Exploration of factors influencing crop protection against wireworm herbivory (Coleoptera: Elateridae) using *Beauveria bassiana*

by

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Abstract

Pest wireworms (Coleoptera: Elateridae) negatively affect crops worldwide. Traditionally controlled by chemical insecticides, wireworms now have limited control options due to new regulations stemming from environmental and health concerns. The use of biological control agents, such as entomopathogenic fungi, is an alternative strategy. I investigated possible mechanisms of potato protection against wireworms by *B. bassiana*. I found that fungal inoculation did not cause mortality in three wireworm species. Wireworms avoided *B. bassiana* as granules and spores, and tissue from inoculated plants. Fungal inoculation of *Agriotes lineatus* caused up to 33% decreased feeding time in the lab; however, tubers from inoculated plants were not protected from herbivory. *Beauveria bassiana* was detected inside plants, but plant performance was not affected. The wireworm internal bacterial load increased after *B. bassiana* inoculation, and there were shifts in community members, without affecting mortality. This study provides insight into the non-lethal effects of *B. bassiana.*

Keywords: *Beauveria bassiana*; wireworm; biological control; behaviour; colonization; microbiome

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Chapter 1.

Introduction

Entomopathogens are microorganisms that are pathogenic to insects and comprise fungi, bacteria, nematodes, and viruses. They play a vital role in ecosystems as natural enemies of insects and can effectively regulate insect population levels, leading to their exploitation as biological control agents to manage insect pests. The relationship between insects and fungi has a long and intricate history. From mutualistic interactions such as fungus-farming bark beetles, where gardens of fungi are grown and protected for food (Joseph & Keyhani, 2021), to parasitic interactions such as in zombie ants, where *Cordyceps* fungi use mind-control to march ants to their deaths (Hughes et al., 2011), insects and fungi have evolved together and their roles in ecosystems are inextricably linked. An important interaction is the role that fungi have as disease-causing agents for insects, and in reverse, that insects act as nutrient sources and dispersal agents for fungi (Boucias & Pendland, 1998; Shah & Pell, 2003; Stone & Bidochka, 2020).

1.1. Entomopathogenic fungi

Entomopathogenic fungi are widely distributed in a diverse range of taxa in the phyla Microsporidia, Chytridiomycota, Entomophthoromycota, Basidiomycota, and Ascomycota (Araújo & Hughes, 2016). There are an estimated 700 species of entomopathogenic fungi in about 90 genera— the most widespread of which are the orders Hypocreales and Entomophthorales (Roberts & Humber, 1981). The hosts of these fungi are diverse and are distributed within 20 orders of insects and in all developmental stages (Araújo & Hughes, 2016). Fungal entomopathogens are distinct from other kinds of entomopathogens because they can infect hosts through their cuticle, thus bypassing the requirement of ingestion or entering through an orifice. In general, fungal entomopathogens must kill their host to sporulate and transmit to other hosts. Host specificity can range from extremely specific, such as some clades in the Entomophthorales, to very broad, such as for *Beauveria bassiana* (Vega et al., 2012).

In nature, entomopathogenic fungi are found at the highest concentrations on mycosed insect hosts where the fungus has sporulated. The infective fungal propagules, spores, then spread passively in the soil, wind, and water. Spores can remain there or settle onto plant surfaces where they can survive for up to several weeks depending on the amount of UV exposure, which degrades the spores (Sinha et al., 2016; Thompson et al., 2006). Entomopathogenic fungi also thrive in the carbohydrate-rich rhizosphere of plants, where their competency varies by plant and fungal species, as well as by fungal isolate (Bruck, 2010). Some entomopathogenic fungi can colonize plants and exist within their tissues as endophytes (reviewed in Vega, 2018, see Chapter 3).

Biological control, the regulation of plant and animal numbers by natural enemies, is used in pest management to reduce dependency upon, or as an alternative to, chemical pesticides. The use of fungal entomopathogens for insect pest control has been studied for more than a century (Roberts & Hajek, 1992). Negative environmental and health impacts from chemical pesticides have led to increasingly strict regulations, especially for organophosphates and neonicotinoids (Health Canada, 2022a, 2020b; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017; McGrath, 2014). Biological control methods do not pose the same risks to human health or the environment. Programs such as Canada's Pesticide Risk Reduction Program aims to increase the adoption of integrated pest management and the use of biopesticides by growers (Health Canada, 2004).

Fungal entomopathogens can be used both for above-ground and below-ground pests and can be applied in various ways. Conidia can be mass-produced *in vitro* and suspended in a liquid or mixed with a powder carrier and sprayed as a mist or dust using conventional chemical insecticide equipment. They can be applied in oil suspensions for use in bodies of water to combat mosquitoes (Bukhari et al., 2011), for use in dry environments for locusts (Scanlan et al., 2001), and in dry formulations, such as in granules for band applications (Behle & Goett, 2016). Although entomopathogenic fungi have the potential for use as classical biocontrol agents (Hajek et al., 2021), most research focuses on their use as inundative agents to achieve a rapid reduction of the pest (Butt et al., 2001). Important factors in the viability of a fungus for biological control are its ease of mass production, consistent efficacy, production cost, and stability. Of the many entomopathogenic fungi, most of the researched and commercially produced

species are of the genera *Beauveria*, *Metarhizium*, *Lecanicillium*, and *Isaria* (reviewed in Lacey et al., 2015; Vega et al., 2012).

1.2. *Beauveria bassiana*

Beauveria bassiana is an ascomycete entomopathogenic fungus that occurs naturally in soils worldwide and is found in both temperate and tropical areas (Zimmermann, 2007). *Beauveria bassiana* has a large host range and has been observed to infect 707 species within 149 insect families (Hall & Papierok, 1982; Li, 1988). As of November 2021, there are 3492 records of *B. bassiana* isolated from various insect hosts listed in the USDA ARS Entomopathogenic Fungus collection (U.S. Department of Agriculture, 2016). Even though *B. bassiana* has a large host range, individual isolates can have differences in virulence to specific insects (Cottrell & Shapiro-Ilan, 2003; Li et al., 2014).

1.2.1. *Beauveria bassiana* in biocontrol

Beauveria bassiana has a broad host range, a cuticular infection mode, an ability to infect most life stages, and can be cultured and mass produced easily, leading its investigation as a control agent against many insect pest species. This includes lepidopterans (Bathina & Bonam, 2020; Kovač et al., 2020), coleopterans (Akello et al., 2008; Batta, 2018; Chałańska et al., 2017), and mosquitoes (Blanford et al., 2011). In Canada, as of September 2022, there were 21 registered products across five strains of *B. bassiana,* primarily for the biological control of greenhouse pests such as whiteflies, aphids, and thrips (Brownbridge & Buitenhuis, 2019; Health Canada, 2022b).

There are several advantages and disadvantages of using entomopathogenic fungi such as *B. bassiana* as control agents compared to other natural enemies of insect herbivores. A benefit is their ability to survive as spores during unfavourable environmental conditions. They are not mobile and depend on passive transportation, which prevents loss due to dispersal; however, this also makes them vulnerable to ultraviolet (UV) degradation. Under simulated sunlight, *B. bassiana* conidia are inactivated within minutes to hours, depending on the strain (Krieg et al., 1981; Morley-Davies et al., 1996). This vulnerability has resulted in various strategies to protect conidia from UV in microbial control agents, such as the incorporation of sunscreens into

formulations or the use of lignin coatings (Inglis et al., 1995; Leland & Behle, 2005). Fungal entomopathogens are also sensitive to low humidity conditions, requiring high humidity to germinate. Strategies such as including moisture-retaining substances in the formulation of bioinsecticides are used to overcome this constraint. However, the longevity of fungal conidia is decreased at higher humidities (Clerk & Madelin, 1965); therefore, there is a compromise between optimizing germination versus shelf life in bioinsecticide products (Boyetchko, 2020). The efficacy of *B. bassiana* can also be improved by incorporating it with other insecticides, such as spinosad, resulting in synergistic effects (Bourdon et al., 2021; Ericsson et al., 2007).

Beauveria bassiana infection in insects can result in non-lethal effects that can contribute to crop protection. This includes behavioural effects, such as reduced feeding rates (Hussain et al., 2015; Tefera & Pringle, 2003; see Chapter 2), as well as physiological effects, such as reduced fecundity (Toledo et al., 2007), and reduced survival of subsequent generations (Torrado-León et al., 2006). For example, the offspring of *B. bassiana* – treated parental whiteflies (*Bemisia tabaci*) and western flower thrips (*Frankliniella occidentalis*) exhibited moulting problems (Torrado-León et al., 2006) and had a lower population growth rate and reproductive rate (Zhang et al., 2015), respectively.

Like any insecticide released into the environment, entomopathogenic fungi can potentially affect non-target organisms. Although *B. bassiana* can infect non-target arthropods including beneficial insects in laboratory settings, it does not appear to happen in typical field settings (Goettel et al., 2021; Hajek & Goettel, 2007, Hanif et al., 2020; Zimmermann, 2007). There is no evidence that *B. bassiana* is toxic to vertebrates. In humans, exposure to high levels of *B. bassiana* spores may trigger allergic reactions (Westwood et al., 2005), but *B. bassiana* infections in humans are very rare and are not a concern. There is also the potential for the displacement of non-target microorganisms, but the impact of interference with the resident environmental microbiota is not well understood. When an exotic strain of *B. bassiana* was introduced to control a pest insect, Wang et al. (2004) found that indigenous strains of *B. bassiana* remained dominant. There is also the risk of genetic exchange between indigenous and exotic *B. bassiana* strains, but Castrillo et al. (2004) found that it is unlikely due to its large number of vegetative compatibility groups.

1.2.2. Insect resistance to *B. bassiana*

The reproduction of *B. bassiana* depends on its ability to invade arthropod hosts. Once the conidia encounter the host, they attach to the host cuticle through hydrophobic forces, and the attachment is further strengthened by fungal enzymes (Boucias & Pendland, 1991). Once attached, *B. bassiana* will germinate and produce several cuticle-degrading enzymes including proteases, chitinases, and lipases, depending on the strain (Boucias & Pendland, 1998). Germination usually starts 10 h after attachment and is completed by 20 h at 20-25°C. Afterwards, *B. bassiana* penetrates the cuticle and epidermis, usually near the joints, between segments, or the mouthparts (Zimmermann, 2007), and then grows towards the hemocoel. The successful adhesion, germination, and penetration of the host cuticle depend on many factors, including host cuticle proteins and environmental factors such as temperature, humidity, and UV irradiation. The age of the host and life stage also influence success; younger insect larvae are generally more susceptible than older ones, and the ability to infect adults or moulting insects varies by strain (Boucias & Pendland, 1998).

Insects have a variety of defence mechanisms against fungal infection, firstly with a physical barrier, followed by innate immune responses including cellular and humoral immunity (Qu & Wang, 2018). The insect cuticle, made of chitin and proteins, is the most effective protective barrier to fungal infection; however, the hydrophobic nature of the insect cuticle is favourable to the adhesion of fungal conidia. Adaptations against fungal adhesion include the production of fatty amides, lipids, and aldehydes, as well as volatile glandular secretions (reviewed in Sinha et al., 2016). The production of cuticular molecules plays a vital role in determining which strains of *B. bassiana* are pathogenic to the insect (Lecuona et al., 1997). Moulting is important in insect resistance as well, as the fungal conidia are cast off before they can penetrate the cuticle (Vey & Fargues, 1977).

Once a fungus enters the hemolymph, the insect's innate defence response initiates. The cellular immune response is mediated by hemocytes and involves phagocytosis, encapsulation, and nodulation (Lu et al., 2015; Sinha et al., 2016). The humoral immune response involves various effector molecules that trigger the phenoloxidase (PO) cascade and produce anti-microbial peptides, antifungal compounds, and protease inhibitors (Tsakas & Marmaras, 2010). Melanization is

activated through the PO cascade which functions in wound healing near the penetration site and limits the growth of fungal hyphae within the hemocoel. In *Drosophila melanogaster*, humoral responses to fungi are largely mediated by the Toll pathway, which leads to the induction of antifungal peptides into the hemolymph. This pathway is upregulated in response to *B. bassiana* infection, but often has little effect (Lu et al., 2015). There is also evidence that the Jak/STAT pathway is involved in fungal infection response (Dong et al., 2012).

Once the fungus successfully penetrates the hemolymph, it will produce blastospores which are distributed passively in the hemolymph to invade other host tissues. Blastospores are produced within 48 h of infection. Death of the host is due to nutrient depletion, dehydration, and toxins produced by the fungus (Boucias & Pendland, 1998). The incubation period of the fungus depends on the host, the fungal strain, and environmental conditions, and can take from days to weeks. Once the host dies and environmental conditions are suitable, *B. bassiana* will erupt from the host body and produce conidia, appearing as a cloud of white spores. These spores are then able to be transmitted to other hosts through passive environmental processes or by insect vectors (Dromph, 2003; Kreutz et al., 2004; Toledo et al., 2007).

Insect resistance to insect pathogens such as the bacterium *Bacillus thuringiensis*, which involves one or more toxins, or the codling moth granulovirus. a highly specific pathogen, is typically conferred by a single mechanism of resistance from a small number of key mutations (reviewed in Cory, 2017). Contrastingly, the development of resistance against fungal entomopathogens such as B. bassiana is unlikely because they are generalists which employ a variety of virulence factors. Resistance would likely involve both cellular and humoral immunity; therefore, there is a high fitness cost for the insects to develop multiple modes of resistance. Furthermore, fungal insecticides are often used in combination with other control measures, such as chemical insecticides (reviewed in Kubicek & Druzhinina, 2007), and their vastly different modes of action allows for reduced selection pressure for the development of resistance, which occurs due to repeated exposure to an insecticide. There have not been any reports of insects developing resistance against B. bassiana when used for pest management.

1.2.3. *Beauveria bassiana* toxins

Beauveria bassiana produces metabolites that have antibacterial, antifungal, cytotoxic, and insecticidal effects to aid in its invasion of hosts (Boucias & Pendland, 1998; Wang et al., 2021). The major metabolites produced are the cyclic peptides beauvericin and bassianolide, and the pigments bassianin and tenellin. Beauvericins dissolve within lipid bilayers and increase the permeability of cell membranes to ions, and this change in ion transport disrupts the function of cells and organelles (Wang & Xu, 2012). Bassioanolides are toxic to insects and are a significant virulence factor (Patocka, 2016). Bassianin and tenellin inhibit erthyrocyte membrane ATPases, but little is known about their function. Other metabolites include oosporein, oxalic acid, and when grown in culture, cyclosporin A. Oosporein blocks the insect immune system and suppresses bacterial growth after the death of the host so the fungus can fully utilize all nutrients (Feng et al., 2015), and oxalic acids are important because they can solubilize specific cuticular proteins (Butt et al., 2001). Cyclosporein A has immunosuppressive properties (Ptachcinski et al., 1986). Recently, a new virulence factor was discovered in *B. bassiana* in the form of a ribotoxin (Yuan et al., 2020); it inhibits insect host immunity by modulating the response to reactive oxygen species and suppressing antimicrobial peptide production. Several novel *B. bassiana* toxins have also been discovered with various insecticidal properties (Wang et al., 2021).

1.3. Fungal entomopathogens interact with plants

Fungal entomopathogens interact with other species in their communities, whether it be directly or indirectly, leading to tri-trophic interactions among fungi, plants, and insects (Cory & Ericsson, 2010; Elliot et al., 2000; Mantzoukas & Lagogiannis, 2019; Qin et al., 2021; Ramirez-Rodriguez & Sanchez-Pena, 2016; Shrivastava et al., 2015; Silva et al., 2020; Vega, 2018; Zitlalpopoca-Hernandez et al., 2017). Plants produce a wide range of secondary metabolites that protects them against herbivores and plant pathogens, but they can have side effects on insect pathogens as well; differences in metabolite production between plant species dictate their relationships with insect pathogens such as fungal entomopathogens (Elliot et al., 2000; Erb et al., 2021; Mann & Davis, 2020; Souza-Moreira et al., 2019). Plants can directly affect fungal entomopathogens through their structural topology, architecture, surface chemistry, and

leaf modifications that affect the survival of fungal spores (reviewed in Cory & Ericsson, 2010). Plants can also indirectly influence the susceptibility of insects to entomopathogenic fungi: plant defences that are sublethal to the insect, or poor plant nutritional quality, can reduce the growth and vigour of the insect, impacting disease resistance and resulting in a greater chance of mortality (reviewed in Bala et al., 2018; Cory & Hoover, 2006). Slower growth can result in a prolonged window of vulnerability to natural enemies and therefore greater mortality overall (Feeny, 1976; Poprawski & Jones, 2001; Shikano et al., 2018; Uesugi, 2015). Plant structural differences can also influence insect behaviour and thus indirectly affect spore contact rates (Inyang et al., 1998).

Entomopathogenic fungi can also form close relationships with plants, whether it be through mycorrhizae-like interactions, whereby fungi derive carbon from the rhizosphere while transporting nutrients (derived from a parasitized insect cadaver) to the plant (Behie et al., 2012), an interaction in the rhizosphere, or an endophytic relationship. Endophytes are microorganisms, typically bacteria and fungi, that reside within living plant tissue without causing disease. They provide a range of benefits to plants, such as providing protection against insect herbivores by activating plant defences (see Chapter 3). In a meta-analysis, Gange et al. (2019) found that insects of the orders Hemiptera, Thysanoptera, and Hymenoptera, especially those with galling and sucking lifestyles, were negatively affected by entomopathogenic endophytes; likely through indirect effects mediated by plant defences. Although fungal entomopathogens reduce insect herbivory, their lack of mobility means that they cannot be recruited to insect-damaged plants in the same way as other natural enemies such as predators or parasitoids. However, plants can influence their microbiota through the production of certain root exudates, as has been demonstrated with bacteria (Cotton et al., 2019; Mattoo & Nonzom, 2021), and with *B. bassiana* (McKinnon et al., 2018).

1.4. Wireworms

The larvae of Elaterid beetles are commonly called wireworms and are one of the most diverse insect families. They are found in a broad range of habitats, residing in soil and forest litter, and feeding on plants, animals, and organic matter. Some species are subterranean agricultural pests that feed on root systems and burrow into stems, roots, and tubers, reducing crop yields, and causing agricultural products to become

unmarketable. Wireworms are often hard-bodied, segmented, and golden yellow (Figure 1.1).

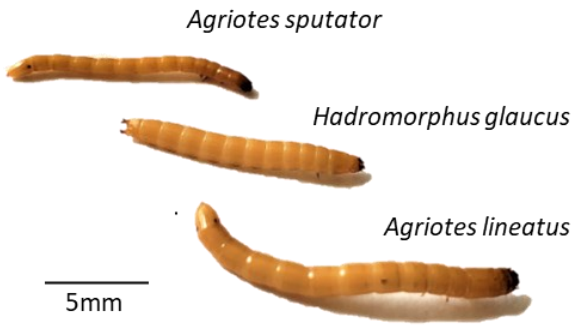

Figure 1.1. *Agriotes sputator*, *Hadromorphus glaucus*, and *Agriotes lineatus* wireworms.

1.4.1. Distribution and species in Canada

There are approximately 10,000 species of Elaterid beetles in 400 genera worldwide (Johnson, 2002) and of these, at least 100 species are considered economic pests (Vernon & van Herk, 2022). The main distinguishing feature of this family is the adult beetles' ability to flex and snap themselves into the air to avoid predators and to right themselves, resulting in their common name 'click beetles'. In Canada, approximately 400 species have been described (Bousquet et al., 2013) and both native and invasive species of wireworms are pests. In British Columbia, Nova Scotia, and Newfoundland*, Agriotes obscurus* and *A. lineatus* are the most prominent pest species, and *A. sputator* is the major species in Nova Scotia and Prince Edward Island (Agriculture and Agri-Food Canada, 2018). The introduction of the invasive *Agriote*s species from Europe to BC was likely through the nursery trade between 1895 and 1900 (Wilkinson, 1963), and introduction to the eastern provinces likely was from ship ballasts as early as the 1850s (Eidt, 1953). In the prairies, all wireworm pest species are native, and the two most widespread are *Hyponoidus bicolor and Selatosomus aeripennis destructor* (van Herk et al., 2021). Other species include *Limonius californicus, Aeolus mellillus*, and *Hadromorphus glaucus* (van Herk et al., 2021).

1.4.2. Life cycle

The life cycles of click beetles vary by species. Generally, click beetles lay eggs in the spring and early summer just below the soil surface, and wireworms hatch in about 4-6 weeks depending on temperature. Neonates are about 1.5mm long and larvae can grow up to 25mm, depending on the species (Parker & Howard, 2002). As neonates, they are not yet damaging to crops due to their small size and at this stage, they are very vulnerable to starvation, cannibalism, and environmental extremes. After their first year, they become resilient to stress and unfavourable conditions, surviving at least three years without feeding and even moulting to a smaller size to delay pupation during poor conditions (Strickland, 1939). Wireworms age very slowly and pass through 1-5 instars per year. *Agriotes* wireworms have 8-14 instars and pupate within 2-5 years depending on the species and environmental conditions.

Soil temperature is the major determinant of moulting time, but soil moisture content and food quality play a role as well (Evans & Gough, 1942; Furlan, 1998). *Agriotes* wireworms will reach maturity in the summer to early fall. To pupate, the wireworms will hollow out pupation cells 5-30cm into the soil where they will pupate for 3-4 weeks, after which the adults will remain through the winter (Parker & Howard, 2002). In early spring, adults will emerge and will immediately mate, then, oviposition occurs for several weeks, after which they will die. Most species including *S. a. destructor* and *Agriotes* spp. require mating to reproduce, but some populations of *H. bicolor* are parthenogenic (Catton et al., 2021).

1.4.3. Feeding

Wireworms feed on a variety of crops including cereals, legumes, maize, and potatoes (Saguez et al., 2017; Vernon & van Herk, 2022). They do not appear to prefer specific crops (Griffiths, 1974; Kruger, 1933; Schallhart et al., 2012). Wireworms are attracted to carbon dioxide emitted from plant roots (Doane et al., 1975), as well as other root volatiles (Gfeller et al., 2013; la Forgia et al., 2020). Wireworms prefer feeding on thin and soft roots (Johnson et al., 2010) and their attraction to specific plants can change seasonally (Staudacher et al., 2013).

In temperate regions, wireworms have two distinct feeding periods. The first period begins when soil temperatures warm to approximately 10°C in the spring, at which point wireworms, likely attracted by germinating plants, move upwards from deep soil to feed (Edwards & Evans, 1950). This period coincides with the planting of crops. Wireworm damage in potato seed tubers and roots typically does not affect crop establishment and wireworm damage is often undetectable aboveground. However, wireworm feeding of newly planted seed crops will cause seedling mortality and reduced stands. Wireworm damage to cereal crops appears as hollowed-out seeds and damaged stems, resulting in the wilting or yellowing of the early crop (Catton et al., 2021). The second feeding period occurs in the late summer to early fall. In potatoes, wireworms feed and tunnel into the daughter tubers creating unsightly feeding holes, which gives other soil organisms access to feed or cause disease (Keiser et al., 2012). Furthermore, feeding on young tubers causes deformities (Vernon & van Herk, 2022). The longer potatoes are left in the ground, the more opportunities wireworms have to cause damage, although it is unknown whether this is due to continued feeding by the same wireworms, or to increasing numbers of feeding wireworms (Vernon & van Herk, 2022). Adult beetles feed on nectar, pollen, flowers, fruit, and leaves, and their feeding is not considered harmful to crops.

1.4.4. Control methods

Wireworms are challenging to manage and study due to their subterranean habitat, patchy distributions, and long life cycle. To manage wireworms, there are multiple cultural control methods available, such as avoiding infested fields, cultivar selection, crop rotation, and the use of trap crops (reviewed in Barsics et al., 2013). Chemical control methods are the most effective method of wireworm management. Following the ban of the highly effective but toxic organochlorines, organophosphates and carbamates became the first line of defence for managing wireworm damage in potatoes (Parker & Howard, 2002). However, toxicity remains a point of concern. The organophosphate most widely used in potatoes in Canada, phorate, has been withdrawn from use since 2015 in part due to raptor poisonings (Elliott et al., 1996). Most organophosphates are now obsolete or facing de-registration in North America (Health Canada, 2020a). The current market standard of protection of cereals and pulses from wireworms is neonicotinoid insecticides. However, they are unable to kill wireworms and

instead cause temporary intoxication. Neonicotinoids are harmful to pollinators and are currently under re-evaluation (Health Canada, 2020b). Since 2020, broflanilide, a novel meta-diamide, has been available in Canada for use in cereals, corn, and potatoes. However, new methods of control must constantly be researched due to risks such as insecticide resistance, and future safety re-evaluations.

There are several promising candidates for the biological control of wireworms, such as bacterial, a relatively new area of research, and entomopathogenic nematodes. Wireworms succumb to a range of different bacterial pathogens such as species of *Pseudomonas* and *Bacillus* (Kleespies et al., 2013; Traugott et al., 2015). Some bacterial strains isolated from diseased wireworms, as well as other known *Bacillus* isolates, have the potential for biocontrol (Danismazoglu et al., 2012). Entomopathogenic nematodes have a broad host range, are very active, and can quickly kill hosts. They enter the wireworm through natural openings, after which they release *Photorhabdus* and *Xenorhabdus* bacteria. These bacteria produce toxins that kill the larvae within a couple of days and produce other secondary metabolites to suppress other microbes (Dillman & Sternberg, 2012). The nematodes then feed on the liquifying host and bacteria. Nematodes can cause wireworm mortality in the lab (Ansari et al., 2009; Öğretmen et al., 2020) but field experiments show mixed results (Ester & Huiting, 2007; Öğretmen et al., 2020). Though encouraging, more research must be performed.

More promising is the use of entomopathogenic fungi for the biocontrol of wireworms. *Metarhizium brunneum (*formerly *M. anisopliae* var. *anisopliae*) is a naturally occurring soil fungus that has a broad host range and whose virulence towards different insect orders is dependent on the strain. It has been shown to be effective in wireworm control in field studies against both invasive European species as well as indigenous Canadian species (Ritter & Richter, 2013). Because wireworms reside within the soil, strategies such as in-furrow sprays and food baits are used. Mixed results have been observed by using *M. brunneum* against wireworms in cover crops and crop rotations (Rogge et al., 2017), as well as directly into crop fields (Kabaluk & Ericsson, 2014; Reddy et al., 2014). *Beauveria bassiana* is an entomopathogenic fungus that is commonly used in biocontrol and has potential for use against wireworms. It has shown promise in both lab and field studies (Antwi et al., 2018; Ester & Huiting, 2007; Reddy et al., 2014), and will be discussed further in Chapter 2.

1.5. Research objectives

The protection of plants from insect pests by *B. bassiana* can occur by causing direct mortality, or by altering pest behaviour to reduce herbivory. However, industrial field trials also observed that *B. bassiana* strain PPRI5339, first isolated from a tortoise beetle larva (*Conchyloctenia punctate*) in South Africa (European Food Safety et al., 2018), provided a reduction in wireworm feeding damage at harvest, but apparently without causing wireworm mortality (BASF, internal data). The mechanism behind this protection is not known. This thesis investigates the possible mechanisms by which *B. bassiana* PPRI5339 could reduce wireworm feeding damage in potatoes through laboratory and greenhouse experiments. In Chapter 2, the susceptibility of different sizes and species of wireworm to *B. bassiana* is investigated, as well as the sub-lethal behavioural effects of *B. bassiana* inoculation, including avoidance, movement ability, and feeding activity. In Chapter 3, the effects of seed inoculation of *B. bassiana* on potato performance are examined, as well as its effects on plant defence hormone levels in the presence of wireworms, and whether it protects against wireworm feeding damage. In Chapter 4, the effects of *B. bassiana* on the bacterial abundance, diversity, and composition in the wireworm internal microbiome are examined.

Research questions:

- Are wireworms susceptible to *B. bassiana* infection, and does the response differ between inoculation methods, wireworm size, or wireworm species?
- Do wireworms avoid *B. bassiana*?
- Does *B. bassiana* cause changes to wireworm movement or feeding behaviours?
- Does *B. bassiana* inoculation of potato plants affect plant growth, tuber production, levels of plant defence hormones, or protect tubers from wireworm herbivory?
- What are the effects of *B. bassiana* inoculation on the wireworm internal microbiome, specifically, on the bacterial load, community composition, and diversity?

1.6. References

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Chapter 2.

The effects of *Beauveria bassiana* on wireworm survival and behaviour

2.1. Abstract

The use of *Beauveria bassiana*, a fungal entomopathogen, as a biological control agent against wireworms is of interest due to the limited availability of effective and sustainable control options. Wireworms, the larvae of elaterid beetles, are major soilborne pests of crops worldwide. A reduction in wireworm feeding damage due to *B. bassiana* has been observed, but the mechanism of protection is unknown. In this chapter, I examined the susceptibility of wireworms to *B. bassiana*, and if the response differed between larval sizes, or among the species *Agriotes lineatus, A. sputator*, and *Hadromorphus glaucus.* The effects of *B. bassiana* on wireworm behaviour were investigated using choice experiments and window bioassays. *Beauveria bassiana* inoculation did not affect wireworm survival, irrespective of the size or species. Wireworms avoided *B. bassiana* and daughter tubers of B. bassiana-inoculated potato plants. *Agriotes lineatus* wireworms that were inoculated with *B. bassiana* spent slightly less time feeding. The apparent protection of crops from *B. bassiana*-exposed wireworms is not primarily due to mortality, avoidance, or behavioural effects on the insect.

Keywords: *Beauveria bassiana*; entomopathogen; wireworm; biological control; avoidance; insect behaviour

2.2. Introduction

The use of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent to combat agricultural pests is a successful strategy in a variety of different crop systems (reviewed in Zimmermann, 2007). The reduction of crop damage by insect pests after exposure to *B. bassiana* is typically caused by direct mortality; however, changes to insect behaviours such as avoidance and reduced food consumption have the potential to reduce damage to plants, as seen with other crop protection agents

(Copping & Duke, 2007; Koul, 2008). This chapter examines whether the infection route of *B. bassiana* influences wireworm (larvae of elaterid beetles) susceptibility and if susceptibility is influenced by the age or species of the wireworm. Furthermore, I examine how *B. bassiana* may be affecting wireworm behaviours that can result in reduced potato feeding damage.

2.2.1. Two factors affecting *B. bassiana* infection success

The successful infection of *B. bassiana* depends on the ability of the fungus to recognize the insect, attach to the cuticle, penetrate it, and resist the insect immune system (Zimmermann, 2007). Among many others, two factors that influence the susceptibility of an insect to *B. bassiana* are the infection route and the age of the insect. Infection of insects by fungal entomopathogens typically occurs through the cuticle, but ingestion is an alternate route of infection that bypasses the need for cuticle penetration. *Beauveria bassiana* shares many genes with non-fungal insect pathogens that infect orally such as *Bacillus thuringiensis* (Mannino et al., 2019), which may aid in overcoming unfavourable insect gut conditions including digestive enzymes and the microbiota. Yanagita (1987) found that *B. bassiana* was able to germinate in the digestive juice of silk moth (*Bombyx mori*) larvae and that oral inoculation of the larvae was able to cause mortality, albeit at a lower level than from cuticular infection. Insects that were successfully invaded after oral *B. bassiana* inoculation include *Solenopsis richteri* ants (Broome et al., 1976), *Aedes aegypti* mosquitoes (Miranpuri & Khachatourians, 1991), *Melanoplus sanguinipes* grasshoppers (Jeffs et al., 1997), and *Sitophilus granariu*s weevils (Batta, 2018). Insect age affects susceptibility to mycosis, with a trend that shows older adults being more resistant to infection compared to younger adults (reviewed in Ben-Ami, 2019), but with exceptions (Maniania & Odulaja, 1998). Similarly, larval age also impacts fungal disease susceptibility, with older instars having a lower susceptibility compared to younger instars (Levchenko et al., 2020; Yubak Dhoj et al., 2008).

2.2.2. Repellency of *B. bassiana*

Fungi produce a large variety of volatile organic compounds which are involved in different biological processes (reviewed in Hung et al., 2015), including in the

attraction and/or repellency of insects. The application of fungi that have attractive or repellent properties on crops can therefore affect the level of insect pest damage.

The ability to detect enemy semiochemicals is an important behavioural defence mechanism of many insects, and co-evolution between insects and entomopathogens and their ability to detect and avoid detection, respectively, drives their relationship (Baverstock et al., 2010). The behavioural response of termites to *M. anisopliae* or *B. bassiana* is directly related to fungal virulence, with the more virulent strains being more likely to be avoided (Mburu et al., 2009). Repellency caused by *B. bassiana* has been observed in a variety of insects including termites (Osipitan et al., 2016), ladybirds (Ormond et al., 2011), mole crickets (Thompson & Brandenburg, 2005), and palm weevils (Jalinas et al., 2016) in laboratory studies. In beneficial insects, *B. bassiana* has also been shown to have repellency effects on the predatory mite *Phytoseiulus persimilis*, but it is short-lived and was not shown to affect predation rates (Wu et al., 2018). Entomopathogenic fungi can colonize plants and produce volatiles, or induce plants to produce volatiles, that repel insects. Grape, onion, tomato, cotton, and corn colonized by *B. bassiana* have been shown to repel, respectively, vine weevils (Rondot & Reineke, 2017), thrips (Muvea et al., 2015), whiteflies (Wei et al., 2020), aphids (Gurulingappa et al., 2011), and grasshoppers (Pelizza et al., 2017).

The repellency of fungal biocontrol agents is beneficial if its intent is to make crops less desirable for insects, but it may pose a problem if the aim is to cause mortality by infection. In these instances, strategies such as introducing a food bait may reduce the repellency. Kabaluk and Ericsson (2007) found that when a food bait was introduced, the rate of emigration of *Agriotes obscurus* wireworms from *Metarhizium* spore-contaminated soil was reduced. Similarly, Wang and Powell (2004) incorporated *M. anisopliae* spores into cellulose bait, which reduced the repellency of the termites by the fungus.

2.2.3. Anti-feeding behavioural effects of *B. bassiana*

When compared to chemical pesticides, fungal entomopathogens often take longer to kill insects. However, infected insects tend to have reduced food consumption, which decreases the amount of damage caused by their herbivory during the incubation period. *Beauveria bassiana* infection of insects, or its colonization in plants, results in

reduced food consumption in insects: for example, in lepidopterans (Hussain et al., 2015; Kovač et al., 2020; Russo et al., 2019; Tefera & Pringle, 2003), orthopterans (Mohammadbeigi & Port, 2015; Pelizza et al., 2017; Sieglaff et al., 1997; Srygley & Jaronski, 2010), coleopterans (Ekesi, 2001; Fargues et al., 1994; France et al., 2002), and mosquitoes (Blanford et al., 2011; Darbro et al., 2012). Reduced food consumption may be attributed to toxins secreted by the fungi, or the physical disruption of insect tissues by mycelial growth. It may also be partly due to impacts on insects' ability to detect food odour cues after fungal infection (George et al., 2011). Interestingly, Noma and Strickler (2000) found that *Lygus hesperus* (Hemiptera) inoculated with *B. bassiana* resulted in greater feeding damage compared to their control group. The authors speculate that this increased feeding activity may be in response to a nutrient deficiency resulting from the fungus drawing nutrients from the host's hemolymph.

2.2.4. Wireworms

Click beetle larvae (Coleoptera: Elateridae), commonly called wireworms, are important agricultural pests worldwide. Feeding upon a variety of crops, these subterranean insects cause plant mortality and unsightly, costly feeding holes in produce (Barsics et al., 2013; Vernon & van Herk, 2022). Controlling wireworms is difficult due to their patchy distribution and their long larval stage. In western Canada, wireworms of the genus *Agriotes* are the most important species of economic concern (van Herk et al., 2021). Traditional chemical insecticides used to control wireworms are being phased out in many countries including Canada due to undesirable environmental and health impacts (Health Canada, 2022). Although the meta-diamide insecticide broflanilide has recently been introduced into the Canadian market, there is still the need for the development of alternative control methods to chemical insecticides, such as the use of biological control agents against wireworms.

The use of entomopathogenic fungi in wireworms

A variety of entomopathogenic fungi have been recorded as infecting *Agriotes* sp. larvae (Kleespies et al., 2013), and the two most notable species are *M. brunnuem* and *B. bassiana*. *M. brunneum* (formerly *M. anisopliae var. anisopliae*) is an entomopathogenic fungus that has been well-studied for the biocontrol of wireworms worldwide. It is virulent toward wireworms in laboratory experiments (Ansari et al., 2009;

Razinger et al., 2018) and is effective at protecting crops in field experiments to protect crops (Brandl et al., 2017; Kabaluk, 2020; Thien et al., 2021). Studies on the efficacy of *B. bassiana* for wireworm control have had mixed results: some laboratory experiments have found that *Agriotes* wireworms do not die from *B. bassiana* inoculation (Ansari et al., 2009; Razinger et al., 2018); others have found moderate mortality, depending on dosage (Chałańska et al., 2017; Sufyan et al., 2017). Mixed results have also been found in field experiments using *B. bassiana* against wireworms (Ansari et al., 2009; Antwi et al., 2018; Ester & Huiting, 2007; Ladurner et al., 2009; Reddy et al., 2014; Schepl et al., 2010; Sufyan et al., 2017).

2.2.5. Objectives

Early field studies found that the application of *B. bassiana* strain PPRI5339 (BASF), originally isolated from a tortoise beetle larva (*Conchyloctenia punctate*) in South Africa (European Food Safety et al., 2018), provided a reduction in wireworm feeding damage at harvest without apparent wireworm mortality (BASF, internal data). In this chapter I investigate the effects of *B. bassiana* strain PPRI5339 inoculation on wireworm survival and behaviour in the aim of understanding the causal mechanism(s) for reduced wireworm herbivory.

Firstly, I examined wireworm survival after inoculation with *B. bassiana*. To ensure the highest probability of infecting larvae, I compared three inoculation methods: (i) wireworms brushed directly with spores, (ii) dipped in a spore suspension, and (iii) fed with granules. I then compared the susceptibility of large wireworms to small wireworms after *B. bassiana* inoculation. The susceptibilities of wireworms from three distinct geographical locations to *B. bassiana* were examined: the two major invasive European wireworm species in Canada, *A. lineatus* from the western coast, and *A. sputator* from the eastern coast, as well as the indigenous prairie species *Hadromorphus glaucus*. Secondly, I investigated wireworm avoidance of *B. bassiana* spores directly or when formulated as granules, as well as avoidance of tissue from inoculated plants. Finally, I examined wireworm movement and feeding behaviours after *B. bassiana* inoculation using different inoculation methods and compared the three wireworm species. Evaluating the possible mechanisms of reduced wireworm feeding damage caused by *B. bassiana* is vital in its development as a biocontrol agent.

2.3. Materials and Methods

Agriotes lineatus, A. sputator, and *H. glaucus* wireworms were sourced from Vancouver Island, Prince Edward Island, and Alberta, respectively. Wireworms were kept in a 4°C cooler and fed with a piece of potato until use. To select active feeders, wireworms were placed in 25°C and provided half a potato on the soil surface and collected from the potato after 6 h. The soil used was a silt-loam mixture collected at the Agassiz Research and Development Centre, BC. All soil used was sifted through a 1.18mm screen and autoclaved at 121°C for 50 min at 16psi. Soil moisture was adjusted to 16-18% moisture content.

Beauveria bassiana PPRI5339 (BASF) was mass-produced using solid-state fermentation on rice (Jaronski, 2014) and stored in vacuum-sealed bags at 4°C. *Beauveria bassiana* spore viability was determined by plating 50µl of a 1×10^6 spores/mL suspension on potato dextrose agar and counting the number of germinated spores out of 100 after 24 h at 27°C twice. Spore viability was 97% or greater for all experiments.

2.3.1. Experiment 2-1: Are wireworms susceptible to *B. bassiana*?

Three experiments were carried out to investigate the impact of *B. bassiana* inoculation on the survival of wireworms. Firstly, three fungal inoculation methods were tested to determine which method, if any, were most effective at causing wireworm mortality. Secondly, the effect of larval size on wireworm susceptibility to *B. bassiana* was evaluated. Thirdly, the response of three wireworm species to *B. bassiana* inoculation was compared.

2.3.1.1 Experiment 2-1a: B. bassiana inoculation methods

Agriotes lineatus mortality was compared using three *B. bassiana* inoculation methods: (i) direct brushing with spores, (ii) dipping in a spore suspension, (iii) feeding with formulated granules, and (iv) a non-inoculated control group. Wireworms in the brushed treatment (i) were held by soft tweezers and brushed with a paintbrush which had been dipped into sporulated rice granules (1×10^{10} spores/g rice), until saturated with white spores. The wireworm was dropped twice from a height of 30cm onto a solid surface to dislodge loose spores. Wireworms in the dipped treatment (ii) were

submerged for 10 s in a suspension of 0.05% Tween 80 containing 2×10^7 spores/mL. This spore concentration was chosen based on experience using *M. brunneum*. Wireworms in the fed treatment (iii) were placed in soil and fed with *B. bassiana*-formulated granules (proprietary formula, BASF). Wireworms in the brushed, dipped, and non-inoculated control group were placed into soil with blank granules. All granules were mixed into the soil at a density of 0.043g granules/g dry soil. The number of spores (dose) inoculated onto wireworms through the brush and dip treatments was measured by submerging treated wireworms in 1mL of 0.1% Tween 80. Five replicates (individual wireworms) per treatment were then vortexed for 30 s, placed in an ultrasonic bath for 30 s, and then the number of spores per ml was counted twice under 400x magnification using a hemocytometer and the mean was taken.

2.3.1.2 Experiment 2-1b: Wireworm sizes

The largest *A. lineatus* wireworms were compared to the smallest wireworms available, with the size being a proxy for wireworm age (and instar), to evaluate whether larval age impacts susceptibility to *B. bassiana*. The length and weight of the wireworms were measured before they were inoculated, or mock-inoculated, by the brush method (see 2.3.1.1). The four treatment groups consisted of large non-inoculated wireworms, large inoculated wireworms, small non-inoculated wireworms, and small inoculated wireworms. The sizes of the groups are described in Table 2.1. Wireworms in the small size group corresponds approximately to the larval instars L2-L6, and the large size group corresponds approximately to the larval instars L8-L11+ (Sufyan et al., 2014).

Table 2.1. Weight, size range, and length of the *A. lineatus* wireworms within the large and small size groups used in the experiment examining their susceptibility to *B. bassiana* inoculation (2-1b).

Experimental replicate	Size group	Mean weight (g)	Size range (g)	Length range (mm)
1	Large	0.037 ± 0.0054	0.031-0.048	
1	Small	0.0049 ± 0.0022	0.0028-0.012	
2	Large	0.037 ± 0.0051	0.028-0.049	16.16-22.84
2	Small	0.0044 ± 0.0017	0.0019-0.0085	4.10-10.08

2.3.1.3 Experiment 2-1c: Wireworm species

Wireworms of three species: *Agriotes lineatus*, *Agriotes sputator*, and *Hadromorphus glaucus* were either mock-inoculated or inoculated with *B. bassiana* by brushing (see 2.3.1.1) giving a total of six treatment groups.

2.3.1.4 General methods

For all survival experiments, wireworms were transferred individually from their incubation containers to 1oz plastic cups containing 14 ± 0.2g of soil. Insects of similar sizes were used in all experiments, except in experiment 2-1b, where wireworms were grouped as large and small. Cups were arranged on a tray at random. Wireworms were fed with one germinated wheat seed that was replaced monthly, and the soil was kept moist. Mortality of the wireworms was evaluated once weekly for 1 month, followed by twice weekly until two months at 25°C. After two months, they were placed into a 15°C growth chamber and evaluated for mortality and health (see below) monthly for 4 months (experiment 2-1a) and 6 months (experiments 2-1b & 2-1c). In each treatment group, there were 15 replicates (individual wireworms), except for experiment 2-1c, where there were six in the *H. glaucus* group in the second experiment replicate due to insufficient numbers. Experiments 2-1b & 2-1c were conducted twice.

To measure wireworm health, their mobility was examined using arenas constructed with standard Petri dishes lined with a moistened 9cm filter paper with 1cm circles drawn radiating from the center (adapted from Vernon et al., 2008). Wireworms were introduced into the centre of the arena, given two min. to move freely, and the distance travelled was recorded. Wireworm health was ranked on a scale of 1-3: the rank of 3 ("fast") comprised wireworms that travelled 6cm or more; the rank of 2 ("slow") included wireworms that travelled less than 6cm; the rank of 1 ("not crawling") were wireworms that did not move from the center. Wireworms that were dead, moulting, or pupating were excluded from the health analysis.

2.3.2. Experiment 2-2: Do wireworms avoid *B. bassiana*?

In experiment 2-2, wireworm avoidance of *B. bassiana* was examined with the following research questions:

- 2-2a) Are blank granules attractive to wireworms?

- 2-2b) Do wireworms avoid granules formulated with *B. bassiana*?
- 2-2c & 2-2d) Do wireworms avoid *B. bassiana* spores at low and/or high concentrations in soil?
- 2-2e) Do wireworms avoid potatoes from *B. bassiana* inoculated plants?

Two-choice soil olfactometers were constructed using two 35-mm film canisters with caps that had a 10mm hole drilled in the center (Figure 2.1a,b). The canisters were filled with the appropriate treatment (Table 2.2) and fitted into the two opposite ends of a 48.26 mm T-shaped connecting PVC pipe. The pipe was filled with autoclaved soil and the apparatus was arranged so that the opening of the PVC pipe faced upwards. The experimental setups were left for 2 days to generate a CO_2 gradient and meanwhile, wireworms were transferred from the cooler into soil at 25°C without food in a group. After 2 days, a wireworm was introduced head-downwards in to the opening of the PVC pipe and was left for 24 h. The apparatus was then disassembled, and the location of the wireworm was determined. Wireworms that remained in the PVC connector were considered unresponsive. The apparatuses were sanitized in 3% bleach after each experiment. *A. lineatus* wireworms were used once for experiments 2-2a-c, and *A. sputator* wireworms were used once for experiments 2-2d-e. The treatment groups for experiments 2-2a-d are described in Table 2.2. The blank and *B. bassiana*-formulated granules (BASF) is a proprietary formula whose placement in soil generates CO_2 to attract wireworms.

In experiment 2-2e, we determined whether daughter tubers of plants inoculated with *B. bassiana* affected wireworm avoidance using a 3-choice apparatus (see plant inoculation methods in Chapter 3). An upward facing 48.26 mm PVC elbow was attached to one end of a 48.26 mm PVC cross, and three canisters containing each treatment were attached to the remaining three ends (Figure 2.1c). Treatments were i) soil control, ii) tuber from the control plant, and iii) daughter tuber from *B. bassiana*-inoculated plant (see 3.3.2). Potato cores of the size 12 ± 0.25g were taken using a coring tool (size 23.8mm), added to the canister, and soil was filled to a final weight of 28 ± 1g. The soil control canister was also filled to 28 ± 1g. The arrangement of canisters on the apparatus was randomized. Feeding *A. sputator* wireworms of similar sizes were selected, weighed, and then introduced head-first into the open end. After eight days at 25°C, the apparatuses were disassembled, and the location of the wireworm and the number of feeding holes in the tubers were recorded.

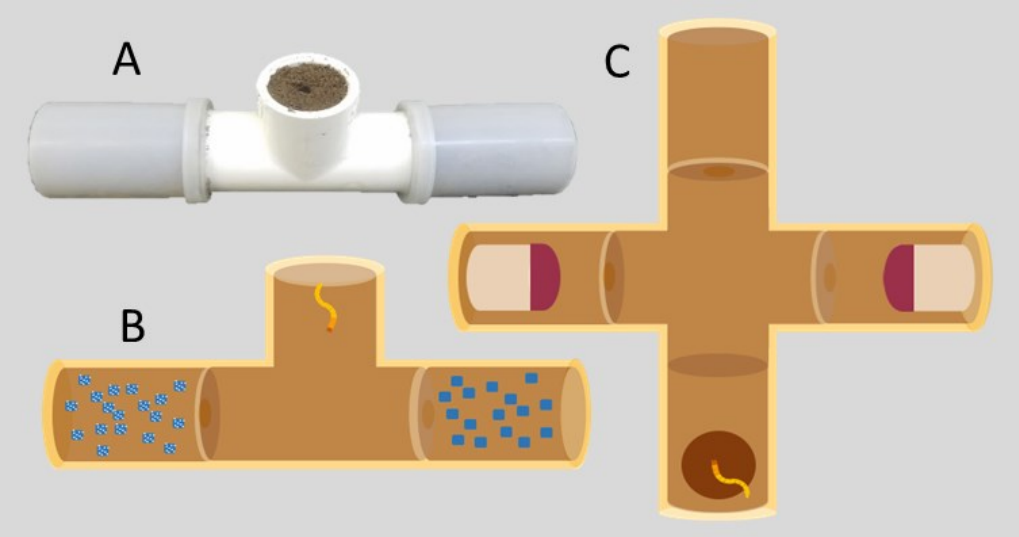

Figure 2.1. **A) Photograph and B) diagram of the two-choice apparatus used to examine the response of wireworms to *B. bassiana* treatments and the respective control (experiments 2-2a to 2-2d). C) Diagram of three-choice apparatus (aerial view) used to examine the response of wireworms to potato tubers inculated with *B. bassiana* versus the control (experiment 2-2e). 35mm film canisters containing each treatment closed with caps that had a 10mm hole drilled in the center and then fitted to a 48.26mm PVC connector. A PVC elbow was attached to the connector in C).**

Table 2.2. **Treatment parameters of two-choice experiments examining the response of wireworms to *B. bassiana* treatments and the respective control (experiments 2-2a to 2-2d). Treatments A and B describe the contents of each canister and the amounts added. The number of replicates is listed in column "n".**

Experiment	Treatment A	Amount added (g)	Treatment B	Amount added (g)	n
2-2a	Blank granules	0.5 ± 0.03 + 30 ± 0.5 soil	Soil control	35 ± 0.5 soil	30
2-2b	*B. bassiana* granules	0.036 per g dry soil	Blank granules	0.036 per g dry soil	44
2-2c	*B. bassiana* spores (2× 10^6/g soil)	30±0.5 + 0.5 ± 0.03 blank granules	Soil control	30 ± 0.5 + 0.5 ± 0.03 blank granules	37
2-2d	*B. bassiana* spores (2× 10^7/g soil)	30 ± 0.5 + 0.5 ± 0.03 blank granules	Soil control	30 ± 0.5 + 0.5 ± 0.03 blank granules	25

2.3.3. Experiment 2-3: Are wireworm movement or feeding behaviours affected by *B. bassiana* inoculation?

Wireworm movement and feeding behaviours were examined with three sets of experiments to answer the following questions:

- 2-3a) How do *A. lineatus* wireworms behave after *B. bassiana* inoculation?
- 2-3b) Do different inoculation methods of *B. bassiana* matter in the behavioural response of *A. lineatus*?
- 2-3c) How do the behaviours of *A. lineatus, A. sputator*, and *H. glaucus* compare after *B. bassiana* inoculation?

Behaviour was examined using vertical “window” apparatuses, consisting of two plexiglass sheets sandwiching a thin layer of autoclaved soil (190 x 150 x 3mm) (Figure 2.2). A hinged door in one face of the window allowed soil to be added (75 ± 5g) and the wireworm was introduced through a 3mm hole at the bottom of the window. A circle, 24mm in diameter, of slightly moistened, ground rolled oats (0.7 ± 0.02g) was placed in the soil in the upper right quadrant. Windows were prepared three days before wireworm introduction. Wireworms were inoculated by brushing (see 2.3.1.1) and fed with a slice of potato except in experiment 2-3b, where wireworms in the "fed" treatment group were exposed to *B. bassiana*-formulated granules at a density of 0.043g granules/g dry soil, and the other treatment groups were given blank granules. After inoculation, wireworms were transferred into soil and left in a dark room at room temperature for 4 weeks (experiment 2-3b) and 3 weeks (2-3a, 2-3c). On the day of introduction, wireworms were weighed, a single insect was inserted, and the hole into which the wireworm was introduced was blocked. The experiments were run in a dark room at 25°C and red lights were used to observe wireworm movements.

Wireworm locations were determined visually, and their position was marked on the window with a permanent marker at the head if visible (Figure 2.2). Wireworm locations were determined at 0.5, 1, 2, 3, 5, 7, 9, 12, 15, 18, 21, 24, 27, 30, and 52 h post introduction. Observation points within 12mm or less to the circle of food were deemed as feeding. A single wireworm was used for each window. Each experiment was repeated twice for a total of three experimental replicates. In experiment 2-3a, there were 10 windows per treatment group in each experiment’s replication in time for the first

two replicates, and 12 in the third. In experiment 2-3b, there were 8 windows per treatment group, and in experiment 2-3c, there were 5 windows per treatment group for all three experimental replicates. Windows were washed and sanitized in 2% bleach after each run.

Seven metrics were measured:

- Number of responding wireworms (moved more than 15mm from the entry point)
- Number of feeding wireworms (observed 15mm or closer to the oats at least once)
- The proportion of observations that a wireworm spent at the oat food source
- Time to reach the food
- Distance to reach the food
- Total linear distance from the food
- Total path distance travelled

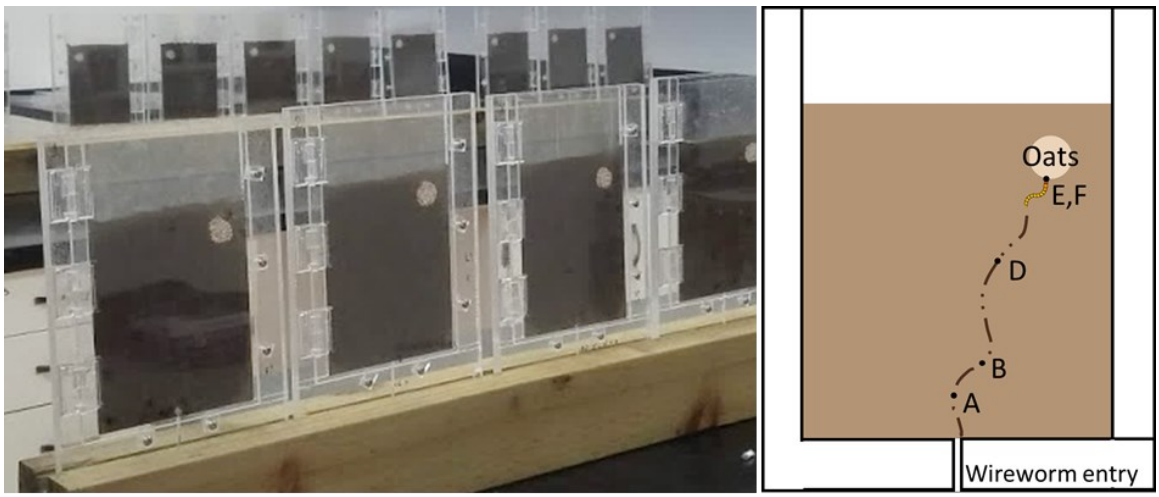

Figure 2.2. Window apparatus setup and diagram showing an example of a wireworm path. The soil was sandwiched between two sheets of plexiglass (190 x 150 x 3mm) with a circle of rolled oats in the upper right corner. Wireworms are introduced from the bottom, and dots and dashes simulate their movement path. Letters represent each observation point.

2.3.4. Statistical analyses

All analyses were performed using R version 4.1.2. The significance level was designated as $\alpha \leq 0.05$.

Experiment 2-1: To compare *B. bassiana* inoculation methods (2-1a), wireworm survival was analyzed using a Cox proportional hazards model from the "survival" package. The treatment group and the weight of the wireworms as a co-variate were fixed effects. The effect of size and species were analyzed using a survival analysis from the “survival” package with an exponential Weibull distribution, due to violations of the Cox proportional hazards assumptions. In the size experiment (2-1b), models included *B. bassiana* treatment, wireworm size, experimental replicate, and interaction between treatment and size as fixed effects. In the species experiment (2-1c), the full model included species, *B. bassiana* treatment, wireworm weight as a continuous variable, experimental replicate, and interaction between species and treatment as fixed effects. Non-significant terms were removed until the minimal model was obtained, and pairwise differences were obtained using the function “pairwise_survdiff”. Kaplan-Meier plots for all three experiments were created using the "survminer" package. Hazard ratios, the ratio of the death rate of the two treatments, are reported.

Health data were analyzed using analysis of variance (ANOVA) based on linear models on the mean health rank across each evaluation point using the same variables as in the regression models. The model only included data from wireworms that survived until the end of the experiment. Pairwise differences were determined using the “TukeyHSD” function.

Experiment 2-2: Wireworm choice in the two-choice bioassays was evaluated using exact binomial tests, and chi-squared tests in the three-choice bioassays. The number of wireworm feeding holes in potato tubers was compared using a Wilcoxon rank sum test.

Experiment 2-3: *B. bassiana* treatment and experimental replicate were fixed effects in all analyses. Analysis of the number of responsive wireworms and the number of feeders was performed using a logistic regression with a logit-link function, followed by an ANOVA using the "car" package. Analysis of wireworm feeding events was performed using logistic regression with a logit-link function followed by pairwise contrasts using the package “emmeans”.

Several metrics were analyzed with ANOVA with the following data transformations: time and distance to the food data were log-transformed, total linear

distance data were normalized using a square root transformation (2-3a) and log-transformed (2-3b & 2-3c), and total path distance was square root transformed. Weight was included as a covariate in the analyses in experiment 2-3c due to their variable sizes. When applicable, pairwise differences were analyzed with the package "emmeans".

2.4. Results

2.4.1. Experiment 2-1: Are wireworms susceptible to *B. bassiana*?

There were no effects of *B. bassiana* inoculation on wireworm survival, regardless of the inoculation method ($\chi^2_3 = 4.71$, $p = 0.19$, Figure 2.4a). Wireworms that were fed with *B. bassiana* granules tended to have the lowest survival probability compared to the other treatments, but the difference was not significant (HR = 3.97, CI = (0.747, 19.33), $p = 0.11$). Wireworms brushed with *B. bassiana* received a higher spore load ($5.4 \times 10^6 \pm 8.3 \times 10^5$ spores/wireworm) compared to wireworms dipped in a spore suspension ($6.4 \times 10^5 \pm 4.5 \times 10^5$ spores/mL) ($t(4) = 8.51$, $p \leq 0.001$) (Figure 2.3). As there were no significant differences in percentage survival among any of the *B. bassiana* treatments in experiment 2-1a, the brushing method was chosen for the following experiments due to the ease of application and the high number of spores that are applied (Figure 2.3). The response to *B. bassiana* inoculation was not affected by wireworm size ($\chi^2_1 = 0.39$, $p = 0.53$) or species ($\chi^2_2 = 2.13$, $p = 0.34$).

There were differences in survival time between the sizes of the wireworms, where small wireworms had an increased risk of death (HR = 4.35, CI = (1.87,10.10), $p \leq 0.001$) (Figure 2.4). The survival of *A. lineatus* was significantly lower compared to *A. sputator* (HR = 2.69, CI = (1.40,5.18), $p = 0.0025$) and the survival of *H. glaucus* was also significantly lower compared to *A. sputator* (HR = 3.73, CI = (1.87,7.44), $p \leq 0.001$) (Figure 2.4). There was no difference between the survival of *H. glaucus* compared to *A. lineatus* (HR = 1.39, CI = (0.79,2.42), $p = 0.26$). Cadaver sporulation was not formally assessed in these experiments, but it was observed that about ¼ of the cadavers eventually sporulated with either green or white spore masses.

The movement ability of wireworms was measured as a proxy for their health status. *Beauveria bassiana* inoculation had no effects on wireworm movement ability

($F_{(3,43)}$ = 2.54, p = 0.07, Figure 2.5b).). Neither the weight of the wireworms ($F_{(1, 43)}$ = 3.48, p = 0.066), nor their size ($F_{(1, 83)}$ = 0.53, p = 0.47) influenced their health. However, wireworm species did differ in their health ($F_{(1, 192)}$ = 61.04, p ≤ 0.001) with both *A. sputator* and *H. glaucus* exhibiting a higher mean health rank compared to *A. lineatus* (p ≤ 0.001), but not each other (Figure 2.5c, 2.6). *Agriotes lineatus* wireworms tended to become slower and less responsive over time (Figure 2.5).

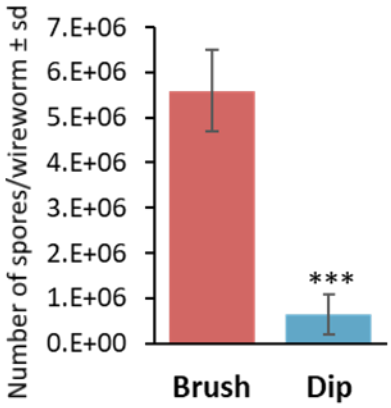

Figure 2.3. The number of spores recovered from *A. lineatus* wireworms when inoculated with *B. bassiana* by brush application (1 × 10^{10} spores/g rice granules), or by dipping in a spore suspension (2 × 10^{7} spores/mL). Error bars denote standard deviation. (N = 5, t(4) = 8.51, *** p ≤ 0.001)

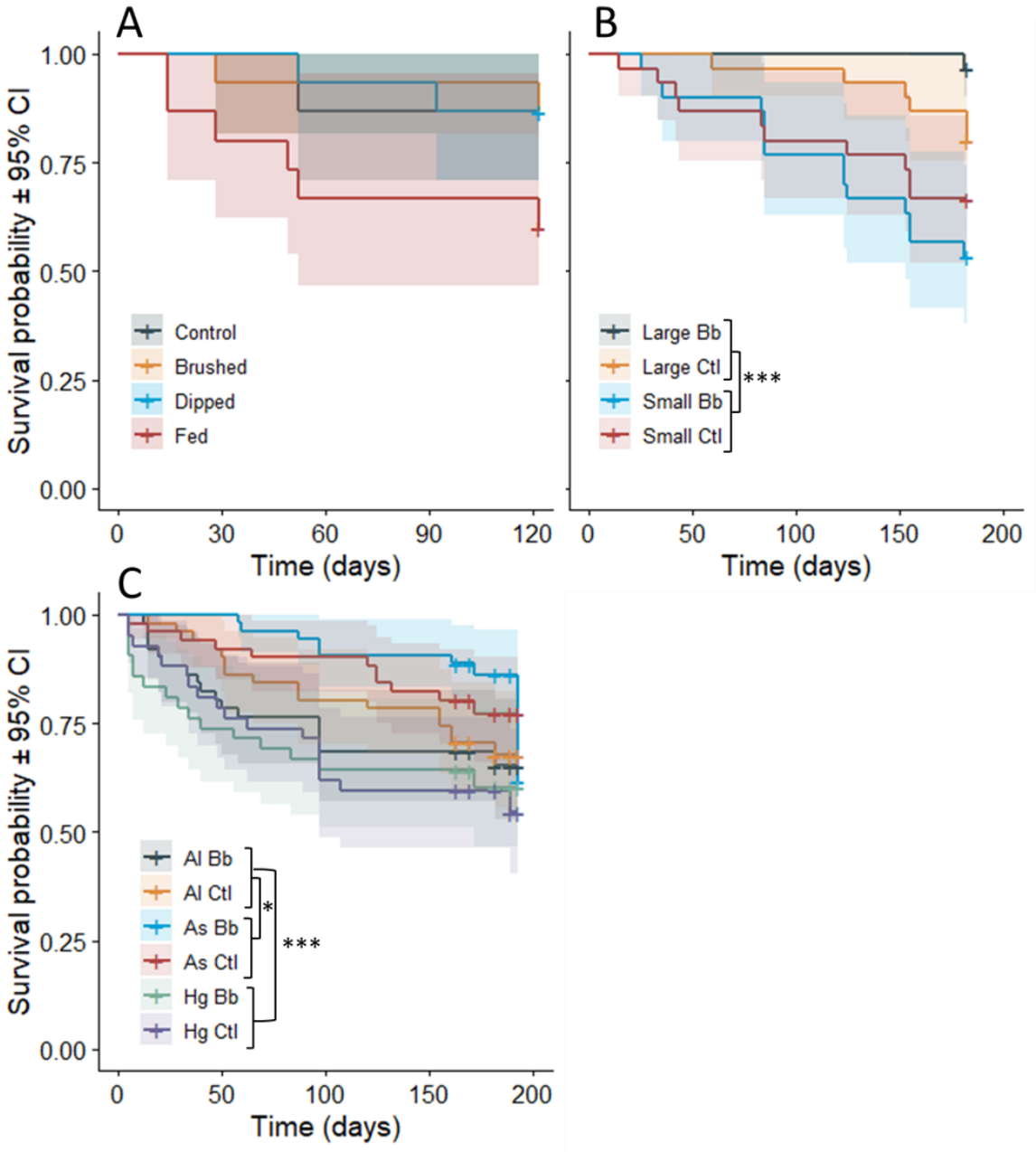

Figure 2.4. **Kaplan-Meier plots of A) the survival probability of *A. lineatus* wireworms inoculated with *B. bassiana* by brushing, dipping, or feeding, compared with the non-inoculated control. B) The survival probability of large and small *A. lineatus* wireworms inoculated by *B. bassiana* by brush (Bb), and the non-inoculated controls (Ctl). C) Survival probability of *A. sputator* (As), *A. lineatus* (Al), and *H. glaucus* (Hg) wireworms inoculated by *B. bassiana* by brush, and the non-inoculated controls. Shading depicts the 95% confidence interval. * $p \leq 0.05$, *** $p \leq 0.001$**

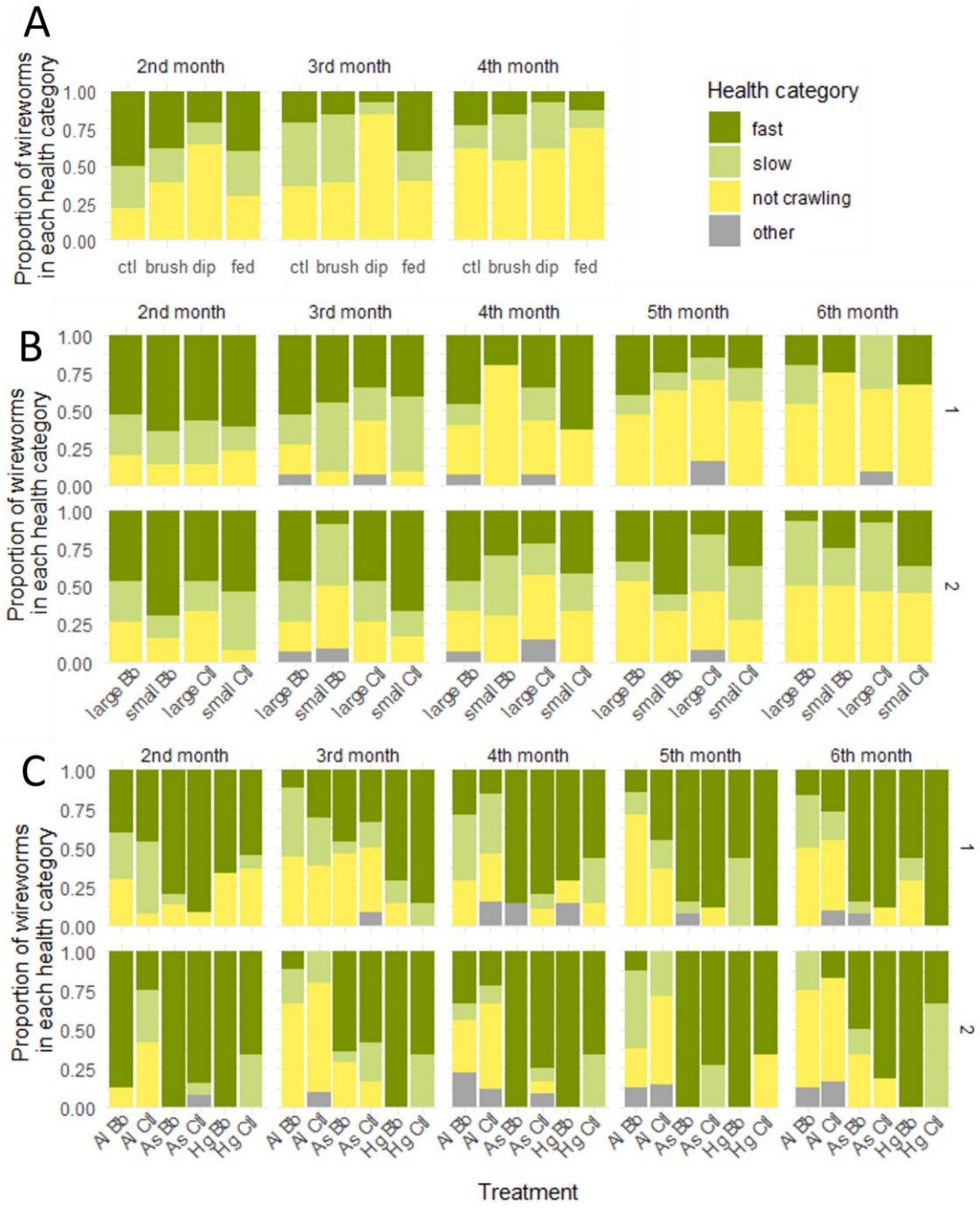

Figure 2.5. **Proportion of surviving wireworms in each health category over time. A) *A. lineatus* inoculated with *B. bassiana* in the brushed, dipped, fed, or non-inoculated control treatment. B) Large and small *A. lineatus* wireworms, inoculated with *B. bassiana* (Bb) or non-inoculated control (Ctl). C) *A. sputator* (As), *A. lineatus* (Al), and *H. glaucus* (Hg) wireworms inoculated with *B. bassiana* and non-inoculated control treatments. The "other" category contains moulting and pupating wireworms. The top rows (1) show the first experimental replicate, and the bottom rows (2) show the second experimental replicate.**

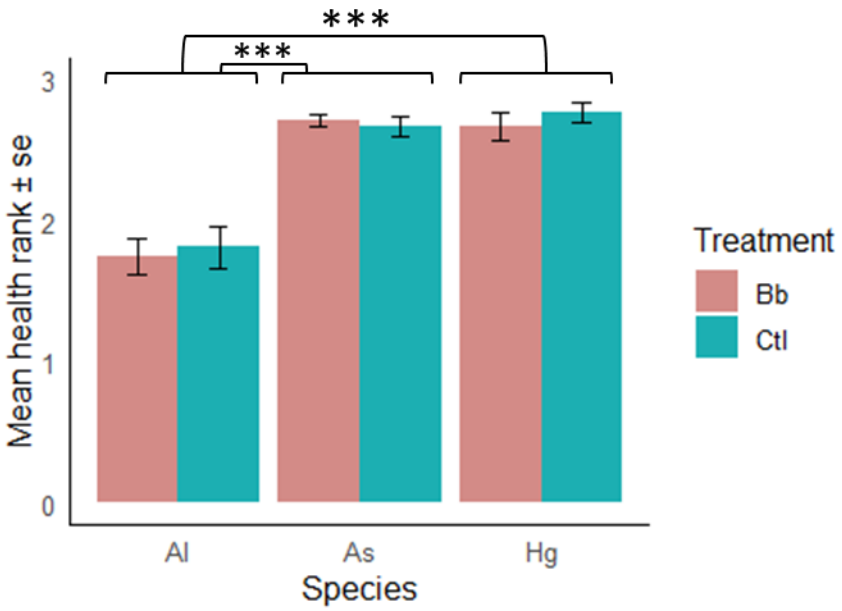

Figure 2.6. **The mean health rank of *A. lineatus* (Al), *A. sputator* (As), and *H. glaucus* (Hg) wireworms inoculated with *B. bassiana* (Bb) and the non-inoculated control (Ctl) over 6 months. Error bars denote standard error. *** p ≤ 0.001**

2.4.2. Experiment 2-2: Do wireworms avoid *B. bassiana*?

Wireworms preferred the blank granules compared to granules formulated with *B. bassiana* (p = 0.043, CI (0.51, 0.85), Figure 2.7), with 70% of responding wireworms choosing the blank granule treatment. Blank granules were confirmed to be attractive to wireworms when compared to soil (p ≤ 0.001, CI (0.84, 1), Figure 2.7).

Wireworms had no significant preference between the 2×10^6 spores/g soil ("low") treatment and the control treatment (p = 0.66, CI (0.34, 0.78)), but did show a preference for the untreated control at the higher concentration (2×10^7 spores/g; p = 0.035, CI (0.52,0.96)), where 80% of responding wireworms chose the control (Figure 2.7).

When the wireworms were presented potatoes and soil, none chose the soil and there was a small, but significant, preference for the control tubers versus those treated

with *B. bassiana* (p = 0.047, CI (0.50, 0.82), Figure 2.7). There was no difference in the number of wireworm feeding holes in the potatoes between the *B. bassiana* treatment and the control (W = 513.5, p = 0.27).

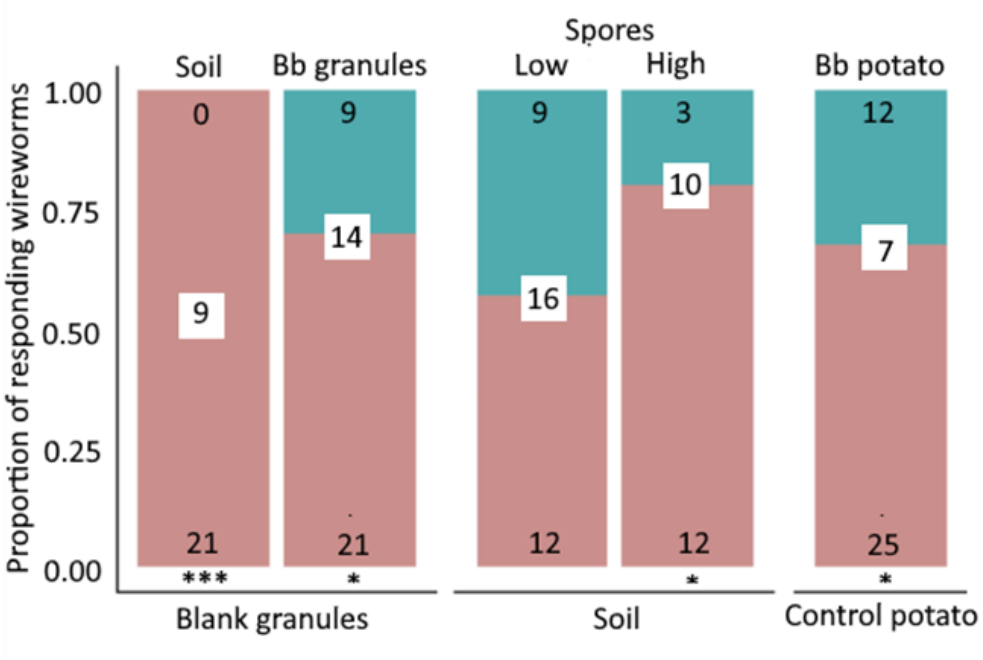

Figure 2.7. Responses of wireworms in choice experiments. Treatments were: soil control versus blank granules; blank granules versus *B. bassiana* (Bb) granules; soil control versus low concentration of *B. bassiana* spores; soil control versus high concentration of *B. bassiana* spores; tubers from non-inoculated plants versus tubers from *B. bassiana*-inoculated plants. Treatments and the numbers of responsive wireworms are depicted at the top and bottom of the bars. The numbers of non-responders are shown in the white boxes. * p ≤ 0.05 *** p ≤ 0.001

2.4.3. Experiment 2-3: Are wireworm movement or feeding behaviours affected by *B. bassiana* inoculation?

Beauveria bassiana inoculation did not affect the number of responding wireworms (Table 2.3). Fewer wireworms fed in the *B. bassiana* treatment compared to the untreated control (χ^2_1 = 4.37, p = 0.037), but only in experiment 2-3a examining *A. lineatus* that were brushed with spores. There was no difference in the number of feeding wireworms among *B. bassiana* inoculation methods in experiment 2-3b (Table 2.3). There was a difference between wireworm species, but there was no effect from *B. bassiana* inoculation (Figure 2.8, Table 2.3).

A 26% reduction in the number of feeding occasions was recorded for *B. bassiana*-inoculated insects compared to the control ($\chi^2_1 = 6.18$, $p = 0.013$). Both inoculation methods resulted in reduced feeding time (brush: $z = -3.54$, $p \leq 0.001$, fed: $z = 4.90$, $p \leq 0.001$, Figure 2.9). Feeding time differed between wireworm species ($\chi^2_2 = 69.13$, $p \leq 0.001$) with *A. sputator* feeding the most (As-Al: $z = -3.057$, $p = 0.0063$; As-Hg: $z = 7.83$, $p \leq 0.001$), and *H. glaucus* feeding the least (Hg-Al: $z = 3.081$, $p = 0.0059$) (Figure 2.9), but there was no effect from *B. bassiana* inoculation (Figure 2.9, Table 2.3).

Wireworm time and distance to the food, total linear distance from the food, and total path distance did not differ between the *B. bassiana*-inoculated and non-inoculated control treatments in any experiment (Table 2.4). *Agriotes sputator* found the food most rapidly, followed by *A. lineatus*, and lastly *H. glaucus* (Table 2.5).

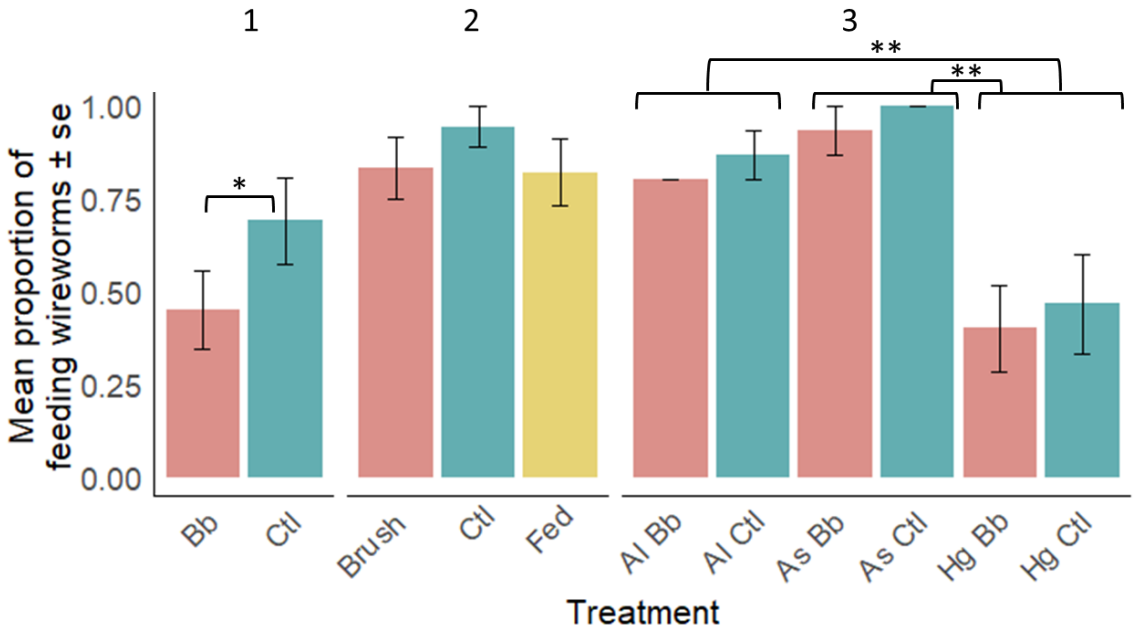

Figure 2.8. **The mean proportions of wireworms that were observed feeding during at least one time point. Error bars denote standard error. 1) Experiment 2-3a: *B. bassiana* (Bb) – inoculated and control (Ctl) *A. lineatus* (N = 32); 2) Experiment 2-3b: *A. lineatus* wireworms inoculated with *B. bassiana* by brush, fed granules, and the non-inoculated control (N = 24); 3) Experiment 2-3c: *A. lineatus* (Al), *A. sputator* (As), and *H. glaucus* (Hg) wireworms inoculated *by B. bassiana* by brush, and the non-inoculated controls (N = 15). * $p \leq 0.05$ ** $p \leq 0.01$**

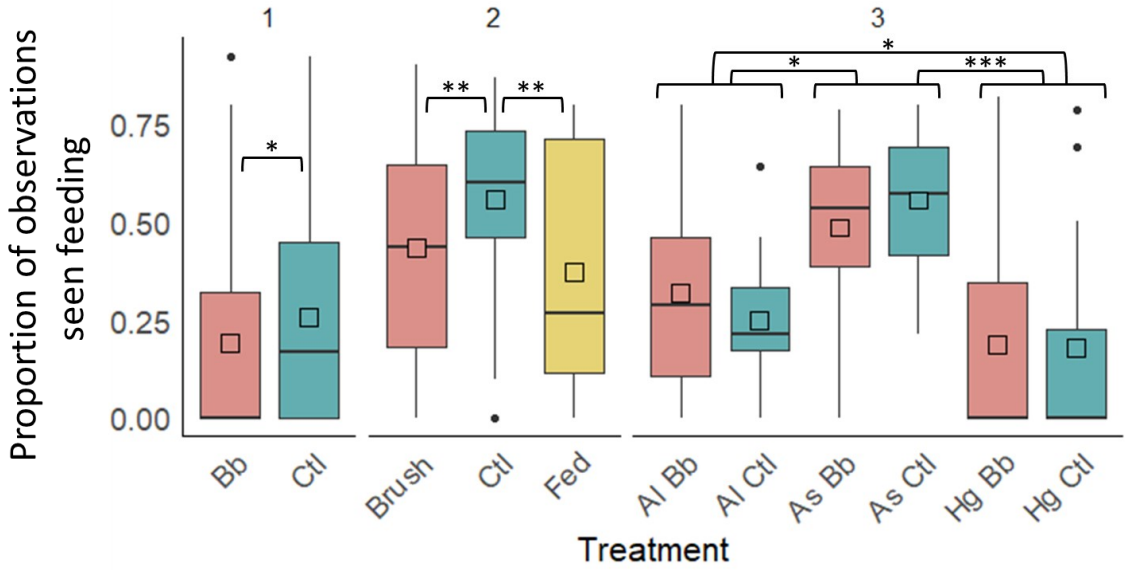

Figure 2.9. **The proportion of observations that wireworms of each treatment spent at the food source. Squares depict the means. 1) *B. bassiana*–inoculated (Bb) and control (Ctl) *A. lineatus* (N = 32); 2) *A. lineatus* wireworms inoculated with *B. bassiana* by brush, fed granules, and the non-inoculated control (N = 24); 3) *A. sputator* (As), *A. lineatus* (Al), and *H. glaucus* (Hg) wireworms inoculated by brush with *B. bassiana* and the non-inoculated controls (N = 15). * p ≤ 0.05 ** p ≤ 0.01 *** p ≤ 0.001**

Table 2.3. **Statistical analysis of behaviour experiments (Exp 2-3a, 2-3b, and 2-3c). Table shows analysis of variance results from the logistic regression of wireworm behaviour responses to *B. bassiana* (Bb) treatment in window bioassays. Exp 2-3a examined responses in *A. lineatus*. Exp 2-3b examined two different Bb inoculation methods in *A. lineatus*. Exp 2-3c examined the responses between three wireworm species. Significant p values are highlighted in bold.**

Exp		df	N	X^2	p value
2-3a	**Number of responding wireworms**				
	Bb Treatment	1	3	2.56	0.11
	Experimental replicate	1	3	4.83	**0.028**
	Number of feeding wireworms				
	Bb Treatment	1	3	4.37	**0.037**
	Experimental replicate	1	3	3.95	**0.047**
	Time spent feeding				
	Bb Treatment	1	32	6.18	**0.013**
	Experimental replicate	1	32	37.21	**0.001**
2-3b	**Number of responding wireworms**				
	Bb Treatment	2	3	2.30	0.32
	Experimental replicate	2	3	2.48	0.11
	Number of feeding wireworms				
	Bb Treatment	2	3	2.13	0.34
	Experimental replicate	2	3	2.18	0.14
	Time spent feeding				
	Bb Treatment	2	24	25.83	**≤ 0.001**
	Experimental replicate	2	24	2.66	0.10
2-3c	**Number of responding wireworms**				
	Bb Treatment	1	3	0.23	0.63
	Species	2	3	4.49	0.11
	Experimental replicate	2	3	1.64	0.44
	Number of feeding wireworms				
	Bb Treatment	1	3	0.72	0.39
	Species	2	3	25.69	**≤ 0.001**
	Experimental replicate	2	3	0.16	0.92
	Time spent feeding				
	Bb Treatment	1	12	0.015	0.90
	Species	2	24	69.13	**≤ 0.001**
	Experimental replicate	2	24	1.19	0.55

Table 2.4. **Statistical analysis of behaviour experiments (Exp 2-3a, 2-3b, and 2-3c). Table shows analysis of variance results from the linear regression of wireworm behavioural responses to *B. bassiana* (Bb) treatment in window bioassays. Exp 2-3a examined responses in *A. lineatus*. Exp 2-3b examined two different Bb inoculation methods in *A. lineatus*. Exp 2-3c examined the responses between three wireworm species. Log transformations were used on the time and distance to the oats data. Total linear distance data were log-transformed in Exp 2-3b and 2-3c, and square root transformed in Exp 2-3a. Total path distance data were square root transformed. Significant p values are highlighted in bold.**

Exp		df	F value	p value
2-3a	**Time to oats**			
	Bb treatment	1	1.8	0.19
	Experimental replicate	1	0.12	0.74
	Residuals	33		
	Distance to oats			
	Bb treatment	1	0.83	0.37
	Experimental replicate	1	1.85	0.18
	Residuals	33		
	Total linear distance			
	Bb treatment	1	0.5	0.48
	Experimental replicate	1	21.1	**≤ 0.001**
	Residuals	61		
	Total path distance			
	Bb treatment	1	2.104	0.152
	Experimental replicate	1	0.005	0.945
	Residuals	61		
2-3b	**Time to oats**			
	Bb treatment	2	0.32	0.73
	Experimental replicate	1	6.39	**0.014**
	Residuals	55		
	Distance to oats			
	Bb treatment	2	0.847	0.43
	Experimental replicate	1	1.08	0.3
	Residuals	55		
	Total linear distance			
	Bb treatment	2	1.74	0.19
	Experimental replicate	1	0.56	0.46
	Residuals	64		
	Total path distance			
	Bb treatment	2	0.30	0.74
	Experimental replicate	1	11.30	**≤ 0.001**
	Residuals	64		

2-3c	**Time to oats**			
	Bb treatment	1	1.00	0.32
	Species	2	14.30	**≤ 0.001**
	Experimental replicate	2	1.56	0.22
	Weight	1	4.36	**0.035**
	Residuals	60		
	Distance to oats			
	Bb treatment	1	0.05	0.82
	Species	2	3.01	0.06
	Experimental replicate	2	1.94	0.15
	Weight	1	0.06	0.81
	Residuals	59		
	Total linear distance			
	Bb treatment	1	0.17	0.68
	Species	2	4.00	**0.024**
	Experimental replicate	2	0.49	0.62
	Weight	1	0.00	0.98
	Residuals	78		
	Total path distance			
	Bb treatment	1	0.028	0.867
	Species	2	44.50	**≤ 0.001**
	Experimental replicate	2	2.27	0.11
	Weight	1	2.71	0.10
	Residuals	78		

Table 2.5. **Statistical analysis of the behaviour experiment comparing three wireworm species (Exp 2-3c). Table shows the pairwise comparisons of wireworm behaviour responses in window bioassays between *A. sputator* (As), *A. lineatus* (Al), and *H. glaucus* (Hg) wireworms. Significant p values are highlighted in bold.**

	Contrast	df	t ratio	p value
Time to the oats	Al-As	78	-2.69	**0.023**
	Al-Hg	78	3.72	**≤ 0.001**
	As-Hg	78	8.17	**≤ 0.001**
Total linear distance	Al-As	78	1.36	0.36
	Al-Hg	78	-0.47	0.89
	As-Hg	78	-2.48	**0.041**
Total path distance	Al-As	78	-2.68	**0.023**
	Al-Hg	78	3.72	**≤ 0.001**
	As-Hg	78	8.17	**≤ 0.001**

2.5. Discussion

The results of this study show that *B. bassiana* does not cause mortality to wireworms regardless of inoculation method, wireworm age, or wireworm species. Wireworms show a slight avoidance of *B. bassiana* and of tissue from inoculated plants. *Beauveria bassiana* inoculation of *A. lineatus* wireworms resulted in a slight reduction in feeding time.

2.5.1. *Beauveria bassiana* does not impact wireworm survival, and the response does not differ between wireworm sizes or species

Several inoculation methods of *B. bassiana* were tested: wireworms directly brushed with spores, dipped in a spore suspension, and fed with fungal granules; these methods did not result in increased wireworm mortality. This result supports previous studies that found no significant mortality in *A. lineatus* after *B. bassiana* inoculation of strains ATCC 74040 and Botanigard through dipping (Ansari et al., 2009; Razinger et al., 2020). However, Chałańska et al. (2017) found that *A. lineatus* dipped in a *B. bassiana* spore suspension (10^6 spores/mL) resulted in up to 30% mortality after 5 weeks. In *Hypolithus bicolor* and *Ctenicera destructor* wireworms, inoculation of *B. bassiana* via direct spore application to the cuticle, contact with spore-infested soil, and feeding on spore-coated wheat seeds did not result in high levels of mortality (Zacharuk and Tinline 1968). Regarding oral inoculation, Sufyan et al. (2017) saw that *B. bassiana* ATCC 74040 caused 50% *Agriotes* mortality in the lab at high wireworm densities using wheat seeds coated with *B. bassiana* spores. It is unclear whether *B. bassiana* can infect insects through the gut, or rather, by feeding, since the spores are brought to vulnerable sites on the head and mouthparts for infection (Allee et al., 1990; Jeffs et al., 1997). Green or white fungal sporulation was observed in roughly ¼ of the cadavers, signifying that *Metarhizium* was present either inside the wireworms (Kabaluk et al., 2017) or in the soil. The presence of fungi other than *B. bassiana* in these experiments was possible, as they were not performed under sterile conditions. Further investigations should examine the effects of different spore doses, and measure levels of fungal sporulation in cadavers.

Wireworm size and species did not influence wireworm susceptibility to *B. bassiana*. This does not support Zacharuk and Tinline (1968), who found that larger

wireworms were more resistant to fungal infection. On the other hand, van Herk and Vernon (2011) found that larger wireworms were more likely to die from *M. anispoliae* infection compared to smaller wireworms. Studies in other insects found that larval instar influences susceptibility to fungal disease, but its relationship does not show a clear trend: in some studies, older instars are less susceptible to fungal entomopathogens compared to younger instars (Levchenko et al., 2020; Rosengaus & Traniello, 2001; Yubak Dhoj et al., 2008) but in other studies, younger instars are less susceptible to disease (Mohamed et al., 1977; Tang et al., 1999).

Differences in susceptibility to fungal disease between insect ages and species may be attributed to differences in cuticle composition, insect behaviour, or immune system strength. The insect cuticle can have mechanical or chemical barriers due to the structure and composition of the cuticle. Larger larvae have more accumulated resources and therefore, theoretically, more can be allocated toward the immune system. However, a larger body size also results in a higher surface area for fungal pathogens to attach to. Small wireworms may have intrinsically lower survival rates due to other factors, such as being more prone to starvation, dehydration, or other infections. These factors may have played a role in exacerbating fungal infections in other studies, though it was not seen in these experiments. The insect microbiome is another player in these interactions, as it has been shown that *B. bassiana* infection can cause dysbiosis in insects and may accelerate insect death (Wei et al., 2017, see Chapter 4).

2.5.2. Wireworms avoid *B. bassiana*

Beauveria bassiana, in the form of granules and as a high concentration of spores in soil, causes avoidance behaviours in wireworms. These results support previous research in various insects showing avoidance of *B. bassiana* spores. *Beauveria bassiana* repelled 55% of *Macrotermes bellicosus* termites in a treated paper assay (Osipitan et al., 2016). Adult *Coccinella septempunctata* ladybirds avoided contact with leaf surfaces and soil inoculated with *B. bassiana*, as well as with mycosed cadavers (Ormond et al., 2011). In a Y-tube olfactometer study involving *B. bassiana* and *Rhynchophorus ferrugineus* (Coleoptera), *B. bassiana* repelled females, and two specific volatile organic compounds were identified (Jalinas et al., 2016). Wireworms were repelled by entomopathogenic fungi-contaminated soil, and their repellency rate increased with spore concentration but slowed when a food source was present

(Kabaluk & Ericsson, 2014). Rate-dependent repellency was observed in these experiments as well, where the soil with a higher concentration of spores caused wireworm avoidance, but the lower concentration did not. The experiments here all used a food source as a lure, if a food source was absent, perhaps the *B. bassiana*-contaminated soil would have been more aversive to the wireworms. It is unclear whether the avoidance seen is due to volatile compounds, or from ingestion, and further choice experiments using exclusion barriers are needed to determine this.

Wireworms avoid potato tubers from plants inoculated with *B. bassiana*, suggesting that *B. bassiana* inoculation on potato seed tubers had a physiological effect on daughter tubers, and supporting previous studies. For example, avoidance of *B. bassiana* inoculated plants was seen in *Otiorhynchus sulcatus* weevils and grapevines (Rondot & Reineke, 2017), *Thrips tabaci* and onion plants (Muvea et al., 2015), *Bemisia tabaci* (Hemiptera) and tomato plants (Wei et al., 2020), *Aphis gossypii* and cotton plants (Gurulingappa et al., 2011), and *Dichroplus maculipennis* and corn plants (Pelizza et al., 2017). The avoidance effect may be due to the production of plant secondary metabolites induced by *B. bassiana*, but further research is needed to understand the mechanism. There was no difference in the number of feeding holes between the *B. bassiana* treatment and the control, showing that although wireworms avoided the *B. bassiana* treatment, it did not prevent them from feeding on the tubers. Wireworm feeding on potatoes is examined further in Chapter 3.

2.5.3. *Beauveria bassiana* has limited effects on wireworm feeding time

Agriotes lineatus wireworms inoculated with *B. bassiana* spent less time feeding. This result supports previous studies, for example, *B. bassiana* inoculation via immersion in a spore suspension of *Ocinara varians* silk moth larvae resulted in reduced food consumption of 39–45%, reduced dietary utilization of food of 55–61%, and reduced relative growth (Hussain et al., 2009). Other lepidopterans found to exhibit reduced feeding after *B. bassiana* inoculation are *Chilo partellus* larvae (Tefera & Pringle, 2003), and *Dendrolimus pini* larvae (Kovač et al., 2020). In orthopterans, *B. bassiana* inoculation of *Uvarovistia zebra* grasshoppers resulted in a 60% reduction in food consumption (Mohammadbeigi & Port, 2015). *Beauveria bassiana* infection through topical inoculation caused a 17% weight loss in a variety of Mormon crickets relative to

the controls and is thought to be caused by reduced food consumption, metabolic rate increase, or reduced nutrient absorption from the gut (Srygley & Jaronski, 2010). Similar feeding reductions after *B. bassiana* inoculation have been observed in other coleopterans, for example, in *Leptinotarsa decemlineata* (Fargues et al., 1994), and *Asynonychus cervinus* (France et al., 2002). Effects are dependent upon fungal strain: Ekesi (2001) tested four different isolates of *B. bassiana* against *Ootheca mutabilis* and found that only isolate CPD3 caused a significant reduction in leaf consumption. Experiments involving the blood-feeding activity of mosquitoes after *B. bassiana* inoculation have shown mixed results (Blanford et al., 2011; Darbro et al., 2012). A reduction in wireworm feeding may reduce seedling mortality in vulnerable crops in the field; however, even a small number of feeding holes in potato tubers would result in the rejection of the product, so these small anti-feeding effects do not protect potatoes against wireworm herbivory.

The ability of wireworms to orient to and travel to a food source was not affected by *B. bassiana* inoculation. This does not support work done in mosquitoes, where *B. bassiana*- infected female *A. stephensi* showed a reduction in behavioural and neuronal responsiveness to host odour cues as seen through reduced upwind flight host-seeking responses and reduced olfactory receptor neuron sensitivity (George et al., 2011). *Beauveria bassiana* inoculation had minimal effect on wireworm activity levels, unlike Thompson and Brandenburg (2005), who found that in mole crickets (*Scapteriscus borellii*), infection by *B. bassiana* reduced activity levels. Limitations by the experimental design, such as the inability to measure distances travelled between observation points, result in only approximate measures in this study.

2.5.4. Conclusions

In conclusion, wireworms were not killed by *B. bassiana* inoculation and wireworms showed limited behavioural effects in response to *B. bassiana* inoculation under these laboratory conditions. In laboratory experiments, wireworms avoided *B. bassiana* in granule form, as a high concentration of spores in soil, and in tubers of inoculated potato plants. This slight avoidance is not likely to contribute to crop protection because there were no differences in the number of wireworm feeding holes in potato tubers. There were small effects of *B. bassiana* inoculation on the amount of time *A. lineatus* wireworms spent feeding, but there were no effects due to inoculation

when the three wireworm species were compared. A combination of avoidance and anti-feeding behaviours may contribute to crop protection. It is important to understand the lethal and sublethal effects of an entomopathogenic fungus that has the potential to be used as a biological control agent. Further research is needed to determine if, and in what ways, *B. bassiana* may be providing reducing wireworm herbivory. Through this process, we gain understanding of the relationships between insects, entomopathogenic fungi, and the crops that we aim to protect.

2.6. References

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Chapter 3.

Seed tuber inoculation of potatoes with *Beauveria bassiana* does not influence potato performance or wireworm feeding damage

3.1. Abstract

Beauveria bassiana is a fungal entomopathogen widely used as a biological control agent against insect pests. There are limited effective control options for wireworms, larval elaterids, in potatoes because of new restrictions on chemical pesticides due to negative environmental and health impacts. The use of *B. bassiana* against wireworms is a potential option. In potatoes, wireworms feed upon the tubers, causing disfiguring holes and impacting marketability. This study investigates if a seed tuber inoculation of *B. bassiana* affects the growth and productivity of potato plants and/or wireworm feeding damage in greenhouse and laboratory experiments. *Beauveria bassiana* was detected in plant tissue, but *B. bassiana* treatment did not affect plant biomass or tuber production. There were no effects of *B. bassiana* treatment on levels of plant defence hormones, and no effects on wireworm feeding damage. The inoculation of potato seed tubers by *B. bassiana* does not protect daughter tubers from wireworm herbivory under greenhouse conditions.

Keywords: *Beauveria bassiana*; wireworm; biological control; colonization; potato; defence hormones

3.2. Introduction

Plants and fungi have evolved together from their earliest beginnings and shape the ecosystems in which they exist. They interact in many ways, from parasitic interactions causing disease to symbiotic relationships exchanging nutrients (Southworth, 2012; Zeilinger et al., 2016). The most important fungal-plant interaction,

the mycorrhiza, is the most prevalent symbiosis on the planet, and this vital exchange of nutrients has been speculated to have been the prerequisite to terrestrial plant life (Selosse & Le Tacon, 1998). Plants commonly encounter and interact with soil fungi through the root system, forming the biodiverse rhizosphere. Beneficial root microbes can aid with siderophore synthesis (Barra-Bucarei et al., 2020; Press et al., 2001), boost plant growth through plant hormone production (Contreras-Cornejo et al., 2009; Cosme et al., 2016), through nutrient transfer (Behie & Bidochka, 2014), and provide disease resistance (Disi et al., 2019; Hu et al., 2020). They can also penetrate plant tissue, travel throughout the plant xylem, and become endophytic (Wagner & Lewis, 2000). Endophytes are microorganisms, typically bacteria and fungi, that reside inside living plant tissues for at least part of their life cycles without causing disease. Different endophytes occupy different areas of plant tissues, which are arranged in multiple layers (Alam et al., 2021). Endophytes provide many of the same benefits as beneficial rhizosphere microbes and improve plant stress tolerance (Brotman et al., 2013; Waller et al., 2005). The benefits of associations with microbes in the soil alone versus associations when these microbes become endophytic have not been clearly defined.

Fungal entomopathogens such as those in the genera *Metarhizium* and *Beauveria* can endophytically colonize a wide range of plants. *Metarhizium* is more closely related to endophytes and plant pathogens than to animal pathogens (Stone & Bidochka, 2020), and it is speculated that entomopathogenic fungi may have obtained the genes involved in insect pathogenicity through the evolution of plant colonization genes or horizontal transfer from a bacterium (Screen and St. Leger 2000). These fungi were plant symbionts that gained the ability to infect insects as an adaptation possibly to access a greater source of nutrients (Wang & Wang, 2017). *Beauveria bassiana* evolved into an insect pathogen independently of the *Metarhizium* lineage but showed a similar expansion of gene families important for insect and plant interactions, such as proteases, chitinases, and hydrophobins, demonstrating convergent evolution (Xiao et al., 2012). An analysis of various *B. bassiana* genes shows that there are differences in the expression of invasion-related genes during insect parasitism versus endophytism (Al Khoury, 2021). Both *Metarhizium* and *Beauveria* use similar mechanisms to invade insect cuticles and to colonize plants, such as conidia adherence and the use of appressoria (Xiao et al., 2012). *Beauveria bassiana* has been recorded as endophytically colonizing many different plant families including crops such as maize,

cocoa, banana, sorghum, tomato, squash, and rapeseed (reviewed in Vega, 2018; Zimmermann, 2007). A range of host tissues can be colonized by *B. bassiana*, with differences in presence between plant species; it has been observed in both parenchyma and vascular tissues, and intracellularly as well as in intercellular spaces (Vega, 2018; Wagner & Lewis, 2000). Endophytic colonization success varies between and even within species due to fungal strain and experimental methods.

Endophytic entomopathogens generally have positive or neutral effects on their plant hosts (Vega, 2018). Similar to other fungal endophytes, endophytic entomopathogenic fungi can promote growth, which is thought to be due to the production of plant growth-promoting hormones (Liao et al., 2017), nutrient solubilization (Barra-Bucarei et al., 2020; Mehta et al., 2019), a transfer of insect-derived nitrogen by the mycelia to the plant (Behie et al., 2012), and/or protection from disease and herbivory through the induction of plant defences (reviewed in McKinnon et al., 2017; Vega, 2018). They can influence plant-herbivore systems through the production of secondary metabolites (reviewed in Kumar & Kaushik, 2012), and more importantly, induce the plants to produce secondary metabolites (Rasool et al., 2021; Shrivastava et al., 2015). Plant secondary metabolites are compounds produced by plants in response to tissue damage and are an important defence response against insect herbivores. They have a range of different effects and can affect herbivore feeding, growth and fecundity (Gurulingappa et al., 2010, 2011; Pelizza et al., 2017; Russo et al., 2019).

Endophytic *B. bassiana* protects plants against both fungal and bacterial plant pathogens. For example, it protects tomato and cotton against *Rhizoctonia solani* and *Pythium myriotylum* fungi (Ownley et al., 2008), cotton against *Xanthomonas axonopodis pathovar malvacearum* bacteria (Griffin, 2007), and tomato and chilli pepper against *Botrytis cinerea* fungi (Barra-Bucarei et al., 2020). Protection against insects by endophytic *B. bassiana* has also been widely observed. Endophytic *B. bassiana* can protect plants by causing direct mortality to the pest (Akello et al., 2008; Mantzoukas & Lagogiannis, 2019; Omukoko, 2020; Ramos et al., 2020). Other studies found that endophytic *B. bassiana* caused significant pest mortality but not due to direct infection by *B. bassiana,* rather, mortality was likely due to fungal or plant metabolites (Qin et al., 2021; Sánchez-Rodríguez et al., 2018; Silva et al., 2020). *Beauveria bassiana* boosts plant growth, and this may play an important role in their defence against insects because robust plants are more tolerant to herbivory (Zitlalpopoca-Hernandez et al.,

2017). Herbivore repellency is another mechanism of defence provided by endophytic entomopathogenic fungi to plants (Rondot & Reineke, 2017).

Induced systemic resistance (ISR) is the mechanism by which plant defences are primed against pathogens and insect herbivores by non-pathogenic microbes such as bacteria and entomopathogenic fungi. Plants primed by ISR exhibit a faster and stronger defence response after pathogen or herbivore damage. ISR is triggered by the activation of the jasmonic acid/ethylene (JA/ET) pathway but may also affect the salicylic acid (SA) pathway which subsequently affects gene expression and the biosynthesis of various plant defensive compounds (Rashid & Chung, 2017; Vallad & Goodman, 2004). The dual defence against both plant pathogens and insect herbivores at the same time by *B. bassiana* is provided by the induction of plant defence responses and affects both salicylic acid (SA) and jasmonic acid (JA) defence pathways (Qin et al., 2021). This leads to the expression of a variety of defence genes, such as PR1, PR2, and ERF-1 (Batool et al., 2020; Jensen et al., 2020), and modulation of plant secondary metabolites (Gautam et al., 2016; González-Mas et al., 2021; Rasool et al., 2021).

The study of fungal endophytes in potatoes is lacking. Some studies show enhanced plant growth and disease resistance, but most involve bacterial endophytes. For example, Pavlo et al. (2011) found that endophytic bacteria *Pseudomonas* sp. and *Methylobacterium* sp. enhanced potato plant growth and *Pseudomonas* sp. increased disease resistance against the bacterial pathogen *Pectobacterium atrosepticum.* Growth promotion was not correlated with disease resistance. They also found activation of ISR and SAR genes after pathogen challenge in *B. bassiana* inoculated *Arabidopsis* plants. The colonization of potato tubers by *Beauveria brongiartii* was successful in a greenhouse study but the fungus was not detectable in the field (Abendstein et al., 2000). In a recent study, Zhang et al. (2022) showed that potato plants can be endophytically colonized by *B. bassiana*; it protected plants against the potato tuber moth, *Phthorimaea operculella*, whose larvae feed on tubers. Feeding on the foliage killed the larvae, and they also saw negative effects on their growth, development, and reproduction.

Wireworms are the soil-dwelling larvae of elaterid beetles and are pests of many crop systems worldwide. In potatoes, they make disfiguring feeding holes in the tubers, which results in an unmarketable crop. Traditionally, they are controlled by chemical

insecticides, but due to negative effects on human and environmental health, the use of these is now more restricted. Therefore, there is interest in developing alternative methods of control, such as the use of entomopathogenic fungi. *Beauveria bassiana* provides control against various insect pests and can establish itself as a plant endophyte. In field trials, Sufyan et al. (2017) and Ladurner et al. (2009) saw effective control of wireworms in potatoes using the commercial strain *B. bassiana* strain ATCC 74040; however, Schepl et al. (2010), using the same strain, saw no significant reduction in wireworm feeding damage in potatoes. Here, I investigated if *B. bassiana* strain PPRI5339, originally isolated from a tortoise beetle larva (*Conchyloctenia punctate)* in South Africa (European Food Safety et al., 2018), can protect potatoes against wireworm herbivory through seed tuber inoculation in a greenhouse experiment. I investigated effects on plant growth, tuber yield, wireworm feeding damage, and plant hormone responses. Furthermore, I conducted a laboratory feeding experiment to determine if direct inoculations of wireworms with *B. bassiana* affect feeding damage.

3.3. Materials and Methods

3.3.1. Insects and fungus

Agriotes lineatus and *A. sputator* wireworms were sourced from Vancouver Island and PEI, respectively. They were kept in a 4°C cooler and fed with a piece of potato which was replenished as needed. Wireworms were used within 12 months. To select active feeders, wireworms were placed in 25°C and provided half a potato on the soil surface and collected from the potato after 6 h. *Beauveria bassiana* PPRI5339 (BASF) was mass-produced using solid-state fermentation on rice (Jaronski, 2014) and stored in vacuum-sealed bags at 4°C*. Beauveria bassiana* spore viability was determined on potato dextrose agar after 24 h at 27°C and viability was ≥ 97% for all experiments (see Chapter 2).

3.3.2. The effect of *B. bassiana* inoculation on potato performance and wireworm herbivory

A greenhouse experiment was designed to investigate whether *B. bassiana* influences the performance of potato plants and if it reduces wireworm herbivory damage on tubers. Red Chieftain seed tubers (Pacific Potato Corp., Delta, BC) were

stored in a cooler at 5°C prior to use. They were washed and left to sprout at 25°C three weeks before planting. Large seed tubers were cut into approximately 45g pieces with at least two good sprouts and left to suberize for three days before planting. Field soil was mixed with potting soil at a 50:50 ratio to simulate more natural conditions for wireworms, and to reduce the porosity of the potting soil. Field soil from Agassiz, BC was sifted through a 10mm mesh, soil organisms were picked out manually, and 1.55-gallon pots filled and fitted with a 0.8mm mesh netting on the bottom to prevent wireworm escape.

There were four treatment groups in a 2x2 factorial design:

i) *B. bassiana* inoculated (+), wireworms (+)
ii) *B. bassiana* inoculated (+), wireworms (-)
iii) *B. bassiana* inoculated (-) wireworms (+)
iv) *B. bassiana* inoculated (-) wireworms (-).

Potatoes were surface sterilized using a 0.5% bleach solution for 10 min and randomly assigned to the treatment groups. The potatoes in the *B. bassiana*-inoculated treatment groups were submerged in a 0.1% Tween 80 suspension containing 3×10^7 spores/mL. The non-inoculated treatments were submerged in a 0.1% Tween 80 solution. Potatoes in suspension were then agitated on a VWR OS-500 Shaker at speed 1 for 2 h. One potato per pot was then planted at a soil depth of 7cm and the sprouts were lightly covered with soil. Three plants in each of the four treatment groups were randomly placed in a 3 x 4 grid on five benches. Plants of similar sizes were placed on the same bench. They were kept in a greenhouse under a 16:8 light-dark schedule at 20°C and under 60% humidity. Plants were watered through drip irrigation using a fertigation mixture of 18-6-20 (nitrogen-phosphorus-potassium). Once the plants were at least 16cm tall, the potatoes were thinned to one stem, and soil was added to the pots to a height of 24cm to simulate hilling.

Plant height and the number of leaves per plant were recorded at three weeks post-planting. At the start of blooming, when potato tubers start to develop, seven large *A. lineatus* wireworms were added to each plant in the wireworm (+) treatment groups. Wireworms were given two weeks to feed, and then the plants were sampled for hormone analysis by collecting the terminal triplet of the second leaf from the terminal

bud of each plant, plunging it immediately in liquid nitrogen, and storing it at -80°C (Figure 3.1). Leaf samples from three plants of the same treatment were pooled together, ground into a powder in liquid nitrogen, and then stored at -80°C. These samples were taken from three benches from the second experimental replicate for a total of 3 pooled samples per treatment.

At 3.5-4 months, when the plants began to senesce, they were cut at the base of the stem and the above-ground portion of the plant was collected in perforated plastic bags. Samples of the cross-section of the plant stem from the second experimental replicate were taken for endophyte detection. The fresh mass of the plants was measured, and then the plant material was dried at 60°C for 4 days and then weighed. Potato tubers were harvested, washed, counted, and weighed. The total number of holes found on all the tubers of each plant was counted. The experiment was performed twice, once starting in November 2021, and the second starting in February 2022.

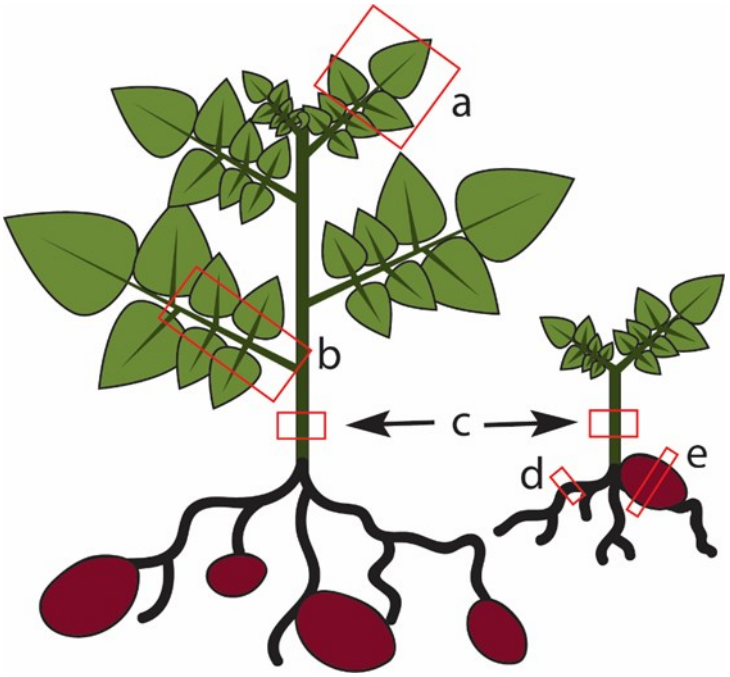

Figure 3.11 Sampling locations of the potato plant: a) terminal triplet of the third leaf, for metabolite analysis; b) the first leaf, prioritizing the petiole, for DNA extraction; c) cross-section of the plant stem, for culture; d) cross section of root, for culture; e) cross section of inoculated seed tuber, for culture.

3.3.3. Endophyte detection

On the same sampling date that the plant hormone leaf samples were taken, the first visible leaf above the soil was collected and surface sterilized in 70% EtOH for 2 min for endophyte detection via PCR (Figure 3.1). This location was chosen because endophytic fungi are speculated to move upward from the inoculation site (Bing & Lewis, 1992). The leaf was sliced into small pieces, prioritizing the petiole, and stored at -20°C before DNA extractions. A leaf imprint and final wash-water were plated on entomopathogenic fungi-specific CTC media (Fernandes et al., 2010), incubated for 7 days, and evaluated for no fungal growth.

To verify that *B. bassiana* can endophytically colonize the potato plants, a separate mini-experiment with an additional 10 seed tubers was established. The tubers were inoculated using the same method as described above, planted, and left to grow for 16 days under the same conditions. Samples were then taken at the cross-section of the potato sprout, the seed tuber tissue, and the potato root (Figure 3.1). These samples, as well as the stem cross-section samples from the main experiment (3.3.2), were surface sterilized in 70% ethanol for 2 min, cut into 5 thin sections per sample with a sterile scalpel, plated on CTC media, and incubated for 10 days at 27°C. Final wash water and tissue imprints were plated to check sterilization efficacy. Fungal outgrowth resembling *B. bassiana* was sub-cultured at 27°C for 7 days.

DNA extractions of plant leaf samples, as well as the fungal subcultures, were performed using the ZymoBIOMICS^{TM} DNA Miniprep Kit. Half of the ceramic beads in each lysis tube were substituted by 2mm ZR Bashingbeads to better handle the tough plant tissue. For the positive control, *B. bassiana* was grown on a PDA medium and DNA was extracted using FastDNA SPIN Kit (MPBio). The PCR was performed using the Hot Start PCR-To-Gel Taq Master Mix (VWR). Multiple primers specific to *B. bassiana* were tested: P1/P3 (Hegedus & Khachatourians, 1996), EF3-5 (McKinnon et al., 2018), and modified ITS (Griffin, 2007). Cycling conditions were initial denaturation for 3 min at 94°C, followed by 25 cycles of 94°C for 30 sec, 50°–60°C for 30 sec, and 72°C for 45 sec, and a final 10-min extension at 72°C. 1.5% TAE gels were used to separate the DNA fragments. The P1 (5'AAGCTTCGACATGGTCTG) and P3 (5'GGAGGTGGTGAGGTTCTGTT) primers amplifying a 524-bp region of the *B. bassiana*-specific probe pBb22 were chosen for all subsequent analyses.

3.3.4. Plant hormone detection

The plant defence hormones measured were SA, JA, as well as their conjugated forms, conjugated SA, and JA-isoleucine. Quantitative analysis of these compounds was performed by the National Research Council of Canada using UPLC ESI-MS/MS with a modified procedure described in Murmu et al. (2014). The UPLC/ESI-MS/MS utilized a Waters ACQUITY UPLC system equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. The chromatographic traces for each phytohormone and respective deuterium-labelled internal standard was quantified using the QuanLynx v4.1 software (Waters Inc).

3.3.5. Wireworm feeding experiments

In these laboratory experiments, the soil used was a silt-loam mixture collected at the Agassiz Research and Development Centre, BC. The soil was sifted through a 1.18mm screen and autoclaved at 121°C for 50 min at 16psi. Soil moisture ranged from 16-18% moisture content.

Two experiments were performed. Firstly, I investigated the effects of direct *B. bassiana* inoculation on wireworm feeding. Actively feeding *A. sputator* wireworms were directly inoculated with *B. bassiana* spores by saturating a paintbrush with spores and brushing the wireworm until it was covered in white, or mock-inoculated with a brush with no spores. They were then dropped at a height of 30cm onto a hard surface to knock off loose spores and put into a container of autoclaved soil at room temperature for 3 weeks. Daughter tubers of control plants from the greenhouse experiment (3.3.2) were cut in half, adjusted to 50 ± 5g, and placed cut side down into a container filled with 50 ± 1g of autoclaved soil. Five wireworms were weighed together and then introduced into the container. Twenty-two reps of each treatment (inoculated wireworms and non-inoculated wireworms) were then randomly arranged and left at 25°C. The number of wireworm feeding holes and the weight of the five wireworms were measured after one week. The cut side of the potato with the feeding holes was sliced so that no more holes were visible, then the potato and wireworms were placed back into the container and assessed once more the following week.

Secondly, I investigated whether the inoculation of potato plants affected wireworm herbivory in a laboratory setting using daughter tubers of *B. bassiana*-inoculated and non-inoculated plants from 3.3.2. They were handled exactly as described above, and five non-inoculated *A. sputator* wireworms were placed in each container. There were 20 reps of each treatment (tubers from inoculated plants and tubers from non-inoculated plants). Data collection was the same as described above.

3.3.6. Statistical analysis

All analyses were performed using R version 4.1.2. The plant height and the number of leaves at 5 weeks post-planting, the fresh biomass, the dry biomass, the weight of potatoes, and the number of tubers produced were analyzed using linear mixed effects models followed by an analysis of variance (ANOVA) by removing non-significant terms. The packages “car” and “lmerTest” were used. *Beauveria bassiana* treatment, wireworm treatment, *B. bassiana* and wireworm treatment interaction, and experimental replicate number were included as fixed effects. Bench number was included as a random effect. The wet and dry biomass, the weight of potatoes, and the number of potatoes were log-transformed to satisfy ANOVA assumptions. The total number of feeding holes in the tubers of each plant was analyzed using a negative binomial regression due to overdispersion using the same terms. The SA and JA levels were analyzed using ANOVA based on a linear mixed effects model as well by removing non-significant terms. *Beauveria bassiana* treatment, wireworm treatment, and *B. bassiana*:wireworm treatment interaction terms were included as fixed effects. Bench number was included as a random effect.

In the laboratory feeding experiments, the number of wireworm feeding holes and the combined weights of the five wireworms were analyzed with repeated measures ANOVA using the package “car”. *Beauveria bassiana* treatment was a fixed effect and the week number was an error term. Correlations between the number of wireworm feeding holes and plant characteristics were measured using the Kendall rank correlation test.

3.4. Results

3.4.1. Plant productivity and wireworm herbivory

Neither *B. bassiana* inoculation nor the addition of wireworms influenced any plant growth metric measured (Table 3.1, A.1). There were significant differences in the number of leaves, wet biomass weight, dry biomass weight, number of potatoes, and potato weight between experimental replicates, but not plant height (Table 3.1, A.1). The data between replicates show the same trend, but with differences in magnitude. There were higher values in the first replicate for wet and dry biomass, potato weight, and potato count, and a higher number of leaves in the second replicate (Table 3.1, A.1). During the first replicate, plants overgrew to the point of falling over and becoming damaged, so fertilization frequency was reduced in the second replicate, which was likely the major cause of the reduced plant and potato biomass. Potatoes of one plant in the *B. bassiana* (-) wireworm (-) treatment of the second experimental replicate suffered tuber rot and all data relating to tubers was removed for this plant.

The number of wireworm feeding holes was only affected by the addition of wireworms ($\chi^2_1 = 53.67$, $p \leq 0.001$; *B. bassiana**wireworm: $\chi^2_1 = 0.44$, $p = 0.51$) (Figure 3.2), but not *Beauveria bassiana* treatment ($\chi^2_1 = 0.57$, $p = 0.45$). Experimental replicate did not influence wireworm damage ($\chi^2_1 = 2.19$, $p = 0.14$), indicating that that the differences in plant biomass between experimental replicates was not important in reducing wireworm damage.

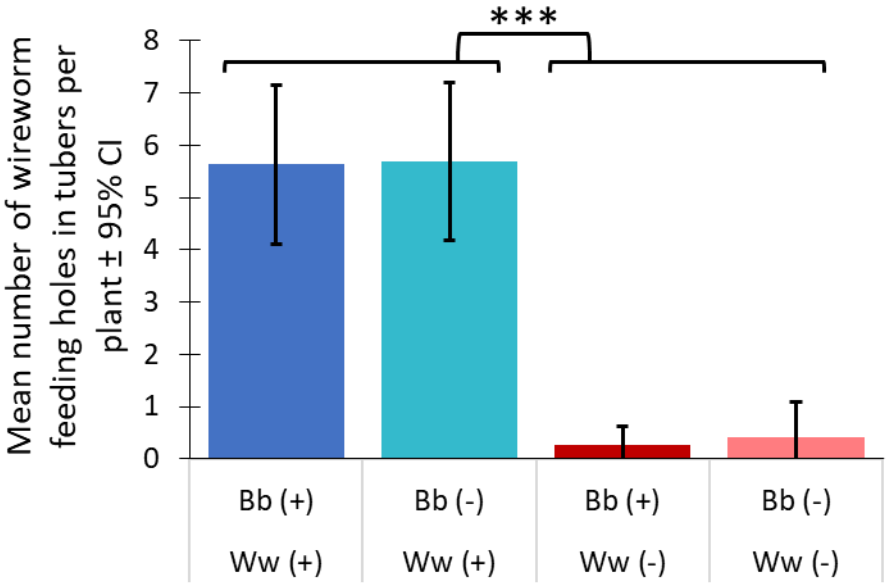

Figure 3.2. **The total number of wireworm feeding holes found in all of the tubers of each plant in the *B. bassiana* (Bb) and wireworm (Ww) treatment groups of the greenhouse experiment. *** $p \leq 0.001$**

Table 3.1. **Statistical analysis of the greenhouse experiment comparing plants inoculated with *B. bassiana* and non-inoculated, and plants with wireworms introduced, and without wireworms. Table shows the analysis of variance results of plant performance data examining the effects of *B. bassiana* seed tuber inoculation of potatoes and wireworm herbivory. Significant p values are highlighted in bold.**

	df	F value	p value
Height of plant			
B. bassiana treatment	1	0.01	0.93
Wireworm treatment	1	1.03	0.31
Experimental replicate	1	0.13	0.72
B. bassiana:wireworm interaction	1	0.13	0.72
Residuals	111		
Number of leaves			
B. bassiana treatment	1	2.08	0.15
Wireworm treatment	1	0.57	0.45
Experimental replicate	1	5.78	**0.02**
B. bassiana:wireworm interaction	1	0.04	0.84
Residuals	111		
Wet weight of biomass			
B. bassiana treatment	1	0.20	0.65
Wireworm treatment	1	0.33	0.57
Experimental replicate	4	7.46	**<0.001**
B. bassiana:wireworm interaction	1	1.13	0.29
Residuals	111		
Dry weight of biomass			
B. bassiana treatment	1	0.16	0.69
Wireworm treatment	1	0.84	0.36
Experimental replicate	1	464.86	**<0.001**
B. bassiana:wireworm interaction	1	0.27	0.61
Residuals	111		
Number of potatoes			
B. bassiana treatment	1	0.73	0.39
Wireworm treatment	1	0.30	0.59
Experimental replicate	1	171.67	**<0.001**
B. bassiana:wireworm interaction	1	1.27	0.26
Residuals	110		
Potato weight			
B. bassiana treatment	1	0.06	0.81

Wireworm treatment	1	1.40	0.24
Experimental replicate	1	509.61	**<0.001**
B. bassiana:wireworm interaction	1	1.31	0.26
Residuals	110		

3.4.2. Plant hormones

Salicylic acid, conjugated salicylic acid, and jasmonic acid were detected in the potato leaf tissue, but not jasmonic acid isoleucine. There were no significant differences in plant hormones between *B. bassiana* inoculated plants and non-inoculated plants, and no differences between wireworm treatments (Table 3.2). There was no interaction between *B. bassiana* and wireworm treatment. Plants with introduced wireworms had a slightly higher JA level and slightly lower conjugated SA level, but not significantly (Table A.2).

Table 3.2. **Statistical analysis of plant defence hormones from the greenhouse experiment comparing plants inoculated with *B. bassiana* and non-inoculated, and plants with wireworms introduced, and without wireworms. Table shows the analysis of variance results of jasmonic acid and salicylic acid levels in the leaves of potato plants collected two weeks after flower bloom and wireworm addition.**

	df	F	p value
Jasmonic acid			
***B. bassiana* treatment**	1	1.19	0.32
Wireworm treatment	1	1.19	0.32
***B. bassiana*:wireworm interaction**	1	0.05	0.83
Residuals	6		
Salicylic acid			
***B. bassiana* treatment**	1	0.02	0.90
Wireworm treatment	1	0.12	0.74
***B. bassiana*:wireworm interaction**	1	2.21	0.19
Residuals	6		
Conjugated salicylic acid			
***B. bassiana* treatment**	1	0.70	0.44
Wireworm treatment	1	3.97	0.09
***B. bassiana*:wireworm interaction**	1	1.38	0.28
Residuals	6		

3.4.3. *Beauveria bassiana* endophyte detection

Beauveria bassiana was not detected from the leaf imprint or the last wash water cultures, indicating that surface sterilization was successful. All three primer pairs tested were successful in amplifying the *B. bassiana* positive control (Figure 3.3). *Beauveria bassiana* was not detected in the samples taken from leaf petioles that underwent direct DNA extraction. Potato plant stem cross-sections that were taken at 104 days were cultured, and *B. bassiana* was found in 10% of samples from *B. bassiana*-inoculated plants. No *B. bassiana* was detected in control plants. In the separate mini-experiment (see 3.3.3), 40% of *B. bassiana*-inoculated plant stems and 40% of plant roots were positive for *B. bassiana* when sampled at 16 days. *Beauveria bassiana* was not detected in the seed tubers or the control plants.

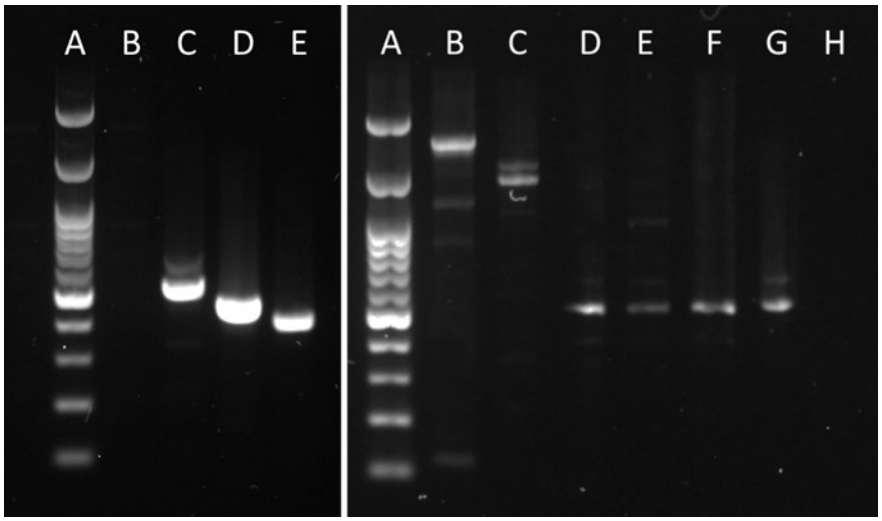

Figure 3.3. Left: Agarose gel of the three primer pairs for detecting *B. bassiana*. A) 100-bp DNA ladder; B) negative sample; C) P1/P3 primers (expected band size 524bp); D) modified ITS primers (421bp); E) EF3-5 primers (406bp). Right: examples of positive plant subcultures for *B. bassiana*. A) 100-bp DNA ladder; B-C) negative samples; D-F) positive samples; G) positive control; H) negative control.

3.4.4. Laboratory feeding experiments

Beauveria bassiana inoculation of wireworms did not affect the number of wireworm feeding holes in potato tubers ($F_{(1,41)} = 0.54$, $p = 0.47$). There were no effects on the growth rate of wireworms due to *B. bassiana* treatment ($F_{(1,41)} = 1.44$, $p = 0.24$). *Beauveria bassiana* inoculation of potato plants likewise, did not affect the number of wireworm feeding holes in potato tubers ($F_{(1,37)} = 0.20$, $p = 0.66$), nor in changes in wireworm weights ($F_{(1,36)} = 0.02$, $p = 0.88$). There were no correlations between the number of wireworm feeding holes and any measured characteristic in either experiment ($-0.01 <$ Kendall's tau < 0.01, $p > 0.05$).

3.5. Discussion

Beauveria bassiana was detected inside potato plant tissue, but *B. bassiana* inoculation did not affect potato plant growth, tuber production, or wireworm feeding damage in either the greenhouse or laboratory experiments. There were no effects of *B. bassiana* inoculation on salicylic acid or jasmonic acid levels in plants.

3.5.1. *Beauveria bassiana* colonized potato plants

Beauveria bassiana was detected at low levels in the inoculated potato plants. It was not detected in the lowest leaf when samples were directly used for DNA extraction but was found in the cross-section of the plant stem closest to the soil that was plated on media before DNA extraction. This suggests that using the methods in this study, either the likelihood of *B. bassiana* colonization of plants is low, or the level of colonization is low resulting in a low likelihood of detection. The detection of endophytes is difficult and often not consistent because fungal colonization is sparse and not uniform, so future experiments should take advantage of more sensitive detection methods such as using nested PCR and qPCR (Landa et al., 2013).

Several factors influence the detection rate of endophytic *B. bassiana,* including the fungal isolate and the location of the plant sampled (Akutse et al., 2013; Gurulingappa et al., 2010), as well as the plant species and inoculation method (Sánchez-Rodríguez et al., 2018). For example, soil inoculation worked in wheat, but failed in cotton, where only foliar inoculation was successful (Gurulingappa et al., 2010). Leaf sprays were the most successful in wheat, corn, and soybean, compared to seed inoculation and root immersion (Russo et al., 2015). In coffee, direct stem injection was more successful compared to foliar sprays or soil drenches (Posada et al., 2007), and in maize, seed inoculations were more effective compared to soil drenching (Batool et al., 2020). In this study, *B. bassiana* was able to colonize potato plants in non-sterile mixed soil. Soil sterility and composition contribute to successful colonization: Parsa et al. (2018) saw more variable endophytic colonization in their sand:soil:peat substrate compared to vermiculite, and soil sterilization was a major factor in colonization. In contrast to my finding, Tefera and Vidal (2009) found that sorghum seed inoculation with *B. bassiana* conidia did not result in colonization in non-sterile soil but succeeded in vermiculite and sterile soil. Differences in soil microbes can cause antagonistic effects on the entomopathogen (Lingg & Donaldson, 1981), which can result in differences in colonization success.

In this study, the colonization rate of potato plants was lower at the later sampling date compared to the earlier date. This supports Zhang et al. (2022) who detected *B. bassiana* in potatoes and found that colonization rates at 7 days post-inoculation (dpi) were 90% in the lower leaves, but at 50 dpi, it dropped to 18%. Several studies in other

plants also found that the colonization rate of endophytic *B. bassiana* declined over time. Sánchez-Rodríguez et al. (2018) found that in bread wheat, *B. bassiana* colonization rates after leaf spray and seed dressing peak at 10-13 days after sowing and decrease steadily until almost undetectable at 31 days. Similar decline rates were seen in beans, corn, wheat, soybeans, and pumpkins (Gurulingappa et al., 2010; Russo et al., 2015). It is unclear what causes the decline of endophytic colonization over time, but competition from other endophytes may be a contributing factor (Posada et al., 2007), or because the plant outgrew the fungus, leading to more uncolonized plant tissues.

The establishment of *B. bassiana* as a systemic endophyte may not be necessary for growth and protective effects, as *B. bassiana* can be localized to its inoculation location or exist in the rhizosphere. Tall and Meyling (2018) found that *B. bassiana* promoted the growth of corn, but no endophytic establishment was detected at 6 weeks, suggesting that the fungus-plant interaction was independent of endophytic establishment. Alternatively, endophytic colonization may have been transient and therefore could not be detected at their sampling time.

3.5.2. *Beauveria bassiana* did not affect plant performance or wireworm feeding damage

Neither *B. bassiana* nor wireworm treatment affected potato plant growth or potato production. This result does not support studies which report on the growth-promoting effect of endophytic colonization of *B. bassiana*, for example, in tomatoes (Barra-Bucarei et al., 2020), beans (Afandhi et al., 2019), tobacco (Qin et al., 2021), and wheat (Sánchez-Rodríguez et al., 2018). This study supports others, such as Vera et al. (2022), who found that *Beauveria vermiconia* colonized ryegrass roots but did not affect aboveground biomass. Rasool et al. (2021) did not find effects on wheat or bean plant height due to *B. bassiana* inoculation; however, they found that inoculation affected the dry root weights of both plants in the presence of aphids, but not in the absence of aphids. Nutritional availability can also influence whether *B. bassiana* acts as a growth promoter: Tall and Meyling (2018) found that the seed treatment of maize with *B. bassiana* resulted in growth promotion only when grown at high nutrient conditions but not at low nutrient conditions.

The promotion of plant growth can allow plants to compensate for insect herbivory. Zitlalpopoca-Hernandez et al. (2017) found that *B. bassiana* treatment did not cause mortality to *Phyllophaga vetula* scarab beetle larvae, as only 5% were infected with *B. bassiana*; however, when in the presence of mycorrhizal fungi, they found increased plant growth and nitrogen concentration in the shoots, which increased plant tolerance to root herbivory. This effect is not relevant to wireworm herbivory on potato plants, where plant growth is not affected by wireworm feeding. Indeed, in this study, there was no correlation between wireworm herbivory damage and plant growth characteristics, possibly because herbivory was very light.

Beauveria bassiana protects plants against insect herbivores by causing insect mortality (Akello et al., 2008; Mantzoukas & Lagogiannis, 2019; Ramos et al., 2020); however, protection need not be only through direct infections. Silva et al. (2020) found that endophytic *B. bassiana* caused 90% mortality of tomato pinworm (*Tuta absoluta*) in tomatoes; however, no fungal sporulation was observed on the cadavers. Similarly, Sánchez-Rodríguez et al. (2018) found that endophytic *B. bassiana* caused up to 57% mortality of cotton leafworm larvae (*Spodoptera littoralis*) in wheat, but no fungal sporulation was observed in the cadavers. Other studies that have found mortality but no sporulation in cadavers include endophytic *B. bassiana* on tobacco with *Myzus persicae* aphids (Qin et al., 2021), *Iraella luteipes* gall wasp larvae and opium poppy (Quesada et al., 2018), and *Tetranychus evansi* spider mites and tomato (Omukoko, 2020). The protective effects in these cases are likely to be feeding changes, reproductive changes, and non-mycosis-related mortality, possibly from the production of secondary metabolites by the fungus or plant. Rasool et al. (2021) found no correlation between endophytic colonization and plant protection, suggesting that it is systemic effects, likely due to induced defence responses, not local effects, that are protecting plants.

Beauveria bassiana treatment of seed tubers did not protect potato tubers from wireworm feeding damage. This finding does not support other studies, which show that plant endophytic colonization with *B. bassiana* results in anti-feeding effects against insects. For example, feeding assays using endophytic *B. bassiana* in soybean plants resulted in reduced consumption by *Helicoverpa gelotopoeon* bollworm moths (Russo et al., 2019). Corn plants with endophytic *B. bassiana* negatively affected the feeding and food preference of *Dichroplus maculipennis* grasshoppers (Pelizza et al., 2017). The repellent effect may be due to metabolites produced by the fungi or plant metabolites

that are induced by the fungi. In our study, *B. bassiana* may have resulted in slight anti-feeding effects; however, once a wireworm begins feeding, it blemishes the tuber. The tolerance for wireworm feeding holes is very low in commercial markets, so slightly reduced feeding would not greatly affect crop protection.

3.5.3. Plant defence hormones were not affected by *B. bassiana*

No differences in SA or JA levels were detected in potato plants after *B. bassiana* treatment, aligning with the absence of wireworm protection. This supports Raad et al. (2019) who found neither protection by *B. bassiana* in *Arabidopsis thaliana* against *M. persicae* aphids or *Plutella xylostella* caterpillars, nor higher concentrations of JA or SA. Interestingly, they did see changes to genes related to plant defence including JA and SA signalling pathways in inoculated plants and protection against the pathogen *Sclerotinia sclerotiorum.* Induced plant protection by microbes, including *B. bassiana*, is typically triggered by SA or JA pathways (Kunkel & Brooks, 2002; Rashid & Chung, 2017); for example, Qin et al. (2021) found that *B. bassiana* protected tobacco against the pathogens *Alternari alterana*, *Botrytis cinerea*, and *Ralstonia solanacearum*, as well as from *Myzus persicae* aphids by triggering both SA and JA defence pathways. However, other signalling pathways than those requiring SA and JA are likely involved in the plant as well, as evidenced by studies that show *B. bassiana* protection against pests, but no differences in SA or JA levels (Pus, 2017; Raad et al., 2019). Regardless of the pathway, *B. bassiana* inoculation has been shown to influence the expression of defence genes and proteins including *PRI1, PR2*, *ERF-1, Pti-5, Pi1,* and reactive oxygen species, resulting in protection against both pathogens and insect pests (Gupta et al., 2022; Jensen et al., 2020; Senthilraja et al., 2013).

Beauveria bassiana inoculation may have other effects unmeasured in this study, including effects on plant secondary metabolite production. For example, Shrivastava et al. (2015) found that while endophytic *B. bassiana* in tomatoes did not lead to beet armyworm (*Spodoptera exigua*) mortality, it resulted in enhanced levels of monoterpenes and sesquiterpenes, which may have contributed to lower larval weight of larvae when fed with inoculated plants. Furthermore, González-Mas et al. (2021) found that endophytic *B. bassiana* in melon and cotton plants resulted in a different emission of volatile compounds compared to non-colonized plants. Some emitted compounds found are associated with the attraction of herbivore natural enemies, and others are

associated with anti-microbial effects. Metabolites induced by *B. bassiana* inoculation may have negative effects on wireworms and other insects that warrant further investigation.

3.5.4. Conclusions

In conclusion, potato plants were endophytically colonized by *B. bassiana* using a seed tuber treatment, but no effects on growth parameters nor wireworm herbivory damage were observed. Salicylic acid and jasmonic acid levels were not affected by *B. bassiana* or wireworm treatment. It is unlikely that *B. bassiana* provides potatoes protection against wireworm feeding damage through endophytic colonization or root association. Plant-fungal-insect interactions are complex and important to understand to develop biological control strategies. Further investigation is needed to examine if, and how, *B. bassiana* protects potatoes from wireworm feeding damage.

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Chapter 4.

Beauveria bassiana alters the bacterial load of wireworms

4.1. Abstract

The insect microbiome has an important role in disease prevalence by modifying interactions between host and pathogen. Entomopathogenic fungi infect insects through the cuticle and have been shown to interact with insect gut microbiota, but their interactions are not well understood. Wireworms, the larval stage of elaterid beetles, are subterranean generalists and are pests of a variety of crops worldwide. This chapter examines if the inoculation of wireworms with the fungal entomopathogen *Beauveria bassiana* influences the abundance and/or diversity of bacteria in the internal insect microbiome through bacterial culture and 16S sequencing. There was a significant increase in the bacterial load after *B. bassiana* inoculation, but no change in bacterial diversity. After *B. bassiana* inoculation, the relative abundance of bacterial phyla shifted from primarily proteobacteria to primarily actinobacteria, and the genera *Mycobacterium, Skermania, Bacillus,* and *Tissierella* increased in relative abundance. Interestingly, these shifts occurred without detectable *B. bassiana* infection of wireworms.

Keywords: *Beauveria bassiana*; entomopathogen; wireworm; behaviour; dysbiosis; microbiome

4.2. Introduction

The insect microbiome consists of all the microorganisms that live within or upon a host, with the cuticle and the gut being the habitats where most microbes reside. The composition of the microbiome is highly variable and is influenced by many factors including the host diet and their environment (Jang & Kikuchi, 2020; Yun et al., 2014). Members of the microbiome can have a major influence on the fitness of their hosts and

drive their evolution through impacts on various factors including host nutrition, fecundity, stress resistance, and resistance towards pathogens and parasites (Feldhaar, 2011). Some examples include, providing essential amino acids (Lu et al., 2016), detoxifying plant defensive compounds (Ceja-Navarro et al., 2015), and producing anti-microbials to protect against pathogens (Shao et al., 2017). Insect microbes can even suppress plant defence responses to herbivory (Acevedo et al., 2017). However, not all interactions are beneficial – the insect microbiome also contains members that are opportunistic pathogens (Wei et al., 2017). Pathogenic interactions between insects and their microbiome have been recent subjects of interest, especially regarding the biological control of insects using pathogens (Bai et al., 2022; Wei et al., 2017; Zhang et al., 2018). The study of the insect microbiome has been focused primarily on *Drosophila* and other flies (Sharon et al., 2010; Yuan et al., 2020; Zhang et al., 2021), lepidopterans (Bai et al., 2022; Polenogova et al., 2019; Wang et al., 2022; Wu et al., 2020), aphids (Douglas & Prosser, 1992; Oliver et al., 2003; Scarborough et al., 2005; Schmid et al., 2012), and mosquitoes (Barnard et al., 2019; Ramirez et al., 2018; Wei et al., 2017).

Members of the microbiome can live in symbiosis with their host and provide several benefits, and a well-known example is nutrient provisioning in phloem-feeding insects like aphids. These insects have specialized diets that result in the lack of the essential amino acid tryptophan, which is not present in the phloem, and therefore they rely on their intracellular symbiotic gut bacterium *Buchnera* aphidicola to produce this amino acid (Douglas & Prosser, 1992). Microbes also serve a protective function to their hosts, for example in thermal stress tolerance (Dunbar et al., 2007). *Wolbachia*, a genus of ubiquitous intracellular bacteria, is notorious for lowering host fitness due to reproductive parasitism; however, they have also evolved to be beneficial to their host by increasing fecundity, survival, and nutrient provisioning (reviewed in Zug & Hammerstein, 2015). In these ways, the microbiome can influence the evolution of its hosts and can even influence mating behaviours (Sharon et al., 2010). Another benefit of the microbiome is the detoxification of plant defence compounds, for example, of caffeine by the gut microbiota of coffee berry borer (*Hypothenemus hampei*) (Ceja-Navarro et al., 2015). On a similar note, they can also help insects by detoxifying insecticides and contributing to insecticide resistance (Barnard et al., 2019; Pietri & Liang, 2018).

Microbes can protect insects against both parasitoids and pathogens, albeit through different mechanisms. The bacteria *Hamiltonella defensa* protects aphids from parasitoid wasps by disabling wasp larval development, and this is dependent upon the infection of *H. defensa* with bacteriophages (Oliver & Higashi, 2019). *Wolbachia* protects *Drosophila* against RNA viruses and the suggested mechanisms are through the activation of host immunity and/or the competition with the virus for cellular resources (Pimentel et al., 2020). Gut microbiota can also contribute to insect defence against opportunistic or pathogenic microbes through competition and the production of antimicrobials (Boucias et al., 2018; Moraes et al., 2014; Shan et al., 2014; Zhang et al., 2018). The secretion of anti-microbial compounds can selectively affect pathogenic bacteria and not harm other microbiome residents, contributing to the overall stability of the microbiome (Shao et al., 2017). There is potential in the manipulation of the insect microbiome to the detriment of a pest insect as a part of pest management. By disrupting the microbiome, insects may lose these fitness benefits and may make insects more susceptible to various stressors including entomopathogen infection (Wang et al., 2022; Zhang et al., 2018) and insecticides (Tang et al., 2021).

Wireworms are the larvae of elaterid beetles and are soil-dwelling pests of various crops worldwide. Due to tightening restrictions for conventional chemical control methods due to environmental and health toxicity, there is interest in developing alternatives, such as biological control. In wireworms, biological control agents such as entomopathogenic nematodes and parasitoids have not been effective at controlling populations, and bacteria with pathogenic potential have been identified, but not developed (Danismazoglu et al., 2012; Kleespies et al., 2013). Wireworm biocontrol has found better success with entomopathogenic fungi, such as *Metarhizium brunneum* (Kabaluk et al., 2007; Razinger et al., 2020; Rogge et al., 2017). *Beauveria bassiana* is an entomopathogenic fungus that is successful in biocontrol programs against a variety of insects (Charnley & Collins, 2007). In laboratory assays, *B. bassiana* was suppressed by bacterial isolates from insect microbiomes (Moraes et al., 2014; Toledo et al., 2011; Wang et al., 2022). Furthermore, *in-vivo* studies show that insects with intact microbiomes survive better after *B. bassiana* infection compared to insects with disrupted microbiomes (Wang et al., 2022; Zhang et al., 2018; Zhou et al., 2019). *Beauveria bassiana* infection can also affect the host microbiome to accelerate host death by disrupting the gut, possibly manipulating opportunistic bacteria, increasing gut

bacterial load, and altering the bacterial community structure (Bai et al., 2020; Polenogova et al., 2019; Ramirez et al., 2018; Wei et al., 2017; Xu et al., 2019). Whether the microbiome of wireworms is impacted by, or interacts with, *B. bassiana* has yet to be examined.

I investigated the effects of *B. bassiana* inoculation on the *A. lineatus* internal whole-body microbiome. I measured the culturable bacterial load through dilution plating and examined the effects on bacterial diversity and community composition using 16S sequencing. This study is the first to examine the effects of entomopathogen inoculation on the wireworm microbiome. My research questions are:

1. Can *B. bassiana* penetrate the wireworm cuticle?
2. Does *B. bassiana* inoculation increase the culturable bacterial load of the wireworm internal microbiome?
3. Does *B. bassiana* inoculation affect the bacterial composition or diversity of the wireworm internal microbiome?

4.3. Materials and Methods

Agriotes lineatus wireworms were collected from an organic farm on Vancouver Island. All experiments contained two treatment groups: wireworms inoculated with *B. bassiana* (Bb), and the non-inoculated control group (Ctrl). Larvae in the *B. bassiana*-inoculated group were brushed with a paintbrush saturated with *B. bassiana* PPRI5339 spores (BASF), obtained by solid-state fermentation on rice (Jaronski, 2014) until all surfaces were coated with a white dusting of spores. The control group was brushed with a clean paintbrush. Wireworms were dropped twice from a height of 30cm onto a solid surface to knock off loose spores, then transferred into autoclaved soil (121°C for 50 min at 16psi). They were fed with a slice of potato and incubated at 25°C for three weeks. Following this, the wireworms were put into 0.8% water agar for 24h to let unattached conidia be passively groomed off their bodies as they tunnel through the media (Kabaluk et al., 2017). They were transferred into 3% bleach for 1h, then put into sterile water for 24h to distend their integuments. Afterwards, they were washed in 0.1% Tween 20 for 2 min, vortexed, and rinsed in sterile water.

4.3.1. Detection of *B. bassiana* within wireworms

The ability of *B. bassiana* to invade the bodies of the wireworms was assessed using culture-dependent and molecular methods. The culture-dependent assessment utilized 10 wireworms each in the Bb and the Ctrl groups. Following inoculation, incubation, and surface sterilization as described, wireworms were macerated individually in 1040uL of sterile water and 75uL of this was spread-plated onto fungal entomopathogen-specific CTC media (Fernandes et al., 2010). Plates were monitored for 14 days for the presence of fungal colonies resembling *B. bassiana.*

To further verify the presence or absence of *B. bassiana* within wireworms, 15 wireworms each in the Bb and the Ctrl groups were subjected to inoculation, incubation, and surface sterilization. Individual DNA extractions were then performed using Quick-DNA Tissue/Insect Microprep Kit (Zymo Research) and then polymerase chain reaction (PCR) was performed using the Hot Start PCR-To-Gel Taq Master Mix (VWR). For the positive control, mycelia from a pure culture were collected and DNA was extracted with MP Biomedicals™ FastDNA™ SPIN Kit. The primers P1 (AAGCTTCGACATGGTCTG) and P3 (GGAGGTGGTGAGGTTCTGTT) (Hegedus & Khachatourians, 1996) were used to amply a 524-bp region of the *B. bassiana*-specific probe pBb22. Cycling conditions were initial denaturation for 3 min at 94°C, followed by 25 cycles of 94°C for 30 sec, 50°–60°C for 30 sec, and 72°C for 45 sec, and a final 10 min extension at 72°C. A 1.5% TAE gel was used to separate the DNA fragments.

4.3.2. Bacterial abundance

To examine the effects of *B. bassiana* inoculation on the bacterial load of culturable species, wireworms in the Bb and the Ctrl groups were inoculated, incubated, and surface sterilized. The whole body was then ground using a pestle in 500uL of sterile water. Samples of individual larvae were each spot plated in a series of 6 dilutions from 0 – 10^{-5} in 100uL of sterile water. Three spots of 10uL per sample per dilution were plated onto a plate of potato dextrose agar per sample and were incubated at 25°C for 48 h, after which colony-forming units of the most legible dilution were counted. The mean of the CFU counts was taken across the three spots for each wireworm. There were three experimental replicates, and the number of insects in each *B. bassiana* and control treatment group are: 1) n = 10, 2) n = 12, 3) n = 18.

4.3.3. Bacterial composition and diversity

The same wireworm DNA samples used in 4.3.1 were sequenced (www.mrdnalab.com, Shallowater, TX, USA) for the 16S rRNA gene V4 variable region to examine bacterial composition and diversity. The PCR primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) were used in single-step 35-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) on an Illumina NovaSeq with methods via the bTEFAP® DNA analysis service. The conditions were: 95°C for 5 min, followed by 30-35 cycles of 95°C for 30 s, 53°C for 40 s and 72°C for 1 min, and a final elongation step at 72°C for 10 min. Samples were multiplexed and pooled equally based on molecular weight and DNA concentrations, then were purified with calibrated solid phase reversible immobilization beads. The samples were sequenced using Illumina NovaSeq chemistry following the manufacturer's guidelines.

The sequence data were processed using a ribosomal and functional gene analysis pipeline (MR DNA, Shallowater, TX, USA). Primers were removed from sequences, and sequences less than 150bp and those with ambiguous base calls were removed. Final zero-radius OTUs were taxonomically classified using BLASTn against the NCBI database (www.ncbi.nlm.nih.gov). The relative percentage of each genus refers to the relative proportion of sequences with each sample that map to the classification.

4.3.4. Statistical analysis

Colony-forming unit counts were analyzed in R version 4.1.2 using analysis of variance (ANOVA) based on a zero-inflated negative binomial regression from the packages *pscl* and *car* with *B. bassiana* treatment and experimental replicate as fixed effects. Relative abundance was compared between taxa using two-sample T-tests, and only taxa whose relative abundances across all samples were ≥ 0.1% were considered. Microbial diversity analysis was performed by MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using Qiime2 (Bolyen et al., 2019), NCSS 2007, NCSS 2010, XLstat, and “R”. Alpha diversity was measured using the number of amplicon sequence variants, as well as Shannon Diversity indices, and differences were compared using Kruskal-Wallis pairwise comparisons. The microbial community structure was analyzed

using a pairwise analysis of similarities (ANOSIM) of the weighted UniFrac distance matrix to determine if there were any significant differences between the microbial communities. The community structure was visualized with a principal coordinate analysis plot.

4.4. Results

4.4.1. Detection of *B. bassiana* within wireworms

Beauveria bassiana was not detected inside the body of wireworms either through culture methods or by PCR. One sample in the control treatment was lost during DNA extraction, resulting in a reduced sample size of 14. No wireworm mortality was observed due to *B. bassiana* inoculation during the 3-week incubation periods.

4.4.2. Bacterial abundance

Beauveria bassiana inoculation of wireworms resulted in an increased abundance of culturable bacteria in individual wireworm internal microbiomes by an average of 201% (rep 1), 763% (rep 2), and 218% (rep 3) three weeks after inoculation ($\chi^2_1 = 10.01$, $p = 0.0016$, Figure 4.1). The numbers of CFUs between experimental replicates were significantly different ($\chi^2_1 = 13.61$, $p \leq 0.001$, Figure 4.1).

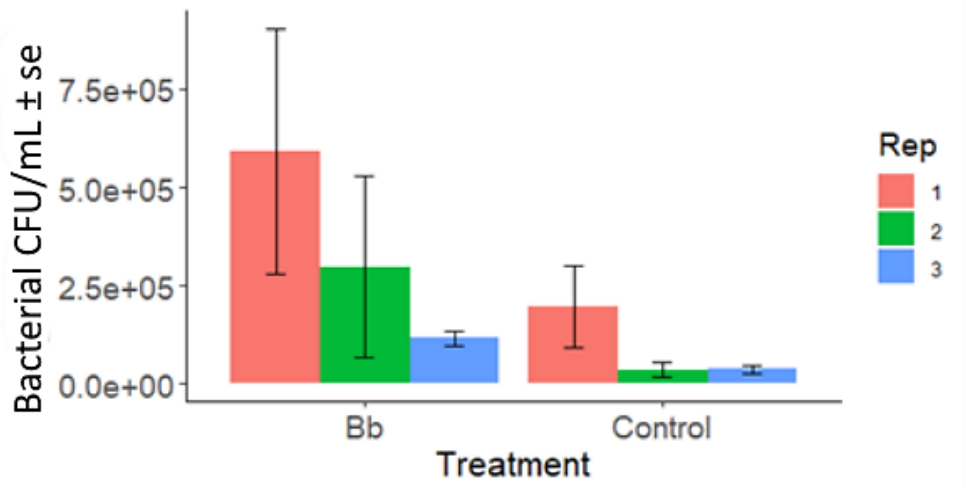

Figure 4.14 **Number of bacterial colony-forming units counted in the internal microbiome of *A. lineatus* wireworms in the *B. bassiana* treatment (Bb) and the untreated control over the three experimental replicates (Rep) after a three-week incubation. Sample sizes in each treatment group are Rep 1) n = 10, 2) n = 12, 3) n = 18. Error bars denote the standard error.**

4.4.3. Bacterial composition and diversity

There was a total of 239 genera matches in this study and 44 genera of which had relative abundances of ≥ 0.1% (Table B.1). Of these genera, four were significantly higher in the *B. bassiana* group compared to the control group: *Mycobacterium, Skermania, Bacillus,* and *Tissierella* (Table 4.2). The top 30 most abundant genera are displayed in Figure 4.2, and the 18 genera whose relative abundances were ≥ 1% are depicted in Figure 4.3. *A. lineatus* wireworm microbiomes consist primarily of proteobacteria (46%), followed by Actinobacteria (18%), Firmicutes (15.3%), Tenericutes (11.8%), Bacteroidetes (4.5%), and Cercozoa (3.9%) (Table 4.1). When inoculated with *B. bassiana*, wireworms exhibited a significant increase in the proportion of Actinobacteria (T-test: p = 0.049) (Table 4.1).

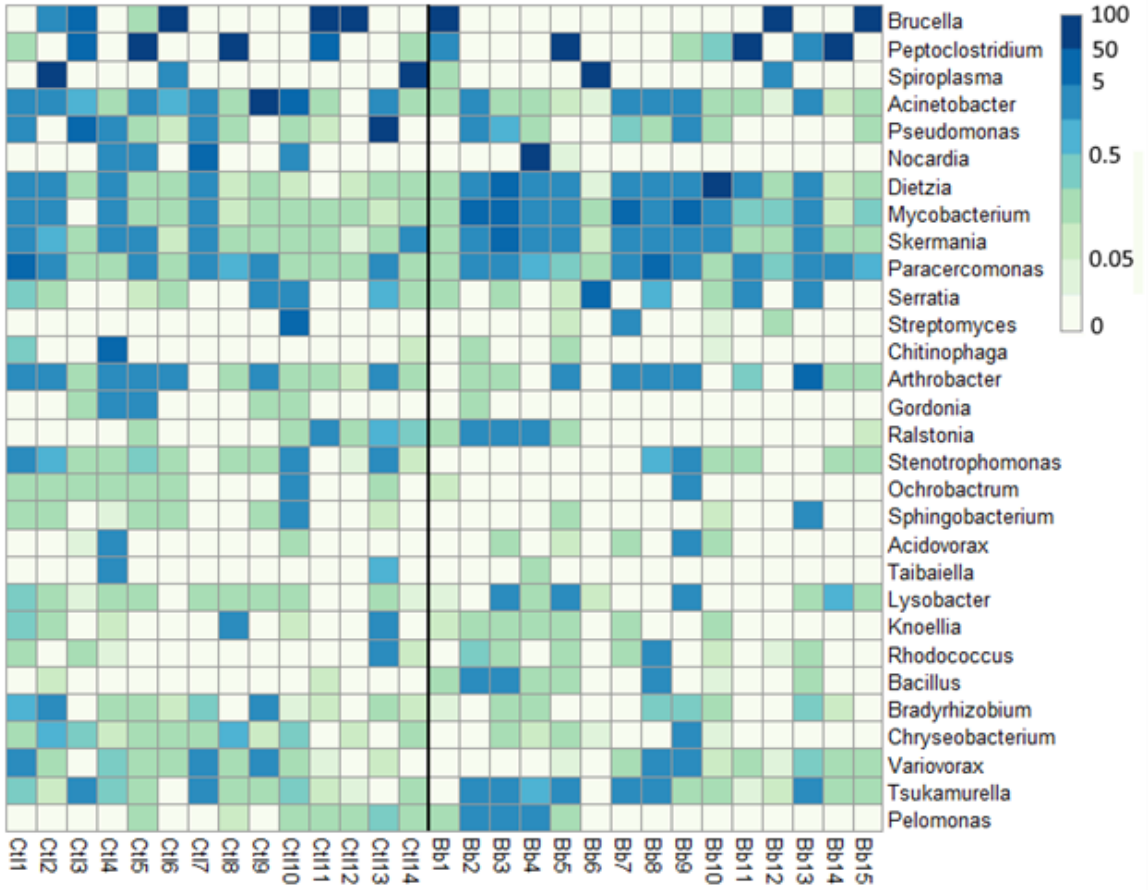

Figure 4.2. **Heat map of the relative abundance (%) of the top 30 most abundant bacterial genera in the internal microbiome of *A. lineatus*. Each column represents an individual wireworm. Individuals of the control (Ctl) treatment are grouped on the left, and individuals of the *B. bassiana*-inoculated (Bb) treatment are grouped on the right.**

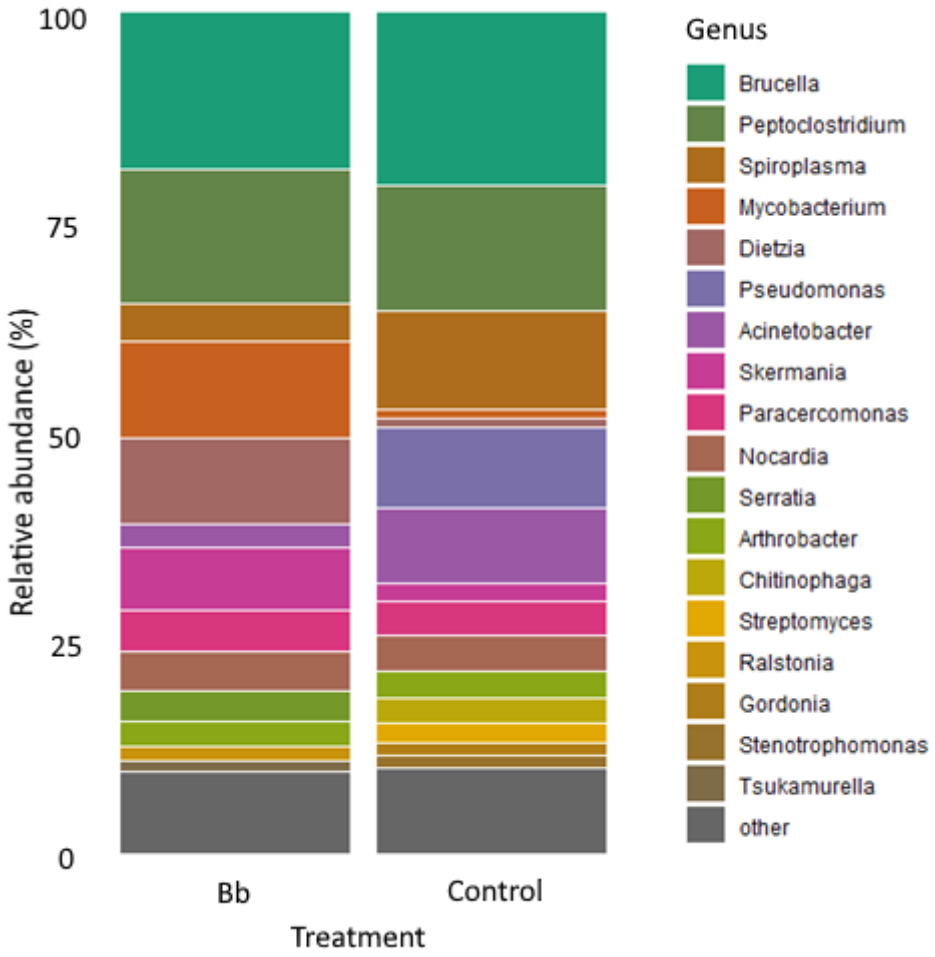

Figure 4.3. **Relative abundances of bacterial genera (≥1%) in the internal microbiome of *A. lineatus* wireworms in the *B. bassiana* (Bb) inoculated treatment and the non-inoculated control treatment. The "other" category represents genera with less than 1% relative abundance.**

Table 4.1. **Mean relative abundances (%) of each phylum found within *A. lineatus* wireworms in the control (n = 14) and the *B. bassiana* (n = 15) treatment groups and the p-value between these two groups using two-sample T-tests (alpha = 0.05, N.s. = no significance).**

Phylum	Control	*B. bassiana*	p-value
Proteobacteria	46.03	31.80	N.s.
Actinobacteria	17.56	39.49	0.049
Firmicutes	15.33	17.91	N.s.
Tenericutes	11.75	4.47	N.s.
Cercozoa	3.95	5.03	N.s.

Bacteroidetes	4.49	0.45	N.s.

Table 4.2. **Mean relative abundances (%) of each bacterial genus with significant differences ($p \leq 0.05$, Two-sample T-test) between within *A. lineatus* wireworms in the control (n = 14) and *B. bassiana* (n = 15) treatment groups.**

Genus	Control	*B. bassiana*	p-value
Mycobacterium	1.00	11.62	0.0048
Skermania	2.03	7.38	0.02
Bacillus	0.01	0.37	0.04
Tissierella	0.02	0.18	0.04

Beauveria bassiana did not affect the bacterial diversity of the wireworm internal microbiome, as there were no differences in the amplicon sequence variants between the *B. bassiana* treatment group and the control group (Table 4.3). Analysis of the Shannon diversity indices also showed that the alpha diversity within *B. bassiana* and control groups is not statistically significant (Table 4.3). The beta diversity of the microbiome was not different due to *B. bassiana* inoculation either ($R = -0.007$, $p = 0.42$, Figure 4.4).

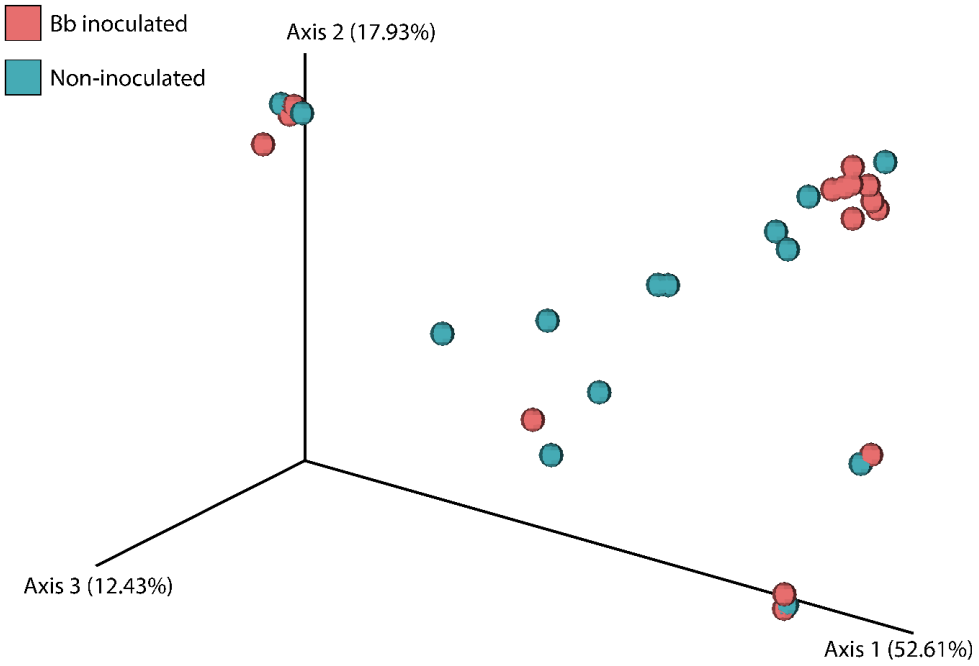

Figure 4.4. **Principal coordinate plot of weighted UniFrac data of the internal microbiome of the *B. bassiana* (Bb) inoculated group and the non-inoculated control group of *A. lineatus* wireworms. The primary vector explains 52.6% of the variation between the groups and the first 3 vectors together exhibit 82.9% of the variation among the groups.**

Table 4.3. **Alpha diversity of the internal microbiome of *A. lineatus* wireworms in the *B. bassiana*-inoculated (Bb) and the control groups (Ctl). Differences were compared using Kruskal-Wallis pairwise comparisons.**

		H	p-value	q-value
Observed features	Bb (n = 15) vs Ctl (n = 14)	0.03	0.86	0.86
Shannon diversity	Bb (n = 15) vs Ctl (n = 14)	0.43	0.51	0.51

4.5. Discussion

In this study, I investigated whether *B. bassiana* can invade the bodies of *A. lineatus* wireworms and whether the inoculation of *B. bassiana* results in a change in the abundance or diversity of their internal microbiome. The results show that *B. bassiana* does not invade the body of wireworms but causes an increase in the abundance of

culturable bacteria. The microbial community of wireworms was altered slightly, but *B. bassiana* inoculation did not affect the alpha or beta diversity of the wireworm internal microbiome.

4.5.1. *Beauveria bassiana* caused increased bacterial load in wireworms but did not influence bacterial diversity

Although wireworm survival was not affected by *B. bassiana*, *B. bassiana* inoculation resulted in an increased abundance of culturable bacteria in the internal wireworm microbiome, but no differences in diversity. Opportunistic bacteria are members of the microbiome that normally do not harm their host but can cause disease when the host's resistance is lowered. Recent work has shown that infections by entomopathogenic fungi tend to result in a decrease in the diversity of microbiota and an increase in the bacterial loads, especially of opportunistic bacteria, contributing to insect deaths (Kryukov et al., 2021; Wu et al., 2020). In other insects, similar effects have been found: *B. bassiana* increased the abundance of microbiome bacteria in *Anopheles stephensi* mosquitoes (Wei et al., 2017), *Galleria mellonella* moths (Polenogova et al., 2019), and *Dendroctonus valens* beetles (Xu et al., 2019). Furthermore, the community structure was altered, and there was reduced bacterial diversity in the microbiomes of *D. valens* beetles (Xu et al., 2019), *Musca domestica* house flies (Zhang et al., 2021), and *Monochamus alternatus* pine sawyer beetles (Deng et al., 2022) after *B. bassiana* inoculation. Potentially opportunistic gut bacterial genera that increased in abundance after *B. bassiana* inoculation are: *Serratia* (Deng et al., 2022; Polenogova et al., 2019; Wei et al., 2017)*, Erwinia* (Xu et al., 2019)*, Pantoea, Cyanobacteria* (Zhang et al., 2021)*,* and *Pseudomonas* (Deng et al., 2022)*.* In our experiment, we detected low levels of *Serratia* in the wireworm microbiome, and after *B. bassiana* inoculation, the relative abundance increased (non-significantly) by 86%. *Pseudomonas* levels decreased by 94% in relative abundance in *B. bassiana*-inoculated wireworms. *Bacillus* bacteria have been found to be associated with disease in wireworms (Danismazoglu et al., 2012; Lacey et al., 2007), and there was an increased relative abundance by 97% in *B. bassiana*-inoculated wireworms. Other wireworm bacterial genera found in this study, such as *Brucella, Peptoclostridium*, *Mycobacterium, Dietzia, and Tissierella,* include members that have been observed as opportunistic bacteria in humans (Aujoulat et al., 2012; Caméléna et al., 2016; Koerner et al., 2009; Mehta & Shamoo, 2020), but little is known about their roles in insect pathology. In *Anopheles stephensi* mosquitoes, *B.*

bassiana caused the downregulation of midgut immune responses due to the production of the metabolite oosporein. This caused the translocation of *Serratia marcescens,* an opportunistic gut bacterium in mosquitoes, into the hemocoel, where they became pathogenic and accelerated insect death by 16% (Wei et al., 2017). Further studies of the wireworm microbiome could investigate the role of metabolites like oosporein in wireworm immune responses and the roles of possible opportunistic bacteria in wireworms.

4.5.2. Members of the microbiome may increase resistance against fungal disease

The wireworm microbiome may contribute to the resistance of wireworms to infection by *B. bassiana*. The insect microbiome aids in insect resistance against fungal disease by inhibiting fungal growth (Deng et al., 2022), and several bacterial genera with anti-fungal effects have been found in the wireworms in this study: *Pantoea, Bacillus, Pseudomonas*, and *Acinetobacter* (Table B.1). *Pantoea*, previously detected in the microbiomes of oriental fruit moth (*Grapholita molesta),* Colorado potato beetle (*Leptinotarsa decemlineata*), and *Agriotes* wireworms, have inhibitory effects against fungal entomopathogens (Blackburn et al., 2008; Kabaluk et al., 2017; Wang et al., 2022). *Bacillus*, detected in various hemipterans and *Blattella germanica* cockroaches, as well as *Pseudomonas*, isolated from Colorado potato beetle (*Leptinotarsa decemlineata*) and *Blattella germanica* cockroaches, have inhibitory effects against *B. bassiana* (Blackburn et al., 2008; Toledo et al., 2011; Zhang et al., 2018). *Acinetobacter* had inhibitory effects against *B. bassiana* in *Blattella germanica* (Zhang et al., 2018). Other genera found within the wireworms in this study that have been associated with insect disease resistance are *Mycobacterium*, *Pandorea, Nocardia*, *Serratia*, *Sphingobacterium,* and *Stenotrophomonas* (Kabaluk et al., 2017; Moraes et al., 2014; Zhou et al., 2019). *Mycobacterium* levels in this study significantly increased due to *B. bassiana* treatment, and wireworms were resistant to infection, suggesting that this bacterium may play a role in insect-pathogen interactions. This supports Kabaluk et al., (2017), who found an association between *Mycobacterium* and levels of *Metarhizium brunneum* in *Agriotes* wireworms. Further research should be done to understand the roles of these bacteria on the resistance of wireworms to *B. bassiana*. Several bacterial genera including *Pseudomonas, Bacillus*, and *Serratia* may include species that are opportunistic pathogens, but also species that are associated with disease resistance. In

the future, the accurate identification of bacteria to the species level will aid in research to understand the roles of microbiome members in insect disease.

4.5.3. *Beauveria bassiana* does not invade wireworms

Beauveria bassiana was not detected inside the wireworms, so it is unclear how it influenced the wireworm internal microbiome. A possible mechanism is through the production of secondary metabolites with antimicrobial properties (Patocka, 2016). Fungal conidia may have attached to the wireworm cuticle or passed through its gut, triggering immune responses that then had secondary effects on its microbiome. In *Aedes aegypti* mosquitoes and *Lymantria dispar* moths, the increase in gut bacterial load due to *B. bassiana* infection is attributed in part to reduced gut reactive oxygen species (ROS) activity (Bai et al., 2020; Ramirez et al., 2018). This reduction of ROS was also observed in Colorado potato beetle (*Leptinotarsa decemlineata*) after *Metarhizium robertsii*, but there were no effects on the gene expression of immune pathways (Kryukov et al., 2021). Further research, possibly to investigate ROS, is needed to understand how *B. bassiana* influenced the wireworm microbiome without infection.

4.5.4. Wireworm microbiome composition

The microbiome of *A. lineatus* wireworms found on the west coast of British Columbia was dominated by Proteobacteria (46%), Actinobacteria (18%), Firmicutes (15%) and Tenericutes (12%), with low levels of Cercozoa (4%) and Bacteroidetes (5%). Similar compositions have been observed in other insects: Yun et al. (2014) investigated the gut microbiomes of 218 species of insects in 21 orders using 16S rRNA sequencing and found that the insect gut microbiota was dominated by Proteobacteria (62% of the classified sequences) and Firmicutes (21%), followed by Bacteroidetes (6%), Actinobacteria (5%), Tenericutes (2%), and unclassified bacteria (3%). Jang and Kikuchi (2020) examined studies, including Yun et al. (2014), involving a diverse set of insect orders and found that the predominant phyla present in the guts of insects are Proteobacteria (66%) and Firmicutes (7%), Bacteroidetes (10%), Actinobacteria (11%), others (7%). These authors found that the composition and diversity of the insect microbiome are dependent upon various factors including life stage, environmental habitat, and diet. For example, the larval gut microbiota is more diverse than those of

adults, and those of omnivores are more diverse compared to herbivores or carnivores (Yun et al., 2014). There are differences in the proportions of bacterial phyla in insects of different feeding styles: for example, leaf-feeders are dominated by proteobacteria, but carnivores have a mix of proteobacteria, firmicutes, and actinobacteria (Jang & Kikuchi, 2020). From the findings of these previous studies, wireworms have a microbiome characteristic of wood-feeding and omnivorous insects.

4.5.5. Conclusion

In conclusion, the inoculation of *B. bassiana* on wireworms caused dysbiosis of the internal microbiome, namely, an increased bacterial load, and shifts in the relative abundances of several members. However, no invasion of the wireworm body by *B. bassiana* was observed. The role of the insect microbiome in insect disease is not yet well understood and has many implications for pest management opportunities. Some bacterial symbionts are important for the resistance of insects towards entomopathogens, but others are opportunistic members of the microbiota that progresses insect mortality after pathogen infection. Wireworms were found to be resistant to *B. bassiana* infection, and further research should be conducted to understand the role of microbiome members, especially those identified to shift in abundance, in fungal resistance. The understanding of fungal-insect-microbiome interactions is important in the optimization of and development of new biological control strategies.

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Chapter 5.

Concluding summary

Earlier studies found that potato plants were protected from wireworm feeding damage in the field by *B. bassiana* application, but wireworm mortality was not observed (BASF, internal data). This thesis aimed to uncover how *B. bassiana* protects potato plants from wireworm herbivory. I examined the effects of *B. bassiana* inoculation on wireworm survival, avoidance, and movement and feeding behaviours in Chapter 2 in a series of laboratory bioassays. In Chapter 3, I examined whether the seed tuber inoculation of potato plants protected against wireworm herbivory in the greenhouse. In Chapter 4, I examined the effects of *B. bassiana* inoculation on the wireworm internal microbiome.

Beauveria bassiana inoculation did not result in significant mortality of *A. lineatus, A. sputator*, *or H. glaucus* wireworms over 6 months. Therefore, unlike in other crop systems protected by *B. bassiana,* causing mortality is not likely a mechanism of action for crop protection against wireworms. Further research may investigate how wireworms are resistant to *B. bassiana* infection such as examining the properties of the cuticle, and wireworm immune responses.

Crop protection need not only be through causing mortality - behavioural changes, such as anti-feeding behaviours and repellency can reduce plant damage (Muvea et al., 2015; Pelizza et al., 2017). For example, crops like corn are more likely to survive wireworm herbivory if they are protected during their most vulnerable early stages (Waliwitiya et al., 2005). I observed small effects of *B. bassiana* on wireworm avoidance and feeding time reduction. However, it did not translate into reduced wireworm herbivory in potato tubers, where tolerance for feeding damage is low for commercial markets. It is possible that the combination of small effects of avoidance and reduced feeding time may translate into reduced feeding damage in field settings, and this warrants further investigation.

Wireworms avoided potato tuber tissue from plants inoculated with *B. bassiana* and there was an absence of the fungus in the tuber tissue. This indicates that *B. bassiana* induced a detectable change, possibly through the production of secondary

metabolites, in the potato plants. Although this avoidance may reduce wireworm herbivory on the tubers, this may be problematic if these metabolites are present in the tubers when harvested for human consumption. Further work is needed to detect and identify the causal agent for wireworm avoidance in potato tissue from *B. bassiana* inoculated plants.

Endophytic entomopathogenic fungi can induce plant defences and protect plants against both pathogens and insect herbivores (Vega, 2018). This was not observed in greenhouse experiments using a seed tuber treatment of *B. bassiana* on potato plants*. Beauveria bassiana* was detected inside of plant tissue, but the inoculation of plants had no effects on plant performance, levels of plant defence hormones, and they did not prevent wireworm feeding holes in tubers. Plant inoculation methods can result in differences in colonization rates (Russo et al., 2015) and this may impact their success in plant protection. Future studies may test different inoculation methods of *B. bassiana* on potatoes*,* as well as the use of other fungal strains. Experiments conducted in field conditions may also show different results due to differences in biotic and abiotic factors.

The wireworm species tested are resistant to *B. bassiana* infection, and no fungus was detected inside the body of wireworms, indicating that the insect cuticle was impenetrable to *B. bassiana*. However, fungal inoculation was somehow able to influence wireworm microbiomes. Perhaps, the wireworm immune system was alerted when the conidia contacted the wireworm cuticle or if it passed through the gut, and dysbiosis was a side effect of an immune response. Dysbiosis affects insect health, especially regarding the development of disease (Hamdi et al., 2011), but it is unknown whether it can influence insect behaviour. It is possible that the reduced feeding time observed in wireworms may be attributed to dysbiosis. Additionally, several members of the microbiome shifted in abundance after *B. bassiana* inoculation, but it is unknown if these members contribute to the resistance of wireworms to fungal infection. Further research is needed to understand the effects of fungal entomopathogens on insect microbiomes, and how this may translate to crop protection.

In this thesis, I cast a wide net in the hope of determining the mechanism(s) behind the reduction in wireworm feeding damage by *B. bassiana*. *Beauveria bassiana* affected insect behaviour and this, along with other possible factors, may contribute to

crop protection. Although the exact answer is still elusive, this work narrows down some possibilities, opens avenues for further research, and provides insight into the complexity of insect-plant-microbe interactions.

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Appendix A.

Supplementary tables for Chapter 3

Table A.1. **Summary statistics of metrics measured in greenhouse experiments examining the effects of *B. bassiana* seed tuber inoculation of potatoes and wireworm herbivory. Height is measured in centimetres and weights are measured in grams.**

Experimental replicate 1	n	mean	sd	Experimental replicate 2	n	mean	sd
***B. bassiana* (-) wireworm (-)**				***B. bassiana* (-) wireworm (-)**			
height	15	8.5	5.5	**height**	15	8.2	3.8
leaves	15	5.9	1.7	**leaves**	15	5.8	2.1
wet weight	15	1211.1	175.6	**wet weight**	15	309.2	150.0
dry weight	15	102.5	13.6	**dry weight**	15	29.9	13.7
feeding hole count	15	0.1	0.4	**feeding hole count**	14	0.7	2.7
potato weight	15	1301.0	197.6	**potato weight**	14	484.0	91.7
potato count	15	12.3	3.5	**potato count**	14	5.2	2.1
***B. bassiana* (+) wireworm (-)**				***B. bassiana* (+) wireworm (-)**			
height	15	8.5	4.7	**height**	15	8.8	2.9
leaves	15	5.6	1.5	**leaves**	15	6.7	1.4
wet weight	15	1201.7	278.2	**wet weight**	15	318.0	245.1
dry weight	15	101.5	15.2	**dry weight**	15	32.9	25.5
feeding hole count	15	0.5	1.4	**feeding hole count**	15	0.0	0.0
potato weight	15	1177.8	222.6	**potato weight**	15	520.6	137.8
potato count	15	10.9	2.4	**potato count**	15	6.1	3.3
***B. bassiana* (-) wireworm (+)**				***B. bassiana* (-) wireworm (+)**			
height	15	8.3	4.4	**height**	15	7.6	2.6
leaves	15	5.3	1.5	**leaves**	15	5.9	1.4
wet weight	15	1128.7	129.7	**wet weight**	15	303.0	116.4
dry weight	15	100.7	13.0	**dry weight**	15	30.9	11.4
feeding hole count	15	7.1	3.5	**feeding hole count**	15	4.3	4.6
potato weight	15	1138.5	350.7	**potato weight**	15	500.3	88.0
potato count	15	12.4	2.4	**potato count**	15	4.9	2.2
***B. bassiana* (+) wireworm (+)**				***B. bassiana* (+) wireworm (+)**			
height	15	7.0	3.2	**height**	15	8.6	2.9
leaves	15	5.6	0.9	**leaves**	15	6.4	1.2
wet weight	15	1198.2	136.0	**wet weight**	15	359.3	197.8
dry weight	15	101.3	10.4	**dry weight**	15	36.0	19.0

feeding hole count	15	6.1	4.9	**feeding hole count**	15	5.2	3.7
potato weight	15	1202.6	259.5	**potato weight**	15	507.5	84.5
potato count	15	16.5	8.5	**potato count**	15	5.2	2.0

Table A.2. **Plant hormone levels of jasmonic acid (JA) and salicylic acid (SA) in potato plants that were inoculated with *B. bassiana* [Bb (+)] and the non-inoculated control [Bb (-)], with wireworms added [Ww (+)] and without [Ww (-)].**

		ng/g tissue	sd
JA			
Bb (+)	Ww (-)	6.0	3.6
Bb (+)	Ww (+)	7.0	2.0
Bb (-)	Ww (-)	5.3	1.2
Bb (-)	Ww (+)	6.0	1.7
SA			
Bb (+)	Ww (-)	65.7	4.9
Bb (+)	Ww (+)	72.7	1.2
Bb (-)	Ww (-)	75.7	15.9
Bb (-)	Ww (+)	64.3	15.9
Conjugated SA			
Bb (+)	Ww (-)	17459	9309
Bb (+)	Ww (+)	10336	1060
Bb (-)	Ww (-)	12938	4267
Bb (-)	Ww (+)	11104	2528

Appendix B.

Supplementary table for Chapter 4

Table B.1. Relative abundances (%) of genera (≥0.1%) found in the internal microbiomes of non-inoculated (Control) and *B. bassiana* – inoculated *A. lineatus* wireworms.

Genus	Control	*B. bassiana*
Acidobacterium	0.08	0.21
Acidovorax	0.23	0.35
Acinetobacter	9.08	2.75
Aeromicrobium	0.03	0.19
Arthrobacter	3.12	3.11
Bacillus	0.01	0.37
Bradyrhizobium	0.30	0.17
Brucella	20.60	18.57
Chitinophaga	2.93	0.04
Chryseobacterium	0.28	0.18
Dietzia	1.11	10.13
Gordonia	1.50	0.01
Knoellia	0.35	0.12
Labrys	0.23	0.04
Luteolibacter	0.27	0.00
Lysobacter	0.23	0.49
Mycobacterium	1.00	11.62
Nocardia	4.38	4.50
Nocardioides	0.13	0.11
Nothotsuga	0.00	0.22
Ochrobactrum	0.19	0.54
Pantoea	0.19	0.02
Paracercomonas	3.95	5.03
Pelomonas	0.12	0.57
Peptoclostridium	14.80	16.00
Phyllobacterium	0.21	0.08
Planifilum	0.03	0.18
Pseudomonas	9.53	0.60
Ralstonia	0.23	1.61
Rhodococcus	0.19	0.17
Serratia	0.53	3.65
Shinella	0.21	0.04
Skermania	2.03	7.38
Sphingobacterium	0.48	0.09

Sphingomonas	0.01	0.28
Spiroplasma	11.75	4.47
Stenotrophomonas	1.45	0.17
Streptomyces	2.50	0.13
Taibaiella	0.31	0.02
Thermoactinomyces	0.04	0.25
Tissierella	0.02	0.18
Tsukamurella	0.65	1.33
Variovorax	0.61	0.79
Others (<1%)	4.11	3.27