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Preface

This master thesis was carried out in collaboration between the Laboratory of Chemical and Behavioural Ecology, Gembloux Agro-Bio Tech, TERRA, University of Liege, and the Mycothèque of the Université catholique de Louvain (BCCM/MUCL, Belgium). The work was conducted within the framework of the Trans-Pest project, which is part of the Biocontrol 4.0 project portfolio, funded by the Interreg VI France-Wallonie-Vlaanderen cooperation program. This master thesis is presented in article format. The formatting rules applied follow those of the Biocontrol Science and Technology journal.

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I would first like to express my sincere gratitude to Professor François Verheggen for welcoming me into his laboratory and giving me the opportunity to carry out this master's thesis. I am especially grateful to him for believing in this project, which aims to develop new control strategies that could provide farmers with additional tools to manage the Colorado potato beetle. I also wish to thank him for the time he devoted to the many discussions surrounding this project and for his careful review of the manuscript.

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I would also like to thank the Mycothèque of the Université catholique de Louvain, as well as Professor Stephan Declerck, for their collaboration in this work. I would like to express my special gratitude to Ismahen Lalaymia, who taught me the fundamentals of microbiology and guided me through the implementation and development of the entire section devoted to entomopathogenic fungi. Her availability, patience, and expertise were invaluable throughout this important stage of the thesis.

I would also like to thank my fellow thesis students, Victor, Louise, Juliette, and Éloïse. Without you, this experience would not have been the same. It is often said that the best ideas emerge during coffee breaks, and I believe this proved especially true over the past six months. Thank you for the discussions, the shared moments of doubt, the encouragement, and the many enjoyable moments that made this work far more pleasant to carry out.

It would be impossible to write these acknowledgements without thinking of my uncle, whom I would like to thank for passing on to me his passion for agriculture, and especially for potatoes. Since I was twelve years old, you have taught me an immense amount of knowledge and practical skills related to this crop, which undoubtedly inspired me to pursue these studies and ultimately complete my academic journey with a thesis focused on an issue directly connected to it. The many hours spent on the farm have given me a form of knowledge that no book can truly provide, knowledge that is learned above all in the field, alongside passionate and experienced people.

Finally, I would like to end these acknowledgements with what matters most: my family. I thank my grandparents for their support and for always encouraging me to challenge my assumptions and continue improving. I also thank my father and mother for the same reasons, as well as for

their constant support throughout my studies, including during the difficult examination periods. Lastly, I thank my girlfriend, Juliette, for supporting me in the projects I pursue today and in those we hope to build together in the future. Your support has undoubtedly been of immense importance to me.

Résumé

Le doryphore de la pomme de terre, *Leptinotarsa decemlineata*, demeure l'un des ravageurs les plus destructeurs de la culture de la pomme de terre à l'échelle mondiale et a développé une résistance à de nombreux insecticides, rendant nécessaire le développement de stratégies de lutte alternatives. Dans le cadre d'une première étape vers le développement d'une stratégie « attract-and-kill » basée sur des billes d'alginate, nous avons évalué des candidats sémiochimiques et des champignons entomopathogènes pour la gestion de ce ravageur. Quatre attractifs potentiels, comprenant des extraits de pomme de terre et des mélanges synthétiques de composés organiques volatils, ont été testés au moyen d'essais comportementaux en arène sur des adultes, tandis que quatre souches de *Metarhizium* et une souche de *Beauveria* ont été évaluées pour leur virulence. Aucun des attractifs testés n'a induit d'attraction significative après encapsulation, indiquant que l'optimisation de la composante attractive reste nécessaire. En revanche, la virulence des champignons variait fortement selon les souches. Une souche belge de *Metarhizium anisopliae* a provoqué une mortalité cumulée de 97 % avec un LT50 de 6 jours, tandis que *Beauveria bassiana* a atteint une mortalité de 90 % avec un LT50 de 11 jours. Dans les conditions de laboratoire, ces deux souches ont agi plus rapidement que le témoin positif traité avec un insecticide. Globalement, cette étude apporte une preuve de concept pour la composante fongique de la stratégie « attract-and-kill » et identifie une souche locale de *M. anisopliae*, hautement virulente, comme un candidat prometteur pour un développement ultérieur. Les travaux futurs devront se concentrer sur la quantification de la libération des composés volatils à partir des billes d'alginate, l'évaluation de nouveaux sémiochimiques, notamment en combinaison avec la phéromone d'agrégation, ainsi que la validation de la formulation complète en conditions de terrain.

Mots-clés : *Leptinotarsa decemlineata*, biocontrôle, bille d’alginate, *Metarhizium anisopliae*, tests comportementaux.

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Development of an “Attract & kill” control strategy for Colorado potato beetle: integrating semiochemicals and entomopathogenic fungi

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Abstract

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, remains one of the most destructive potato pests worldwide and has evolved resistance to numerous insecticides, creating an urgent need for alternative control strategies. As a first step toward the development of an alginate bead-based attract-and-kill strategy, we evaluated candidate semiochemicals and entomopathogenic fungi for CPB management. Four candidate attractants, including potato-derived extracts and synthetic volatile blends, were tested in behavioural arena assays with adult CPB, while four fungal strains of *Metarhizium* and one of *Beauveria* were screened for virulence. None of the tested attractants induced significant attraction after encapsulation, indicating that further optimisation of the attractive component is required. In contrast, fungal virulence differed markedly among strains. A Belgian *Metarhizium anisopliae* strain caused 97% cumulative mortality and showed a short LT50 of 6 days, whereas *Beauveria bassiana* reached 90% mortality with an LT50 of 11 days. Both strains outperformed the insecticide positive control in terms of speed of action under laboratory conditions. Overall, this study provides proof of concept for the fungal component of the attract-and-kill strategy and identifies a highly virulent, locally relevant *M. anisopliae* strain as a promising candidate for further development. Future work should focus on quantifying volatile release from alginate beads, evaluating additional semiochemicals, including combinations with the aggregation pheromone, and validating the complete formulation under field conditions.

Keywords:

***Leptinotarsa decemlineata*, biocontrol, alginate bead encapsulation, *Metarhizium anisopliae*, behavioural assay**

1. Introduction

Despite more than a century of intensive chemical control, the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (*Coleoptera*: *Chrysomelidae*), remains the most devastating defoliating insect pest of potato (*Solanum tuberosum* L.) (Hare, 1990; Weber,

2003). Severe infestations can lead to yield losses of up to 80% when left uncontrolled, highlighting the major agronomic threat posed by this species (Sablon et al., 2013; Eski et al., 2022). Native to Mexico, the CPB spread globally throughout the 20th century and is now considered the most widespread insect pest of potato (Sablon et al., 2012; Eski et al., 2022). Its remarkable capacity to defoliate crops, combined with its high reproductive potential and adaptability, makes it particularly difficult to manage (Alyokhin et al., 2022). In late spring, overwintered adults emerge from the soil and colonise potato fields, initially by walking and subsequently by flight if suitable host plants are not found nearby (Harcourt, 1971; Weber, 2003). Importantly, the CPB life cycle involves both aboveground and belowground stages, with larvae entering the soil to pupate and adults overwintering underground (Hare, 1990; Weber, 2003). After mating, females lay clusters of approximately 20-30 eggs on the underside of potato leaves (Harcourt, 1971). Larvae develop through four instars over 3-4 weeks, feeding intensively throughout this period (Walgenbach and Wyman, 1984; Boiteau and LeBlanc, 1992). A single larva can consume up to 40 cm^2 of foliage during its development, while adults ingest approximately 10 cm^2 per day (Ferro et al., 1985). Beyond its destructive feeding behaviour, the CPB has a high reproductive potential: females lay 300-800 eggs over their lifetime, and one to three generations may develop per year depending on climatic conditions (Harcourt, 1971; Ferro et al., 1985; Walgenbach and Wyman, 1984). Climate change is expected to exacerbate this issue, as modelling studies suggest that warming conditions will increase the frequency of years allowing the completion of two generations in regions where the pest is already established, thereby enhancing population growth and spread (Pulatov et al., 2014). Although potato is its primary host, the CPB feeds on a wide range of *Solanaceae*, including tomato, pepper, eggplant, and wild nightshades. These alternative hosts may serve as reservoirs that sustain populations between cropping seasons and facilitate the spread of infestations (Eski et al., 2022).

The management of the CPB has historically relied predominantly on chemical control (Casagrande, 1987; Eski et al., 2022). The use of insecticides began as early as 1864 and remains the primary control strategy in many regions today (Mota-Sanchez et al., 2006; Eski et al., 2022, Chiarini et al., 2026). However, intensive and repeated applications have led to the rapid evolution of resistance, which now represents a major constraint in CPB management (Alyokhin et al., 2008; Zichová et al., 2010; Szendrei et al., 2012; Kadoić Balaško et al., 2020). Known resistance mechanisms in the CPB include enhanced metabolic detoxification, target-site insensitivity, reduced cuticular penetration, increased excretion, and behavioural avoidance (Alyokhin et al., 2008). To date, CPB populations have developed resistance to 57 insecticidal compounds across all major chemical classes, including organophosphates, carbamates, pyrethroids, neonicotinoids, and spinosad (Michigan State University Arthropod

Pesticide Database, 2026). Beyond resistance issues, environmental and toxicological concerns associated with broad-spectrum insecticides have led to regulatory restrictions, particularly within the European Union, where several organophosphates, carbamates, and neonicotinoids have been limited or banned (Marchand and Robin, 2019). As a result, the number of approved active substances is steadily declining, further narrowing the chemical options available for CPB management (Konopická et al., 2024). Taken together, the widespread development of resistance and the progressive reduction in available chemical tools highlight the urgent need to develop sustainable and effective alternative strategies for CPB management. This trend is further reflected by the temporary emergency authorisation of an RNA interference (RNAi)-based insecticide for CPB management in Belgium, which illustrates the growing interest in innovative control technologies (SPF Santé publique, 2026).

Although cultural practices such as crop rotation can reduce infestations (Sexson and Wyman, 2005), their effectiveness is limited by the strong dispersal capacity of adults, which can fly over distances exceeding one kilometre (Huseth et al., 2012). Biological control using natural enemies, including predators and parasitoids, has shown potential (Sablon et al., 2012). Microbial control agents such as *Bacillus thuringiensis*, entomopathogenic fungi (EPF) and entomopathogenic nematodes are increasingly considered environmentally friendly alternatives, with several studies demonstrating efficacy against insecticide-resistant CPB strains (Eski et al., 2022; Baki et al., 2021). However, reduced susceptibility to *Bacillus thuringiensis* var. *tenebrionis* has been reported under laboratory selection conditions, highlighting the potential for resistance development under sustained selection pressure (Whalon et al., 1993). Therefore, among these microbial control agents, EPF have emerged as particularly promising tools for CPB management (Konopická et al., 2024). Several fungal species have shown efficacy against this pest, notably *Beauveria bassiana* (Hypocreales: *Cordycipitaceae*) and *Metarhizium* spp. (Hypocreales: *Clavicipitaceae*). However, their virulence varies considerably depending on the fungal strain. Under laboratory conditions, the most virulent strains have achieved cumulative mortalities approaching 100%, with median lethal times (LT50) as low as 5-6 days following exposure (Wraight and Ramos, 2015, Baki et al., 2021; Zemek et al., 2021; Keçili et al., 2022; Yildirim et al., 2024). Given their documented effectiveness and widespread use in integrated pest management programs, these fungi represent a relevant and realistic alternative for sustainable CPB control (Cook et al., 2007). These fungi offer several practical advantages over chemical insecticides: they leave no persistent residues in the environment or on harvested crops, and the risk of resistance development is considered lower than for synthetic compounds (Gupta and Dikshit, 2010). Naturally present in the environment, they infect their hosts through conidial adhesion to the cuticle, followed by penetration, internal colonisation and subsequent sporulation, allowing

horizontal transmission among individuals (Gao et al., 2011). However, their field adoption remains limited by a short shelf life, high production costs, and variable efficacy under fluctuating environmental conditions, particularly humidity, temperature, and UV exposure (Dar et al., 2011; Sharma et al., 2023). To overcome these limitations and enhance their efficacy, EPF can be formulated in alginate beads to provide a protective environment for the survival and growth of the micro-organism (Brandl et al., 2017).

To further improve the efficacy of biological control strategies, targeting pest behaviour represents an interesting complementary approach. Semiochemical-based strategies have been widely investigated for pest monitoring and control, particularly through the manipulation of insect behaviour using host plant-derived volatile organic compounds (VOCs) (Sablon et al., 2012). Several studies have shown that CPB adults rely on olfactory cues to locate host plants, responding to specific blends of volatiles emitted by potato foliage (Dickens, 2000; Dickens, 2002). Notably, adults have been shown to respond to green leaf volatiles and monoterpenes emitted by damaged plants (Dickens, 2000; Sablon et al, 2012). Two blends in particular elicited strong behavioural responses, with 80-85% of adults exhibiting oriented movement towards the source: (Z)-3-hexenyl acetate combined with linalool, and (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol combined with linalool (Dickens, 2000; Dickens, 2002). Despite this well-documented behavioural responsiveness, the practical implementation of semiochemical-based control strategies against CPB remains limited. Attempts have been made to develop attracticide formulations combining attractive blends with insecticides (Martel et al., 2007). In this study, the formulations were applied directly onto potato rows using a tractor-mounted, CO_2-driven applicator that distributed droplets along the crop canopy (Martel et al., 2007). However, their efficacy under field conditions is constrained by the need for prolonged insect exposure to treated surfaces and by the difficulty of achieving uniform foliar coverage, both of which reduce the likelihood of effective intoxication (Martel et al., 2007). In addition, the reliance on chemical insecticides in such systems limits their relevance in the current context of widespread resistance and increasing regulatory restrictions. Effective attractant blends have thus been identified, but their translation into operational control tools requires alternative delivery systems that bypass the limitations of conventional attracticide approaches.

Combining semiochemical attraction with entomopathogenic fungal infection in an integrated Attract-and-Kill (A&K) strategy therefore offers a promising path forward for CPB. This strategy relies on the co-localisation of an attractant cue and a biological control agent to actively guide insects towards a source of infection, thereby increasing host-pathogen contact (Todd Kabaluk et al., 2015; Delahaut et al., 2025). In contrast to conventional inundative applications of EPF, which depend on high propagule densities to ensure random encounters, the A&K approach enhances infection efficiency by spatially concentrating both components

within a targeted formulation (Meyling and Eilenberg, 2007; Brandl et al., 2017). More broadly, the A&K concept has been successfully implemented against several agricultural pests, particularly aboveground insects such as noctuid moths and tephritid fruit flies (Gregg et al., 2018). These successes demonstrate that the A&K concept can effectively enhance pest control when coupled with an appropriate killing agent and delivery system. A particularly relevant example of this approach is its successful application against wireworms, where EPF conidia (*Metarhizium brunneum)* were encapsulated with a carbon dioxide-producing source (*Saccharomyces cerevisiae*) in calcium alginate beads, resulting in increased pest-pathogen interactions in the soil (Brandl et al., 2017). In this context, alginate encapsulation not only provides a protective microenvironment that supports fungal survival and persistence but also allows the simultaneous release of semiochemicals and infective propagules (Brandl et al., 2017; Delahaut et al., 2025). Adapting this concept to CPB management appears particularly relevant given both the ecology and behaviour of the pest. Alginate beads containing EPF and an attractant could be applied to the soil to target overwintered adults during spring emergence, when they actively search for host plants (Hare, 1990; Weber, 2003). Contact with the formulation at this early stage of population establishment may promote fungal infection before extensive crop colonisation occurs. In addition, larvae entering the soil for pupation, as well as newly emerged adults of the subsequent generation, may also encounter the formulation, increasing the likelihood of infection across multiple life stages (Hare, 1990; Weber, 2003). By combining behavioural manipulation with microbial biocontrol in a single formulation, the A&K strategy therefore aims to optimise host-pathogen interactions, improve control reliability, and reduce the need for high-dose fungal applications.

The objective of the present study is to develop and evaluate an A&K approach combining semiochemical attraction and EPF for CPB management in potato cropping systems, building on the encapsulation technology reviewed by Vemmer and Patel (2013). CPB adults were selected as the target life stage because they actively forage at the soil surface during spring emergence and are therefore likely to encounter soil-applied alginate beads. For the “attract” component, this study aims to identify an effective attractant compatible with encapsulation in alginate beads. Four candidate attractants will be tested : (1) a potato tuber extract previously used in A&K formulations targeting wireworms (Smeets et al., 2022; Delahaut et al., 2025); (2) an extract from aerial potato vegetation reflecting host plant cues encountered under field conditions; and (3-4) two synthetic blends of potato VOCs ((Z)-3-hexenyl acetate and linalool; (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol and linalool) previously shown to elicit strong behavioural responses in CPB adults (Dickens, 2000). For the synthetic blends, we will first test three concentrations of one blend to determine the most effective dose, which will then be used to evaluate the second blend. We hypothesise that at least one of these attractants will induce

significant attraction in CPB adults, and that encapsulation in alginate beads will modulate VOC release without suppressing behavioural attractiveness.

In parallel, for the “kill” component, EPF belonging to the genera *Beauveria* and *Metarhizium*, which are known to infect CPB, will be investigated (Wraight and Ramos, 2015; Baki et al., 2021; Keçili et al., 2022; Yildirim et al., 2024). Five fungal strains obtained from the Mycothèque of UCLouvain (MUCL) will be screened to identify the most effective candidate for future integration into an A&K formulation. We hypothesise that significant differences in virulence will occur among MUCL strains and that at least one strain will cause substantially higher mortality and faster disease progression in CPB adults than the others.

Overall, this study is structured around two complementary objectives: (i) to identify an effective attractant for CPB, and (ii) to select a highly virulent entomopathogenic fungal strain. By independently identifying and optimising both the “attract” and “kill” components, this study provides the essential groundwork for the rational development of a biodegradable A&K strategy for sustainable CPB management.

2. Materials and methods

2.1. Biological material

CPB (*Leptinotarsa decemlineata* (Say)) were obtained from an existing laboratory colony maintained at Ghent University. Immature stages were separated by cohort and maintained in plastic containers (15 x 10 x 5 cm) under controlled laboratory conditions (22 ± 1°C; 65% relative humidity; 16:8 h light:dark photoperiod). All immature stages were fed *ad libitum* with fresh, untreated potato foliage (*Solanum tuberosum*, mixed of cultivars “Jazzy” and “Markies”), which was renewed three times per week. Upon emergence, adults were transferred to a mesh cage (60 x 40 x 40 cm) containing a potato plant (cultivar “Jazzy”) to allow mating and egg production. The plants were replaced once a week, and eggs were collected daily to obtain cohorts of known age. Adults used in the behavioural experiments were maintained separately from those used exclusively for colony reproduction.

Five entomopathogenic fungal strains were selected from the Mycothèque of the Université catholique de Louvain (BCCM/MUCL, Belgium) based on three criteria: (i) species previously reported as pathogenic to the CPB; (ii) geographical origin, with preference given to strains originating from Belgium or neighbouring regions to maximise their relevance under local environmental conditions; and (iii) ecological origin, favouring strains isolated from insects or agricultural soils representative of habitats likely to be encountered by CPB. The selected strains comprised three strains of *Metarhizium anisopliae*, one strain of *Metarhizium brunneum*, and one strain of *Beauveria bassiana* (Table 1).

Strain (MUCL)	Code name	Species	Isolation source	Habitat	Country
MUCL 6859	Ma6859	*Metarhizium anisopliae*	Root	Forest	Belgium
MUCL 9815	Ma9815	*Metarhizium anisopliae*	Soil	Corn and bean field	Brazil
MUCL 58549	MAN	*Metarhizium anisopliae*	Wireworm	Organic crop	Belgium
MUCL 9645	Mb9645	*Metarhizium brunneum*	Wireworm	Forest grove	USA
MUCL 39817	Bb 39817	*Beauveria bassiana*	Dead insect	Not mentioned	Cuba

Table 1. *Origin and characteristics of the five entomopathogenic fungal strains selected from the BCCM/MUCL collection for this study.*

Strains were preserved by cryopreservation at −130°C and reactivated by culturing on sterilized (121°C, 15 min) potato dextrose agar (PDA) in 90 mm Petri dishes (Sarstedt, Germany). Cultures were incubated at 24 ± 1°C in darkness for 21 days to allow fungal growth and conidia production.

2.2. Preparation of the attract formulation

The attract formulations consisted of biodegradable alginate beads produced at the Chemical and Behavioural Ecology Laboratory (Gembloux, Belgium) following a protocol adapted from La Forgia et al. (2023). Beads were prepared from sodium alginate (2% w/v) (Sigma-Aldrich, Belgium) and α-tocopherol (0.7% w/v) (Sigma-Aldrich, Belgium), polymerised in a 0.1 M calcium chloride ($CaCl_2$) (Supelco, Belgium) solution, and used as carriers for either natural potato extracts or synthetic volatile blends. To minimize potential degradation of volatile compounds, all beads were stored at 4°C and used within one week of preparation.

Natural extract beads*:* Natural extracts from potato tubers (TE) and aerial vegetation (VE) (50% v/v; cultivar “Jazzy”; Belgium) were used as odour source. The extracts, obtained using a juice press machine, were incorporated into the alginate mixture before bead formation. Beads were formed by polymerisation in a 0.1 M $CaCl_2$ solution for 30 min, rinsed with distilled water, and air-dried under a chemical hood for 20 min. Control beads were prepared using distilled water instead of natural extracts.

Synthetic blend beads*:* Beads containing synthetic volatile blends were prepared using a modified version of the above protocol. The volatile blends were formulated according to Dickens (2000, 2002). The first blend (B1) consisted of (Z)-3-hexen-1-ol (Sigma-Aldrich, Belgium; purity 98%), (E)-2-hexen-1-ol (Sigma-Aldrich, Belgium; purity 96%), and linalool at a ratio of 1:1:10, prepared as a 2.5% (v/v) solution in paraffin oil. The second blend (B2) consisted of linalool (Sigma-Aldrich, Belgium; purity 97%) and (Z)-3-hexenyl acetate (Sigma-Aldrich, Belgium; purity 98%) mixed in equimolar proportions and prepared as a 2.5% (v/v)

solution in paraffin oil. Stock solutions were subsequently diluted ten-fold with paraffin oil to obtain the 0.25% (v/v) working solutions used for bead preparation. For both blends, 4 mL of the working solutions were incorporated into a mixture containing sodium alginate (2% w/v), paraffin oil (20% v/v), and α-tocopherol (0.7% w/v). The resulting mixture was homogenized using a high-shear emulsifier (FSH-2A, 18 000 rpm for 15 s) prior to bead formation to obtain an emulsion. Beads were then polymerised in a 0.1 M $CaCl_2$ solution for 24 h. Three loading levels were tested (1, 10, and 100 ng per bead), resulting in the following formulations: B1-10, B1-100, and B1-1000 for B1, and B2-1000 for B2. The formulation names refer to behavioural assays performed with 10 beads loaded with 1, 10, and 100 ng per bead, respectively. These loading levels were selected based on electroantennographic data available for (Z)-3-hexen-1-ol in CPB antennae, which indicate reliable antennal responses over a nanogram range concentration (Weißbecker et al., 1997). When compound proportions differed within a blend, these values refer to the compound present at the highest concentration. Control beads were prepared using the same protocol but without the addition of the synthetic blend.

2.3. Screening of the attractant cue

Behavioural bioassays aimed to evaluate the attractiveness of the different bead formulations and to identify the most effective attractant among the tested modalities. The experimental procedure was identical for all four bead types. Adult *Leptinotarsa decemlineata* were sexed prior to the experiments and isolated individually without food for 2 h before each behavioural assay to standardise their hunger level. Each beetle (n = 30 per treatment) was then placed in the centre of a glass crystalliser (Ø = 19 cm) lined with moist filter paper and maintained under an isolation chamber (Ø = 4.5 cm) for 5 s to ensure a stable starting position and minimise handling stress (Figure 1). A metal mesh (2 x 2 mm) was placed on top of the crystalliser to maintain an odour gradient while preventing insect escape. After acclimation, the isolation chamber was removed, and the insect was allowed to choose between two plastic lids (Ø = 5 cm): one containing control beads and the other containing one of the tested bead modalities. Each lid contained 10 beads, and the positions of the odour source and the control were randomised across replicates to avoid positional bias.

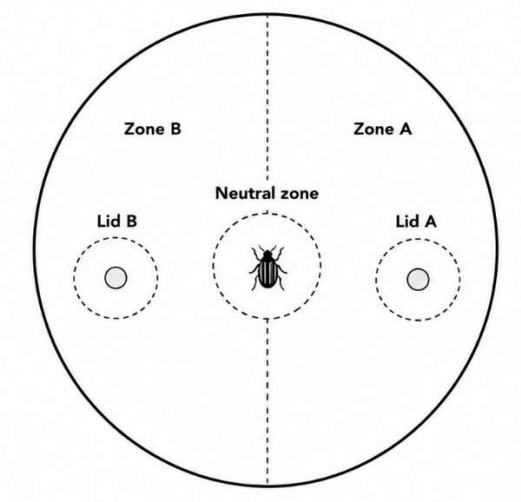

Figure 1. *Experimental arena used for the behavioural bioassays. Top view of the circular arena showing the two choice zones (Zones A and B), the central neutral zone where each adult Leptinotarsa decemlineata was released, and the two lids containing 10 beads each.*

Behavioural assays were conducted over several experimental days, with trials distributed between morning and afternoon sessions to minimise potential effects of circadian variation in CPB activity. A positive control consisting of a fresh potato leaf versus an empty lid was included to confirm that the bioassay was able to record attraction. A negative control, consisting of paraffin oil-loaded beads on both sides of the arena, was used to verify the absence of any inherent directional bias. The following bead formulations were tested: natural tuber extract (TE), natural aerial vegetation extract (VE), three loading levels of the first synthetic blend (B1-10, B1-100, and B1-1000), and the second synthetic blend at the highest loading level (B2-1000). After each trial, the crystalliser was thoroughly cleaned with hexane (Thermo Scientific, Germany) to remove residual chemical cues.

Assays were conducted at 20 ± 1°C under controlled lighting conditions. The arena was uniformly illuminated from below using a diffuse LED light panel (Crafts & Co.), whereas the surrounding environment remained dark to minimise external visual cues. Each trial was recorded for 10 min using a digital camera (Vahoiald, China). Video recordings were analysed using EthoVision XT17 (Noldus Information Technology, Wageningen, The Netherlands). The software was used to track beetle position throughout the assay and to calculate the time spent in predefined zones of the arena. Two behavioural parameters were extracted: (i) time spent in each choice zone (treatment vs. control) and (ii) time spent within each lid zone.

2.4. Screening of the most virulent entomopathogenic fungi

The protocol for screening the most virulent EPF strains was adapted from Regnier et al. (2022). Once fungal cultures were reactivated, conidial production was standardised using a preliminary growth assay in multiwell plates. Aerial conidia were harvested by scraping using

a sterile loop and suspended in sterile water. A volume of 5 µL of this suspension, adjusted to 1 x 10^6 conidia mL^{-1}, was used to inoculate 1000 µL of potato dextrose agar (PDA) in each well of a 12-well plate (Sarstedt, Germany). Three independent 12-well plates were prepared for each fungal strain. Inoculated plates were incubated at 24 ± 1°C in darkness for 21 days before conidial production was assessed. At sampling time, the entire surface of the well was scraped using a sterile loop and resuspended in 1 mL of sterile water, followed by vortexing for at least 30 s to obtain a homogeneous suspension. Conidial concentration was then determined using a Thoma haemocytometer (BRAND, Germany). For each strain, three wells were sampled and each suspension was counted twice. Based on these measurements, wells showing homogeneous and abundant sporulation were selected for subsequent bioassays. The mean conidial concentration available for insect exposure were approximately 14.5 x 10^6 conidia per well for Mb9645, 7.1 x 10^6 for Bb39817, 9.7 x 10^6 for Ma6859, 13.3 x 10^6 for Ma9815, and 9.8 x 10^6 for MAN.

The virulence of each strain was evaluated using a direct contact bioassay. Adults CPB (n = 30 per treatment) were individually placed in a well in direct contact with the fungal cultures for 5 h, ensuring exposure to both conidia and actively growing mycelium. During the exposure period, the plates were covered with their lids to prevent insect escape and maintain consistent exposure conditions. A negative control (PDA medium only), and a positive control (Tracer®, 480 g L^{-1} Spinosad, Corteva Agriscience Netherlands B.V.) were included, following the same procedure. The Spinosad commercial formulation was diluted to reach the recommended Belgian field application rate (24 g a.i. ha^{-1}), and 306 µL of the resulting solution was applied to each well, to ensure homogenous coverage of the well surface. After exposure, insects were individually transferred to plastic boxes and maintained under controlled conditions (22 ± 1°C; 16:8 h light:dark photoperiod). Individuals were fed with fresh, untreated potato leaves throughout the experiment. Mortality was recorded daily over a period of 21 days. Insects were considered dead when no movement was observed following gentle stimulation. To confirm that mortality was caused by fungal infection, cadavers were surface sterilized. Each cadaver was successively immersed with gentle vortexing in 2% sodium hypochlorite (2 min), then 70% ethanol (2 min) in sterile 1.5 mL microtubes (VWR International). After three rinses in demineralised water, insects were individually placed on sterile, moist filter paper (Whatman No. 1) in sterile 90 mm Petri dishes (VWR International) and incubated at 22°C in darkness. Fungal outgrowth was monitored regularly and confirmed by morphological identification. Two independent experimental replicates, 25 days apart, were conducted with fresh insects (n = 15 insects per replicate) and newly prepared material in each time.

2.5. Statistical analysis

Data processing was performed using Microsoft Excel for Microsoft 365 (version 2606). All statistical analyses were conducted using R software (version 4.4.2) and RStudio (version 2024.12.1). The following R packages were used: survival, survminer, lsmeans, dplyr, tidyr, car, RVAideMemoire, multcompView, readxl, ggplot2, ggsci, glmmTMB and DHARMa.

For the behavioural arena assays, the time spent in each choice zone and within each lid zone was expressed as a proportion of the total trial duration (600 s). As these proportion data included a substantial number of observations at the boundaries (0 and 1), reflecting individuals that remained exclusively on one side of the arena throughout the trial, they were analyzed using generalized linear mixed models (GLMM) with an ordered beta distribution (Kubinec, 2023) and a logit link function, implemented in the glmmTMB package (Brooks et al., 2017). For graphical presentation, however, the behavioural data are shown as raw durations in seconds.

For each treatment, separate GLMMs were fitted for the choice zone and lid zone analyses. In these models, the response variable was the proportion of total trial time spent in the focal zone. The explanatory variables included stimulus (treatment vs. control), sex and time of day (morning vs. afternoon) as fixed effects, whereas individual identity was included as a random intercept to account for the paired structure of the design. For the aerial vegetation extract treatment, bead age at the time of testing was also recorded but was not included as an explanatory variable in the statistical models. Model assumptions were validated using simulated residuals from the DHARMa package, including tests for zero-inflation, dispersion and residual quantile deviations. P-values for the stimulus effect were corrected for multiple testing across all treatments and measures (choice zone and lid) using the Benjamini–Hochberg false discovery rate procedure.

For the screening of EPF, survival outcomes between the two temporal replicates were first compared for each treatment using Fisher’s exact tests. As no significant differences were detected between temporal replicates for the fungal treatments, data from both replicates were pooled for subsequent analyses. The positive control from the first replicate was excluded from the pooled dataset because an unintended dilution error during its preparation resulted in an abnormally low mortality.

Survival analyses were performed using Cox proportional hazards regression (package survival). The effect of treatment and sex on CPB survival was assessed independently by comparing the null model (intercept only) with models including the factor of interest using likelihood ratio tests. The proportional hazards models were confirmed using Schoenfeld residuals.

To identify the most virulent fungal strain, a second Cox proportional hazards regression was performed after excluding the negative and positive controls, with the most virulent strain (MAN) used as the reference level. Kaplan-Meier survival curves were generated using the survminer package, and differences in survival among treatments were assessed using the Mantel-Cox log-rank test.

3. Results

3.1. Screening of the attractant cue

All CPB displayed exploratory behaviour and moved within the arena during the 10-min assay. However, not all individuals entered the lid zones, and the time spent in contact with the lids was generally short, with median values remaining below 100 s across all treatments (Figure 2).

The proportion of time spent in the choice zones showed substantial inter-individual variability, with values ranging from 0 to 600 s, indicating that some CPB remained almost exclusively on one side of the arena throughout the trial. Despite this variability, the boxplots suggested a visual shift in median time towards the treatment side for the positive control (C+). By contrast, the negative control (C-) and the other tested stimuli displayed broadly overlapping treatment and control distributions. Time spent in the lid zones was generally low and concentrated near zero for most observations, although a few individuals exhibited prolonged contacts with the lids.

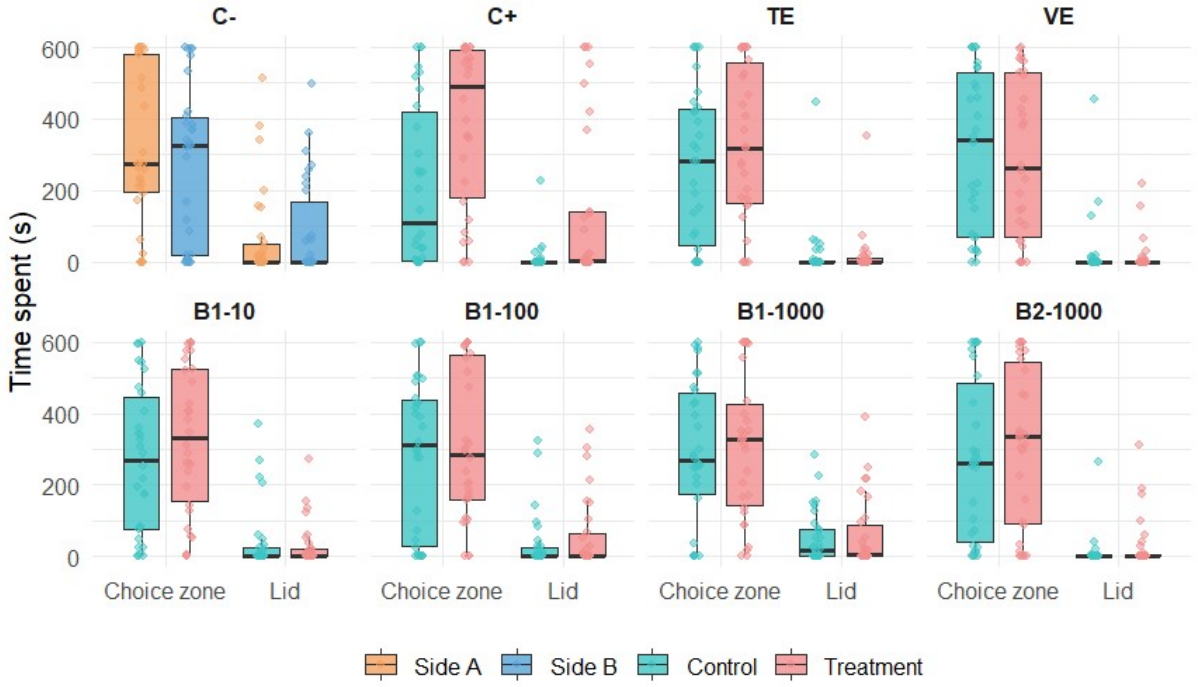

Figure 2. *Time spent by adult Leptinotarsa decemlineata in the choice zone and in contact with the lid according to treatment (C-, C+, TE, VE, B1-10, B1-100, B1-1000, B2-1000) (n = 30 per treatment). In the C- treatment, "Side A" and "Side B" correspond to the two untreated sides. Points represent individual observations*

These observations were confirmed statistically using a GLMM with an ordered beta regression (Table 2). CPB spent significantly more time in the choice zone (estimate = 1.178, SE = 0.336, p adj = 0.007) and on the lid (estimate = 1.249, SE = 0.426, p adj. = 0.027) containing a potato leaf (C+), compared to the control. This confirms the ability of the experimental setup to detect an attraction response. Conversely, no significant difference between sides was observed for the negative control (C-), either in the choice zone (estimate = -0.261, SE = 0.314, p adj. = 0.810) or on the lid (estimate = 0.123, SE = 0.315, p adj = 0.820), indicating the absence of a systematic bias in the arena design.

None of the tested volatile blends elicited a significant attraction response, regardless of the compound or concentration tested (Blend 1 at 10, 100 and 1000 ng; Blend 2 at 1000 ng; all p adj. > 0.69 for both the choice zone and the lid). Similarly, neither the tuber extract (TE) nor the vegetation extract (VE) induced significant attraction for the insects (all p adj. > 0.69). Sex and time of day (morning vs. afternoon) had no significant effect on the proportion of time spent on the treatment side across all models (all p-values > 0.05).

Treatment	Measure	Estimate	SE	p	p adj.	Sig.
C+ (positive control)	Choice zone	1.178	0.336	0.0005	0.0073	**
	Lid	1.249	0.426	0.0034	0.0269	*
C- (negative control)	Choice zone	-0.261	0.314	0.4050	0.8100	ns
	Lid	0.123	0.315	0.6956	0.8195	ns
Blend 1 - 10 ng	Choice zone	0.310	0.300	0.3020	0.6949	ns
	Lid	-0.092	0.309	0.7640	0.8195	ns
Blend 1 - 100 ng	Choice zone	0.324	0.300	0.2800	0.6949	ns
	Lid	0.219	0.322	0.4970	0.8195	ns
Blend 1 - 1000 ng	Choice zone	-0.141	0.280	0.6150	0.8195	ns
	Lid	-0.084	0.276	0.7610	0.8195	ns
Blend 2 - 1000 ng	Choice zone	0.311	0.298	0.2960	0.6949	ns
	Lid	0.664	0.483	0.1690	0.6949	ns
Tuber extract (TE)	Choice zone	0.304	0.296	0.3040	0.6949	ns
	Lid	-0.077	0.371	0.8360	0.8360	ns
Vegetation extract (VE)	Choice zone	0.101	0.299	0.7350	0.8195	ns
	Lid	-0.122	0.414	0.7683	0.8195	ns

Table 2. *Effect of stimulus (treatment vs. control side) on the proportion of time spent in the choice zone and in contact with the lid, for each of the eight treatments tested in the behavioural arena assay. Estimates (on the logit scale), standard errors (SE) and p-values were derived from generalized linear mixed models with an ordered beta distribution. Significance codes: ** p adj. < 0.01; * p adj. < 0.05; ns = not significant (p adj. ≥ 0.05).*

3.2. Screening of the most virulent entomopathogenic fungi

Every EPF strain induced mortality in CPB adults, although virulence varied markedly among fungal strains, with cumulative mortality ranging from 3% to 97% at 21 days post-inoculation (Figure 3). The highest final mortalities were observed for MAN (97%) and Bb 39817 (90%), whereas the positive control reached 60% mortality. In contrast, Ma6859, Ma9815 and Mb9645 caused only limited mortality (17%, 13% and 3%, respectively), close to the level observed in the negative control (10%). The estimated LT50 values were approximately 6 days for MAN, 11 days for Bb 39817 and 17 days for the positive control, while LT50 could not be estimated for Ma6859, Ma9815 and Mb9645 because mortality never reached 50%.

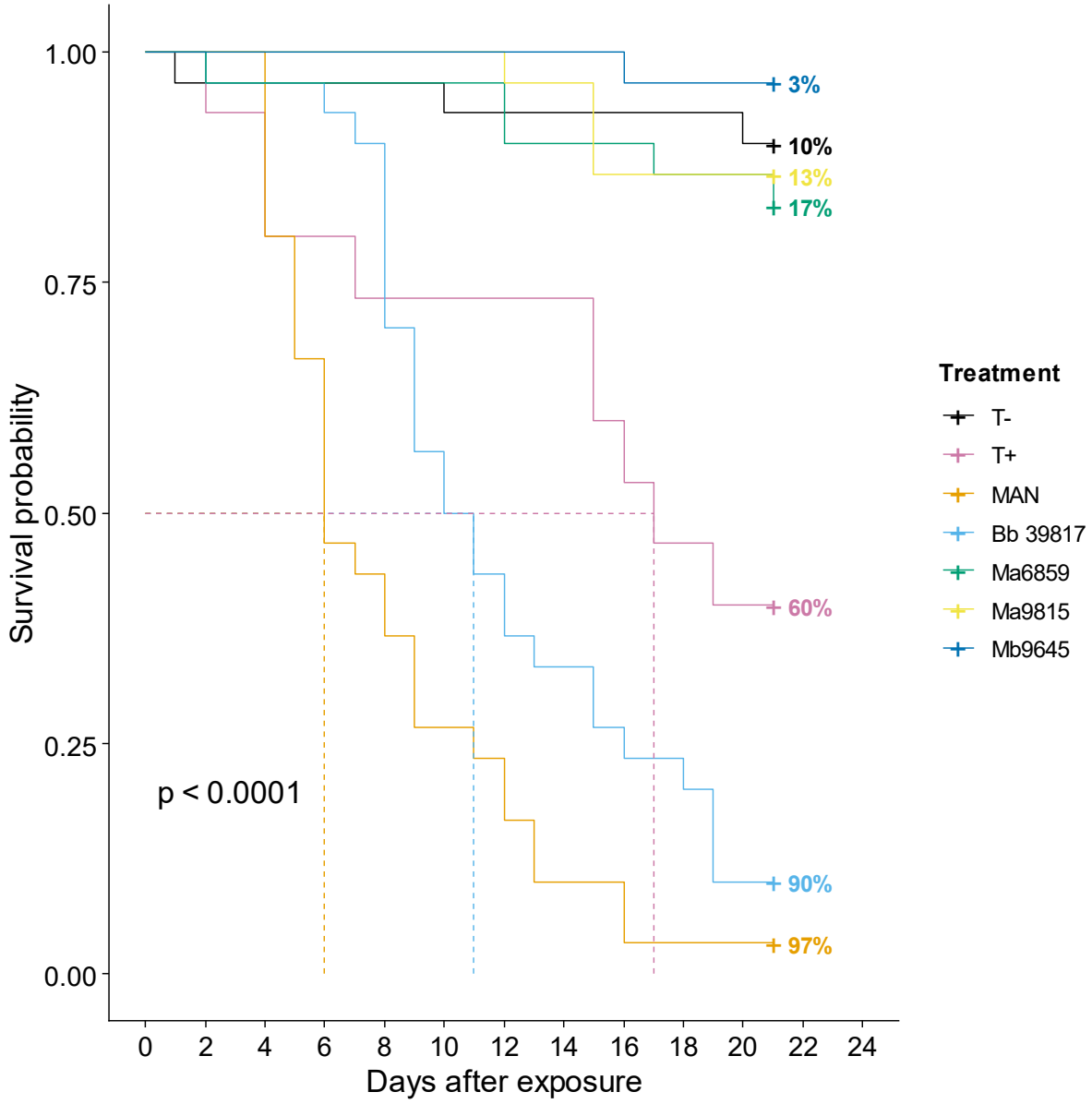

Figure 3. *Kaplan-Meier survival curves of Leptinotarsa decemlineata exposed to different entomopathogenic fungal strains and controls over 21 days (n = 30 per treatment, excepted for T+: n = 15). Survival curves differed significantly among treatments (Mantel-Cox log-rank test: $\chi^2 = 187$, df = 6, $p < 0.001$).*

Cox proportional hazards regression revealed a highly significant effect of fungal treatment on CPB survival (likelihood ratio test: $\chi^2 = 146.83$, df = 6, $p < 0.001$). The sex of CPB had no significant effect on their survival probability, regardless of the treatment ($\chi^2 = 0.14$, df = 1, $p = 0.706$). The EPF strains Bb 39817 ($p < 0.001$), MAN ($p < 0.001$) and the positive control ($p = 0.001$) significantly increased the mortality risk of CPB compared to the negative control. In contrast, the strains Ma6859 ($p = 0.46$), Ma9815 ($p = 0.70$) and Mb9645 ($p = 0.32$) did not differ significantly from the untreated control (Table 3). Relative to the negative control, MAN showed the strongest effect, increasing the mortality hazard by nearly 40-fold (hazard ratio (HR) = 39.92), followed by Bb 39817 with an approximately 19-fold increase (HR = 19.38). The

positive control also significantly increased mortality risk, with an almost 9-fold higher hazard than the negative control (HR = 8.76). In contrast, Ma6859 (HR = 1.71), Ma9815 (HR = 1.34) and Mb9645 (HR = 0.32) did not differ significantly from the untreated control (Table 3).

Treatment	Hazard Ratio	95% CI	p-value
T- (reference)	1.00	-	-
Bb 39817	19.38	[5.82 – 64.53]	< 0.001
Ma6859	1.71	[0.41 – 7.15]	0.46
Ma9815	1.34	[0.30 – 6]	0.70
MAN	39.92	[11.89 – 134.06]	< 0.001
Mb9645	0.32	[0.03 – 3.05]	0.32
T+	8.76	[2.37 – 32.39]	0.001

Table 3. *Effect of fungal entomopathogenic strain on Leptinotarsa decemlineata survival. Bb 39817, MAN and the positive control significantly increased mortality risk relative to the negative control (T-), whereas Ma6859, Ma9815 and Mb9645 did not differ significantly from it. Hazard ratios and 95% confidence intervals were estimated using a Cox proportional hazards model.*

To identify the most virulent fungal strain among all tested, a second Cox proportional hazards regression was performed after excluding the negative and positive controls. Using MAN as the reference, all remaining fungal strains showed significantly lower virulence. Bb 39817 exhibited a 53% lower mortality hazard (HR = 0.47, p = 0.006), whereas Ma6859, Ma9815 and Mb9645 showed much lower hazards, corresponding to approximately 96%, 97% and 99% reductions, respectively (Ma6859: HR = 0.042, p < 0.001; Ma9815: HR = 0.033, p < 0.001; Mb9645: HR = 0.008, p < 0.001). These results confirm MAN as the most virulent strain tested (Table 4).

Treatment	Hazard Ratio	95% CI	p-value
MAN (reference)	1.00	-	-
Bb 39817	0.47	[0.28 – 0.81]	0.006
Ma6859	0.042	[0.016 – 0.11]	< 0.001
Ma9815	0.033	[0.011 – 0.096]	< 0.001
Mb9645	0.008	[0.001 – 0.058]	< 0.001

Table 4. *Comparative virulence of entomopathogenic fungal strains against Leptinotarsa decemlineata, excluding negative and positive controls. Hazard ratios and 95% confidence intervals were estimated using a Cox proportional hazards model, with MAN set as the reference level.*

4. Discussion

This study aimed to begin the development of an A&K control strategy for CPB, combining an olfactory attractant encapsulated in alginate beads with a fungal biocontrol agent. Two complementary objectives guided this work: (i) identifying an effective attractant among four candidates (a tuber extract, an aerial plant extract, and two synthetic VOC blends), and (ii) selecting, among five strains of EPF (*Beauveria* and *Metarhizium*), the one showing the highest virulence against CPB adults.

Across the eight treatments tested in the behavioural arenas, only the potato leaves (positive control) induced significant attraction of CPB adults, with none of the candidate attractants statistically differing from their respective control. These results therefore do not support our initial hypothesis that at least one of the tested attractants would induce significant attraction in CPB adults after encapsulation in alginate beads. This finding contrasts with previous study, which reported CPB adults' orientation rates of 80-85% towards these same VOC blends (B1 and B2) (Dickens, 2000). Several factors may account for this discrepancy. First, the synthetic blends were tested at only nanogram loading levels whereas previous studies reported kairomone release rates in the microgram range under field conditions (Martel et al., 2007). This nanogram range was nonetheless deliberately chosen based on electroantennographic (EAD) calibration data showing that the CPB antenna can detect (Z)-3-hexen-1-ol down to picogram amounts, with a reliable dynamic response spanning from approximately 10 pg to 100 ng before signal saturation occurs (Weißbecker et al., 1997). However, these EAD data concern only (Z)-3-hexen-1-ol, and the resulting concentration range may not be directly extrapolated to the behavioural response elicited by the complete VOC blends tested in the study. Moreover, the absence of attraction at the highest loadings tested here (100 and 1000 ng per bead formulation) may not necessarily reflect a simple saturation effect: behavioural responses to semiochemicals are often dose-dependent and may decline when release rates exceed a biologically relevant range (Byers, 2013). Importantly, the lowest loading (10 ng), which remained below the reported saturation threshold for (Z)-3-hexen-1-ol, also failed to induce a behavioural response, suggesting that the absence of attraction cannot be explained solely by sensory overstimulation. In addition, the amount of volatile compound effectively perceived by the insect was probably lower than the amount initially encapsulated in the alginate beads. Encapsulation may have altered diffusion kinetics, and the actual release rate of each VOC under the experimental conditions was not quantified. Consequently, the concentration surrounding the beetle may have been substantially lower than the nominal loading of the beads. Future work should therefore characterize VOC release from alginate beads using analytical techniques such as headspace GC-MS to determine the concentrations effectively emitted and available for insect perception.

Beyond this dose-related consideration, a more fundamental explanation likely lies in the behavioural device itself. Dickens (2000, 2002) relied on a Y-tube olfactometer, in which beetles were subjected to a binary choice between two airstreams under a light source placed above the apparatus. In contrast, the arena system used in the present study allowed beetles to move freely and explore their surroundings, more closely reflecting natural host-seeking behaviour. This choice was deliberate, as the present work aims to develop a field-applicable A&K strategy rather than to identify attractive compounds under highly controlled, forced-choice conditions. While a Y-tube design maximises the detectability of a compound's intrinsic attractiveness by constraining the insect's behavioural options, an open arena introduces additional degrees of freedom, such as walking speed, exploration pattern and orientation persistence, that may dilute or delay a measurable attraction response, particularly if the tested cue is only moderately attractive. We hypothesis that the absence of a significant effect in the present study may therefore not necessarily reflect a lack of biological activity of the tested attractants, but rather the lower sensitivity of a free-choice arena compared with a forced binary olfactometer in detecting such effects.

Regarding the plant-derived extracts, the absence of attraction towards the tuber extract may reflect the fact that CPB adults rely on the odour of damaged foliage rather than to below-ground plant tissues. The lack of significant attraction towards the aerial plant extract is more surprising, as this extract was expected to contain volatile compounds more representative of the odour emitted by potato foliage under natural conditions. Interestingly, a change in bead age was observed to modify the attractiveness of these plant-based extracts during the experiment, with beetles appearing more attracted to older beads, a trend visible on the corresponding boxplot (Appendix 1). This effect did not reach statistical significance in the interaction test, which may be attributable to insufficient statistical power: although 30 individuals were tested for the plant extract treatment overall, this sample was not further stratified by bead age, resulting in a comparatively small number of individuals within each age category. Consequently, an effect of bead age on the attractiveness of natural extracts cannot be ruled out and would warrant further investigation, particularly given its potential relevance for the eventual field deployment of the A&K formulation, where beads are likely to age between application and beetle contact.

The most striking result of this study was the identification of highly virulent EPF strains against CPB adults. Among the five strains tested, MAN (*Metarhizium anisopliae*, Belgian strain) and Bb 39817 (*Beauveria bassiana*, strain from Cuba) caused very high cumulative mortalities (97% and 90%, respectively) and relatively short LT50 values (6 and 11 days), indicating that effective fungal candidates can be identified for the kill component of the A&K strategy. These results contrast sharply with the limited mortality induced by Ma6859, Ma9815

and Mb9645, highlighting the strong heterogeneity of pathogenicity among strains. Such marked differences in virulence are consistent with previous studies showing that the efficacy of *Beauveria bassiana* and *Metarhizium* spp. against CPB depends strongly on the fungal strain rather than on the species alone (Wraight and Ramos, 2015; Baki et al., 2021; Keçili et al., 2022; Yildirim et al., 2024). Interestingly, these differences in virulence cannot be readily attributed to differences in conidial concentration alone. The fungal cultures used for exposure contained between approximately 7.1 x 10^6 and 14.5 x 10^6 conidia per well, representing an approximately two-fold variation among strains. MAN did not have the highest conidial concentration, whereas Mb9645, which had the highest concentration, was the least virulent strain. Moreover, the two most effective strains, MAN and Bb 39817, were not those associated with the highest conidial concentrations. These observations lead us to hypothesise that the strong differences in mortality were primarily related to differences in strain virulence rather than inoculum size. Although the conidial concentrations were not fully standardised, this variation does not appear to have driven the overall ranking of the strains under the conditions of this screening assay.

The performance of the two most virulent strains was particularly noteworthy because it exceeded that of the positive control (Tracer®, 480 g L^{-1} Spinosad, Corteva Agriscience Netherlands B.V.) under the conditions of this laboratory assay. Although direct comparisons between EPF and chemical insecticides should be interpreted cautiously, Tracer® is described by the manufacturer as acting through both contact and ingestion. In the present assay, CPB adults were directly exposed to a Spinosad solution, providing contact exposure but potentially limiting ingestion compared with exposure under more natural feeding conditions. Consequently, the efficacy of the positive control may have been underestimated. The positive control was nevertheless expected to induce mortality more rapidly than the fungal treatments. The shorter LT50 values observed for MAN and Bb 39817 therefore represent a particularly striking result. While these findings cannot be directly extrapolated to field performance, they suggest that highly virulent fungal strains may constitute valuable alternatives to conventional plant protection products. In the context of the well-documented evolution of resistance in CPB populations (Michigan State University Arthropod Pesticide Database, 2026), these results further highlight the need to develop complementary and alternative control strategies.

Both MAN and Bb 39817 appear to be biologically relevant candidates for further development. However, MAN was retained as the primary candidate for the A&K strategy because it combined the highest mortality, the shortest LT50, and greater ecological relevance. Isolated from Belgium, MAN is considered more suitable for the intended application than Bb 39817, which originated from a geographically distant source (Cuba). The use of a local strain is particularly advantageous because it avoids introducing an exotic one into Belgian

agroecosystems and could potentially increase the likelihood of persistence and efficacy under local environmental conditions.

The added value of the present study is therefore not the demonstration that EPF can infect CPB, which is already well established, but the identification of a locally relevant *Metarhizium* strain combining very high virulence with compatibility for future integration into an alginate bead-based A&K formulation. This compatibility is supported by previous work showing that the same MAN strain can be successfully incorporated into alginate beads and remains effective against wireworms (Delahaut et al., 2025), another major potato pest. Identifying a single fungal strain capable of targeting two economically important potato pests is particularly interesting from an applied perspective, as it may facilitate the development of future integrated control strategies with a broader spectrum of activity and potentially increase the agronomic value of alginate bead-based delivery systems for potato protection.

Beyond the A&K approach, MAN may also have potential for more conventional inundative applications of fungal spores in suspension. A spatially targeted A&K formulation appears particularly relevant early in the season, when overwintered adults are actively foraging at the soil surface and vegetation cover is still limited. As the potato canopy develops, beetles are expected to have less contact with soil-applied beads, making the A&K approach potentially less effective at later stages. Under these conditions, a conventional foliar application of fungal spores could represent a complementary curative tool against residual or established populations. The two application modes should therefore be regarded as complementary components of an integrated management strategy rather than interchangeable alternatives. Compared with broad, non-targeted spraying, however, an A&K formulation would retain greater selectivity by concentrating the infectious agent in specific areas of beetle activity, thereby potentially reducing exposure of non-target organisms.

In addition, some broader constraints of the present study should also be acknowledged. All behavioural and virulence assays were conducted under controlled laboratory conditions. This is appropriate for a proof-of-concept study, but it does not capture the environmental variability encountered under field conditions, including fluctuations in temperature, humidity and soil properties. In addition, all experiments were performed using a single laboratory-reared CPB population. Testing additional populations from different geographic or genetic backgrounds would help determine whether the observed patterns of olfactory responsiveness and susceptibility to MAN are consistent across populations.

From a formulation perspective, the behavioural assays evaluated a selected set of attractants chosen from previous literature and from ecologically relevant potato-derived cues directly in their encapsulated form. Although none of these candidates induced significant

attraction under the conditions tested, the results help refine the selection of attractants for future A&K formulations rather than undermining the concept itself. A key unresolved question is the actual concentration of volatile compounds released by the alginate beads, which was not quantified in the present study. Future work should therefore characterize VOC emission from the beads using analytical approaches such as headspace GC-MS, to determine the concentrations effectively emitted and available for insect perception. Additional semiochemicals should also be investigated, particularly the male-produced aggregation pheromone (S)-CPB I, alone or in combination with host-plant volatiles. Previous laboratory and field studies have shown that combinations of the aggregation pheromone with potato volatile blends can be more attractive than plant volatiles alone (Dickens, 2006). This suggests that synergistic interactions between insect and plant-derived cues may represent a promising direction for improving the efficacy of the encapsulated A&K formulation.

As a broader perspective, the recent emergence of novel control technologies, such as RNAi-based insecticides, raises the question of how EPF-based strategies should be positioned within future CPB management programs. In Belgium, Calantha®, a RNAi-based insecticide targeting an essential CPB gene, recently received a 120-day emergency authorisation for CPB control (SPF Santé publique, 2026), illustrating the growing interest in RNAi technologies. However, this development does not necessarily reduce the relevance of EPF-based approaches. Within an integrated pest management framework, diversifying the available control tools rather than relying on a single mode of action remains essential for reducing selection pressure and delaying resistance evolution, a major issue in CPB management (Alyokhin et al., 2008). In this context, the present study supports the continued development of EPF-based A&K formulations as a complementary biological approach that could be integrated alongside emerging technologies such as RNAi, rather than viewed as a competing alternative.

Finally, this study provides a proof of concept for the development of an A&K strategy against the CPB based on the combination of semiochemicals and entomopathogenic fungi delivered in alginate beads. The behavioural assays showed that the tested potato-derived extracts and VOC blends did not induce significant attraction after encapsulation, indicating that the attractive component of the formulation still requires optimisation. In contrast, the virulence screening identified a highly effective and locally relevant *Metarhizium anisopliae* strain (MAN), which caused very high mortality and a short LT50 in CPB adults, supporting its selection as the fungal candidate for the kill component of the strategy. Together, these results validate the biological potential of the fungal component while refining the selection of attractants for future formulations.

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Declaration of interest statement

No potential conflict of interest was reported by the author(s)

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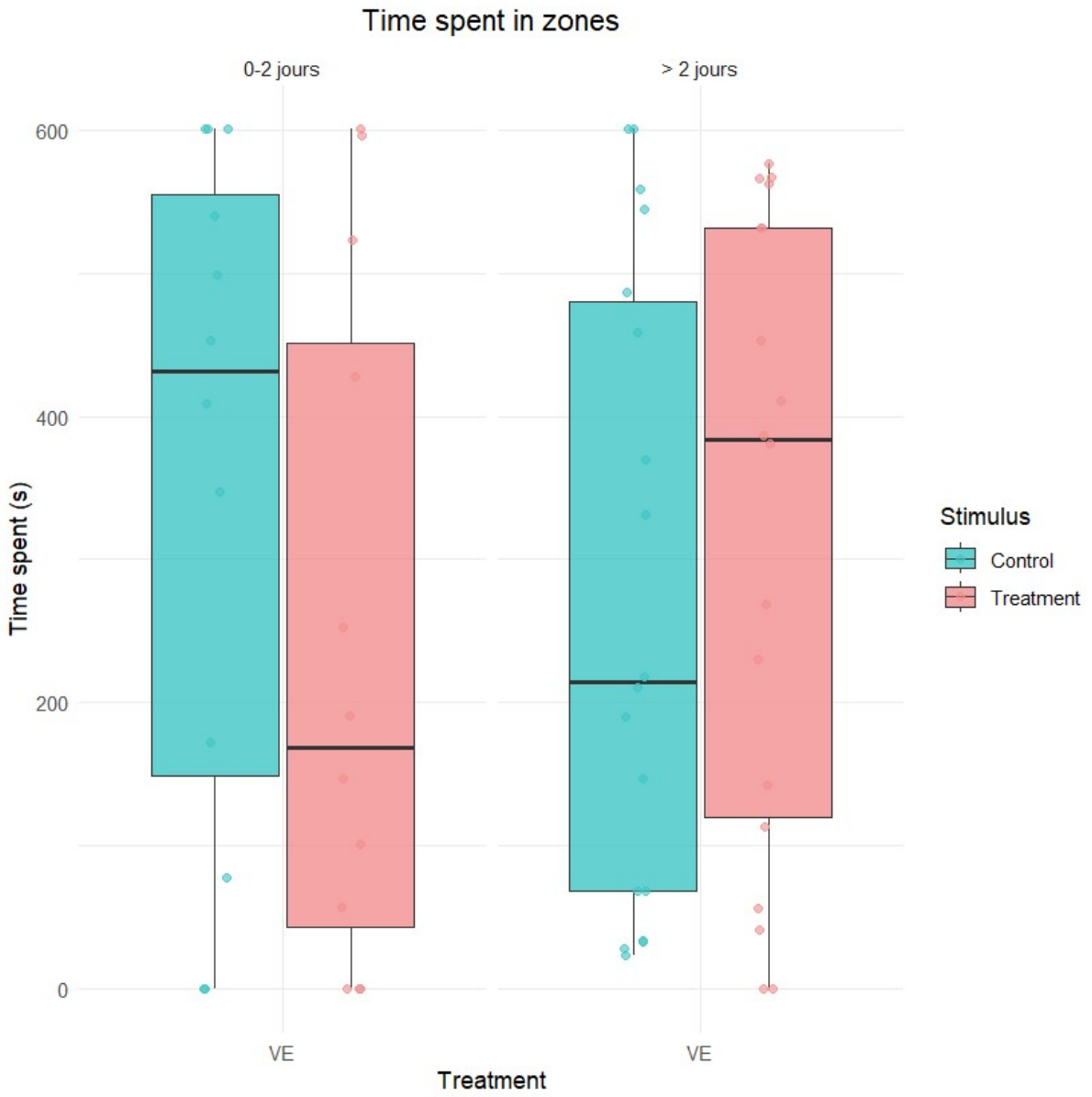
Appendices



Appendix 1. *Time spent in the treatment and control zones during the 10-minute behavioural assay with the VE attractant, according to bead age (0–2 days vs. > 2 days since encapsulation).*

Personal contribution

During the six-month master thesis carried out within the Laboratory of Chemical and Behavioural Ecology, I was personally involved in all major stages of the project.

First, I established the Colorado potato beetle rearing system at Gembloux. Before the official start of the thesis (December 2025), I travelled to Ghent to collect beetles and learn the rearing procedures used there. I subsequently maintained and monitored the colony throughout the experimental period. In parallel, I established potato crops in the greenhouse and took care of their maintenance to provide fresh host plant material for the insects.

Then, I also conducted an extensive literature review to identify the most relevant entomopathogenic fungal (EPF) strains and semiochemicals to be tested in the context of an attract-and-kill strategy. With the scientific guidance of Fanny Ruhland and François Verheggen, I evaluated several behavioural bioassay systems before selecting the arena assay used for data acquisition. I was responsible for preparing the experimental setup, conducting the behavioural tests, and collecting the corresponding data.

For the EPF experiments, I designed and implemented the bioassays with methodological advice from Ismahen Lalaymia and Fanny Ruhland. This part of the work required a strong personal commitment, as I carried out two independent repetitions, each involving a daily follow-up over 21 days. In total, this represented 42 days of continuous monitoring, including weekends.

Regarding the use of artificial intelligence, it was employed as a writing-support tool, mainly to help improve English phrasing and identify appropriate scientific wording. It was also used as an aid for constructing statistical code and for exploring potentially suitable statistical models for the data. However, the statistical analyses were not performed by artificial intelligence alone. The final analytical choices were made following a consultation with the Gembloux statistical support office and through numerous discussions and recommendations provided by Fanny Ruhland.

Overall, this thesis involved substantial personal investment in insect rearing, greenhouse management, literature review, experimental design, data collection, statistical interpretation, and scientific writing.