraldehyde, a more generic compound characteristic of fruits such as apples, reflects a chemical strategy that is probably adapted to interaction with a diversity of organisms in varied environments (Biard, 2009). The laboratory conditions of this study simplify natural ecosystems, where many factors could influence wireworm behaviour.

The non-entomopathogenic fungus *Trichoderma harzianum* was attractive for wireworms and has a distinct chemical profile marked by specific volatile organic compounds (VOCs). *Trichoderma* is characterised by 1-octen-3-ol and 3-octanone which could play a crucial role in attracting wireworms. These compounds are known for their olfactory properties, which can attract various organisms, such as

entomopathogenic nematodes (*Steinernema carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora*) at low concentrations (Tonks et al., 2023). On the other hand, a repellent effect was observed on *D. suzukii*, *Cornu aspersum* Muller (garden snail) and *Derocerus reticulatum* Muller (grey field slug) (Cini et al., 2012; Khoja et al., 2019). Moreover, 3-octanone is an alarm pheromone in ants (Hughes et al., 2001) and is also associated with fungal aromas (Lee et al., 2016). 1-octen-3-ol is a volatile alcohol commonly produced by fungi, known for its characteristic mushroom-like aroma (Zawirska-Wojtasiak, 2004). The attraction of wireworms to *T. harzianum* could be a consequence of their search for food sources. Further studies in real-world environments would help confirm these findings.

After being selected, the effectiveness of *M. anisopliae* NEW combined with an attractant in an A&K formulation was tested in a more complex environment in the presence of sugar beet plants and wireworms. However, we showed that the formulation in semi-controlled environments, had no significant impact on the emergence of sugar beet plants or on their dry biomass, either for leaves or roots. Firstly, plant development was strongly affected by the presence of leaf miners, which caused damage to the plant canopy. These insects are known to reduce root growth and sugar beet yield by reducing photosynthesis (Hillman, 2022). In addition, the boxes used for the experiment had been pre-filled several months before sowing, resulting in significant soil compaction. This situation hampered the germination and development of the plants, regardless of the treatment applied. As shown by Draycott et al. (1970), compacted soil can reduce seedling density and root yield, which is consistent with our low emergence rates. In addition, soil compaction may also have masked the potential effects of our formulation on wireworms. Indeed, Razinger et al. (2020) have shown that this parameter directly affects the behaviour and populations of wireworms. An earlier harvest, at BBCH 12, might have allowed the first signs of wireworm damage to the roots to be detected (Viric Gasparic et al. 2021). These results highlight the complexity of bio-insecticide use and underline the need for future research to optimise experimental conditions while exploring the interactions between biotic and abiotic factors influencing formulation efficacy.

The difference observed in leaf dry weight in the field, confirms the potential efficacy of our formulation. This results are in line with Kabaluk (2018) and Kabaluk (2008) whose previously demonstrated that the use of *Metarhizium anisopliae* increased yield and reduced damage to potato tubers and carrots. Similarly, Kabaluk et al. (2007) showed that treating maize seed with *Metarhizium anisopliae* (F52) conidia significantly increased plant emergence density and yield. The choice of phenological stage for evaluation (BBCH 19-20) may have limited the ability to detect the effect of wireworm damages on roots. Indeed, the major damage caused by *Agriotes* spp. larvae generally occurs at earlier stages (BBCH 12), where it causes crop thinning and reduced yield (Viric Gasparic et al., 2021). An analysis carried out at a more juvenile stage could have provided more accurate information on the direct impact of the formulation on root health and wireworm damage. The reduction in root damage could also be linked to suppression of *Rhizoctonia solani*, a pathogen present in the trial plot. Mimma et al. (2023) showed that *M. anisopliae* (MetA1) improves plant growth while reducing the incidence of *R. solani* through biocontrol mechanisms and stimulation of

plant defences. These results highlight that the impact of MAN in our study could go beyond simple control of wireworms. However, certain limitations of our study need to be recognised: the number of samples collected could be increased to fully capture the variability of the data. On the other hand, it would be relevant to examine the condition of the roots in more detail, particularly the bite marks at an earlier stage. A reduction in the number of bites in the presence of the formulation could indicate a reduction in damage, which would be an interesting avenue to explore. In addition, the hypothesis that the fungus exerts a direct biostimulant effect on the plant deserves to be tested in in-depth greenhouse studies. These trials would make it possible to assess the interactions between the plant and the fungus in the absence of the insect pest. The formulation shows promising potential for stimulating beet leaf growth and limiting pests, justifying further research at juvenile stages.

Another aim of this study was to verify the safety of the formulation developed on carabid beetles, as well as its impact on the behaviour of this non-target species. The formulation had no impact on the mortality of carabid beetles and these conclusions are consistent with the work of Kabaluk et al. (2005), who found no mortality attributable to *Metarhizium anisopliae* in several carabid species, including *Pterostichus melanarius*. This species' resistance to conidia could therefore explain the low mortality rate observed in our study, despite prolonged exposure to the fungus. However, a slight side effect of fungal exposure was observed, with weight variation occurring between days 14 and 21. This variation could be explained by physiological processes, such as a higher egg-laying by the females between those days, leading to a reduction in weight after a peak in weight gain. Our results also showed that the formulation did not alter the locomotor behaviour of the carabid beetles. However, our experiments had their limitations. Firstly, the cannibalism observed in the boxes (up to 26% in certain conditions) complicates the interpretation of the results on mortality and morbidity. This phenomenon could be minimised in future studies by isolating the carabid beetles in individual boxes, although this would preclude the study of reproduction or social interactions.

The behavioural test on carabid beetles enabled us to examine the safety of the product for these insects and to determine whether the formulation could alter their behaviour when in contact with it. Carabid beetles spent significantly more time in the area containing the attractant, suggesting that the attractant (i.e. potato juice) targeting wireworms exerts a significant attractive effect on the carabid beetles. This supports previous observations that certain attractants can influence the spatial movements of insects. For instance, *Pterostichus melanarius* females are attracted by the combined odour of cabbage and white clover (Tréfás et al. 2001). Carabid beetles were not specifically attracted or repelled in the presence of the entomopathogenic fungus. Notably, beetles chewed more frequently on control beads (A-K-) than on treated beads (A+K-, A-K+), suggesting an aversion or reduced interest in treated beads. This behaviour may result from chemical or olfactory signals associated with the formulation composition, potentially altering beetle feeding responses. These results are in line with those of similar studies which have shown that the addition of certain attractive or repellent compounds to the insects' environment can modify their foraging behaviour. McKemey et al. (2004) demonstrated that *Pterostichus melanarius*

is able to detect dead slugs, highlighting its sensitivity to olfactory signals. Analysis of antenna cleaning behaviour revealed a significantly higher frequency of cleaning in the fungus and attractant zone compared with the control zone. This response could be interpreted as the insect reacting to an unnatural or potentially harmful stimulus, such as contamination or chemical interaction with the fungus. The results, although interesting, are disparate and unexpected, particularly regarding the combined effect of the fungus and the attractant. This study opens promising prospects for future research, as in-depth exploration of the behavioural and physiological responses of non-target species at larval stages could provide essential information for optimising bio-insecticide formulations. Indeed, although the bio-insecticide was tested here on adult carabid beetles, it would be relevant, in future experiments, to conduct safety tests with continuous exposure on carabid beetle larvae. These larvae evolve in the soil, in the same place as the wireworms, and are therefore directly exposed to the formulation. In addition, during the continuous exposure of adult carabid beetles, carabid beetle eggs and larvae were observed in the substrate, although the individuals were not sexed. These observations suggest the feasibility of collecting and testing these larvae to assess their sensitivity to the formulation, which is an experimental prospect to be explored. In conclusion, although the bio-insecticide does not affect mortality, its influence on behaviours, such as a preference for areas containing the fungus-free attractant, highlights the importance of investigating potential side effects on non-target species.

The results obtained confirm the promising potential of the attract and kill formulation developed in the laboratory, particularly with the use of *Metarhizium anisopliae* NEW. To maximise the effectiveness of the formulation, several options could be considered in the future. A first step would be to explore the combination of several entomopathogenic fungi in a single formulation for greater overall efficacy. Research has already demonstrated the successful association of entomopathogenic nematodes with fungi (Shapiro-Ilan et al., 2004; Bueno-Pallero et al., 2018), leading to the hypothesis that the integration of several entomopathogenic fungi could also enhance their ability to control wireworms. The identification of new attractant compounds through our volatolomic analyses could further improve the A&K formulation by potentially improving the attraction of wireworms and thus its effectiveness in controlling wireworms. In addition, long-term studies are needed to validate the durability of the results obtained. Field tests to assess the effectiveness of the attract and kill formulation should be carried out over several cropping seasons, in order to gain a better understanding of the impact of environmental and agricultural factors on the effectiveness of the formulation. To get closer to real field conditions, it would be relevant to conduct experiments with non-sterile soil, incorporating a diversity of natural micro-organisms. This would make it possible to assess the interaction between the fungus and the soil microbiome. In addition, the interaction between the entomopathogenic fungus and the plant should be studied in greenhouse trials. The objectives of this study have been achieved, paving the way for promising new research. In terms of fundamental research, future work could explore in greater depth the interactions between EPFs and their hosts via VOCs. In terms of applied research, these results will provide opportunities to develop and optimise formulations for integrated pest management (IPM).

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Annexes

Annex 1: Classification performance metrics for the PLS-DA models based on the odour profile (Confusion Matrix)

Reference	Metarhizium	Ophiocordyceps	Trichoderma
Metarhizium	5	0	0
Ophiocordyceps	0	5	0
Trichoderma	0	0	5
Sensitivity	1	1	1
Specificity	1	1	1

Annex 2: Personal contribution

During these eight months, I carried out a large number of varied tasks, either independently or with the help of Fanny Ruhland, my supervisor during this end-of-studies project. To begin with, I completed extensive bibliographical research on the ecology of wireworms, entomopathogenic fungi and biological control methods. I also played an active role in the rearing of wireworms and their collection in the field, both at the larval and adult stages. I also developed a carabid beetle and mealworm breeding system to use in my innocuity tests. These rearing operations, which require a considerable amount of time, were conducted in parallel with the other tests done throughout the TFE.

I prepared the potato juice-based attract beads myself, which were needed for different types of tests: mesocosms, field tests, behavioural tests and innocuity tests. The fungi beads, as well as the strains used, were produced in Louvain-la-Neuve by Ismahen Lalaymia.

The biocidal tests on wireworms were carried out in Louvain-la-Neuve and monitored by Ismahen Lalaymia. However, the raw data was sent to me and I analysed it myself. I carried out the orientation tests, with the help of Fanny Ruhland and Andrea Chacon-Hurtado, who were my technicians during these two intensive days of work. I then collected and processed the data myself. The development of the odour sampling method had been done by Fanny Ruhland before the start of my TFE. I prepared the odour samples: filling the olfactometers with the soil and the fungi beads, integrating the chromatographs on GC/MS, using GC Aligner, creating a matrix on Excel, and processing the data statistically on RStudio.

For the mesocosm tests, although the protocol had already been established by Fanny Ruhland, I set up the experiment: planting the sugar beet, introducing the wireworms and applying the ‘attract and kill’ formulation. I collected and processed the data from the mesocosms. For the field trials, I drew up the experimental design and drafted the protocol, which were then validated by Fanny and Mr Verheggen. I visited the field several times to place the formulation and then harvest the sugar beet plants and the data. The data was also statistically processed in Rstudio by myself, following Fanny and Mr Brostaux's advice on the statistical tests to be run.

For the carabid beetle tests, I designed the protocol based on scientific articles on similar experiments. The protocol was validated and improved by Fanny Ruhland and Mr Verheggen.

I then implemented the whole experiment, collected the various data over several weeks and processed them in RStudio. For the behavioural tests, I designed the protocol, which was then validated, recorded the 120 videos, which I then analysed using BORIS software before processing the data on RStudio.

Finally, I wrote my report in parallel with the experiments. My writing was first reviewed by Fanny Ruhland, then validated by Mr Verheggen.