The evaluation of *Beauveria* isolates for virulence to diamondback moth (*Plutella xylostella* L.)

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Horticultural Science

at

Lincoln University

by

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Lincoln University

2021

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Beauveria is a genus of entomopathogenic fungi that exhibits traits suitable for the development of commercial biopesticide products to control various arthropod pests, as well as plant pathogens. The literature describes thousands of strains of *Beauveria*, largely limited to strains of *Beauveria bassiana* that have been isolated and screened for their ability to control pests around the world. Some of these pests, such as diamondback moth (DBM), the two-spotted spider mite, green peach aphid and housefly, are highly resistant to chemical insecticides. This study was conducted to find at least one New Zealand derived *Beauveria* isolate able to control DBM. The first question of this study was, "do other *Beauveria* species have the potential to control DBM"? Thus, 14 isolates from four *Beauveria* species were assessed for their control of DBM under *in vitro* conditions. Before the assessment, all 14 isolates were taxonomically identified using both morphological and DNA-based methods. The *in vitro* bioassays identified three isolates of *B. bassiana* (Mo1, CTL20, and CTA20) and *B. pseudobassiana* (FRhp, FW Mana, and I12 Damo) that successfully killed 50% of the DBM larvae (median lethal dose LD_{50}) at a low dosage while isolates from two other species could not achieve LD_{50} at a higher rate. This is the first report that details *B. pseudobassiana* as a potential species for DBM control.

Combining two or more fungal isolates may improve the efficacy of a biological control agent. The second question examined was, "do combinations of *Beauveria* isolates result in a synergistic or an antagonistic interaction"? *Beauveria* isolates were grouped according to their previously assessed virulence towards DBM. Under *in vitro* conditions, combining the low virulent isolates together resulted in a higher and faster DBM mortality rate than when these isolates were applied separately. Combining the three species of *Beauveria* together resulted in antagonistic interactions between the fungi, with lower mortality rates than when these isolates were applied separately.

Diet can sometimes affect the susceptibility of an insect to entomopathogenic fungi. The third question of this study was "is there any difference in the susceptibility of DBM larvae to fungal infection by selected *Beauveria* isolates when raised on different brassicas"? DBM larvae were fed on four

different brassicas with predicted high (broccoli and cabbage) and low (cauliflower and radish) glucosinolate levels. Glucosinolates have been implicated in the resistance of brassica hosts to plant pathogens. *In vitro* results showed that DBM larvae fed on broccoli and cabbage were more susceptible to infection by *B. pseudobassiana* I12 Damo than larvae raised on cauliflower and radish. Conversely, DBM larvae raised on cauliflower and radish were more susceptible to infection by *B. bassiana* CTL20 than larvae fed on broccoli and cabbage.

Secondary metabolites produced by *Beauveria* spp. have previously been found to have insecticidal properties and can kill a wide range of insects, including DBM. The fourth question investigated was, "was there evidence that an isolate of *Beauveria* spp. produces toxins that kill DBM larvae directly"? *B. pseudobassiana* FW Mana was selected as it caused high DBM mortality in previous bioassays but exhibited less sporulation from DBM cadavers compared to the other isolates within the study. The filtered supernatant of *B. pseudobassiana* FW Mana was used for assays along with toxins from two other less-lethal isolates of *Beauveria*. As expected, the supernatant from *B. pseudobassiana* FW Mana caused significantly higher mortality than the supernatant from the other two isolates assessed. Overall, this study found several *Beauveria* isolates that have the potential to be developed and commercialised as biopesticides to control DBM.

Keywords: biopesticides, brassicas, combined isolates, DBM, glucosinolates, median lethal rate (LD_{50}), median lethal time (LT_{50}), metabolites, screening, virulence

Acknowledgements

Firstly, I would like to express my sincere gratitude to my main supervisor, Professor Travis Glare, for his incredible support, guidance, and motivation. I am thankful to my associate supervisors, Professor John Hampton and Dr Stuart Card, for their constructive feedback to make my writing more concise. Special thanks to my advisor Jenny Brookes for the lab guidance and her availability whenever needed. These amazing people are my academic guardians.

Hearty thanks to the New Zealand government through the Ministry of Foreign Affairs and Trade (MFAT) for the financial support. Without this support, I would not be here at all. Special thanks to Sue Bowie, Jayne Borrill, and Mandy Buller for taking care of me throughout my time in New Zealand.

I would like to thank Dave Saville for the statistical advice. My appreciation to Josefina Narciso, Norma Merrick, Artemio Mendoza, and Hossein Alizadeh for their guidance during my molecular experiments.

My thanks to all staff at the BPRC, Andrew, Sandy, Brian, Claire, Wadia, Michelle, Ashley, and Nicola, for their help and support. Thanks to my officemate Tauseef for the academic discussion and for sharing some exciting papers. Thank you to Alvand, Francisco, Karla, Corzzie, Nghia, and Sunita for many fun times and talks.

I would like to thank my flatmates Uykim and Yuthnea for being like my brothers in New Zealand. A big thank you to Bong Samnang, Bong Samneang, Phee Aon, Phee Lek, Bong Verak, and Bong Heang who treated me as a younger brother and cooked many delicious meals. Thank you to uncle Kimsay for his kindness and advice. My thanks to the Cambodian community in Christchurch for treating me as a relative.

Sincere thanks to my parents, siblings, and all relatives in Cambodia for their encouragement and support from a distance. Last but special, thanks to my beloved girlfriend, Sokunthea Nhien, for her love and patience.

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Chapter 1

General Introduction

1.1 Brassica crops

Vegetables are a vital source of nutrients for billions of people around the world. They are a significant commodity that can enhance small-scale growers' benefits and fulfil nutritional values for people's daily livelihoods in most developing countries of Africa and Asia (Srinivasan et al., 2019). Brassica, a genus of the family Brassicaceae, has 37 economically significant species, including three diploids: *Brassica nigra* (black mustard), *B. oleracea* (cabbage), *B. rapa* (turnip) and three amphidiploids: *B. carinata* (abyssinian cabbage), *B. juncea* (mustard), and *B. napus* (rapeseed). These species are utilised for the production of oilseed, as forage, condiments, and eaten as vegetables (Cartea et al., 2011b). In 2017, vegetable brassicas were produced by more than 150 countries, occupying over 5.5 million hectares worldwide with an annual production of approximately 140 million tonnes. Asia accounts for more than 70% of the world's brassica vegetable production, while China is the world's biggest producer, with more than 30 million tonnes (FAOSTAT, 2017). A decade ago, brassica vegetables were valued globally at more than USD 26 billion, with production increasing every year (Furlong et al., 2013). The market value reached a peak at USD 43.7 billion in 2016, with an annual growth rate of 3.4%. A production decline was experienced in 2018 to USD 39.4 billion, but the market is still expected to rise at 1.4% annually within a seven-year period from 2019-2025 (Research&Markets, 2021). In New Zealand, around 105,000 tonnes of broccoli, cauliflower, and cabbage with a gross value of approximately USD 57 million were produced in 2018. Almost all products from these crops were for the domestic market (FreshFacts, 2018).

Brassica crops have been recognised widely for their human health benefits as they contain many essential nutrients and phytochemicals that may offer protection against certain diseases, especially cancers (Francisco et al., 2017) and heart diseases (Cartea et al., 2011a). Health-enhancing substances include vitamins, carotenoids, flavonoids, polyphenols, minerals, and their distinctive compounds, widely known as glucosinolates (Cartea et al., 2011b). Glucosinolates and their products have been shown to exhibit anticancer, anti-bacterial, anti-fungal, anti-oxidative, and allelopathic properties (Bhandari et al., 2015; Faller & Fialho, 2009; Verhoeven et al., 1996).

Abiotic and biotic factors play significant roles in reducing brassica yields worldwide. Abiotic factors such as salinity, extreme temperatures, drought, and flooding can cause up to a 50% reduction in brassica crop yields in many countries (Kayum et al., 2016). Generally, the optimal temperature for most brassica crops ranges from 15^{o}C to 25^{o}C, while unfavourable temperatures are above 35^{o}C and below 10^{o}C (Angadi et al., 2000). Biotic stresses caused by pathogenic microbes, arthropods, animals, and weeds can cause yield reductions of up to 40% (Oerke & Dehne, 2004). Plant pathogens and arthropod pests alone contribute to around 25% of crop losses worldwide (Savary et al., 2012). The

most important pathogens of *Brassica* include *Fusarium oxysporum* (causing Fusarium wilt), *Leptosphaeria maculans* (causal agent of stem canker), *Plasmodiophora brassicae* (the causer of club root), *Pectobacterium carotovorum* (causing soft rot), and *Xanthomonas campestris* pv. *campestris* (causal agent of black rot) (Kayum et al., 2016).

Invertebrate pests also contribute considerably to brassica crop destruction across the globe. The most devastating insect pests of cruciferous vegetables are *Plutella xylostella* (diamondback moth), *Helula undalis* (cabbage webworm), *Pieris brassicae* (cabbage moth), *Trichoplusia ni* (cabbage looper), *Myzus persicae* (green peach aphid), *Thrips tabaci* (thrips), *Phyllotreta striolata* (flea beetle), and *Brevycoryne brassicae* (cabbage aphid) (Mpumi et al., 2020). These can cause yield losses of up to 100% if there are no controlling actions (Srinivasan et al., 2019).

1.2 Diamondback moth

1.2.1 Insect taxonomy and ecology

Order: Lepidoptera

Family: Plutellidae

Subfamily: Plutellinae

Genus: *Plutella*

Species: *Plutella xylostella* (L.)

Common name: Diamondback moth (CABI, 2020)

Diamondback moth (DBM) is an extraordinarily destructive and predominant pest species on brassica crops worldwide (Li et al., 2019; Li et al., 2012; Srinivasan et al., 2019). It is a highly invasive pest species and is universally distributed (Talekar & Shelton, 1993). The origin of the moth may be from Europe, as brassica crops have been planted in this continent for a longer time than other continents (Hardy, 1938). DBM has become widespread globally due to its strong ability to migrate and be wind-dispersed over long distances. Some publications have shown that DBM is capable of moving up to 3,000 km in several days from low to high altitudes; however, there is a lack of substantial evidence to prove how the pest can survive during this migration (Liu et al., 2007; Niu et al., 2014; Talekar & Shelton, 1993). DBM is a wind-born migrated insect and, therefore, the moth can move by using wind-aided power to fly without using much energy (Chapman, 2002; Dosdall et al., 2001). Temperature is a significant factor influencing the development of DBM populations. The optimal temperature for DBM ranges between 17–25^{o}C (Niu et al., 2014; Talekar et al., 1986). However, it can survive under extreme weather at a low temperature of 4^{o}C (Chen, 2004) and high temperatures of up to 49^{o}C (Chang, 2012). At optimal temperatures, 19 generations per year can be produced compared to as low as three under sub-optimal conditions (Harcourt, 2012; Perry, 2019).

1.2.2 Insect life cycle

As its life cycle depends heavily on temperature, the generation time can be varied accordingly. A generation typically takes approximately three weeks to complete at a constant temperature of 23°C (CABI, 2020). If the temperature is not optimal for the insect, the development can be as long as six weeks.

- **Egg**

The egg is laid within two hours of mating and deposited randomly on the underside of a plant leaf. Initially, eggs are small (0.44 × 0.26 mm) and slightly yellow, then they turn brown before hatching within 4–8 days (Harcourt, 2012). Eggs are laid separately or in small clusters of 2 to 8 eggs per group. On average, a mated adult female can oviposit up to 200 eggs in a lifetime (Capinera, 2001).

- **Larva**

There are four larval stages. Generally, the larvae fully develop in 15–21 days (Perry, 2019). As with all the other stages, larval growth depends heavily on temperature. At 17.5–32.5^{o}C, all larval stages are completed within 7.8–19.5 days (CABI, 2020).

- **Pupa**

The fourth instar larvae produce loose cocoons for pupation underneath plant leaves, but pupae can also be found inside the cauliflower and broccoli curd. The pupa is yellow at first, then turns brown, is 7–9 mm in length and has a pupation period of 5–15 days (Capinera, 2001). This duration is shorter in some tropical countries (CABI, 2020).

- **Adult**

The adult moth is greyish brown with white zigzags in the shape of a diamond on its back, hence its common name. The moths are tiny compared to other brassica moths and slender with pronounced antennae. These adults can usually reproduce within two hours after hatching from the cocoon (CABI, 2020; Capinera, 2001). The oviposition and flight occur around midnight, with adults feeding mainly on nectar. Females can live up to 16 days, laying eggs for around ten days, while males survive only around 12 days after emergence. Adult moths can usually sustain short flights but can migrate long distances by wind-aided movement (Capinera, 2001). Figure 1.1 shows the life cycle of DBM and the time period for each stage.

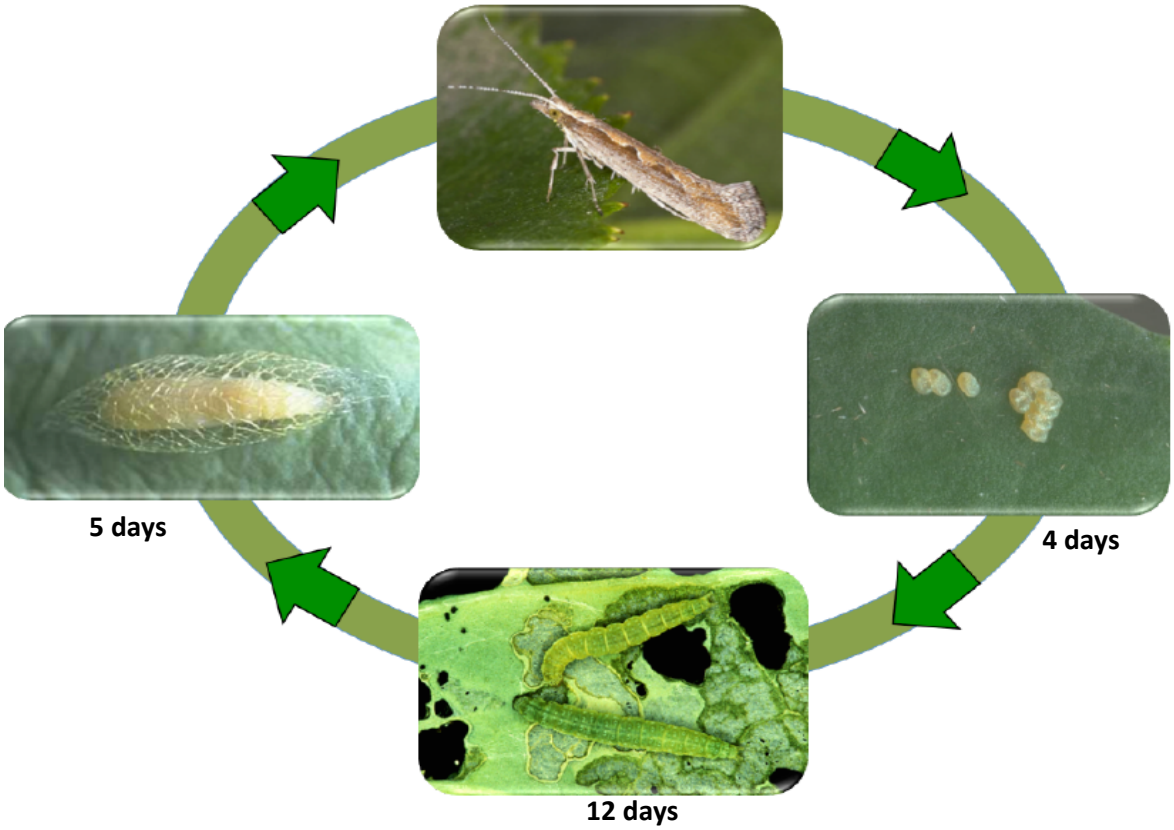


Figure 1.1: Life cycle of the diamondback moth, *Plutella xylostella* (L.). At a temperature of 23°C, the complete life cycle takes around 21 days (CABI, 2020). Picture sourced from Harvey-Samuel et al. (2015)

1.2.3 Host plants

DBM is an herbivorous insect that feeds on leaves of all brassica and glucosinolate containing plants in the Brassicaceae family (CABI, 2020; Dosdall et al., 2001; Furlong et al., 2008; Talekar & Shelton, 1993). A study on host preference of wild crucifers revealed that charlock mustard was preferred to treacle mustard and shepherd's purse for egg depositing and survival rate (Sarfraz et al., 2011). A review on potential trap crops (those plants that can attract agricultural pests away from nearby crops) for DBM showed that adult moths laid 18 times more eggs on *B. juncea* (brown mustard), 12 times more on *Barbarea vulgaris* var. *arcuate* (yellow rocket) and three times more on *Capsella bursa-pastoris* (shepherd's purse) than on *B. oleracea* var. *capitata* (cabbage) (Badenes-Perez, 2004). Similarly, Badenes-Perez (2006) reported that DBM laid 28% more eggs on yellow rocket than on cabbage and on young leaves rather than old leaves on the same crop. Surprisingly, in Kenya, a strain DBM-P also infests pea plants, particularly sugar pea, *Pisum sativum* var. *macrocarpon,* when there is no cabbage (Henniges-Janssen et al., 2011).

1.2.4 The economic significance of DBM

DBM contributes significantly to economic losses across the globe. These total billions of dollars due to the direct damage and control expenses (Table 1.1). In South Africa, crop losses due to DBM range from 31% to 100% if no control actions are taken (Machekano et al., 2017). This species has also severely attacked cruciferous crops in some countries in Southeast Asia, such as Thailand and Vietnam (CABI, 2020).

Table 1.1: Estimation of financial loss across the globe and some leading producers of brassicas caused by DBM

Country	Year	Loss	Reference
Worldwide	1993	USD 1 billion	(Talekar & Shelton, 1993)
Worldwide	From 2012-2019	USD 4-5 billion	(Gurr et al., 2018; Li et al., 2019; Srinivasan et al., 2017; Zalucki et al., 2012; Zhu et al., 2018)
China	From 2012-2019	USD 770 million	(Li et al., 2019; Li et al., 2012; Zalucki et al., 2012)
India	2011	USD 168 million	(Uthamasamy, 2011)
USA	2001	USD 100 million	(Shelton, 2001)
Australia	NA	AUD 26 million	(CABI, 2020)
Canada	2001	CD 52 million	(Dosdall et al., 2001)

1.2.5 Resistance to insecticides

DBM is a destructive pest on cruciferous vegetables in leading producer countries, including China, the USA, Canada, Mexico, Australia, New Zealand, Russia, Brazil, almost all countries in Europe, and many in Southeast Asia (Shelton, 2001). DBM has developed resistance to many synthetic insecticides, including organophosphates, carbamates, pyrethroids, indoxacarb, avermectins, cyclodiene organochlorines, phenylpyrazoles, spinosyns, nereistoxin analogues, neonicotinoids, chlorfenapyr, benzolureas, diacylhydrazines, azadirachtin, anthranilic diamides (Furlong et al., 2013; Li et al., 2016; Li et al., 2019; Sarfraz & Keddie, 2005), and lambda-cyhalothrin (Walker, 2012). Additionally, DBM has shown resistance to several biopesticides based on *Bacillus thuringiensis* (Bt). It was placed second in 2016 by Zhu et al. (2016) in terms of resistance development, in which two-spotted spider mite was the first. Table 1.2 shows the top 20 highly resistant arthropods in 2021 with the number of compounds that they have developed resistance to and the number of locations where this resistance was recorded.

Table 1.2: List of 20 most highly resistant arthropods in 2021 (Mota-Sanchez & Wise, 2021) ranked from high to low according to the number of compounds to which they are resistant

Rank	Scientific name	Common name	Compounds	Locations
1	*Plutella xylostella*	Diamondback moth	101	558
2	*Tetranychus urticae*	Two-spotted spider mite	96	261
3	*Myzus persicae*	Green peach aphid	81	245
4	*Musca domestica*	Housefly	65	248
4	*Bemisia tabaci*	Whitefly	65	430
6	*Leptinotarsa decemLineata*	Colorado potato beetle	56	144
7	*Rhipicephalus microplus*	Southern cattle tick	50	405
7	*Aphis gossypii*	Cotton aphid	50	163
9	*Helicoverpa armigera*	Corn bollworm	48	421
9	*Panonychus ulmi*	European red mite	48	66
11	*Blattella germanica*	German cockroach	43	112
11	*Spodoptera exigua*	Beet armyworm	43	491
13	*Spodoptera litura*	Climbing cutworm	42	421
14	*Culex quinquefasciatus*	Southern house mosquito	41	146
15	*Aedes aegypti*	Yellow fever mosquito	37	450
16	*Culex pipiens pipiens*	House mosquito	36	79
17	*Heliothis virescens*	Tobacco budworm	35	20
18	*Phorodon humuli*	Hop aphid	34	14
19	*Tribolium castaneum*	Red flour beetle	33	79
20	*Spodoptera littoralis*	African cotton leafworm	31	6

Some DBM possess natural resistance to a single or several pesticides, so they can pass on their resistant genes to the next generation if they mate. As a result, these insects can become resistant to those insecticides if the same insecticide is sprayed over time (Fathipour & Mirhosseini, 2017). Lin et al. (2020) reported that several completed and conserved signalling pathways such as Toll, IMD, JNK, and JAK-STAT were found in the midgut of DBM. These pathways may activate the expression of antimicrobial peptide (AMP) genes involved in enhancing immune responses to fight against a microbial-based biopesticide. Metabolic resistance mechanisms coexisting with individuals' resistance lead to a wide range of active compounds resistance of DBM (Furlong et al., 2013). Xie et al. (2017) found that DBM inherits a well-known family of enzymes called carboxylesterases, which enhance its immune ability to reduce the toxicity of some synthetic insecticides. Additionally, the short life cycle of DBM is another critical factor contributing to the rapid development of resistance to newly developed insecticides (Hurst et al., 2019). For instance, the larva is the only feeding stage that can be

completed within a short time; they can survive and enter the pupal stage even though they have eaten some insecticide. Therefore, hatching adults from this generation already have some resistant genes, so when mating with other moths, they diversely pass on the gene to the next generation. As a result, DBM will become accumulatively resistant from part to fully resistant over a period of time.

The wide availability of suitable food sources, a high level of resistance to several synthetic insecticides, the lack of natural enemies, and adaptation to multiple climatic conditions have led to an abundance of DBM worldwide (Chapman, 2002; Perry, 2019; Sarfraz & Keddie, 2005; Talekar & Shelton, 1993). One of the most common factors contributing to the resistance of DBM to many synthetic insecticides is the aggressive use of chemicals for control (Hurst et al., 2019; Kumar et al., 2016). Applying synthetic pesticides without the awareness of their impacts on nontarget organisms can lead to the degradation of natural enemies. DBM becomes abundant, and resistance develops rapidly when there are no competitors (Talekar & Shelton, 1993). Using the same synthetic chemical compounds and sprays repeatedly leads DBM to quickly develop resistance (Furlong et al., 2013; Li et al., 2016). For instance, in South-East Asia's intensive cultivation of brassica plants, farmers sprayed at 3-5 day intervals for a total of 12–16 times per growing season (Hurst et al., 2019; Mazlan & Mumford, 2005; Soth, 2016). Some articles report that using the same insecticides for two to three years can cause the insect to develop resistance (Furlong et al., 2013; Sayyed & Wright, 2006; Wang & Wu, 2012). Providing abundant and permanent host plants can drive insects to develop resistance to insecticides (Dermauw et al., 2018) because having food all year-round, DBM can produce new generations consecutively without pausing the cycle, which can pass on resistant genes diversely. For instance, in some parts of Asia, farmers usually cultivate their cruciferous crops all year-round, which provides food for DBM in all seasons (Hurst et al., 2019). Some alternative measures must be taken to respond to the abundance of DBM in some regions of the world. The aim is to implement integrated pest management (IPM), particularly biological control, to reduce insecticide use, minimise the negative impact on the environment, and save a significant amount of money (Zalucki et al., 2012).

1.3 Biopesticides

1.3.1 Background

Biopesticides are an environmentally friendly approach that has been used to reduce the application of synthetic pesticides (Glare et al., 2012). Biopesticides (or biological pesticides) are types of pesticides produced from plants, microorganisms and/or their metabolites, as well as from other natural materials such as pheromones (Gupta & Dikshit, 2010; Sporleder & Lacey, 2013). They are also a potential tool utilised within integrated pest management approaches (Seiber et al., 2014; Sporleder & Lacey, 2013). Globally, the plant protection market was valued at USD 51.44 billion in 2017 and is projected to register a compounded annual growth rate of 3.1%, to reach USD 57.6 billion by 2023. Synthetic pesticides shared the largest proportion of this market, whereas biopesticides were

comparatively small (AbsoluteReports, 2018), comprising only 6% of the world pesticide market (Zaki et al., 2020). The demand for biopesticides has increased six-fold, from sales of USD0.68 billion in 2005 to USD 4 billion in 2016, with an annual growth rate of 12 to 14.1% (Glare et al., 2016). According to Research&Markets (2020), globally, the biopesticide market is forecasted to increase to USD 12.23 billion by 2025, with a compounded annual growth rate of 14.8% from 2017 to 2025. The registration cost for a new conventional synthetic pesticide has become expensive, and the discovery of new chemical substances has declined. According to McDougall (2016), the overall cost for R&D of a new synthetic product rose around 88% from USD 152 million in 1995 to USD 286 million in 2010-2014. For these reasons, large agrichemical companies have become involved in biopesticide development, largely through company acquisitions and licensing deals. For example, some commercial biopesticides have been developed by Bayer CropScience, BASF, Valent BioSciences, and Syngenta (Beer, 2012; Glare et al., 2016). The cost for the development of a new biopesticide is around USD 3-5 million and requires roughly three years for the prototype to be available on the market (Glare et al., 2012). In 2008, approximately 72 microbial active ingredients were registered by the USA-EPA. The registered microbial biopesticides included 35 bacterial-based products, 16 fungal-based products, six non-viable microbial pesticides, eight plant-incorporated protectants, one protozoan, and six viruses (Shukla, 2020). In New Zealand, some current biopesticide products are based on the bacterium *Serratia entomophila* (formulated as Invade and BioShield by Biostart), the fungus *Beauveria bassiana* (commercial name Beaugenic and Beaublast by Biotelliga), *Lecanicillium lecanii* (commercial name eNTokill and eNtoblast developed by Biotelliga), and fungal endophytes in the genus *Epichloë* (commercialised by Grasslanz Technology Ltd) (Glare & O'Callaghan, 2019). Potential candidates for future biopesticides in New Zealand are *Yersinia entomophaga* (Hurst et al., 2011), *Brevibacillus laterosporus* (van Zijll de Jong et al., 2016), and the nematode *Phasmarhabditis hermaphrodita* (Wilson et al., 2016). The registration processes for new biopesticides in New Zealand is described in Glare and O'Callaghan (2019).

1.3.2 What are biopesticides?

Biopesticides can be alive biocontrol agents or their bioactive ingredients that target specific organisms (Gross et al., 2018; Sporleder & Lacey, 2013). Whatever definition of biopesticides is used, they are based on compounds that occurred or are derived from nature, are low risk to nontarget organisms, applicable in organic farming, biodegradable, pose fewer risks to mammals, have low impacts on the farmer and consumer health, utilise green techniques, and have a widely accepted usage (Seiber et al., 2014). The US Environmental Protection Agency (EPA) has divided these products into three main categories: (1) microbial organisms (bacteria, fungi, virus, or protozoa) and entomopathogenic nematodes; (2) Plant-incorporated protectants (PIPs), plant substances produced

by genetically modified plants or; and (3) biochemical pesticides based on naturally occurring substances that control pests by non-toxic mechanisms, e.g. pheromones (Morán-Diez & Glare, 2016).

Among all potential microbes for integrating into microbial pesticides, entomopathogenic fungi (EPF) are the most promising candidates for controlling herbivorous pests. Many entomopathogenic fungi belong to two phyla: Ascomycetes and Zygomycetes (Goettel et al., 2010). EPF-based insecticides make up 27% of biopesticides sold worldwide (Mascarin & Jaronski, 2016; Mishra et al., 2016), an increase of 7.6% within five years. The characteristics of EPF, such as their pathogenicity to invertebrate pests without needing to be ingested, their broad-spectrum activity, which can result in killing pest complexes, their ability to form self-sustaining populations, their abundance and effective performance in various environmental conditions, and the ability to be mass-produced on cheap media, make them the most attractive agents for formulating as biopesticides (Chandler, 2017; Cherry et al., 2005; Litwin et al., 2020; Mascarin & Jaronski, 2016; Sala et al., 2020). Recently, the genus *Beauveria* has become the most exploited fungal genus for mycoinsecticides (Mascarin & Jaronski, 2016).

1.4 *Beauveria* spp.

1.4.1 Background

Many EPF species belong to the Ascomycetes, including the genera *Beauveria*, *Metarhizium*, *Isaria*, and *Lecanicillium* (Faria & Wraight, 2007). Among these EPF, the genus *Beauveria* (Balsamo) Vuillemin is recognised as the most important fungal genus for controlling a wide range of agricultural, veterinary, and forestry arthropod pests (Mascarin & Jaronski, 2016). *Beauveria* has a cosmopolitan distribution, a simple life cycle, and a broad host range of more than 700 susceptible invertebrate species (Rehner & Buckley, 2005). *B. bassiana* has been found as a potential candidate for the control of over 70 arthropod pests. One of them is the diamondback moth, the invertebrate that has become resistant to a wide range of insecticides (Batcho et al., 2018).

1.4.2 Fundamental aspects of *Beauveria* spp.

a. History

The history of the fungus is related to the sericulture industry around the world. As the silk industry was established primarily in China around 500 B.C in the Han dynasty, the Chinese ruler gave capital punishment to those who trafficked silkworm eggs and mulberry seeds outside of China as well as those who revealed silk-making methods to outsiders. However, it was introduced to the Persian Empire after Buddhist monks illegally transported the silkworm eggs. Then, the sericulture industry moved to western countries (Feltham & Mair, 2009). The biggest challenge for the industry was when caterpillars started dying of a muscardine disease, which occurred first in Italy (1805) and then in France (1841) (Cheong, 2015). The Italian entomologist, Agostino Bassi, in 1835, began

investigating the disease and identified that the disease was contagious and caused by a parasitic fungus, which turned infected caterpillars into white mummies (Lord, 2005). The study of Agostino Bassi saved the silkworm industry by recommending the use of disinfectants, separating feeding caterpillars to lower the spread of the disease and destroying infected caterpillars. He provided insight into the germ theory that microbes cause disease before the work of Louis Pasteur and Robert Koch. The species *bassiana* was named to honour Bassi. The identification work was done by both Agostino Bassi and Giuseppe Gabriel Balsamo-Crivelli (1800-1874), but the species was named *Beauveria bassiana* by a French physician, Jean-Paul Vuillemin (1861-1932), in honour of the French scientist Jean Beauverie (1874-1938) (Vega et al., 2009). Currently, the genus *Beauveria* is one of the most studied entomopathogenic fungal groups due to its pathogenicity to more than 700 species of arthropods, including class Acari and Insecta (Zimmermann, 2007). Globally, more than a thousand isolates of *B. bassiana* have been examined in different parts of the world for the potential control of pest insects (Rehner et al., 2011). Figure 1.2 shows the number of publications and citations about *Beauveria* from various aspects, including biological, industrial, and pharmaceutical importance over 20 years from 2000 to 2021 (Clarivate, 2021).

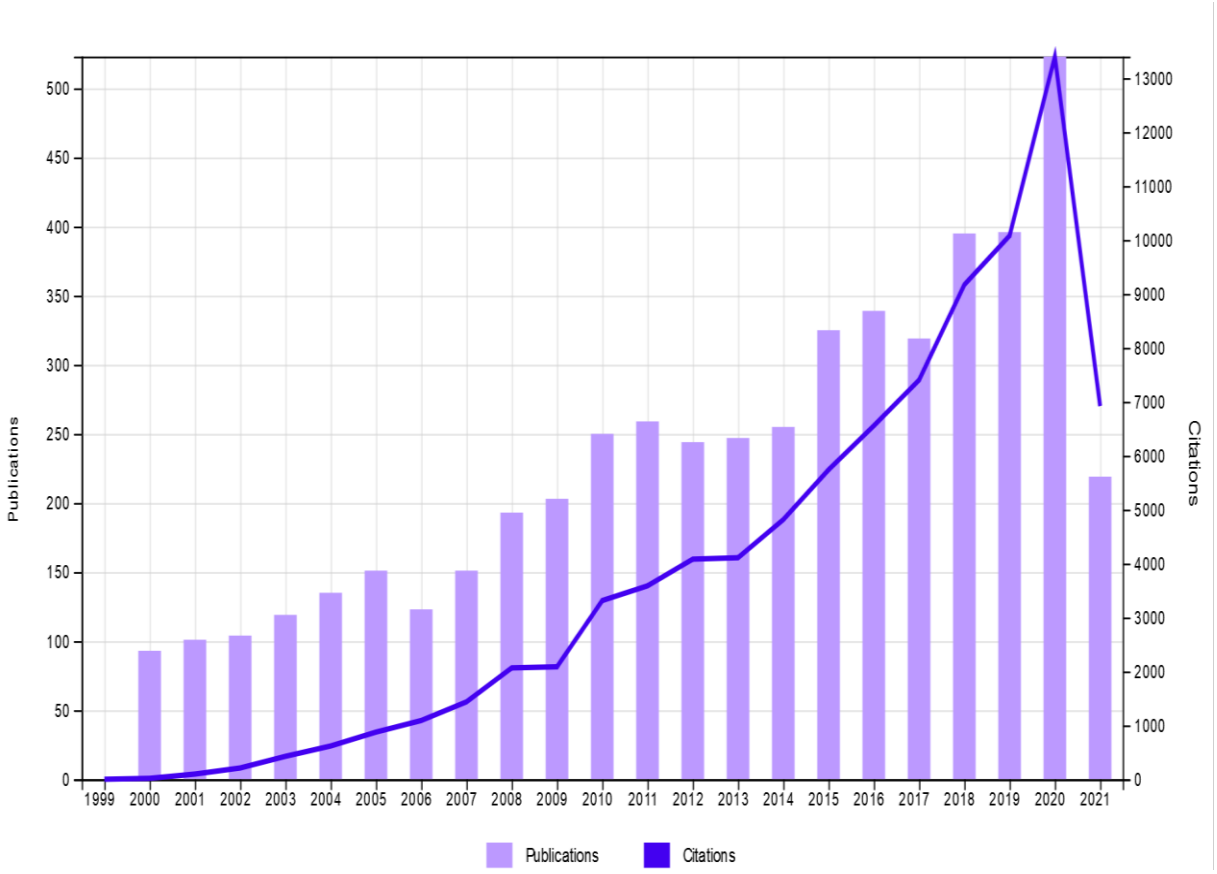


Figure 1.2: Number of publications (5,144 papers) and citations (32,646 citing articles) about *Beauveria* from 2000 to 2021 (Clarivate, 2021)

b. Genus taxonomy

The genus *Beauveria* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is one of the most abundant genera of soil-borne entomogenous moulds (Rehner & Buckley, 2005). According to the current phylogenetic sequence, *Beauveria* is a monophyletic genus within the family Cordycipitaceae (Hypocreales) and is closely phylogenetically related to *Cordyceps* species (Rehner et al., 2011). According to CABI (2019), the taxonomic classification of the genus *Beauveria* is:

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Cordycipitaceae

Genus: *Beauveria* (Balsamo) Vuillemin (1912)

c. Morphological characteristics

Beauveria species are white filamentous moulds that produce dry and white powdery conidia on many media and arthropod hosts. Conidia are often ball-shaped, produced on conidiogenous cells called the rachis. The conidia are single-celled, haploid, and hydrophobic, with diameters ranging from 2.4 to 3.6 µm for most species (Talaei-Hassanloui et al., 2006). For *Beauveria* species in New Zealand, the morphological characteristics are white hyphae, which are floccose in appearance when infecting arthropods, and colonies that are white to pale yellow or occasionally red-pigmented on the reverse. Conidiogenous cells are formed in clusters, resembling fluffy balls below lower power magnification under a microscope, within the aerial hyphal mass. The conidiophores are flask-shaped, swollen at the base, extending up to 20 µm in a slender rachis, geniculate or irregularly bent or denticulate. Conidia with a diameter larger than 3 µm are found in *B. brongniartii,* while *B. bassiana* conidia are more spherical (Glare & Inwood, 1998). In culture, species of *Beauveria* typically develop slowly compared to other ascomycetes, appearing lanose, rarely structuring synemata, white or yellowish, occasionally pinkish (*B. malawiensis*). Aerial conidia are hyaline, smooth- and thin-walled, loose, or sometimes fasciculate. Old colonies turn powdery due to the dense hyphae of different structures, from globose, subglobose, and ellipsoid to a cylindrical shape (Imoulan et al., 2017; Rehner et al., 2011). At least 28 species in the genus *Beauveria* have been described with respect to their morphological characteristics (colony colour, conidiogenous cell shape and length, conidial size and shape), habitat, and geographic distribution (Khonsanit et al., 2020).

d. *Beauveria* life cycle

The infection of insects by *Beauveria* spp. generally commences when the asexual spores of the fungus physically attach to the epicuticle of the host. The spores germinate and subsequently start to penetrate the cuticle with the aid of hydrolytic enzymes (i.e., proteases, lipases and chitinases), mechanical pressure, and other factors (Ortiz-Urquiza & Keyhani, 2013). When the developing hyphae reach the nutrient-rich haemolymph, the fungus forms clustered groups of single-celled, yeast-like blastospores that are specialised structures that proliferate and exploit nutrients rapidly, colonise internal tissues and evade the host immune system (Hesketh et al., 2010). During the colonising process, the fungus produces considerable antimicrobial peptide compounds that help suppress host immune defence, destroy internal tissues, and absorb nutrients that eventually leads to host death. The pathogenesis and virulence of *Beauveria* are related to the internal toxic metabolites, cuticle degradation, anti-oxidant enzymes, and active vegetative growth of hyphae that cause host insects to die of nutrient depletion (Mascarin & Jaronski, 2016; Zimmermann, 2007). As the yeast-like blastospores contain hydrophobin constituents or hydrophobic rodlet layers, they can kill the host by evading immune defences and exploiting nutrients. The electrostatic charges present on the blastospores surface are also involved in the fungal pathogenicity interaction (Holder et al., 2007). As a result, airborne or submerged conidia may provide less virulence than blastospores to different invertebrate species (Mascarin et al., 2015).

After the insect host is killed and all nutrients have been consumed, hyphae grow out of the cadaver, particularly at the margins of the intersegmental regions, and produce conidia that promote the spread of the fungus. Several means of spore dispersal are known, including passive dispersal through water, wind, or secondary agents (insects or other adjacent arthropods, etc.). From first attachment to death of an arthropod requires 3 to 20 days, depending on factors such as insect species; host conditions (starvation, food types, overcrowding, and stresses that lead the host to become vulnerable to the infection); the number of spores on the host; fungal species and strain; and the ecological conditions (Hesketh et al., 2010; Mascarin et al., 2015). Pre-mortality symptoms can be observed within 1 to 4 days after inoculation when the infected insect starts to have a poor appetite, becomes inactive, and stays still (Roy et al., 2006). Figure 1.3 shows a completed life cycle of a *Beauveria* species infecting an insect species.

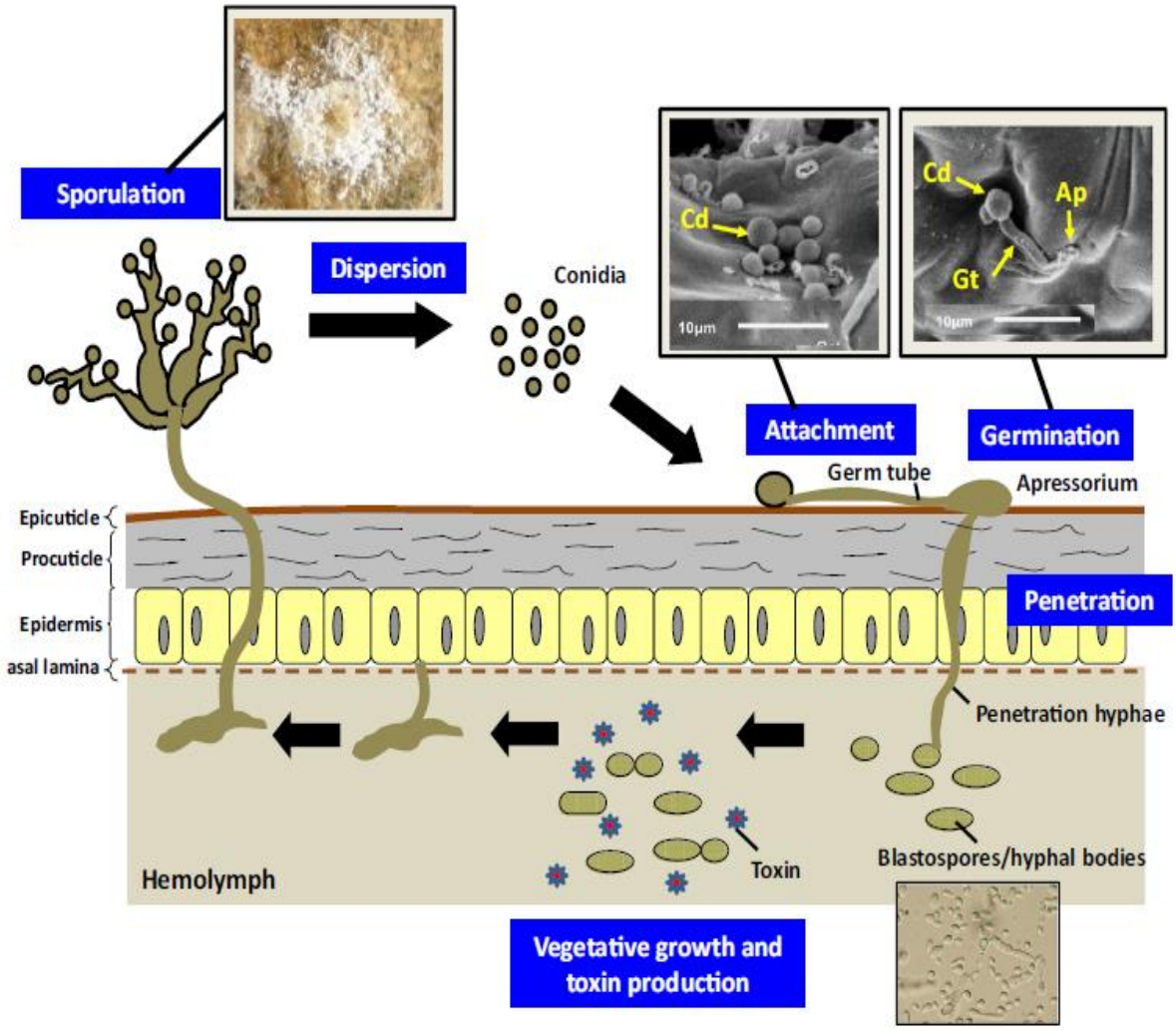


Figure 1.3: Life cycle or pathogenesis of *Beauveria bassiana* in an insect species (Mascarin & Jaronski, 2016)

e. Effect of environmental conditions

Abiotic and biotic factors significantly impact the dispersal, penetration, virulence, germination, propagation, and survival of *Beauveria* species. The primary environmental conditions are temperature, humidity, rainfall, and solar radiation, which play a significant role in the biological processes of *Beauveria* (Fernandes et al., 2015; Jaronski, 2010). The optimal temperature for germination ranges from 25°C to 30°C with ideal humidity of more than 95%, but germination can occur at temperatures between 15°C and 35°C under saturating moisture conditions (Luz & Fargues, 1997). Fungal growth is delayed above 35°C and completely inhibited at 40°C (Sabbour et al., 2011). As *Beauveria* are a ubiquitous soil microbiota component, soil conditions are the most critical factor for fungal survival (Chandler, 2017). Soil is a shield to protect fungal conidia from ultraviolet exposure, and extreme temperatures, provides water for facilitating passive-spore movement, and provides suitable growing conditions and pathogenic processes (Vega et al., 2009). Some soil conditions, such as soil texture (sandy, silty, or clay), soil temperature, and soil moisture, significantly affect fungal

growth. In contrast, soil pH, cation exchange capacity, and soil minerals have no measurable impacts (Jaronski, 2010). An experimental study on the germination of *B. bassiana* under artificial environmental conditions demonstrated that this species grows well on PDA medium, with nutritional sources including sucrose and calcium nitrate. However, the same study showed that the fungicide Benlate is extremely toxic and can kill the fungus at ≥6.25 ppm, with some insecticides also negatively impacting mycelial growth (Sabbour et al., 2011).

Survival of *B. bassiana* in soils and its infectivity for managing pest insects also depends significantly on biotic factors. As soil is a primary habitat for many microbes, the interaction of these microbes can inhibit or accelerate *Beauveria* fungal development (Jaronski, 2010). The fungistatic impact of soil infected with *Penicillium urticae* showed that this fungus produces water-soluble metabolites that can actively inhibit the growth of *B. bassiana* (Lingg & Donaldson, 1981). As the plant is a secondary habitat for entomopathogenic fungi in some cases, plant volatiles and plant surface chemistry can also potentially affect fungal development (Cory & Ericsson, 2009). Different plant species could also provide different efficacies of fungal growth. For example, when applying the same strain of *B. bassiana* to control western flower thrips, this insect was six times more susceptible to fungal infection when spraying on *Phaseolus vulgaris* than on *Impatiens walleriana* (Ugine et al., 2005). The age of the host insects, their level of susceptibility, and particular behaviour also contribute to the impact of fungal development (Jaronski, 2010). A study on the influence of soil inhabitants on fungal plant pathogens demonstrated that some species of Collembola feed on plant-pathogen fungi hyphae, so they can also eat *Beauveria* conidia if they are present in soil (Friberg et al., 2005).

1.4.3 Biopesticides for control of diamondback moth

Due to the fact that DBM has developed resistance to most synthetic insecticides, future management of this insect may depend on biocontrol agents. Generalist predators such as mantis, lady beetles, spiders, lacewings and ants contributed to suppressing the DBM population (Ma et al., 2005; Pagore et al., 2021; Reddy et al., 2004). Some egg parasitoids in the genus *Trichogramma* (Talekar & Shelton, 1993), larval parasitoids such as *Diadegma insulare* (Munir et al., 2015), *D. semiclausum* (Talekar, 2004), *Cotesia plutellae* (Shi, 2002), and pupal parasitoids like *D. collaris* and *D. subtilicornis* (Delvare, 2004) were found to have the potential for the control of DBM. Some entomopathogenic nematodes such as *Steinernema carpocapsae* (Schroer & Ehlers, 2005; Sunanda et al., 2013), *Steinernema thermophilum* (Somvanshi et al., 2006), and *Heterorhabditis bacteriophora* (Correa-Cuadros et al., 2016; Zolfagharian et al., 2016) also showed potential for use as biocontrol agents for DBM. Some microbial-based insecticides including bacteria: *Yersinia entomophaga* (Hurst et al., 2011), *Brevibacillus laterosporus* (van Zijll de Jong et al., 2016), fungi: *Lecanicillium muscarium* (Kuchár et al., 2019), *Beauveria bassiana* (Narciso et al., 2019; Vandenberg et al., 1998a), *Metarhizium anisopliae*

(Poornesha & Ramanujam, 2021; Shehzad et al., 2021), and viruses: granulovirus (Malik et al., 2020), baculovirus (Hauxwell, 2018) were also found to be potential candidates for biological control of DBM.

Some constraints when using biocontrol agents such as slowness to kill, inconsistent field performance, narrow host spectrum, complex life cycles, lack of handling technologies for ongoing viability, and registration or regulatory challenges are factors that limit the development of biopesticides (Agboyi et al., 2020; Glare et al., 2012; Glare et al., 2016). The two major scientific constraints are 1) a relatively slow time to kill the pest and 2) inconsistent field results compared to commonly used synthetic insecticides. Principally, using living agents, biological control agents, to control invertebrate pests requires several days to kill. However, this constraint is largely true for most biological control agents compared to a conventional chemical control programme. For instance, most entomopathogenic fungi take 5 to 10 days after infection for insect mortality to occur, in which time the infected pests can still manage to severely damage the crop (St Leger et al., 1996). Additionally, while many microbes are promising candidates for pest control, their uses are limited because living organisms can have reduced shelf life compared to synthetic pesticides and can be vulnerable to environmental conditions (Sporleder & Lacey, 2013). For example, Botanigard ES and Naturalis L (both based on *B. bassiana*) were examined for their field effectiveness. Even though these products contain the same species, Botanigard ES caused higher mortality than Naturalis L (Prince & Chandler, 2020).

Typically, end-users want biopesticides that rapidly kill target pests, provide broad-spectrum activity against complex pests, are cheap, and give consistent results under various climatic conditions. This is similar to the expectation from conventional synthetic pesticides (Butt et al., 2016). To fulfil these needs, genetic manipulation can be an alternative transformational technology to using wild-type organisms directly, producing viable mutants that can have high virulence and an expanded range against pests (Glare et al., 2012; Lovett & Leger, 2018). Other approaches have also been applied to improve abiotic stress tolerance, particularly from ultraviolet radiation and extreme temperatures, which are significant challenges for fungal-based biopesticides under field conditions (Tseng et al., 2011). Adding a chitinase (Bbchit1) or a subtilisin-like protease (Pr1A) homolog CDEP1 to *B. bassiana* resulted in increased virulence with a lower lethal rate (LD_{50}) and lethal time (LT_{50}) (Fang et al., 2005). Parallel results revealed that combining two cuticle-degrading enzymes (CDEP1:Bbchit1) provided 25% of LT_{50} faster and 60% of LD_{50} lower than the wild-type (Fang et al., 2009). Using *B. bassiana* as a vector to carry Bt vegetative insecticidal protein (Vip) Vip3A, an insecticidal metabolite that kills lepidopteran pests quickly, provides the dual functions of cuticle penetration and oral toxicity (Qin et al., 2010). Some strains of *B. bassiana* can produce chitinase-like proteins, an insecticidal protein potentially toxic to lepidopterans (Fuguet et al., 2004). Some other toxic compounds, such as beauvericin and bassianolide, are also produced by *Beauveria* spp. and these play a significant role as secondary metabolites after penetrating host insects (Zimmermann, 2007). However, methods for ramping up the production of these metabolites require expertise and years of experience. Moreover, these

genetic manipulation approaches are complicated to some extent, and the release of genetically modified organisms is highly restricted in most countries, including New Zealand. The only practical strategy is to screen indigenous or wild type *Beauveria* spp. for controlling a specific pest.

1.4.4 *Beauveria* as biopesticides for DBM control

Generally, the requirements for biopesticide development from fungal entomopathogens, particularly *B. bassiana,* are cost-effective production, reasonably long shelf-life (remain viable and infective during storage), consistent efficacy under field conditions, as well as ease of application (Jaronski, 2010). As *B. bassiana* is a promising candidate for incorporation into mycoinsecticides, it was considered as a potential candidate for DBM control (Shelton et al., 1998; Vandenberg et al., 1998a). Globally, some isolates of *B. bassiana* have been examined as potential candidates for controlling DBM under different conditions, with mortality of up to 100% (Table 1.3). However, most of these isolates were examined under controlled environmental conditions and have not yet been formulated for specific field control of DBM.

Table 1.3: Some potential isolates of *Beauveria bassiana* tested for diamondback moth control in different parts of the world

Isolate	Country	Authors	Mortality rate (%)	Day after spraying	Conidia/mL	Conditions
J18	New Zealand	(Narciso et al., 2019)	90	4	1.7×10^7, 3.9×10^8	*In vitro* assay
Bba5653	Benin	(Godonou et al., 2009)	94	6	1×10^8	*In vitro* assay
MG-Bb-1	Japan	(Yamada et al., 2009)	100	4	1×10^6	*In vitro* assay
SG8702	China	(Tian & Feng, 2006)	95	8	4×10^8, 2×10^7, 1×10^8	*In vitro* assay
IBCB01, 18, 66	Brazil	(Duarte et al., 2016)	80-100	7	1×10^7	*In vitro* assay
Bb 3	India	(Agrawal et al., 2017)	87	3	2.1×10^8	*In vitro* assay
Bb 11	Togo	(Agboyi et al., 2020)	93	4	1×10^4- 1×10^9	Field
Myco-Jaal	India	(Ghosh et al., 2011)	70	14	6×10^6	Field
GHA	USA	(Vandenberg et al., 1998b)	Up to 100	4	5×10^3, 1.4×10^4	*In vitro* assay
ESALQ-447	Brazil	(Rondelli et al., 2013)	40 Larvae, 60 Pupae	8	5×10^6	Greenhouse
CS-1	South Korea	(Yoon et al., 1999)	86	2	1×10^8	*In vitro* assay
GHA	Australia	(Furlong, 2004)	100	4	3×10^6	*In vitro* assay

DBM has developed resistance to *Bacillus thuringiensis* (Bt)-based biopesticides (Furlong et al., 2013; Mota-Sanchez & Wise, 2021) as this strategy is reliant on a single toxin for most of the bioactivity. As fungal biopesticides, particularly *Beauveria* spp., kill the insect by direct cuticle penetration and use multiple modes of action, it is harder for the insect to develop resistance (Mascarin & Jaronski, 2016). As demonstrated in Table 1.3, *Beauveria* species have the potential for future DBM control products.

1.5 Research rationale

1.5.1 Fungal identification

Specific identification of *Beauveria* isolates is a crucial part of any study to ensure the correct isolate is used for further investigation. Morphological characteristics and DNA-based techniques have previously been used for this task (Robène-Soustrade et al., 2015). The morphological characterisation can be used for the identification of some species, in which some features such as colony morphology on culturing media, conidial shape and sizes are used for characterisation (Imoulan et al., 2017). However, these features may change depending on colony age, conidial density, and media, which can lead to misidentification. As some species in the genus *Beauveria* are cryptic lineages, species confirmation using morphological features is difficult (Fisher et al., 2011). However, morphological identification can be the first insight into species in the genus (Imoulan et al., 2017). Therefore, this approach is included in the identification section.

The nuclear ribosomal internal transcribed spacer (ITS) primers have been developed for fungal amplification (Rehner & Buckley, 2005; White et al., 1990). However, ITS provides unequal variability among some fungal species, which may result in ambiguous data, which is particularly problematic in some species-rich genera, including *Beauveria* (Baturo-Cieśniewska et al., 2020). RNA polymerase II largest subunit (*RPB1*), RNA polymerase subunit (*RPB2*), translation elongation factor-1α (*TEF*), and the nuclear intergenic region BLOC are now also commonly used for genealogical concordance phylogenetic analysis for *Beauveria* (Fisher et al., 2011; Imoulan et al., 2017; Kepler et al., 2017a; Khonsanit et al., 2020; Rehner et al., 2011; Wang et al., 2020). Thus, to obtain the most reliable result, molecular identification is required. Therefore, the first aim of this study was to use the morphological and molecular approaches for the identification of all 14 isolates used.

1.5.2 Screening assay

Different isolates or species of *Beauveria* can work differently in controlling insect pests under diverse conditions, so screening under different conditions is necessary to select good candidates for DBM control. For instance, a screening study of 43 *B. bassiana* isolates to control lepidopteran pests, including DBM, showed that all of them provided significantly positive results, but only some gave high larval mortality (Wraight et al., 2010). This evidence proves that even within a

species, different isolates show different efficacies. Might there be another species that is more efficacious than *B. bassiana*? Additionally, rate and time contribute significantly to fungal infection. The mortality of DBM larvae was found to be up to 96%, with a 10^9 conidia/mL application rate on the 7^{th} day after spraying (Nithya et al., 2019). Providing 100% humidity for the first 24 hours can also enhance fungal establishment during insect cuticle penetration (Wraight et al., 2010).

Bioassays are a type of *in vitro* experiment that tests the effectiveness of a stimulus treatment to a living organism under controllable conditions (Bruck, 2009). This kind of experiment provides insight into the target organism's susceptibility, which then underpins decisions on whether or not to conduct semi-control (greenhouse) and open field experiments that can save a large amount of money and time. Thus, they must be carefully designed to obtain statistically valid biological data (Robertson et al., 2017). As this experimental study is a pilot for screening suitable candidates to control DBM specifically, the bioassay test is the most appropriate approach. Therefore, the second aim of this study was to screen suitable isolates and application rates to control DBM using bioassay experiments.

1.5.3 Use of multiple strains

The combination of different *Beauveria* strains within a species or across species may result in synergistic interactions, although strain mixing within a genus has rarely been reported. Mixing different species has been reported; for example, a mixture of *B. thuringiensis* and *B. bassiana* (ratio 3:1) was found to provide higher DBM mortality than the individual organisms (Xue et al., 2018). Additionally, the combination of a conidial spray of *B. bassiana* with its insect-toxic metabolites has been found to provide the best control of horticultural pests in New Zealand (Glare & O'Callaghan, 2019). As some strains of *Beauveria* produce high quantities of lepidopteran toxic compounds (Fuguet et al., 2004), it may be beneficial to combine these isolates for better control of DBM. For instance, an experimental study found that when combining the three less virulent strains of *B. bassiana*, the beetle's mortality reached 93%, while single isolates caused around 60% mortality or less (Cruz et al., 2006). These studies suggest investigating further the synergism of combining *Beauveria* isolates to achieve the optimal outcome. However, the combination of high virulent isolates was found to be antagonistic rather than synergistic in some studies (Bayman et al., 2021). Cruz et al. (2006) studied different isolates within the same species, but this experimental study combined different species to look for any synergistic interactions. Therefore, this study's third aim was to investigate synergistic results when combining different species of *Beauveria* spp. on DBM larvae mortality.

1.5.4 Effect of diet on susceptibility

Plants are sometimes an alternate habitat for entomopathogens, and in some cases, plant volatiles and plant surface chemistry can potentially affect fungal development (Cory & Ericsson, 2009), which could provide different susceptibility of an insect to fungal application. For example, when using the same strain of *B. bassiana* to control western flower thrips, *Frankliniella occidentalis*, this insect was six times more susceptible to fungal infection when feeding on kidney beans than on garden impatiens (Ugine et al., 2005). Different cruciferous species contain various secondary plant metabolites, and they may have diverse effects on *Beauveria* development and insect susceptibility to fungal infection. For instance, a study on glucosinolate contents of eight brassica crops, namely broccoli, Brussel sprout, cabbage, cauliflower, kale, kimchi cabbage, mustard, and radish, found that cauliflower and radish contain lower glucosinolates than other species (Kim et al., 2020). DBM has been found to have a strong relationship with levels of glucosinolates in brassica plants (Badenes-Pérez et al., 2020; Robin et al., 2017). These studies give rise to the hypothesis that *Beauveria* species might have different efficacy when applied to DBM larvae fed on higher and lower glucosinolate brassicas. Thus, this study's fourth aim was to observe any effects of different brassicas as a diet on the susceptibility of DBM larvae to infection by *Beauveria* isolates.

1.5.5 Toxic metabolites

The infection pathway of *Beauveria* on a particular insect can involve multiple modes of action (Mascarin & Jaronski, 2016). Some *Beauveria* spp. produce different levels of insect toxins to kill hosts rather than killing by direct penetration and infection. A study on the toxicological properties of *B. bassiana* and *B. pseudobassiana* revealed that these species exhibited different metabolite profiles and produced different amounts of these metabolites when grown on different types of synthetic media (Berestetskiy et al., 2018; Wang et al., 2020). Cheong (2015) provided detailed information about insecticidal compounds extracted from an isolate of *B. bassiana* and used these extracts for direct insect control. This experimental study found the isolate killed more aphids than DBM larvae. A 37% mortality of DBM larvae was found when using extracted toxins of a *B. bassiana* isolate (Gao et al., 2012). Some *Beauveria* species might produce more toxins than others resulting in faster killing. Resquín-Romero et al. (2016) found the mortality of a lepidopteran pest ranged from 50% to 67% using isolates from two species of *Beauveria*. Therefore, the fifth aim of this study was to extract metabolites from fast-killing isolates, the isolates selected depending on the screening bioassay chapter, and to use these toxins for bioassays.

Chapter 2

Morphological and molecular identification of *Beauveria* isolates

2.1 Introduction

Microorganisms are ubiquitous, and this creates a potential problem with many techniques and protocols used in the discipline of microbiology. Contamination, defined as the presence of a constituent, impurity, or some other undesirable element that spoils, corrupts, infects, makes unfit, or makes inferior a material, physical body, natural environment, workplace, can occur within the discipline unless certain techniques (e.g., aseptic technique) are strictly adhered to. Contamination, mostly by other airborne fungi, can happen during the cultivation of entomopathogenic fungi (Shi et al., 2019). It is essential to ensure that the fungal target is not contaminated by other unknown living organisms (excluding mycoviruses, which are more challenging to detect and remove). If possible, the isolate should be pure to confirm identification and characterisation. As most hypocrealean fungi, including *Beauveria,* sporulate prolifically, and hyphae can grow on unspecialised agar media containing a broad-spectrum antibacterial agent (e.g. an antibiotic), the individual colony can generally be cultured free of bacteria (Inglis et al., 2012). The two methods commonly used for fungal purification are single-spore isolation (Mwamburi, 2016) and hyphal tip isolation methods (Inglis et al., 2012). The single-spore isolation method is generally more applicable, as it is not always possible to isolate a single hypha (Inglis et al., 2012). The most commonly used media for the cultivation of *Beauveria* spp. are potato dextrose agar (PDA) (Wang et al., 2020) and Sabouraud dextrose agar (SDA) (Mwamburi, 2016). Both media can be supplemented with a broad-spectrum antibiotic (e.g. 50 mg/L of tetracycline chloride and 350 mg/L of streptomycin sulphate), as described by Raad (2016) when isolating the fungus from soil.

Morphological identification can be used to distinguish individual species of *Beauveria* using a light microscope at high magnification to compare vegetative and reproductive characteristics (Raad, 2016). The most important morphological feature used to distinguish between species of *Beauveria* are their asexual spores or conidia (Cummings, 2009). The shape and size of these conidia can be assessed at 400x magnification after culturing the fungus on PDA (Glare et al., 2008). For definitive species identity, however, DNA-based techniques are required in order to avoid misidentification (Imoulan et al., 2017). For molecular analysis, methods such as polymerase chain reaction (PCR) and specific region sequencing followed by phylogenetic analysis are required. Published studies have used various DNA extraction methods in the past, including readily available commercial kits, e.g. Genomic DNA Purification Kit (Qiagen) (Wang et al., 2020), PrepMan Ultra Sample Preparation Reagent® (Fisher et al., 2011; Rehner & Buckley, 2005; Rehner et al., 2011), REDExtract-N-Amp™ (Sigma-Aldrich) (McKinnon et al., 2018), cetyltrimethylammonium bromide (CTAB) (Khonsanit et al., 2020), and

DNeasy Plant Kit (Qiagen, Hilden, Germany) (Glare et al., 2008; Raad, 2016). These extraction methods all involve transferring DNA from one tube to another that contains different reagents, and that may introduce contamination. Furthermore, many kits are relatively expensive for processing large samples compared to the Chelex-100 method (Walsh et al., 2013). The DNA extraction protocol using Chelex-100 is simple, fast, requires a single transfer of DNA (after centrifugation), therefore reducing the possibility of contamination, and contains no organic solvents (Singer-Sam, 1989). This method has been found efficient for forensic-type samples (Walsh et al., 2013) and microbial DNA extraction (Alizadeh et al., 2017; Hennequin et al., 1999).

Molecular methods using phylogenetic analyses are now commonly used in contemporary fungal taxonomic studies (Baturo-Cieśniewska et al., 2020; Jayasiri et al., 2015). Since the establishment of advanced research on molecular identification of entomopathogenic fungi, there have been well-established methods for the identification of *Beauveria* (Berestetskiy et al., 2018). The use of the nuclear ribosomal internal transcribed spacer (ITS) sequence helps determine the molecular phylogenetic diversity of fungal species. The benefits of using this sequence are the high probability of the correct identification and the highly conserved regions of DNA for each primer (Rehner & Buckley, 2005; Rehner et al., 2006b; White et al., 1990). However, using ITS can provide unequal variables among some fungal groups, resulting in ambiguous data. This issue is particularly problematic in some species-rich genera, including *Beauveria* (Baturo-Cieśniewska et al., 2020), where its usefulness at the species level is limited (Imoulan et al., 2017). Most recent studies on *Beauveria* taxonomy used multiple-gene sequences to examine combining regions such as RNA polymerase II largest subunit (*RPB1*), RNA polymerase subunit (*RPB2*), translation elongation factor-1α (*TEF*), and the nuclear intergenic region (BLOC) for genealogical concordance phylogenetic analysis (Fisher et al., 2011; Imoulan et al., 2017; Kepler et al., 2017a; Khonsanit et al., 2020; Rehner et al., 2011; Wang et al., 2020). These four loci types are suitable for phylogenetic binding of fungi and are useful in clavicipitaceous phylogenetic studies (Rehner et al., 2011). While several studies have built phylogenetic trees based on the single BLOC locus (Cabrera-Mora et al., 2019; Khun et al., 2020; Ramos et al., 2017) or EF1-α (Cabrera-Mora et al., 2019; González-Mas et al., 2019; Rehner & Buckley, 2005), others have combined several markers (Bustamante et al., 2019; Khonsanit et al., 2020; Rehner et al., 2011). Building a phylogenetic tree using combined sequence data is more reliable for supporting species boundaries of *Beauveria* (Bustamante et al., 2019).

Single spore isolation was undertaken to obtain a pure colony of each *Beauveria* isolate used in this current study. At the same time, morphological and DNA-based techniques were utilised to determine species identity.

2.2 Materials and Methods

2.2.1 Sources of isolates

Fourteen isolates of *Beauveria* spp. derived from New Zealand habitats were investigated (Table 2.1).

Table 2.1: The species identities of the 14 *Beauveria* isolates used in this phylogenetic study

Isolate	Habitat/Host	Originated from	Isolated by
F615	Organic soil	Lincoln University	Jenny Brookes
J2	*Plutella xylostella*	BPRC growing chamber	Jenny Brookes
Mo1	*P. xylostella*	BPRC growing chamber	Marsha Ormskirk
TPP-H	*Bactericera cockerelli*	BPRC growing chamber	Jenny Brookes
J18	Maize cultivar 38V12	Ashburton	Jenny Brookes
FRh2	*Hylastes* or *Hylurgus*	Riverhead	Jenny Brookes
FRhp	Lab contaminant	Lincoln University	Sereyboth Soth
Bweta	*Hemideina femorata*	Westland	Jenny Brookes
FW Mana	*Anagotus fairburni*	Mana Island	Colin Miskelly
I12 Damo	*Coccinella undecimpunctata*	Lincoln University	Damien Bienkowski
F532	*Hylastes ater*	Riverhead	Travis Glare
O2380	*Brassica rapa*	Palmerston North	Stuart Card
CTL20	*P. xylostella* larva	BPRC growing chamber	Jenny Brookes
CTA20	*P. xylostella* adult	BPRC growing chamber	Jenny Brookes

Note: BPRC = the Bio-Protection Research Centre, Lincoln University

2.2.2 Purification and identification methods

a. Single-spore culture

For the single-spore culture of all isolates, the method of Mwamburi (2016) was followed. In summary, PDA (Oxoid) was used as a growth medium for the fungal purification process. A sterile metal needle was used to transfer single-spores of each isolate to fresh PDA. All culture plates were incubated at 23^{o}C ± 1^{o}C with a photoperiod of 12:12 D/L for 14 days, at which time conidia were collected for long-term storage and mycelium was utilised for genomic DNA extraction. Conidia were immersed within 20% glycerol (Univar, Ajax Finechem) and frozen at -80^{o}C for future use.

b. Morphological characteristics

At 14 days of growth, each Petri plate's upper and bottom sides containing the cultured fungi were photographed using a Canon camera (PowerShot G2). A metal loop was used to transfer conidia from a plate onto a glass microscope slide, stained with 10 µL of cotton blue dye (diluted 1:1 with dH_2O) and observed under an optical microscope, Leica DM2500 with cellSens software (Olympus) at 400x magnification. The length and width of three random spores were determined using the measurement function within the software (Figure 2.1).

c. DNA extraction

Fungal genomic DNA was extracted from a pure culture of each *Beauveria* isolate. The Chelex® 100 protocol was used for DNA extraction (Alizadeh et al., 2017; Hennequin et al., 1999; Walsh et al., 2013). Chelex-100 solution preparation was 5% or 2 g of Chelex-100 in 40 mL of sterilised water. In summary, mycelia were released from each culture by gently scraping the fungal colony with a metal spatula into a 1.7 mL centrifuge tube, followed by pipetting 500 µL of Chelex-100 solution into the tube and incubating on ice for 30 minutes. Around 200 µL of liquid nitrogen was poured, and a sterile pestle was used for crushing the mycelium while the solution was defrosting. The defrosted solution was vortexed and incubated in boiling water for 12 minutes (starting the timing when the water began to boil), followed by cooling down to room temperature for 10 minutes. The solution was then centrifuged at 1300 rpm for 20 minutes, and 200 µL of the top clear solution pipetted into a new 1.7 mL centrifuge tube before storing at 4°C. Genomic DNA solution was diluted with nanopure water (ratio 1:1) for amplification of all isolates.

d. PCR

In this study, the elongation factor EF1-α and BLOC regions were targeted for PCR amplification (Table 2.2). PCR amplification was performed in a total volume of 25 µL including 2 µL genomic DNA extract, 2 µL (2.5mM) dNTPs, 1 µL (10mM) of forward and 1 µL (10mM) of reverse primers (Integrated DNA Technology) (Table 2.2), 2.5 µL reaction buffer $MgCl_2$ (2mM), 0.25 µL of FastStart *Taq* polymerase (0.7U/reaction) (Roche Diagnostics GmbH), 0.5 µL of BSA, and 15.75 µL of PCR water. Amplification was conducted in a PCR thermal cycler (Kyratech) under 1 cycle of denaturation for 5 min at 95°C, 1 cycle of run for 45 sec at 95°C, annealing 40 cycles for 45 sec at 57°C, extending for 2 min at 72°C (Glare et al., 2008; Reay et al., 2008), and concluding with 7 min incubation at 72°C (Rehner & Buckley, 2005; Rojas et al., 2010). Positive (a previously amplified sample) and negative (dH_2O only) controls were included in each PCR run. For the electrophoretic gel run, 1 g of powder of Multi-purpose Agarose gel (HyAgarose™) with 100 mL of buffer solution (1x TAE), after being well-mixed, was microwaved for 2 min to make the solution clear. When the mixture had cooled to around 50°C, 2 µL of nucleic acid staining solution, Redsafe (iNtRON), was added and shaken to mix

before pouring into a gel tray with a 24-comb frame. Once the gel had solidified, and the frame was removed, 3 µL of gel staining buffer and 6 µL of PCR product were pipetted onto Parafilm to mix before loading into each well. The electrophoretic gel was run using 1x TAE buffer. A power supply system (Midicell® Primo EC330) was set up at 100 volts, 177 milliamperes and 16 watts for 45 minutes. After completing the run, the gel was captured for bands using the gel documentation system (Uvidoc HD6).

Table 2.2: Primers used in this study

Marker	Primer name	Sequence (5'-3')	Reference
EF1-α	983F	CARGAYGTBTACAAGATYGGTGG	(Cummings, 2009; Glare et al., 2008; Rehner & Buckley, 2005)
	2218R	CCRAACRGCRACRGTYYGTCTCAT	
BLOC	B22U	GTC GCA GCC AGA GCA ACT	(Fisher et al., 2011; Korosi et al., 2019; Rehner et al., 2006b)
	B822L	AGA TTC GCA ACG TCA ACT T	

e. PCR clean-up

NucleoSpin PCR clean-up columns (Macherey-Nagel, 2017) were used to remove impurities from PCR products before sequencing. DNA was diluted (20 µL DNA + 80 µL of H_2O), then 200 µL of NTI buffer was added and incubated at room temperature (around 20°C) for 1 min. The solution was pipetted into the NucleoSpin PCR clean-up column, placed into lidless 2 mL collection tubes and centrifuged at 11,000 g for 30 sec. The supernatant was discarded, and 700 µL of NT3 buffer was added into the column and centrifuged for 30 sec at 11,000 g. This last step was repeated. After washing two times, the column and collection tube were combined and centrifuged for another 2 min at 11 000 g to remove the NT3 buffer. The tube, with the lid open, was dried at 70°C for 3 min to evaporate the ethanol. DNA was eluted using 30 µL of NE buffer and incubated for 1 min at room temperature before centrifugation for 1 min at 11,000 g.

f. Sequencing PCR products

PCR products were sequenced using the MagBio HighPrep dye terminator removal (DTR) Clean-up protocol at the Bio-Protection Research Centre, Lincoln University, sequencing facility, using a Hitachi ABI Prism 3130xl Genetic Analyser with a 16 capillary 50 cm array installed, and using Performance-Optimised Polymer 7 (POP7). Typically, sequencing using thermal capillaries can produce a faster and higher resolution of separation through a higher electric field (Dovichi, 1997). The total volume of 10 µL of the sample including 6µL of Nanopure water, 0.5 µL of big dye terminator, 2 µL of 5x sequence buffer, 0.5 µL of each direction primer (10mM) (Table 2.2), and 1 µL of the PCR product. After preparing, all samples were run in a PCR thermal cycler (Kyratech) for 1 cycle of denaturation for 1 min at 95°C; 25 cycles for 10 seconds at 96°C, 5 seconds at 50°C, and 2 min at 60°C; and indefinitely

at 4°C. The HighPrep™ DTR reagent is a paramagnetic bead-based system designed to remove unincorporated terminators from Sanger sequencing reactions (MAGBIO, n.d.). The protocol consists of selective binding of DNA to the HighPrep™ DTR particles, followed by washing off nucleotides, primers and non-targeted amplicons, and finally elution of pure DNA to be directly used in downstream applications. All the procedures can be found in MAGBIO (n.d.).

g. Phylogenetic analysis

Sequences of the 14 *Beauveria* isolates from this study were compared with previous studies utilising both EF1-α and BLOC markers (Table 2.3). Sequence data were initially imported into the software package Geneious Prime® 2021.0.1 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012) as applied biology information (ABI) files. The forward and reverse directions from the same sequence were assembled using *de novo* assembly to combine contigs into larger consensus reads (Bridgeman et al., 2020), where regions of the poor match were trimmed using the Trim ends function before extracting the consensus files. The consensus result of each isolate was first confirmed using Nucleotide Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990). After blasting using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), all consensus data from EF1-α and BLOC were then aligned using the software Clustal Omega as it is quick for larger alignments and precise for protein alignment (Sievers & Higgins, 2018). After aligning, longer sequences were manually trimmed to adjust to the length of the shortest sequence. Trimmed results were then joined manually for EF1-α, and BLOC, respectively, then extracted as combined consensus data. Additional comparison sequences were obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) (Rehner et al., 2011), from Peru (originally isolated in Peru) (Kocacevik et al., 2016) (this isolate is phylogenetically similar to *B. bassiana*) and from New Zealand (Glare et al., 2020) through the National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). Only isolates that had accessions for the two regions were downloaded (Table 2.3). All 15 additional isolates were also manually combined and extracted as combined consensus data. All isolates (from this study and NCBI) were multiple-aligned using MUSCLE alignment, distance measured using khmer4_6, clustering using the Neighbor-joining method, and tree rooting using the pseudo method. The combined isolates downloaded from NCBI were then trimmed manually to adjust the length of the New Zealand isolates in this study. The consensus tree was built from the combined sequence alignment by Neighbor-Joining as nucleotide substitution (Saitou & Nei, 1987) using Jukes-Cantor (Jukes & Cantor, 1969) as phylogenetically mathematics distance model, no outgroup, with 1000 replications Bootstrap and a support threshold of 50% in Geneious tree builder (Biomatters, 2020).

Table 2.3: The 29 *Beauveria* isolates used in this phylogenetic study

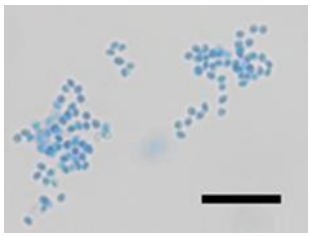
Species	Country of origin	Isolate code	Host or habitat	Bloc sequence	EF1- α sequence	Reference or original collection
B. amorpha	Australia	ARSEF4149	Coleoptera: Scarabaeidae	HQ880735	HQ881006	(Rehner et al., 2011)
B. australis	Australia	ARSEF4580	Orthoptera: Acrididae	HQ880719	HQ880994	(Rehner et al., 2011)
	Japan	ARSEF1040	Lepidoptera: Bombycidae	HQ880689	AY531881	(Rehner et al., 2011)
	Italy	ARSEF1564	Orthoptera: Arctiidae	HQ880692	HQ880974	(Rehner et al., 2011)
	Japan	ARSEF7518	Hymenoptera: Pamphiliidae	HQ880693	HQ880975	(Rehner et al., 2011)
	New Zealand	K4	Coleoptera: Scolytidae	MW030951	MW030949	(Glare et al., 2020)
	New Zealand	O2380	*Brassica rapa*	MZ703290	MZ703304	AgResearch Limited
	New Zealand	CTA20	Lepidoptera: Plutellidae	MZ703289	MZ703303	BPRC collection
B. bassiana	New Zealand	CTL20	Lepidoptera: Plutellidae	MZ703288	MZ703302	BPRC collection
	New Zealand	F615	Organic soil	MZ703287	MZ703301	BPRC collection
	New Zealand	FRh2	Coleoptera: Scolytidae	MZ703286	MZ703300	BPRC collection
	New Zealand	J2	Lepidoptera: Plutellidae	MZ703285	MZ703299	BPRC collection
	New Zealand	J18	Endophyte of *Zea mays*	MZ703284	MZ703298	(Brookes, 2017)
	New Zealand	Mo1	Lepidoptera: Plutellidae	MZ703283	MZ703297	BPRC collection
	New Zealand	TPP-H	Hemiptera: Triozidae	MZ703282	MZ703296	BPRC collection
B. brongniartii	Japan	ARSEF7516	Coleoptera: Scarabidae	HQ880697	HQ880976	(Rehner et al., 2011)
	New Zealand	FRh1	Coleoptera: Scolytidae	MW030952	MW030947	(Glare et al., 2020)
B. caledonica	Denmark	ARSEF8024	Coleoptera: Scarabaeidae	HQ880749	HQ881012	(Rehner et al., 2011)
	New Zealand	F532	Coleoptera: Scolytidae	MZ703281	MZ703295	BPRC collection
B. kipukae	USA	ARSEF7032	Homoptera: Delphacidae	HQ880734	HQ881005	(Rehner et al., 2011)
	Australia	ARSEF4755	Soil	HQ880754	HQ881015	(Rehner et al., 2011)
B. malawiensis	New Zealand	Bweta	Orthoptera: Anostostomatidae	MW030953	MW030946	(Glare et al., 2020)
	New Zealand	Bweta	Orthoptera: Anostostomatidae	MZ703280	MZ703294	BPRC collection
B. peruviensis	Peru	UTRF21	Coleoptera: Curculionidae	MN094752	MN094767	(Bustamante et al., 2019)
	USA	ARSEF3529	Lepidoptera: Lymantriidae	HQ880726	HQ880998	(Rehner et al., 2011)
B. pseudobassiana	Canada	ARSEF1855	Coleoptera: Scolytidae	HQ880727	HQ880999	(Rehner et al., 2011)
	New Zealand	FRhp	Lab contamination	MZ703279	MZ703293	This study
	New Zealand	FW Mana	Coleoptera: Curculionidae	MZ703278	MZ703292	BPRC collection
	New Zealand	I12 Damo	Coleoptera: Coccinellidae	MZ703277	MZ703291	BPRC collection

Note: BPRC = the Bio-Protection Research Centre

2.3 Results

2.3.1 Morphology

Colonies of the 14 *Beauveria* isolates were white to pale yellow or pink to red in colour. While isolates F615, J2, Mo1, TPP-H, J18, FRh2, FRhp, FW Mana, I12 Damo, O2380, CTL20, and CTA20 were fluffy white when observing on the upper plate, they were pale yellow when viewed from the base of the Petri plate. For isolate F532, both sides of the Petri plate were white, while isolate Bweta was pink on the top and slightly red on the bottom. Conidia ranged in shape from globose, subglobose, or broadly ellipsoid for isolate F615, J2, Mo1, TPP-H, J18, FRh2, FRhp, FW Mana, I12 Damo, O2380, CTL20, and CTA20 to ellipsoid, oblong, or cylindrical for isolate F532 and isolate Bweta (Figure 2.1). The conidial sizes ranged from 2.00 to 2.94 µm in length and 1.76 µm to 2.27 µm in width (average 2.48 x 2.01) with a ratio (length/width) of 1.11 µm to 1.37 µm for isolates F615, J2, Mo1, TPP-H, J18, FRh2, O2380, CTL20, and CTA20. Relatively similar to these isolates, the size of conidia of isolates FRhp, FW Mana, and I12 Damo ranged from 2.07 to 2.27 µm in length and 1.8 to 1.9 µm in width (average 2.16 x 1.8 µm). Isolate F532 had, comparatively, quite big conidia, 3.6 µm in length and 2.14 µm in width, with a ratio of 1.68 µm. Conidia of isolate Bweta were larger than all the other isolates in the study and measured 3.8 µm in length and 2.00 µm in width with a ratio of 1.90 µm (Table 2.4).



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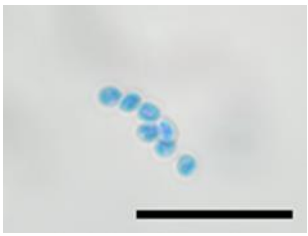
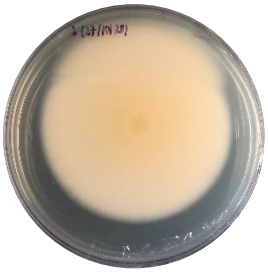
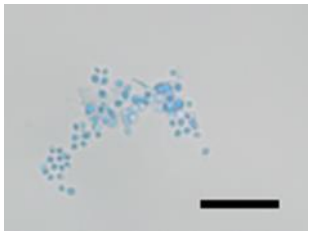


Mag: x 50, scale: 20 µm

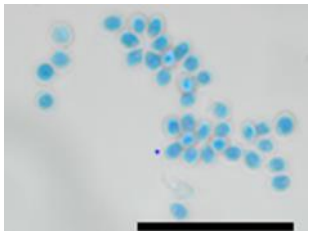
Isolate F615

Upper **Bottom** **Conidia** **Conidiogenous cells**



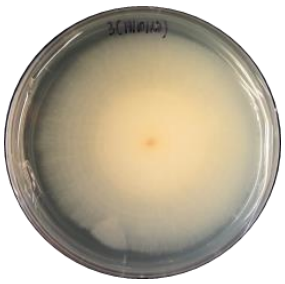
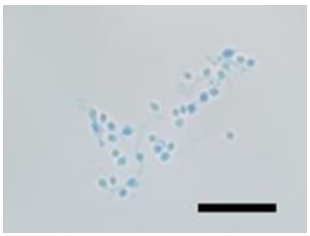
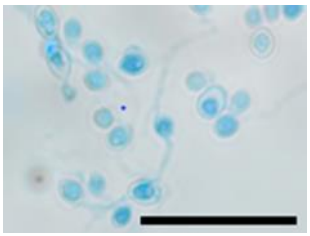


Mag: x 20, scale: 20 µm

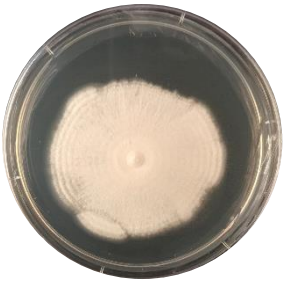
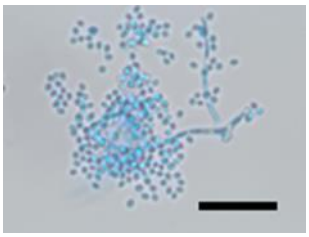
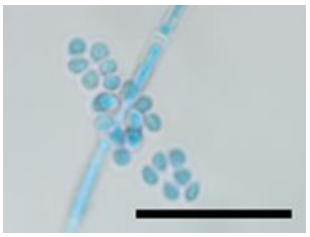


Mag: x 50, scale: 20 µm

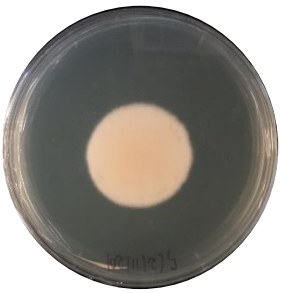
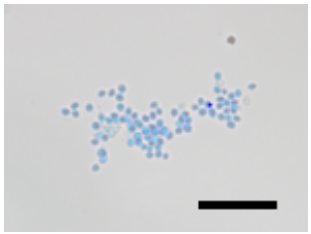
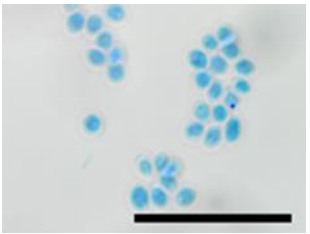
Isolate J2

Upper	Bottom	Conidia	Conidiogenous cells
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

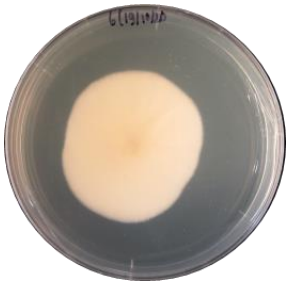
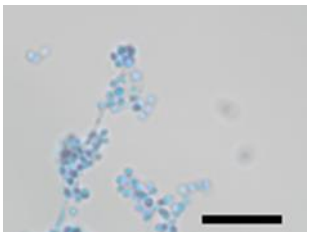
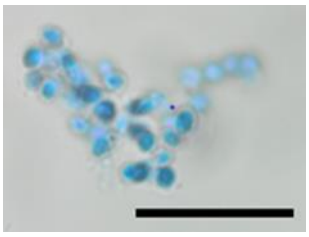
Isolate Mo1

Upper	Bottom	Conidia	Conidiogenous cells
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

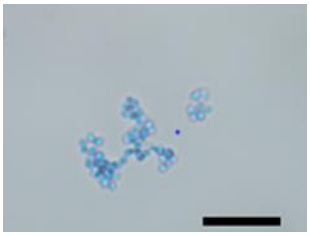
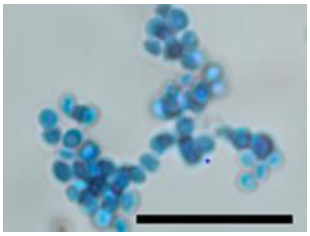
Isolate TPP-H

Upper	Bottom	Conidia	Conidiogenous cells
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

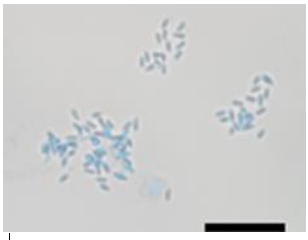
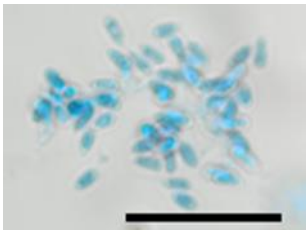
Isolate J18

Upper	Bottom	Conidia	Conidiogenous cells
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

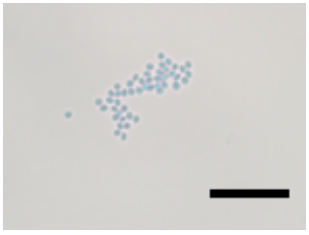
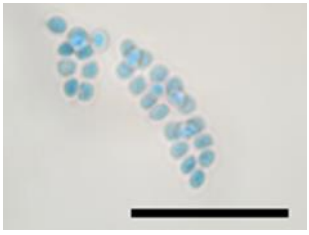
Isolate FRh2

Upper	Bottom	Conidia	Conidiogenous cell
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

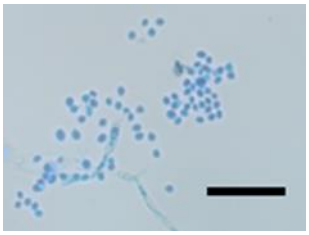
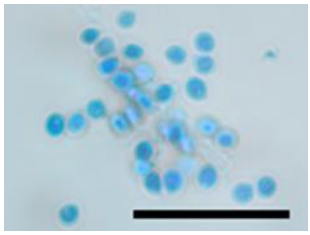
Isolate FRhp

Upper	Bottom	Conidia	Conidiogenous cell
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

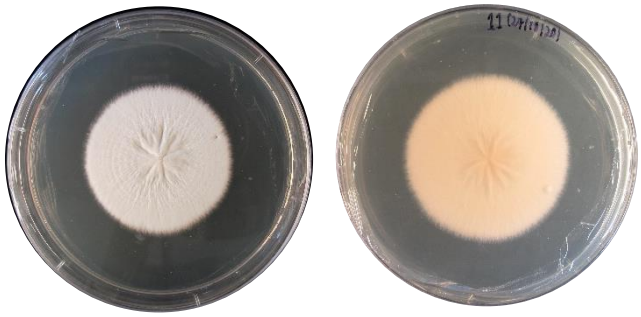
Isolate BWeta

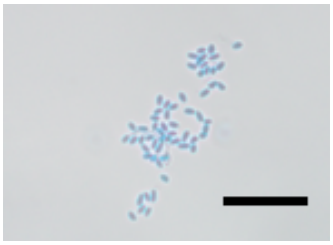
Upper	Bottom	Conidia	Conidiogenous cell
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

Isolate FW Mana

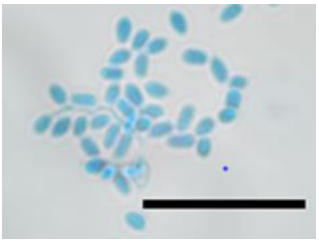
Upper	Bottom	Conidia	Conidiogenous cell
		 Mag: x 20, scale: 20 µm	 Mag: x 50, scale: 20 µm

Isolate I12 Damo





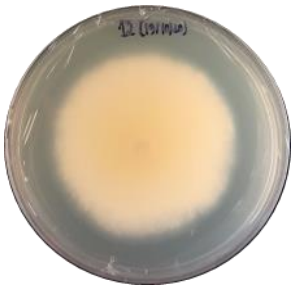
Mag: x 20, scale: 20 µm

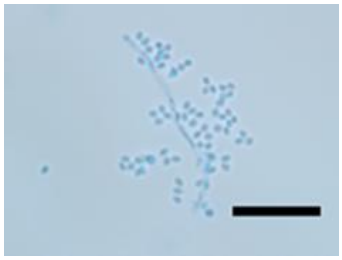


Mag: x 50, scale: 20 µm

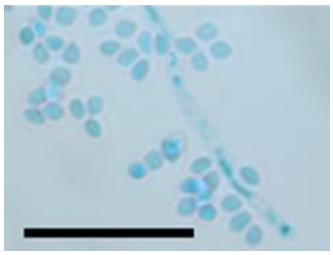
Isolate F532

Upper **Bottom** **Conidia** **Conidiogenous cell**





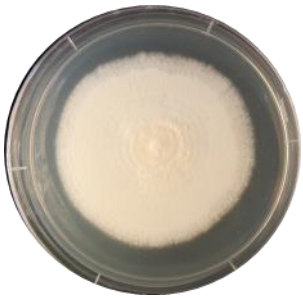
Mag: x 20, scale: 20 µm

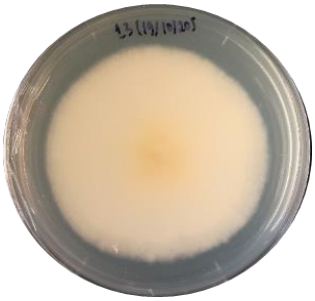


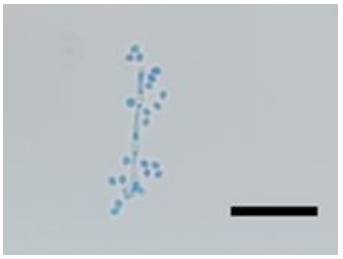
Mag: x 50, scale: 20 µm

Isolate O2380

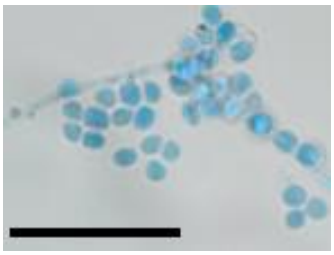
Upper **Bottom** **Conidia** **Conidiogenous cell**







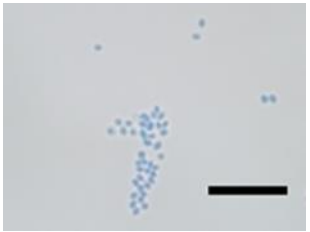
Mag: x 20, scale: 20 µm



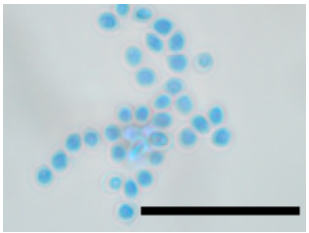
Mag: x 50, scale: 20 µm

Isolate CTL20

Upper **Bottom** **Conidia** **Conidiogenous cell**



Mag: x 20, scale: 20 µm



Mag: x 50, scale: 20 µm

Isolate CTA20

Figure 2.1: Colonies and conidia of all 14 *Beauveria* isolates utilised in this study (after incubation at 23°C ± 1°C on PDA within a photoperiod of 12:12 D:L for 14 days) staining using blue cotton dye

Table 2.4: Conidial size (the average of three spores) of the 14 *Beauveria* isolates measured using an optical microscope Leica DM2500

Isolate	Length (µm)	Width (µm)	Ratio L/W (µm)
F615	2.00	1.76	1.14
J2	2.57	2.07	1.24
Mo1	2.48	1.94	1.28
TPP-H	2.54	2.27	1.12
J18	2.61	2.00	1.31
FRh2	2.94	2.14	1.37
FRhp	2.07	1.80	1.15
Bweta	3.80	2.00	1.94
FW Mana	2.14	1.90	1.13
I12 Damo	2.27	1.80	1.26
F532	3.60	2.14	1.68
O2380	2.67	2.14	1.25
CTL20	2.40	1.94	1.24
CTA20	2.07	1.86	1.11

2.3.2 EF1-α and BLOC sequencing

Consensus data from forward and reverse directions of these markers consisted of 539 and 353 bp in the aligned positions for EF1-α and BLOC, respectively. After deleting gaps and ambiguous alignments using MUSCLE alignment, 527 and 308 bp of EF1-α and BLOC sequences remained, respectively (Appendix B2.1). Both sequences showed similar results, in which the 14 isolates used in this study were homologically aligned with four species of *Beauveria*, namely: *B. bassiana*, *B. caledonica*, *B. malawiensis*, and *B. pseudobassiana*. Isolates CTA20, CTL20, J2, J18, and Mo1 (all except J18, were isolated from DBM larvae) were identical and phylogenetically similar to K4 *B. bassiana* isolate from New Zealand, and the isolate O2380 aligned with this same group. The isolate TPP-H was nearly identical to isolate ARSEF1040, while isolates FRh2 and F615 were aligned with the isolates ARSEF518 and ARSEF1564. The isolate F532 was genetically similar to FRh1, the *B. caledonica* isolate from New Zealand and the isolate ARSEF8024. The isolate Bweta was the same as Bweta from New Zealand and ARSEF4755, which are both *B. malawiensis*. Isolates FRhp, I12 Damo, and FW Mana were aligned with the same group as *B. pseudobassiana* ARSEF1855 and ARSEF3529 (Figure 2.2).

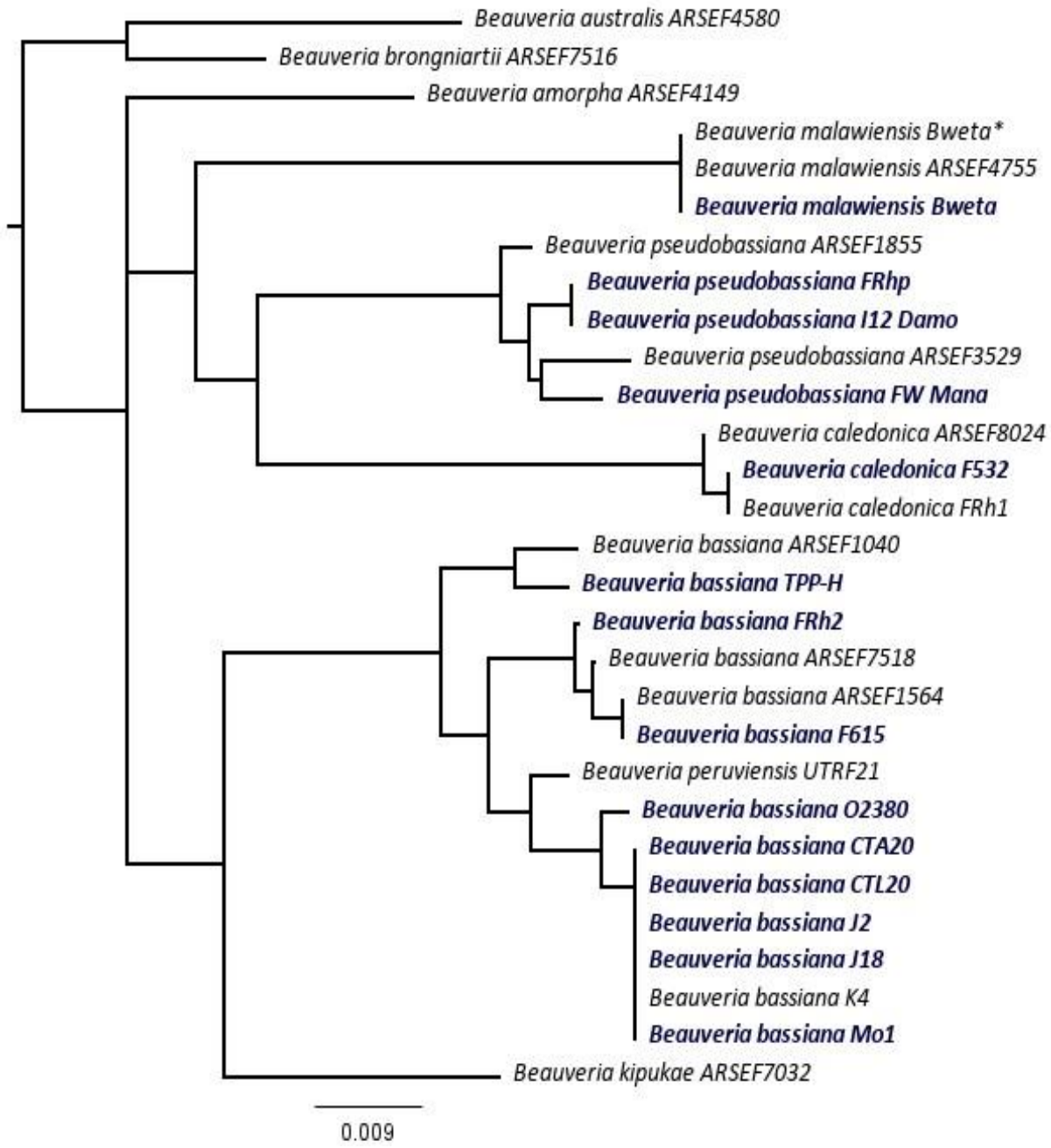


Figure 2.2: Phylogenetic tree of the combined-sequence data for EF1-α and BLOC based on the Neighbor-Joining method using Juke-Cantor as a phylogenetically mathematical distance model, no outgroup, with 1000 replicates Bootstrap and the support threshold of 50% in Geneious tree builder.

2.4 Discussion

The identities of all the *Beauveria* isolates utilised within this study were confirmed using both traditional morphological and molecular DNA-based techniques. For example, the morphological result showed that two species, *B. bassiana* and *B. pseudobassiana,* were similar with respect to colony colour and conidial shape, but *B. pseudobassiana* was, on average, smaller in terms of conidial size. Imoulan et al. (2017) showed that colonies of these two species could be distinguishable from each

other as *B. pseudobassiana* colonies were dark yellow to light yellow while colonies of *B. bassiana* were white to off-white and became powdery when old. In the current study, *B. pseudobassiana* colonies ranged from pale yellow to yellow to white. This colouration is similar to the description of *B. pseudobassiana* given by Rehner et al. (2011) and Wang et al. (2020). Other examples include *B. caledonica* and *B. malawiensis* that produced colonies that were distinguishable as *B. malawiensis* produced a pink pigment and developed conidia of larger size compared with *B. caledonica*. This result fits with the description of Cummings (2009) and Glare and Inwood (1998). While *B. bassiana, B. caledonica*, and *B. malawiensis* displayed unique morphological characteristics, which allowed them to be distinguishable from other species within the genus, *B. bassiana* and *B. pseudobassiana* were still very similar in appearance and were difficult to identify using only colony morphologies. Previous studies have established that the morphological approach is not dependable enough to classify species-rich genera such as *Beauveria* (Cummings, 2009; Imoulan et al., 2017), and with the advent of DNA-based approaches, more sophisticated methods are now available to answer these research questions around species identity.

Identification using molecular approaches is the most reliable method to track the evolution of *Beauveria* species, where morphological methods cannot be used. For instance, in this study, isolates J2, Mo1, CTL20, and CTA20 were isolated from infected DBM cadavers, likely through infection of the DBM colony by isolate J18. Thus, tracking descendent can only be achieved using the molecular method (Bustamante et al., 2019). Many significant studies on the evolution of *Beauveria* used the molecular approach (Kepler et al., 2017a; Khonsanit et al., 2020; Rehner et al., 2011; Wang et al., 2020). DNA extraction using the Chelex-100 method was convenient, and it was easy to get *Beauveria* DNA for molecular experiments. Previous studies, including Alizadeh et al. (2017) and Hennequin et al. (1999), found that using this method was effective for extracting microbes' genomic DNA. However, the first attempt resulted in sequencing results of poor quality, possibly due to the Chelex-100 bead matrix inhibiting the reaction (Walsh et al., 2013). By using PCR clean up, the resulting sequences were of high quality with sharp peaks and fewer misreads. Therefore, cleaning up PCR products before sequencing is an important step worth implementing (Korosi et al., 2019; Macherey-Nagel, 2017).

DNA sequencing results using the EF1-α and BLOC markers categorised all 14 isolates into four different *Beauveria* species, which were *B. bassiana* (isolates F615, J2, Mo1, TPP-H, J18, FRh2, O2380, CTL20, and CTA20), *B. caledonica* (isolate F532), *B. malawiensis* (isolate Bweta), and *B. pseudobassiana* (isolates FRhp, FW Mana, and I12 Damo). Based on the dendrogram in Appendix B2.1, nucleotide data obtained from PCR fragments amplified using the primer pair B22U and B822L of BLOC showed more distinctive individual species than primers 983F and 2218R of EF1-α. The latter lack introns often useful in distinguishing Hypocreales (Rehner & Buckley, 2005). However, when integrating with a suitable nested PCR protocol, the EF1-α region was highly sensitive and specific for detecting *Beauveria* isolates in maize and non-sterile soil (McKinnon et al., 2018). BLOC primers are explicitly designed for the

identification *of Beauveria* (Fisher et al., 2011; Rehner et al., 2006b); therefore, they gave specific introns for each species. Both the MUSCLE and Geneious alignment methods were suitable for the multiple alignments of large nucleotides within the combined DNA regions, where Clustal Omega could not separate best pairs into individual sequences. Different alignment methods can impact the phylogenetic relationship results (Abou-Shaara, 2020).

Building a phylogenetic tree using multi-locus phylogenetic analysis is the most reliable strategy for tracing *Beauveria* evolution. The use of multi-locus sequence data is necessary to establish robust species boundaries of *Beauveria*, as this genus has simple structures and lacks distinguishable phenotypic features (Bustamante et al., 2019; Chen et al., 2018; Kepler et al., 2017b; Rehner et al., 2011; Wang et al., 2020). Based on Figure 2.4, the combined loci tree showed that every isolate was well supported in its species. Geneious tree builder using the Neighbor-Joining method with the Juke-Cantor model provided high quality and fast tree generation. This tree building method has been commonly used with evolutionary data (Boardman et al., 2020; Jung et al., 2021; Skantar et al., 2020; Wan et al., 2020). Therefore, building a phylogenetic tree based on multi-locus data using Geneious tree builder is a simple, quick, and reliable method for the identification of *Beauveria*.

2.5 Conclusion

The species identities of 14 *Beauveria* isolates were confirmed using morphological and molecular methods. Single-spore isolation is a practical and straightforward approach for fungal purification, and the Chelex-100 method is a convenient and easy method for fungal genomic DNA extraction. Colonies of the 14 *Beauveria* isolates ranged in colour from white to pale yellow and pink to red, while their conidial shapes ranged from globose, subglobose to broadly ellipsoid to ellipsoid, oblong or cylindrical. Conidial sizes ranged from 2.48 x 2.01 µm for *B. bassiana*, 2.16 x 1.8 µm for *B. pseudobassiana*, 3.6 x 2.14 µm for *B. caledonica*, and 3.8 x 2.00 µm for *B. malawiensis*. The primers EF1-α (983F and 2218R) and BLOC (B22U and B822L) were confirmed as reliable markers suitable for *Beauveria* amplification. Phylogenetic tree building using combined sequence data in Geneious tree builder was confirmed as a simple, quick, and reliable method. The integration of morphological and molecular techniques is a convenient way for *Beauveria* identification.

Chapter 3

Assessing the virulence of selected *Beauveria* isolates towards diamondback moth larvae

3.1 Introduction

Primary infection of *Beauveria* spp. generally commences when the asexual spores of the fungus physically attach to the insect cuticle. The spores germinate and subsequently start to penetrate the cuticle with the aid of hydrolytic enzymes (i.e., proteases, lipases and chitinases), mechanical pressure, and other factors (Ortiz-Urquiza & Keyhani, 2013). During the colonising process, the fungus produces antimicrobial compounds that help suppress host immune defence, destroy internal tissues, and absorb nutrients that eventually leads to host death. The pathogenesis and virulence of *Beauveria* are related to the internal toxic metabolites, cuticle degradation, anti-oxidant enzymes, and active vegetative growth of hyphae that cause host insects to die of nutrient depletion (Mascarin & Jaronski, 2016; Zimmermann, 2007). Most *Beauveria* spp. produce toxic secondary metabolites to kill their insect hosts. Two common insecticidal metabolites produced by *Beauveria* species are beauvericin and bassianolide (Grove & Pople, 1980; Singh et al., 2015; Xu et al., 2009a; Xu et al., 2009b; Zimmermann, 2007). *B. bassiana* and *B. pseudobassiana* produce different metabolite profiles (Wang et al., 2020) and different amounts of these metabolites when grown on different types of synthetic laboratory media (Berestetskiy et al., 2018). A study that screened 43 isolates for their ability to control lepidopteran pests, including DBM, showed that while all the isolates provided significant mortality compared to the control, only some of them gave high mortality (Wraight et al., 2010). These results demonstrated that the different isolates of *Beauveria* provided diverse efficacies in controlling targeted insects.

Innate immune responses play a significant role in protecting insects, including lepidopteran arthropods (Casanova-Torres & Goodrich-Blair, 2013), against infection by bacteria, fungi, and viruses (Xia et al., 2015; Zhang et al., 2020). For instance, several conserved signalling pathways such as Toll, IMD, JNK, and JAK-STAT found in the midgut of diamondback moth larvae (DBM) may activate the expression of antimicrobial peptide (AMP) genes involved in enhancing immune responses to fight against a bacterial-based biopesticide (Lin et al., 2020). Thus, understanding the interactions between a particular pest's innate immune system and its associated entomopathogens is crucial for developing new biopesticides (Xia et al., 2015). Some strains of *Beauveria* spp. may possess different mechanisms of action that can kill target pests using their unique characteristics such as penetration speed and production of toxins that can overcome the immune system (Jaronski, 2010). Godonou et al. (2009) in the Republic of Benin showed that *B. bassiana* Bba5653 caused a significantly higher level of mortality

than other isolates of the same species indicating much diversity within this species. Furthermore, reports show that isolates of *B. bassiana* and *B. pseudobassiana* isolated from Coleoptera caused higher mortality for coleopteran insects than ones isolated from other habitats, and this was also true for lepidopteran pests (Chen et al., 2015; Huang et al., 2014; Wang et al., 2020). This may indicate a level of host specificity for this entomopathogen.

DBM larvae were found dead on the 7th day after spraying with 10^9 conidia/mL (Nithya et al., 2019). Providing 100% humidity for the first 24 hours is necessary to allow fungal infection (Wraight et al., 2010). Bioassays are a type of *in vitro* experiment that tests the effectiveness of a stimulus treatment to a living organism under controllable conditions (Bruck, 2009). This kind of experiment provides insight into the susceptibility of the target organism, which then underpins decisions on whether or not to conduct semi-control (greenhouse) and open field experiments. Therefore bioassays can save a large amount of money and time. Thus, they must be carefully designed to obtain statistically valid biological data (Robertson et al., 2017). To get accurate data, the bioassay experiment must include randomisation test subjects, appropriate control for comparison, sufficient replicates, and reliable methods (Scharf, 2008).

The primary objective of the experiments reported in this chapter was to assess and rank 14 selected isolates of *Beauveria* with respect to their pathogenic potential towards DBM larvae.

3.2 Materials and methods

3.2.1 Source of DBM larvae

Third instar larvae (light green with dark brown head) of DBM were obtained from a laboratory colony maintained at the Bio-Protection Research Centre, Lincoln University, New Zealand. In 2014, DBM moths were firstly collected from a cabbage field at Lincoln University and used to establish a colony. All colonies were routinely fed on cabbage (six-week-old cabbage leaves were used). The colony was kept within controlled temperature rooms at a temperature of 25 ± 2^{o}C with a 12:12 day:night light period). A soft brush was used to collect larvae from cabbage leaves for the following assays. All larvae collected were of a similar size and healthiness (i.e., they were all active, feeding and green in colour) (Figure 3.1).

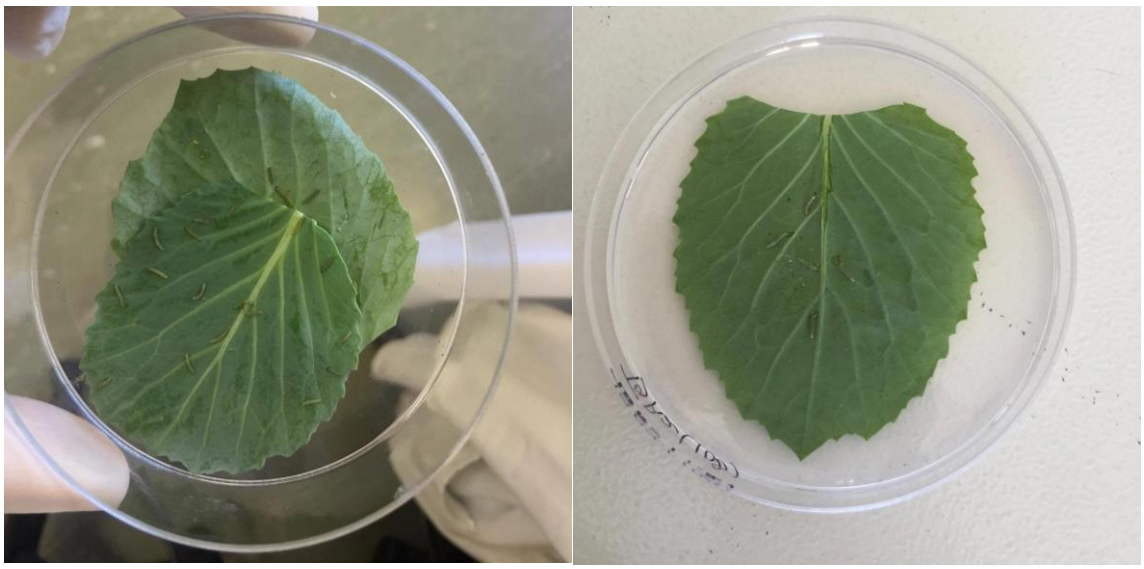


Figure 3.1: Third instar larvae of DBM collected for the assay in a deep Petri dish (left) and five larvae in a standard Petri dish on a cabbage leaf resting on water agar ready for the assay (right)

3.2.2 Fungal isolates

Eleven *Beauveria* isolates were obtained from the Lincoln University culture collection and maintained on PDA. One isolate was obtained from AgResearch, Palmerston North, and two isolates were isolated directly from dead larvae (CTL20) and adults (CTA20) of DBM from the CT rooms at Lincoln University. For further information on these isolates, refer to Table 2.1. The 14 isolates were sub-cultured at a temperature of 23 ± 1^{o}C and 12:12 D/L on PDA, within Petri plates, several times until fungal colonies were growing uniformly. After colonies had been produced from single-spore isolations, a further subculture was undertaken, and spores from the subsequent three-week-old colonies were harvested for the following bioassays.

3.2.3 Bioassay procedure

Spores of *Beauveria* were harvested by pipetting 4 mL 0.01% Triton X -100 onto the colonies and scraping gently with a metal hockey stick. The resulting crude suspension was gently poured from the Petri plates into a 15 mL centrifuge tube and shaken vigorously by hand until well mixed. Spore concentrations were estimated by using a Neuman Bayer Haemocytometer Chamber. For these bioassays, all full-strength solutions from each isolate were diluted 100 times using Triton X -100. The conidial suspensions were immediately placed at 4^{o}C until required.

Six-week-old cabbage leaves (cv. Arisos NS, South Pacific Seeds) were collected from plants growing in a glasshouse and washed with 0.01% Triton X-100 before being placed within a Petri plate containing water agar (WA), prepared at 12 g/L. This medium kept the detached cabbage leaves fresh and stopped them from drying out (Figure 3.1). Five DBM larvae at the third instar stage were transferred to the surface of each leaf, and the plates were covered by breathable lids. A Potter Spray

Tower (designed by C. Potter, Burkard Manufacturing Col Ltd., England) was used to spray conidia onto the Petri dish (lids removed) (Figure 3.2) (Herron et al., 1995). The apparatus is capable of providing good replication and an equal distribution on a circular area of 9 cm in diameter (Potter, 1952). Before spraying, the liquid reservoir tube of the tower was cleaned with 1.2 mL of 70% ethanol and rinsed twice with 1.2 mL of 0.01% Triton X-100 with the fan on. A 0.05 cm diameter atomiser was used, and the height of the spray table was adjusted to achieve an even distribution over the Petri dishes. Plates were sprayed with a 600 µL conidial suspension from each isolate from low to high rates of each isolate (i.e. from 10^5 to 10^7, and to 10^8 conidia/mL) while the control treatment was sprayed with 600 µL of Triton X-100. As the spray was only 600 µL out of 1 mL, the actual application rates were 6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray. The tower was cleaned between each isolate using the same procedure as before spraying. Each rate and control had two replicates. After spraying, all Petri dishes were placed on plastic trays (Figure 3.3) alongside a soaked tissue towel and covered by a plastic bag to retain high humidity. Trays were incubated at 23°C (12:12 D/L). Mortality assessments of DBM were undertaken daily using an Olympus SZX12 stereomicroscope connected to an Olympus camera with a cell Sens software system after inoculation for seven days. DBM larvae were considered dead when they did not respond to touch and had changed colour from green to brown or yellow. The assessment of mycosed cadavers to confirm that fungi had killed the larvae was conducted by placing them on glass slides on water agar for 24 hours (at 23°C under 12:12 D/L) and using an Olympus SZX12 stereomicroscope (Figure 3.4).

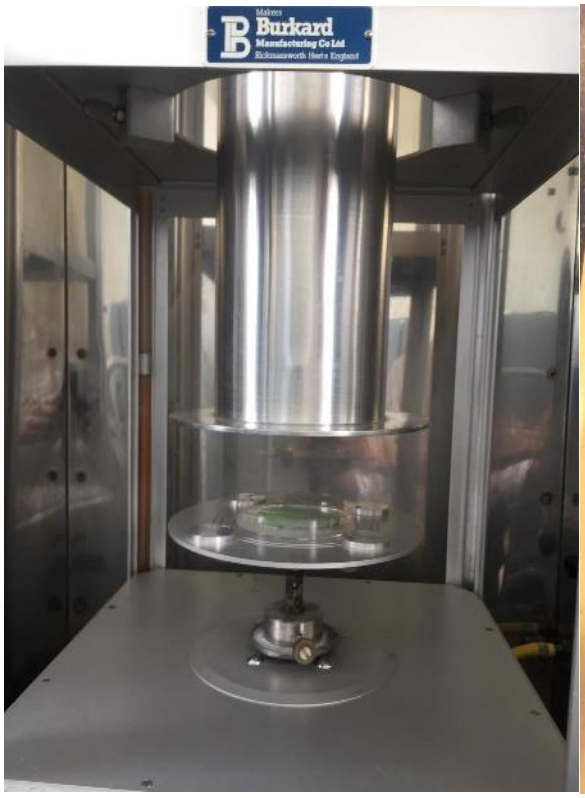


Figure 3.2: Potter spray tower

Figure 3.3: Plastic tray containing 15 Petri dishes

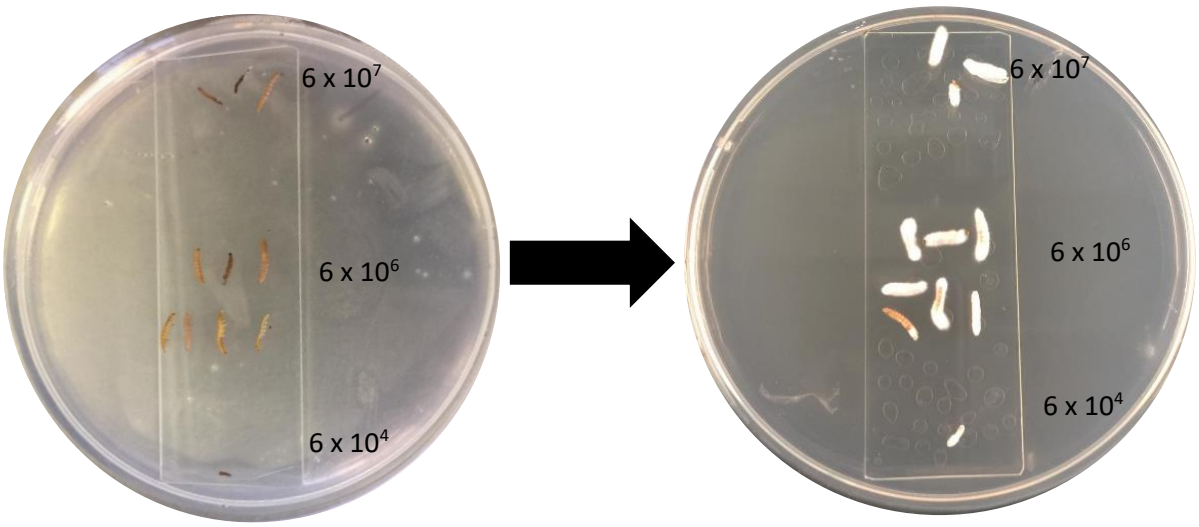


Figure 3.4: Example of a mycosed cadaver assessment for isolate *B. pseudobassiana* I12 Damo, after 24 hours on water agar at 23°C under 12:12 D/L

3.2.4 Experimental design

The experiments were repeated three times to confirm the consistency of each isolate and application rate (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray). For each experiment, the isolates were prepared fresh and in a different order. Within a total of 330 Petri dishes, 1650 larvae were used in these three assays (550 larvae per assay). The design was randomised trays in the incubator, with one isolate with two replicates and three application rates followed by two controls before starting another isolate.

3.2.5 Statistical analysis

All data collected was inputted in Microsoft Excel 2016 and analysed using Genstat software (20th Edition). The larval mortality was analysed by Probit analysis using the Genstat 20th Edition (Payne et al., 1987) to examine the lethal concentration for 50% (LD_{50}) and 95% (LD_{95}) of the population (Wheeler et al., 2006). Before performing the Probit analysis, the logarithm to base 10 was used to transform rates into a normal distribution dataset to obtain the familiar bell-shaped curve (Glaser & Biggs, 2010). Natural mortality was assessed using the control treatment (0.01% Triton X-100), and less than 20% was acceptable (Glare et al., 2008) or otherwise, the assay was repeated. Then the natural mortality was corrected using Abbott's formula to adjust the data. Corrected Mortality = $\frac{\%MT-\%MC}{100-\%MC} \text{x } 100$, where %MT is mortality in the treatment, %MC is mortality in the control (Abbott, 1925). For multiple comparisons of LD_{50} and LD_{95}, each replicate was analyzed separately before organizing for ANOVA analysis. Tukey's honestly significant difference (HSD) test was used to compare individual treatment means (Williams & Abdi, 2010). The median lethal time (LT_{50}) was calculated to

test for the speed of kill of each isolate. Some isolates caused lower than 50% mortality within the experimental time, so the expected days for them to achieve LT_{50} could not be calculated using the formula $LT_{50} = [ND_{50} \times (M_{50} - 1) + DB \times (NM_{50} - M_{50})]/(NM_{50} - 1)$, where ND_{50}: number of days that mortality reached 50%; M_{50}: 50% mortality of tested larvae; DB: the day started to die before 50% mortality; and NM_{50}: total mortality on ND_{50} (personal communication, Dave Saville, 20 April 2021). Therefore, for some isolates LT_{50} prediction were determined using the formula $LT_{50} = (M_{50} \times ND)/MD$, where M_{50}: 50% mortality of tested larvae; ND: number of days tested; and MD: total mortality of days tested.

3.3 Results

3.3.1 Mortality results

All the *Beauveria* isolates assessed were virulent to DBM larvae, with the mortality rate ranging from 4% to 100% within seven days after inoculation. Mortality caused by the 14 isolates was significantly different (df = 28, deviance = 605.3) (Table 3.1). *B. bassiana* Mo1, *B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, *B. pseudobassiana* I12 Damo, *B. bassiana* CTL20, and *B. bassiana* CTA20 at 6 x 10^7 conidia/spray killed up to 100% of larvae. Interestingly, *B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, *B. pseudobassiana* I12 Damo, and *B. bassiana* CTL20 achieved 100% kill at the lower rate of 6 x 10^6 conidia/spray (Figures 3.5 & 3.6). After mortality was corrected using Abbott's formula, these four isolates also provided 100% mortality (Table 3.2).

Table 3.1: Statistical analysis of all 14 isolates, Logistic Regression, Probit test

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	28	605.3	21.62	21.62
Residual	136	273.7	2.01	
Total	164	879.0	5.36	

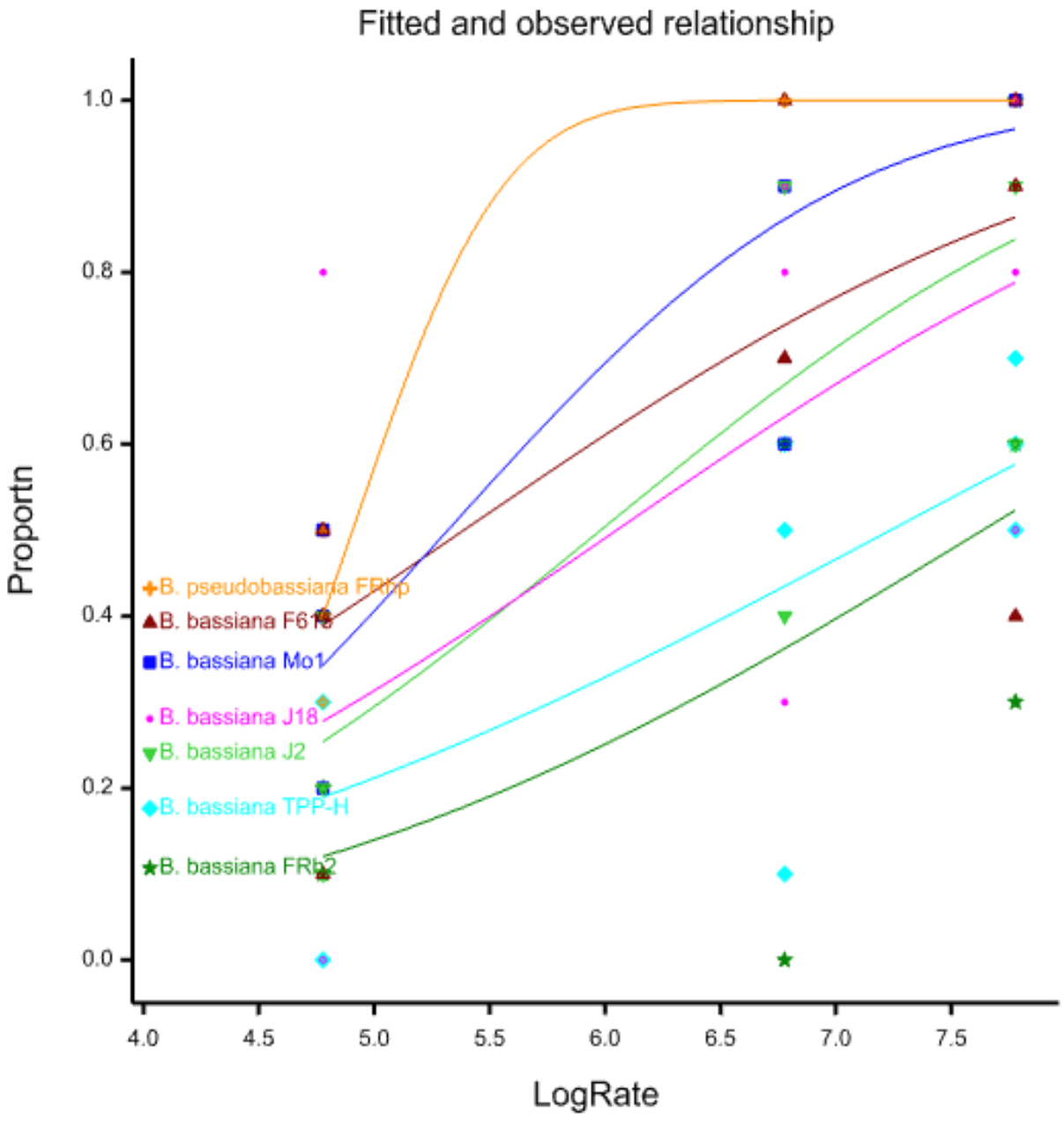


Figure 3.5: Mortality response curves for seven *Beauveria* isolates with three application rates; rates were transformed with Log to base 10 in Logistic Regression, Probit test

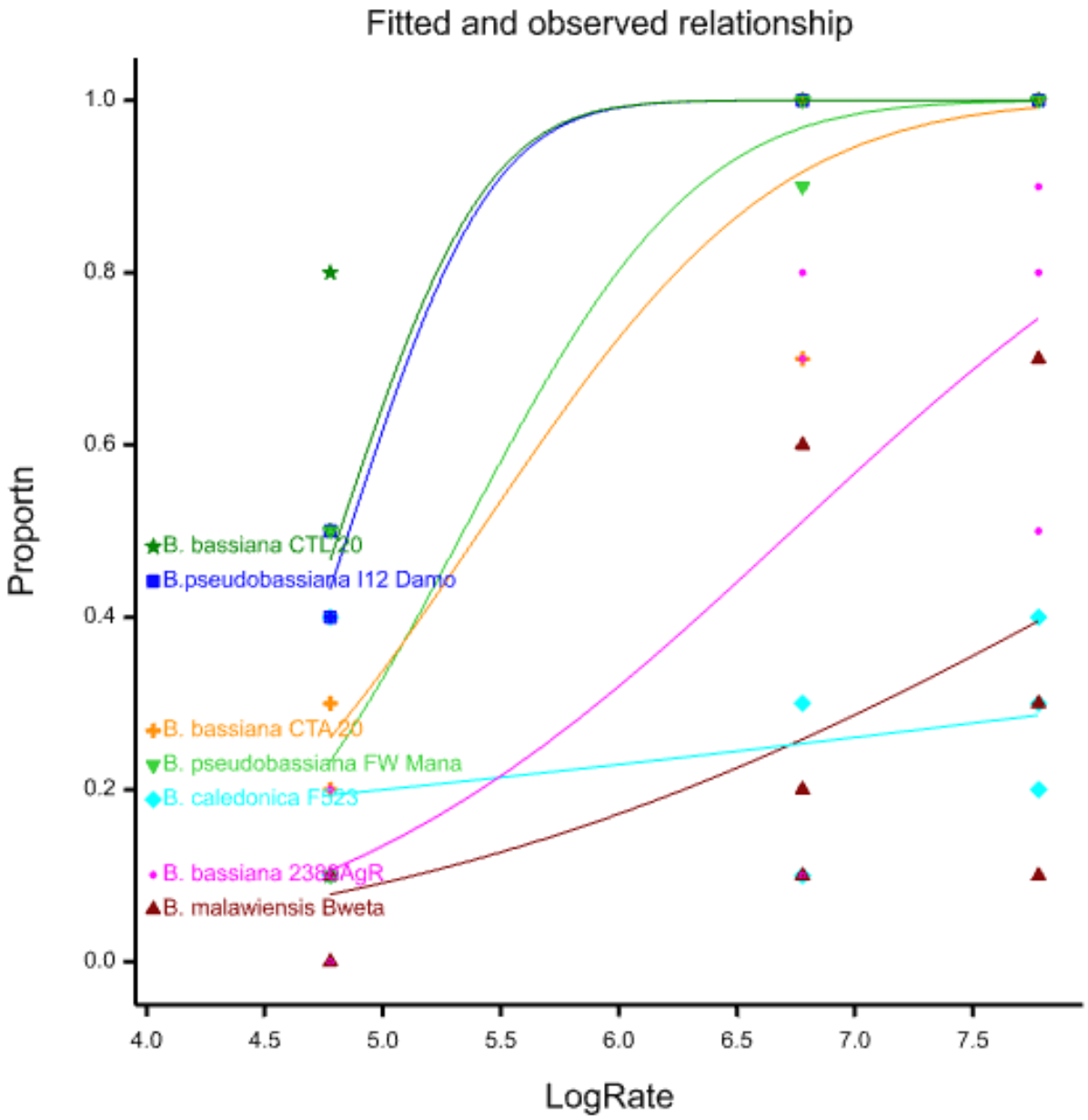


Figure 3.6: Mortality response curves for seven *Beauveria* isolates with three application rates; rates were transformed with Log to base 10 in Logistic Regression, Probit test

Table 3.2: Mortality (%) after correcting for control mortality using Abbott's formula

Isolate	Inoculum (conidia/spray)		
	6×10^4	6×10^6	6×10^7
	Mean ± SE	Mean ± SE	Mean ± SE
B. bassiana F615	36 ± 1.46	89 ± 3.51 d	79 ± 3.05 ab
B. bassiana J2	25 ± 0.87	79 ± 2.37 cd	79 ± 3.60 ab
B. bassiana Mo1	36 ± 1.32	79 ± 3.73 cd	100 ± 4.02 b
B. bassiana TPP-H	14 ± 0.55	36 ± 1.57 abc	57 ± 1.59 ab
B. bassiana J18	25 ± 0.76	68 ± 2.37 bcd	79 ± 2.90 ab
B. bassiana FRh2	14 ± 0.37	14 ± 0.55 a	68 ± 2.12 ab
B. pseudobassiana FRhp	36 ± 1.91	100 ± 4.19 d	100 ± 4.08 b
B. malawiensis Bweta	4 ± 0.03	25 ± 0.37 ab	36 ± 1.93 a
B. pseudobassiana FW Mana	25 ± 0.87	100 ± 3.76 d	100 ± 4.46 b
B. pseudobassiana I12 Damo	46 ± 2.87	100 ± 2.31 d	100 ± 4.30 b
B. caledonica F532	14 ± 0.28	25 ± 1.20 a	25 ± 1.30 a
B. bassiana O2380	4 ± 0.41	57 ± 2.22 abc	79 ± 3.64 ab
B. bassiana CTL 20	46 ± 1.60	100 ± 4.02 d	100 ± 3.05 b
B. bassiana CTA 20	25 ± 1.26	89 ± 4.76 d	100 ± 3.84 b
F-value	1.15	10.54	6.01
P-value	0.364	<.001	<.001

Note: Letters after each % represent significance groups. There were no significant differences at 6×10^4 conidia/spray (Tukey multiple comparisons, general ANOVA test)

3.3.2 Cumulative mortality

The mortality of DBM larvae began two days after inoculation for most of the 14 isolates at all application rates. At 6×10^7 conidia/spray, almost all isolates caused mortality faster than at 6×10^6 and 6×10^4 conidia/spray. At 6×10^4 conidia/spray, mortality ranged from 4% to 46% by day seven (Figure 3.7). Increasing the application rate to 6×10^6 conidia/spray allowed seven isolates to achieve 80% mortality or greater by day seven, with *B. pseudobassiana* I12 Damo and *B. pseudobassiana* FRhp achieving 100% mortality day six post-treatment (Figure 3.8). At 6×10^7 conidia/spray, isolate *B. pseudobassiana* I12 Damo, *B. pseudobassiana* FRhp, and *B. bassiana* CTA20 caused 100% mortality within six days (Figure 3.9).

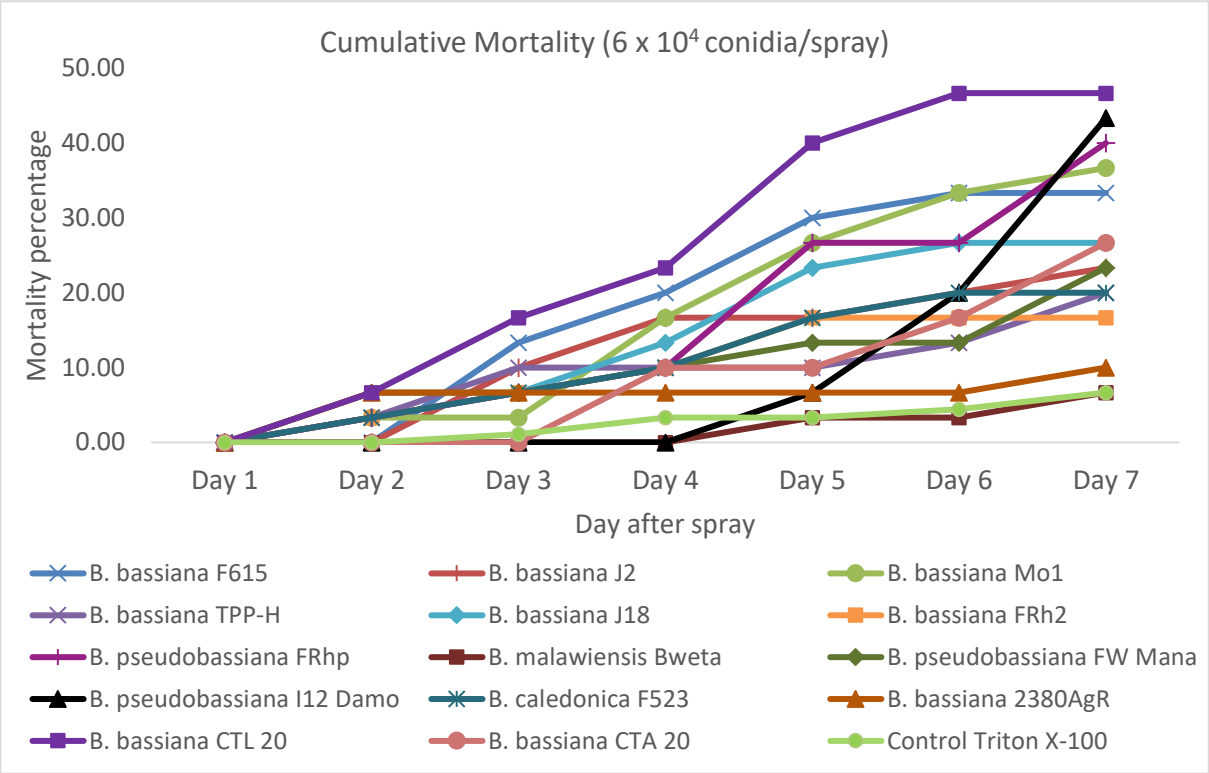


Figure 3.7: Cumulative mortality of DBM larvae over seven days after spraying at 6 x 10^4 conidia/spray

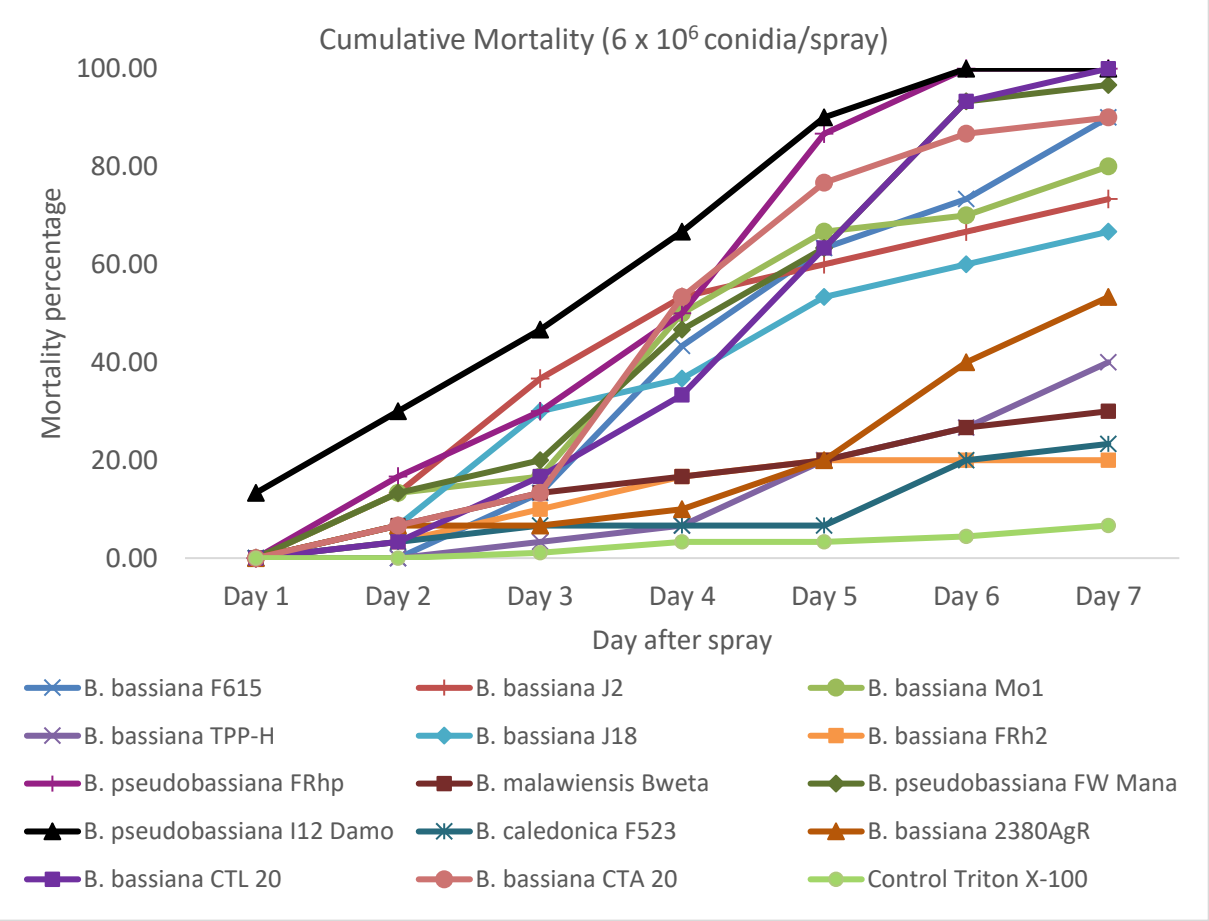


Figure 3.8: Cumulative mortality of DBM larvae over seven days after spraying at 6 x 10^6 conidia/spray

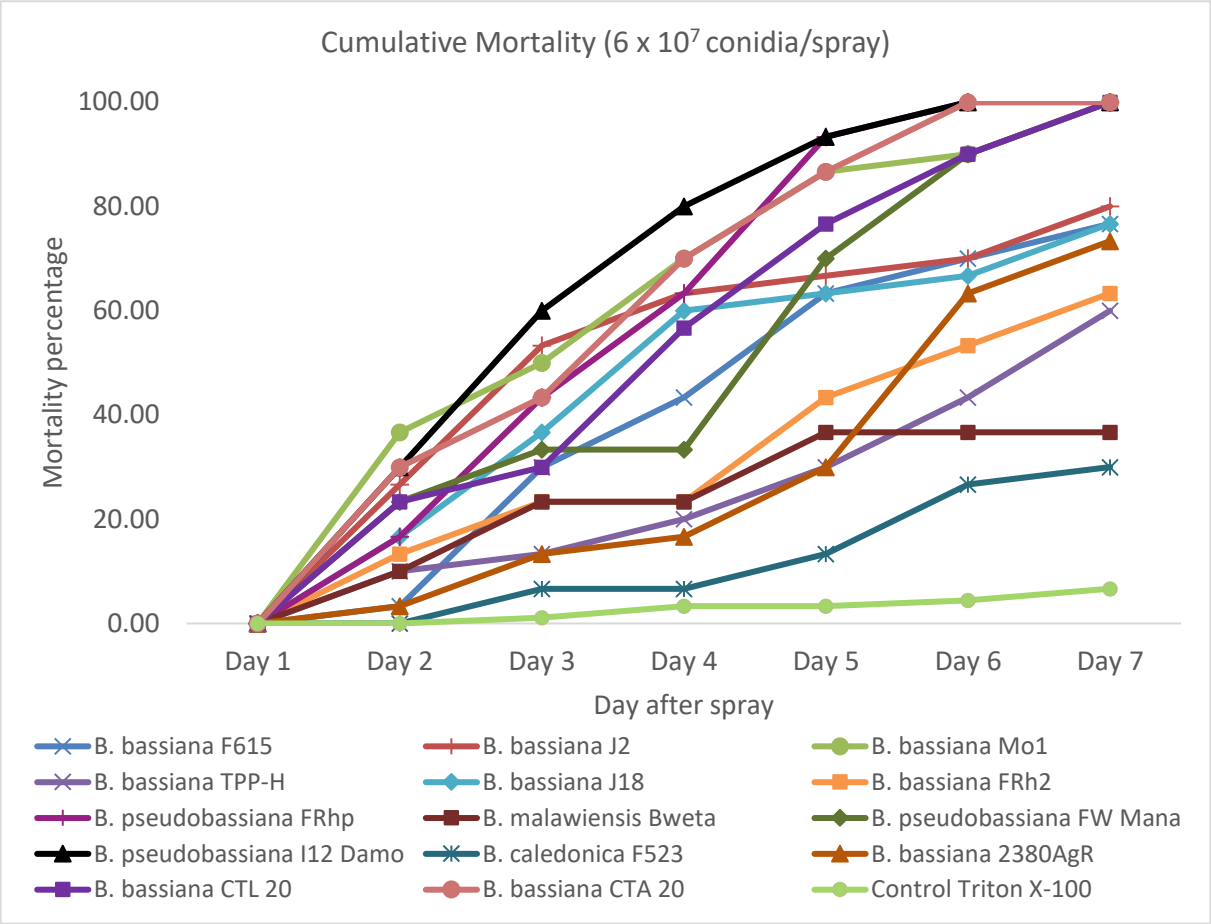


Figure 3.9: Cumulative mortality of DBM larvae over seven days after spraying at 6 x 10^7 conidia/spray

3.3.3 LD_{50} and LD_{95}

The most virulent isolates were *B. bassiana* Mo1, *B. pseudobassiana* FRhp, *B. pseudobassiana* I12 Damo, *B. pseudobassiana* FW Mana, *B. bassiana* CTA20 and *B. bassiana* CTL20. These were ranked as the top-performing group (a to abc in Figure 3.10), and each required application rates around 10^4 to 10^5 conidia/spray to achieve the LD_{50} (Figure 3.10). The second-ranked group of isolates included *B. bassiana* F615, *B. bassiana* J2, *B. bassiana* J18 and *B. bassiana* O2380 (group bcd), and these required an application rate of 10^6 conidia/spray to achieve a 50% mortality of DBM. The third-ranked group (cd and de) included isolates *B. bassiana* TTP-H and *B. bassiana* FRh2 that each needed an application rate of around 10^7 conidia/spray to kill 50% of the DBM. The least effective isolates were *B. caledonica* F532 and *B. malawiensis* Bweta, ranked in the fourth and last group e, which required around 10^8 conidia/spray to achieve the LD_{50} (Figure 3.10). Statistical significance data are also provided in Table B.1.1 (Appendix B).

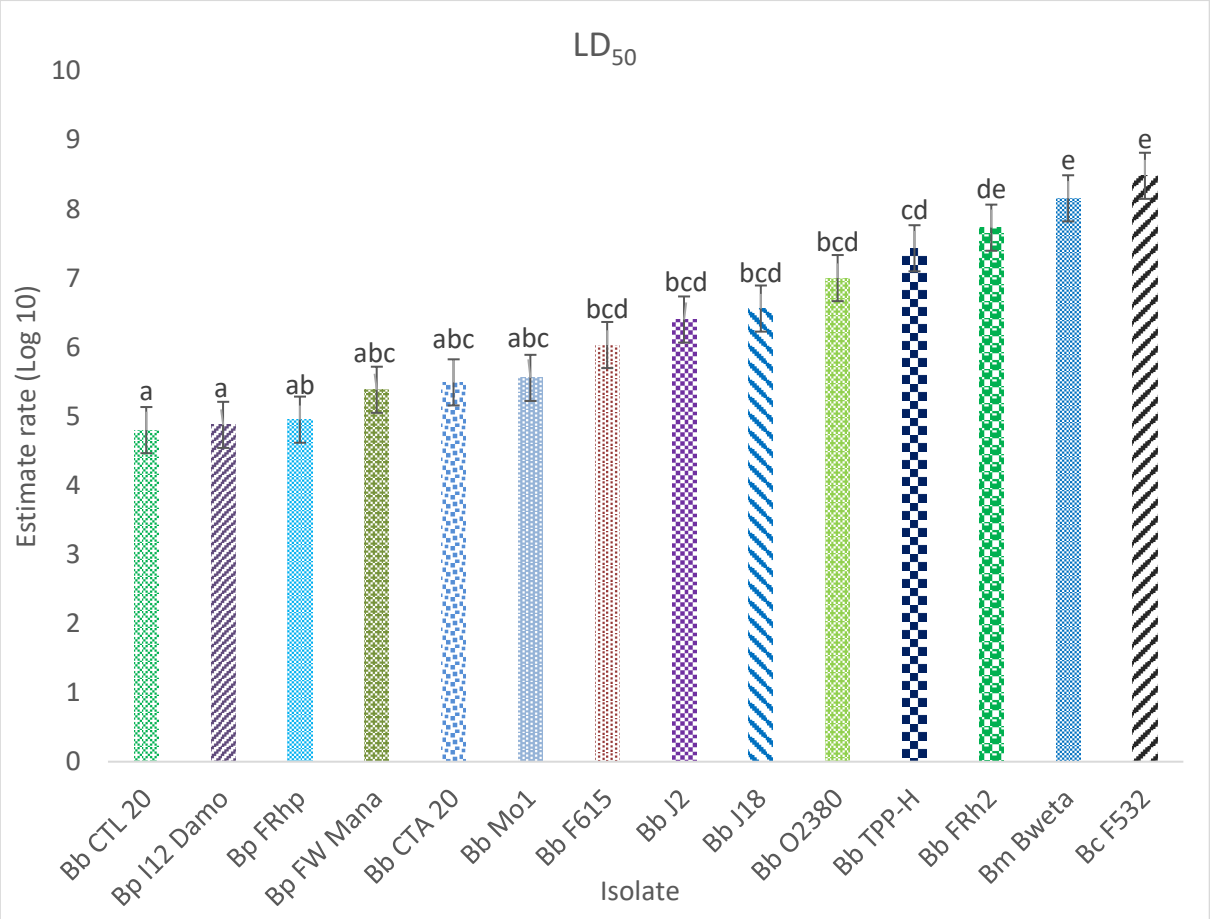


Figure 3.10: LD_{50} for all 14 isolates divided into five significance groups, for which the natural scale estimation was transformed by Log10, ANOVA test (Error bar based on standard error, grouped using LSD 95%, multiple comparisons using Tukey test)

*** Note: Bb:** *Beauveria bassiana*, **Bc:** *Beauveria caledonica*, **Bm:** *Beauveria malawiensis*, **Bp:** *Beauveria pseudobassiana*

The lethal dose required to kill 95% of larvae (LD_{95}) for each isolate differed slightly from the LD_{50} results (Figure 3.11). The most effective isolates, *B. bassiana* Mo1, *B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, *B. pseudobassiana* I12 Damo, *B. bassiana* CTL20, and *B. bassiana* CTA20 (group a and ab), required around 10^6 conidia/spray to 10^7 conidia/spray to reach LD_{95}. The second group of isolates *B. bassiana* F615, *B. bassiana* J2, and *B. bassiana* J18 (group bcd and cd) required 10^8 conidia/spray to cause 95% mortality. Isolates *B. bassiana* TPP-H *B. bassiana* FRh2 and *B. bassiana* O2380 were placed in the third group (group cde and de), which needed around 10^9 conidia/spray to achieve LD_{95}. The least virulent isolates *B. caledonica* F532 and *B. malawiensis* Bweta (group e), required 10^{10} conidia/spray to cause 95% mortality (Figure 3.11). Statistical significance data are also presented in Table B.1.2 (Appendix B).

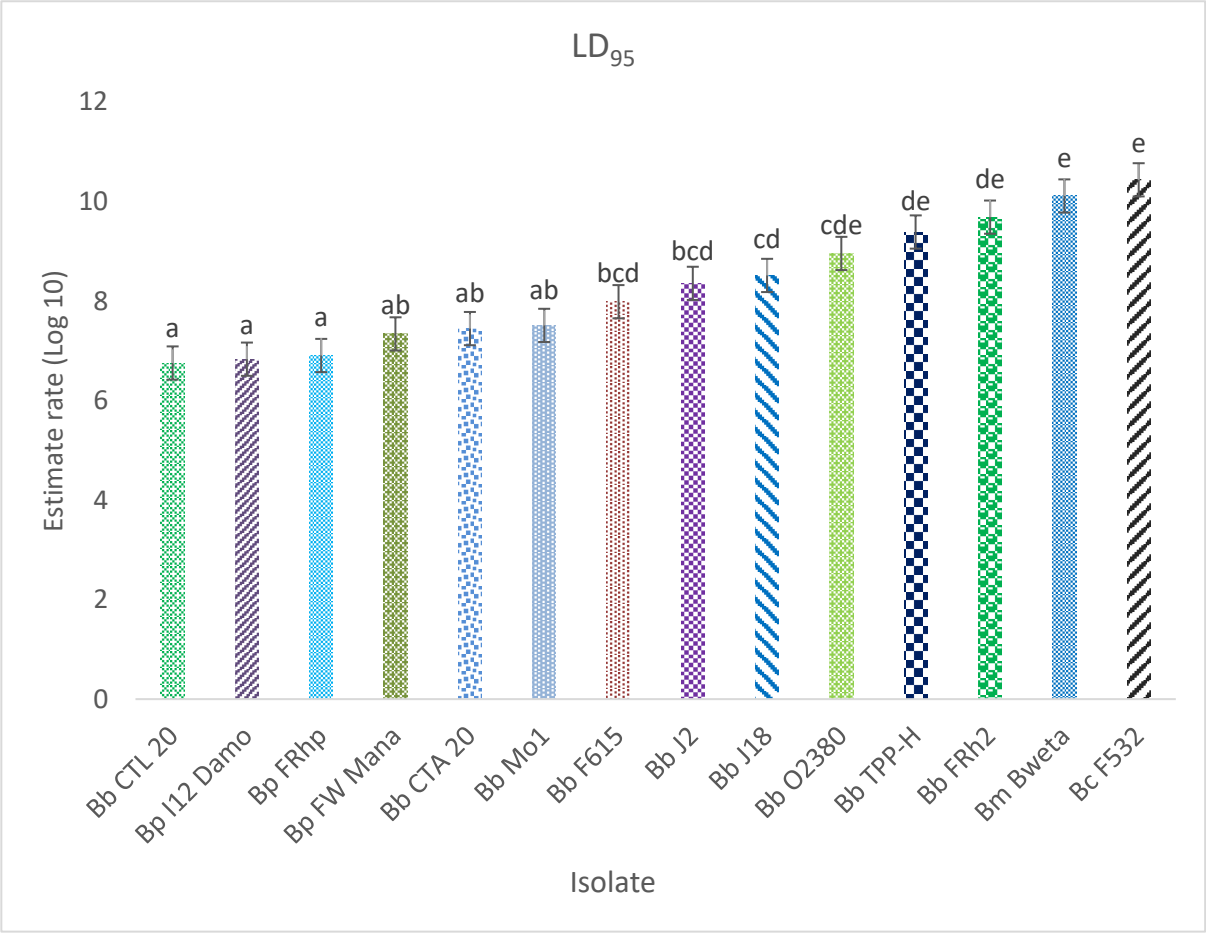


Figure 3.11: LD_{95} for all 14 isolates divided into significance groups, for which the natural scale estimation was transformed by Log10, ANOVA test (Error bar based on standard error, grouped using LSD 95%, multiple comparisons using Tukey test)

*** Note: Bb:** *Beauveria bassiana*, **Bc:** *Beauveria caledonica*, **Bm:** *Beauveria malawiensis*, **Bp:** *Beauveria pseudobassiana*

3.3.4 Median lethal time to kill (LT_{50})

At the application rate of 6 x 10^4 conidia/spray, all isolates tested did not differ significantly for LT_{50} ($F = 2.10$, $df = 13$, $p = 0.053$). The LT_{50} was statistically different at the application rate of 6 x 10^6 conidia/spray ($F = 2.33$, $df = 13$, $p = 0.032$). The isolates F615, Mo1, FRhp, FW Mana, I12 Damo, CTA20, and CTL20 needed around three to five days to reach 50% mortality and isolates J2 and J18 needed five to six days to kill 50%. These nine isolates were placed in the first group (a and ab). Isolates O2380, TPP-H, FRh2, Bweta, and F532 were placed in the second group (group b), which required longer than 15 days to achieve LT_{50}. At the application rate of 6 x 10^7 conidia/spray, the LT_{50} was significantly different ($F = 2.68$, $df = 13$, $p = 0.016$). Isolates J2, Mo1, FRhp, I12 Damo, CTL20, and CTA20 required two to three days to achieve LT_{50} and the remaining isolates required four to six days (group a and ab), except for isolate Bweta, which was placed in the second group (group b), which

needed longer than 17 days to cause 50% mortality (Figure 3.12). Overall statistical differences are described in Table B.1.3 (Appendix B).

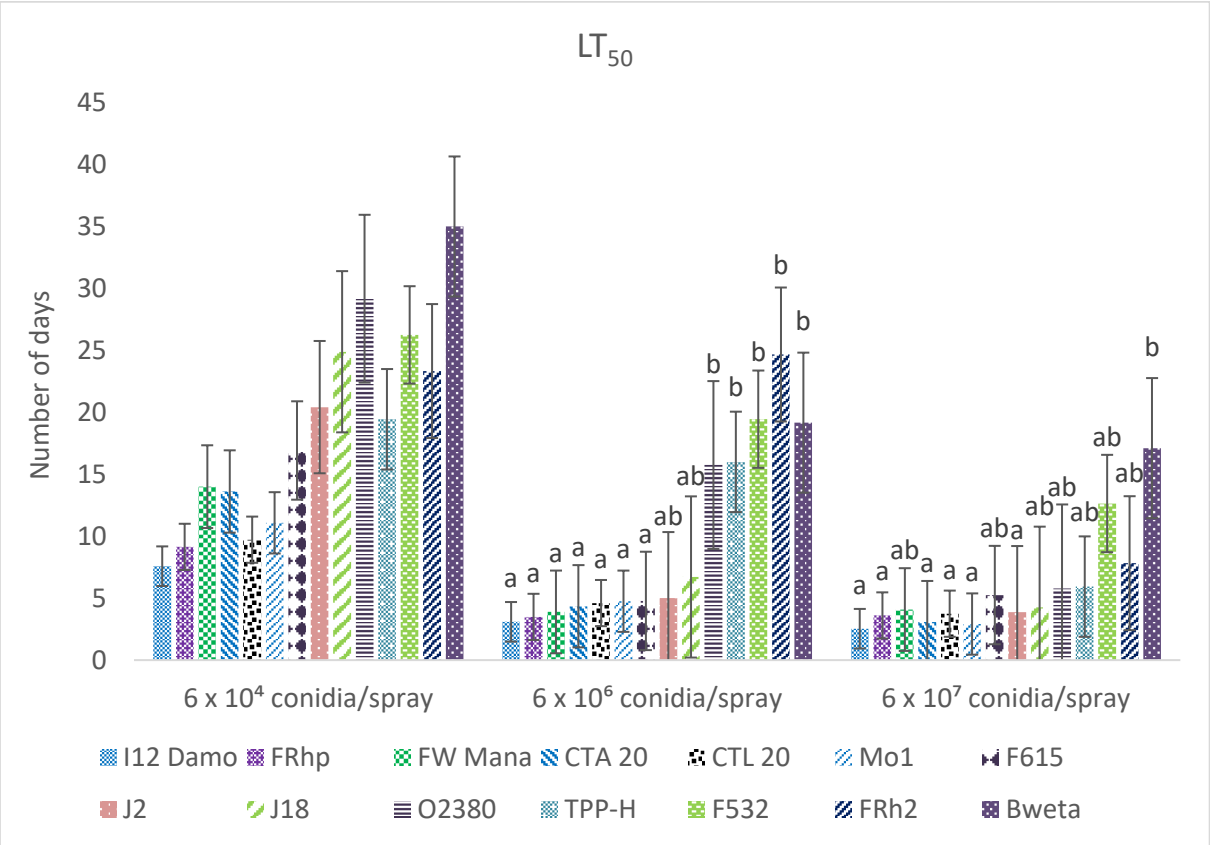


Figure 3.12: LT_{50} of the 14 isolates across the three application rates (Error bar based on standard error, grouped using ANOVA LSD 95%, multiple comparisons using Turkey test)

3.3.5 Percentage of cadavers supported sporulation

All cadavers killed by isolates *B. pseudobassiana* I12 Damo and *B. bassiana* CTA20 at all three application rates supported sporulation within 24 hours. The other most infective isolates, *B. bassiana* CTL20, *B. bassiana* Mo1, *B. pseudobassiana* FRhp, and *B. pseudobassiana* FW Mana, only had 100% mycosed cadavers for the two highest application rates (6 x 10^6 and 6 x 10^7 conidia/spray). Surprisingly, isolate *B. bassiana* FRh2, the least effective isolate based on mortality, had 100% sporulation on cadavers at rates of 6 x 10^6 and 6 x 10^7 conidia/spray, while another less pathogenic isolate, *B. caledonica* F532, had 100% sporulation on cadavers at 6 x 10^4 conidia/spray application rate rather than the 6 x 10^6 and 6 x 10^7 conidia/spray application rates (Figure 3.13).

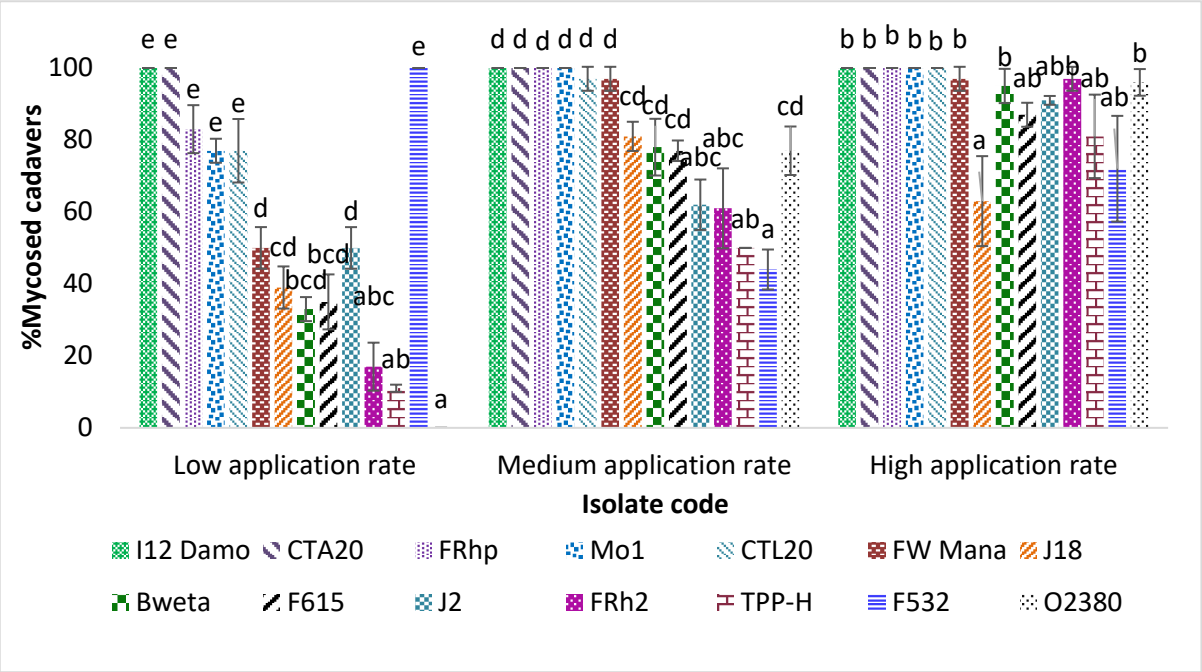


Figure 3.13: The percentage of diamondback moth larval cadavers that supported fungal sporulation after exposure to 14 selected isolates of Beauveria at three application rates (Error bars represent ANOVA, multiple comparisons using Tukey's HSD test)

3.4 Discussion

This study showed that all 14 *Beauveria* isolates assessed were virulent against DBM larvae. Six isolates (*B. bassiana* Mo1, *B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, *B. pseudobassiana* I12 Damo, *B. bassiana* CTL20, and *B. bassiana* CTA20) killed 100% of DBM larvae at 6 x 10^7 conidia/spray within seven days within the laboratory assays. These results align with previous studies with *B. bassiana* that showed up to 100% mortality of DBM larvae for isolates MG-Bb-1 in Japan (Yamada et al., 2009), IBCB01 in Brazil (Duarte et al., 2016), Bb11 in Togo (Agboyi et al., 2020), Bba5653 in the Republic of Banin (Godonou et al., 2009), GHA in the USA (Vandenberg et al., 1998a) and Australia (Furlong, 2004), and J18 in New Zealand (Narciso et al., 2019). Some of the isolates within this study were, however, much less virulent towards DBM. This finding was similar to the study of Wraight et al. (2010), who screened 43 isolates of *B. bassiana* and demonstrated different efficacies against lepidopteran pests, including DBM. In the present study, isolates *B. caledonica* F532 and *B. malawiensis* Bweta caused significantly lower mortality than the other 14 isolates. The study of Reay et al. (2008) found almost similar mortality with *B. caledonica* F532 when testing on *Hylastes ater* (Coleoptera), but a different result for isolate *B. malawiensis*, which caused high mortality against *H. ater*.

The fungal isolates that were originally derived from DBM larvae, *B. bassiana* Mo1, CTA20, and CTL20, caused higher mortality than other *B. bassiana* isolates derived from other invertebrate hosts. As these three isolates were obtained from infected DBM cadavers from the laboratory culture, they potentially derived accidentally from isolate J18. Recent studies have demonstrated it is possible that

passage through host insects can change the methylation patterns of entomopathogens to increase virulence. An isolate of *Metarhizium* was found to have improved virulence when passaging it through wax moth larvae (Hu & Bidochka, 2020). Studies indicate that *B. bassiana* isolates are generally more effective when applied to the same host as they were initially isolated from, which means these species inherit the characteristics of specification (Chen et al., 2015; Huang et al., 2014). While most of the current publications have only assessed isolates of *B. bassiana* against DBM (Agboyi et al., 2020; Duarte et al., 2016; Narciso et al., 2019; Wraight et al., 2010), our study also incorporated native New Zealand *B. pseudobassiana* isolates, and these outperformed most *B. bassiana* in terms of percentage mortality, killing speed, and lower application rate required. The three *B. pseudobassiana* isolates, FRhp, FW Mana, and I12 Damo, caused 100% mortality of DBM larvae within six days post-spray at rates of 6×10^6 and 6×10^7 conidia/spray. The same species was found to cause 100% mortality within six days after inoculation on two species of bark beetles (Kocacevik et al., 2016). Using genetically different isolates of *B. pseudobassiana* from different host insects, Wang et al. (2020) proved that those isolated from Lepidoptera species caused significantly higher mortality of silkworm compared to those from isolates derived from coleopterans, while those isolated from species of Coleoptera caused higher mortality of mealworm larvae. This study's results showed that *B. pseudobassiana* isolated from different coleopteran insects caused higher mortality of DBM larvae, which is a lepidopteran species, compared to flax weevil, the coleopteran host (Glare & Brookes, 2017). The opposite finding to this result indicated that coleopteran-derived isolates caused lower mortality than lepidopteran-derived isolates in virulence to *Pissodes nemorensis*, a coleopteran pest (Romón et al., 2017).

Berestetskiy et al. (2018) showed that both *B. bassiana* and *B. pseudobassiana* produce different amounts of toxins when cultured on different media, and Wang et al. (2020) showed that these two species could produce diverse metabolite profiles on different media. Therefore, based on the current result, the three *B. pseudobassiana* isolates may have produced more insecticidal properties against DBM than other species, or they may contain some genes that can overcome the insect immune responses. For instance, an experimental assessment of the virulence level of *B. bassiana* and *B. pseudobassiana* to *P. nemorensis* showed that *B. pseudobassiana* induced more positive expression of virulence genes such as chitinase and a protein kinase than *B. bassiana* to the insect's immune response genes, which resulted in higher mortality (Romón et al., 2017).

The six most effective isolates from this study belonged to *B. bassiana* and *B. pseudobassiana,* which caused 100% mortality of DMB. Furthermore, these isolates also caused 50% mortality of DBM at the lowest application rate. For instance, they required only around 10^5 conidia/spray for an LD_{50}, a result similar to previous findings by Vandenberg et al. (1998a), Nguyen et al. (2007) and Duarte et al. (2016) for DBM and other lepidopteran pests (Pour et al., 2021; Swathi et al., 2017). For *B. pseudobassiana* I12 Damo, the result was almost the same as the isolate *B. pseudobassiana* ARSEF 9271 when tested on two coleopteran pests of pine (Kocacevik et al., 2016), which required an

application rate of 10^5 conidia/mL for 50% mortality. Furthermore, among the six most effective isolates of this study, four were more prolific spore producers and produced up to 2 x 10^9 conidia/mL within three weeks while cultured on PDA at 12:12 D:L and 23 ± 1°C. From an economic perspective, these efficacious and high sporulating isolates show promise as commercial biopesticides (Litwin et al., 2020; Mascarin & Jaronski, 2016).

The median lethal time (LT_{50}) at the 6 x 10^7 conidia/spray application rate ranged from two days for highly virulent isolates to 17 days for least virulent isolates (Figure 3.15). At the same application rate, Nithya et al. (2019) found the most effective isolate required 3.62 days to achieve LT_{50} against DBM larvae, which was similar to isolates FRhp, CTL20, and J2 but slower than isolates I12 Damo, Mo1, and CTA20 that reached LT_{50} in less than three days. However, when testing on another lepidopteran pest, Kirubakaran et al. (2014) found that the *B. bassiana* isolate MTCC7690 needed 4.64 days to achieve the LT_{50}, which was slower than the isolates FRhp, CTL20, and J2. At the application rate of 6 x 10^6 conidia/spray, three *B. pseudobassiana* isolates (FRhp, FW Mana, and I12 Damo) required around three days to achieve 50% mortality, while other isolates needed four days (CTA20, CTL20, Mo1, F615, and J2), six days (J18), 16 days (O2380 and TPP-H), 19 days (Bweta and F532), and 24 days (FRh2). Sun et al. (2007) found an LT_{50} of three days for highly virulent and four days for less virulent *B. bassiana* isolates. Compared to the *B. pseudobassiana* isolates in my study, this finding revealed the same $LT_{50,}$ but these authors used relatively higher conidial concentration. Furthermore, the LT_{50} of *B. bassiana* isolates in the present study were the same as the least virulent isolate of the study of Sun et al. (2007). Additionally, at the same rate of 10^7 conidia/mL, Medo et al. (2021) showed that a highly virulent *Beauveria* isolate achieved a 50% mortality of *Ostrinia nubilalis* at 5.5 days post-treatment, while the least virulent isolates required up to 21.3 days. As the current study screened more species of *Beauveria*, including *B. bassiana* and *B. pseudobassiana* than the study by Wraight et al. (2010), these isolates achieved the LT_{50} 1-2 days faster than achieved for most other *B. bassiana* and *B. pseudobassiana* isolates. However, at the application rate of 6 x 10^4 conidia/spray, all isolates in this study did not achieve LT_{50} within seven days after spraying. The most virulent *B. bassiana* isolate caused 50% mortality at 4.6 days at the rate of 1.3 x 10^4 conidia/mL, when assessed for activity against *Helicoverpa armigera* under laboratory conditions (Fite et al., 2020).

All isolates used sporulated on DBM cadavers, most sporulating when inoculated at a concentration of 6 x 10^7 conidia/spray. Among them, isolates I12 Damo and CTA20 gave 100% sporulation of cadavers across the three spore concentrations (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray). An electron microscopy study on the colonisation of dead second instar larvae of *Duponchelia fovealis* by *B. bassiana* showed that the hyphae of the fungus started to form reproductive structures six hours after inoculation. Within 54 hours, the fungus had completely colonised the cadaver (Baja et al., 2020). The ability of these entomopathogenic fungi to colonise a cadaver is highly dependent on species and genotype, which can impact their sporulation rate and the speed of cadaver

penetration and proliferation (Nguyen et al., 2007). Isolates Mo1, FRhp, FW Mana, and CTL20 resulted in 100% sporulation on cadavers for the 6 x 10^6 and 6 x 10^7 conidia/spray application rates.

3.5 Conclusion

All isolates assessed in this study were virulent to DBM larvae with a mortality rate ranging from 4% to 100% at application rates from 6 x 10^4 to 6 x 10^7 conidia/spray. Six isolates were highly virulent to DBM larvae, achieving 100% mortality within seven days post-treatment. Among these six, four isolates managed to kill 100% of larvae with an application rate of 6 x 10^6 conidia/spray. While *B. bassiana* is the only species previously reported as pathogenic to DBM, the results of this study proved that *B. pseudobassiana* isolates were also promising candidates for future DBM control. Most of the high virulence isolates had a lower LD_{50} and were at least partly able to reinfect in a population, as most sporulated quickly on cadavers. Surprisingly, those high virulent isolates required a shorter time than previously reported to reach LT_{50}. This characteristic makes them potential candidates for the development of commercial biopesticides for DBM control. Following these bioassay experiments, a greenhouse experiment is suggested for future work to confirm the bioassay results before field testing.

Chapter 4

Combining isolates of *Beauveria* for the control of diamondback moth larvae

4.1 Introduction

Combining two or more microorganisms in a single biopesticide application can improve the biological control agent's efficacy, commonly through a synergistic interaction. Synergy occurs when two or more agents give higher potency than the sum of the separate agents (Cedergreen, 2014; Roell et al., 2017). Using combined agents for synergistic effects is a relatively new approach for effective control of an invertebrate pest. For example, mixtures of *Bacillus thuringiensis* and *B. bassiana* provided better control than either individual organism for DBM (Xue et al., 2018), the cabbage looper (*Trichopluisa ni*) (Sayed & Behle, 2017), the Colorado potato beetle (*Leptinotarsa decemlineata*) (Wraight & Ramos, 2005; Wraight & Ramos, 2017), and the house fly (*Musca domestica* L.) (Mwamburi et al., 2009). However, combinations of *B. bassiana* (J18) with entomopathogenic bacteria such as *Yersinia entomophaga* (MH96) and *Brevibacillus laterosporus* (1951 and 1821) were effective only within *in vitro* laboratory bioassays, and there was no significant control improvement under field conditions (Narciso et al., 2019). A similar concept of synergy was reported as an effective method to control the grain weevil (*Sitophilus granaries* L.) (Coleoptera: Curculionidae) and rice weevil (*S. oryzae* L.) (Coleoptera: Curculionidae) using the integration of *B. bassiana* with *Isaria fumosorosea* (Mantzoukas et al., 2019). Additionally, the combination of *B. bassiana* and *Metarhizium anisopliae* (Iqbal et al., 2019) and the microsporidia *Paranosema locustae* (Tan et al., 2020) produced a synergistic effect against pulse beetle (*Callosobruchus chinensis*) and grasshopper (*Locusta migratoria*), respectively.

Super- and co-infection models have been proposed for mathematical models to predict efficacy when mixing two or more biocontrol strains together and can help predict the interaction between two microorganisms. The coinfection model can be predicted when the combined isolates cooperate to infect a host. However, in the superinfection or antagonism model, one strain may dominate another for infection of the host, which means that the weaker strain may provide no contribution to the effect and is therefore not a contributing factor to insect mortality (Nowak & Sigmund, 2002). The coinfection model may provide a faster kill or higher mortality rate than the sum of the single isolates due to competition to proliferate within the infected host (Kada & Lion, 2015). With the superinfection model, it is predicted that the combined strains can result in less virulence than the single strains (Massey et al., 2004).

Most studies on the use of entomopathogenic fungi have examined a combination of different microbial species, but different genotypes of the same species can also be combined, resulting in more significant effects. An experiment using strain mixtures of *B. bassiana* provided antagonistic and synergistic control of coffee berry borer (*Hypothenemus hampei*) (Benavides et al., 2012), while a further study found that combinations of three strains of *B. bassiana* resulted in a coffee berry borer mortality rate of 93% compared to around 57% mortality of single isolates (Cruz et al., 2006). This same study also found that combining genetically similar *B. bassiana* strains gave no synergistic interaction, but antagonism and synergistic effects were obtained when genetically diverse strains of the same species were mixed.

The compatibility of entomopathogenic strains of *Beauveria*, when grown in culture, is also useful if considering multiple strain-based products. The *in vitro* approach of assessment is generally termed dual culture and is commonly utilised in the biological control of plant pathogens whereby microbial candidates can be assessed for their antagonistic potential against a plant pathogen (Bunbury-Blanchette & Walker, 2019; Hajieghrari et al., 2008; Kunova et al., 2016; Malathi & Mohan, 2011; Pliego et al., 2011). The dual culture or multiple-culture method has not yet been practised for assessing antagonistic effects between strains of *Beauveria.*

In this Chapter, research was conducted that assessed the interaction of multiple isolates from four species of *Beauveria* through the dual culture method, and the combinations were then ranked. Based on the result in Chapter 3, four combinations were used against DBM larvae: (1) a combination of less virulent isolates, (2) a combination of medium virulent isolates, (3) a combination involving different species of *Beauveria*, and (4) a combination of highly virulent *Beauveria* isolates. The main objective of this Chapter was to evaluate the combinations of *Beauveria* strains against DBM larvae.

4.2 Materials and methods

4.2.1 *In vitro* dual culture assay

Conidia from eight isolates (Mo1, FRhp, Bweta, FW Mana, I12 Damo, F532, CTL20, and CTA20), from four *Beauveria* species, were randomly selected from three-week-old colonies grown on PDA (subculture from the previous chapter) and assessed for their antagonism within a dual culture assay within Petri plates containing PDA (dissolved 39 g of Oxoid powder in 1 L of distilled water, autoclaved at 15 psi for 15 min). All eight isolates were cultured singly (single culture) to be used for growth diameter comparison (Table 4.1). The base of the Petri plates was marked with a permanent marker pen with three spots equally spaced apart. Each mark was then numbered with the isolate name before inoculating, and a metal needle was used to transport a conidium to the spot (Figure 4.1). Then, all Petri plates were incubated at 23 ± 1^{o}C with a 12:12 D:L photoperiod for up to a month. Fungal growth was assessed weekly by observing and measuring any zones of inhibition using a dissecting

microscope. Growth diameter was measured using a millimetre ruler for three weeks when the colony diameter of single cultures had reached the full size of the Petri dish.

Table 4.1: The eight isolates used in the dual culture experiment; all of them were grown as single cultures for growth diameter comparison

Treatment	Isolate
Dual culture 1	*B. bassiana* Mo1
	B. pseudobassiana FRhp
	B. malawiensis Bweta
Dual culture 2	*B. malawiensis* Bweta
	B. pseudobassiana FW Mana
	B. caledonica F532
Dual culture 3	*B. malawiensis* Bweta
	B. caledonica F532
	B. bassiana CTL20
Dual culture 4	*B. pseudobassiana* I12 Damo
	B. caledonica F532
	B. bassiana CTA20

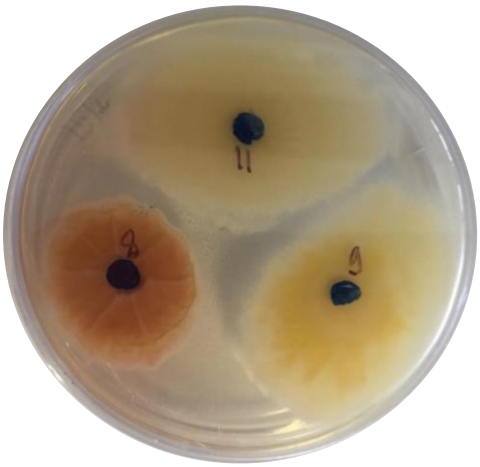


Figure 4.1: An example of marking at the bottom of a Petri dish for dual culture

4.2.2 Bioassays against DBM

Nine fungal isolates were used in the combined-isolate assays (Table 4.2). Insects were obtained from colonies maintained in the controlled temperature rooms at Lincoln University (25 ± 2^{o}C and 12:12 D/L) as described in Chapter 3. For these bioassays, fungal spore harvesting, spore counting, and insect preparation were accomplished as described in Chapter 3. The spores from the

nine isolates were harvested, and the resulting spore suspensions were adjusted to 10^5, 10^7, and 10^8 conidia/mL. Combinations of these isolates were then prepared by pipetting 1 mL of each isolate into a one 15 mL centrifuge tube, all at the same concentration (i.e. 1 mL of 10^5 conidia/mL of *B. bassiana* F615, 1 mL of 10^5 conidia/mL of *B. pseudobassiana* I12 Damo, and 1 mL of 10^5 conidia/mL of *B. caledonica* F532). Then, 600 µL of the mixture was pipetted for each spray application (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray). The inoculum was applied in a Potter Tower, as previously described in Chapter 3. Before spraying, the centrifuge tubes containing the conidial suspensions were gently shaken vigorously for around 1 min to mix. Treated insects were maintained at 23 ± 1°C and under 12:12 D/L as described in Chapter 3. The percentage mortality and percentage of cadavers that supported sporulation were also assessed as described in Chapter 3.

4.2.3 Experimental design

From the dual culture experiment, the isolate *B. malawiensis* Bweta showed the characteristic of antagonising other isolates. Thus, this isolate was not included in the bioassay, and only three species were available for the combination of species. According to the results in Chapter 3, four different treatments were designed (Table 4.2). All assays were conducted twice. With four treatments, three rates, two replicates, and two times, the experiment required 60 plates and 300 larvae (5 larvae per plate).

Table 4.2: Isolates used in the four combinations (less virulent, medium virulent, high virulent, and three species combinations) grouped based on the mortality result in Chapter 3

Type of combination	***Beauveria* species**	**Isolates**	**% mortality results after 7 days from Chapter 3 application rate (conidia/spray)**		
			6 x 10^4	**6 x 10^6**	**6 x 10^7**
Less virulence	*B. bassiana*	TPP-H	14	36	57
	B. bassiana	FRh2	14	14	68
	B. caledonica	F532	14	25	25
Medium virulence	*B. bassiana*	F615	36	89	79
	B. bassiana	J2	25	79	79
	B. bassiana	J18	25	68	79
High virulence	*B. pseudobassiana*	FW Mana	25	100	100
	B. pseudobassiana	I12 Damo	46	100	100
	B. bassiana	CTL20	46	100	100
Three species	*B. bassiana*	F615	36	89	79
	B. caledonica	F532	14	25	25
	B. pseudobassiana	I12 Damo	46	100	100

4.2.4 Statistical analysis

Data were analysed using the statistical software package, Genstat (VSN International, 20th Edition). Data were analysed using the same methods described in Chapter 3. Results of the nine isolates individually reported in Chapter 3 were used to compare with the results obtained with the combinations of these same isolates. Data were organised into separate runs that included control mortality for all combinations and individual isolates used for comparison using the Probit test.

4.3 Results

4.3.1 *In vitro* dual culture

The *in vitro* dual culture results showed that the colony sizes resulting from dual cultures were relatively smaller than for single cultures, except for the dual culture 4 combination. Growth of those isolates cultured alongside *B. malawiensis* Bweta was significantly ($p < 0.05$) slower than the single isolates at week 2, especially isolate *B. caledonica* F532 that grew 45% less in dual culture 2 and 30% less in dual culture 3. When *B. bassiana* CTA20 and *B. caledonica* F532 were cultured alongside *B. pseudobassiana* I12 Damo, the colonies of these isolates grew as large as when they were grown in PDA in single cultures. At week 3, isolate *B. caledonica* F532 was 52% and 39% slower than the single culture in dual cultures 1 and 2. *B. pseudobassiana* FW Mana and *B. bassiana* CTL20 were 40% and 30% smaller than when these isolates were grown as single cultures. The colonies of *B. malawiensis* Bweta were 36% smaller in dual culture 2 and 19% smaller in dual culture 3, smaller than single culture. In dual culture 4, the three isolates were 17%, 22%, and 18% smaller for isolate *B. pseudobassiana* I12 Damo, *B. caledonica* F532, and *B. bassiana* CTA20, respectively (Table 4.3). Figure 4.2 shows inhibition of isolates FW Mana and F532 by isolate Bweta. For all diameter, measurement data refer to Table B.2.1 (Appendix B.2).

Table 4.3: Percentage growth of eight isolates in multiple cultures compared with eight isolates in single culture (week 4 data were not included as all isolates, except isolate Bweta in single cultures, grew to the full size of the Petri dish)

Treatment	Isolate	% Growth compared to single isolates		
	week	1	2	3
Dual culture 1	*B. bassiana* Mo1	92	88	97
	B. pseudobassiana FRhp	94	91	91
	B. malawiensis Bweta	86	90	80
Dual culture 2	*B. malawiensis* Bweta	98	77	64
	B. pseudobassiana FW Mana	93	79	60
	B. caledonica F532	90	55	48
Dual culture 3	*B. malawiensis* Bweta	94	83	81
	B. caledonica F532	92	70	61
	B. bassiana CTL20	98	77	70
Dual culture 4	*B. pseudobassiana* I12 Damo	100	91	83
	B. caledonica F532	94	100	78
	B. bassiana CTA20	91	84	82

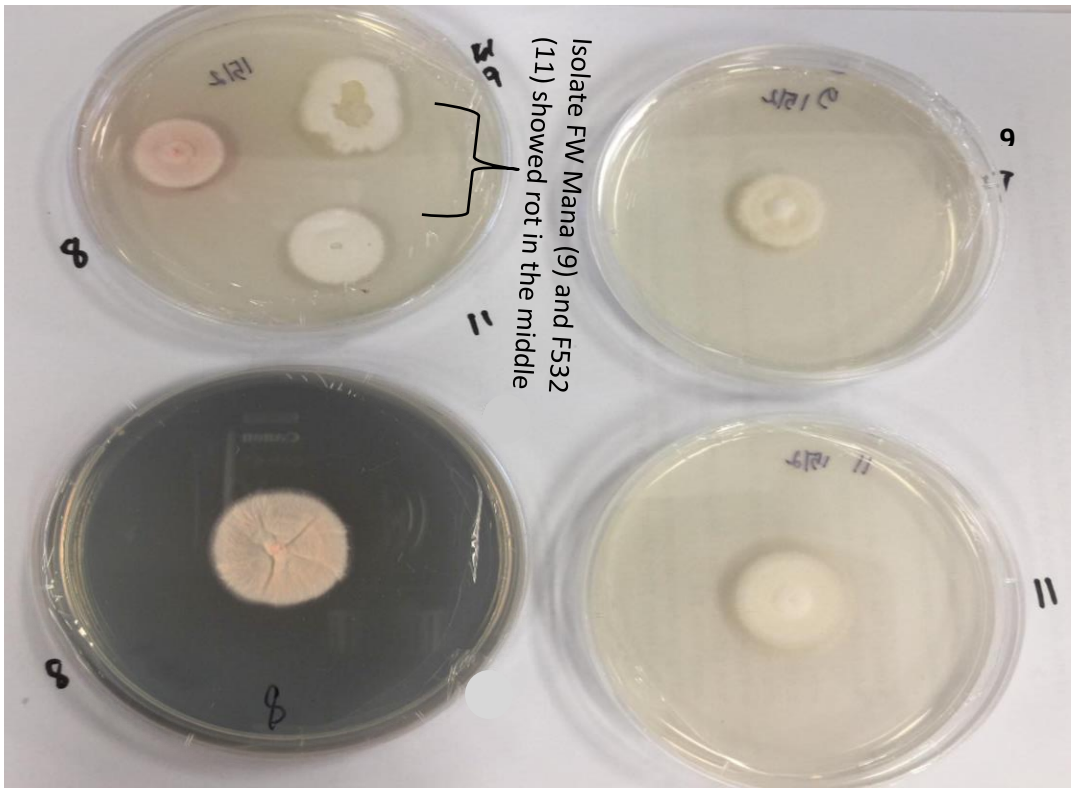


Figure 4.2: Fungal colonies of *Beauveria* growing on PDA on the first week following inoculation showing the inhibition by isolate Bweta of the other two isolates; 8 = *B. malawiensis* Bweta, 9 = *B. pseudobassiana* FW Mana, 11 = *B. caledonica* F532

4.3.2 Mortality of DBM larvae

Mortality was between 22% and 100% for the four combinations across the three application rates (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray). Overall mortality did not differ significantly among the four combinations of *Beauveria* (df =4, deviance =50.061) (Table 4.4). Overall, at the highest application rate of 6 x 10^7 conidia/spray, the less virulent (*B. bassiana* TPP-H + *B. bassiana* FRh2 + *B. caledonica* F532) treatment, the three species (*B. bassiana* F615 + *B. pseudobassiana* I12 Damo + *B. caledonica* F532) treatment, and the high virulent (*B. pseudobassiana* FW Mana + *B. pseudobassiana* I12 Damo + *B. bassiana* CTL20) treatment caused 100% mortality, while the medium virulent (*B. bassiana* F615 + *B. bassiana* J2 + *B. bassiana* J18) treatment killed 94% of the DBM larvae (Table 4.5, Figure 4.3).

At the application rate of 6 x 10^7 conidia/spray, the less virulent combination caused 100% mortality (Table 4.4), while the results for each of these isolates, when applied individually, resulted in less than 50% mortality for DBM (Table 3.2). Similarly, the medium virulent combination resulted in 94% mortality for DBM; for these three isolates applied individually, there was less than 80% mortality for DBM (Figure 4.4). However, the high virulent mixture resulted in less mortality at 6 x 10^4 conidia/spray than any of the isolates when they were applied individually (Figure 4.5 compared to Figure 3.7). Multiple comparisons using LSD showed that the application rate of 6 x 10^6 conidia/spray for the high virulent combination did not differ significantly from the less and medium virulent combinations, while compared to the three species combination, it was significantly (at $p < 0.036$) more effective (Table 4.4).

Table 4.4: Statistical analysis of all 14 isolates, Logistic Regression, Probit test using Genstat

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	4	50.061	12.5153	12.52
Residual	7	6.551	0.9358	
Total	11	56.612	5.1465	

Table 4.5: Percentage mortality of DBM following application rates of 6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray after correcting for control mortality using Abbott's formula (Tukey test, ANOVA)

Isolate	Application rate (conidia/spray)		
	6 x 10^4	**6 x 10^6**	**6 x 10^7**
	Mean ± SE	**Mean ± SE**	**Mean ± SE**
Less virulent combination (TPP-H, FRh2, F532)	28 ± 2.18	78 ± 3.89 de	100 ± 8.41 b
Medium virulent combination (F615, J2, J18)	33 ± 2.77	83 ± 4.74 de	94 ± 4.97 b
High virulent combination (FW Mana, I12 Damo, CTL20)	28 ± 3.62	100 ± 3.85 e	100 ± 8.34 b
Three species combination (F615, I12 Damo, F532)	22 ± 2.30	56 ± 2.10 bcd	100 ± 3.85 b
B. bassiana F615	36 ± 1.46	89 ± 3.51 de	79 ± 3.05 ab
B. bassiana J2	25 ± 0.87	79 ± 2.37 cd	79 ± 3.60 ab
B. bassiana TPP-H	14 ± 0.55	36 ± 1.57 abc	57 ± 1.59 ab
B. bassiana J18	25 ± 0.76	68 ± 2.37 bcd	79 ± 2.90 ab
B. bassiana FRh2	14 ± 0.37	14 ± 0.55 a	68 ± 2.12 ab
B. pseudobassiana FW Mana	25 ± 0.87	100 ± 3.76 e	100 ± 4.46 b
B. pseudobassiana I12 Damo	46 ± 2.87	100 ± 2.31 e	100 ± 4.30 b
B. caledonica F532	14 ± 0.28	25 ± 1.20 ab	25 ± 1.30 a
B. bassiana CTL 20	46 ± 1.60	100 ± 4.02 e	100 ± 3.05 b
F-value	0.63	11.61	6.53
P-value	0.783	<.001	0.001

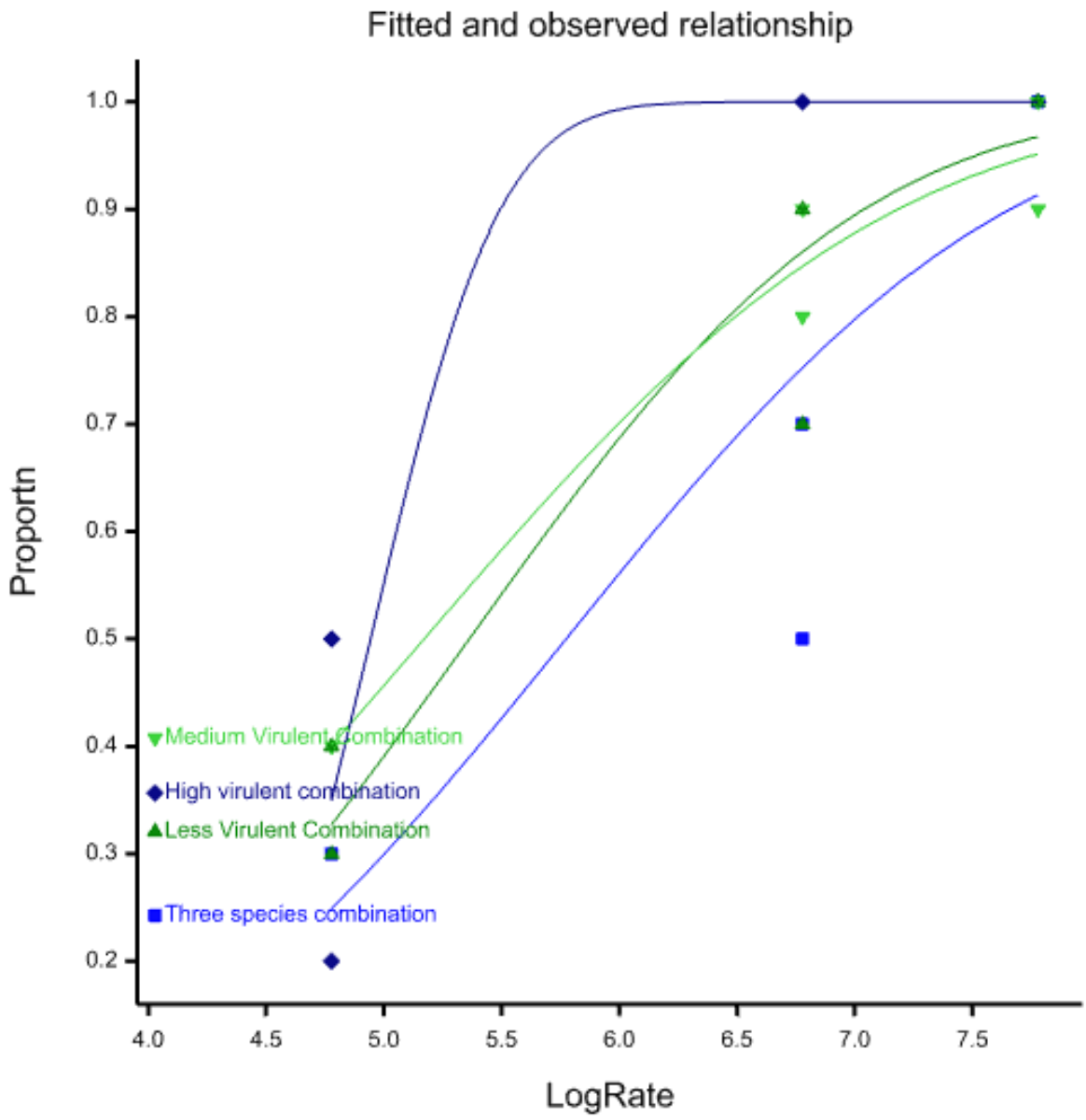


Figure 4.3: Mortality response curves of the four combinations of *Beauveria* isolates at three application rates of 6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray. Rates were transformed with Log to base 10 in Logistic Regression and Regression Analysis using Probit test using Genstat.

Note: *B. bassiana* TPP-H + *B. bassiana* FRh2 + *B. caledonica* F532 (less virulent), *B. bassiana* F615 + *B. bassiana* J2 + *B. bassiana* J18 (medium virulent), *B. pseudobassiana* FW Mana + *B. pseudobassiana* I12 Damo + *B. bassiana* CTL20 (highly virulent), and *B. bassiana* F615 + *B. pseudobassiana* I12 Damo + *B. caledonica* F532 (three species)

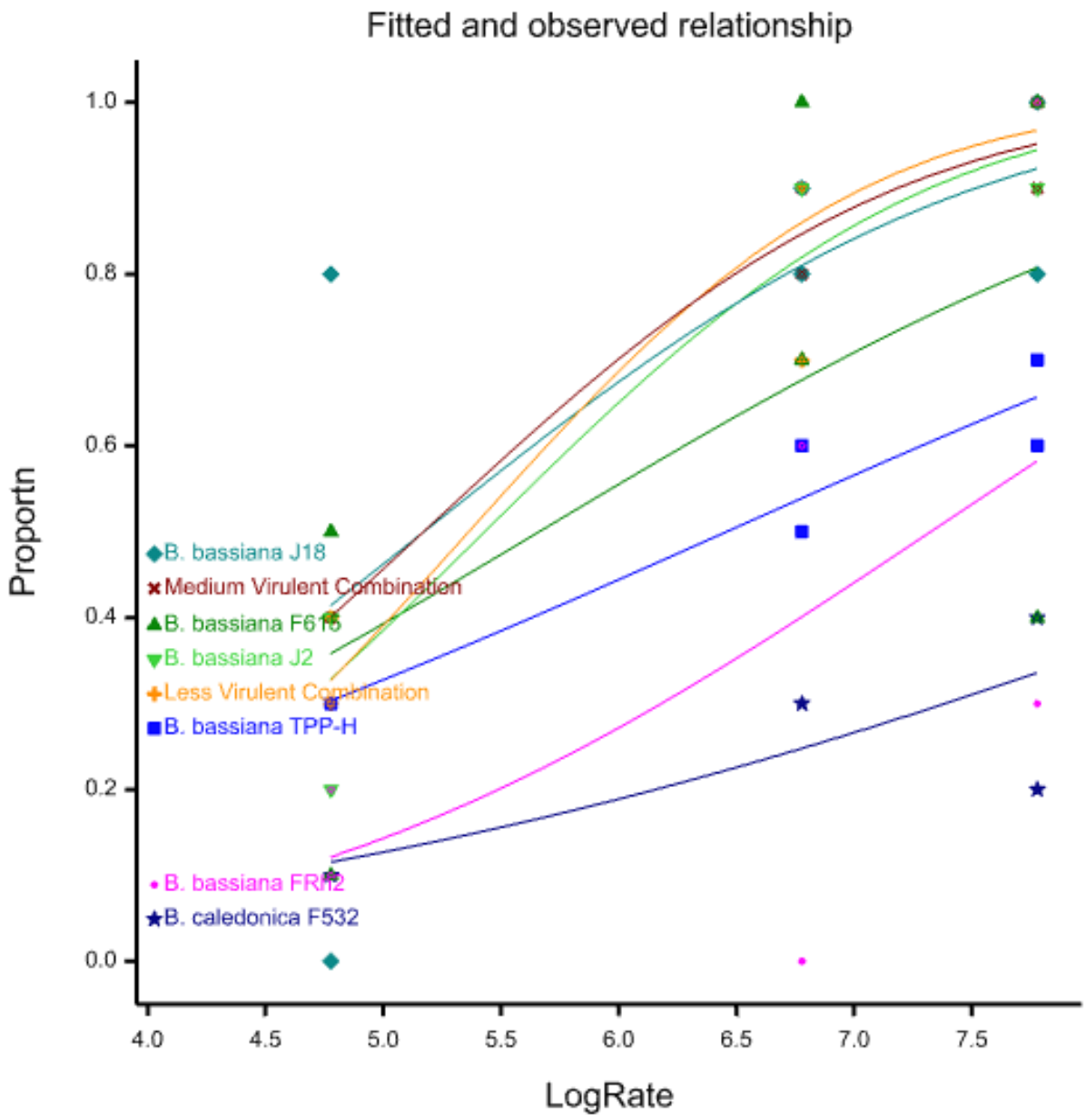


Figure 4.4: Mortality response curves of the less and medium virulence combinations of *Beauveria* isolates at three application rates of 6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray compared to the single isolates bioassay from Chapter 3. Rates were transformed with Log to base 10 in Logistic Regression and Regression Analysis using Probit test using Genstat.

Note: *B. bassiana* TPP-H + *B. bassiana* FRh2 + *B. caledonica* F532 (less virulence), *B. bassiana* F615 + *B. bassiana* J2 + *B. bassiana* J18 (medium virulence), **Bb:** *Beauveria bassiana*, **Bc:** *Beauveria caledonica*

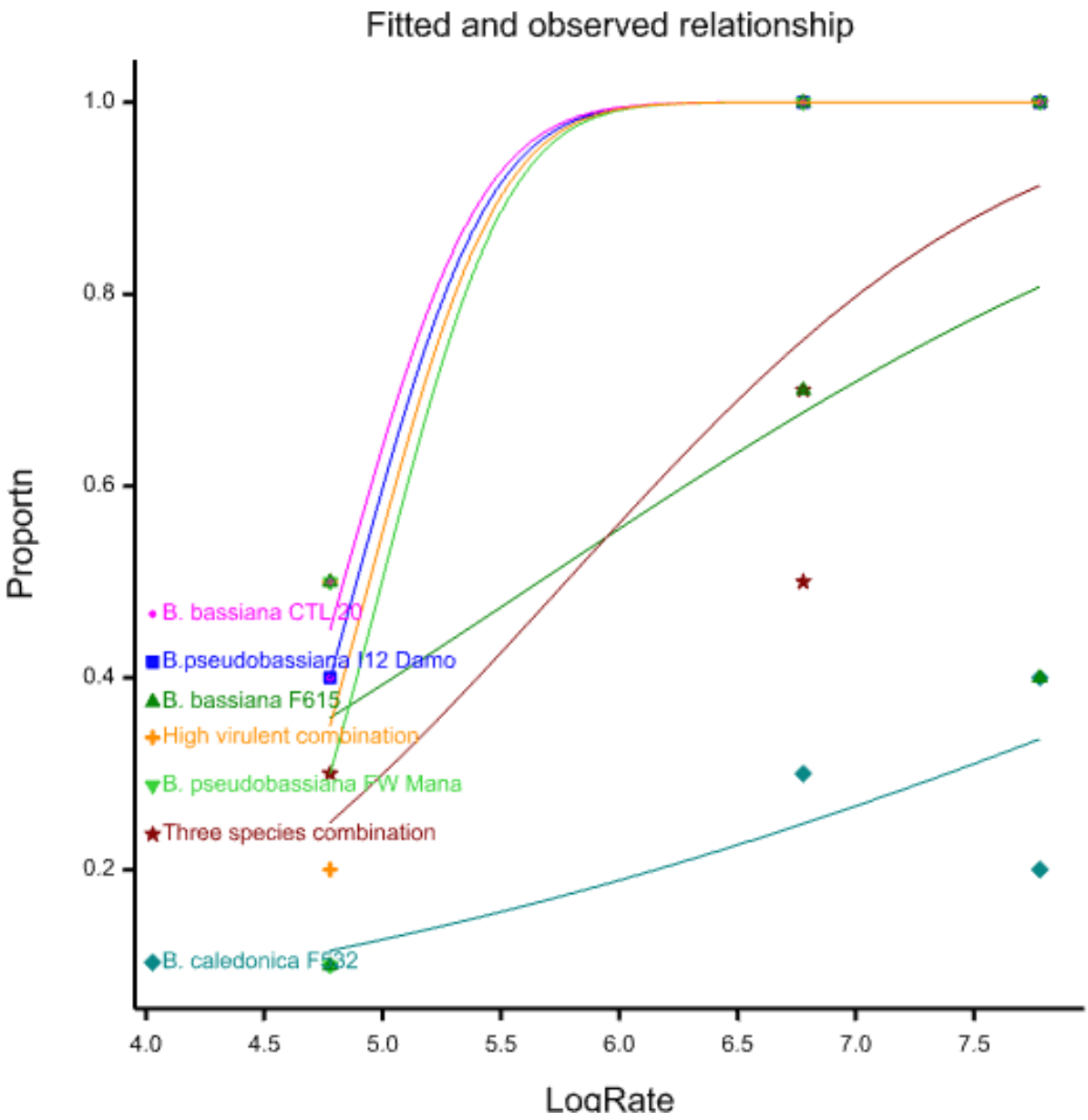


Figure 4.5: Mortality response curves of the high virulence and three species combinations of *Beauveria* isolates at three application rates of 6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray compared to single isolates bioassays from Chapter 3. Rates were transformed with Log to base 10 in Logistic Regression and Regression Analysis using Probit test, using Genstat.

Note: *B. pseudobassiana* FW Mana + *B. pseudobassiana* I12 Damo + *B. bassiana* CTL20 (high virulence), and *B. bassiana* F615 + *B. pseudobassiana* I12 Damo + *B. caledonica* F532 (three species), **Bb:** *Beauveria bassiana*, **Bc:** *Beauveria caledonica*, **Bp:** *Beauveria pseudobassiana*

4.3.3 Mortality over time

DBM larval mortality began two days after inoculation with the *Beauveria* isolates for most of the four combinations at all application rates. At 6 x 10^4 conidia/spray, DBM mortality ranged from 30% to 40% by day seven after inoculation (Figure 4.6). Increasing the inoculum to 6 x 10^6 conidia/spray increased mortality from 60% to 100%, with only the highly virulent isolate combination (*B. pseudobassiana* FW Man + *B. pseudobassiana* I12 Damo + *B. bassiana* CTL20), resulting in 100% mortality of DBM (Figure 4.7). At 6 x 10^7 conidia/spray, the medium virulent combination reached 94% mortality for DBM, while the other three combinations caused 100% mortality within seven days (Figure 4.8).

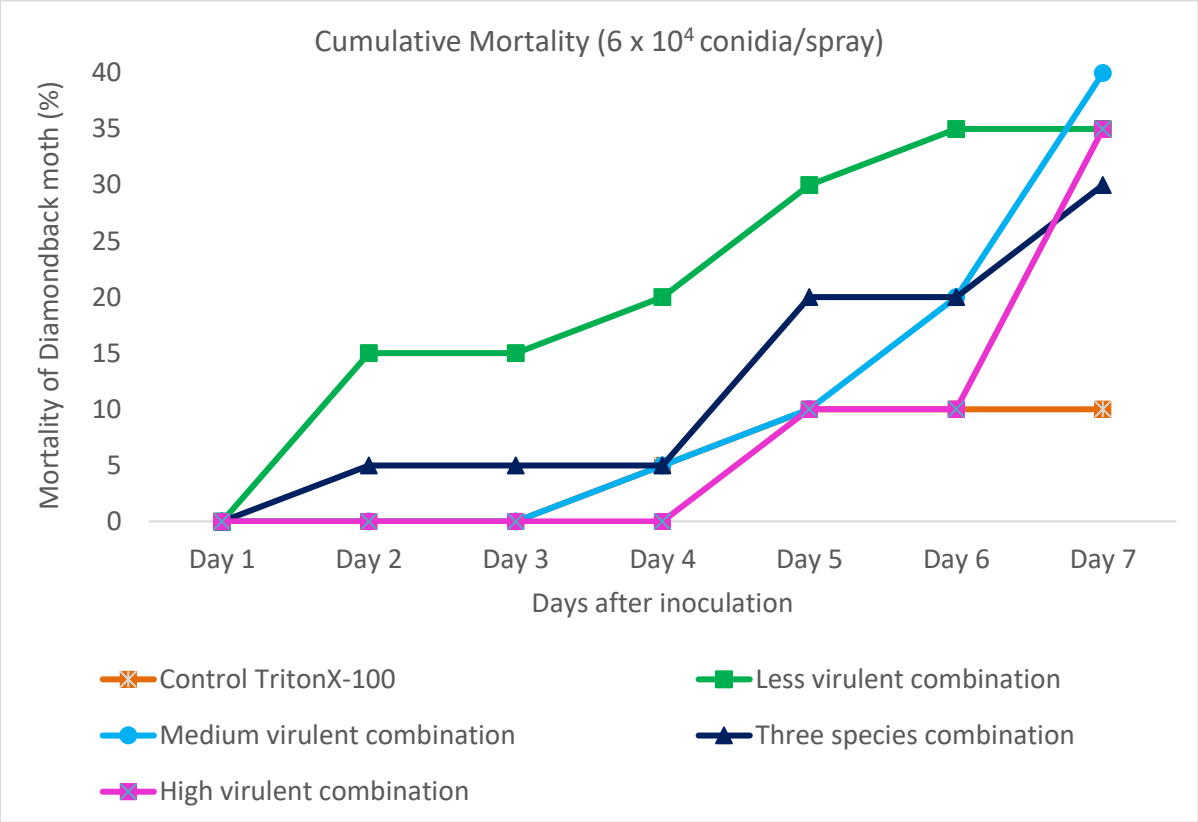


Figure 4.6: Cumulative mortality of DBM larvae seven days after spraying with four combinations of *Beauveria* at the application rate of 6 x 10^4 conidia/spray

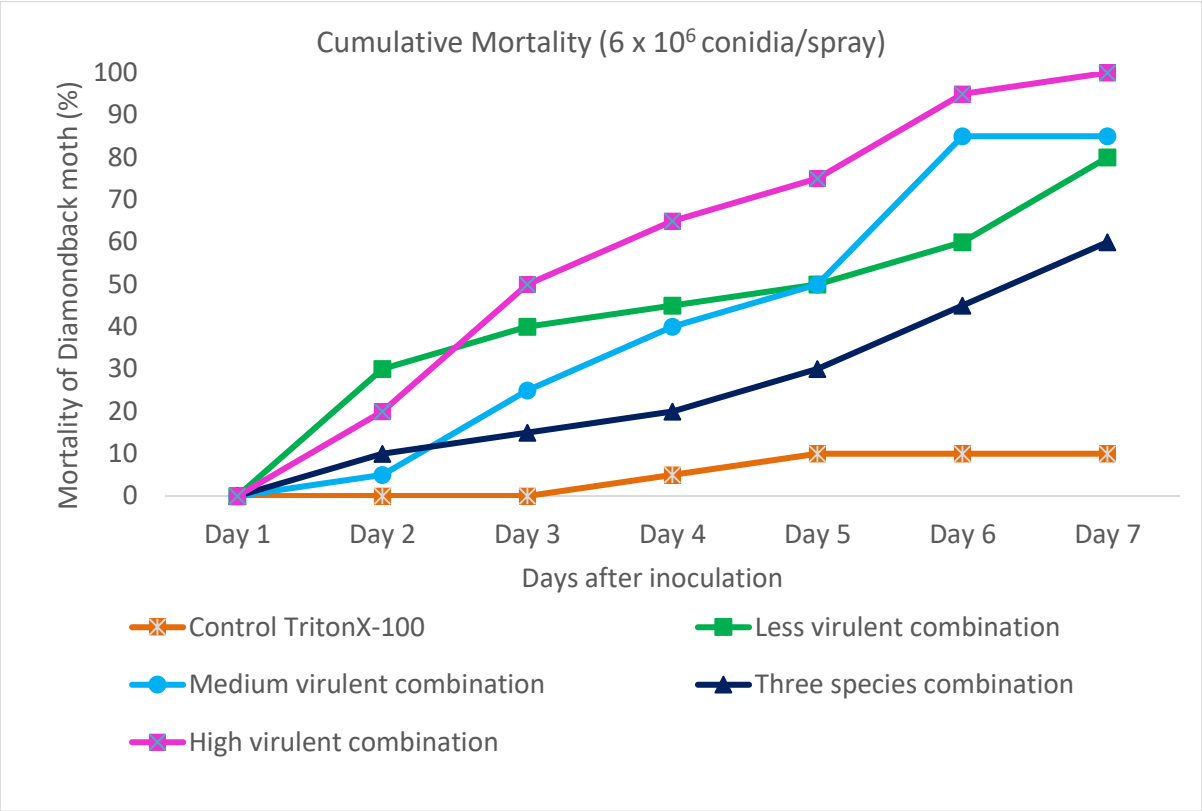


Figure 4.7: Cumulative mortality of DBM larvae seven days after spraying with four combinations of Beauveria at the application rate of 6 x 10^6 conidia/spray

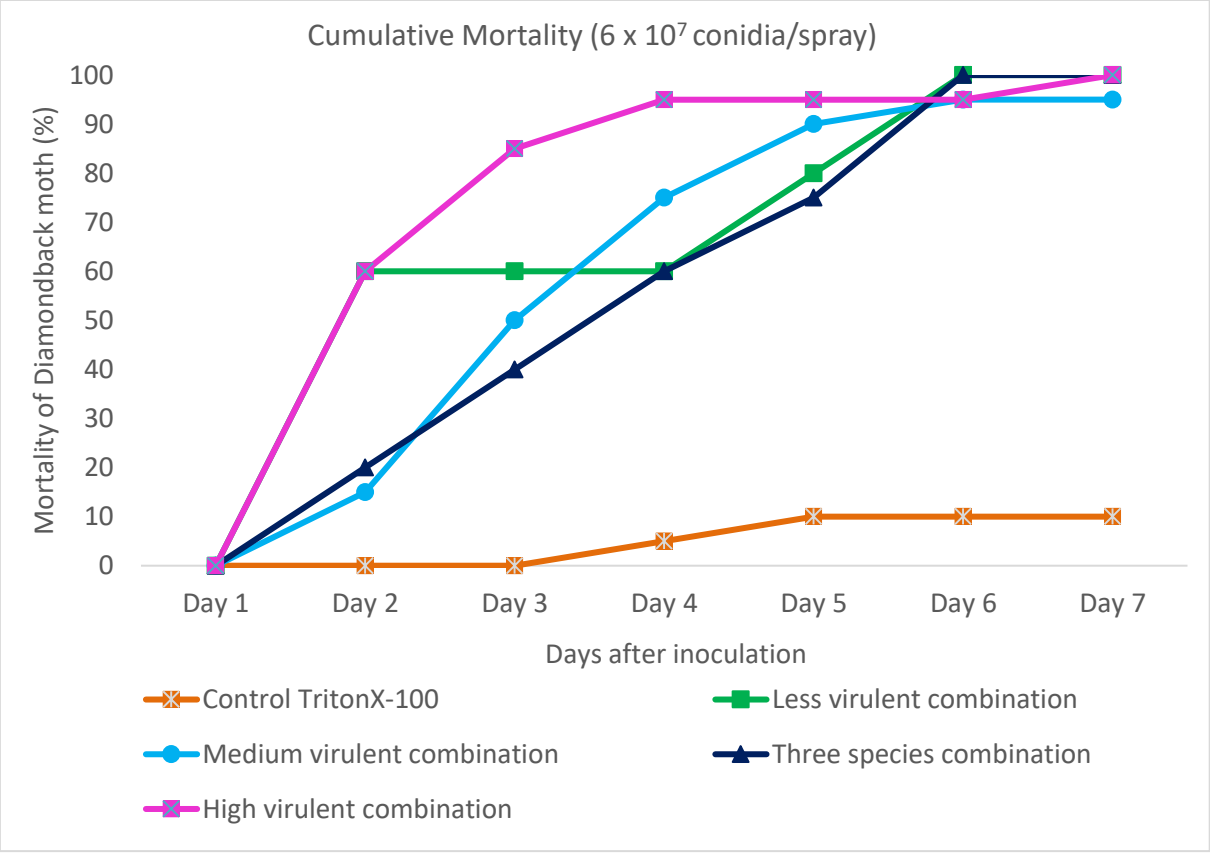


Figure 4.8: Cumulative mortality of DBM larvae seven days after spraying with four combinations of *Beauveria* at the application rate of 6 x 10^7 conidia/spray

4.3.4 The median lethal rate (LD_{50}) and time (LT_{50})

Results of all combinations and single isolates bioassay results from Chapter 3 were used for comparisons. LD_{50} for the high virulent combination (FW Man + I12 Damo + CTL20), medium virulent (F615 + J2 + J18), and less virulent combination (TPP-H + FRh2 + F532) did not differ from that of the high virulent isolates I12 Damo and CTL20 used individually in Chapter 3 (statistically similar groups a and ab in Figure 4.9). This first group required around 10^5 conidia/spray to cause 50% mortality. The three species combination (F615 + I12 Damo + F532) was placed in the group (b and bc), which required roughly 10^6 conidia/spray to achieve LD_{50}. In comparison to single isolates, the less virulent combination needed lower inoculum rates. The three species did not differ from medium and less virulence isolates, FW Mana, F615, J18, and J2, which required around 3-8 x 10^6 conidia/spray to achieve 50% mortality. Statistical significance of LD_{50} refers to Figure 4.9 and Table B.2.2 (Appendix B.2).

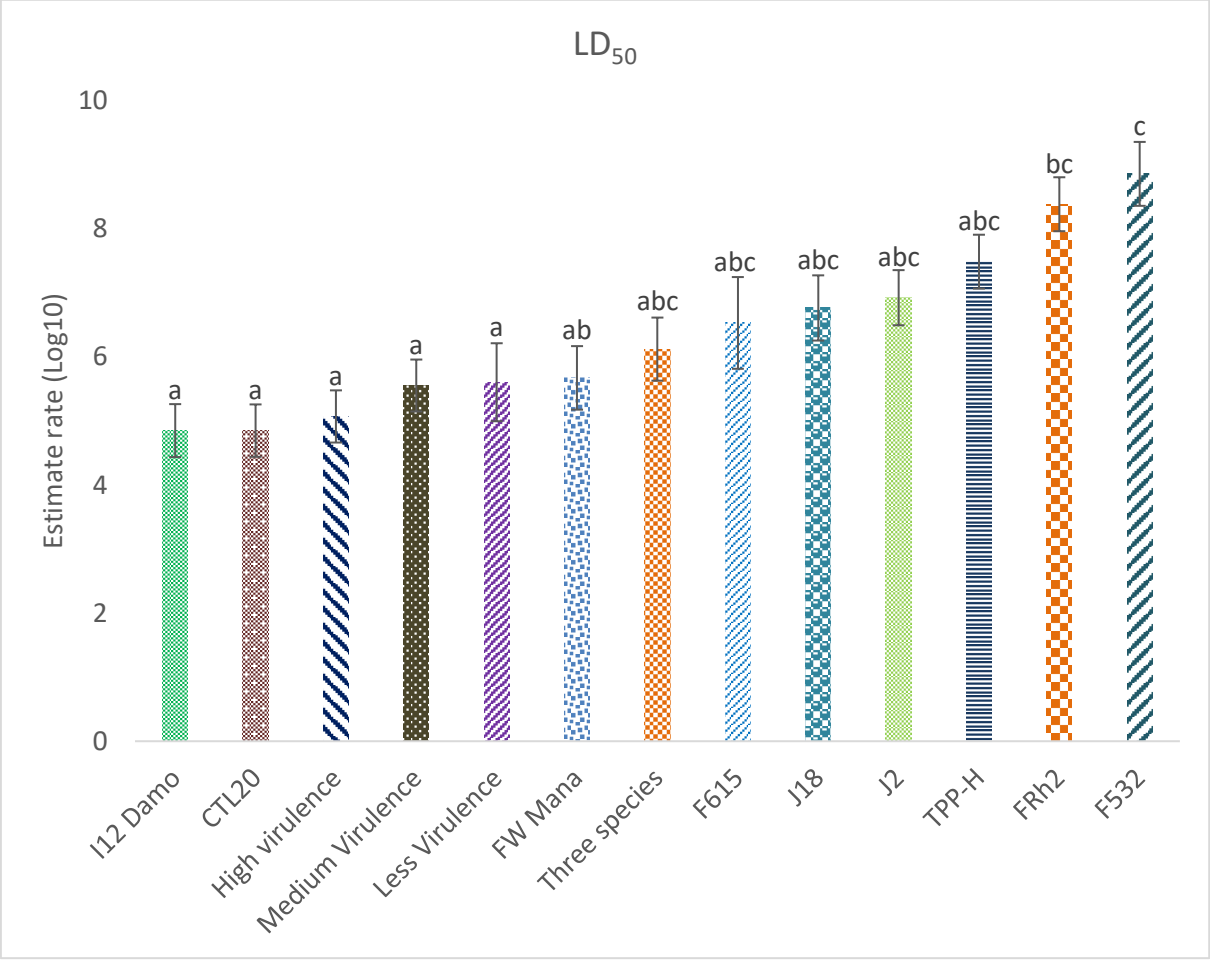


Figure 4.9: LD_{50} for the four combined isolates compared to the individual isolates (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, using Genstat). a to b or b to c: significantly different, a to ab or bc to c: not significantly different

At the application rate of 6 x 10^4 conidia/spray, medium and less virulent combinations of *Beauveria* reached the LT_{50} at almost the same time as the isolates I12 Damo and CTL20 when gown individually. All four combinations took four to five days to achieve 50% mortality at the application rate of 6 x 10^6 conidia/spray. This result was as quick as the high virulent isolates such as I12 Damo, FW Mana, and CTL20 when applied individually (Figure 4.10). Compared to single isolates, the less virulent combination was significantly faster (at $p < 0.002$). At 6 x 10^7 conidia/spray, the high virulent combination reached LT_{50} faster than the other three combinations. Statistically, all four combinations were not significantly different from the individual isolates, but the less virulent combination was significantly quicker. Statistical significance of LT_{50} refers to Figure 4.10 and Table B.2.3 (Appendix B.2).

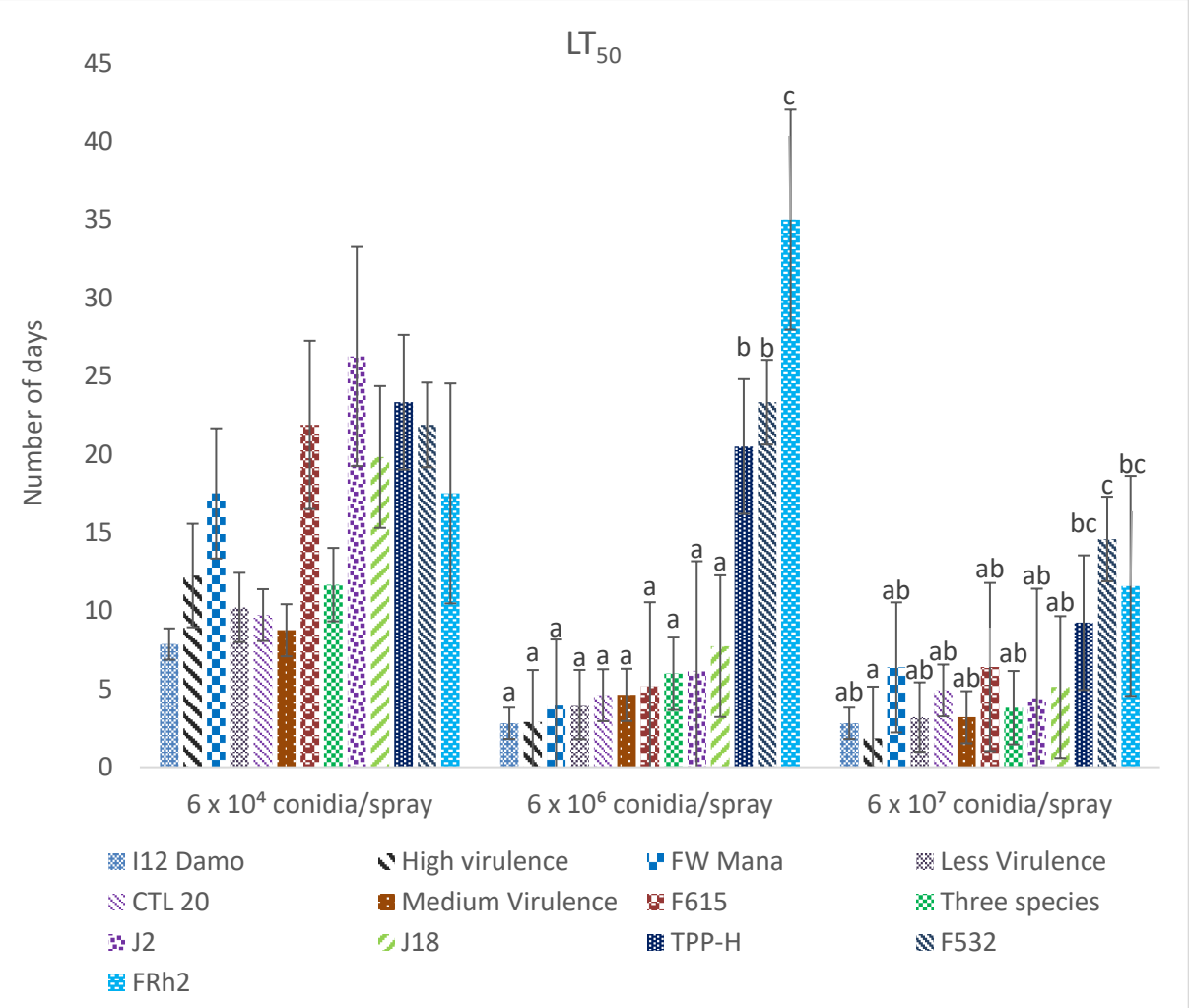


Figure 4.10: LT_{50} for the four combined *Beauveria* and the individual isolates (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, using Genstat). a to b or b to c: significantly different, a to ab or bc to c: not significantly different

4.3.5 Percentage of cadavers supporting sporulation

The percentage of cadavers that supported sporulation by the *Beauveria* isolates ranged from 42% to 95% for all four combinations across the three application rates. The high virulence combination resulted in 95% of cadavers supporting sporulation at an application rate of 6 x 10^7 conidia/spray followed by medium virulent (90%), three species (85%), and less virulent (75%) combinations. At the application rate of 6 x 10^6 conidia/spray, the medium virulent combination showed 83% of the cadavers assessed resulted in *Beauveria* sporulating on them, followed by less virulent (75%), high virulent (70%), and three species (61%) combinations. At the 6 x 10^4 conidia/spray application rate, the high virulent treatment resulted in 80% sporulation, followed by medium virulent (75%), three species (67%), and less virulent (42%) combinations (Figure 4.11). There was a statistically significant difference between the high virulent and less virulent combinations (F = 12.66, df = 3, p = 0.033).

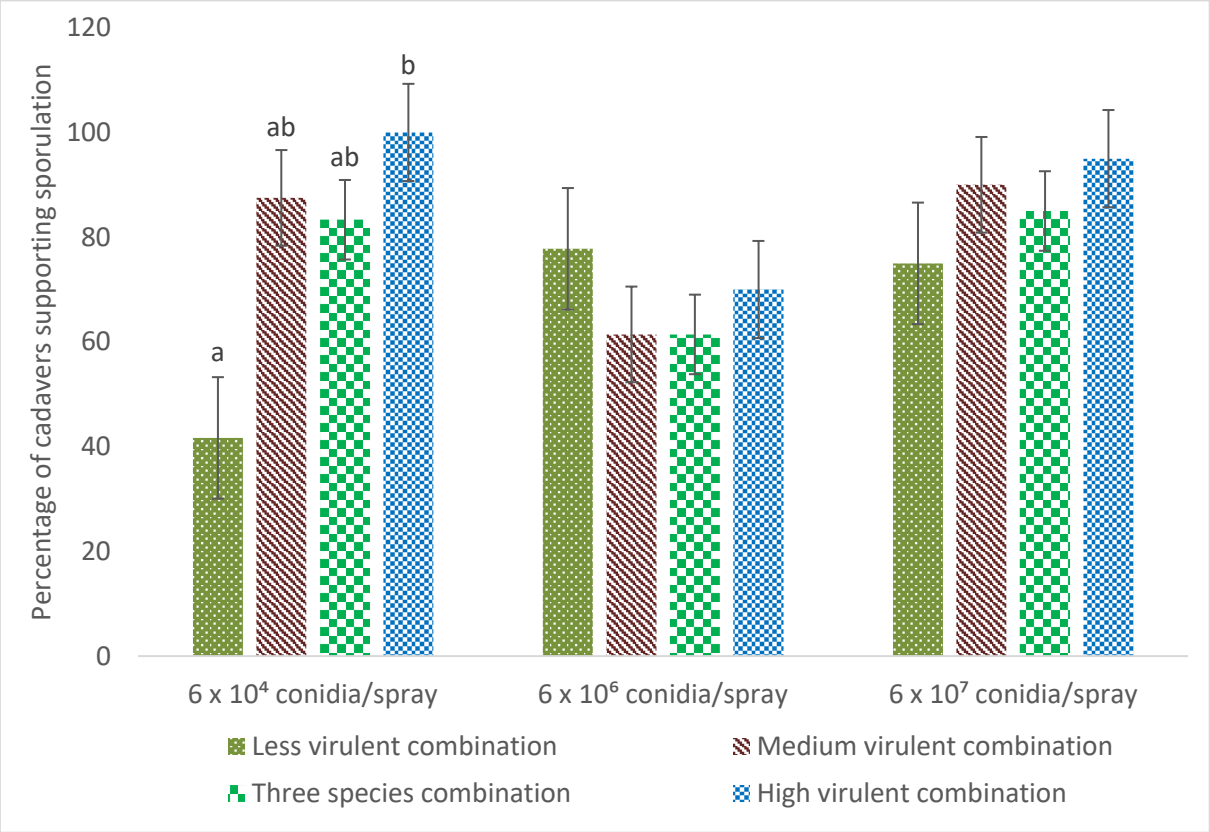


Figure 4.11: Percentage of DBM cadavers supporting sporulation of *Beauveria* following application of four combined treatments at three application rates (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray), (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, using Genstat)

Note: Less virulent (*B. bassiana* TPP-H + *B. bassiana* FRh2 + *B. caledonica* F532), medium virulent (*B. bassiana* F615 + *B. bassiana* J2 + *B. bassiana* J18), highly virulent (*B. pseudobassiana* FW Mana + *B. pseudobassiana* I12 Damo + *B. bassiana* CTL20), and three species (*B. bassiana* F615 + *B. pseudobassiana* I12 Damo + *B. caledonica* F532)

4.4 Discussion

In vitro dual culture results provided reasonable indications for predicting the efficacy of an application of combined isolates. Christensen (1996) used this method to predict the efficacy of two fungal endophytes against plant pathogenic fungi. However, diverse efficacies can be found when testing under *in vivo* and *in vitro* conditions. Growing *Beauveria* isolates from different species reduced colony diameter. When culturing with *B. malawiensis*, the colony diameter of *B. bassiana, B. pseudobassiana,* and *B. caledonica* was reduced by 13%, 15%, and 30%, respectively, while growing these three species together produced less than 10% reduction. Mortality of DBM from combining three species indicated an alignment of virulence with the *in vitro* dual culture results. This is the first report of parallel outcomes between dual culture and DBM mortality when combining *Beauveria* species under *in vitro* conditions.

This study showed synergistic and antagonistic interactions when combining genetically different isolates and species of *Beauveria* for DBM larval mortality. These results can be aligned to both the coinfection and superinfection models described by Nowak and Sigmund (2002) and intraspecific and interspecific competition (Cruz et al., 2006). The less virulent and medium virulent combinations of *Beauveria* resulted in 100% and 94% mortality of DBM larvae, respectively. From Chapter 3, two *B. bassiana* isolates (TPP-H and FRh2) of the less virulent combination caused less than 60% larval mortality, and the isolate *B. caledonica* (F532) killed less than 30%, while the three *B. bassiana* isolates (F615, J2, and J18) in the medium virulent combination, gave less than 80% mortality. Combining the less and the medium virulent isolates of *B. bassiana* enhanced the fungal infection of DBM larvae compared to the single isolates. A previous study that combined three low virulent isolates of *B. bassiana* showed that although only one isolate genetically differed, they were found to provide synergistic results against the coffee berry borer when combined (Cruz et al., 2006). Synergism occurs when two or more compatible microbial agents combine to give a higher level of pest control than if these isolates were used individually (Cedergreen, 2014; Roell et al., 2017). The study of Nowak and Sigmund (2002) combined two main factors of the coinfection model, which were virulence and time to kill. Based on cumulative data from this study, at the application rate of 6 x 10^7 conidia/spray, the least virulent and medium virulent combinations caused 100% and 95% mortality, respectively, within six days post-treatment, while the individual isolates of these two combinations caused less than 60% (less virulence) and 79% (medium virulence) mortality within seven days post-treatment. Therefore, the less virulent and medium virulent combinations could be placed in the coinfection model.

Within this study, the combination of three *Beauveria* species (*B. bassiana*, *B. pseudobassiana* and *B. caledonica*) resulted in antagonism. The antagonistic model happens when a highly virulent isolate outcompetes a less virulent one for host infection (Nowak & Sigmund, 2002). Even though the DBM mortality of this combination was 100%, the killing speed was even slower than isolate *B. pseudobassiana* I12 Damo alone. When mixing two isolates to control coffee berry borer in Puerto

Rico, Bayman et al. (2021) found competitive activity between the chosen isolates and no coinfection of the pest. Furthermore, the combination of these isolates did not lead to a higher level of borer mortality than when these same isolates were used individually. In this study, isolate I12 Damo alone worked faster and required a lower application rate than this three species combination. The highly virulent strains may compete with the least virulent strains dominating food resources, subsequently reducing the speed of host infection (Benavides et al., 2012; Cruz et al., 2006). Therefore, the isolate I12 Damo might have inhibited the other strains before starting host invasion, leading to a delay in host infection. This phenomenon was also found when mixing *B. bassiana* with *M. anisopliae* to control the desert locust (Thomas et al., 2003), *B. bassiana* with *Steinernema ichnusae* in the haemocoel of *Bombyx mori* (Tarasco et al., 2011), and when combining *B. bassiana* with *Brevibacillus laterosporus* against DBM larvae (Narciso et al., 2019). These studies indicated that combinations of entomopathogenic fungi with other microbes could reduce pathogenic ability. As already known, isolate *B. pseudobassiana* I12 Damo in this study caused high mortality. The expectation was that the two combinations with this isolate would achieve this mortality in less time, but this did not occur. This result indicated that biopesticides containing highly virulent isolates of *Beauveria* might not be improved by combining them with additional genetically distinct strains of the same species (Bayman et al., 2021; Boucias et al., 2000). As the superinfection model included both virulence and time (Nowak & Sigmund, 2002), the three species and high virulent combinations were within this model.

The diversity of genetically different isolates enhances the fungal tolerance to some diverse climatic effects, improving virulence against a particular pest under different conditions. Under field conditions, the application of the combination of three *B. bassiana* strains (Bb9001, Bb9024 and Bb9119), commercial name Cenicafé, reduced a coffee berry borer population by up to 75% (Vera et al., 2011). When combining Cenicafé strains with a strain of *M. anisopliae*, the mortality of this coffee pest reached 94% (Jaramillo et al., 2015). The efficacy of our combined-isolate treatments may be the result of gene recombination through sexual mating-type. For instance, when observing Masson pine caterpillar larvae, Wang et al. (2004) proved some *B. bassiana* isolates could breed or join together through sexual mating to infect the insect under natural conditions, which provided synergism. However, the teleomorph characteristic of *B. bassiana* was found to be a rare occurrence in nature, and it is even difficult to investigate in the laboratory (Li et al., 2001). When *Beauveria* spp. were described as *Cordyceps* spp.*,* Yokoyama et al. (2006) reported that mating-type genes (MAT1-1-1 and MAT1-2-1) were amplified better than the 18S rRNA gene using phylogenetic base analysis. Some sexual mating genes found in the fungi *Aspergillus nidulans* and *Neurospora crassa* were observed in *B. bassiana* (Xiao et al., 2012). The absence of meiotic recombination Spo11-like protein means sexual reproduction in *Beauveria* strains rarely happens (Panizza et al., 2011). These reports showed *Beauveria* spp. also have sexual mating characteristics, and the lack of Spo11 protein resulted in a lower frequency of this reproductive type.

The four combinations of *Beauveria* assessed in this study required 10^5 conidia/spray application rates or above to achieve an LD_{50} for DBM larvae. Compared to single isolates that needed around 10^8 conidia/spray for LD_{50}, the less virulent combination required only 10^5 conidia/spray, which was almost half of the rate. Additionally, the medium virulent combination needed the same application rate as the least virulent combination, while the individual isolates required 10^6 conidia/spray to achieve LD_{50}. When testing on coffee berry borer, the application rate of 10^6 conidia/mL of three isolates of *B. bassiana* caused 93% mortality (Cruz et al., 2006). In contrast, while the high virulence combination required a similar application rate as single isolates at 10^4 conidia/spray, the three combined species needed around 10^6 conidia/spray, which was a higher rate than the isolate *B. pseudobassiana* I12 Damo. Similarly, using a single isolate at the application rate of 10^4 conidia/mL to test on DBM, the studies of Nguyen et al. (2007) and Duarte et al. (2016) showed the same result. The LD_{50} of DBM for one isolate was previously found to be 10^6 conidia/mL (Rondelli et al., 2013; Yamada et al., 2009). Requiring lower application rates and providing high mortality are two positive characteristics for fungal isolates for potential biopesticide development (Chandler, 2017; Chandler et al., 2011; Mascarin & Jaronski, 2016).

All four combinations took from around eight to twelve days, four to six days, and from two to four days to cause 50% mortality at rates of 6×10^4, 6×10^6, and 6×10^7 conidia/spray, respectively. Compared to the individual isolate results in Chapter 3, the less virulent combination reached LT_{50} significantly faster at high rates, comparable to the high virulent isolates. Xue et al. (2018) found that when combining *B. bassiana* with Bt, the mixture caused 50% mortality of DBM larvae on the second and third days after inoculation. The four combinations used in this study reached LT_{50} on day two and day three, which was similar. When testing on coffee berry borer, Cruz et al. (2006) showed the highly effective mixture took 5.58 days to cause 50% mortality. Another study on the same insect in Puerto Rico found LT_{50} at 6.7 to 6.8 days after inoculation for all treatment tested, including six combined treatments (Bayman et al., 2021). These studies took longer because they tested on a coleopteran pest, which required more time to kill (Padın et al., 2002; Steinwender et al., 2010).

From the result of this study, the combined isolates may compete for growth, resulting in killing faster but reducing sporulation on the host. For instance, the co-inoculum of *B. bassiana* and *B. brongniartii* revealed different metabolites were produced compared to single isolates sprayed separately (Canfora et al., 2017). Thus, the result of these combined isolates may also produce metabolites to kill DBM larvae while killing by spores simultaneously. Another rationale is that when fungi compete, they may reduce their ability to sporulate as the host's immune systems can inhibit growth even if they have already died (Vilcinskas & Wedde, 1997). An opportunist bacteria, *Serratia marcescens,* that lives associated with insects can also be involved in this inhibition (Ishii et al., 2014). In this study, some cadavers that did not support sporulation were found to have red pigment and were identified as having *Serratia marcescens*. Therefore, the lower percentage of cadavers, which

supported sporulation, might also be related to this bacteria. The four combinations, which caused high mortality with low sporulation, may be good characteristic for industry. Companies may want a biopesticide that provides significant mortality with low sporulation so that they can sell more products.

4.5 Conclusion

The four combinations of isolates resulted in both synergistic and antagonistic results in bioassays against DBM larvae. Super- and co-infection are good mathematic prediction models for the application of combined isolates because these models included both LD_{50} and LT_{50} for the prediction. The combination of less and medium virulent isolates provided potentially synergistic effects in terms of mortality percentage and speed of kill and required a lower application rate than the individual isolates. The combination of three high virulent isolates and three different species showed antagonism, where the kill speed was slower, and the percentage of cadavers supporting sporulation was less than for their single isolates. Under laboratory conditions, combining the less virulent isolates was as effective as the high virulence combination and isolates. This combination may be suitable for field conditions as diversified genotypes may enhance the fungal pathogenicity under variable conditions. Further trials with different insects and conditions should be conducted to assess the response consistency of this combination. The same species combination is more likely to give synergism rather than antagonism. Therefore, for any future combinations of *Beauveria*, the same species is likely to be the most suitable option.

Chapter 5

The effect of diet on the susceptibility of diamondback moth larvae to *Beauveria* infection

5.1 Introduction

Understanding the interaction between plants, insect pests, and entomopathogenic fungi is important for developing biological control strategies. In some cases, volatile plant metabolites and plant surface chemistry can impact fungal development and, ultimately, the efficacy displayed by the entomopathogen (Cory & Ericsson, 2009). When assessing *B. bassiana* for control of *Blissus leucopterus* (Chinch bug) on four different kinds of cereal (barley, maize, sorghum, and wheat), Ramoska and Todd (1985) found that bugs reared on barley and wheat were more susceptible to the infection of the fungus than those reared on maize and sorghum. Legumes, such as kidney beans, are usually rich in nitrogen and barley and wheat rich in carbohydrates (Gholizadeh et al., 2014; Reinprecht et al., 2020). Mehta et al. (2012) reported that supplies of carbon and nitrogen were crucial for mycelial growth and sporulation of *Beauveria*.

Brassica crops and other species within the order Brassicales are widely known for the production of glucosinolates within their vegetative organs and seeds (Ishida et al., 2014). Glucosinolates are a large group of plant-derived secondary metabolites that contain sulphur and nitrogen (Barba et al., 2016). These metabolites function as plant protection agents against pathogens and invertebrate pests (Neugart et al., 2020). Their pungent odour plays a significant role in defending plants against leaf chewing and sap-sucking insects (Bekaert et al., 2012; Bidart-Bouzat & Kliebenstein, 2008; Hopkins et al., 2009; Santolamazza-Carbone et al., 2016). However, some specialist insects are biochemically adapted to the glucosinolate-containing plants, including *Pieris rapae* (cabbage white butterfly) (Wittstock et al., 2004) and *Plutella xylostella* (diamondback moth) (DBM) (Ratzka et al., 2002). The DBM gut contains sulfatase enzymes that deactivate the toxic glucosinolate metabolites produced by brassicas as plant defence compounds (Ratzka et al., 2002).

Cultivars of brassica crops containing high glucosinolate levels have been more resistant to damage caused by DBM larvae than those containing low levels or where glucosinolate is absent (Robin et al., 2017; Santolamazza-Carbone et al., 2014). The pungency of these glucosinolates is released if the plant is damaged (e.g. by chewing or cutting) and has multiple negative effects on invertebrates. These compounds can act directly as a repellent and an antifeedant (Santolamazza-Carbone et al., 2016) and indirectly by attracting parasitoids that feed on the invertebrate pests (Hopkins et al., 2009). Robin et al. (2017) showed that the concentration of glucosinolates did not determine a plant's susceptibility to DBM, but specific glucosinolates, including glucobrassicin, glucoiberin, and

glucoiberverin, were heavily involved. Furthermore, Hamilton et al. (2005) found that DBM laid more eggs on cabbage cv. Savoy King than cv. Green Coronet, but hatching larvae fed on cv. Green Coronet grew faster and survived longer than those raised on cv. Savoy King. These findings showed different cultivars of cabbage might have different levels of glucosinolates. Interestingly, Badenes-Perez (2006) reported that when DBM moths laid more eggs on a plant containing high glucosinolate, the hatching rate was low.

Glucosinolates have been reported to have the ability to inhibit the growth of fungal pathogens on brassicas (Poveda et al., 2020; Sotelo et al., 2015; Teng et al., 2021). However, glucosinolates have not been reported to have such interaction with entomopathogenic fungi. A study on plants, insects and entomopathogenic fungi interaction by Razinger et al. (2018) still separated the assays into insect-fungi and plant-fungi, not all three together. Raad et al. (2019) showed two isolates of *B. bassiana* had no effect on DBM larvae when used as endophytes in a brassica plant, but these fungal isolates inhibited other fungal pathogens. This study did not examine glucosinolates in the brassica plant, but it provided a complete interaction among a glucosinolate-containing plant, DBM, and a *B. bassiana* isolate. Additionally, this study showed that glucosinolates do not affect *Beauveria* isolates' ability to control other plant pathogens. Still, broader interactions among several glucosinolate-containing plants, DBM, and other *Beauveria* isolates using conidial spray may provide more insights.

There is a lack of scientific articles published to compare the susceptibility of DBM larvae to *Beauveria* infection feeding on different brassica species. This study was conducted to investigate the effect of diet (i.e., different brassica subspecies) on the susceptibility of DBM larvae to two highly virulent *Beauveria* isolates.

5.2 Materials and methods

5.2.1 Fungal isolates

Two fungal strains, *B. pseudobassiana* I12 Damo and *B. bassiana* CTL20, were selected from those investigated in the previous studies (see Chapter 3) based on their high virulence towards DBM larvae. In summary, the fungi were sourced from the BPRC culture collection, kept at -80C, defrosted at room temperature and 100 µL of inoculum transferred to a Petri plate containing PDA (Oxoid). The plate was incubated at 23 ± 1 ^{o}C for 21 days. Fungal spore harvesting, spore counting, and concentration adjustment were accomplished as described in Chapter 3.

5.2.2 Brassica plants and DBM larvae

Seed of the predicted high glucosinolate group: broccoli, cv. De Cicco was provided by Wharehouse seed, cabbage cv. Arisos NS, provided by South Pacific Seeds (New Zealand) Limited; and the predicted low glucosinolate group: cauliflower, cv. All Seasons, and radish, cv. Red Cherry were supplied by Wharehouse seed. Seeds of each cultivar were sown, 10 to a pot (1 L) with a total of 24

pots per cultivar (240 seeds per cultivar in total) within a mixture of 400 kg of potting mix + 100 kg of pumice + 500 g of agricultural lime + 500 g of Hydraflo + 1500 g of Osmocote (16-3.9-10). Plants were grown in a glasshouse (average temperature during experiment 23 ± 5^{o}C) and watered twice a day for six weeks before they were transferred to a controlled temperature room for rearing DBM moths. Glucosinolate levels in the plants were not assessed at the time of the experiment, but this assessment is currently underway in a replicate experiment.

DBM moths used for setting up new colonies were those hatched from larvae that routinely fed on cabbage (six-week-old cabbage plants were used) and were kept within controlled temperature rooms at a temperature of 25 ± 2^{o}C with a 12:12 day:night light period. Then, third instar DBM larvae (hatching from the second and third generations) were collected from plants belonging to the four different brassicas.

5.2.3 Experimental protocols

Six-week-old brassica plants were used for maintaining DBM colonies on different plants. Five plastic pots containing ten brassica plants each were transferred to a cage made of fibre net (mesh size 1 mm x 1mm). A total of 12 cages were used for these two assays (four cages for colony establishment, four cages for the first assay, and four cages for the second assay). Around 100 healthy DBM adult moths were transferred from colonies maintained on cabbage cv. Arisos NS to each of the four brassica cages. The DBM larvae that hatched first were maintained on the selected brassica cultivars until they developed into adults, and then these adults (more than 100 moths) were transferred to new colonies on the same four brassicas. The second and third generations of DBM larvae were used for the bioassays (and these experiments were repeated twice). The *Beauveria* conidial suspensions, application, incubation, mortality of DBM and the number of cadavers supporting sporulation were assessed as described in Chapter 3. For the bioassays, larvae from the four different brassicas were placed in a Petri dish that contained a single detached cabbage leaf supported on 1.2% water agar.

5.2.4 Experimental design

The two isolates of *Beauveria, B. pseudobassiana* I12 Damo and *B. bassiana* CTL20, were grown on PDA, and the resulting conidial suspensions were prepared at three application rates (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray). Each application rate and brassica had two replicates. In total, there were 104 Petri dishes (2 isolates of *Beauveria* x 3 application rates x 4 brassicas x 2 replicates x 2 times + 8 controls) and 520 larvae (5 larvae/Petri dish) used in this experimental study. There was a two-week difference between the first and second assays. For the experimental treatments refer to Table 5.1.

Table 5.1: Experimental treatments for two *Beauveria* isolates applied at three rates to DBM larvae fed on four brassicas

Treatment	Isolate	Larvae fed on	Application rate (conidia/spray)
Control	-	Cabbage	0.01% Triton X-100
T1	I12 Damo	Broccoli	6×10^4, 6×10^6, and 6×10^7
T2	I12 Damo	Cabbage	6×10^4, 6×10^6, and 6×10^7
T3	I12 Damo	Cauliflower	6×10^4, 6×10^6, and 6×10^7
T4	I12 Damo	Radish	6×10^4, 6×10^6, and 6×10^7
T5	CTL20	Broccoli	6×10^4, 6×10^6, and 6×10^7
T6	CTL20	Cabbage	6×10^4, 6×10^6, and 6×10^7
T7	CTL20	Cauliflower	6×10^4, 6×10^6, and 6×10^7
T8	CTL20	Radish	6×10^4, 6×10^6, and 6×10^7

5.2.5 Statistical analysis

All data collected were prepared and analysed using the same method as described in Chapter 3. The ANOVA test was performed to differentiate mortality across the four brassicas, based on LD_{50} and LT_{50} as well as the percentage of cadavers that supported sporulation. The Tukey test was used to rank significant differences among treatments. Mortality results from Chapter 3 for isolates *B. pseudobassiana* I12 Damo and *B. bassiana* CTL20 (for which larvae were fed on cabbage) were used for statistical comparison.

5.3 Results

5.3.1 Mortality percentage

The mortality of larvae ranged from 40% to 100% across all four brassicas for isolate I12 Damo. There were no significant differences ($p > 0.05$) among the mortality of larvae fed on the four brassicas compared to that observed for isolate I12 Damo in Chapter 3, where larvae fed on only cabbage ($F = 1.77$, $df = 4$, $p = 0.191$) (Table 5.2). At the 6×10^4 conidia/spray application rate, isolate I12 Damo killed 78%, 40%, 61%, and 56% of larvae from broccoli, cabbage, cauliflower and radish, respectively. When increasing the application rate to 6×10^6 conidia/spray, the isolate caused 94%, 100%, 94%, and 94% mortality of larvae from broccoli, cabbage, cauliflower, and radish, respectively. At the 6×10^7 conidia/spray rate, the isolate killed 100% of all larvae on all four brassicas (Figure 5.1).

Table 5.2: Statistical analysis of DBM mortality on four brassicas caused by *Beauveria pseudobassiana* isolate I12 Damo (General ANOVA, multiple comparisons using Tukey test, in Genstat)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	0.8333	0.8333	2.06	
Brassica	4	2.8667	0.7167	1.77	0.191
Dose	2	120.8667	60.4333	149.31	<.001
Brassica.Dose	8	11.1333	1.3917	3.44	0.021
Residual	14	5.6667	0.4048		

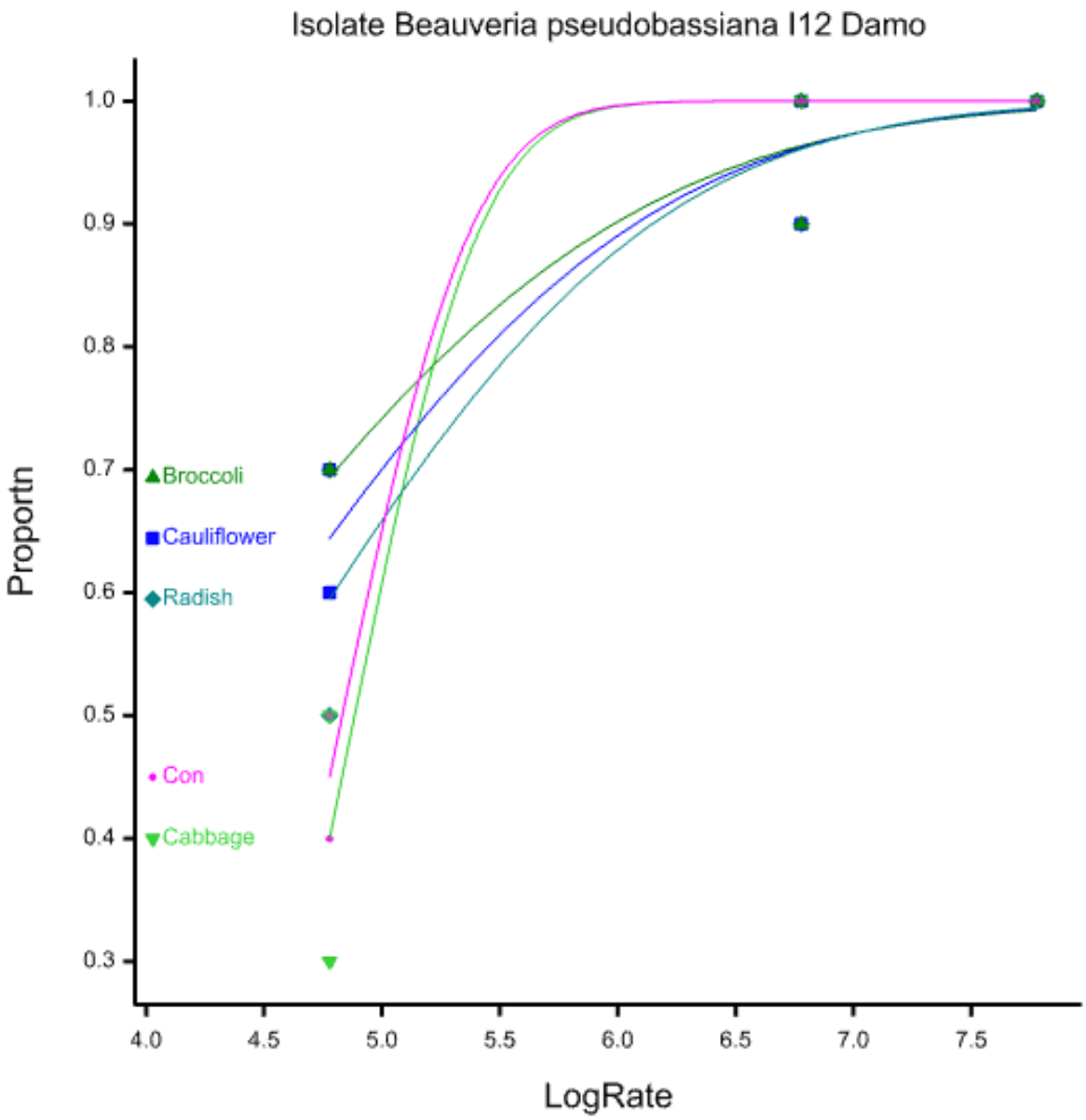


Figure 5.1: The percentage DBM larvae mortality fed on four different brassicas caused by isolate I12 Damo with three application rates (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray) (Logistic regression, Probit test using Log10), Con = the mortality result of isolate I12 Damo from Chapter 3 (larvae raised on cabbage)

The mortality of DBM larvae ranged from 33% to 100% across all larvae fed on all four brassicas for isolate CTL20. There was a statistically significant ($p < 0.05$) difference between mortality of larvae fed on the four brassicas compared to the cabbage treatment (the result of isolate CTL20 in Chapter 3) ($F = 3.50$, $df = 4$, $p = 0.04$) and the three application rates ($F = 158.50$, $df = 2$, $p < .001$) (Table 5.3). The mortality of larvae fed on radish was significantly ($p < 0.05$) higher than on broccoli but not from cabbage and cauliflower at the application rate of 6×10^4 conidia/spray. There were no significant differences at application rates of 6×10^6 and 6×10^7 conidia/spray. DBM larvae raised on cauliflower exhibited higher mortality than broccoli and cabbage when treated with isolate CLT20, ranging from 39%, 100%, and 100% across the three rates. The mortality of DBM larvae fed on broccoli and cabbage was similar, ranging from 33%, 83%, and 94% for broccoli and 45%, 94%, and 94% for cabbage for 6×10^4, 6×10^6 and 6×10^7 conidia/spray, respectively (Figure 5.2). Mortality data from both isolates and four brassicas were corrected using Abbott's formula Table 5.4.

Table 5.3: Statistical analysis of the mortality of DBM from four brassicas caused by *Beauveria bassiana* isolate CTL20 (General ANOVA, multiple comparisons using Tukey test, in Genstat)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	4.0333	4.0333	7.56	
Brassica	4	7.1333	1.7833	3.34	0.04
Rate	2	166.8667	83.4333	156.44	<.001
Brassica.Rate	8	5.4667	0.6833	1.28	0.327
Residual	14	7.4667	0.5333		

Table 5.4: Mortality rate after correcting for control mortality using Abbott's formula caused by both *Beauveria pseudobassiana* isolate I12 Damo and *Beauveria bassiana* isolate CTL20

Isolate	Brassica	Application rate (conidia/spray)		
		6×10^4	6×10^6	6×10^7
		Mean ± SE	Mean ± SE	Mean ± SE
I12 Damo	Broccoli	78 ± 7.00	94 ± 5.55	100 ± 0.00
I12 Damo	Cabbage	40 ± 5.00	100 ± 0.00	100 ± 0.00
I12 Damo	Cauliflower	61 ± 6.00	94 ± 5.55	100 ± 0.00
I12 Damo	Radish	56 ± 5.00	94 ± 5.55	100 ± 0.00
F-value		6.01	1.00	NA
P-value		0.087	0.500	NA
CTL20	Broccoli	33 ± 11.00 a	83 ± 4.00	94 ± 6.00
CTL20	Cabbage	45 ± 5.55 ab	94 ± 6.00	94 ± 6.00
CTL20	Cauliflower	39 ± 4.00 ab	100± 0.00	100 ± 0.00
CTL20	Radish	61 ± 3.00 b	100 ± 0.00	100 ± 0.00
F-value		9.22	8.03	1.00
P-value		0.050	0.060	0.500

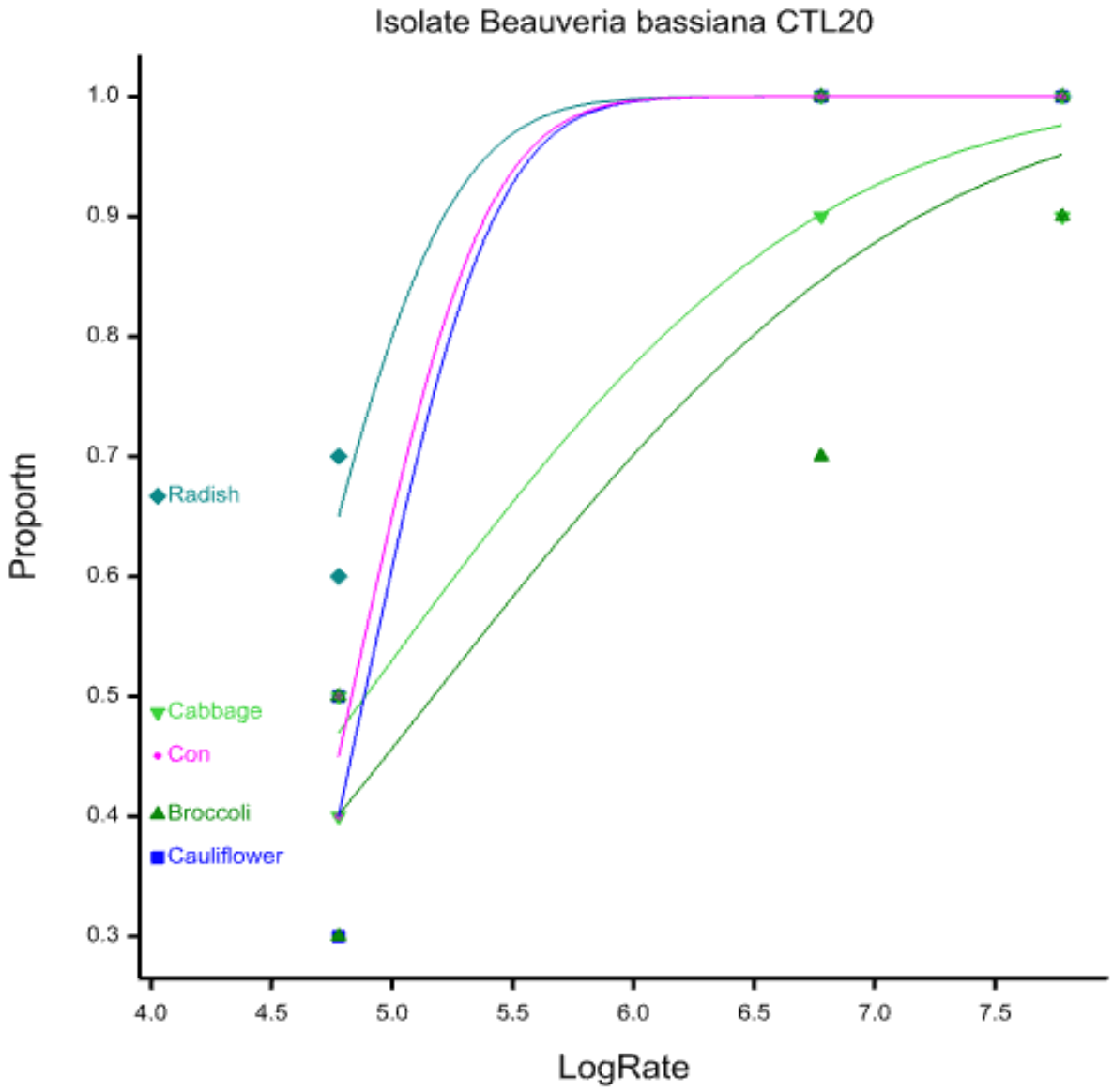


Figure 5.2: The percentage mortality of DBM larvae fed on four different brassicas caused by isolate CTL20 with three application rates (6 x 10^4, 6 x 10^6, and 6 x 10^7 conidia/spray) (Logistic regression, Probit test using Log10), Con = the mortality result of isolate CTL20 from Chapter 3 (larvae raised on cabbage)

The conidial application rate required to cause 50% mortality of DBM larvae by isolate I12 Damo ranged from around 10^4 conidia/spray for broccoli, cauliflower and radish to 10^5 conidia/spray for cabbage. There were no significant ($F = 1.21$, $df = 4$, $p = 0.428$) differences among the groups (Figure 5.3). Statistical significance of LD_{50} of isolate I12 Damo refers to Table B.3.1 (Appendix B). While the application rate of isolate I12 Damo required to cause 50% mortality on larvae fed on broccoli was 10^4 conidia/spray, isolate CTL20 required 10^5 conidia/spray, which was significantly ($F = 6.3$, $df = 4$, $p = 0.050$) more than the DBM larvae required when reared on the other three brassica subspecies. Chronologically, larvae fed on radish, cauliflower, and cabbage needed 10^4 conidia/spray to achieve LD_{50}, and only larvae raised on broccoli required 10^5 conidia/spray for 50% mortality (Figure 5.4). Statistical significance of isolate CTL20 refers to Table B.3.2 (Appendix B).

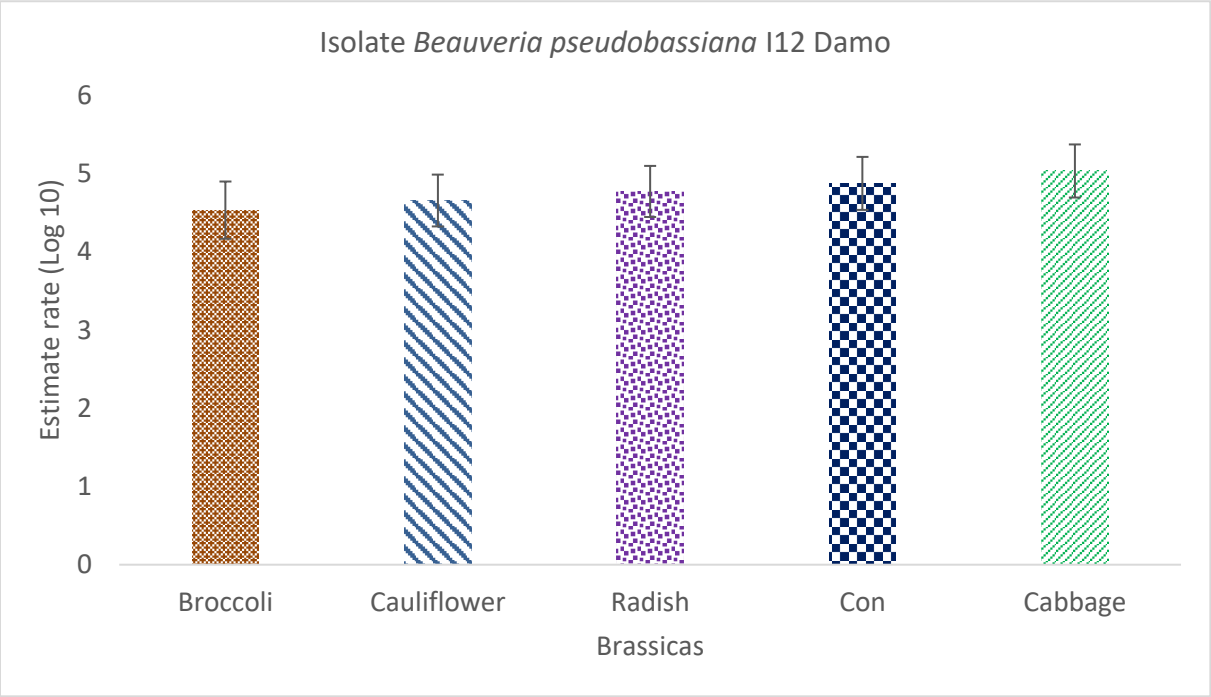


Figure 5.3: Application rate of isolate I12 Damo required to kill 50% of larvae fed on the four different brassicas (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, in Genstat), Con = LD_{50} result of isolate I12 Damo in Chapter 3 (larvae raised on cabbage)

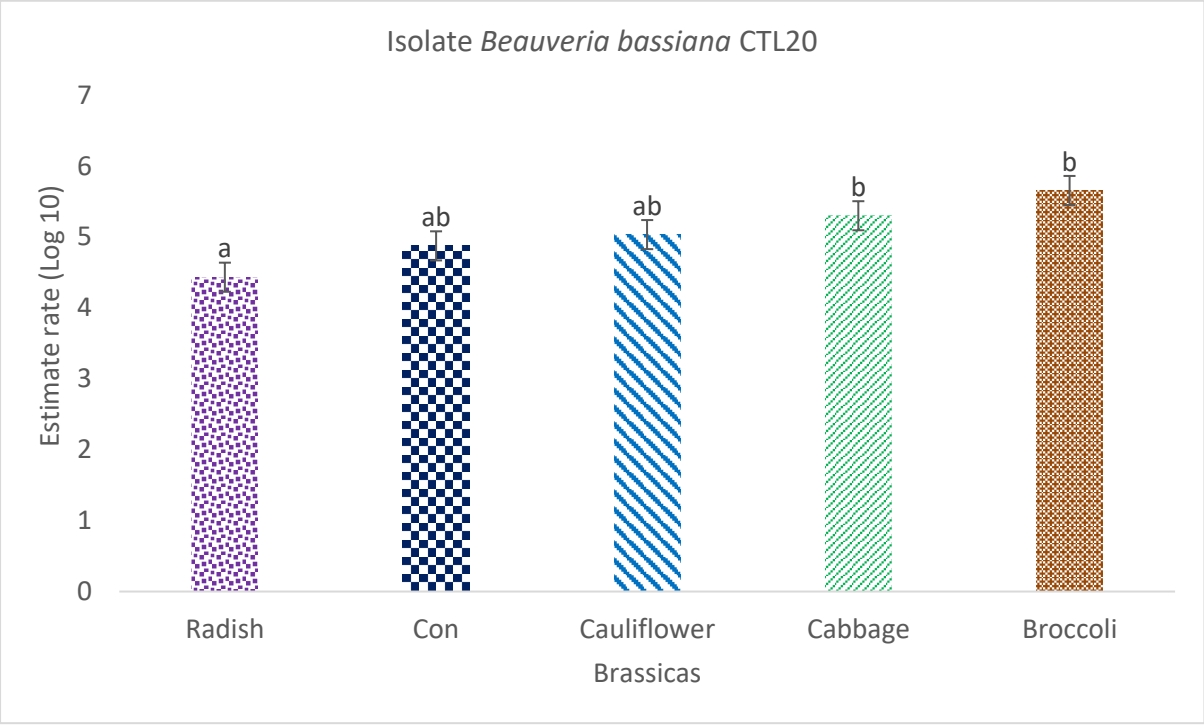


Figure 5.4: Application rate of isolate CTL20 required to kill 50% of larvae fed on the four different brassicas (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, in Genstat)**,** Con = LD_{50} result of isolate CTL20 in Chapter 3 (larvae raised on cabbage)

5.3.2 Median lethal time (LT_{50})

The LT_{50} of DBM larvae fed on the four brassicas ranged from 4.8 to 7.25 days at the application rate of 6 x 10^4 conidia/spray, 2.62 to 3.80 days at 6 x 10^6 conidia/spray, and from 2.05 to 2.80 days at 6 x 10^7 conidia/spray for isolate I12 Damo. Larvae raised on broccoli, cauliflower, and radish reached the LT_{50} within seven days at the application rate of 10^5 conidia/mL, while for those on cabbage, it took longer. At the 6 x 10^6 conidia/spray application rate, larvae fed on cabbage and cauliflower took less than three days to achieve LT_{50}, and for those on broccoli and radish, it took less than four days to reach 50% mortality. Larvae fed on all four brassicas had 50% mortality after around two days at the 6 x 10^7 conidia/spray application rate (Figure 5.5). There was no statistical difference among the four brassicas (F = 2.21, df = 4, p = 0.120). Statistical significance of LT_{50} for isolate I12 Damo refers to Table B.3.3 (Appendix B).

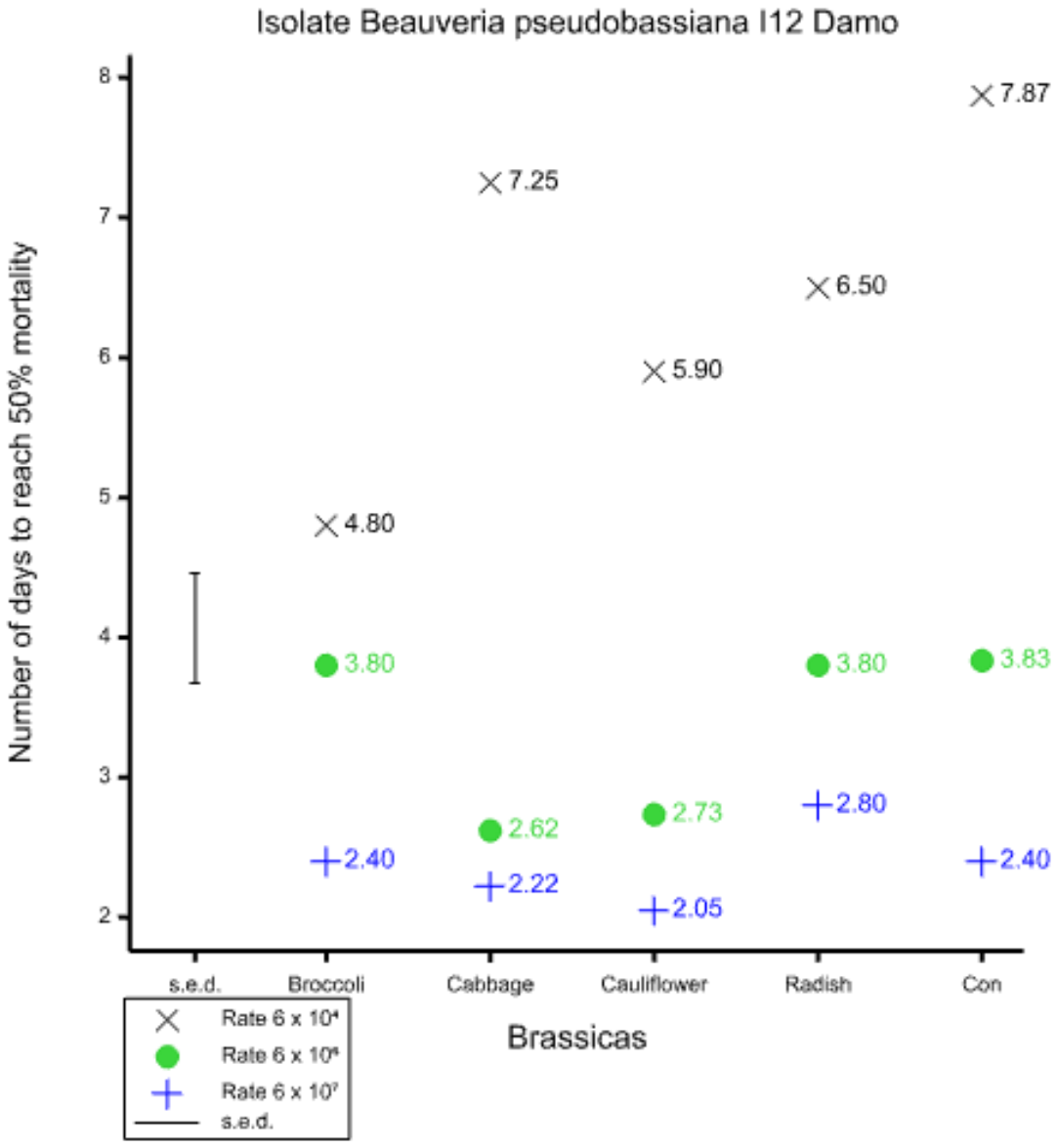


Figure 5.5: LT_{50} of DBM larvae fed on four brassicas across three application rates of isolate I12 Damo (General analysis of variance, Rate*Brassica interaction, Genstat)**,** Con = LT_{50} result of isolate I12 Damo in Chapter 3 (larvae raised on cabbage)

For isolate CTL20, only larvae raised on cabbage and radish achieved LT_{50} within seven days at the application rate of 6 x 10^4 conidia/spray. Within the group, there were significant differences between radish and cauliflower (F = 24.54, df = 4, p < 0.001). LT_{50} took less than five days at the application rate of 6 x 10^6 conidia/spray and less than four days at 6 x 10^7 conidia/spray across all four brassicas (Figure 5.6). There was no significant difference among groups of brassicas at application rates of 6 x 10^6 conidia/spray (F = 1.49, df = 4, p = 0.353) and 6 x 10^7 conidia/spray (F = 0.45, df = 4, p = 0.772). Statistical significance of LT_{50} for isolate CTL20 refers to Table B.3.4 (Appendix B).

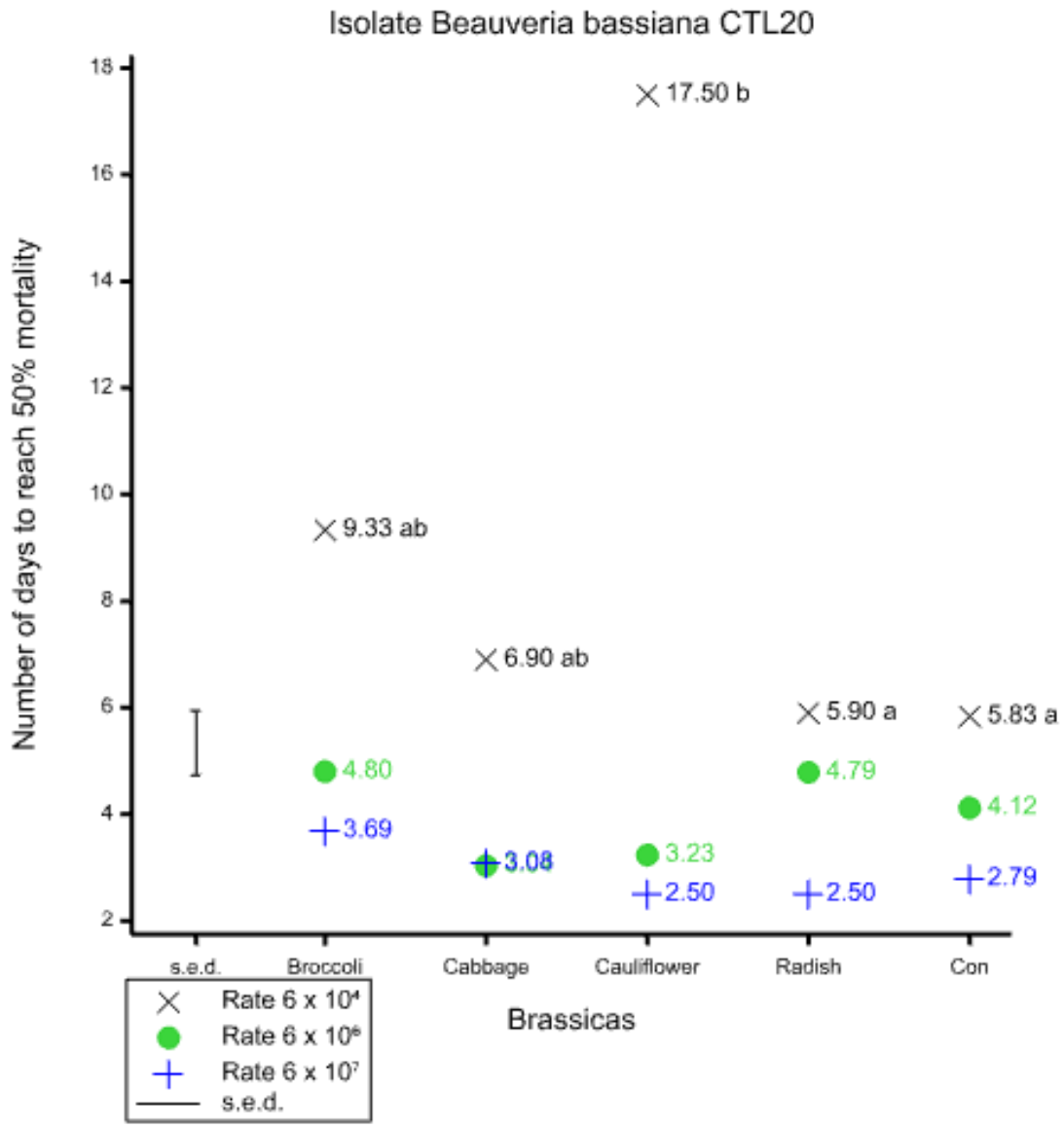


Figure 5.6: LT_{50} of DBM larvae fed on four brassicas across three application rates of isolate CTL20 (General analysis of variance, Rate*Brassica interaction, Genstat)**,** Con = LT_{50} result of isolate CTL20 in Chapter 3 (larvae raised on cabbage)

5.3.3 Cumulative mortality

Larval mortality began on the second day post-treatment at the application rate of 6 x 10^6 and 6 x 10^7 conidia/spray and the third day at 6 x 10^4 conidia/spray for isolate I12 Damo across the four brassicas. At the 6 x 10^4 conidia/spray rate, the mortality of larvae fed on broccoli was consistently higher than the other plants, followed by cauliflower, radish and cabbage, respectively (Figure 5.7). Statistically, among the four brassicas, there was no significant difference. The result changed slightly at the application rate of 6 x 10^6 conidia/spray. Only for larvae raised on cabbage did 100% die, while the larvae on the other three brassicas had less than 100% mortality (Figure 5.8). At the 6 x 10^7 conidia/spray application rate, 100% of larvae from all four brassicas were killed. Larvae fed on cabbage reached 100% mortality within four days after spraying, while larvae raised on broccoli and cauliflower took six days and on radish took up to seven days (Figure 5.9).

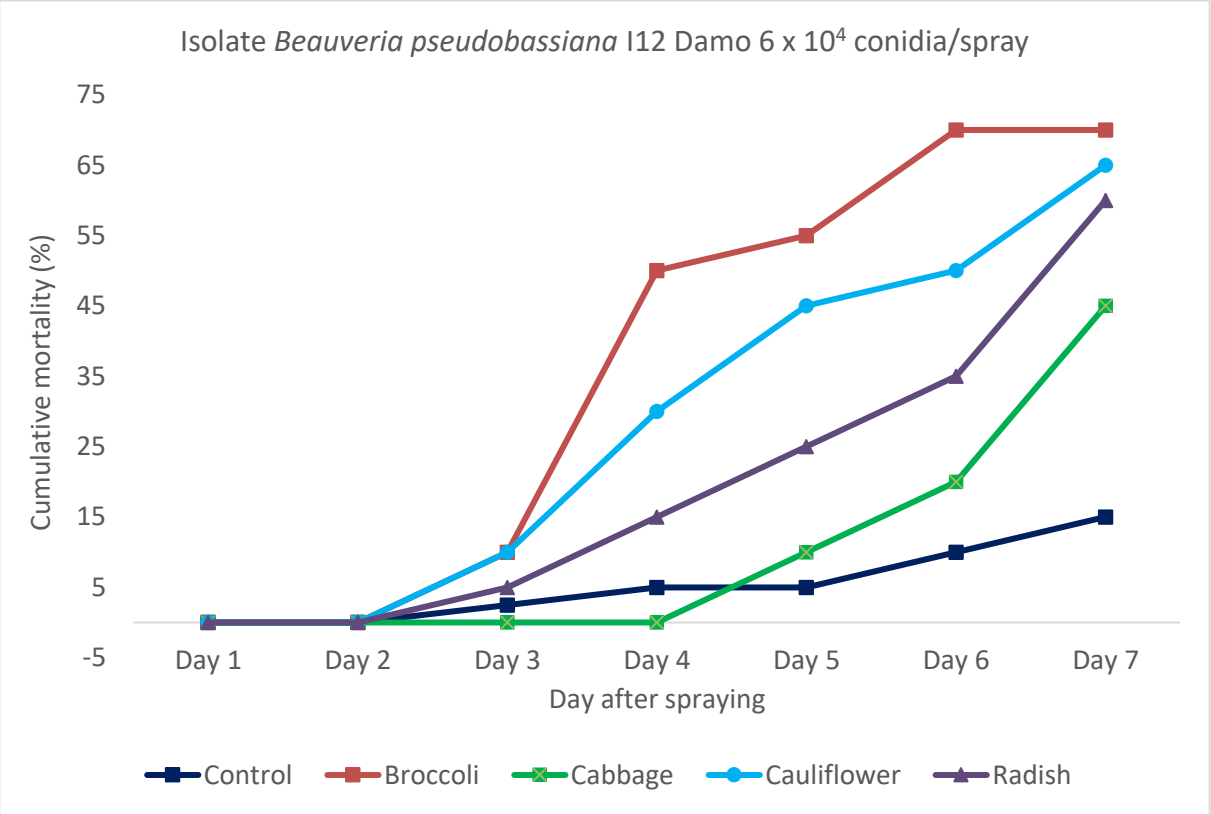


Figure 5.7: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate I12 Damo at the application rate 6 x 10^4 conidia/spray, Control = 0.01% Triton X-100

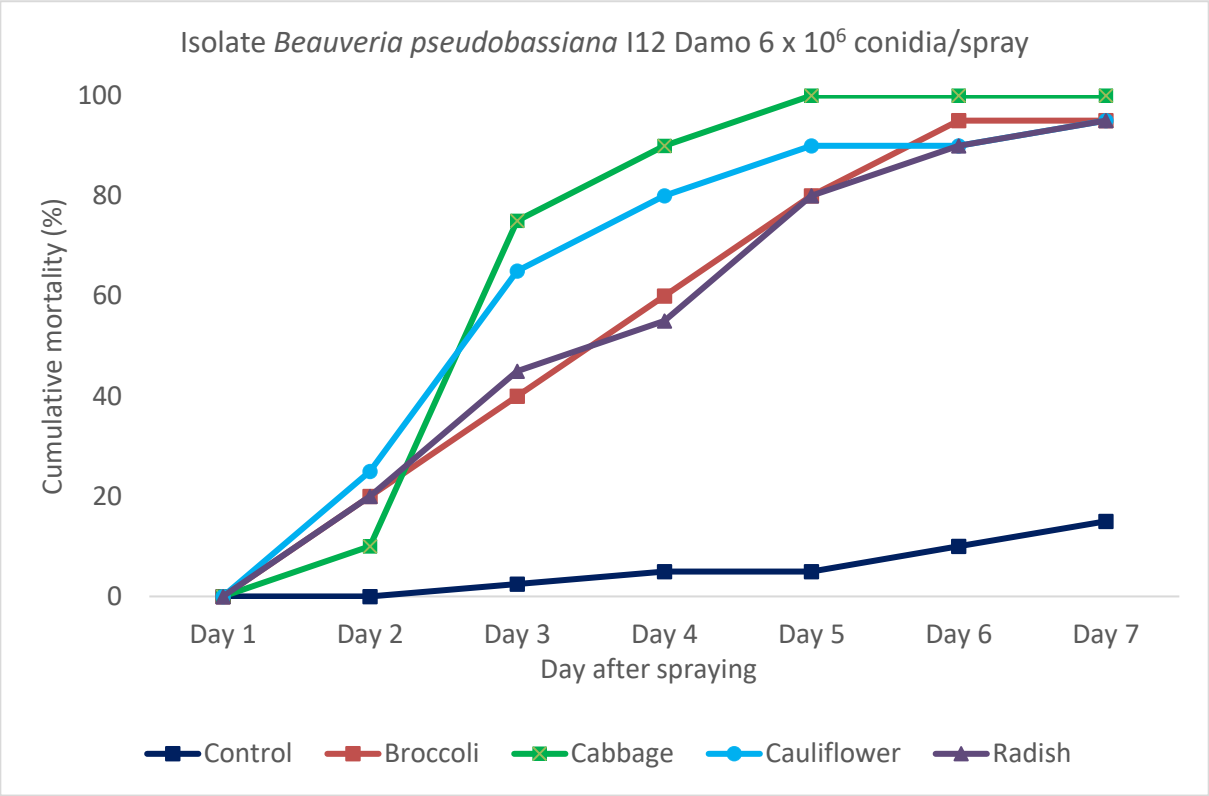


Figure 5.8: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate I12 Damo at the application rate of 6 x 10^6 conidia/spray, Control = 0.01% Triton X-100

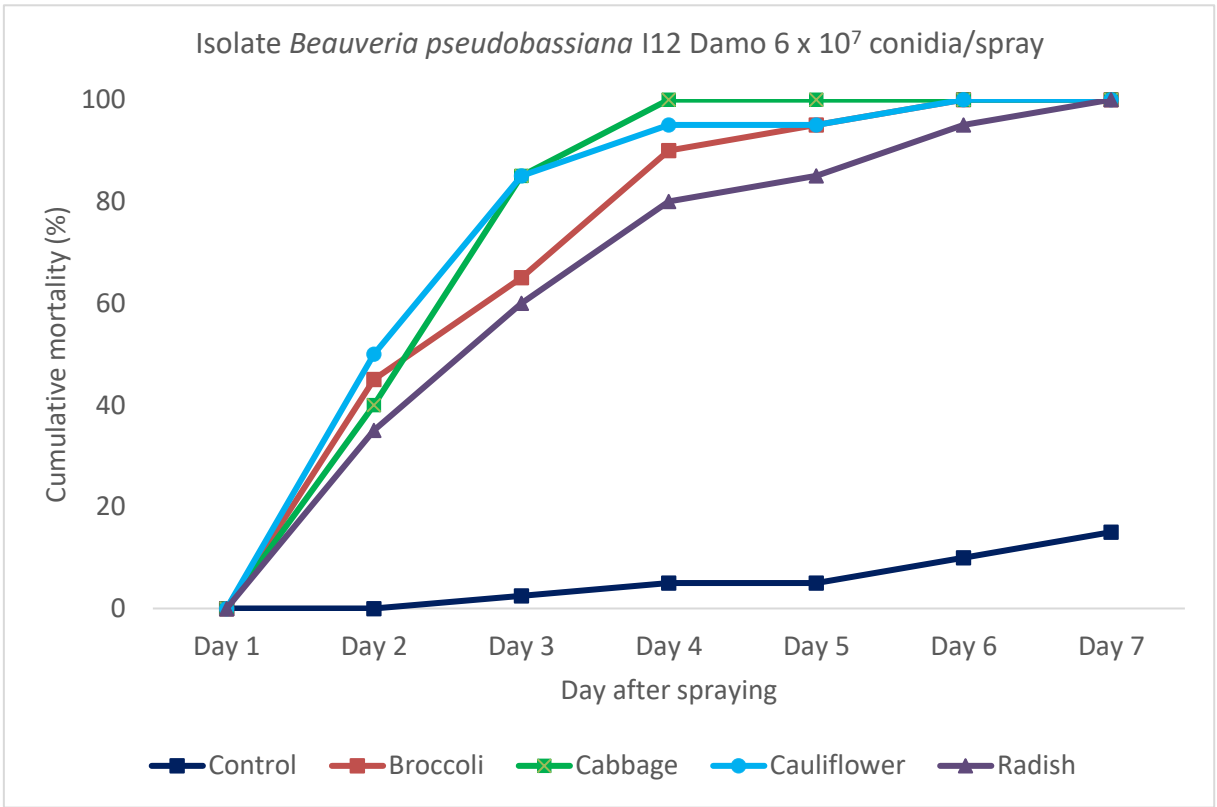


Figure 5.9: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate I12 Damo at the application rate of 6 x 10^7 conidia/spray, Control = 0.01% Triton X-100

For isolate CTL20, results were the opposite of isolate I12 Damo. At the application rate of 6 x 10^4 conidia/spray, there was more mortality for larvae fed on radish (up to 65%) than cabbage (55%), broccoli (40%), and cauliflower (40%), respectively (Figure 5.10). When the rate increased to 6 x 10^6 conidia/spray, 100% of DBM larvae raised on cauliflower and radish were killed, while 85% and 95% of larvae from broccoli and cabbage died by day seven post-spray (Figure 5.11). At the rate of 6 x 10^7 conidia/spray, all larvae fed on cauliflower had been killed by the fifth day, followed by radish by day seven. In contrast, larvae from broccoli and cabbage reached only 95% mortality by day seven (Figure 5.12).

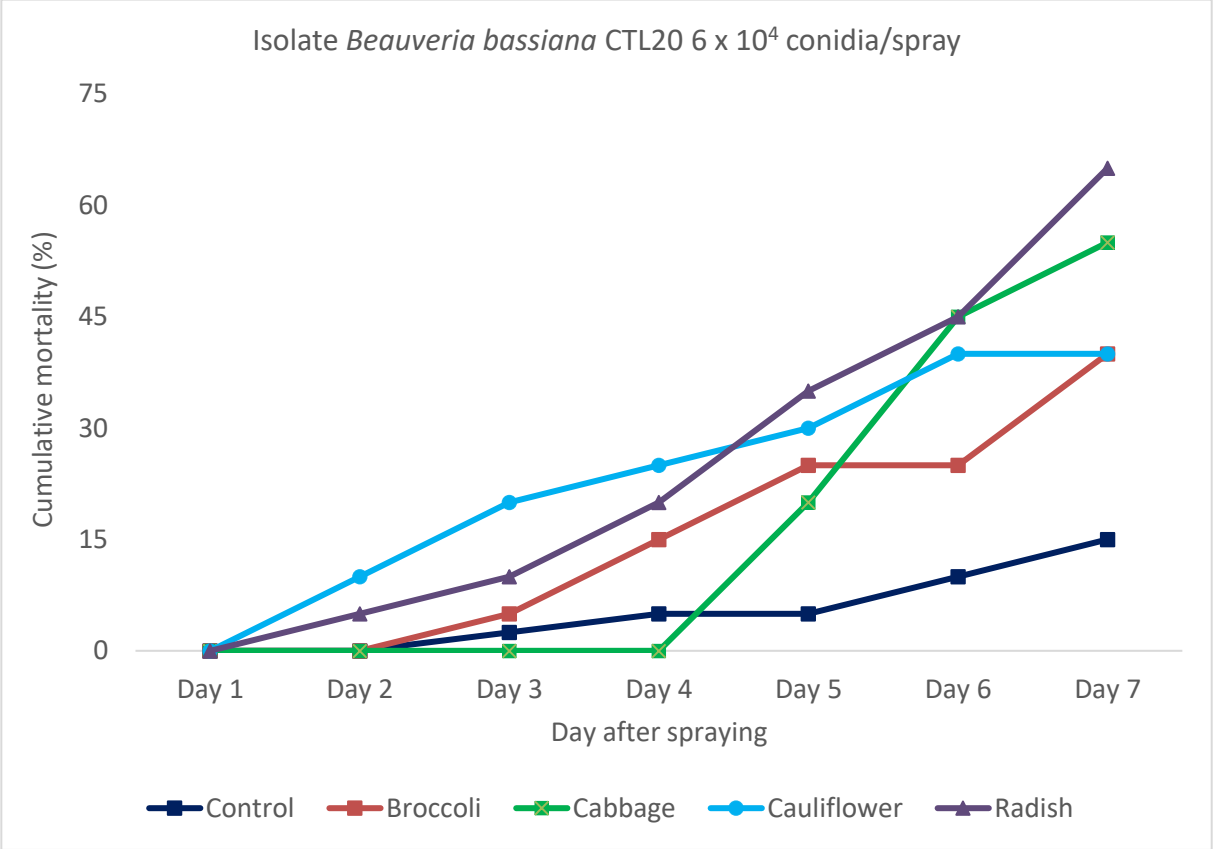


Figure 5.10: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate CTL20 at the application rate of 6 x 10^4 conidia/spray, Control = 0.01% Triton X-100

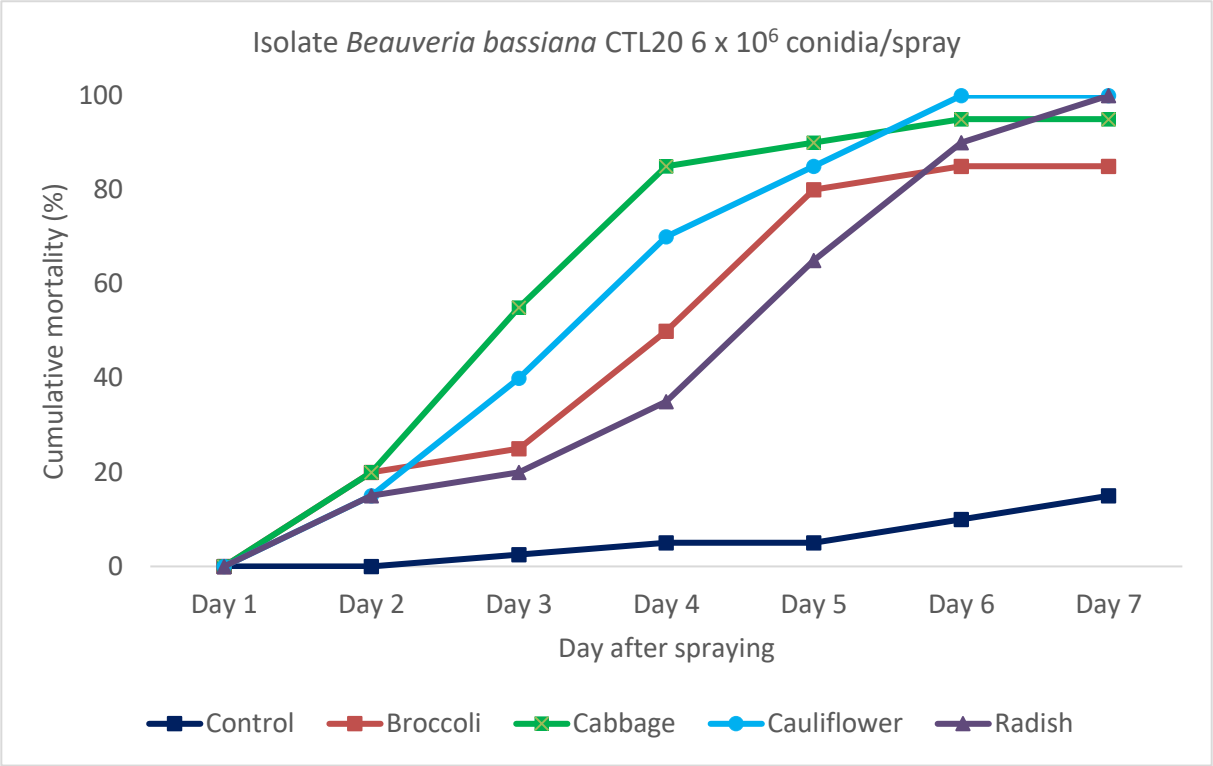


Figure 5.11: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate CTL20 at the application of rate 6 x 10^6 conidia/spray, Control = 0.01% Triton X-100

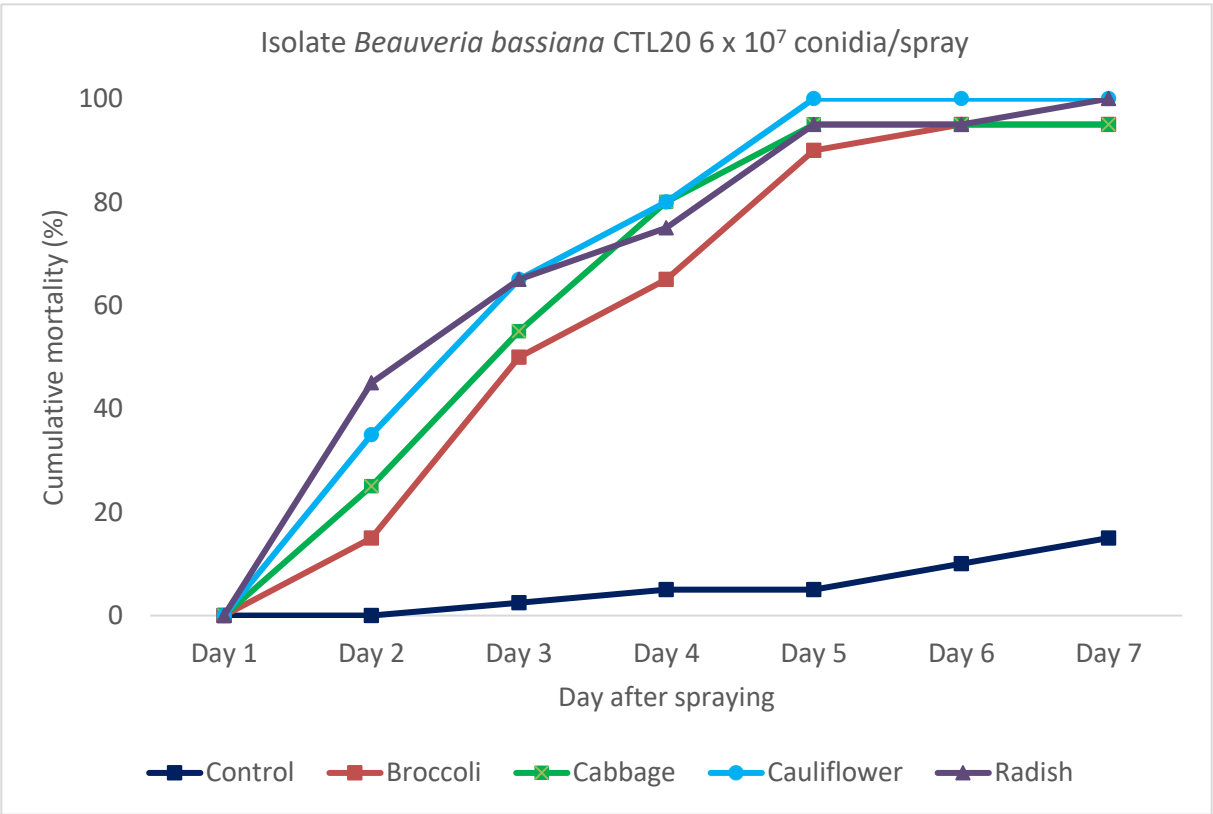


Figure 5.12: Cumulative mortality of DBM larvae raised on four brassicas caused by isolate CTL20 at the application rate of 6 x 10^7 conidia/spray, Control = 0.01% Triton X-100

5.3.4 Percentage of cadavers that supported sporulation

Compared to the result in Chapter 3, the number of cadavers that supported sporulation of isolate I12 Damo was statistically significantly different at the rate of 6 x 10^4 conidia/spray (F = 19.08, df = 4, p = 0.007). Cadavers of larvae fed on broccoli and radish supported less sporulation than those raised on cabbage and cauliflower. There was no significant difference within brassicas at the rates of 6 x 10^6 and 6 x 10^7 conidia/spray (Figure 5.13). Percentage sporulation results for isolate CTL20 showed no significant difference among all rates and brassicas compared with the result in Chapter 3 (Figure 5.14).

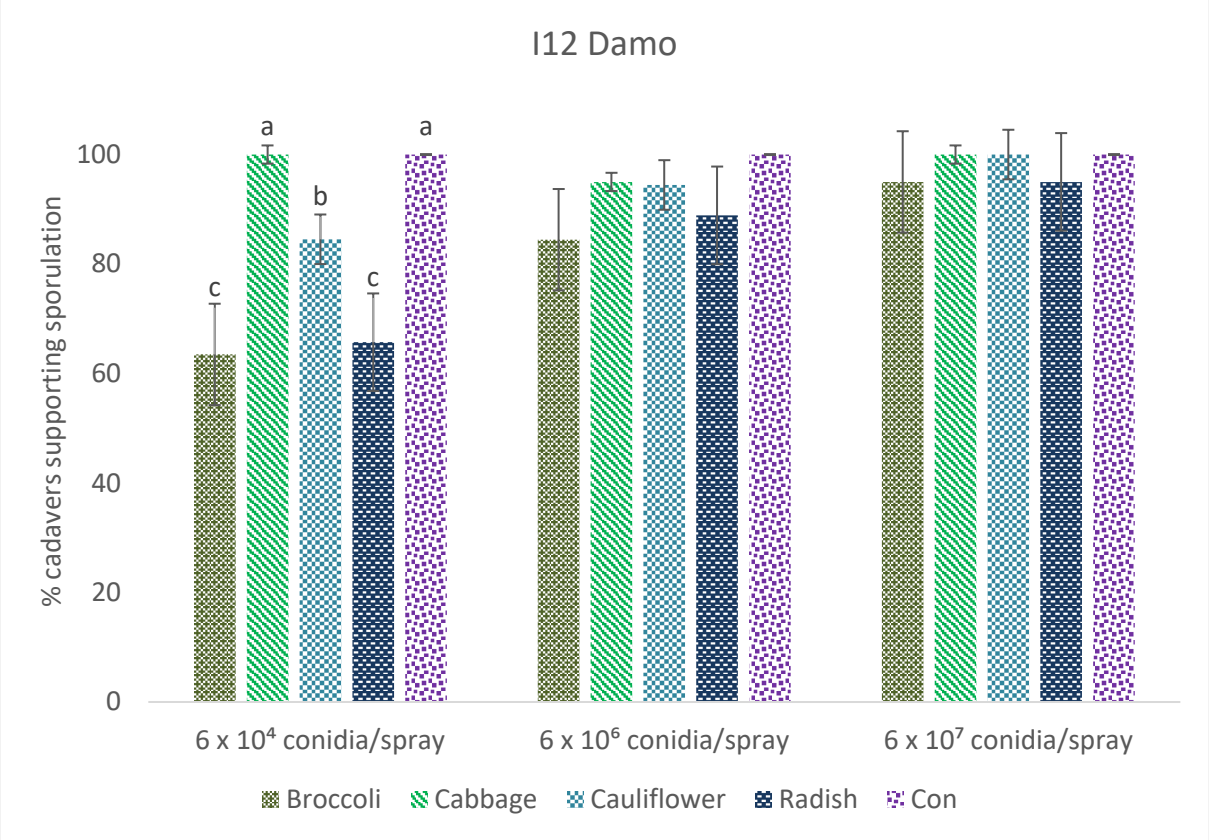


Figure 5.13: Percentage of cadavers that supported sporulation after larvae fed on four brassicas treated with three rates of isolate I12 Damo (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, in Genstat)**,** Con = percentage sporulation result of isolate I12 Damo in Chapter 3 (larvae raised on cabbage)

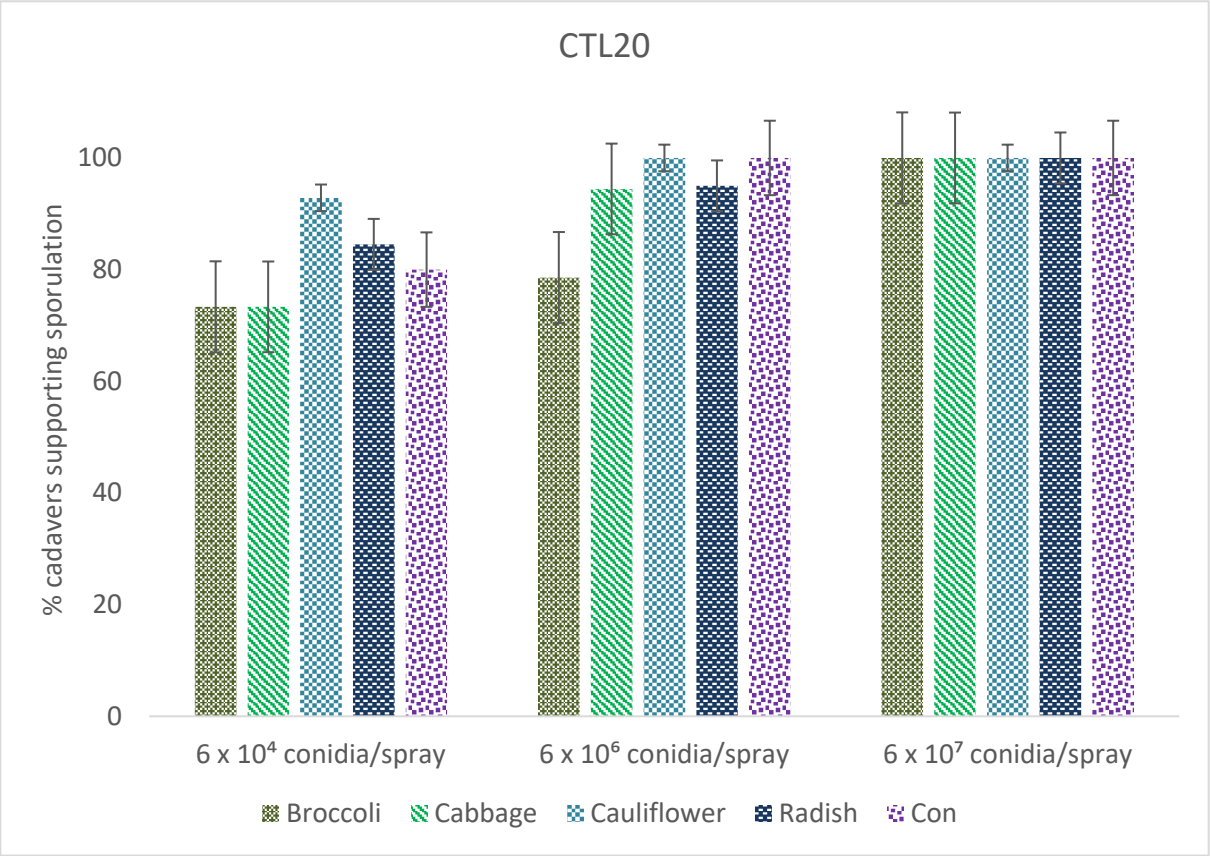


Figure 5.14: Percentage of cadavers that supported sporulation after larvae fed on four brassicas treated with three rates of isolate CTL20 (Error bar based on standard error, grouped using General ANOVA, multiple comparisons using Tukey test, in Genstat), Con = percentage sporulation result of isolate CTL20 in Chapter 3 (larvae raised on cabbage)

5.4 Discussion

The two highly virulent isolates of *B. bassiana* at all three application rates resulted in 33% to 100% mortality of DBM larvae fed on four different brassicas. Mortality of DBM larvae fed on both the predicted high and low glucosinolate brassica groups did not differ for isolate *B. pseudobassiana* I12 Damo for any of the three application rates. By comparing percentage mortality and median lethal time for trends rather than statistical significance, larvae fed on broccoli had higher mortality and died faster than those fed on the other three brassicas. Different cruciferous species display different secondary metabolite profiles; they may have diverse effects on *Beauveria* development and insect susceptibility to the fungus. Studies on glucosinolate contents of eight brassica species, broccoli, Brussel sprout, cabbage, cauliflower, kale, kimchi cabbage, mustard, and radish, found that cauliflower and radish contain lower quantities of glucosinolates than the other species (Hwang et al., 2019; Kim et al., 2020). However, different cabbage cultivars also had different glucosinolates levels, and those cultivars containing low glucosinolates were more susceptible to DBM larvae than those containing higher glucosinolates (Robin et al., 2017). Additionally, studies revealed that the insect-resistant

properties of brassica were related to the glucosinolate metabolites rather than the total amount of glucosinolates in the plant (Robin et al., 2017; Santolamazza-Carbone et al., 2014; Sarosh et al., 2010). For example, at least 120 of these metabolites were reported in many members of the *Brassicaceae* family (Fahey et al., 2001). Among these 120 metabolites, three hydrolysis compounds, isothiocyanates, thiocyanates, and nitriles, are known to play a significant role in plant protection against herbivores (Halkier & Gershenzon, 2006). Therefore, in the current study, feeding DBM larvae broccoli and cabbage may have decreased their fitness, making them more susceptible to infection by isolate *B. pseudobassiana* I12 Damo.

B. bassiana CTL20 produced higher mortality of DBM larvae raised on radish at the rate of 6 x 10^4 conidia/spray, while the two higher rates showed no increase in DBM mortality among the four brassicas used in this study. DBM larvae fed on cauliflower and radish reached 100% mortality at application rates of 6 x 10^6 and 6 x 10^7 conidia/spray. According to Kim et al. (2020), cauliflower and radish contain lower glucosinolate contents than the other two brassicas used in this study, broccoli and cabbage. This study showed that DBM larvae fed on low glucosinolate brassicas were more susceptible than high glucosinolate brassicas to the isolate *B. bassiana* CTL20. A study on food utilisation and consumption of DBM using eight cultivars of cabbage showed no relationship between low and high glucosinolate groups (Karmakar et al., 2021). These reports indicate that DBM can feed on high and low glucosinolate plants without adverse effects on its health fitness. However, the result of *B. bassiana* CTL20 in this study showed that infection by this fungal isolate might be related to glucosinolate content, as DBM larvae fed on brassicas with lower glucosinolate compounds were more susceptible to fungal infection than those raised on higher glucosinolate plants. This result contradicts previous results that found high glucosinolate plants caused larvae to be more vulnerable to the infection of insect pathogens (Hopkins et al., 2009; Robin et al., 2017; Santolamazza-Carbone et al., 2016). However, Robin et al. (2017) reported that the total amount of glucosinolates did not determine plant responses to DBM damage, but rather it was some specific glucosinolates. With this regard, radish may contain some specific compounds that give suitable conditions for isolate *B. bassiana* CTL20 to infect the host, but this is yet to be determined.

The levels of glucosinolates in the plant types used in the current study are still being assessed. Grouping brassicas crops according to their levels of glucosinolates, both Hwang et al. (2019) and Kim et al. (2020) took samples from leaves of these crops in South Korea. As these experiments in this study used leaves of four brassicas purchased in New Zealand, they may contain various levels of glucosinolates. Some studies revealed that growing cabbage crops from different cultivars showed low and high levels of glucosinolates (Hamilton et al., 2005; Robin et al., 2017; Robin et al., 2016). These findings indicate brassica cultivars within may have different specific glucosinolates. Additionally, brassica crops produced more glucosinolates in response to abiotic stresses, so growing them under different conditions may impact levels of glucosinolates in plants (Antonious et al., 2009). This

chemical diversity would therefore have significant impacts on biological control of DBM when applying *Beauveria* as they may impact fungal growth. This study showed that *B. pseudobassiana* I12 Damo killed a higher number of DMB larvae when assessed on brassica plants that were expected to contain high glucosinolate levels compared to plants containing low levels. However, a second isolate showed the opposite result. Therefore, the findings of this study showed that different *Beauveria* isolates worked differently on DBM larvae raised on brassicas reported to contain low and high glucosinolates. Future study to analyse levels of glucosinolates in the four brassicas used in this experimental study should be conducted.

The percentage of DBM cadavers that supported sporulation from *B. pseudobassiana* I12 Damo applied at a low application rate to the DBM larvae was lower than previously reported in Chapter 3. No difference was found for isolate *B. bassiana* CTL20 between this current study and that undertaken in Chapter 3. Glucosinolate metabolites inhibit grow of fungal pathogens on brassicas (Teng et al., 2021). These metabolites may have reduced the percentage sporulation of isolate *B. pseudobassiana* I12 Damo on infected cadavers. Three-way interactions among plants, arthropods, and entomopathogenic fungi contribute significantly to the development of each dimension (Biere & Tack, 2013). For example, the production of newly formed conidia of *B. bassiana* was considerably higher on cadavers of sweet potato whitefly fed on melon than on cotton (Santiago-Álvarez et al., 2006). Additionally, Zafar et al. (2016) reported that *B. bassiana* Bb-01 caused higher mortality of insects reared on cotton than three Solanaceous species when testing the cotton whitefly species. Vega et al. (1997) showed allelochemicals produced by plants inhibited the germination of blastospores of the fungus *Paecilomyces fumosoroseus* on a plant leaf, on insect cuticle, and in the haemolymph. At the application rate of 6 x 10^4 conidia/spray, the percentage sporulation of isolate *B. pseudobassiana* I12 Damo was greater on DBM fed on cabbage and cauliflower than on broccoli and radish. Moreover, as this isolate gave consistent mortality at the rate of 6 x 10^6 and 6 x 10^7 conidia/spray, there was no significant difference in sporulation across the four brassicas. The efficacy assessment of *B. bassiana* in controlling two-spotted spider mite on five species of plants found the mortality was high on beans and cucumber, but the conidia viability was the same, as was persistence ability (Gatarayiha et al., 2010). This finding was similar to isolate *B. bassiana* CTL20 that showed a significant difference in mortality among the four brassicas tested but not a different sporulation rate on cadavers. Even though sporulation of both isolates in this study was reduced, this could be a good characteristic for industry to invest in as they may want a biopesticide that provides significant mortality with low sporulation, reducing the subsequent infections so that they can sell more product.

This study showed specific isolates of *Beauveria* work better in controlling DBM on specific brassica crops depending on their predicted glucosinolate profiles. Consideration could be given to using isolate *B. pseudobassiana* I12 Damo to control DBM larvae damage on broccoli and cabbage, and isolate *B. bassiana* CTL20 to control DBM larvae damage on cauliflower and radish because they

require a lower application rate and shorter time than previous studies (Batta et al., 2010; Furlong, 2004; Kirubakaran et al., 2014; Medo et al., 2021; Nithya et al., 2019). Another technique to improve DBM control is to integrate the fungal application with natural insecticides based on glucosinolate metabolites. Some glucosinolate profiles, particularly isothiocyanates, improved toxicity to brassica specialists, *P. xylostella* (DBM) and *Pieris rapae* (White cabbage butterfly) through myrosinase activities (Agrawal & Kurashige, 2003; Li et al., 2000). Thus, a biopesticide based on isothiocyanates may be potentially incorporated with a fungal spray to optimize the application.

5.5 Conclusion

Isolate *B. pseudobassiana* I12 Damo and *B. bassiana* CTL20 showed different effects on mortality of DBM larvae when fed on predicted low and high glucosinolate brassicas. When feeding on broccoli and cabbage, DBM larvae were susceptible to infection by isolate *B. pseudobassiana* I12 Damo. Conversely, those larvae raised on cabbage and radish were more vulnerable to infection by isolate *B. bassiana* CTL20. The use of these two isolates should consider their performance based on glucosinolate contents of each brassica (if higher, apply *B. pseudobassiana* I12 Damo, if lower, apply *B. bassiana* CTL20). Further investigation should differentiate levels of glucosinolates of the four brassicas used in this study to give the findings a plausible conclusion. A study of the combination of natural insecticide based on isothiocyanates with fungal spray should be conducted for assessing synergistic responses. The same isolates should be used to test against DBM fed on the same brassicas under glasshouse or field conditions to examine the consistency of activity.

Chapter 6

The potency of *Beauveria*-derived toxins on diamondback moth larvae

6.1. Introduction

Biopesticides are an alternative control option to synthetic pesticides for the control of agricultural pests or can be incorporated alongside synthetic pesticides within well-developed integrated pest management systems. However, high production and research costs, slowness to kill, inconsistent field performance, narrow host spectrum, complex life cycles, lack of handling technologies for ensuring ongoing viability, and registration or regulatory challenges are factors limiting the development and deployment of new biopesticides (Agboyi et al., 2020; Glare et al., 2012; Glare et al., 2016; Kim et al., 2010). The two major problems of using biological control agents are slowness to kill and inconsistent field results (Glare et al., 2016; Wraight et al., 2001). Generally, it takes several days for any living agent, such as an entomopathogen, to infect and kill an invertebrate pest. At this time, the infected pests are still able to severely damage the plant/crop (St Leger et al., 1996), while many synthetic pesticides can take just a few hours. Growers want biopesticide products that rapidly kill target pests, provide broad-spectrum activity against pest complexes, are cheap, and give consistent results under a range of climatic conditions, similar to conventional synthetic pesticides (Butt et al., 2016).

Many entomopathogenic fungi, including *Beauveria*, produce secondary metabolites that can kill insects (Kim et al., 2013; Vey et al., 2001). These toxic compounds, such as beauvericin and bassianolide produced by *Beauveria* spp., are the fundamental mechanism by which these entomopathogens function after the fungus has penetrated the host. Zimmermann (2007) reported that *B*. *bassiana* and *B. brongniartii* produce multiple metabolites that are effective across a diverse range of invertebrate species. These findings led to the hypothesis that some species of *Beauveria* may rely on their insecticidal metabolites to kill their hosts before conidial infection. Therefore, some species of *Beauveria* may kill an insect species faster if they have a specific compound to overcome the host's immune responses. *In vitro* studies investigating the toxicology properties of *B. bassiana* and *B. pseudobassiana* revealed that these species could produce different metabolite profiles and increased amounts of certain toxins depending on the type of media they are cultured on (Berestetskiy et al., 2018; Wang et al., 2020). If the required nutrients can be determined for this increase, it may be possible to select mutants of these species in the laboratory that have higher mortality rates and speed of kill than their wild types.

The integrated application of insecticidal metabolites with fungal conidia may improve fungal ability to cause high mortality under diverse conditions. Biotelliga Ltd (Auckland, New Zealand) have developed commercial products based on insecticidal metabolites produced by *B. bassiana*. These

were found to control insect pests on glasshouse vegetables better when applied as a combination with fungal conidia (Glare & O'Callaghan, 2019). This evidence showed that metabolites produced by *Beauveria* might cause the insect to be susceptible to conidial infection. For example, secondary metabolites of *Beauveria* species play a role as a repellent, antifeedant, inhibiting growth, and de-functioning enzyme activities of *Helicoverpa zea* (corn earworm) (Leckie, 2002). As corn earworm is a lepidopteran pest, insecticidal metabolites produced by *Beauveria* species may also provide the same results. An example is the secondary metabolites produced by *Isaria fumosorosea* that were found to have dual functions, able to kill directly and reduce feeding activities of DBM larvae (Freed et al., 2012). Therefore, the combination application between fungal conidia and metabolites may be a suitable option for DBM control.

The liquid fermentation process (submerged culture) is suitable for producing secondary metabolites from *Beauveria* species. In submerged culture, these fungal species produce many insecticidal metabolites, including oxalic acid, bassianin, beauvericin, oosporein, and tenellin (Seger et al., 2005a; Seger et al., 2005b; Strasser et al., 2000; Strasser et al., 2007; Vey et al., 2001). This method can be tailored to provide nutrients to increase the production of some of these metabolites and produce a highly potent cocktail of secondary metabolites from *Beauveria* species. Furthermore, an evaluation of the supernatant produced by a Korean *B. bassiana* isolate showed that penetration-aided enzymes possessed high aphicidal activity (Kim et al., 2010). When using supernatants derived from *B. bassiana* broth cultures to spray on green peach aphid (*Myzus persicae*), the insects showed symptoms of toxicity and died within a day (Cheong et al., 2020). These observations suggest that an alternative strategy could be developed for the application of *Beauveria*-based biopesticides using non-viable solutions containing fungal-derived secondary metabolites instead of viable conidial suspensions. This strategy may exhibit multiple advantages over the use of viable conidial suspensions (e.g., faster insect kill rate and reduce consistency problems linked to fluctuating climatic conditions) and be more comparable to using a contact synthetic pesticide.

The vast majority of research conducted on non-viable biopesticides has been based on the secondary metabolites of *Isaria* spp., *Metarhizium* spp., *Lecanicillium lecanii*, and *B. bassiana*, with a few studies based on *B. pseudobassiana*. Methods for the extraction of fungal toxins from the axenic cultures of *Beauveria* spp. include thin-layer chromatography, high-performance liquid chromatography, crude soluble protein extraction, ethyl acetate extraction, and the use of filtration (Berestetskiy et al., 2018; Cheong et al., 2020; Gurulingappa et al., 2011; Kim et al., 2013; Kim et al., 2010; Ortiz-Urquiza et al., 2010; Quesada-Moraga et al., 2006). Using filtration techniques to extract toxic compounds from axenic cultures of *M. anisopliae*, Mohanty et al. (2008) found that the extracts caused high mortality when applied to colonies of mosquitoes. However, Quesada-Moraga et al. (2006) found the insecticidal activities of *M. anisopliae* did not change when growing on different media types. When testing for virulence and toxicogenic activities of some *B. bassiana* strains under *in vivo*

and *in vitro* conditions, Quesada-Moraga and Vey (2003) showed metabolite production was considerably decreased when growing on Sabouraud Dextrose Agar (SDA), while growing in Malt Agar (MA) significantly boosted the activity. Having said that, when culturing in potato dextrose broth (PDB), Kim et al. (2013) found the mortality of *M. persicae* was significantly higher than when treated with other fungi. Therefore, the PDB can be a suitable medium for culture extract to test on DBM larvae.

Obtaining a crude extract from fungal cultures using a filtration method is preferred over other extraction methods (e.g., crude soluble protein extraction and ethyl acetate extraction) as it easily excludes fungal hyphae (Kim et al., 2013). The main objective of this chapter was to assess secondary metabolites extracted from three *Beauveria* isolates to control DBM under laboratory conditions.

6.2. Materials and Methods

6.2.1. Insect collection

Third instar larvae of DBM were obtained from the laboratory colony raised on cabbage (cv. Arisos NS, provided by South Pacific Seeds (New Zealand) Limited) and maintained within the controlled temperature rooms (25 ± 2^{o}C, 12:12 D/L) at the Bio-Protection Research Centre, Lincoln University, New Zealand. Soft brushes were used to transfer larvae from plants to plastic boxes (cleaned cabbage leaves were placed in the box to keep larvae inside during collecting) for the assays. Collected larvae were actively feeding, green in colour and approximately the same size.

6.2.2. Preparation of *Beauveria* spp.

Three genetically diverse isolates of *Beauveria* (*B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, and *B. bassiana* CTA20) were selected from the culture collection held at the Bio-Protection Research Centre (BPRC), Lincoln University, New Zealand. *B. pseudobassiana* FW Mana was selected as this isolate showed a faster kill rate of DBM compared to other related strains but was slow to sporulate on DBM cadavers indicating that it used toxins to kill this insect. Isolate *B. pseudobassiana* FRhp (which showed almost the same speed of kill and percentage sporulation on cadavers as FW Mana) and isolate *B. bassiana* CTA20 (which killed slower but had higher sporulation on cadavers) were used for comparison. The three *Beauveria* isolates were removed from the -80 freezer, defrosted at room temperature and cultured on PDA (Oxoid) at 23 ± 1^{o}C 12:12 (D:L) until use.

Fungal suspensions were prepared by flooding individual 14-day-old *Beauveria* cultures with 10 mL aqueous Triton X-100 solution (10 µL in 1000 mL sterile water) and scraping gently with a sterile loop. One hundred microliters of each suspension were inoculated into 100 ml of Potato Dextrose Broth (PDB) within a 250 ml Erlenmeyer flask. The neck of the flask was sealed with a cotton plug and tin foil. Flasks were incubated at 24 ± 1^{o}C on an orbital shaker (Cocono, Taiwan) set at 150 rpm under a photoperiod of 12:12 D/L for seven days. The subsequent suspension was filtered via a pre-sterilised vacuum-driven disposable filtration system (0.22 µm, Millipore, Express^{TM}PLUS, Stericup

and Steritop) to remove viable fungal structures (Figure 6.1). The filtered suspensions were then kept at 4°C until use. The full-strength suspension was diluted with 0.01% Triton X-100 50:50 to obtain half-strength suspensions.

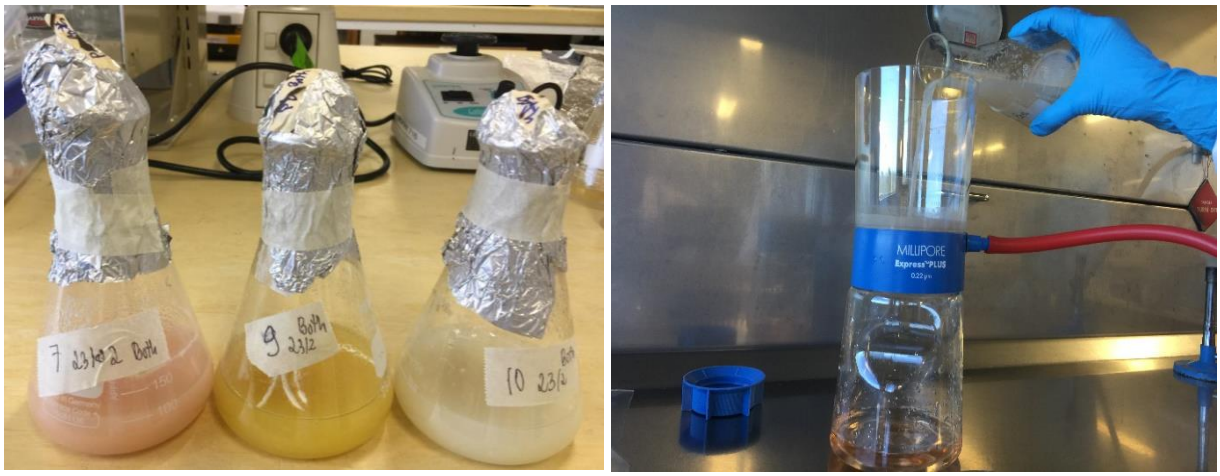


Figure 6.1: The three isolates cultured in PDB and filtered using a pre-sterilised vacuum

***Note:** Flasks marked with 7= B. pseudobassiana FRhp, 9= B. pseudobassiana FW Mana and 10= B. bassiana CTA20

6.2.3. Bioassay protocol

The experimental design was: treatment 1 (isolate *B. pseudobassiana* FRhp half-strength), treatment 2 (*B. pseudobassiana* FRhp full-strength), treatment 3 (isolate *B. pseudobassiana* FW Mana half-strength), treatment 4 (*B. pseudobassiana* FW Mana full-strength), treatment 5 (isolate *B. bassiana* CTA20 half-strength), and treatment 6 (*B. bassiana* CTA20 full-strength). Each treatment had two replicates, and the assays were repeated twice. After the extracting process, 600 µL of the filtered supernatant was pipetted for each spray application. The inoculum was applied in a Potter Tower as previously described in Chapter 3. As these assays used metabolites in liquid, trays were not covered with plastic bags after inoculation, and the assessment of the number of larvae supporting sporulation was unnecessary. Application of the filtered suspensions started with half-strength followed by full-strength, after which the equipment was cleaned, then two controls were sprayed before starting the subsequent isolate. Some isolates caused lower than 50% mortality within the experimental time limit, and therefore, the LT_{50} could not be calculated using the previously used formula $LT_{50} = [ND_{50} \times (M_{50} - 1) + DB \times (NM_{50} - M_{50})]/(NM_{50}-1)$, where ND_{50}: number of days that mortality reached 50%; M_{50}: 50% mortality of tested larvae; DB: the day started to die before 50% mortality; and NM_{50}: number of total mortality on ND_{50} (personal communication, Dave Saville, 20 April 2021). Therefore, the LT_{50} was determined using the formula LT_{50} prediction = $(M_{50} \times ND)/MD$, where M_{50}: 50% mortality of tested larvae; ND: number of days tested; and MD: total mortality of days tested.

6.2.4. Statistical analysis

All data were analysed using Genstat software (20th Edition) (Payne et al., 1987). General analysis of variance (ANOVA) was used for statistical comparison, and Tukey test was used for multiple comparisons. The median lethal time (LT_{50}) was used to test for the speed of kill of each isolate.

6.3 Results

Liquid cultures were pink, yellow, and white for isolates *B. pseudobassiana* FRhp, *B. pseudobassiana* FW Mana, and *B. bassiana* CTA20, respectively (Figure 6.1). The filtered supernatants from the three *Beauveria* isolates, at the two different concentrations (half and full-strength), resulted in a 20% to 65% mortality of DBM larvae within seven days post-treatment. The application of *B. pseudobassiana* FW Mana resulted in higher mortality of DBM than the other two isolates at both application rates. At the half-strength application rate, *B. pseudobassiana* FW Mana killed 35% and 20% more DBM larvae than isolates *B. pseudobassiana* FRhp and *B. bassiana* CTA20, respectively, which was statistically significant ($F = 71.75$; $df = 3$; $p < 0.003$). At the full-strength concentration, isolate *B. pseudobassiana* FW Mana killed 25% more DMB larvae than both isolates FRhp and CTA20, which was statistically significant ($F = 113.56$; $df = 3$; $p < 0.001$) (Table 6.1). *B. bassiana* CTA20 killed 35% of the DBM larvae at the half-strength application rate and 40% at the full-strength rate. The least toxic strain towards DBM larvae was *B. pseudobassiana* FRhp, which killed 20% of the DBM larvae at the half-strength application rate and only 40% at the full-strength concentration (Table 6.1). Statistical difference of DBM mortality at both half and full-strength refers to Table B.4.1 and B.4.2 (Appendix B).

Median lethal time (LT_{50}) ranged from 5.4 days to 13.1 days after inoculation at the half and full-strength concentrations. At half-strength concentration, isolate *B. pseudobassiana* FW Mana achieved an LT_{50} seven and four days faster ($F = 180.28$, $df = 2$, $p < 0.006$) than isolates *B. pseudobassiana* FRhp and *B. bassiana* CTA20, respectively. At full-strength concentration, this isolate achieved an LT_{50} four and three days quicker than *B. pseudobassiana* FRhp and *B. bassiana* CTA20, respectively. There was a statistical difference between isolates *B. pseudobassiana* FW Mana and *B. pseudobassiana* FRhp but no significant difference between isolates *B. pseudobassiana* FW Mana and *B. bassiana* CTA20 ($F = 68.08$, $df = 2$, $p < 0.014$). Isolates *B. pseudobassiana* FRhp and *B. bassiana* CTA20 could not reach an LT_{50} within the seven-day assessment period. However, the mathematical model for the LT_{50} for these two isolates predicted *B. bassiana* CTA20 would reach the LT_{50} within 8.8 and 10.4 days for full and half strength application rates, respectively, while *B. pseudobassiana* FRhp would reach the LT_{50} within 9.5 and 13.1 days, respectively (Table 6.1). Statistical difference of LT_{50} at both half and full-strength refers to Table B.4.3 and B.4.4 (Appendix B).

Table 6.1: Percentage mortality of DBM larvae seven days after spraying using filtered supernatants from fungal colonies and the LT_{50} of the three isolates at two different concentrations (General ANOVA, Tukey test in Genstat)

Concentration	Isolate	%Mortality	Predicted LT_{50} (day)
Triton X-100	Control	4 ± 1.8 a	-
	B. pseudobassiana FRhp	20 ± 2.9 ab	13.1 ± 4.4 c
Half strength	*B. bassiana* CTA20	35 ± 5.1 b	10.4 ± 1.6 b
	B. pseudobassiana FW Mana	55 ± 7.4 c	6.4 ± 0.6 a
F-value		71.75	180.28
P-value		0.003	0.006
Triton X-100	Control	4 ± 1.8 a	-
	B. pseudobassiana FRhp	40 ± 5.8 b	9.5 ± 2.5 b
Full strength	*B. bassiana* CTA20	40 ± 5.6 b	8.8 ± 0.1 a
	B. pseudobassiana FW Mana	65 ± 10.7 c	5.4 ± 0.4 a
F-value		113.56	68.08
P-value		0.001	0.014

6.4 Discussion

The results from this study showed that the culture filtrates of *B. pseudobassiana* FW Mana achieved a higher mortality rate for DMB larvae than previously published studies using non-viable extractions. For example, culture filtrates of *B. bassiana* Bb-2, from China, achieved an accumulative mortality rate for DBM larvae of only 37% (Gao et al., 2012). Compared to isolate *B. bassiana* CTA20 of this study, isolate *B. bassiana* Bb-2 of Gao et al. (2012) caused similar DBM mortality. This current result was the first report to show that filtered supernatant of an isolate of *B. pseudobassiana* killed more DBM larvae than a *B. bassiana* isolate. A study investigating the insecticidal activity of *B. pseudobassiana* and *B. bassiana* towards *Spodoptera littoralis* (cotton leafworm) identified fungal isolates that achieved mortalities of up to 67% while one of the *B. bassiana* isolates did not kill any insects (Resquín-Romero et al., 2016). The understanding from the result of this study is that at least one isolate of *B. pseudobassiana* may produce a higher amount of insecticidal metabolites or enzymes than *B. bassiana* against DBM larvae.

A study by Kim et al. (2013) investigating the insecticidal activity of *B. bassiana* towards green peach aphid (*Myzus persicae*) showed that their chosen isolates achieved 15% to 79% aphid mortality. A recent study using supernatants of *B. bassiana* against *M. persicae* found a mortality rate between 70% and 30% when extracting from different media and fermentation times (Cheong et al., 2020). By comparing the mortality between aphid and DBM, Gao et al. (2012) found that the aphid mortality was

higher than DBM, which reached up to 48% for aphids by using the filtrated supernatant of *B. bassiana* Bb-2. These findings indicate that the mortality rate of DBM larvae was lower than for aphids using filtered supernatant. *Beauveria* metabolites may only have a mode of action through the digestive pathway, and DBM larvae can develop resistance to many insecticidal compounds, which leads to low mortality. In New Zealand, a biopesticide producing company, Biotelliga Ltd, showed the combination of secondary metabolites with conidial spray lead to the optimisation of the application (Glare & O'Callaghan, 2019). Thus, the application of isolate *B. pseudobassiana* FW Mana may be improved through the integration of its metabolites and conidial spray.

As expected, isolate *B. pseudobassiana* FW Mana killed DBM larvae using toxic metabolites; the supernatant of this isolate gave significantly greater mortality than the two other isolates. This result showed that *B. pseudobassiana* could kill DBM larvae through toxins more effectively than the *B. bassiana* isolates tested. Previously an assessment of metabolite profiles between these two species revealed differences (Wang et al., 2020), which may assist in overcoming the immune system of a particular insect. Two enzymes, namely chitinase and protease, have been found to have dual functions: aiding fungal infection and toxicity to some insects (Kim et al., 2013; Montesinos-Matías et al., 2011). However, Cheong et al. (2020) showed no correlation between their production and mortality, and enzymes did not affect the fungal ability to kill the green peach aphid with metabolites from an isolate of *B. bassiana*. The aphicidal compounds have yet to be identified. Based on the results in Chapter 3 and herein, the assessment of percentage sporulation was an important indication of whether larvae were killed by conidia or toxins produced by the fungi.

Different extraction methods of fungal metabolites lead to different efficacies. Comparing leaf dipping and topical spray methods, the extracted supernatant of *Cordyceps militaris* using the methanol extraction method caused 100% and 0% mortality of DBM larvae, respectively. This result shows that ingestion was the likely pathway rather than contact virulence (Kim et al., 2002). Cheong (2015) found a crude mycelial extract of *B. bassiana* K4B3 gave 96% mortality, while no larvae died in a filtered supernatant treatment. The mycelial extract using methanol extraction was found to be larvicidal against the mosquito *Aedes aegypti* larvae, where the supernatant caused 86% mortality within two days (Daniel et al., 2017). Similarly, methanolic extracts of *B. bassiana* caused greater mortality of *Aphis gossypii* (Gurulingappa et al., 2011) and two *Spodoptera* pests (Villegas-Mendoza et al., 2019) than conidia spray. The secondary metabolites of *B. bassiana* may be enhanced or activated through the methanolic extract, causing significant mortality (Villegas-Mendoza et al., 2019).

The three isolates' median lethal time (LT_{50}) ranged from 5.4 days to 13.12 days after spraying, with only FW Mana achieving an LT_{50} within seven days (5.4 days for full strength, 6.42 days for half strength). Previously, DBM mortality was 27% and 36% for one and two days after spraying, respectively, when testing with *B. bassiana* Bb-2 (Gao et al., 2012). Under a growing temperature of 25^{o}C, Herlinda et al. (2020) found the LT_{50} was longer than seven days when testing extracted

metabolites of two *B. bassiana* isolates against *Spodoptera litura*. Assaying on green peach aphid using extracted supernatant of *B. bassiana* isolates*,* aphid mortality reached 78.6% within 2.7 days after treatment for a most effective isolate, while the less effective isolates required 9.8 days (Kim et al., 2013). When using crude protein extracted from *I. fumosorosea* to assay on DBM, Freed et al. (2012) found the LT_{50} was six days post-treatment, but earlier with a protein concentration, which may enhance the antifeedant characteristic. Spraying *S. littoralis* using extracted supernatant of a *B. pseudobassiana* isolate achieved LT_{50} by day four after treatment, while a *B. bassiana* isolate had no effect (Resquín-Romero et al., 2016). However, the study found promising results when combining both conidia and extracted metabolites in the spray, giving 100% mortality within four to five days after application. In the current study (Chapter 4) combinations of conidia from different isolates could result in synergy. It is possible that the combination of conidia with insect toxins of the same isolate may give synergistic rather than antagonistic interactions, but this is yet to be integrated.

6.5 Conclusion

This study showed the potential use of *Beauveria* metabolites to assist the control of DBM. The isolate *B. pseudobassiana* FW Mana is the most suitable candidate for managing this insect by exported metabolites. Methanol extraction should be used as a method for this isolate for greater mortality. Growing in different types of liquid media to find a suitable medium for isolate FW Mana toxin extraction should be undertaken. Testing of FW Mana isolate on other insect pests and under diverse conditions will be necessary to confirm the efficacy and consistency, as well as non-host safety. Combining conidia and extracted toxins of the same isolates may potentially give an additional effect or synergistic results for DBM control.

Chapter 7

General Discussion

The ultimate objective of this study was to find highly effective *Beauveria* isolates for future development into *Beauveria*-based biopesticides to control DBM. Some *Beauveria*-based products have been registered and approved for use in many countries. Many previous studies have focused on the use of *Beauveria bassiana* against various pests; studies that involved screening different species of *Beauveria* seemed to be limited. An investigation of interactions of *Beauveria* species with DBM after feeding on different brassicas, and strategies to improve the efficacy of those species, such as the use of combined strains and extracted toxins to overcome diverse environmental factors, are also very limited. Therefore, this experimental study was conducted to better understand these understudied areas. The ultimate goal of this study was to find at least one suitable *Beauveria* candidate for the development of a biopesticide to control DBM.

Initially, it was vital to confirm the identities of all isolates before using them for screening assays. Both morphological and molecular approaches were used (Chapter 2) to confirm all isolate identities. Colony and conidial morphology were the two main features used for morphological identification. Growing cultures were white to pale yellow (isolates from *B. bassiana* and *B. pseudobassiana*) and pink to red (isolates from *B. caledonica* and *B. malawiensis*). These findings aligned with previous studies (Glare & Inwood, 1998; Rehner et al., 2006a; Rehner et al., 2011; Wang et al., 2020). While it is difficult to distinguish between *B. bassiana* and *B. pseudobassiana* using morphological features, it is possible to differentiate these two species from *B. caledonica* and *B. malawiensis* based on colony colour and conidial shape and size. Thus, morphological characteristics initially still play an important role in fungal *Beauveria* taxonomy.

Using the Chelex-100 method with the aid of liquid nitrogen during the crushing process for DNA extraction (Alizadeh et al., 2017; Hennequin et al., 1999) was suitable for isolating fungal DNA from *Beauveria* species. Operationally, this method was simple, quick, straightforward and ideal for a large number of samples. However, sequence results from the PCR products without cleaning up contained too many misreads, possibly because Chelex-100 matrix beads acted as inhibitors (Walsh et al., 2013). Good quality sequences were achieved using PCR clean-up methods (Korosi et al., 2019). PCR clean-up using the NucleoSpin column following the protocol of Macherey-Nagel (2017) was a fast, simple, and effective way to remove impurities in PCR products. The molecular results were improved by using this method. Sequence results contained very few misreads, and peaks of each base were sharp. Therefore, when extracting DNA using the Chelex-100 method, cleaning up the PCR product before sequencing is recommended.

The two standard DNA regions, EF1-α (primers 983F and 2218R) and BLOC (primers B22U and B822L) were suitable for *Beauveria* identification. These two primer sets resulted in high DNA yields at the same annealing temperature of 57°C. Previous studies had run under an annealing temperature for BLOC primers of 56°C (Fisher et al., 2011; Rehner et al., 2006b), and another study increased that up to 63°C (Korosi et al., 2019). Previously, the annealing temperature for the EF1-α primers was run at 58°C (Glare et al., 2008), 56°C (Cummings, 2009; Rehner & Buckley, 2005), and 55°C (Brownbridge et al., 2012). This study found BLOC showed more discrimination between isolates than EF1-α, which was found to lack introns that are often useful to distinguish species-rich genera of Hypocreales (Rehner & Buckley, 2005). BLOC provided more distinction because it was designed specifically for identifying *Beauveria* species (Fisher et al., 2011; Rehner et al., 2006b). Building a phylogenetic tree using Geneious Prime software made the job easier and faster than MEGA, which required more complicated steps. This study found MUSCLE alignment was the most suitable method for multiple-aligning large nucleotides with combined DNA regions, which provided the accuracy and high throughput required (Edgar, 2004). Building a phylogenetic tree based on multi-locus data using Geneious tree builder is a simple, quick, and reliable method for *Beauveria* identification. This method has been previously used by Bustamante et al. (2019), Boardman et al. (2020), Skantar et al. (2020), and Wang et al. (2020).

The molecular characterisation showed isolates F615, J2, Mo1, TPP-H, J18, FRh2, O2380, CTL20, and CTA20 were *B. bassiana*; isolates FRhp, FW Mana, I12 Damo belonged to *B. pseudobassiana*; isolate F532 was *B. caledonica*, and Bweta was *B. malawiensis*. Isolate J18 was originally isolated from maize (Brookes, 2017), and isolates Mo1, CTL20, and CTA20 were isolated from DBM larvae in the colony maintained at Lincoln University. It is possible that these isolates are derivatives of J18, accidentally introduced into the DBM colony (Jenny Brookes, personal communication). These isolates are phylogenetically identical to J18 (Figure 2.4 and Appendix A.4) using the two regions examined. Isolate F615 was originally derived from organic farm soil at Lincoln University, isolate TPP-H was derived from a cadaver of a tomato-potato psyllid in the controlled temperature rooms of the Bio-Protection Research Centre at Lincoln University, and isolate FRh2 was isolated from a cadaver of a bark beetle from Riverhead, North Island, New Zealand. Isolate O2380 was found as a cabbage endophyte in Palmerston North by Stuart Card, AgResearch Ltd. Isolate FRhp was isolated from a colony labelled as *B. caledonica* FRh1, grown on PDA. After repeating the molecular characterisation several times, this isolate was confidentially placed in *B. pseudobassiana*. Isolate FW Mana was isolated from cadavers of flax weevil from Mana Island in New Zealand that was found to cause high mortality of this insect. Isolate I12 Damo was derived from a cadaver of the eleven-spotted ladybird at Lincoln University. Isolate Bweta was isolated from a cadaver of a Weta from Westland, New Zealand. Isolate F532 was derived from an infected pine bark beetle from the Riverhead forest. The sources of the isolates used in this study were reported by Brookes (2017), Glare and Brookes (2017), Glare et al.

(2008), McKinnon et al. (2018), and Reay et al. (2008). Future molecular studies to identify *Beauveria* spp. should combine DNA regions such as EF1-α, BLOC, RPB1, and RPB2 for species identification in the genus. Among the 14 isolates used in this study, isolates I12 Damo, FRhp, and CTL20 produced more spores than other isolates on standard media, reaching around 10^9 conidia/mL per plate within three weeks. Isolate Bweta produced fewer spores than the other isolates, and two plates usually needed to be harvested to obtain 10^8 conidia/mL. The speed of spore production is also an important component for the mass production of fungal isolates into biopesticides (Mascarin & Jaronski, 2016). Thus, isolate I12 Damo, FRhp, and CTL20, as fast-growing isolates, would be more suitable for mass production if further investigations confirmed their virulence in the field.

The most significant chapter of this experimental study is Chapter 3, which reported the screening of 14 isolates from four different species of *Beauveria* looking for highly virulent isolates to control DBM. Highly virulent isolates were those that provided high mortality at a low application rate (low LD_{50}), fast-killing speed (high LT_{50}), and, ideally, a high sporulation rate on infected cadavers. This study found that among four species and 14 isolates, only two species and six isolates gave 100% mortality at 6 x 10^6 and 6 x 10^7 conidia/spray. Wraight et al. (2010) found different efficacies among 43 *B. bassiana* isolates in killing lepidopteran pests, including DBM. The three highly virulent isolates of *B. bassiana* (isolate Mo1, CTL20, and CTA20) in the present study were obtained from infected DBM cadavers from the laboratory culture, potentially deviated accidentally from isolate J18. Recent studies have demonstrated it is possible that passage through host insects can change the methylation patterns of entomopathogens to increase virulence. An isolate of *Metarhizium* was found to have improved virulence when passaging it through wax moth larvae (Hu & Bidochka, 2020). Isolate J18 caused lower mortality than isolates Mo1, CTL20, and CTA20, which may be because these three isolates were already adopted to the host's immune systems. To confirm that these isolates are derivatives of J18, genome sequencing would need to be undertaken as alignment over less than 2 Kb of DNA is not enough evidence.

In a cropping system, multiple species of pests occur and attack target crops (Glare et al., 2016). Thus, the adaptation of a fungal isolate to infect a particular host insect could be beneficial if they can also control a wide range of other insect pests. The capability of a biological agent to control a broad range of insect pests on a particular crop, such as brassica, is also an important characteristic for a developed biopesticide to compete with chemical pesticides (Litwin et al., 2020). For example, an isolate of *B. bassiana* was found to have an effect on some leaf-chewing and sap-sucking pests, which increased its utility (Arthurs & Dara, 2019). The evaluation of side effects of *Beauveria* spp. found no detrimental significances of these fungi on other beneficial insects and animals (Zimmermann, 2007). Investigation of our highly virulent isolates of *Beauveria* on other arthropods, including beneficial insects, would be needed to prove them safe to use in New Zealand and to examine their potential for control of other pests.

Three of the highly infectious isolates were *B. pseudobassiana,* suggesting that this species is also a potential species for future DBM control. Studies in other parts of the world have mostly tested *B. bassiana* isolates against DBM (Agboyi et al., 2020; Duarte et al., 2016; Furlong, 2004; Godonou et al., 2009; Narciso et al., 2019; Vandenberg et al., 1998a; Yamada et al., 2009). This result was the first report to show that *B. pseudobassiana* isolates from New Zealand were more suitable candidates than the other *Beauveria* species tested in this study in killing DBM larvae. All three isolates of *B. pseudobassiana* used in the assays caused 100% mortality at the application rates of 6 x 10^6 and 6 x 10^7 conidia/spray. A *B. pseudobassiana* isolate was found highly virulent to two species of bark beetles using the application rate of 10^8 conidia/mL (Kocacevik et al., 2016), and *B. pseudobassiana* isolates derived from lepidopteran and coleopteran hosts were found highly virulent to silkworm and mealworm, respectively (Wang et al., 2020). However, the three *B. pseudobassiana* isolates used in this study were isolated from coleopteran hosts but found to work well on DBM, a lepidopteran pest. Romón et al. (2017) found the reverse in that those isolates derived from a lepidopteran host caused significantly higher mortality than isolates derived from coleopteran insects to control *Pissodes nemorensis*, a coleopteran pest. Testing these isolates against DBM under greenhouse and field conditions will be necessary to see how consistent these isolates are and their potential for control of this insect. Results in Chapter 2 showed that two of the isolates of *B. pseudobassiana* (FRhp and I12 Damo) produced more spores than other isolates on media, which will be beneficial for future biopesticide development.

Isolate CTL20 and I12 Damo required a low application rate to kill 50% and 95% of larvae. These two isolates required the same rates as reported for a commercially developed isolate GHA (Furlong, 2004; Vandenberg et al., 1998a), isolate IBCB01 (Duarte et al., 2016), and isolate ARSEF9271 (Kocacevik et al., 2016), but lower rates than isolate MG-Bb-1 (Yamada et al., 2009), and isolate ESALQ-447 (Rondelli et al., 2013). The isolate *B. bassiana* GHA is, to date, the most successful isolate developed into commercial products of the genus. It has been widely used to control lepidopteran pests, including DBM (Wraight et al., 2010), Colorado potato beetle (Wraight & Ramos, 2017), emerald ash borer (Liu & Bauer, 2008), tobacco whitefly (Wari et al., 2020), plant bugs (Leland et al., 2005), coffee berry borer (Wraight et al., 2021), house fly (White et al., 2021), western cherry fruit fly (Yee, 2020), and fall armyworm (Kuzhuppillymyal-Prabhakarankutty et al., 2021). As isolate CTL20 and I12 Damo required the same reported application rate as this popular isolate (GHA), they are potential candidates for future biopesticide formulation. Wraight et al. (2010) found isolate 1200, an isolate indigenous to Ontario, New York (where their experiment was conducted), caused significantly higher mortality of DBM larvae than the isolate GHA. This finding revealed that some wild type *Beauveria* isolates might have more potential to control particular pests than those isolates already developed or introduced from other locations. As the present results were from highly artificial laboratory bioassays, greenhouse and field trials must be conducted to evaluate the consistent effects of these two isolates.

Therefore, isolate CTL20 and I12 Damo showed two positive characteristics: (1) requiring a low application rate to kill DBM larvae, and (2) growing fast on culturing medium. These two characteristics are crucial for the development of fungal-based mycopesticides (Faria & Wraight, 2007; Mascarin & Jaronski, 2016).

The killing speed of isolate CTL20 and I12 Damo was around three and two days post-spraying, respectively. Compared to isolate TM MH590235 (Nithya et al., 2019), isolate Bb9205 (Sáenz-Aponte et al., 2020), isolate SG8702 (Tian & Feng, 2006), and isolate GHA (Wraight et al., 2010), the two isolates used in this study killed faster. Fast to kill is an essential component for a biological agent to compete with chemical pesticides. A case study in Brazil showed the failure of a newly developed biopesticide was because of slow killing speed. Panicked farmers started to apply chemical pesticides after biopesticide application when they did not see any effects within several days (Moscardi et al., 2011). Using living agents to control invertebrate pests generally requires several days to kill. However, this constraint is largely true for most biological control agents compared to a conventional chemical control programme (Glare et al., 2016; Sporleder & Lacey, 2013). For instance, most entomopathogenic fungi take around a week after inoculation for insect mortality to occur, in which time the infected pests can still manage to severely damage the crop (St Leger et al., 1996). The DBM is a short life cycle insect (CABI, 2020); if the agent takes a long time to kill, the pest may have already destroyed the entire plant. This study's two most highly virulent isolates took less than six days after spraying to cause 100% mortality of DBM larvae. Therefore, they are very suitable for controlling this insect.

The two highly virulent isolates, CTL20 and I12 Damo, also resulted in a high percentage of sporulation on infected cadavers. Under laboratory conditions (12:12 D/L & 23±1^{o}C) on water agar for 24 hours, most cadavers supported the sporulation of these two isolates. This efficient colonisation of the cadaver and sporulating is an indication of the strongly self-sustaining characteristic of these isolates. From a profitability point of view, growers need biopesticides that can regenerate their mode of action to infect target pests for more than one generation (Glare et al., 2012). As the primary infection by most fungi is by penetration, they can replicate their generations through both horizontal and vertical transmissions, which may lead to long-term suppression of treated pest populations without a need to repeat the application (Chandler, 2017; Sporleder & Lacey, 2013). The high percentage of sporulation from infected cadavers is an indication that the effective isolates in this study could be somewhat self-sustaining on the DBM population, which could be an important characteristic of effectiveness. However, testing these two isolates under glasshouse and field conditions against DBM for self-sustaining characteristics is very important. The result of this study demonstrated the highly prolific sporulation of the most effective isolates. However, it is also essential to look for appropriate transmission technologies to avoid any possible infections of beneficial insects. *Beauveria* spp. have generally proved to be safe for non-target organisms (Colombo et al., 2020; Nunes

et al., 2019; Peng et al., 2020; Queiroz de Oliveira et al., 2011; Zimmermann, 2007). For example, Beaublast, a commercial product based on *B. bassiana*, has been commercialised and used in New Zealand (Glare & O'Callaghan, 2019), establishing a pathway to registration. Thus, if the highly virulent isolates of this study prove to have no negative impacts on beneficial insects, they are the most suitable candidates for *Beauveria*-based biopesticides for DBM control in New Zealand.

In Chapter 4, the *in vitro* challenge results showed compatible and antagonistic characteristics within the genus *Beauveria*. An isolate of *B. malawiensis* appeared to be antagonistic to the isolates from other species. Culturing different isolates in a single plate led to competition for resources, resulting in inhibition (Bayman et al., 2021). When colonising in solanaceous plants, isolates of *B. bassiana* inhibited mycelial growth of the damping-off pathogens *Rhizoctonia solani* and *Pythium myriotylum* (Clark, 2006; Ownley et al., 2008; Ownley et al., 2004). As these plant pathogens are fungi, antagonism among *Beauveria* species is possible. Based on the result in Chapter 4, those isolates cultured with isolate Bweta (*B. malawiensis*) showed separating lines between colonies, and they grew relatively slower than when cultured with other isolates. This result suggests using *in vitro* challenge before combining different *Beauveria* isolates to avoid antagonistic interactions.

Some biocontrol agents have the potential for controlling insect pests, but their uses are limited because they are vulnerable to environmental conditions, which results in inconsistent field performance (Sporleder & Lacey, 2013). Inconsistent performance under diverse climatic conditions is one of the main issues limiting the development of biopesticides (Glare et al., 2012; Jaronski, 2010; Lovett & Leger, 2018). Genetically different strains with different tolerance to some diverse environmental conditions may improve the potential for infection against a particular pest. For instance, when observing the interaction on *Dendrolimus punctatus* larvae, Wang et al. (2004) proved some *B. bassiana* isolates could conjugate through sexual mating to infect the insect under natural conditions which resulted in genetic combination. Combining several isolates, which can also genetically interact synergistically or overcome environmental constraints, may solve the inconsistent field performance issue.

The combination experiment showed two separate scenarios: synergism and antagonism. Usually, combining different isolates results in them cooperating and fighting to kill the host (Cruz et al., 2006; Nowak & Sigmund, 2002). In this study, the best results were from combining three low virulent isolates, as the combination resulted in significantly faster mortality than that of the individual isolates. The low virulent combination caused 100% mortality within six days after spraying, while the individual isolates killed around 50% within seven days post-treatment. Cruz et al. (2006) found combining low virulent isolates showed better results when testing against coffee berry borer than single isolates. Under field conditions, a commercial product that combines three strains, Cenicafé, reduced the population of coffee berry borer emerging from infested beans by up to 75% (Vera et al., 2011), and when combining this commercial product with *M. anisopliae* Ma9236, the insect population

declined by 93.4% (Jaramillo et al., 2015). These findings revealed the consistency of these combined isolates under field conditions. The LD_{50} and LT_{50} of the low virulent combination were as low and quick as a highly virulent isolate like I12 Damo and CTL20 and significantly lower and faster than the individual isolates used in the combination. Further investigation of this combination against DBM under field conditions would be useful. Testing against other insect pests should be conducted to determine if the combination expands the host range.

An antagonistic interaction was found when mixing isolates of three species of *Beauveria*. This combination gave significantly lower mortality than the single species isolates at low application rates, except for isolate F532, which had lower mortality singly anyway. Bayman et al. (2021) found the same result when treating combinations of isolates on coffee berry borer in Puerto Rico. The highly virulent strains may dominate the least virulent strains for food resources, reducing the speed of host infection when combining them in an application (Benavides et al., 2012). As isolate I12 Damo was highly infectious for DBM larvae, this isolate might dominate the others, infecting the host but retarding the speed of the kill. For isolates that cause high mortality for a particular insect, applying as a single application is likely the more effective approach (Boucias et al., 2000). Therefore, we assume the application using only isolate I12 Damo is enough to cause 100% mortality of DBM. The two other combinations provided no obvious interactions. The highly virulent combination caused the same mortality and speed of kill as individual isolates. Even though the medium virulent combination killed relatively more than single isolates, there were no synergistic nor antagonistic results. However, these two combinations may work more consistently under field conditions compared to single isolates as genetic diversity might improve fungal ability to tolerate a range of environments (Wang et al., 2004). The two prediction models of Nowak and Sigmund (2002) helped make an assumption for combining two or more isolates for synergistic and antagonistic results, which included both LD_{50} and LT_{50}.

There was a reduction of sporulation percentage on infected cadavers in all combinations compared to single isolates from these combined experiments. Combining different isolates can result in competition for food resources for invasion inside a host, reducing sporulation (Bayman et al., 2021; Benavides et al., 2012). This may not be a negative effect for a commercial product, as lack of sporulation would mean the product must be applied regularly, increasing sales. Isolating and identifying the isolate of *Beauveria* that emerged from the cadaver infected by combined treatment would provide useful data to track the evolution of isolates. This was not done due to resource limitations.

When using biocontrol agents to control pests, understanding interactions among relevant factors such as the agent, plant, and target insect is vital to optimising the application. Glucosinolate can protect brassica plants against a wide range of insect pests (Furlan et al., 2010). The objective of Chapter 5 was to examine the interactions between two highly infective isolates of *Beauveria* and DBM larvae feeding on four brassicas that were assumed to contained different amounts of glucosinolate.

Infection by tested isolates was dependent on the plant and presumably the glucosinolate content. Previous studies found plants that had low glucosinolates were more vulnerable to the damage of DBM larvae than those that contained high glucosinolates (Robin et al., 2017; Santolamazza-Carbone et al., 2014; Sarosh et al., 2010). On the other hand, some studies showed that feeding activities of DBM on low and high glucosinolate brassicas were not significantly different (Badenes-Pérez et al., 2020; Karmakar et al., 2021; Rapo et al., 2019). For example, Badenes-Pérez et al. (2011) showed a brassica crop (*Barbarea vulgaris*) containing high glucosinolate attracted DBM adults to lay more eggs than on other brassicas. However, the survival of hatching larvae on this plant was reduced (Badenes-Perez et al., 2006). From this evidence, it was hypothesised that larvae fed on high glucosinolate brassica increased the susceptibility rate of DBM larvae to the infection of a *Beauveria* isolate because feeding on high glucosinolate plants may have reduced the health fitness of DBM larvae.

This study discovered that isolate I12 Damo worked better when sprayed on larvae raised on predicted high glucosinolate brassicas (broccoli and cabbage), and isolate CTL20 performed well on larvae fed on low glucosinolate brassicas (cauliflower and radish). While the result of isolate I12 Damo showed the alignment to the hypothesis that larvae feeding on high glucosinolate plants were more vulnerable to the infection of entomopathogens (Santolamazza-Carbone et al., 2014; Sarosh et al., 2010), the isolate CTL20 result aligns with the study of Badenes-Pérez et al. (2020) who found DBM preferred brassicas which contained high levels of glucosinolates. However, the levels of glucosinolates in brassicas grouped by Kim et al. (2020) and Hwang et al. (2019) were sampled from leaves of those plants in South Korea. This study used leaves of brassicas produced in New Zealand, which may vary in glucosinolates. For example, Robin et al. (2016) and Robin et al. (2017) reported that different cabbage cultivars contained different levels of glucosinolates and different resistant levels to attack by DBM larvae. As we raised DBM larvae on different brassicas, levels of glucosinolates in each brassica may be highly diversified. Therefore, grouping levels of glucosinolates in brassicas in New Zealand should be done to give these findings a plausible conclusion, and this is currently underway.

Results in Chapter 5 also showed the consistency of isolate I12 Damo in killing DBM larvae raised on cabbage, whereas isolate CTL20 gave variable results at different times. Consistency under diverse conditions is important for a successful biocontrol agent being formulated into a biopesticide (Chandler, 2017; Faria et al., 2012; Glare et al., 2012). An example is the successful commercial *Beauveria*-based product, BotaniGard, which shows consistent performance at different times under various climatic conditions (Wraight et al., 2018; Wraight et al., 2021). Isolate I12 Damo used in this study provided consistent results under laboratory conditions, and it may also give the same performance under glasshouse or field conditions. Obviously, a potential candidate that can compete with chemical pesticides needs comparable performance in field conditions. Finding methods for delivering isolate I12 Damo to perform better under field conditions is also necessary. Glucosinolate metabolites can be synthesised into natural insecticides through activities of myrosinase enzymes

(Agrawal & Kurashige, 2003). Combining conidial spray with natural insecticide based on glucosinolate metabolites may prove to be beneficial.

Some experimental investigations have been conducted to screen some *Beauveria* isolates and coating technologies that make them tolerant to UV radiation for better field performance (Fernandes et al., 2015; Posadas et al., 2012; Thompson et al., 2006). This is an example of improving the consistency of a fungal isolate by improving the formulation that protects from UV or from other abiotic aspects. Research in Chapter 6 was conducted to look for another potential use of *Beauveria* that could be combined with the spores to improve potential products. The use of secondary metabolites of *Beauveria* isolates to kill DBM larvae was investigated. This method had been found to be effective to control DBM larvae (Gao et al., 2012), *Spodoptera litura* (tobacco cutworm) (Herlinda et al., 2020), two other *Spodoptera* pests (Villegas-Mendoza et al., 2019) and *Myzus persicae* (green peach aphid) (Cheong et al., 2020; Kim et al., 2010). The idea of using filtered supernatant was firstly considered from the results in Chapter 3. When treating with isolate *B. pseudobassiana* FW Mana, DBM larvae were killed relatively quickly, but infected cadavers showed slow or no sporulation, consistent with symptoms of toxicity. To compare the efficacy, isolate *B. pseudobassiana* FRhp (which showed almost the same speed of kill and percentage sporulation as FW Mana) and isolate *B. bassiana* CTA20 (which killed slower but had higher sporulation on cadavers) were used. As expected, the supernatant of isolate *B. pseudobassiana* FW Mana caused significantly higher mortality of DBM larvae than the other two isolates at both full and half-strength. The results of this study showed higher mortality than that reported by Gao et al. (2012), who conducted the same experiment using a *B. bassiana* isolate in China. Resquín-Romero et al. (2016) reported that when testing some *B. pseudobassiana* and *B. bassiana* isolates against *S. littoralis* (cotton leafworm), mortality ranged from 50% to 67% within seven days. Though the present study did not examine what metabolites were present in isolates tested, two commonly known metabolites produced by *Beauveria* species are beauvericin and bassianolide (Grove & Pople, 1980; Singh et al., 2015; Xu et al., 2009a; Xu et al., 2009b; Zimmermann, 2007). Thus, isolate FW Mana may produce more metabolites that are toxic to DBM larvae than isolates FRhp and CTL20. The use of extracted toxins of *Beauveria* species in combination with other controlling methods may be a future biopesticide approach. Further investigation by growing isolate FW Mana in a variety of liquid culture media may show methods to increase the level of toxins produced. Additionally, applying isolate FW Mana to different insects under different conditions should be done to confirm the consistency and safety, which is vital.

In conclusion, this study found several isolates of *B. bassiana* and *B. pseudobassiana* are potential candidates for the control of DBM. Isolates *B. bassiana* CTL20 and *B. pseudobassiana* I12 Damo are the most suitable isolates for a *Beauveria*-based biopesticide to specifically control DBM. These isolates provided consistent results (the high mortality over five different assays), fast killing speed (100% killed within six days or less), high sporulation percentage (100% sporulation on infected

cadavers), and good *in vitro* sporulation on PDA (up to 10^9 conidia/mL per plate within three weeks). Three isolates of *B. pseudobassiana* used in this study all caused 100% mortality. Thus, this is the first report to have shown that New Zealand *B. pseudobassiana* isolates are suitable candidates for DBM control. Combining three low virulent isolates caused high mortality with a faster speed of kill compared to that of the highly virulent combinations and isolates. Combining three *Beauveria* species resulted in antagonism. Brassicas in New Zealand play a significant role in causing DBM larvae to be susceptible to infection by *Beauveria* isolates. Isolate *B. pseudobassiana* I12 Damo caused high mortality of larvae fed on reportedly high glucosinolate brassicas (broccoli and cabbage). Isolate *B. bassiana* CTL20 killed more larvae raised on reportedly low glucosinolate brassicas (cauliflower and radish). While I12 Damo was the most effective isolate using conidia, *B. pseudobassiana* FW Mana produced the most effective toxins in the supernatant. Media that can be produced from cheap and local materials should be trialled for mass production. Integrating highly virulent isolates and combinations into IPM systems may enhance the efficacy under field conditions. To conclude, this study found several *Beauveria* isolates that can be used for future investigations to develop *Beauveria*-based biopesticides to control DBM.

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Appendix A

Supplementary data for molecular results

A.1 Electrophoresis gel image of EF1-α

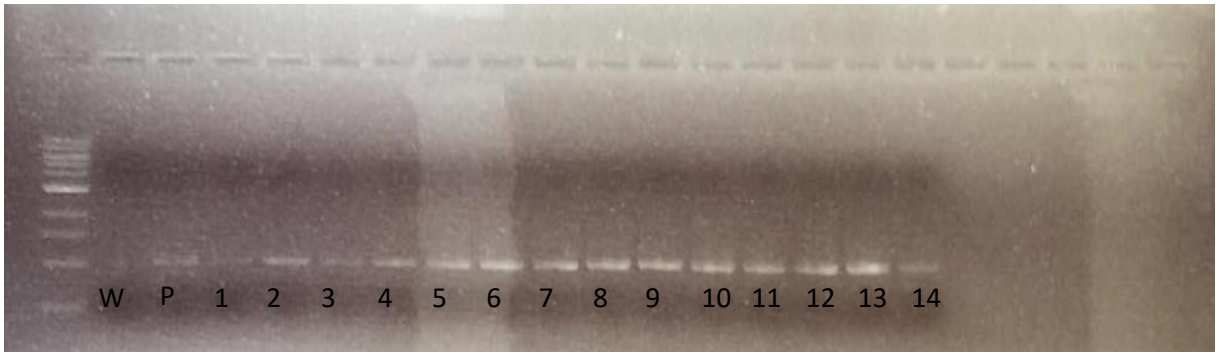

Figure A.1: Electrophoresis gel products received from EF1-α primer (982F & 1822R)

Note: W: water, P: positive control, 1: *B. bassiana* F615, 2: *B. bassiana* J2, 3: *B. bassiana* Mo1, 4: *B. bassiana* TPP-H, 5: *B. bassiana* J18, 6: *B. bassiana* FRh2, 7: *B. caledonica* FRh1, 8: *B. malawiensis* Bweta, 9: *B. pseudobassiana* FW Mana, 10: *B. pseudobassiana* I12 Damo, 11: *B. caledonia* F532, 12: *B. bassiana* 2380AgR, 13: *B. bassiana* CTA20, 14: *B. bassiana* CTL20

A.2 Electrophoresis gel image of BLOC

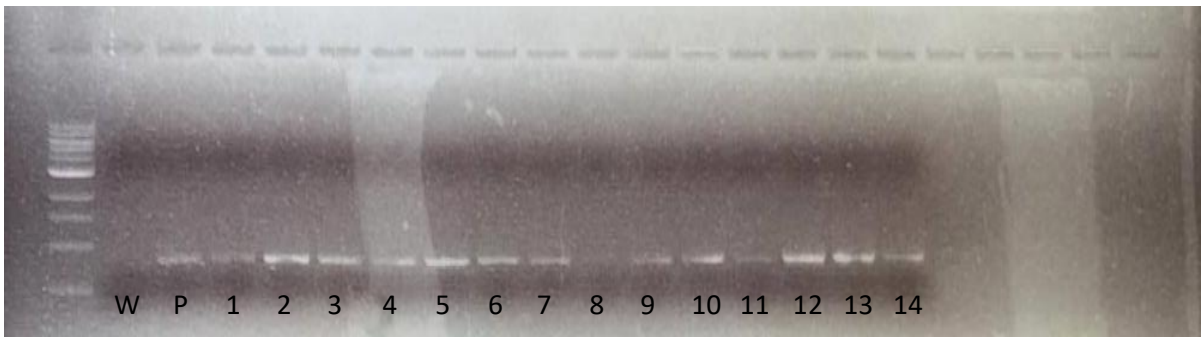

Figure A.2: Electrophoresis gel products received BLOC primer (B22U & B822L)

Note: W: water, P: positive control, 1: *B. bassiana* F615, 2: *B. bassiana* J2, 3: *B. bassiana* Mo1, 4: *B. bassiana* TPP-H, 5: *B. bassiana* J18, 6: *B. bassiana* FRh2, 7: *B. caledonica* FRh1, 8: *B. malawiensis* Bweta, 9: *B. pseudobassiana* FW Mana, 10: *B. pseudobassiana* I12 Damo, 11: *B. caledonia* F532, 12: *B. bassiana* 2380AgR, 13: *B. bassiana* CTA20, 14: *B. bassiana* CTL20

A.3 Sequence dendrogram

Consensus data from forward and reverse directions of these markers consisted of 539 and 353 bp in the aligned positions for EF1-α and BLOC, respectively. After manually deleting gaps and ambiguous alignments using MUSCLE alignment, 527 and 308 bp of EF1-α and BLOC sequences remained, respectively. Though BLOC gave relatively short bp, it showed more distinctive characteristics of each isolate than EF1-α (Figure A.3).

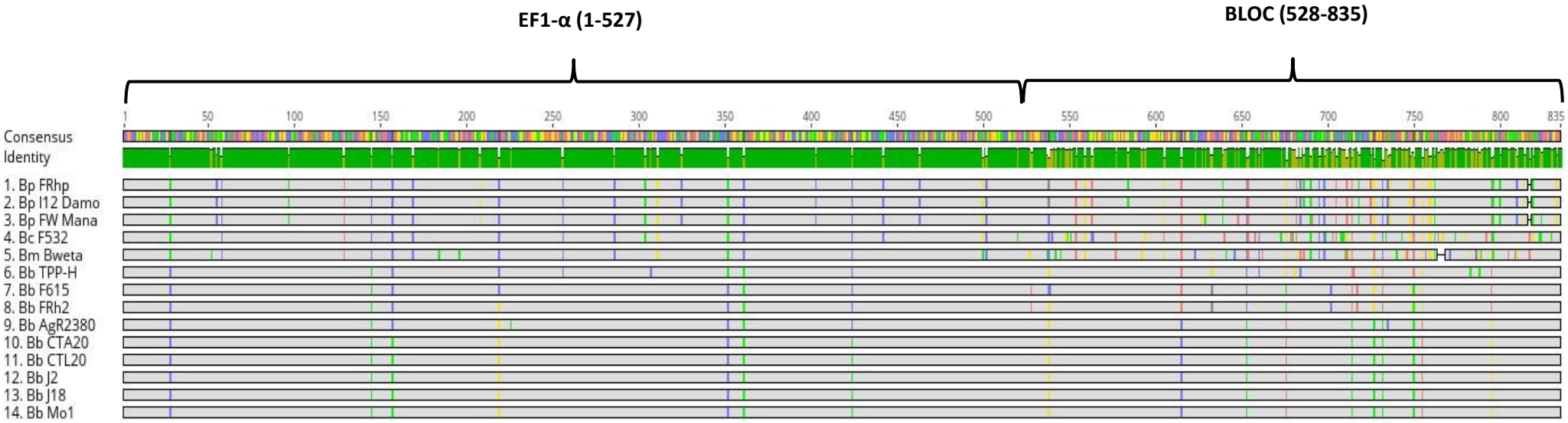

Figure A.3: Sequence alignment of all 14 isolates used in this study (combined consensus data of EF1-α and BLOC, aligned using MUSCLE alignment in Geneious software)

A.4 Sequence matrix

Table A.4: Sequence matrix similarity of all 14 isolates using combined-sequence data for EF1-α and BLOC based on the Neighbor-Joining method using Juke-Cantor

	Bp FRhp	Bp I12 Damo	Bp FW Mana	Bc F532	Bm Bweta	Bb TPP-H	Bb F615	Bb FRh2	Bb O2380	Bb CTA20	Bb CTL20	Bb J2	Bb J18	Bb Mo1
Bp FRhp		100	99.04	93.77	92.69	93.17	92.69	92.57	92.57	92.34	92.34	92.34	92.34	92.34
Bp I12 Damo	100		99.04	93.77	92.69	93.17	92.69	92.57	92.57	92.34	92.34	92.34	92.34	92.34
Bp FW Mana	99.04	99.04		93.77	92.22	92.93	92.46	92.34	92.34	92.1	92.1	92.1	92.1	92.1
Bc F532	93.77	93.77	93.77		91.74	92.1	91.62	91.5	91.26	91.26	91.26	91.26	91.26	91.26
Bm Bweta	92.69	92.69	92.22	91.74		91.98	91.5	91.38	91.26	91.26	91.26	91.26	91.26	91.26
Bb TPP-H	93.17	93.17	92.93	92.1	91.98		97.72	97.84	97.37	97.37	97.37	97.37	97.37	97.37
Bb F615	92.69	92.69	92.46	91.62	91.5	97.72		99.64	97.72	97.72	97.72	97.72	97.72	97.72
Bb FRh2	92.57	92.57	92.34	91.5	91.38	97.84	99.64		98.08	98.08	98.08	98.08	98.08	98.08
Bb O2380	92.57	92.57	92.34	91.26	91.26	97.37	97.72	98.08		99.52	99.52	99.52	99.52	99.52
Bb CTA20	92.34	92.34	92.1	91.26	91.26	97.37	97.72	98.08	99.52		100	100	100	100
Bb CTL20	92.34	92.34	92.1	91.26	91.26	97.37	97.72	98.08	99.52	100		100	100	100
Bb J2	92.34	92.34	92.1	91.26	91.26	97.37	97.72	98.08	99.52	100	100		100	100
Bb J18	92.34	92.34	92.1	91.26	91.26	97.37	97.72	98.08	99.52	100	100	100		100
Bb Mo1	92.34	92.34	92.1	91.26	91.26	97.37	97.72	98.08	99.52	100	100	100	100	

Appendix B

Statistical data for all chapters

B.1 Statistical data for Chapter 3

B.1.1 LD_{50} statistical table

Table B.1.1: Statistical significance for LD_{50}, ANOVA test (LSD 95%, multiple comparisons using Turkey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	2	12.068	6.034	14.42	
Isolate	13	63.4187	4.8784	11.66	<.001
Residual	26	10.8823	0.4185		
Total	41	86.369			

B.1.2 LD_{95} statistical table

Table B.1.2: Statistical significance for LD_{95}, ANOVA test (LSD 95%, multiple comparisons using Turkey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	2	5.6783	2.8392	6.78	
Isolate	13	63.4171	4.8782	11.65	<.001
Residual	26	10.8831	0.4186		
Total	41	79.9785			

B.1.3 LT_{50} statistical table

Table B 1.3: LT_{50} of the 14 isolates across the three application rates, ANOVA test (LSD 95%, multiple comparisons using Turkey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	2	1284.74	642.37	8.95	
Isolate	13	4062.83	312.53	4.36	<.001
Rate	2	4405.35	2202.67	30.7	<.001
Isolate.Rate	26	1283.04	49.35	0.69	0.859
Residual	82	5882.52	71.74		
Total	125	16918.48			

B.2 Statistical data for Chapter 4

B.2.1 *In vitro* challenge result

The *in vitro* challenge result showed that the growing speed of both multiple and single cultures in the first week differed significantly ($F = 5.06$, $df = 19$, $p < .001$). Isolate CTL20 in single culture grew faster, while isolate Bweta in the combination culture 1 grew slower compared to the other isolates. Second week results revealed statistical difference ($F = 57.93$, $df = 19$, $p < .001$). Isolate Bweta was smaller than the others, and those isolates combined with this isolate were also smaller than their single cultures. Interestingly, isolate F532 grew bigger than as a single isolate when combined with isolate I12 Damo and CTA20. At week three, colony diameter of each isolate showed significant differences ($F = 39.41$, $df = 19$, $p < .001$). Isolate Bweta and isolates cultured with this isolate were smaller in diameter than the other combinations and single cultures (Table B.2).

Table B.2.1: Colonial diameters of multiple cultures grown under *in vitro* conditions over three weeks, ANOVA test (LSD 95%, multiple comparisons using Turkey test)

Treatment	Isolate	Mean ± SE (mm)		
	Week	1	2	3
Multiple culture 1	Mo1	15.67 ± 0.33 abc	36.67 ± 1.20 de	46.67 ± 0.88 cdef
	FRhp	17.00 ± 0.58 bcd	32.33 ± 1.45 bcd	42.67 ± 1.76 bcde
	Bweta	14.00 ± 0.58 a	24.33 ± 2.19 a	33.00 ± 1.53 ab
Multiple culture 2	Bweta	16.00 ± 0.58 abcd	20.67 ± 1.20 a	26.33 ± 2.33 a
	FW Mana	17.00 ± 0.58 bcd	25.33 ± 1.45 ab	39.00 ± 5.13 bcd
	F532	15.33 ± 0.33 ab	26.33 ± 3.84 ab	36.67 ± 6.67 abc
Multiple culture 3	Bweta	15.33 ± 0.33 ab	22.33 ± 1.20 a	33.33 ± 0.88 ab
	F532	15.67 ± 0.33 abc	33.67 ± 0.88 cde	46.00 ± 2.08 cdef
	CTL20	18.33 ± 0.58 cd	36.00 ± 0.58 de	55.67 ± 0.67 fgh
Multiple culture 4	I12 Damo	18.00 ± 0.58 bcd	32.00 ± 0.58 bcd	43.33 ± 0.88 bcde
	F532	16.00 ± 0.58 abcd	48.00 ± 1.15 g	59.00 ± 0.58 gh
	CTA20	16.00 ± 0.58 abcd	33.67 ± 0.88 cde	45.67 ± 1.45 cdef
Single	Mo1	17.00 ± 0.58 bcd	40.00 ± 0.58 ef	46.00 ± 0.58 cdef
Single	FRhp	16.67 ± 0.88 abcd	32.00 ± 0.58 bcd	47.00 ± 0.58 defg
Single	Bweta	16.33 ± 0.33 abcd	27.00 ± 0.58 abc	41.00 ± 0.58 bcde
Single	FW Man	18.33 ± 0.88 cd	32.00 ± 0.58 bcd	65.00 ± 0.58 hi
Single	I12 Damo	18.00 ± 0.58 bcd	35.00 ± 0.58 de	52.00 ± 0.58 efg
Single	F532	17.00 ± 0.88 bcd	48.00 ± 0.58 g	76.00 ± 0.58 ij
Single	CTL20	18.67 ± 0.33 d	60.00 ± 0.58 h	80.00 ± 0.58 j
Single	CTA20	17.67 ± 0.33 bcd	47.00 ± 0.58 fg	56.00 ± 0.58 fgh

B.2.2 LD_{50} statistical table

Table B.2.2: LD_{50} for the four combined isolates compared to the individual isolates (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	2.1982	2.1982	3.72	
Isolate	12	33.7414	2.8118	4.76	0.006
Residual	12	7.0824	0.5902		
Total	25	43.022			

B.2.3 LT_{50} statistical table

Table B.2.3: LT_{50} for the four combined isolates and the individual isolates (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	11.33	11.33	0.26	
Isolate	12	1777.52	148.13	3.4	0.002
Rate	2	1644.59	822.3	18.89	<.001
Isolate.Rate	24	685.33	28.56	0.66	0.861
Residual	38	1654.58	43.54		
Total	77	5773.35			

B.3 Statistical data for Chapter 5

B.3.1 LD_{50} of isolate I12 Damo

Table B.3.1: Rate of isolate I12 Damo required to kill 50% of larvae on the four different brassicas (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	0.5333	0.5333	1.67	
Rate	2	115.2667	57.6333	180.64	<.001
Brassica	4	2.1333	0.5333	1.67	0.212
Rate.Brassica	8	9.0667	1.1333	3.55	0.019
Residual	14	4.4667	0.319		
Total	29	131.4667			

B.3.2 LD_{50} of isolate CTL20

Table B.3.2: Rate of isolate CTL20 required to kill 50% of larvae on the four different brassicas (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	3.3333	3.3333	6.09	
Rate	2	173.6	86.8	158.5	<.001
Brassica	4	7.6667	1.9167	3.5	0.035
Rate.Brassica	8	11.7333	1.4667	2.68	0.051
Residual	14	7.6667	0.5476		
Total	29	204			

B.3.3 LT_{50} of isolate I12 Damo

Table B.3.3: LT_{50} of DBM larvae fed on four brassicas across three isolate I12 Damo application rates (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	0.3341	0.3341	0.54	
Brassicas	4	5.4692	1.3673	2.21	0.12
Rate	2	91.1904	45.5952	73.75	<.001
Brassicas.Rate	8	9.6523	1.2065	1.95	0.131
Residual	14	8.6549	0.6182		
Total	29	115.3009			

B.3.4 LT_{50} of isolate CTL20

Table B.3.4: LT_{50} of DBM larvae fed on four brassicas across three isolate CTL20 application rates (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	3.546	3.546	2.41	
Brassicas	4	55.384	13.846	9.39	<.001
Rate	2	217.973	108.987	73.93	<.001
Brassicas.Rate	8	144.891	18.111	12.28	<.001
Residual	14	20.64	1.474		
Total	29	442.435			

B.4 Statistical data for Chapter 6

B.4.1 DBM mortality at both rates

Table B.4.1: Statistical difference of DBM mortality at the half-strength concentration (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	63.28	63.28	4.76	
Isolate	3	2858.59	952.86	71.75	0.003
Residual	3	39.84	13.28		
Total	7	2961.72			

Table B.4.2: Statistical difference of DBM mortality at the full-strength concentration (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	19.53	19.53	1.74	
Isolate	3	3814.84	1271.61	113.56	0.001
Residual	3	33.59	11.2		
Total	7	3867.97			

B.4.2 LT_{50} statistical table

Table B.4.3: Statistical difference of LT_{50} of three isolates at the half-strength concentration (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	2.2407	2.2407	17.76	
Isolate	2	45.4884	22.7442	180.28	0.006
Residual	2	0.2523	0.1262		
Total	5	47.9815			

Table B.4.4: Statistical difference of LT_{50} of three isolates at the full-strength concentration (General ANOVA, multiple comparisons using Tukey test)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run stratum	1	0.54	0.54	3.86	
Isolate	2	19.0633	9.5317	68.08	0.014
Residual	2	0.28	0.14		
Total	5	19.8833			

Appendix C

Photos of cadavers

Larva killed by isolate F615

Larvae killed by isolate J2

Larvae killed by isolate Mo1

Larvae killed by isolate TPP-H

Larva killed by isolate J18

Larvae killed by isolate FRh2

Larvae killed by isolate FRhp

Larva killed by isolate Bweta

Larvae killed by isolate FW Mana

Larvae killed by isolate I12 Damo

Larva killed by isolate F532

Larvae killed by isolate O2380

Larvae killed by isolate CTL20

Larvae killed by isolate CTA20

Larvae killed by isolate FRhp toxins

Larva killed by isolate FW Mana toxins

Larva killed by isolate CTA20 toxins