Developing a biocontrol system for the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), using entomopathogenic fungi

Declan Joseph Perry

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Abbreviations

AIC.....Akaike information criterion

AMPs.....Antimicrobial peptides

Anova.....Analysis of variance

Bt.....*Bacillus thuringiensis*

BVW.....Black vine weevil

CDDs.....Cumulative day degrees

DBM.....Diamondback moth

DD.....Day degree

EPF.....Entomopathogenic fungi

EU..... European Union

GST.....Glutathione S-transferase

GxE.....Genotype by environment interaction

GxG.....Genotype by genotype interaction

GxGxE.....Genotype by genotype by environment interaction

IGRs.....Insect growth regulators

Imd.....Immune deficiency pathway

IPM.....Integrated pest management

ITS.....Internal transcribed spacer

JAK/STAT.....Janus kinase/signal transduction pathway

LC50.....Concentration at which 50% mortality occurs

LD.....Light/dark

LT50.....Time to 50% mortality

PCR.....Polymerase chain reaction

RO.....Reverse osmosis

SDA.....Sabouraud dextrose agar

SE.....Standard error

T0.....Thermal minima

Tmax.....Thermal maxima

Topt.....Thermal optima

Tukey's HSD.....Tukey's honest significance test

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Declaration

This PhD thesis is presented according to the Guide to Examinations for Higher Degrees by Research provided by the Graduate School, University of Warwick. It has been written by myself and has not been submitted for any other degree. All experimental work, analysis, and written work presented here was completed by myself unless otherwise stated.

Summary

The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), is the most important global pest of Brassica crops. It is now prevalent wherever Brassicas are grown and costs the agricultural industry US$ 4-5 billion annually. Traditionally, DBM is controlled by the use of insecticide only applications which has led to wide spread insecticide resistance in DBM populations. As insecticide only control strategies are not viable in the long term, it is the opinion of many experts that integrated pest management (IPM) is the way forward for DBM control. A component of the IPM system which holds great potential is entomopathogenic fungi (EPF). Despite their promise, EPF are not currently routinely used by growers to control DBM. This is mainly because the efficacy of EPF biopesticides tends to vary from one application to the next. The aim of this project was to study interactions between EPF and other elements of the IPM system to understand and address sources of variability. Temperature is the most important determinant of EPF growth, virulence and DBM development. However, there are considerable knowledge gaps when it comes to understanding the effect of temperature on these processes. This is mainly because of the left-skewed nature of the physiological response to temperature. We assessed the suitability of five non-linear models to describe these interactions and found Briere-1 to be the most appropriate model. Cardinal temperatures taken from it were used to develop a day-degree (DD) model which could accurately predict the virulence of EPF against DBM at fluctuating, "field realistic", temperatures. In an IPM system, EPF would be applied with synthetic insecticides. Consequently, it is important to know how co-application of these products would affect the survival of groups of DBM larvae. After applying low-concentrations of various insecticides with EPF we found there to be negligible evidence of antagonism, indicating the insecticides and EPF are compatible for use within an IPM system. Finally, the effect of the age structure of the DBM larval population on efficacy of EPF biopesticides was investigated. We found that, because larvae slough off conidia during ecdysis, time between treatment of EPF and moult had a significant effect on larval mortality. It is hoped that this study will improve growers' confidence in EPF biopesticides, and expedite their use within IPM systems to control DBM.

1 Literature review

1.1 Introduction

DBM is a major pest of Brassica crops across the world. Larvae cause damage to crops through consumption of leaf material (Harcourt, 1957). The pest has a short life cycle, which comprises of four larval instars (Golizadeh et al., 2007), and has a high fecundity, meaning that large populations build up rapidly on Brassica crops (Talekar and Shelton, 1993). Estimates concerning the economic impact of DBM have varied considerably, perhaps because of changes in the abundance and yields of Brassica crops in the last 20 years (FAOSTAT, 2012).

A major contributing factor to the destructive properties of DBM is the propensity of the pest to develop insecticide resistance (Talekar and Shelton, 1993). It is clear that no single strategy will be effective in controlling DBM populations, and for this reason it is the general opinion of experts that integrated pest management (IPM) should be used in control efforts (Furlong et al., 2013). Entomopathogenic fungi (EPF) are considered to have great potential in controlling DBM in the field, but as of yet this potential has not been realised (Grzywacz et al., 2010). EPF are slow acting when compared to synthetic insecticides (Lacey et al., 2001). Additionally, the performance of EPF in field is also highly variable (Thomas and Blanford, 2003). From the point of view of growers, it is the variability of performance of EPF, rather than the slow acting nature of EPF, which prevents uptake in the field.

A major factor which influences the performance of EPF is temperature (Blanford et al., 2005). Consequently, understanding the interaction between temperature and the virulence of EPF is vital in addressing variability observed in the field. However, this interaction is complex: temperature influences the production and secretion of EPF metabolites, growth of fungi, insect immunity and insect growth and development. Surprisingly, there has been little research into the effect of temperature on EPF virulence. The vast majority of studies into EPF virulence have been completed at constant temperatures. It is not currently known whether performance of EPF at

fluctuating temperatures, as seen in the field, can be predicted from experiments completed at constant temperatures.

IPM strategies usually work as a threshold based system: when pest population densities exceed a certain level, synthetic insecticides are used to reduce the pest population density to below the threshold level (Lim, 1992). For this reason, it is likely that EPF would be applied with synthetic insecticides. Consequently, it is necessary to understand the effect of co-application of synthetic insecticides and EPF biopesticides on the survival of DBM larvae. For example, there have been examples in the literature of synergy occurring between insecticides and EPF biopesticides against insect pests (Nian et al., 2015, Farenhorst et al., 2010, Tian and Feng, 2006, Wraight and Ramos, 2005). Synergistic interactions enable lower concentrations of synthetic insecticide to be applied, whilst achieving a similar level of insect control. This has the potential to confer significant environmental and financial savings. Currently, there is lack of understanding of how different elements of the IPM system work together. Experiments in the literature tend to be completed on one element of the IPM system. This has led to a lack of uptake of IPM systems by growers (Lim, 1992).

It has been suggested in the literature that insect larvae can avoid EPF infection by discarding conidia as the cuticle is shed from one instar to the next (Vandenberg et al., 1998b, Vey and Fargues, 1977). Time between treatment of larvae with EPF and moult is an important factor that determines whether conidia are able to penetrate the insect cuticle and cause a systemic infection (Vandenberg et al., 1998a). This is another potential source of variation in EPF performance in the field. In laboratory bioassays EPF are applied to larvae of a fixed age; normally early second instars (Wraight et al., 2010, Altre and Vandenberg, 2001, Godonou et al., 2009, Huang et al., 2010). This allows conidia at 48-72hr to penetrate the cuticle before the moult into the third instar occurs. In the field, populations of DBM are likely to be of a mixed age. Consequently, a certain proportion of larvae will be treated late in the instar; it is likely that these larvae will discard conidia when they transition to the next instar. It is not currently known whether secondary pick-up of conidia from a treated leaf surface can cause infection in those larvae that have discarded conidia in the moulting process.

1.2 DBM as a global pest of Brassica crops

1.2.1 The economic cost of DBM

The annual cost of control of DBM is cited in most papers as being US$ 1 billion (Wang et al., 2004, Farrar et al., 2007, Fahimi et al., 2008, Ebrahimi et al., 2009). This figure was first estimated by Talekar and Shelton (1993) and has continued to be cited since then in papers that broach the subject of DBM control, despite on-going inflation and an increased production of Brassica crops. According to a review by Zalucki et al. (2012), the US$ 1 billion estimate was not based on any sophisticated calculation and was merely an estimate.

Zalucki et al. (2012) estimated the cost of DBM infestations by calculating how much was spent per hectare of Brassica crop on the management of DBM populations, and the extent of crop losses despite this management. Assuming the figure of US$ 1 billion was correct in 1993, then in real terms the figure would be between 1.81 and 2.65 billion in 2012 (when taking into account inflation). Added to this, there have been increases in Brassica crop production, and there is now an additional 12 million hectares of crop that requires protection. This represents an increase of 57% of the global Brassica cropping area since 1993. This further compounds how outdated the 1993 estimated is. Taking this into consideration, a conservative estimate of the annual cost of DBM would be somewhere in the region of US$ 4-5 billion dollars (Zalucki et al., 2012).

1.2.2 Biology of DBM

1.2.2.1 Interactions of DBM with plant hosts

DBM are known to infest any member of the Brassica family. For example, Harcourt (1957) reported that DBM consume leaf material from canola, kohlrabi, kale, Brussel sprouts, broccoli, cauliflower, cabbage and collards (Harcourt, 1957). Early in the season, before arable crops have been sown, cruciferous weeds play an important role in sustaining DBM populations. A key feature of Brassica crops is the use of glucosinolates in the innate immune response. In the event of foliage damage, glucosinolates undergo

hydrolysis to produce toxic by-products (Hopkins et al., 2009). The toxic by-products include the isothiocyanates which are known to be lethal to insects. However, DBM produce a glucosinolate sulfatase enzyme, which cleaves sulfur from glucosinolates, preventing toxic isothiocyanates from being produced (Textor and Gershenzon, 2009). This means that DBM can consume Brassica foliage with impunity. Additionally, DBM benefit from reduced competition from other insect pests which are susceptible to isothiocyanates. Certain compounds derived from glycosinolates can also act as attractants and oviposition stimulants for DBM (Renwick et al., 2006).

1.2.2.2 Geographic distribution of DBM

Initially, Hardy (1938) suggested the moth may have its origins in Europe. However, Kfir (1998) later proposed that DBM originated from South Africa because of the relatively large numbers of parasitoids (which coevolved with DBM) that are indigenous to the area. Using similar arguments, Liu et al. (2007) are of the view that DBM originated in China. This pest is now present wherever its host plants exist and is considered to be the most widely distributed of all Lepidopteran pests (Sarfraz et al., 2005).

DBM has gained such a distribution because of its ability to migrate. The earliest recording of transoceanic migration came from Curtis (1860) who noted the mass migration of DBM into England. Transoceanic migration events have continued to be recorded ever since, as well as their corresponding crop losses (Lokki et al., 1978). Migration allows DBM to move from areas when Brassica crops are grown seasonally to areas where Brassicas are grown all year round. Additionally, DBM often migrate to certain countries when temperatures rise high enough to allow insect growth and development. For example, the use of radar tracking established that a population of DBM migrated from the Netherlands to UK in May 2002 and established a season-long population (Chapman et al., 2002). Similarly, DBM are not able to overwinter in northern Japan because of cold temperatures, so they migrate from the south to the north when temperatures begin to rise in the summer months (Yamada and Umeya, 1972).

1.2.2.3 Life cycle of DBM

The length of the life cycle of DBM is highly influenced by temperature. For example, DBM can take as little as 12 days to develop from egg to adult at temperatures of approximately 30^{o}C (Marchioro and Foerster, 2011). At 10^{o}C DBM takes approximately 70 days to develop from egg to adult (Golizadeh et al., 2007).

The development of DBM is characterized by several moulting stages which indicate the transition between instars, and the transition between the final instar and pupal stage (Marchioro and Foerster, 2011, Bahar et al., 2014, Golizadeh et al., 2007). The insect cuticle is a rigid structure, and must be discarded for the insect to grow larger in size (Wigglesworth, 1948). Typically, before moulting occurs, the insect becomes inactive and ceases feeding (Harcourt, 1954, Harcourt, 1957). The epidermal cells located beneath the insect procuticle and epicuticle then begin to divide and deposit the new cuticle (Richards and Davies, 1977). This event is correlated with a spike in two moulting steroids called ecdysone and ecdysterone which are secreted from epidermal cells (Mordue et al., 1980). As the new cuticle is being formed, the old cuticle becomes folded and separates from the epidermal cells (Wigglesworth, 1948). This process is aided by the secretion of moulting fluid into the cavity between the old and new epidermis. Proteases and chitinases in the moulting fluid allow the old cuticle to be broken down (Fig. 1-1). The remaining portion of the old cuticle that has not been broken down then splits as the insect expands, and ecdysis occurs as the insect extracts itself from the old cuticle (Wigglesworth, 1948). The deposition of the new cuticle and the initiation of ecdyis is correlated with a second, large, spike in ecdysone and ecdysterone concentration in the epidermal cells (Mordue et al., 1980). Just after moult, larvae are darker in color and typically have a softer cuticle (Golizadeh et al., 2007). As the instar progresses there is an increase in cuticle thickness, and an increase in pigmentation (Harcourt, 1954).

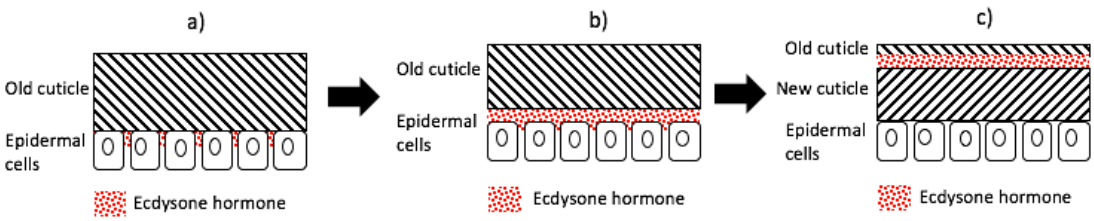


Figure 1-1: Process of ecdysis in DBM larvae. A) Towards the end of the intermoult period epidermal cells secrete ecdysone which triggers the beginning of ecdysis. B) The release of ecdysone triggers epidermal cells to begin synthesizing the new cuticle and causes moulting fluid to build up in the cavity between the epidermal cells and the cuticle. C) Once the new cuticle is formed, the old cuticle can be discarded, this associated with another spike in ecdysone concentration.

Populations of DBM typically migrate from mainland Europe to the UK in the summer months, when temperatures rise high enough for DBM to develop (Chapman et al., 2002, Curtis, 1860). DBM eggs are typically laid on the underside of the leaf in clusters. At 20^{o}C eggs take approximately five days to hatch; just before hatching the egg darkens and neonatal larvae can be seen beneath the surface of the egg (Golizadeh et al., 2007, Marchioro and Foerster, 2011). The newly hatched first instar larva then crawls from the egg on to the underside of the leaf and tunnels through the epidermis (Harcourt, 1957). Larvae remain in these shallow tunnels, feeding on leaf tissue, for the whole of the first instar. At the end of the first instar the larva moves from the tunnel to the outside of the leaf and begins its moult to the second instar. From the second to the final instar, larvae feed on the surface of the leaf. When feeding, larvae do not consume leaf veins or the upper epidermis (Harcourt, 1957). This results in characteristic “windowing” effect often seen in plants that have undergone DBM damage (Fig. 1-2). Larvae moult four times during development: from first to second instar, second to third instar, third to fourth instar and a final moult before pupation occurs (Golizadeh et al., 2007, Marchioro and Foerster, 2011). At 20^{o}C it takes larvae approximately 13 days to move though all the larval instars (Golizadeh et al., 2007). At the end of the fourth instar larvae undergo pupation. Pupae are usually found on the underside of the leaf, or the join

between the leaf and stem (Harcourt, 1957). Before the cocoon is spun, larvae become shorter and wider in size. A cocoon made up of fine fibers is then constructed which is then followed by a period of inactivity known as the pre-pupal stage (Bahar et al., 2014). The larva then undergoes its final moult which indicates it has moved into the pupal stage; the moulted skin remains within the pupae. At 20^{o}C, it takes larvae about seven days to move though the pre-pupal and pupal stages (Golizadeh et al., 2007). After emergence from pupation, mating and oviposition can start almost immediately (Harcourt, 1954). The majority of females begin oviposition on the day of emergence, with the peak period for egg laying being the day after emergence. Females lay an average of 159 eggs over the course of their lifetime. Oviposition can continue for a total of 10 days after emergence, with the female living 13-20 days. Males survive for 10-15 days after emergence (Harcourt, 1957).

Figure 1-2: Characteristic “windows” in cauliflower plant foliage caused by a mixed-age DBM larvae population.

1.2.2.4 Insecticide resistance of DBM populations

A major contributing factor to DBM becoming the most important global Brassica pest has been the insects' propensity to develop insecticide resistance (Talekar and Shelton, 1993, Furlong et al., 2013). Since their introduction in the 1940s, there has been an overuse of broad-spectrum synthetic insecticides in Brassica crop production (Talekar and Shelton, 1993). This is a particular problem for growers in poorer areas of the world, who are unaware or unable to obtain novel and more effective insecticides and so rely on traditional broad spectrum insecticides (Grzywacz et al., 2010). Additionally, in sub-tropical regions, DBM can cycle through one generation every 14 days, meaning that 25 generations of DBM a year may be exposed to synthetic insecticides (Loc and Chi, 2007). This creates a huge selection pressure on DBM populations which inevitably leads to insecticide resistance.

Insecticide resistance of DBM is generally acknowledged to occur from either single site, or multi-site mutations. For example, resistance of DBM to avermectins, is known to occur through the mutation of a single esterase gene (Furlong, 2013). Conversely, resistance to organophosphates is known to occur from resistance of multiple genes, including those coding for glutathione transferase, esterase and mixed-function oxidases (Eziah, 2009). Cross resistance occurs when some key genes cause resistance across more than one insecticide; for example, esterase mutations are known to cause resistance to pyrethroid and indoxacarb insecticides, and resistance to one of these insecticides is likely to precede resistance to the other (Furlong, 2013).

There have been numerous case studies that describe the development of synthetic insecticide resistance in DBM populations (Furlong et al., 2013). For example, in the mid-1980s several novel insect growth regulators (IGRs) were released and used for DBM control in the Cameroon islands. Applications during the growing season normally averaged two sprays per week; within three years there were widespread DBM outbreaks due to the development of resistance to IGRs (Syed, 1992).

Currently, DBM is one of the few insects to have exhibited resistance to all synthetic insecticides applied in the field. DBM was also the first insect pest to show resistance to

Bt-cry toxins (Talekar and Shelton, 1993). The pest has also displayed resistance to other novel insecticides such as spinosad and inoxicarb (Furlong et al., 2013). But resistance is only part of the problem; it has been suggested that the destruction of natural enemies, due to the over use of broad spectrum insecticides, is equally as responsible for DBM infestations (Verkerk and Wright, 1997). Because of this, many insecticides developed over recent decades have been more selective for DBM, in an attempt to preserve natural enemy populations. However, novel selective insecticides are normally sprayed in a cocktail, containing more traditional broad-spectrum insecticides, and evidence suggests this leads to more rapid development of resistance in DBM populations (Sayyed and Wright, 2004). This approach has rendered some selective insecticides redundant.

There is limited knowledge concerning specific mechanisms that lie behind DBM insecticide resistance. Metabolic resistance mechanisms are most commonly reported in the literature. For example, in a study in which pyrethroid resistant DBM were compared with a susceptible population, it was found that there was an up to 1.9 times increase in esterase activity in the resistant strain (Eziah et al., 2009). It was also found that P450 monooxygenases and glutathione S-transferase (GST) activity was significantly higher in resistant field populations, indicating that these metabolic enzymes are likely to be involved in insecticide resistance (Eziah et al., 2009).

1.3 The potential of EPF to control DBM populations

1.3.1 The rise of EPF biopesticides

In 2009, biopesticides (including bacteria, natural predators, viruses, nemotodes, protozoa and EPF) made up 3.5% of the total global agrochemicals market; this grew to a 7.7% market share by 2014 (Glare et al., 2012). The demand for EPF biopesticides has more than doubled over the last decade (Boyetchko and Svircev, 2013). The global market of EPF biopesticides was US$ 113 and US$ 289 in 2009 and 2014, respectively (Glare et al., 2012). The level of growth observed in the biopesticide market is higher than observed in the synthetic insecticide market (Boyetchko and Svircev, 2013). The ratio of novel synthetic insecticides leads to product launches has increased

considerably over this period of time (Glare et al., 2012). This is because of the high cost of bringing a synthetic insecticide to market, when compared to biopesticides. It is estimated that it takes 10 years and costs US$ 250 million to launch a synthetic insecticide product (Glare et al., 2012). In comparison, a biopesticide product can be brought to market in three years at a cost of US$ 5 million (Marrone, 2011). Additionally, several synthetic insecticide active ingredients have been withdrawn over the last 20 years (McGrath, 2014). The registration of biopesticide products is promoted by European Union (EU) policy which favours the use of IPM and low risk control products (EC, 2009b, EC, 2009a). These factors indicate that the number of biopesticide products on the market, and the market growth of biopesticides when compared to synthetic insecticides, is likely to continue to rise in the future.

1.3.2 Benefits of using IPM to control insect pests

It is the prevailing opinion of experts that insecticide-only application is not a long-term option for the control of DBM (Furlong et al., 2013). Integrated pest management (IPM) is considered to hold the most potential in developing an effective control strategy for the pest. IPM systems employ many different products and strategies to achieve control. For example, an IPM system may employ cultural control strategies, behavioural management, biological controls, and careful use of synthetic insecticides (Radcliffe, 2008). In principle, each component of the IPM system is designed to complement the other elements. This has the benefit of promoting a more sustainable system, both economically and environmentally (Radcliffe, 2008).

Within the IPM system, the application of biocontrol agents can be split into three categories (Mahr and Ridgway, 2008). The first, known as conservation biocontrol, involves the use of beneficial farming practices to maintain a population of biological control agents already native to a particular area (McCravy, 2008). The second, known as classical control, involves the release of biological control agents from a different country or geographical region to an area to control an insect pest, which had previously migrated into that area (i.e. a non-native insect pest). In classical biocontrol, the aim is to establish a permanent population of biocontrol agents (Mahr and Ridgway, 2008).

For example, the palm rhinoceros beetle (*Oryctes rhinoceros*) was introduced from Malaysia to many palm tree growing areas in India. The pest causes damage and eventual death of palm trees. A viral pathogen of *O. rhinoceros* was taken from Malaysia and introduced to thirteen areas in India over 21 years which resulted in a huge reduction of the pest population (Huger, 1966, Hajek, 2007).

The third application method, known as augmentation biocontrol, involves the release of a biocontrol agent to temporarily increase their number so that an insect pest can be controlled (Mahr and Ridgway, 2008). Augmentation biocontrol can be split into two categories (Hajek, 2007). The first, known as inoculation control, involves the application of a limited amount of biological control agent which is also a native pathogen at long-term time intervals. The goal of inoculation control is often to trigger an epizootic population crash of insect pests earlier than usual. For example, the native EPF *Entomophaga maimaiga* was released in treatment plots in Maryland, US in 1995/1996 to control populations of gypsy moth; the EPF successfully caused epizootic outbreaks and a reduction in pest density (Hajek and Webb, 1999). The second category, called inundative control, refers to the short-term application of a large amount of biocontrol agent (Hajek, 2007). In IPM, inundative control is most likely to be used for application of EPF biopesticides (Shah and Pell, 2003). Typically, a large amount of EPF biopesticide is applied at regular intervals throughout the growing season which supresses the pest population below a defined threshold level (Lim, 1992).

When compared to insecticide-only control strategies, IPM is underused as a means of DBM control (Grzywacz et al., 2010, Lim, 1992). IPM systems are more difficult to implement by growers and require greater skills and know-how than insecticide only approaches. Decisions need to be made, usually based on the density of the pest population, on which elements of the IPM system to deploy at any one particular time. Although the separate elements of the IPM system are readily available, there is a lack of knowledge of how the different elements work together on a practical level. For example, several papers have reported EPF isolates to be virulent against DBM in the laboratory. However, it is likely that EPF would be applied with a synthetic insecticide.

In a review of IPM research, Lim (1992) reported that a greater focus on how different elements of the IPM system work together would expedite the use of IPM in the field.

Despite these challenges, there are several examples of IPM systems being used against DBM in the field. These strategies normally involve the use of parasitoids of DBM to reduce pest density: once a defined pest density threshold has been exceeded, synthetic insecticides are used to reduce DBM numbers (Lim, 1992). In some cases, Bt is used as a replacement for synthetic insecticides (Grzywacz et al., 2010). Additionally, there has been use of a physical barrier between the crop and pest. Implementing an IPM system can often have financial advantage due to the savings in not buying synthetic insecticides (Butt and Ansari, 2011).

1.3.3 EPF as a component of the IPM system

Numerous EPF species have been shown to be virulent against DBM within laboratory screens, semi-field experiments and field experiments (Wraight et al., 2010, Vandenberg et al., 1998a, Vandenberg et al., 1998b, Xie et al., 2014, Huang et al., 2010). There are approximately 700 species of fungi from 56 genera that have been shown to infect arthropods (Lacey et al., 2001, Chi, 2007). Apart from being able to cause insect mortality, EPF have a range of other benefits. When compared to many synthetic pesticides they are considerably less harmful to many beneficial organisms such as natural predators of DBM, bumble and honey bees, and earth worms (Garrido-Jurado et al., 2011). EPF have also been shown to pose minimal risk to human health (Zimmermann, 2007a, Zimmermann, 2007b, Goettel et al., 2001).

Several studies have shown that EPF are compatible with other commercially available insecticides (Nian et al., 2015, Gomes et al., 2015, Wraight and Ramos, 2005, Ye et al., 2005, Neves et al., 2001). It is likely that EPF would be applied along with synthetic insecticides within an IPM system. For example, Nian et al. (2005) described a predominantly synergistic interaction when an *I. fumosorosea* EPF was applied with Bt to control DBM. EPF formulations can also be applied using the same spraying equipment used to apply synthetic insecticides (Chandler, 2017).

Additionally, it is known that EPF are able to persist in the environment, either on or near pests, after being sprayed (Shah and Pell, 2003). The use of EPF biopesticides is also known to reduce the expression of insecticide resistance in mosquito populations (Farenhorst et al., 2009). This is important considering the propensity DBM have to develop resistance to synthetic insecticides (Talekar and Shelton, 1993).

1.3.4 Biology of EPF

EPF consist of any fungi which are able to infect insects as well as other terrestrial arthropods such as mites, ticks and spiders (Chandler, 2017). There are estimated to be EPF species in at least seven fungal phyla (Kendrick, 1981). Entomopathogenicity is thought to have evolved independently in each phylum. EPF are contained within two main groups; the phylum Entomophthoromycota and the order Hypocreales. EPF from both groups cause infection by attachment of host spores to the insect cuticle, and subsequent growth of mycelium into fissures, causing a systemic infection (Chandler, 2017). Of particular relevance is the order Hypocreales; the largest number of EPF can be found within this order. Fungi from this order have a diverse range of characteristics (Chandler, 2017). EPF from the Hypocreales are known to have evolved from plant parasitic fungi through a process called interkingdom host jumping (Spatafora et al., 2007); for example, an EPF may switch host organisms if their ecological niches overlap (Nikoh and Fukatsu, 2000). Additionally, in some EPF, transitions between similar insect hosts have occurred, and in others the EPF-host connection has been conserved over time (Nikoh and Fukatsu, 2000).

There are three Hypocrealean families: Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae. Clavicipitaceae and Ophiocordycipitaceae are sister taxa, having evolved from a common lineage (Humber, 2012). Fungi from these taxa are not all entomopathogens, as some obtain nutrients from animals or plants. All Cordycipitaceae fungi are entomopathogens (Chandler, 2017). Fungi in these taxa are present in either anamorphic or telemorphic forms. In their anamorphic form, fungi reproduce asexually by the production of haploid conidia and then the growth of filamentous mycelium.

Telemorphs are able to reproduce sexually, and can be characterised by the presence of a fruiting body. Because the anamorphic and telemorphic forms have different morphologies, they often have separate scientific names, despite being the same species (Chandler, 2017).

The Clavicipitaceae and Cordycipitaceae are of particular relevance in this case as they contain the species used in this PhD research. The former contains *Metarhizium* and the latter contains *Beauveria*, *Isaria* and *Lecanicillium* genera (Humber, 2012). The genera most commonly used as biological control products are *Metarhizium* and *Beauveria*. *Beauveria* contains 10 species (Rehner et al., 2011), the most agronomically important of which is *Beauveria bassiana* (as it is the active ingredient in the most biocontrol products). *Metarhizium anisopliae* has been resolved into 10 species (Bischoff and Rehner, 2009).

Beaveria bassiana, Isaria fumosorosea, Metarhizium and *Lecanicillium* EPF can be identified through their physical characteristics (Deacon, 2005). Descriptions of morphological characteristics described here are adapted from Humber (1997). Cultures of *B. bassiana* are white, both in the mycelial growth and sporulation phase. *B. bassiana* produce short ovoid conidia in a sympodial fashion. First, a terminal spore is produced which then elongates and produces further spores. These spores group together to form clusters. Cultures of *Metarhizium* EPF (including the species *M. anisopliae* and *M. brunneum*) are white whilst in the mycelial growth phase. However, *Metarhizium* spores are green and oblong (which changes the colour of the culture) and are grouped together in branched chains. Cultures of *Lecanicillium* (including the species *L. longisporum* and *L. muscarium*) are white, and their spores are white and oblong, but typically smaller than spores produced by *B. bassiana* and *Metarhizium* EPF. Cultures of *I. fumosorosea* are white when in the mycelial growth phase. The colour of the culture changes when sporulation occurs as light-purple fusiform shaped spores are produced. For all Hypocrealean EPF, conidia are produced from structures called phialide which are the basal cell for long chains of conidia (Deacon, 2005).

To complete their life cycle, all EPF must infect a suitable host and overcome the host immune system to cause infection (Hajek and St. Leger, 1994). Insect mortality then occurs; shortly after, the cadaver sporulates. Conidia can then be spread to new susceptible hosts (Furlong and Pell, 2001).

1.3.5 The process of EPF conidial infection of DBM larvae

The infection process of Hypocreales EPF, and the associated insect immune response, is well understood (Hajek and St. Leger, 1994, Ortiz-Urquiza and Keyhani, 2013). The first stage of infection, which involves adhesion of the spore to the insect cuticle surface, a highly dynamic process which involves the interaction of the conidia with the insect cuticle, is critical for the establishment of infection (Oduor et al., 1997). EPF conidia are hydrophobic which allows rapid attachment to the insect cuticle (Chandler, 2017). Typically, a relatively high number of EPF conidia are required to cause lethal infection (Hesketh et al., 2010). Prior to germination, the spore (resting on the cuticle surface) absorbs water through osmosis and begins to swell. Mucus is also secreted, which provides the means by which the spore attaches to the insect cuticle (Boucuas and Pendland, 1991). At this point, the spore is highly dependent on favourable levels of humidity to be maintained for infection to continue (Hajek and St. Leger, 1994). Once resting on the insect cuticle, conidia encounter the first line of insect defence (Leger et al., 1991). Lipids present within the insect cuticle have antifungal properties (Sosa-Gomez et al., 1997). Additionally, there is evidence to suggest that glandular secretions from the cuticle can inhibit fungal growth (Kerwin, 1984, Smith and Grula, 1981). These defence mechanisms, along with the fact that there is little accessible nutrients within the insect cuticle, mean the time-to-germination of conidia on the insect cuticle can be greatly increased (Andersen et al., 2006). However, EPF synthesise and secrete compounds to overcome these defences. Conidia are known to release proteases prior to cuticular penetration which aid the absorption of nutrients, and the break-down of the insect cuticle (Qazi and Khachatourians, 2007). For example, there are at least 16 *B. bassiana* enzymes are associated with absorption of cuticular nutrients (Pedrini et al., 2015).

The process of cuticular penetration involves the use of mechanical forces for mycelium to extend into fissures within the insect cuticle (Chandler, 2017). Fissures are formed through the degradation of the cuticular protein matrix (Hajek and St. Leger, 1994). The extension of mycelium into the insect cuticle causes fissures to widen which may allow the spread of cuticle degrading-enzymes that benefit infection (Brey et al., 1986, Goettel et al., 1989). After full penetration, fungi continue to grow within the insect hemolymph. During this phase of infection, there are two linked insect immune responses; the encapsulation of fungi with hemocytes and the melanisation of the area surrounding the site of infection (Gotz, 1986, Chandler, 2017). Melanisation is known to occur in the cuticle (where it produces the black circular marks which are the first symptom of fungal infection) and within the insect hemolymph within a matter of seconds after invasion (Chambers et al., 2012, Dubovskiy et al., 2013). However, this defence only provides protection against weak pathogens. *B. bassiana*, for example, has been shown to overcome the insect hemocyte response (Hou and Chang, 1985). Hypocrealean EPF evade the hemocyte response by producing small hyphal bodies called blastospores which avoid detection by the insect immune system by rapidly spreading through the hemolymph (Jiang et al., 2010). Additionally, fungi are able to inhibit the production of hemocytes in the period of time after invasion of the cuticle, which allows infection to spread more rapidly (Hung et al., 1993).

The insect also produces a systemic immune response which results in the production and secretion of a range of antifungal molecules (Chandler, 2017). The systemic response is mediated by three interlinked pathways; the Toll pathway, the immune deficiency (Imd) pathway and the Janus kinase/signal transduction and activator of transcription (JAK/STAT) pathway (Chandler, 2017). The Toll pathway is primarily responsible for the fungal systemic immune response, although there is cross-talk between all three pathways. The immune reaction starts with the detection of the fungal pathogen, a cascade reaction then follows which triggers the production of antimicrobial peptides (AMPs) which get secreted into the hemolymph (Chandler, 2017). Several different classes of AMPs exist, all of which have antimicrobial properties (Lemaitre and Hoffmann, 2007). The exact mechanism by which fungi overcome the

systemic immune response is unclear, although it is probable that detection avoidance or Toll pathway signalling interference play important roles (Chandler, 2017)

Once defences have been overcome, insect death can be caused by a variety of means; for example, *M. brunneum* has been shown to secrete destruxins, which affect various organelles and paralyze host cells (Samuels et al., 1988). After 56 hours, hyphae in the haemocoel of the insect cause digestive tract tissues to disintegrate, which is quickly followed by insect death (Xia et al., 2013). In general, the infection process takes between three and seven days to cause mortality. Around 48hr after mortality, cadavers sporulate and conidia are spread to further hosts (Fig. 1-3).

Figure 1-3: *M. brunneum* and *B. bassiana* infected DBM cadavers, 48hr after insect mortality.

1.3.6 Barriers to uptake of EPF in the field

1.3.6.1 A lack of available EPF biopesticide products

Despite the potential that EPF biopesticides hold, there has been a general lack of uptake by growers, which is partly due to a lack of available products on the market designed for the control of DBM. According to a review by Grzywacz et al. (2010) there is at least one product based on an EPF which is currently being used in the field to control DBM, a *B. bassiana* based formulation currently deployed in India; no other

products are mentioned during the review. In a similar vein, Furlong et al. (2013) states that although the biology of many DBM virulent EPF have been well studied, the use of such biocontrol products remains experimental with very few EPF being successfully used in the field.

The lack of EPF biopesticide products which are designed to control DBM is a significant barrier to the uptake of EPF in the field. To increase the number of EPF biopesticide products it is necessary to know which EPF isolates are virulent against DBM. There have been numerous small-scale screens of EPF against DBM; for example, Yoon et al. (1999) applied one isolate of *B. bassiana* to DBM at a concentration of $1x10^8$ conidia ml^{-1} and reported it caused 83.2% mortality, and an LT50 of 3.6 days, in groups of larvae. However, there has been a lack of large-scale screens. Two previous screens have involved more that 20 EPF isolates. Wraight et al. (2010) screened a cohort of 43 *B. bassiana* isolates against groups of DBM larvae, several virulent and non-virulent isolates were found including three novel isolates that had not been previous screened against DBM which caused between 95 and 100% mortality. Additionally, Chui-Chai et al. (2012) applied a group of 25 EPF isolates (made up of *B. bassiana* and *M. anisopliae*) at a concentration of $1x10^8$ conidia ml^{-1} to groups of DBM larvae, five highly virulent isolates were found which displayed LT50s of 25 to 35hr. More screens of Hypocrelean EPF against DBM larvae should be completed to increase understanding of the interaction between DBM and EPF, and identify further DBM virulent EPF isolates.

1.3.6.2 Temperature as a cause of variability of EPF biopesticide performance

Growers lack confidence in EPF biopesticides because they have a level of unpredictability not seen with synthetic insecticides in terms of insect control (Lacey et al., 2001). Isolates which display high virulence in the laboratory can be virtually ineffective in the field (Thomas and Blanford, 2003). Temperature is the most important factor that affects insect development and fungal physiology and is likely to contribute to this unpredictability (Golizadeh et al., 2007, Davidson et al., 2003, Hallman and Denlinger, 1998).

The effect of temperature on DBM development and the effect of temperature on fungal physiology are known as genotype x environment interactions (GxE). These interactions have been investigated in detail and invariably have non-linear responses which are skewed to the left. For example, Golizadeh et al. (2007) determined the development rate of DBM (from egg lay to adult) at seven temperatures, and found there to be a linear increase in development rate between 10 and 30^{o}C, before a sharp drop was observed between 30 and 35^{o}C. The linear portion of the response can be described using a linear regression model; this approach is often used to develop day-degree (DD) models which are used to accurately predict the rate of insect development in the field (Baker et al., 1982, Mohandass, 2001).

However, describing the entire thermal profiles of DBM development and EPF physiology can be problematic due to left-skewed pattern of response. Models based on normal distribution (for example, polynomial models) tend to underestimate thermal optima and are not suitable in this case. To solve this problem, several models were proposed which were designed to fit to left-skewed distributions. For example, models proposed by Logan et al. (1976), Lactin et al. (1995) and Briere et al. (1999) are suitable for describing gentle increases in response up the optimum temperture, and rapid decline between the thermal optima and the maximum temperature. Non-linear models can be used to determine cardinal temperatures with high biological significance, for example: *T0* (the lowest temperature where physiological response occurs) and *Topt* (the optimum temperature of the response). The non-linear models described here generally fit well to DBM development and fungal pysiology data over temperature ranges. For example, the Briere-1 model produced r^2 values of 0.81-0.89 and 0.98 when describing thermal profiles of EPF colony extension and DBM development, respectively.

The more complex process of EPF infection of insect hosts, over a temperature range, can be described as genotype x genotype x environment interaction (GxGxE). EPF and insect genomes interact as EPF conidia attempt to overcome the host immune system to cause a systemic infection (Chandler, 2017). Temperature strongly influences both the host immune response, and fungal virulence (Mishra et al., 2015). The complexity of

this interaction is often overlooked in large-scale screens of EPF biopesticides against DBM, which are typically completed at a fixed temperature. For example, Wraight et al. (2010) completed a screen of 43 *B. bassiana* isolates against DBM larvae at a fixed temperature of 20^{o}C. *B. bassiana* and *M. anisopliae* EPF virulence against insects have shown to be highly influenced by temperature (Vandenberg et al., 1998a, Kuboka, 2013). At any one temperature, the host immune response and fungal physiological processes may respond differently, which could have an influence on the establishment of infection. For example, *Metarhizium* EPF are known to be active at temperatures over 30^{o}C whereas the optima for DBM development is approximately 28^{o}C; this may allow for *Metarhizium* EPF to be more virulent at higher temperatures (Davidson et al., 2003, Golizadeh et al., 2007, Marchioro and Foerster, 2011). For EPF virulence to remain constant across a range of temperatures, insect and EPF would have to be insensitive to temperature or have the same response to temperature over a given range (Thomas and Blanford, 2003). However, when looking at studies into the effect of temperature on EPF virulence, there is typically a positive correlation between temperature and EPF virulence or – if a large enough temperature range is used – a bell-shaped non-linear response (Thomas and Blanford, 2003, Mishra et al., 2015, Vandenberg et al., 1998b). For example, a non-linear response was seen for virulence of *M. anisopliae* against western flower thrips over several temperatures (although this relationship was not explained using non-linear models) (Kuboka, 2013). Unlike GxE interactions, there has been a lack of use of mathematical models (such as those proposed by Logan et al. (1976), Lactin et al. (1995) and Briere et al. (1999)) to describe GxGxE interactions. Consqequently, the effect of temperature on the virulence of EPF is not well understood.

Greater understanding of how temperature effects EPF virulence has many applications within biocontrol. For example, it is often assumed that because a certain EPF germinates or grows well at high temperatures the same isolate will cause insect mortality at high temperatures. For example, Davidson et al. (2003) determined the colony extension thermal profiles of a range of EPF isolates to find suitable high temperature isolates for use in an IPM system to control *Varoea Destructor*, which live at high temperatures. However, it is not known whether using EPF physiology thermal profiles to predict EPF virulence thermal profiles is a reliable procedure. Using the same

non-linear model to describe EPF virulence and physiology profiles would allow cardinal temperatures to be appropriately compared between the two processes, to elucidate whether EPF virulence thermal optima could be predicted from EPF physiology thermal optima.

Additionally, a non-linear model could be used to "match" EPF virulence thermal profiles with the DBM development thermal profile. Because of widespread use of insecticides in South East Asia, and other areas, the population density of pests of DBM, such as parasitoids is constantly low. Without parasitoids acting as a check on DBM populations, DBM population density is likely to be proportional to temperature. DBM is likely to cause the most crop damage at temperatures close to its optima for development because this would allow more generations of DBM to cycle over a period of time (Sarfraz et al., 2005). This would result in more DBM individuals and presumably more crop damage. Therefore, an EPF isolate which shares the same thermal optima as DBM would be most useful in terms of DBM control.

Laboratory bioassays of EPF biopesticides against insect pests are normally completed at constant temperatures (Wraight et al., 2010, Ullah and Lim, 2015, Cui et al., 2014), whereas in the field, temperatures fluctuate in a sinusoidal pattern over the course of a 24hr period (Bannerman and Roitberg, 2014). Consequently, before EPF candidates are taken from the laboratory to the field, it is important to know whether virulence at fluctuating temperatures can be predicted from experiments completed at constant temperatures, however, this is currently unknown. Experiments completed to compare insect development and fungal growth at constant and fluctuating temperatures have produced conflicting results. Bale (1999) reported that development rates of nettle-feeding larvae differed between constant and sinusoidal temperature regimes which shared the same mean temperature, whereas Burgess and Griffin (1967) found that fungal growth rate at fluctuating temperatures could be predicted from fungal growth rates at constant temperatures. Given the complexity of the GxGxE interaction it is possible that predictions of EPF virulence at "field typical" fluctuating temperatures made from data collected at constant temperatures may not hold true, however, this is currently unknown (Thomas and Blanford, 2003). If both host and EPF share the same

thermal optima the effects of temperature can be more simple (Thomas and Blanford, 2003).

1.3.6.3 A lack of understanding of interactions between EPF and other elements of the IPM system

EPF biopesticides take far longer than some synthetic insecticides to cause insect mortality. For example, it is known that pyrethriod based insecticides can cause insect death almost instantaneously, whereas EPF biopesticides take 56hr to cause a systemic infection and usually longer to cause death (Xia et al., 2013). For this reason, EPF biopesticides are likely to be used in combination with other control products in an IPM system (Nian et al., 2015). It is essential to study the interaction between each element of the IPM system to understand how the system will perform in the field. However, a major factor that has reduced the uptake of IPM in the field is the lack of research into these interactions. There has been too much focus on studying elements of the IPM system in isolation when a holistic approach is needed (Lim, 1992). However, there have been a limited number of studies into the compatibility of IPM system elements that have shown promise. Nian et al. (2010) applied a concentration range of *B. bassiana* with and without fixed concentration of a Bt based insecticide to DBM in a laboratory study; a largely synergistic response was found. Studies into these interactions must be done to ensure one element of the IPM system will not inhibit another. There is also a lack of understanding of the mechanisms which cause interactions between biocontrol products. Such an understanding would be beneficial as it may help predict the compatibility of biological control agents when designing IPM systems.

1.3.6.4 DBM larvae of specific ages being less susceptible to EPF infection

The stage of insect growth may also be responsible for observed discrepancies between laboratory and field virulence of EPF isolates. In most cases, initial screens of EPF isolates are completed against a population of DBM larvae at particular developmental stage of a known age. For example, the screen of 43 *B. bassiana* isolates completed by Wraight et al. (2010) involved only early second instar DBM larvae. Time between treatment and

moult has been identified in insects as a means to avoid infection (Vey and Fargues, 1977). Vandenberg et al. (1998) found that if DBM larvae were treated later in the instar, time to death was far longer than if larvae were treated in early instar. The same authors used microscopy to identify conidia on the newly moulted insect cuticle, confirming that if conidia are present on the cuticle at the time of moult, they are likely to be shed. Conidia require approximately 60hr to break through the cuticle and cause a systemic infection (Xia et al., 2013); if the time between treatment and moult is less than this, conidia are likely to be moulted. This means that virulent EPF may be shed from larvae in the field and appear to be non-virulent if applied at the wrong time. However, other routes to infection such as secondary pick-up may cause infection even after shed of conidia by late instar larvae. Secondary pick-up of *M. anisopliae* EPF conidia has been shown to be more important than direct spray when causing infection against the desert locust in an biocontrol system (Langewald et al., 1997). However, there has been no research into how important secondary pick-up is in causing infection to DBM in the field.

1.3.6.5 Summary of barriers to uptake of EPF by growers

The major barrier preventing the uptake of EPF biopesticides by growers is difficulty in predicting their effectiveness in the field (Lacey et al., 2001). From the point of view of the grower, employing an EPF biopesticide system represents a financial risk when compared to other tried and tested techniques, such as synthetic insecticides.

1.4 Aims and objectives

The aim of this project was to increase understanding of why EPF display a variable performance when controlling DBM populations by studying interactions between different elements of the IPM system. Once identified, the sources of variation were addressed to improve consistency of EPF performance. The composite objectives of this PhD project can be split into four broad themes which were addressed in separate chapters:

Chapter III: Assessing the virulence of a cohort of EPF isolates against DBM to increase the understanding of the pest-pathogen interaction (Fig. 1-4)

Chapter IV: Increasing the understanding of the effect of temperature of EPF physiology, DBM development and EPF virulence (environment-pest, environment-pathogen and environment-pest-pathogen interactions (Fig. 1-4)) using non-linear mathematical models, with the ultimate aim of developing a simple predictive model of EPF virulence

Chapter V: Quantifying the effect of coapplication of EPF with commercially available insecticides against groups of DBM larvae, and determining the mechanisms causing any interactions that were found

Chapter VI: Investigating moulting as a means by which DBM larvae avoid EPF infection after a direct application, and then assessing the effectiveness of secondary pick-up of EPF conidia as a route to infection

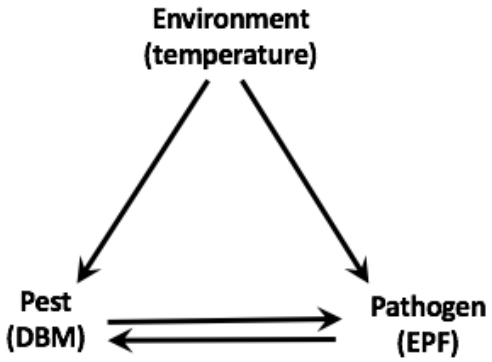


Figure 1-4: Environment-pest-pathogen interaction triangle. Themes (i) and (ii) involved characterizing interactions between three important elements of the IPM system.

2 Materials and methods

2.1 Rearing of DBM population

DBM were reared on cauliflower and Brussels sprouts plants, both part of the *Brassica oleracea* species. 'Skywalker' and 'Doric' varieties were used, respectively (Elsoms Seeds Ltd, Lincoln, UK). Seeds were sewn on vermiculite contained within a clear plastic container (18x12x4 cm) and kept at 20^{o}C (18:6 LD) in a controlled environment room. After one week, individual seedlings were transferred onto compost (M2) in 7 cm deep pots which were kept in the same controlled environment room.

A previously existing stock of DBM (collected on the 18/12/1995, Wellesbourne, Warwickshire) was used to commence the culture to be used for this PhD project. The culture of DBM was divided into two parts:

i. A working culture to maintain the population of insects
ii. A fixed age culture to produce insects for experiments

For the working culture, DBM adults were kept in a breeding cage (30x30x30 cm) with fine mesh sides (Watkins and Doncaster Ltd, Herefordshire, UK) at 20^{o}C (18:6 LD), known as the 'adult cage', in a controlled environment room. Two to three Brussels sprout plants were placed in the adult cage for two to three days so oviposition could take place. The Brussels sprout plants were then placed in larger mesh cage (47.5x47.5x47.5cm) known as the 'larvae cage'. Mature cauliflower (five to ten weeks old) plants were added to the cage twice a week to feed developing larvae. Emerged adults were transferred to the adult cage using a mechanical pooter (Watkins and Doncaster Ltd, Herefordshire, UK). The adult cage was replaced with a clean one once a week.

All bioassays were completed with fixed age populations of DBM larvae which were set up as follows: one Brussels sprouts plant of three to four weeks old (when the third leaf had just emerged) was placed in the adult cage for 24hr so that adults could oviposit on the leaves. The following day, this plant was refrigerated for 24hr whilst another

Brussels was placed in the adult cage. The Brussels plants were then removed from the adult cage and the refrigerator and placed in a larvae cage. Mature cauliflower (five to ten weeks old) plants were added to the cage twice a week to feed developing larvae. When the desired stage of development was reached, larvae were removed from the culture using a fine paintbrush for use in experiments. At this point, the remaining larvae were transferred to the working culture. The adult cage was replaced with a clean one every two to three days.

2.2 Maintenance of EPF cultures

Table 2-1: Description of candidate isolates.

Species	Isolate [†]	Host or source	Origin
B. bassiana	11.98[a]	Click beetle (Coleoptera: Elateridae)	USA
B. bassiana	432.99[b]	*A. grandis* (Coleoptera : Curculionidae)	USA
B. bassiana	433.99[c]	*Bemisia* sp. (Hemiptera : Aleyrodidae)	USA
B. bassiana	1730.08[d]	-	UK
B. bassiana	1757.15 (1850)[e]	*Choristoneura sp.* (Lepidoptera : Tortricidae)	Canada
B. bassiana	1758.15 (3404)[e]	*L. dispar* (Lepidoptera: Lymantriidae)	USA
B. bassiana	1759.15 (3530)[e]	*L. dispar* (Lepidoptera: Lymantriidae)	USA
I. fumosorosea	1761.15 (6799)[e]	*P. xylostella* (Lepidoptera: Plutellidae)	Australia
I. fumosorosea	1762.15 (6800)[f]	*P. xylostella* (Lepidoptera: Plutellidae)	Australia
L. longisporum	1.72[g]	*M. sanborni* (Homoptera: Aphididae)	UK
L. muscarium	19.79[h]	*T. vaporariorum* (Hemiptera : Aleyrodidae)	UK
M. brunneum	275.86[i]	*C. pomonella* (Lepidoptera : Tortricidae)	Germany
M. brunneum	445.99[e]	-	-
M. brunneu	1760.15 (4522)[e]	*P. xylostella* (Lepidoptera: Plutellidae)	USA

[†]Isolate number in the Warwick Crop Centre culture collection (isolate number from culture collection of origin).

(a) Kindly supplied by B. Ownley, University of Tennessee, 2505 E.J. Chapman Drive, 370 Plant Biotechnology Building, Knoxville, TN 37996-4560, USA.

(b) Isolate forms the active ingredient in the proprietary mycopesticide 'Naturalis' (Troy Biosciences Inc., 113 South 47th Avenue, Phoenix, AZ 850433, USA).

(c) Isolate forms the active ingredient in the proprietary mycopesticide 'BotaniGard' (Mycotech Corporation, PO Box 4109, Butte, MT 59702, USA).

(d) Isolate taken from the Warwick Crop Centre culture collection, after being identified during an MSc research project.

(e) Isolate was taken from the ARSEF collection and kindly supplied by Dr Richard A. Humber of The USDA-ARS Biological Integrated Pest Management Research Unit, Robert W. Holley Center for Agriculture and Health, 538 Tower Road, Ithica, USA

(f) Isolate forms the active ingredient in the proprietary mycopesticide Vertalec (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands).

(g) Isolate forms the active ingredient in the proprietary mycopesticide 'Mycotal' (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, Netherlands).

(h) Isolate forms the active ingredient in the proprietary mycopesticide 'Met52' (Novozymes, Krogshoejvej 36, 2880 Bagsvaerd, Denmark).

(i) Isolate forms the active ingredient in the proprietary mycopesticide Bio-Blast (Eco-Science Corporation, 17 Christopher Way, Eatontown, NJ 07724, USA).

14 EPF isolates, taken from four species, were chosen for experimentation (Table 2-1). Eight of the 14 isolates were already in the Warwick Crop Centre (WCC) culture collection. Six isolates were obtained from Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF), these were: *I. fumosorosea* 1761.15 (ARSEF 6799) and *I. fumosorosea* 1762.15 (ARSEF 6800), *M. brunneum* 1760.15 (ARSEF 4522) and *B. bassiana* 1757.15 (ARSEF 1850), 1758.15 (ARSEF 3404) and 1759.15 (ARSEF 3530). Isolates were grown on sabouraud dextrose agar (SDA) (Sigma) for 10-14 days at 23±2°C in darkness. Conidia on SDA plates were then harvested by gentle agitation of 2ml of 0.05% Triton X-100 using a hockey-stick spreader (Fisher Scientific). Suspensions were then filtered using sterile milk filter papers. The filtered suspension was then pipetted onto porous cryotolerant plastic beads with glycerol, as described by Chandler (1994), which were then kept at approximately -80°C.

At the beginning of the PhD, and every six months after this, two beads per isolate were removed from deep freeze and spread on SDA contained in a 30ml Universal tube, known as a slope, using a sterile plastic loop (Fisher Scientific). Slope cultures were grown for 10 to 14 days at 20°C in darkness and then kept at 5°C. Fungal cultures for experiments were grown from slope cultures when required. A sterile loop spreader was used to transfer fungal material from the slope culture to SDA contained within a 9cm Petri dish (Merck). The sterile slope was streaked evenly across the SDA plate. Cultures were grown in the dark at 20°C for 10-14 days (in a Sanyo Gallenkamp cooled incubator) before conidia were harvested for experiments.

2.3 Preparation of EPF conidial suspensions

Conidia from the desired isolate(s) were harvested from 10-14 day old SDA plates. 10ml of 0.05% Triton X-100 was pipetted onto the SDA plate, and a sterile hockey-stick spreader was used to agitate conidia into suspension. Suspensions were filtered using sterile milk filter papers (19cm diameter) (Goat Nutrition Ltd, Kent, UK), then diluted 10-fold in 0.05% Triton X-100. The concentration was then enumerated using an improved Neubauer haemacytometer. Suspensions were then adjusted to the desired concentration.

2.4 Bioassay procedure to determine virulence of EPF isolates against second instar DBM larvae

10ml conidial suspensions (of the desired EPF and concentration) were prepared no more than 24hr before the completion of the bioassay using the procedure described in 2.3 and kept in a 30ml Universal tube at 4^{o}C.

The bioassay protocol used was the same as used in Wraight et al. (2010), the only difference being leaf discs were used to sustain DBM larvae, rather than artificial diet. Early second instar larvae were selected from the fixed age culture (distinguished from other second instars by their smaller size and darker skin) and, using a size one fine paintbrush (Windsor and Newton, UK), transferred to a 9 cm petri dish lid lined with filter paper (10-15 larvae per dish). Petri dishes containing larvae were then refrigerated at 5^{o}C for no longer than 1-2 hours before spraying.

The desired EPF conidial suspension (or a Triton X-100 control) at the desired concentration was then applied to groups of DBM (contained within the petri dish), using a Potter tower air atomising sprayer (Potter, 1952), as follows: before each spray, the petri dish lid containing DBM larvae was sprayed lightly with RO water using a handheld sprayer (VWR International Ltd, UK) until the filter paper was damp. An 18x18 mm coverslip was then placed on the damp filter paper, before being placed in the spray table of the Potter tower and secured below the spraying nozzle using a retractable metal arm. A vortex was then used to shake the 30 ml Universal bottle containing the EPF conidial suspension; 4ml of the conidial suspension was transferred to the spray tube using a pipette and the spray was initiated. Between each experimental spray, RO water, 70% alcohol and Triton X-100 were each run through the spraying equipment separately, to ensure no cross-contamination occurred.

The petri dish lid containing the larvae was then removed from the spray table. A leaf disc (Skywalker, 3 cm diameter) was added to the petri dish, sealed appropriately using Parafilm and incubated overnight at the desired temperature (16:8 LD) in a in a Sanyo MLR-351 versatile test chamber. Overnight, the larvae crawled on to the 3cm leaf disc.

24hr after the spray, a fine paintbrush was used to transfer larvae to a separate leaf disc (6 cm diameter) contained in a separate 9 cm petri dish filled with water agar (7 g agar in 500 ml of RO water) to prevent leaf drying (Fig. 2-1) (appendix 1). The petri dish lid was modified with two 3 cm circular air holes covered with perforated plastic to ensure aeration. Leaf discs were changed every three days for the duration of the experiment.

Larvae mortality was assessed once every 24 hours, at the same time each day after treatment, for seven days. Dead larvae were removed and incubated on damp filter paper within Petri dishes (20 ± 1°C, darkness) for seven days, and inspected for the presence of mycelium on cadavers.

Spore viability was assessed simultaneously to the screen being completed; 20 µl of spore suspension was pipetted onto SDA and incubated for 18 hr at 20 ± 1°C, (darkness). Germination was stopped at this time point using lactophenol cotton blue stain. Numbers of germinated and ungerminated conidia are counted from a group of no less that 100 conidia selected at random and observed under a microscope (Ziess Axioskop Routine Microscope). A conidium was considered germinated if the mycelium exceeded twice the length of the spore.

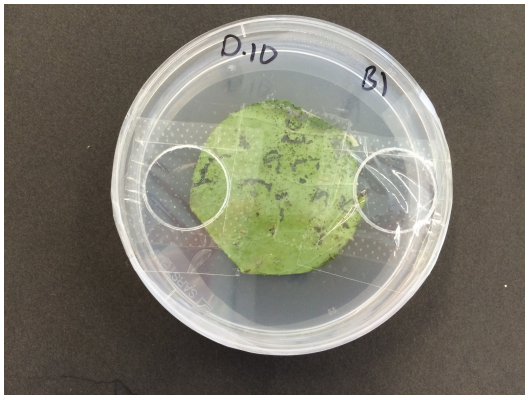


Figure 2-1: Bioassay chamber used in experiments to assess the virulence of EPF isolates against groups of DBM larvae.

2.5 Determining concentration of EPF conidia received after application

2.5.1 Determining the dose of EPF conidia received by individual DBM larvae after treatment

During certain experiments it was necessary to determine the number of conidia present on the insect cuticle after treatment. At the desired time point, an individual larva was removed from the experiment with a fine paintbrush and placed in a 1.5ml Eppendorf containing 1ml 0.05% Triton X-100. The Eppendorf was then shaken vigorously using a vortex shaker for two minutes to dislodge conidia from the cuticle. This solution was then diluted appropriately before 100ul was spread evenly on genera specific media using a hockey-stick spreader (Fisher Scientific). For *M. brunneum* isolates media containing cyloheximide was used (Chase et al., 1986). For *B. bassiana* isolates SDA media containing the fungicide dodine (N-dodecylguanidine) at a concentration of 0.06% was used (adapted from (Inglis et al., 2012)). Reagents for *B. bassiana* and *M. brunneum* specific media can be seen in Appendix I. Plates were left in darkness at 20°C for six days, before colony forming units were counted. The number of spores present on the larval cuticle was then retrospectively calculated.

2.5.2 Determining the concentration of EPF conidia received on the leaf surface after treatment

The concentration of EPF conidia present on leaf material was determined as follows: at the desired time point, a 0.7 cm diameter leaf disc was cut using a cork borer and placed in 1.5ml Eppendorf containing 1ml 0.05% Triton X-100. The Eppendorf was then shaken vigorously using a vortex shaker for two minutes to dislodge conidia from the cuticle. This solution was then diluted appropriately before 100ul was spread on evenly on genera specific media using a hockey-stick spreader (Fisher Scientific). For *M. brunneum* isolates media containing cyloheximide was used (Chase et al., 1986). For *B. bassiana* isolates SDA media containing the fungicide dodine (N-dodecylguanidine) at a concentration of 0.06% was used (adapted from (Inglis et al., 2012)). Reagents for *B. bassiana* and *M. brunneum* specific media can be seen in Appendix I. Plates were left in

darkness at 20°C for six days, before colony forming units were counted. The number of spores present on the leaf surface was then retrospectively calculated.

2.5.3 Determining the concentration of EPF conidia received per unit area completing the larval spray technique

The concentration of EPF conidia present on a cover slip (18x18mm) placed along groups of larvae (when being treated using the larval spray method) was determined as follows: directly after spray, the cover slip was placed in a 30ml Universal tube containing 1ml 0.05% Triton X-100 using sterile forceps. The universal was then shaken vigorously using a vortex shaker for two minutes to dislodge conidia from the coverslip. This solution was then diluted appropriately before 100ul was spread evenly on genera specific media using a hockey-stick spreader (Fisher Scietific). For *M. brunneum* isolates media containing cyloheximide was used (Chase et al., 1986). For *B. bassiana* isolates SDA media containing the fungicide dodine (N-dodecylguanidine) at a concentration of 0.06% was used (adapted from (Inglis et al., 2012)). Reagents for *B. bassiana* and *M. brunneum* specific media can be seen in Appendix I. Plates were left in darkness at 20°C for six days, before colony forming units were counted. The number of spores present on the coverslip was then retrospectively calculated.

2.6 Monitoring the physiology of EPF isolates

2.6.1 Monitoring colony extension of EPF isolates

Conidia suspensions were adjusted to a concentration of 1 x 10^7 conidia ml^{-1} using the procedure described in 2.3. 100ul of the adjusted suspension was spread over an SDA plate using a sterile hockey spreader and left for 48 hours (25°C, darkness). A metal cork borer was used to take 7 mm plugs which were then inverted in the center of a 90mm petri dish containing SDA marked with an x/y axis on the base to measure growth against. Assays were read by using a ruler to measure the size of the mycelial mass at

the center of the plate using the x/y axis (Fig. 2-2). Readings were taken every seven days for a total of 21 days.

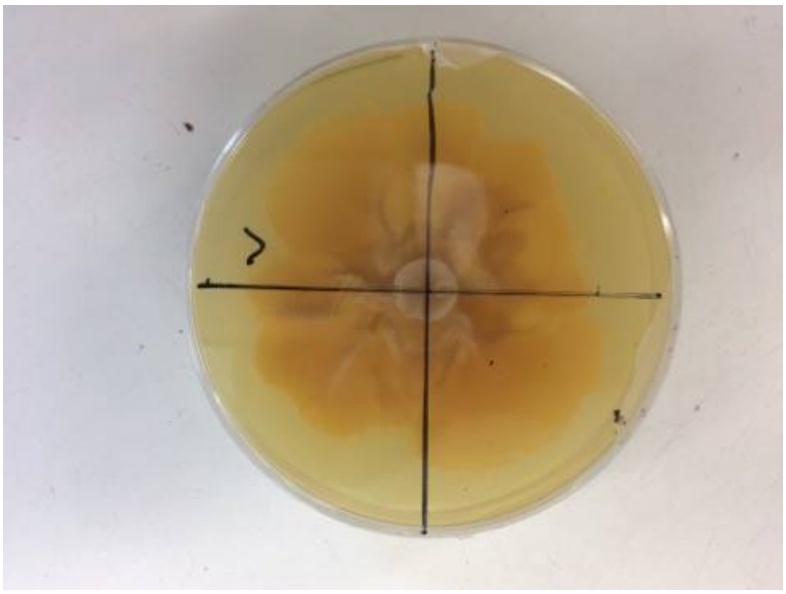


Figure 2-2: The mycelial mass of a *B.bassiana* isolate growing on SDA. The x/y axis is used to monitor the rate of extension.

2.6.2 Recording proportion germination of populations of EPF conidia

Conidia suspensions were adjusted to a concentration of 1 x 10^7 conidia ml^{-1} using the procedure described in 2.3. 20 µl of the adjusted suspension was pipetted onto SDA contained with a 4.5cm diameter petri dish and kept at the desired temperature (in darkness) for the desired length of time. Germination was stopped at the desired time point using lactophenol cotton blue stain. Numbers of germinated and ungerminated conidia were counted from a group of no less that 100 conidia selected at random and observed under a microscope (Ziess Axioskop Routine Microscope). A conidium was considered germinated if the mycelium exceeded twice the length of the spore.

2.7 Statistical analysis of concentration-mortality response experiments

For a number of experiments in this research project concentration mortality response analysis was conducted in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package DRC (version 3.0-1). The protocol for using the software was taken from Cockerton (2013). The purpose of completing concentration response analysis was to derive an estimation of the concentration at which a certain proportion of individuals died. Data of mean uncorrected proportion mortality from six days after treatment for each concentration (plus an untreated control) was analysed. Initially, five common models based on a binomial distribution were tested for suitability; these were two, three and four parameter log-logistic models and Weibull-1 and a Weibull-2 models. Log-logistic models are by far the most commonly used. The generalized log-logisitc model has the following equation:

$$f\big(x,(b,c,d,e)\big) = c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))} \quad (1)$$

With *b* denoting the steepness of the dose-response curve, *c* and *d* representing the upper and lower limits of the response, and *e* representing the LC50 (the concentration at which 50% mortality of groups of larvae occur) (Cockerton, 2013) (Fig. 2-3). In the DRC package, certain parameters can be pre-set at fixed values when running the log-logistic model; this determines the number of parameters within the log-logistic model. In a four-parameter log-logistic model all four parameters are estimated from the experimental data. In a three-parameter log logistic model, *c* is set to 0. In a two-parameter log-logistic model *c* and *d* are set to zero and one respectively. Despite the differing the number of parameters, two-, three- and four-parameter log-logistic models share the same equation.

The Weibull-1 model has the equation:

$$f\big(x,(b,c,d,e)\big) = \ c + (d-c)\exp\left(-\exp\big(b(\log(x)-\log(e))\big)\right) \tag{2}$$

The Weilbull-2 model has the equation:

$$f\big(x,(b,c,d,e)\big) = c + (d-c)\left(1-\exp\left(-\exp\big(b(\log(x)-\log(e))\big)\right)\right) \tag{3}$$

In the Weibull-1 and -2 models, *b* represents the steepness of the curve, *c* and *d* represents the lower and upper limits of the model, and *e* represents the LC50 (Fig. 2-3). Weibull-1 and -2 have an advantage over the log-logistic model in that they can account for asymmetry in the concentration response (Ritz et al., 2016).

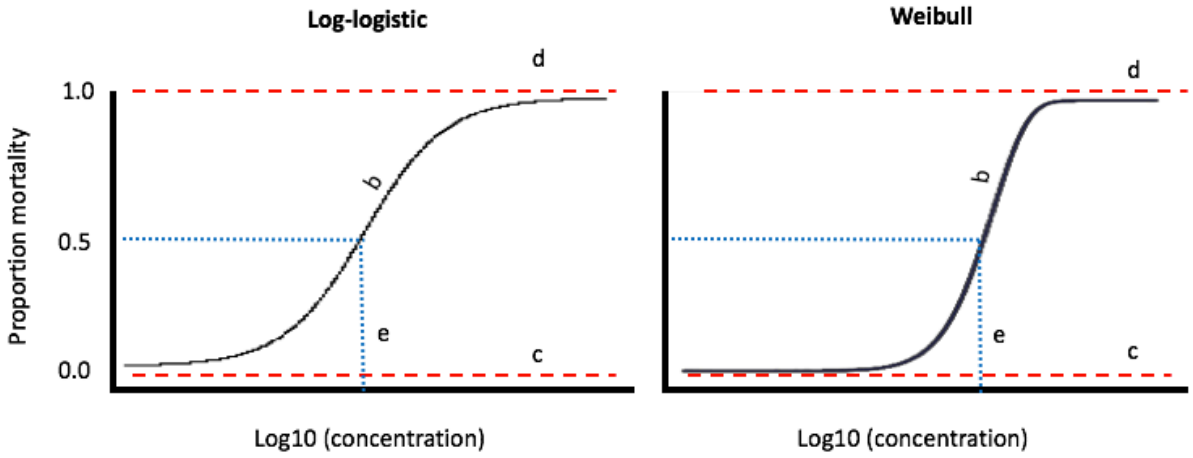


Figure 2-3: An example of a four parameter log-logistic model and a Weibull-2 model. In both models, *b* represents the slope, *c* represents the lower limit, *d* represents the upper limit and *e* represents the LC50.

A lack-of-fit test was used to assess the suitability of these models: it determines whether the residual sum of squares associated with an ANOVA is significantly less than that of the corresponding concentration response model (Cockerton, 2013). A P-value

above 0.05 indicates that the model should be used. If more than one model produced a P-value of >0.05, the model with the highest P-value was used.

Initially, in analyses where a number of treatments were being compared (for example; a concentration response experiment using five isolates), individual parameter estimates were estimated for each treatment. However, the number of parameters was minimized where possible. For example, the slope parameter was constrained to the same value for each isolate and compared using an ANOVA to the model in which slope values were individually estimated for each isolate. If the constrained model was no worse than the unconstrained model the constrained model was used. This same process was completed for the LC50 parameter.

If only one treatment was used in the experiment (for example; a concentration response experiment using one insecticide) the process of reducing the number of model parameters was not required (Cockerton, 2013).

Once an appropriate model had been determined it could be used to derive an estimation of the concentration at which a certain proportion of individuals died (for example, the LC50). The LC50 could then be compared between treatments in the same experiment using a T-test.

2.8 Correcting for control mortality

When completing bioassays against insects it is common for a certain proportion of individuals to die from causes not related to application of the particular substance being studied. To determine the proportion mortality caused by the treatment, the natural mortality must be accounted for. Equations such as Schnieder-Orelli's and Abbot's formulae do this by subtracting the proportion mortality due to natural causes from the overall mortality and then calculating the proportion mortality caused by the treatment in the remainder of the population (Abbot, 1925).

It was decided that Schneider-Orelli's formulae should be used to correct for control mortality, rather than Abbot's formulae (Puntener, 1981, Abbot, 1925). In the experiments analysed here, there were slight differences between the number of individuals in the control and treatment groups, although numbers were always within the range of 10 to 15 individuals. Schneider-Orelli's formulae was preferred to Abbot's formulae because percentage mortality data, rather than numbers of individuals, were required to estimate the corrected mortality. Consequently, corrected mortality could be calculated when there were differences in numbers of individuals in the control and treatment groups. Where the number of individuals were the same in treated and control groups, corrected mortality estimated by Schneider-Orelli's formulae and Abbot's formulae were compared and found to be identical.

When correcting for control mortality, percentage mortality was used from day six of the experiment. Schneider-Orelli's formulae has the equation:

$$Corrected\ mortality\ (\%) = \left(\frac{a-b}{100-b}\right) * 100 \quad (4)$$

Where *a* is the percentage mortality data from the treated group and *b* is the percentage mortality from control group.

3 Developing a bioassay procedure to characterize the virulence of EPF against DBM larvae

3.1.1 Introduction

There are over 700 species of fungi, from 53 genres, that have been shown to infect arthropods (Chi, 2007). When considering the number of potential candidates which may be virulent against DBM, the use of EPF against the pest has not been widespread (Furlong et al., 2013). Reviews by Grzywacz et al. (2010) and Furlong et al. (2013) state that although Hypocrealean EPF isolates have been well studied, they are seldom used to control DBM in the field. There are many reasons for the lack of uptake of EPF biopesticides in by growers, often the mass production fungal spores can be problematic. For example, *Zoopthora radicaans* is known to cause DBM populations to crash in the wild (Furlong and Pell, 1997), additionally laboratory studies have shown that *Z. radicaans* is virulent against DBM (Batta et al., 2011). However, there have been problems with the large-scale production and application of *Z. radicaans* which has prevented the EPF from being used commercially in the field (Furlong et al., 2013). However, Hypocrealean EPF isolates are easier to mass produce, when compared to EPF from the Entomophthoromycota.

Because of the lack of available EPF biopesticide products on the market for DBM control, there is much to be gained from the continuation of research into potential insect-virulent candidates. Not least because the pool of active synthetics toxic to DBM is dwindling due to increases in resistance of DBM populations (Talekar and Shelton, 1993; Furlong et al., 2013) and active chemicals being withdrawn from the market. In terms of speed of kill, EPF are generally not as effective as synthetics, with *B. bassiana* and pyrethroids taking three to five days and under 12hr to cause mortality, respectively (Khaliq et al.). However, there are certain advantages to the use of EPF; after infection insect cadavers sporulate and produce conidia, consequently EPF have an ability to persist in the environment over time (Furlong and Groden, 2001, Furlong and Pell, 2001). Although the likelihood is that, if EPF are regularly used in the field, they would be used in an integrated approach alongside synthetic insecticides.

For the potential of EPF as biocontrol agents to be realized, it is vital for EPF isolates to be screened against DBM to identify virulent candidates. There have been many examples of small scale screens in the literature in which one or two isolates have been applied to DBM. For example, two isolates from Benin were screened against DBM larvae and found to cause over 80% mortality at the highest concentration applied (Godonou et al., 2009). However, there has been a lack of large-scale screens in the literature. To the best of this author's knowledge there have only been two laboratory based screens of over 20 isolates against DBM; Wraight et al. (2010) screened a cohort of 43 *B. bassiana* isolates, finding several virulent candidates and Chui-Chai et al. (2012) screened a selection of Hypocrealean EPF isolates, comprising of many species, against DBM larvae.

Across the literature, the methods of applying EPF to DBM vary considerably. When only taking into account laboratory bioassays of DBM, EPF application methods can be placed into four broad categories (Table 3-1):

i. Treatment by spraying conidia formulation directly onto larvae (from here referred as to as the "larval spray" technique).
ii. Treatment of leaf discs, either by immersion or direct application, with conidia formulation before allowing larvae to feed on treated leaves (referred to as the "leaf application" technique).
iii. Treatment by immersing larvae in conidia formulation (referred to as the "larval immersion" technique).
iv. Showering larvae with actively discharged conidia (which is only relevant to Entomophorales isolates).

After completing a literature search, 14 separate studies were found to involve the treatment of DBM larvae with Hypocrealean EPF in the laboratory. Of these studies, six sprayed larvae directly, four immersed larvae in conidia formulation, and four treated leaves before allowing DBM larvae to feed on them (Table 3-1).

The “larval spray” method was used by Vandenberg et al. (1998) and elsewhere in the literature. Concentration of conidia in suspension is normally standardized, before being applied to larvae (Xia et al., 2013). The way conidia are sprayed varies from study to study; for example, Wraight et al (2010) used an air-atomizing sprayer, whereas other papers do not specify how conidia are sprayed.

The ultimate aim of the first phase of research was to determine the virulence of a range of Hypocrealean EPF isolates against DBM larvae. The Hypocrealean chosen comprised of isolates that had been screened against DBM previously, commercial products which are used to control Lepidopteran pests, and novel isolates that had not been previous assessed for virulence against DBM. But before the screen could be completed an appropriate bioassay system were developed. Several methods of application were investigated, based on protocols stated in previous study. Following the screen of the isolates, a concentration response of selected isolates was completed. The composite aims were as follows:

i. Develop a bioassay for the assessment of virulence of Hypocrealean EPF isolates against DBM larvae.
ii. Complete sequencing to build a phylogenetic tree of all isolates
iii. Use the bioassay system from (i) to screen a range of EPF isolates against second instar DBM larvae.
iv. Selected a range of isolates with which to complete concentration-mortality response experiments against groups of DBM larvae.

Table 3-1. Summary of different methods for the application of fungal isolates to DBM larvae.

Application method				**Group of fungi**		**Number of isolates**		
Larval spray	**Leaf applic-ation**	**Larval immer-sion**	**Spore shower**	**Hypocreales**	**Entomoph-thoromycota**	**<10**	**10-25**	**25-50**
5	4	5	2	14	2	13	1	1
References	(Loc and Chi, 2007, Batta, 2013, Xie et al., 2014, Cui et al., 2014, Wraight et al., 2010, Chui-Chai et al., 2012, Vandenberg et al., 1998b, Selman et al., 1997, Huang et al., 2010, Godonou et al., 2009, Batta et al., 2011, Nian et al., 2015, Morales-Vidal et al., 2013, Altre and Vandenberg, 2001, Yoon et al., 1999, Duarte et al., 2016, Masuda, 2000)							

3.2 Materials and methods

3.2.1 Determining the phylogenetic relationships of candidate EPF strains using internal transcribed spacer (ITS) sequencing

All fourteen candidate isolates were included in this experiment.

To extract DNA, 2-3 “match head” volumes of fungal mycelium were transferred into a 2ml screw cap centrifuge tube containing 10 2.5-3.5mm glass beads and 0.5g of 0.1mm zirconia ceramic beads. Samples were ground in a FastPrep for a total of 60 seconds (three runs of 20 seconds each). 300µl of Acme DNA extraction reagent was added to the grinding tube, which was then manually shaken to disperse the ground material. Grinding tubes were placed in a heat block at 100°C for 10 minutes. Grinding tubes were then spun in a microcentrifuge (13,500 rpm) for a total of 10 minutes (two runs of 5 minutes each). 175µl of the cleared upper supernatant was transferred to a sterile 1.5ml Eppendorf tube. The lysate was diluted 10-fold in sterile water, the concentration of DNA present within samples was estimated using a NanoDrop® (Labtech). 1 µl of the lysate was used as a template in PCR.

The internal transcribed spacer (ITS) primer sequences, as well the reagents and procedures of the polymerase chain reaction (PCR) were taken from White et al. (1990).

For the PCR involving ITS 1 and ITS 4, each PCR reaction had a total volume of 20µl, made up of 10µl of RedTaq (Sigma) along with 1µl of the 10µM ITS-1 primer (5’-TTCGTAGGTGAACCTGCGG- 3’) and 1µl of the 10µM ITS-4 primer (5’-TCCTCCGCTTATTGATATGC-3’). Added to this was 7µl of sterile water and 1µl lysate containing the DNA to make up the reaction volume up to 20µl. The conditions for the reaction cycle were as follows (35 cycles per condition); 2 min at 94°C, 45s at 94°C, 30s at 55°C, 1 min at 72°C, 7 min at 72°C. Following this, 4µl of the amplification product was run on a 1.2% agarose gel (with 2µl per 100ml of GelRed) at 90V for ~1hr.

For the PCR involving ITS 4 and ITS 5, each PCR reaction had a total volume of 20µl, made up of 10µl of RedTaq (Sigma) along with 1µl of the 10µM ITS-5 primer (5'-GGAAGTAAAAGTCGTAACAAGG -3') and 1µl of the 10µM ITS-4 primer (5'-TCCTCCGCTTATTGATATGC-3'). Added to this was 7µl of sterile water and 1µl lysate containing the DNA to make up the reaction volume up to 20µl. The conditions for the reaction cycle was as follows (35 cycles per condition); 2 min at 94°C, 45s at 94°C, 30s at 60°C, 1 min at 72°C, 7 min at 72°C. Following this, 4µl of the amplification product was run on a 1.2% agarose gel (with 2µl per 100ml of GelRed) at 90V for ~1hr (White et al., 1990).

5µl of primer (at a concentration of 5µM) was added to 5µl of the amplification product (at a concentration of 20 -80 ng/µl) in an 1.5ml Eppendorf. Samples were sent to GATC Biotech Ltd. (Germany) to undergo "LightRun Standard Tube Service" sequencing.

Data was used to generate a phylogenetic tree to visualize relatedness of isolates. MEGA 7 was used to determine the best fitting model to generate a phylogenetic tree (Kumar et al., 2015). Maximum likelihood fits for 24 different nucleotide substitution models were completed to do this. Models with the lowest Bayesian information criterion (BIC) scores were determined to have fitted the best.

3.2.2 Developing a standard bioassay procedure for quantifying the effect of EPF isolates on the survival of groups of DBM larvae

DBM larvae were cultured as described in 2.1 were used in this experiment. Groups of early second instar larvae prepared using the procedure described in 2.1 were treated with *B. bassiana* 433.99. *B. bassiana* 433.99 was harvested and suspensions were enumerated to the desired concentration using the procedure described in 2.2 and 2.3, respectively.

For the larval spray method, a Potter tower sprayer (Potter, 1952) was used to apply 4ml of a *B. bassiana* 433.99 conidial suspension (at either $1x10^7$ or $1x10^8$ conidia^{-1}) or

0.05% Triton X-100 to groups of 10-15 larvae on damp filter paper with in a petri dishes (9cm diameter, Merck). Before each spray, the petri dish containing larvae was sprayed with reverse osmosis (RO) water (using a hand sprayer), before being placed in the spray table of the Potter tower and secured below the spraying nozzle using a retractable metal arm. A vortex was then used to shake the 30ml universal bottle containing conidial suspension, 4ml of the conidial suspension was transferred to the spray tube and the spray was initiated. Between each experimental spray, RO water, 70% alcohol and Triton X-100 were each run through the spraying equipment separately, to ensure no cross-contamination occurred. The petri dish lid containing the DBM larvae was then removed from the spray table. A 3 cm diameter leaf disc (cauliflower, variety Skywalker, Elsoms ltd, UK) was added to the petri dish, it was sealed appropriately using Parafilm (Bemis NA, US) and incubated for 24hr at 20^{o}C (16:8 LD). Overnight, the larvae crawled on to the 3cm leaf disc, a fine paint brush was then used to transfer larvae to a separate leaf disc (3 cm diameter) contained in a 3cm deep circular pot with snug fit lid pierced with small holes for aeration. Leaf discs were changed daily.

Four larvae were removed from the experiment (at 0, 6, 12, 24 and 48 hours after treatment) and the concentration of EPF conidia on the insect cuticle was determined using the method described in 2.5.1.

For the leaf application method, 3cm leaf discs were immersed in 10ml of *B. bassiana* 433.99 conidial suspension (at either 1x10^{7} or 1x10^{8} conidia^{-1}) or Triton X-100 (control) for 15 seconds before being left to dry on damp filter paper for 10 minutes. Approximately 30 early second instar larvae were then exposed to the leaf discs (in the same 3cm tubs used in the spray experiment). The same treated leaf disc was left in the tub for the first 48 hours, before being replaced with an untreated leaf disc daily after that.

Four larvae were removed from the experiment (at 0, 6, 12, 24 and 48 hours after treatment) and the concentration of EPF conidia on the insect cuticle was determined using the method described in 2.5.1.

Leaf discs (0.7 cm diameter) were removed from the treated leaf at 0, 6, 12, 24 and 48 hours after treatment. The concentration of EPF conidia on the insect cuticle was determined using the method described in 2.5.2.

For both experiments, larvae mortality was assessed once every 24 hours, at the same time each day after treatment, for seven days. Three replicates of the experiment were completed.

Spore viability was assessed simultaneously to the screen being completed; 20 µl of spore suspension was pipetted onto SDA and incubated for 18 hours at (20 ± 1°C, darkness). Germination was stopped at this time point using lactophenol cotton blue stain. Numbers of germinated and ungerminated conidia were counted from a group of no less that 100 conidia selected at random and observed under a microscope (Ziess Axioskop Routine Microscope). A conidium was considered germinated if the mycelium exceeded twice the length of the spore.

3.2.3 Quantifying the survival of groups of second instar DBM larvae after being treated with candidate EPF isolates

Groups of 10-15 Larvae were sprayed with 4ml of all candidate EPF isolates at a concentration of $1x10^7$ conidia ml^{-1}, or a 0.05% Triton X-100 control, using the Potter tower (as described in section 3.2.2 describing the larval spray application). Larvae were kept at 20°C (16:8 LD) for seven days after treatment. All 14 isolates were applied to larvae in the same block. Five replicates of the experiment were completed.

3.2.4 Quantifying the effect of concentration of EPF conidia on the survival of groups of DBM larvae

B. bassiana 433.99, 1757.15 and *M. brunneum* 275.86, 445.99 and 1760.15 were selected to be used in the concentration-mortality response experiments (*B. bassiana* 1757.18 was not included in this experiment due a contamination issue with the stock

culture). A spray volume of 4ml was applied to groups of 10-15 larvae using the Potter tower using the method described in 2.4 was used to apply to conidial suspensions . EPF isolate suspensions were applied at $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1}, plus an untreated control consisting of 0.05% Triton X-100. DBM larvae were kept at 20°C (16:8 LD) for seven days after application, and mortality was assessed once every 24 hours (as described in 2.4). All EPF isolates (and all concentrations) were applied as one block; this comprised of 25 Petri dishes of 10-15 larvae (five isolates x five concentrations). Three replicates of this block were completed with each replicate completed at a separate time point.

3.2.5 Determining the dose of conidia received by DBM larvae after being treated with selected EPF isolates

Determination of spores present on the insect cuticle was completed for six isolates: *B. bassiana* 433.99, 1757.15 and 1758.15 and *M. brunneum* 275.86, 445.99 and 1760.15. During this experiment, larvae were sprayed with 4ml of conidial suspension using the Potter tower (described in 2.4) with a range of concentrations of each isolate. The conidial concentrations used were $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1}. For each concentration/isolate combination, five early second instar larvae were sprayed. All EPF isolates (and all concentrations) were applied in the same block. Three replicates of the experiment were completed.

Immediately after being sprayed a fine paint brush was used to transfer individual larvae into 1 ml of 0.05% Triton X-100 contained in a 1.5ml Eppendorf, and the concentration of EPF conidia on the insect cuticle was determined using the method described in 2.5.1.

Concentration of EPF conidia received was also determined per unit area (conidia mm^2). To do this, an 18x18mm cover slip was placed alongside larvae during spray application of conidia suspensions. Each coverslip was added to 1ml of 0.05% Triton X-100 in a 30ml Universal tube. Concentration of EPF conidia present on cover slips was calculated using the procedure described in 2.5.3.

3.2.6 General statistical analysis

Data was entered using Microsoft Excel and analyzed using R-studio. For the assessment of 14 candidate isolates, data were analyzed from six days after treatment by using Anovas to find significant differences between mortality caused by different isolates, and genera. Mortality was corrected for control mortality using Schneider-Orelli's formula as described in 2.8 (Puntener, 1981). To calculate the LC50s of selected isolates from the concentration-mortality response experiment, DBM survival data was analyzed using the DRC package, as described in 2.7. For the determination of conidia per larvae, Anovas were used to determine significant differences between number of spores per larvae at each dose. Various polynomials were then fitted to the data, and Anovas used to find the best fitting model.

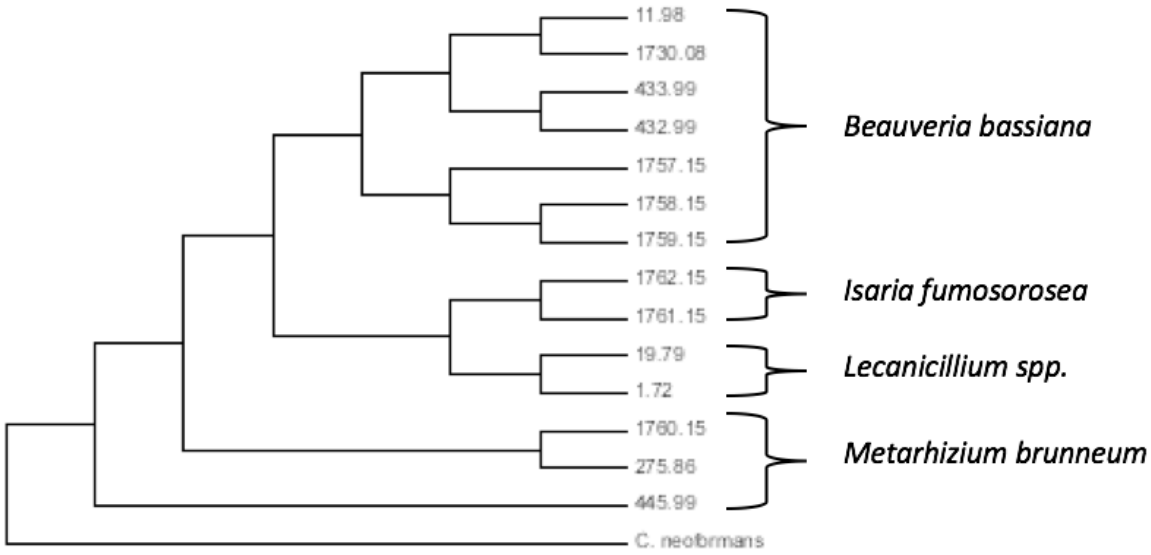
3.3 Results

3.3.1 Determining the phylogenetic relationships of candidate EPF strains using ITS sequencing

The ITS region of all isolates was sequenced. MEGA 7 was used to determine the best fitting model to use to fit a phylogenetic tree (Figure 3-1). Maximum likelihood fits for 24 different nucleotide substitution models were completed to do this. Models with the lowest Bayesian information criterion (BIC) scores were determined to have fitted the best. A Kimura 2-parameter model using a discrete gamma (K2+G) distribution was determined to fit the substitution pattern the best. This model displayed a BIC score of 3774.62. The next best fitting model, a Kimura 2-parameter with a gamma distribution that assumed a certain fraction of sites were evolutionary invariable (K2+G+I), displayed a BIC score of 3776.95.

Using the K2+G model, a phylogeny reconstruction (using maximum likelihood) was completed. The phylogeny was tested using 1000 cycles of boot strapping. As expected, the out group (*Cryptococcus neoformans*) a Basidiomycota, rather than an Ascomycota, was the least related isolate. As expected, *M. brunneum , I. fumosorosea, B. bassiana* and *Lecanicillium* (*L. longisporum* and *L. muscarium*) isolates grouped together within the phylogenic reconstruction. *B. bassiana* isolates were made up of two groups; the first was made up of *B. bassiana* 11.98, 432.99, 433.99, and 1730.15 the second made up of *B. bassiana* 1757.15, 1758.15 and 1759.15. These two groups were more related to each other, than other groups within the phylogeny. *I. fumosorosea* and *Lecanicillium* (*L. longisporum* and *L. muscarium*) isolates were more related to each other, than *M. brunneum* and *B. bassiana* isolates. *M. brunneum* isolates were made up of two groups, the first being made up of *M. brunneum* 275.86 and 1760.15 and the second being made up of *M. brunneum* 445.99.

Figure 3-1. Bootstrap consensus tree, inferred using the Maximum Likelihood model of 14 Hypocrealean isolates plus an out-group made up of *C. neoformans*.



3.3.2 Developing a standard bioassay procedure for quantifying the effect of EPF isolates on the survival of groups of DBM larvae

3.3.2.1 Comparison of larval spray and leaf application techniques using insect mortality

Both "larval spray" and "leaf application" treatment techniques were trialed with *B. bassiana* 433.99 at concentrations of $1x10^7$ and $1x10^8$ conidia ml^{-1}. Control mortality was 2.22±2.22 and 7.14±4.1% for larval spray and leaf application techniques, respectively. Both techniques also produced a similar level of treatment mortality. On six days after treatment, after being sprayed with EPF conidia, percentage mortalities were 61±9.5 and 84±11.6 at $1x10^7$ and $1x10^8$ conidia ml^{-1}, respectively. At the same time point larvae that had placed on treated leaf discs displayed 53.5±14.3 and 83±6.6% mortality at $1x10^7$ and $1x10^8$ conidia ml^{-1}, respectively (Fig. 3-2). No significant differences in mortality were observed between leaf application and larval spray techniques at any concentration used, including the untreated control (Anova, $p>0.05$).

3.3.2.2 Comparison of larval spray and leaf application techniques using concentration of conidia received by insect cuticle and leaf surface

There was a general decrease in conidia present on the leaf surface after being immersed, a decrease in conidia present on the larval cuticle after being sprayed (regardless of concentration used) was also observed. A similar decrease in conidia present on the larval cuticle was observed between 6 and 48hr after treatment, for larvae that had been treated using the leaf application method (regardless of concentration used) (Fig. 3-3). At 0, 6, 12 and 24hr after treatment there were significantly more conidia present on the cuticle surface after being treated with $1x10^8$ conidia ml^{-1} using the larval spray technique. There was no difference in conidia present on the cuticle between the three remaining treatments (leaf application at $1x10^7$ and $1x10^8$ conidia ml^{-1} and larval spray at $1x10^7$ conidia ml^{-1}) at every time point after treatment. By 48hr after treatment there was no significant difference between in conidia present on the cuticle surface regardless of concentration of conidia or

application method used (Anova, P=>0.05). A decrease in conidia present on leaves was seen at both concentrations of conidia at each time point after treatment, with the exception of 12hr after treatment at $1x10^8$ conidia ml^{-1}, where spores were at concentration of $1.4x10^5 \pm 3.6x10^4$ conidia mm^2 on the leaf disc, an increase from $1.0x10^5 \pm 9.3x10^3$ conidia mm^2 at the previous time point, but this difference was not deemed to be significant (Tukey's HSD, p=0.6).

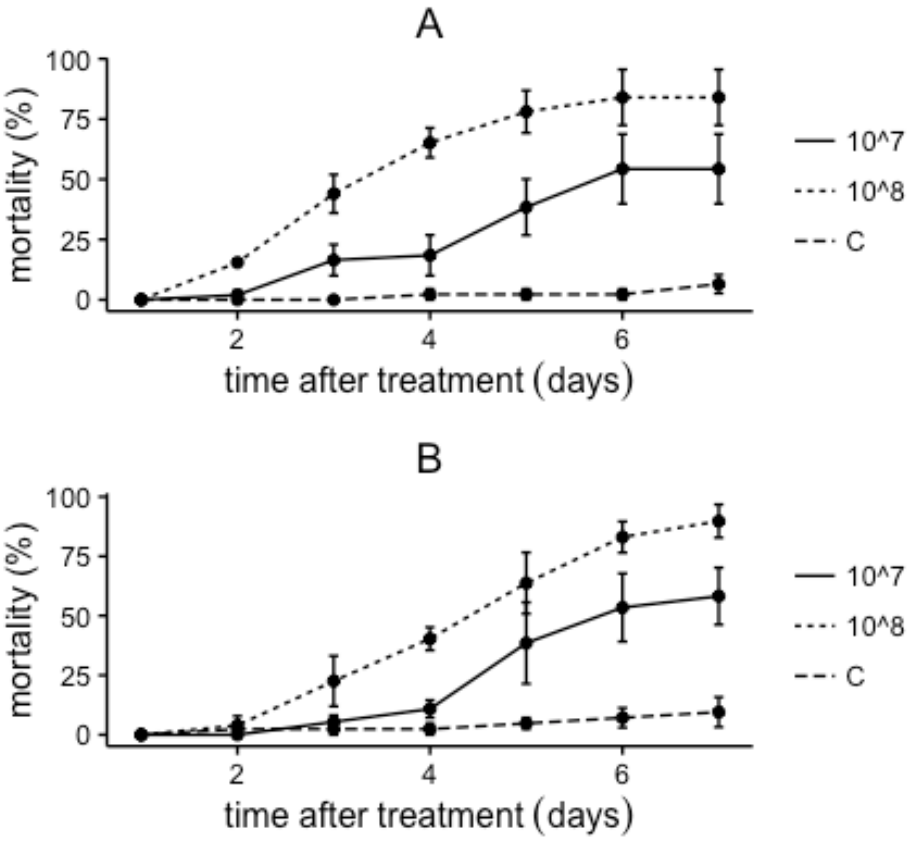


Figure 3-2. Cumulative mortality (%±SE) of groups of larvae over seven days after being treated using larval spray (a) and leaf application (b) techniques, with *B. bassiana* isolate 433.99 at $1x10^7$ and $1x10^8$ conidia ml^{-1}, plus an untreated control.

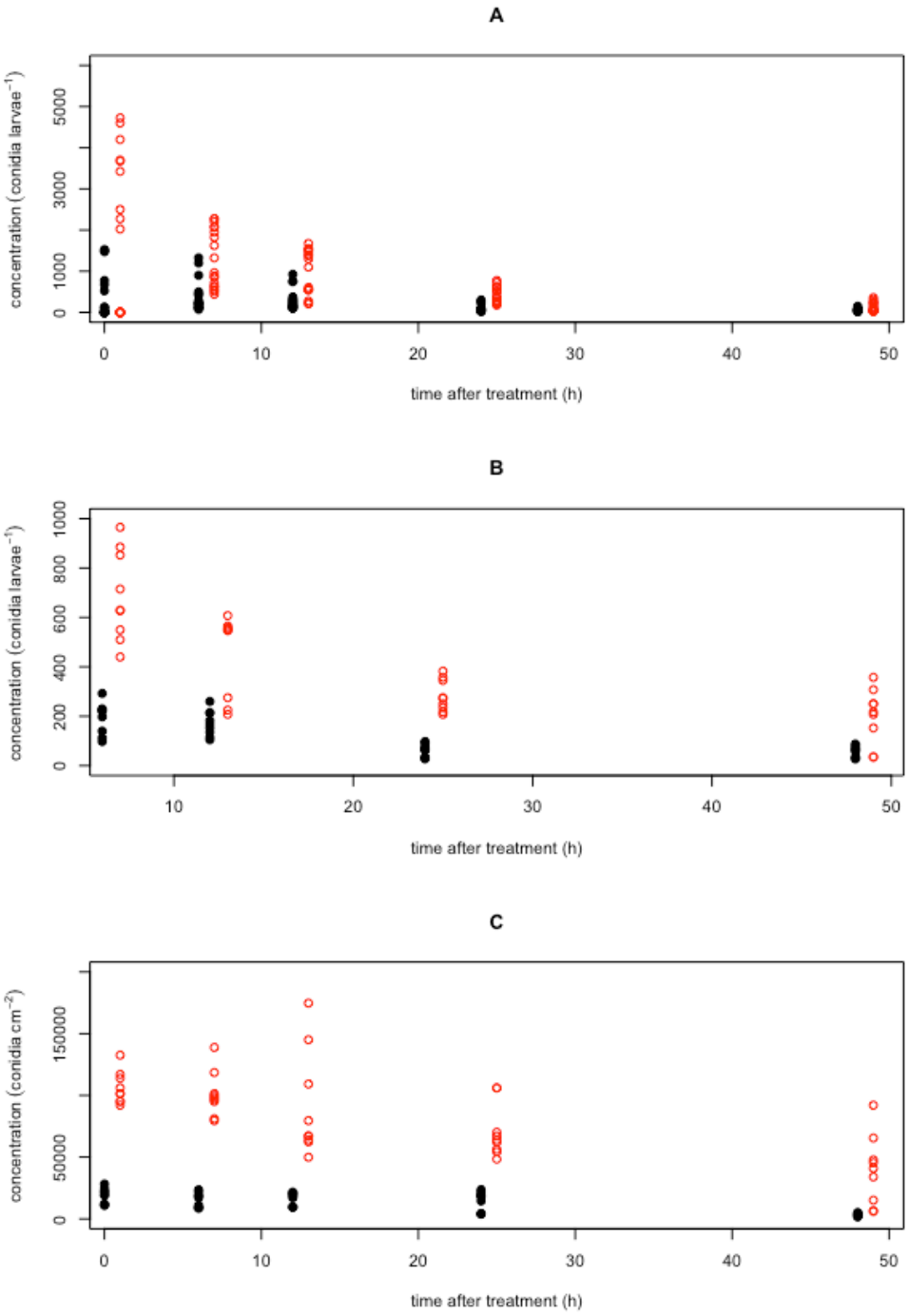


Figure 3-3: A) Conidia (conidia larvae-^{1}) present on the larval cuticle after being treated using the leaf application technique at 1x10^{7}and 1x10^{8} conidia ml^{-1} (displayed by black and red markers, respectively) at 6, 12, 24 and 48hr after being placed on the treated leaf disc. B) Conidia (conidia larvae-^{1}) present on the larval cuticle after being treated using the larval spray technique at 1x10^{7}and 1x10^{8} conidia ml^{-1} (displayed by black and red markers, respectively) at 0, 6, 12, 24 and 48hr after being sprayed. C) Conidia present on leaf disc (conidia mm^{2}) at 0, 6, 12, 24 and 48hr after being immersed in a spore suspension of 1x10^{7} or 1x10^{8} conidia ml^{-1} (displayed by black and red markers, respectively).

3.3.3 Quantifying the survival of groups of second instar DBM larvae after being treated with candidate EPF isolates

Mortality from six days after treatment was analyzed to compare virulence of isolates against DBM. Insect mortality was analyzed after being corrected for control mortality, which was below 15% across all isolates and replicates. Isolates displayed a wide range of virulence ranging from 0% *(L. muscarium* 19.79) to 98.5±3.2% (*B. bassiana* 1758.15), and mortality varied significantly with isolate (Anova, p=<0.001). As well as insect mortality, percentage germination 16-24 hours after application of EPF was assessed. All isolates apart from *M. brunneum* 1760.15 germinated at over 80%. *M. brunneum* 1760.15 displayed a germination percentage of 39±5.

Time to kill analysis was also completed using probit linear regression. The three most virulent isolates in terms of mortality at six days after treatment also displayed the lowest LT50s. *B. bassiana* 1757.15, 1758.15 and 1759.15 took 2.3-2.7 days to cause 50% mortality. In comparison, the next lowest LT50 (4.8 days) was displayed by *M. brunneum* 445.99. *B. bassiana* 433.99 and *M. brunneum* 275.86 had LT50s of below six days. A number of other isolates displayed LT50s of 6-10 days, these were *B. bassiana* 11.98, *B. bassiana* 432.99, *I. fumosorosea* 1761.15, *I. fumosorosea* 1762.15 and *L. longisporum* 1.72. The remaining isolates, plus the untreated control, displayed LT50s of over 10 days (Table 3-2).

The three most virulent isolates, *B. bassiana* 1757.15, 1758.15 and 1759.15, differed from all other isolates and caused upwards of 95% mortality by six days after treatment. The commercial product *B. bassiana* 433.99 (BotaniGard) caused 62.3±6.9% mortality, and had similar virulence to *I. fumosorosea* 1762.15. *M. brunneum* 275.86 and 445.99 caused approximately 50% mortality. The remaining isolates, made up of *L. longisporum, L. muscarium, M. brunneum* and *B. bassiana* species, displayed relatively low mortality. *L. muscarium* 19.79 displayed a lower mortality than the control, this was presumed to be an artifact of the inherent variation in insect mortality (Table 3-2).

Table 3-2: Time to 50% mortality and mean corrected mortality (%) six days after treatment of groups DBM larvae after being treated with one of 14 EPF isolates.

Genera	Isolate	LT50 (days)	Corrected mortality (%)
B. bassiana	11.98	8.1	22.1±13.3 **d**
	432.99	8.7	8.3±15.7 **cd**
	433.99	5.3	62.3±6.9 **bc**
	1730.08	6.5	30.3±19.7 **cd**
	1757.15	2.3	97.4±3.6 **a**
	1758.15	2.7	98.6±3.2 **a**
	1759.15	2.7	92.9±7.1 **ab**
I.fumosorosea	1761.15	7.8	34.7±27.7 **bcd**
	1762.15	6.8	61.8±19.5 **bcd**
L. longisporum	1.72	7.5	19.0±6.4 **d**
L. muscarium	19.79	10.9	0.0±0.0 **d**
M. brunneum	275.86	6.0	41.7±23.8 **cd**
	445.99	4.8	47.9±16.8 **cd**
	1760.15	12.9	24±17 **b**
	Control	9.0	-
		F value	22.9
		P value	<0.0001

3.3.4 Determining the dose of conidia received by DBM larvae after being treated with selected EPF isolates

Concentration of conidia present on the larval cuticle was assessed for six isolates: *B. bassiana* 433.99, 1757.15, 1758.15, and *M. brunneum* 275.86, 445.99 and 1760.15. To begin with, analysis was completed to assess any significant differences in concentration received between different isolate and species. There was no significant difference in both metrics of concentration received (conidia $larvae^{-1}$ and conidia mm^2) between EPF isolates or species at any EPF dose applied. Consequently, concentration received was pooled across all isolates for further analysis.

Dose received by larvae after being sprayed ranged from 7±1 conidia $larvae^{-1}$ at a concentration of $1x10^4$ conidia ml^{-1} to $2.2x10^3$±143 at $1x10^8$ conidia ml^{-1}. A 10-fold increase was not seen in dose received by larvae after every increase in concentration applied, this is indicative a of loss conidia in the spraying process. Nevertheless, a steady increase in dose received was observed as concentration applied increased. However, this relationship was not linear. After comparing linear and two-parameter polynomial models, it was found that the latter was required to explain the relationship between concentration applied and received (Anova, p=<0.001). A two-parameter model (p=<0.001) explained 96.2% of variation with in the data (Fig. 3-4).

As expected, there was also a large degree in variation in the second metric of concentration received, conidia mm^2. Concentration received ranged from 57±4 conidia mm^2 at the lowest fungal concentration to $3.1x10^4$±$1.9x10^3$ at the highest. Again, a less than 10-fold increase in concentration received between every increase in concentration applied suggested that conidia were being lost in the spray process. A three-parameter model was used to describe the data (Anova, p=<0.001), the model fitted the data (p=<0.001) and explained 98.1% of variation with in the dataset (Fig. 3-4).

When assessing the relationship between the two concentration received metrics, a linear relationship was found. Perhaps, because both of these metrics had been influenced by conidia loss during spray and so received the same proportion of spores. The linear model fitted the data significantly (p=<0.001) and explained 96.5% of the variation with in the dataset.

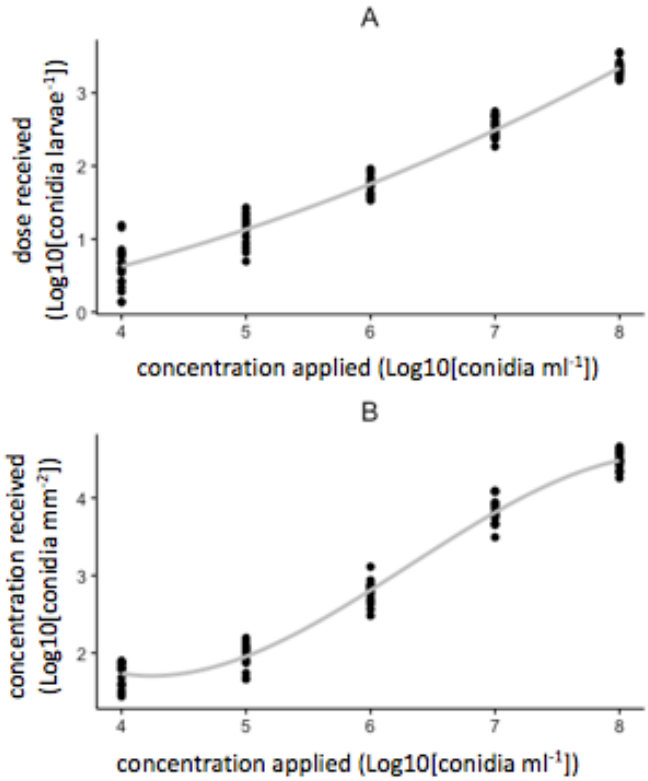


Figure 3-4: A) Dose received by larvae (Log conidia larvae^{-1}) , pooled across all isolates, after being applied with a range of EPF concentrations, along with a two-parameter polynomial model. B) Concentration received on a coverslip (Log10 conidia mm^2), pooled across all isolates, after being applied with a range of concentrations, along with a three-parameter model

Table 3-3: Concentration of EPF conidia received pooled across all isolates (conidia larvae^{-1} and conidia mm^2) after being applied with five fungal doses.

Conc. Applied (conidia ml^{-1})	**Conc. Received±SE**	
	(conidia larvae^{-1})	**(conidia mm^2)**
1x10^4	7±1	57±4
1x10^5	19±2	98±7
1x10^6	59±5	653±52
1x10^7	385±27	7.1x10^3±640
1x10^8	2.3x10^3±143	3.1x10^4±1.9x10^3

3.3.5 Quantifying the effect of concentration of EPF conidia on the survival of groups of DBM larvae

Uncorrected mortality data from six days after treatment was used in analysis. Mortality data was 80nalysed using three metrics of conidial concentration. These were conidia ml-1 (the known concentration of the spore suspension before spraying), plus two measures of effective concentration: conidia larvae-1 (number of conidia present on the cuticle surface of individual larvae) and conidia mm2 (number of conidia present per square mm on a coverslip placed alongside the larvae during the spray). Concentration-mortality response analysis was completed separately, using the DRC package in R studio, for each measure of dose using the same mortality data.

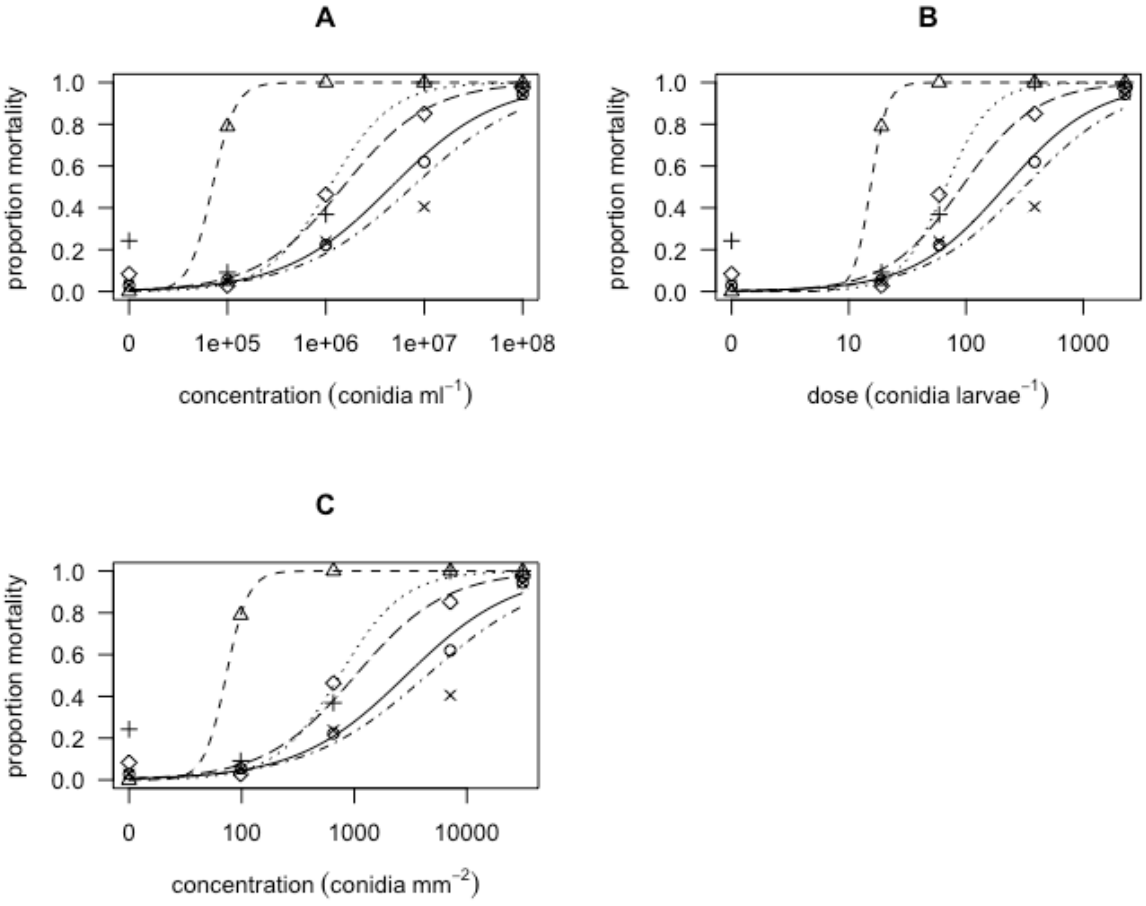


Figure 3-5: Proportion mortality of groups of DBM larvae six days after treatment with one of six isolates, at five concentrations. Three metrics of concentration are used: a) conidia ml^{-1}, b) conidia larvae^{-1} and c) conidia mm^{2}. A 2 parameter log-logistic model is also included. Isolates can be identified as follows:

B. bassiana 433.99 (——o——), *B. bassiana* 1757.15 (- - -△- - -), *M. brunneum* 275.86 (·····+·····), *M. brunneum* 445.99 (– · – x· – ·) , *M. brunneum* 1760.15 (– –◇– –)

Table 3-4: LC50±SE for six isolates measured in three units (conidia ml-^{1}, conidia larvae-^{1} and conidia mm^{2}). Significant differences between LC50s of different isolates are also displayed, different lower case letters with in the same column indicate significant differences are present (multiple T-test).

Genus	Isolate	LC50±SE		
		conidia ml^{-1}	conidia larvae^{-1}	conidia mm^{2}
B. bassiana	433.99	4.6x10^{6} ±1.3x10^{6} **cd**	215±46	2884±742
	1757.15	7.2x10^{4} ± 5x10^{4} **a**	15±5	73±51
M. brunneum	275.86	1x10^{6} ±2.3x10^{5} **b**	67±9	742±145
	445.99	7.6x10^{6} ±2.2x10^{6} **d**	315±69	4414±1158
	1760.15	1.5x10^{6} ±3.8x10^{5} **bc**	92±17	1024±241

Table 3-5: Best fitting models selected after completing DRC analysis to quantify the effect of concentration of EPF conidia on mortality of groups of DBM larvae, for five isolates. Analysis was completed using three metrics of concentration.

Metric of concentration	Model	P-value
Conidia ml^{-1}	LL2	0.72
Conidia larvae^{-1}	LL2	0.63
Conidia mm^{-2}	LL2	0.57

For the analysis using conidia ml^{-1} a two-parameter log-logistic model was used to derive LC50 values. To begin with, two-, three-, and four-parameter log-logistic models and Weibull-1 and -2 models were compared using a lack-of-fit test. The two-parameter log-logistic model provided the highest P-value (0.72) and was selected (Table 3-6). It was then assessed whether individual parameters of the model should be individually estimated for each isolate, or be the same for each isolate. Two Anovas were completed:

one comparing a model with LC50 and slope parameters estimated for each isolate with a model with LC50s estimated for each isolate and a single slope parameter for all isolates, the second Anova compared a model with LC50 and slope parameters estimated for each isolate with a model with a single LC50 estimated for all isolates and individually estimated slope parameters. P-values of 0.0 and 0.032 were generated for each Anova, respectively. This suggested that the model fit was worse if parameters were constrained. Consequently, a two-parameter log-logistic model with slope and LC50 values estimated for each isolate was selected as the best fitting model.

The same process was completed for the analysis using conidia mm^2 and conidia ml^{-1}, it was found that (for both conidia mm^2 and conidia ml^{-1}) a two-parameter log-logistic model was the best fitting model compared to three and four log-logistic models and Weibull-1 and -2 models as it produced a higher P-value after completing a lack-of-fit test (Table 3-6). Anovas were then completed to determine whether the slope or LC50 should be the same for all isolates. For both conidia mm^2 and conidia ml^{-1} analysis it was found that the slope and LC50 should be individually estimated for each isolate ($P < 0.05$ in all comparisons). In summary, a two-parameter log-logistic model, with slopes and LC50 parameters estimated for each isolate was used for analysis (Fig. 3-5).

Multiple T-tests were completed to obtain significant differences in lethal concentrations between different isolates. Separate multiple T-tests were completed for the three metrics of concentration, significant differences were the same across all three metrics of concentration (although T and P-values varied slightly).

LC50 was significantly affected by isolate. *B. bassiana* 1757.15 displayed an LC50 of 15±5 which was lower compared to other isolates, this was true of LC50s in terms of all three metrics of dose. But, for brevity, only conidia $larvae^{-1}$ will be discussed here. Significant differences between isolates were the same for each metric of dose. The next lowest LC50 belonged to *M. brunneum* 275.86 which was 67±9 conidia $larvae^{-1}$, *M. brunneum* 1760.15 displayed a similar LC50 of 92±17conidia $larvae^{-1}$. *B. bassiana* 433.99 and *M. brunneum* 445.99 were observed to be the least virulent, displaying LC50s of 215±46 and 315±69 conidia $larvae^{-1}$, respectively (Table 3-5).

3.4 Discussion

3.4.1 Developing a standard bioassay procedure for quantifying the effect of EPF isolates on the survival of groups of DBM larvae

Several problems were encountered when completing bioassays against DBM larvae (Table 3-7). Initially, mortality was observed to be very variable when second instar larvae were sprayed with EPF. It was proposed that this was due to there being too much variation of age of larvae within the second instar. To combat this, larvae were only selected for experimentation if they were newly molted into the second instar. Newly molted larvae were identified by a visibly darkened cuticle and the presence of the recently molted skin on the leaf surface (Harcourt, 1957). Variation in mortality was reduced by using employing this technique. It was presumed that if EPF isolates were applied to a late instar larva, there may not be enough time for conidia to penetrate the cuticle and cause a systemic infection before being discarded, along with the exuvia, in the molt to the next instar. This mechanism of avoidance has been referred to in previous literature (Vandenberg et al., 1998), and will be investigated in more detail later in this research project.

During the screen, after DBM larvae were sprayed, they were kept in a sealed container containing a leaf disc placed on damp filter paper for seven days while mortality was monitored. Leaf discs were replaced daily, but it was observed that the leaf material would dry out a few hours after being cut from the plant. Self-evidently, this would mean larvae were being starved which may be another cause of mortality other than EPF conidia. Humidity of the bioassay container would also be affected by the drying leaf disc, and may affect the germination of conidia on the insect cuticle (Fargues and Luz, 2000). To address this, during the concentration response experiment, leaf discs were kept on water agar, this essentially stopped the process of leaf drying.

Second instar DBM larvae are around 7mm in length, consequently, escape of insects (both during the initial spraying process, and during the subsequent monitoring phase

of the bioassay) was a problem that had to be solved during the project. The initial bioassay containers, used in the screen of candidate isolates, had “snug-fit” lids, but larvae regularly escaped during the course of the bioassay. Different containers, with tighter fitting lids, were used during the dose response experiment. This effectively stopped larval escape, but insects would regularly become trapped between the lid and the container, resulting in death. For the remainder of the PhD, 9 cm petri dishes (lined with water agar and modified for aeration) securely sealed with Parafilm, were used to hold larvae during bioassays. This reduced the incidences of larval death due to being trapped between lid and bioassay chamber. During the initial bioassays (method development experiment, the screen of 14 isolates and the dose response experiment), it was noticed that larvae would regularly escape during the spraying process. In later bioassays, to reduce incidences of larval escape, larvae were refrigerated for 30 minutes to 2hr before treatment began. However, it was noted that refrigeration may have had an impact on larval susceptibility to conidial infection. To address this, during preliminary experiments, bioassays were completed in which refrigerated and non-refrigerated larvae were treated with the same concentration of *B. bassiana*. There was no significant difference in time-to-mortality and overall proportion mortality between refrigerated and non-refrigerated larvae indicating that refrigeration would not have an impact on bioassay results.

Broadly speaking, there are three main ways Hypocrealean EPF conidia are applied to DBM larvae in the laboratory, these are;

i. Spraying conidia directly on larvae
ii. Applying EPF conidia to a leaf disc before allowing larvae to feed on it.
iii. Directly immersing larvae in a solution of EPF conidia.

Initially, all three types of application were attempted against second instar DBM larvae. However, through preliminary experiments it was found that control mortality for the larval immersion method was both high and unpredictable when compared to the larval spray and leaf application methods. It was presumed the high control mortality was due to the effects of being submersed in an aqueous solution. Furthermore, after plating out

colony forming units from both spayed and immersed larvae, it was found there was no difference in the conidia present on the larval cuticle meaning there was no benefit from using the larval immersion technique. When taking into account the high control mortality, it was decided that the larval immersion method would not be used. Consequently, larval spray and leaf application were assessed as methods to be used as a standard bioassay procedure.

B. bassiana 433.99 was applied using either leaf application or larval spray methods at two concentrations. There was no difference in mortality between the two application techniques. However, it was observed that dose received by larvae was easier to determine when using the "larval spray" technique. When *B. bassiana* 433.99 was applied to the leaf disc, there was a drop off in spore viability over 24hr. In fact, spore viability dropped by over half within the first day after application. When taking this into account, it may be less reliable to equate number of spores present on the leaf surface (directly after treatment with conidia) with dose received by larvae. In comparison, when using the "larval spray" technique, spores received by larvae could be more easily determined as the application was more direct. There was a drop in number of spores present on the larval cuticle over the 48hr after application, but this was presumed to be an artifact of spores attaching, and penetrating the larval cuticle, rather than loss of spore viability (Hajek and St. Leger, 1994). Consequently, dose received by larvae can be more easily equated with conidia per mm. Control mortality was also observed to be lower using the larval spray technique. When taking into account all of these factors, it was decided that the larval spray technique would be used to apply EPF in future experiments.

Table 3-6: Proposed causes and solutions for problems encountered when developing a bioassay system for DBM larvae.

Problem	Possible reason	Solution
Large degree of variation in mortality (both between and within replicates of same EPF concentration)	Age with in instar was not considered when selecting second instar larvae for experimentation	Only early second instar were used
Leaf drying during course of experiment	Lack of moisture	Leaf placed on water agar
Loss of larvae during experiment	Lids to bioassay chambers did not fit snugly enough	Modified petri dishes were used as bioassay chambers, these were sealed with Parafilm

3.4.2 Quantifying the survival of groups of second instar DBM larvae after being treated with candidate EPF isolates

Before the great potential of EPF as biocontrol product against DBM can be realized, there needs to be a better understanding of the interaction between the pest and EPF. This includes improving the knowledge of which isolates are virulent against DBM.

Before the current screen was completed, a group of Hypocrealean EPF isolates were chosen for inclusion. These included; isolates previously screened against DBM and known to have high virulence against the insect (such as *B. bassiana* 1757.15, 1758.15 and 1759.15), commercial products such BotaniGard (the active ingredient of which is *B. bassiana* 433.99), Met52 (*M. brunneum* 275.86), and novel isolates which had not been screened against DBM before, but were isolated from DBM cadavers, making them likely to be virulent against the insect (such as *M. brunneum* 1760.15, *I. fumosorosea*

1761.5 and *I. fumosorosea* 1762.15). Isolates shown to confer virulence against DBM in a previous unpublished mini-project (which led on to the current PhD project) were also included, these included *B. bassiana* 11.98 and *B. bassiana* 1730.08.

In the screen, a number of isolates were found that caused mortality in DBM larvae. The three most virulent isolates were *B. bassiana* 1757.15, 1758.15 and 1759.15, these isolates were also found to be highly virulent against DBM in a previous screen (Wraight et al., 2010). Interestingly, these three isolates caused significantly more mortality than a number of commercial products including BotiniGard (*B. bassiana* 433.99) , Met52 (*M. brunneum* 275.86) and Bio-Blast (*M. brunneum* 445.99). As well as the three highly virulent *B. bassiana* isolates, a number of novel isolates were found to cause mortality. Two *I. fumosorosea* isolates (1761.15 and 1762.15) were found to be virulent, with 1762.15 causing a similar level of mortality to BotiniGard (*B. bassiana* 433.99). The *M. brunneum* isolate 1760.15 was also observed to cause significantly more mortality than the control (T-test, p=0.02). Interestingly, this isolate was also shown to have a relatively low germination rate, when compared to every other isolate in the screen.

Concentration-mortality response experiments were then completed to further investigate the interactions between virulent isolates and DBM. Six isolates were chosen to for inclusion in dose response experiments. A highly virulent *B. bassiana* isolate (1757.15) was included. The commercial isolates *B. bassiana* 433.99 (BotaniGard) , *M. brunneum* 445.99 (Bio-Blast) and *M. brunneum* 275.86 (Met52) were also included to provide a bench mark against which other novel isolates could be compared. *M. brunneum* 1760.15 was included due to the isolate's ability to cause mortality whilst germinating at relatively low rate. The inclusion of *M. brunneum* and *B. bassiana* isolates was also expected to be interesting comparison when the effect of temperature on EPF growth and EPF virulence will be investigated in the next phase of research, considering that previous literature has shown *Metarhizium* isolates to be more suited to high temperatures (Davidson et al., 2003).

Before the concentration response experiment was completed, the number of conidia present on the larval cuticle directly after spray was determined for the six selected

isolates. The number of conidia present was the same regardless of what isolate was sprayed. This indicates that the six isolates have a similar affinity to the larval cuticle, and that differences in virulence between isolates not down to the process of adhesion to the larval cuticle. However, it should be noted that conidia present on the larval cuticle was assessed directly after spray, meaning there could be some differences in conidia presence on cuticle – and therefore, dose received – at different time points during the infection process (Hajek and St. Leger, 1994). This will be investigated later on in the research project.

After completing the concentation response experiment, *B. bassiana* 1757.15 was found to have a far lower LC50 than the other isolates. Observing the LC50 values generated within the context of DBM literature can be challenging, this is because application procedures and measurement of dose received often varies between study. However, *B. bassiana* 1757.15's LC50 (129±20 conidia mm^2 six days after treatment) is comparable to the LC50 (107 conidia mm^2 eight days after treatment) of a *B. bassiana* isolate described as "highly virulent" by Tian and Feng (2006).

The mechanisms which cause isolates such as *B.bassiana* 1757.15 to be more virulent than others in the screen are likely to be due to the isolate's speed of germination of conidia, and its enzyme and secondary metabolite profile (Chandler, 2017). It was noted in the germination experiment that *B. bassiana* 1757.15, 1758.15 and 1759.15 produced longer germ tubes when compared to other isolates, over the same period of time. Presumably this would make the isolates more effective at penetrating the insect cuticle and causing a systemic infection. It was also noted that insect mortality due to *B. bassiana* 1757.15, 1758.15 and 1759.15 occurred two to three days before mycosis occurred. In other isolates mortality occurred hours before the onset of mycosis, indicating that mortality was due to the insect being engulfed by fungal mycelia. The nature of insect death caused by *B. bassiana* 1757.15, 1758.15 and 1759.15 is probably due to specific secondary metabolites being produced during the infection process. For example, *B. bassiana* isolates are known to produce up to seven secondary metabolites which cause effects such as cell paralysis and inhibition of the host immune system (Zimmerman, 2007). It is likely that certain species are particularly susceptible to certain

metabolites because they express the complementary targets (Chandler 2017). To confirm this hypothesis the relevant secondary metabolite genes in *B. bassiana* 1757.15, 1758.15 and 1759.15 would have to identified and knocked-down. Virulence of the knocked-down and wild-type isolates could then be compared. This type of study has seldom been completed in the past, with most investigations in the importance of secondary metabolites involving the direct injection of such metabolites through the insect cuticle (Chandler, 2017). However, a study by Xu et al. (2008) found that a *B.bassiana* isolate with knocked-down secondary metabolite genes was less virulent than wild-type *B. bassiana* against fall army worm and greater wax moth.

There were some differences in virulence observed between the initial screen of the 14 isolates, and the subsequent dose response of the selected isolates. For example, in the original screen *M. Brunneum* 275.86 caused approximately 50% mortality and in the concentration response, at the same dose, the isolate caused approximately 80% mortality. This was assumed to be an artifact of the inherent variation observed with in insect bioassays. Another possible explanation is the slight change in methodology between the screen and the concentration response experiments. Leaf discs dried at a far faster rate in the screen because they were not kept on water agar, as in the dose response experiment. Presumably this would mean humidity would be lower with in the bioassay container in the screen, which may have had the effect of reducing the virulence of the fungi (Fargues and Luz, 2000).

3.4.3 Summary

i. A “larval spray” bioassay protocol was developed to quantify the effect of EPF isolates on the survival of groups of DBM larvae.
ii. This protocol was used to complete a screen of candidate EPF isolates against DBM larvae.
iii. Several highly virulent EPF isolates were found in the screen.

4 Developing a physiological time model to predict EPF virulence against DBM in conditions of fluctuating temperature

4.1 Introduction

DBM is pest with a cosmopolitan distribution, occurring over a wide range of climate zones in many regions of the world (Sarfraz et al., 2005). The rates at which all physiological processes occur within DBM are dependent upon temperature of the external environment. This is because DBM is an ectothermic organism whose body temperature is highly influenced by external sources of heat, while internal sources of heat and behavioural influences have a minor role (Bahar et al., 2012). Consequently, temperature has been proposed as the most important factor effecting the rate of insect development (Hallman and Denlinger, 1998). EPF are also ectothermic organisms, temperature is known to be an important determinant of EPF physiological processes. It is known that EPF of different species can vary immensely in their response to temperature (Davidson et al., 2003). Considering the fact that EPF and DBM are ectothermic organisms, temperature is an important determinant of the EPF infection process of DBM and can influence both speed of kill and over proportion mortality of groups of DBM (Thomas and Blanford, 2003). Three interactions are of particular relevance in this research:

i. The effect of temperature on the rate of DBM development
ii. The effect of temperature on the physiology of EPF isolates *in vitro* (including the colony extension rate of EPF isolates and proportion germination of populations of EPF conidia)
iii. The effect of temperature on the virulence of EPF isolates against DBM larvae

A major barrier preventing the uptake of EPF biopesticides to control DBM infestations is the variability of EPF isolates, in terms of virulence against DBM in the field (Lacey et al., 2001). Temperature is an important cause of this variation (Thomas and Blanford, 2003). Understanding interactions I, ii and iii has the potential to address and resolve

temperature as cause of temperature as cause of variation of EPF virulence against DBM in the field. Interaction (i) can provide accurate predictions on the prevalence and population density of DBM in the field (Baker et al., 1982), growers can then tailor their insecticide applications to these predictions. It has been proposed that investigating interaction (ii) can provide predictions on the thermal virulence profiles of EPF isolates (Smits et al., 2003). For example, if an isolate has an optimum colony extension rate at a high temperature, it is likely that the EPF isolate will also high temperature virulence optima. However, the extent to which thermal virulence optima can be predicted from thermal physiology optima has been debated in the literature (Thomas and Blanford, 2003). Understanding interaction (iii) is vital in predicting which EPF isolates are suited to a particular climate zone.

The effect of temperature on the development of DBM has been extensively studied in the laboratory (Golizadeh et al., 2007, Marchioro and Foerster, 2011, Liu et al., 1995). Typically, this research has two practical applications (Fig. 4-2):

i. The use of non-linear mathematical models to explain the relationship between temperature and development rate for the entire thermal range of the insect to characterise cardinal temperatures such as *T0*, *Tmax* and *Topt*.
ii. Fitting a linear regression model to the development rates at temperatures up to the optima to develop a day-degree (DD) model to predict the development DBM in the field.

Application (i) can be used to estimate the thermal optima of DBM development. A bell-shaped distribution of development rate over temperature is normally observed, invariably, this distribution is skewed to the left (Golizadeh et al., 2007, Marchioro and Foerster, 2011). Consequently, models based on a normal distribution cannot be used to determine thermal optima, as it is likely an under-estimation of thermal optima would occur. Several models have been proposed to counter this problem. Such models include those proposed by Logan et al. (1976), Lactin et al. (1995) and Briere et al. (1999). These models have been shown to describe the relationship between temperature and

DBM development rate accurately. For example, Golizadeh et al. (2007) reported r^2 values for Briere-1, Logan-6 and Lactin-1 models to be over 0.95 when describing DBM development rate, from egg to adult, over seven temperatures.

Application (ii) can be used to develop a DD model, used to predict development rates and population densities of DBM in the field under fluctuating temperatures. For the use of non-linear models the entire thermal range of insect development is used. When using linear models, temperatures over the developmental optima are disregarded. A linear regression model is used to describe the relationship between development rate and temperature. The temperature at which the linear regression crosses the horizontal axis reflects the minimum temperature at which insect development can occur (T0). The slope of the linear regression (*k*) reflects the total number of accumulated DD associated with the development DBM from egg to adult (Hough, 2013). It is known that a DD approach can be used to predict the time required for DBM to develop from egg to adult at fluctuating, field typical, temperatures (Baker et al., 1982). A DD represents the accumulation of heat energy over time (Bryant et al., 1998). DD are only accumulated if the temperature is above the minimum temperature at which insect development occurs (*T0*), and below the optima for insect development (*Topt*). There are several methods used to calculate the number of DD acrewed per day, all these methods use daily maximum and minumum temperature values (taken from the field). The simplist equation is based on taking the mean of the maximum and minimum temperature (Hough, 2013). AIe method, known as The Met. office equation, provides a more accurate estimation of DD. This equation assumes a sino-soidal distrubution of temprature over a 24hr period, which is lilkey to be more ecologicallly relevant (Day, 2006) (Fig. 4-1). In principle, the time between two insect developmental stages (i.e from egg lay to emergence of adult) would occur at a constant DD sum regardless of the temperature the DD were accumulated at. In practice, number of DD accumulated from a specific temporal starting point are calculated. The date at which the number of accumulated DD associated with insect development is accumulated can then be determined and provide a predicted date of insect emergence. Such predictions of time for DBM to develop from egg to adult have proven to be accurate in the literature (Baker et al., 1982).

The effect of temperature on EPF physiology has been investigating using non-linear mathematical modelling (Smits et al., 2003, Davidson et al., 2003). As with the thermal profile of DBM development, distributions are typically bell-shaped and skewed to the left. Various non-linear mathematical models have been used to describe the relationship between EPF physiology and temperature. For example, Smits et al. (2003) evaluated five non-linear mathematical models including Breire and Logan models for describing the relationship between colony extension rate and temperature for five EPF isolates. Consistently high r^2 values and low standard errors for model parameters resulted in Briere being proposed as the most appropriate model to describe the relationship. More sophisticated non-linear models have also been used for this purpose. Davidson et. al (2003) used a model proposed by Schoolfield et al. (1981) to estimate colony extension thermal optima for range of EPF isolates.

Perhaps the most crucial interaction, in terms of biocontrol, is the effect of temperature on EPF virulence. This can be described as a genotype x genotype x environment (GxGxE) interaction as temperature influences both the physiology of DBM and EPF. The interactions between EPF and DBM (a genotype x genotype (GxG) interaction) have been well studied. The effect of temperature on EPF physiology, and the effect of temperature on DBM physiology has also been well studied (both are genotype x environment interactions (GxE)) (Thomas and Blanford, 2003). However, there has been a general lack of study into the effect on temperature on virulence of EPF isolates. The thermal physiology profiles of EPF and DBM can provide useful information on EPF virulence against DBM. However, the most reliable way to understand the effect of temperature on EPF virulence is to complete experiments where insect survival is monitored at several temperatures, ideally over the complete thermal range of both DBM and EPF (Thomas and Blanford, 2003). This is because of the complexity of the GxGxE interaction. Temperature effects the rate of fungal physiological processes related to infection. For example, germination (Hajek and St. Leger, 1994), growth of mycelium (Davidson et al., 2003) and production of virulence determinants (Sajjad, 2010). At the same time, temperature effects the processes associated with the antifungal immune response in DBM. For example, the production of AMPs. Other DBM

physiological processes are also effected which are not explicitly related to fungal defence, but still may have some influence on infection. For example, insect development rate, food consumption, moulting, and movement across the leaf surface (Golizadeh et al., 2007, Marchioro and Foerster, 2011).

The studies that have been conducted on the influence of temperature on EPF virulence have not used non-linear modelling to explain the relationship. For example, Vandenberg et al. (1998) measured proportion mortality of groups of larvae after being treated with *B. bassiana* at five temperatures. Highest proportion morality was found at 25^{o}C, with lowest mortality at 15 and 35^{o}C. Additionally, Mishra et al. (2015) recorded mortality of housefly larvae after being treated with *B. bassiana* at seven temperatures. For both of these studies, non-linear models were not fitted to mortality data, meaning that cardinal temperatures (such as Topt, Tmax and T0) could not be estimated. Cardinal temperatures are vital for predicting which temperatures EPF isolates are best suited to in terms of insect virulence.

The ultimate aim of studying the effect of temperature on EPF virulence was to develop a DD style model to predict proportion mortality on groups of DBM larvae conferred by EPF isolates under conditions of fluctuating temperature. Many authors have expressed doubt on whether data collected under constant temperatures can be used to predict responses under “field typical” fluctuating temperature conditions (Hau et al., 1985, Hagstrum and Milliken, 1991, Bale, 1999). For example, Bale (1999) showed that development rates of nettle-feeding larvae differed between constant and sinusoidal temperature regimes which shared the same mean temperature, presumably because the physiology of the insect an any one temperature is effected by previous temperatures it has experienced. Conversely, Burgess and Griffin (1967) found that the growth rate of fungi experiencing diurnal temperature fluctuations could be accurately predicted from fungal growth rates at constant temperatures. As a result, it was proposed that the growth rate of fungi at any given temperature was unaffected by its previous temperature experience (Burgess and Griffin, 1967). Here, the DD model would be developed based on mortality data collect in the laboratory at constant

temperatures. Consequently, the effectiveness of predicting the response in fluctuating temperature conditions, from data collected at constant temperatures can be assessed.

The process of insect mortality is dependent on the germination of the EPF isolate upon the insect cuticle (Hajek and St. Leger, 1994). In principle, this could be explained by the accumulation of heat units over time as in a DD model (as described above). For example, it is known that a certain number of DD is required for the development of an insect to a certain phenological stage, because insect development is highly influenced by temperature (Golizadeh et al., 2007, Marchioro and Foerster, 2011). It is also known that germination, growth and virulence of EPF are highly influenced by temperature (Thomas and Blanford, 2003, Davidson et al., 2003, Smits et al., 2003). Consequently, it follows that the accumulation of heat units is highly related to germination and growth of fungi on and within insects. DD may, therefore, be useful tool to describe the insect mortality process.

However, there are extra levels of complexity when considering the infection of an insect by EPF, in comparison to the development of an insect. For example, it is likely that different stages of insect infection will vary in their speed. The penetration of the cuticle by conidia may take longer than the proliferation of mycelium with in the insect cuticle. The former process would involve overcoming the insect immune system, whilst the latter would receive less resistance from host immune responses (Hajek and St. Leger, 1994). It also known that spores are shed from insect cuticle larvae in the moult from one instar to the next (Vandenberg et al., 1998), as the infection process is dependent on the spore being attached to the insect cuticle the process of shedding may cause inaccuracies in a DD model to predict virulence. To the best of this author's knowledge this approach has not been attempted when trying to predict the virulence of fungal isolates against DBM.

The aim of this study was to develop a simple forecast model of the virulence of EPF to groups of DBM larvae under fluctuating temperature conditions, to advance the understanding of the thermal biology of EPF. The component objectives were as follows:

i. Quantify the effect of temperature on colony extension and germination of EPF, development rate of DBM and virulence of EPF to DBM.
ii. Compare different statistical regression models for suitability in describing the effect of temperature colony extension and germination of EPF, development rate of DBM and virulence of EPF to DBM.
iii. Use the most appropriate model from (ii) to develop a DD style model, similar to those used to predict pest development rate in field, to predict the virulence EPF isolate in conditions of fluctuating temperature.

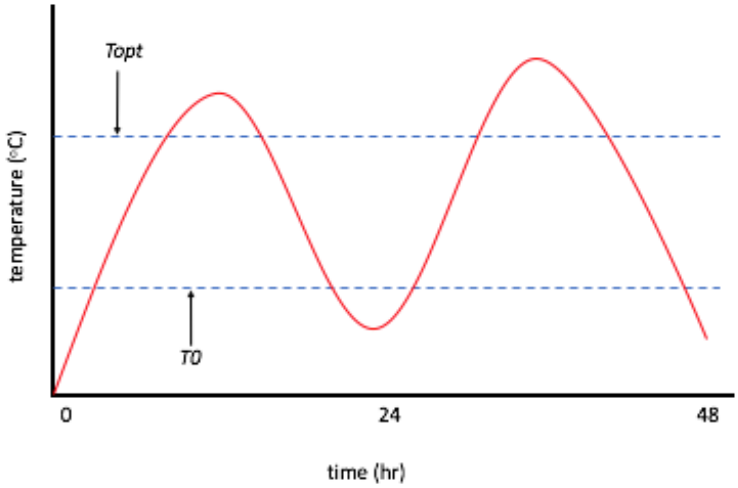


Figure 4-1: The sinusoidal pattern of temperature variation assumed by the Met office formulae for calculating accumulated DD. DD are only accumulated when temperatures are above *T0* and below *Topt*.

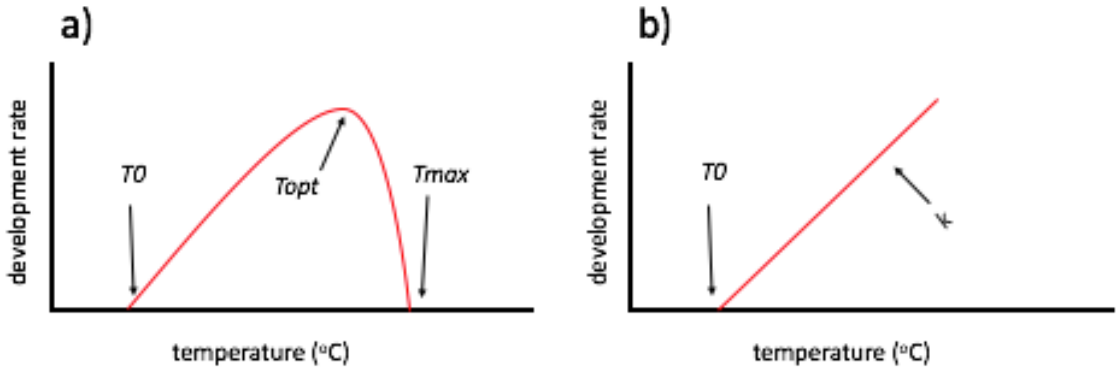


Figure 4-2: Two approaches to using regression models to describe the relationship between insect development rate and temperature. A) A typical non-linear mathematical model describing a left-skewed distribution, cardinal temperatures *T0*, *Topt* and *Tmax* can be estimated from the model. B) A linear regression model fitted to temperatures up to *T0*, parameters *T0* and *k* can be estimated from the model, for use in a DD model.

4.2 Materials and methods

4.2.1 Quantifying the effect of temperature on proportion germination and colony extension rates of candidate EPF isolates

4.2.1.1 Quantifying the effect of temperature on the colony extension rate of EPF isolates

Colony extension rates of isolates were assessed using the procedure described in 2.7.1. Colony extension rates were assessed for all candidate isolates at a range of different temperatures (10, 15, 20, 25, 30 and 33°C) in darkness. The experiment was completed as a block design; with one comprising of 84 Petri dishes (14 isolates x six temperatures). Three blocks were completed in total with each block being completed on a separate occasion.

4.2.1.2 Quantifying the effect of temperature on the proportion germination of populations of candidate EPF conidia

Proportion germination of the of populations of conidia were assessed using the procedure described in 2.7.2. Germination rates were assessed for all candidate isolates after 12 hours of incubation at a range of different temperatures (10, 15, 20, 25, 30 and 33°C, darkness). The experiment was completed as a block design; one block comprised of 84 Petri dishes (14 isolates x six temperatures). Three aliquots of 20µl were pipetted onto each Petri dish. The number of germinated and ungerminated conidia, from a group of no less 100 conidia, were counted from each aliquot of 20µl. In one block, 252 aliquots were counted in each block (three aliquots x 14 isolates x six temperatures). Three blocks were completed in total with each block being completed on a separate occasion.

4.2.2 Quantifying the effect of temperature of the development rate of DBM

DBM larvae were cultured as in 2.1. The development and mortality of DBM was assessed, from egg to adult, over seven temperatures (12.5, 15, 20, 25, 27.5, 30 and 35°C). A three to four weeks old Brussels sprout plant (variety Doric, Elsoms Seeds Ltd, Lincoln, UK) was placed in a small cage (32.5x32.5x32.5cm, Watkins and Doncaster Ltd, Herefordshire, UK) which was kept in a controlled environment room (20^{o}C, 16:8 LD). The cage was flooded with approximately 200 DBM adults over night (~15 hours), to obtain a fixed age population of eggs. 100 eggs per temperature were transferred onto a 3cm diameter leaf disc in a petri dish (3cm, Merck) (containing water agar (14g L^{-1}) to prevent leaf drying) kept at the desired temperature (16:8 hr) maintained with in a Gallenkamp incubator. The petri dish was unsealed, and the lid was modified to provide aeration. Eggs were checked for viability every 24hrs after being laid, if neonatal larval could be seen within the egg using a microscope the egg was assumed to be viable. When viability was easily observed, 30 viable eggs per temperature were transferred to an individual petri dish (3cm, Merck) containing a leaf disc and water agar, which was modified for aeration. Leaf discs were changed every three to five days for the duration of the experiment. Larvae were observed daily and transition between the following life stages were recorded; egg, first instar, second instar, third instar, fourth instar, pre-pupation, pupation and adult. Head capsule size (Table 4-1), appearance of exuvia upon the leaf surface, and the visible darkening of the larvae cuticle directly after moult were used to indicate transition from one instar to the next (Golizadeh et al., 2007, Marchioro and Foerster, 2011). Mortality was also recorded daily, for the duration of the experiment (until the emergence of adult).

Stage of Life Cycle	Mean larvae length (mm)	Mean larval head capsule width (mm)
First instar	1.7	0.16
Second instar	3.5	0.25
Third instar	7.0	0.37
Fourth instar	11.2	0.61

Table 4-1: Mean larvae length (mm) and head capsule width (mm) of larvae taken from the first, second, third and fourth instar (taken from (Harcourt, 1954)).

4.2.3 Determining the effect of temperature on the virulence of selected candidate isolates against groups of DBM larvae

The virulence of isolates was assessed using the procedure described in 2.4. *B. bassiana* 433.99 and 1757.15 and *M. brunneum* 275.86, 445.99 and 1760.15 were applied at a concentration of $1x10^6$ conidia ml^{-1} and kept at one of six temperatures for the duration of the seven day experiment: 10, 15, 20, 25, 30 and 35°C. *M. brunneum* 445.99 and 1760.15 were kept at additional temperature: 36.5°C. For each temperature, a corresponding control was included, which consisted of larvae being treated with 0.05% Triton X-100. The experiment was completed according to a randomized block design. Six blocks of the experiment were completed. Each block consisted of the virulence of all isolates being determined at three temperatures. Each temperature/isolate combination was repeated three times, with mortality of 30-45 larvae being assessed for each temperature/isolate combination (three replicate Petri dishes of 10-15 larvae).

4.2.4 Assessment of virulence of candidate EPF isolates over three variable temperature regimes

Assessment of virulence of four isolates (*B. bassiana* 433.99 and 1757.15 and *M. brunneum* 275.86, 445.99 and 1760.15) was assessed at three variable temperature regimes using the bioassay procedure described in 2.4. Temperature regime one (referred to here as 9-15) began at 9°C and increased by 1°C every 2hr until 15°C was reached at 12hr, the temperature then dropped by 1 °C every 2hr until 10 °C was reached at 24hr. This 24hr cycle was repeated for the seven days of the experiment. Temperature regime one (referred to here as 19-25) began at 19°C and increased by 1°C every 2hr until 25°C was reached at 12hr, the temperature then dropped by 1 °C every 2hr until 19 °C was reached at 24hr. This 24hr cycle was repeated for the seven days of the experiment. Temperature regime three (referred to here as 29-35) began at 29°C and increased by 1°C every 2hr until 35°C was reached at 12hr, the temperature then

dropped by 1 ^{o}C every 2hr until 28 ^{o}C was reached at 24hr. This 24hr cycle was repeated for the seven days of the experiment (Fig. 4-3).

The virulence of all isolates was assessed at 9-15^{o}C. *B. bassiana* 433.99 and 1757.15 and *M. brunneum* 445.99 and 1760.15 were assessed at 19-25, *M. brunneum* 445.99 and 1760.15 were assessed at 29-35. All isolates were applied at 1x10^{6} conidia ml^{-1} using the procedure detailed in 2.4. Experiments involving the three temperature regimes were completed separately. Three replicates of the experiment were completed and appropriate controls were used.

4.2.5 Statistical analysis

4.2.5.1 Preparation of EPF germination, colony extension rate, DBM development rate and EPF virulence data at various temperatures

For colony extension data, for each time point the diameter of the mycelial mass was calculated by taking the mean of the two diameter measurements. This figure was then halved to get the radius of the mycelial mass. The radial rate was calculated by plotting mycelial mass radius against time for each EPF isolate. A linear regression model was used to obtain the radial rate.

For germination data, percentage germination of populations of EPF conidia were calculated from numbers of germinated and ungerminated conidia after 12hr incubation at one of six temperatures.

For DBM development data, the time in days between particular developmental stages (for example, egg and adult), was divided by 1, to produce the development rate. The mean development rate was taken from three replicates of the experiment.

For EPF virulence data, mean mortality of groups of DBM larvae was taken from day six of the experiment. Mortality was analysed in two forms: un-corrected mortality data

and corrected mortality. Mortality was analysed using Schneider-Orelli's formulae as described in 2.8.

4.2.5.2 Modelling the effect of temperature on EPF germination and colony extension rate, insect development and EPF virulence

The aim of this part of the study was to describe the relationship between temperature and the various dependent variables recorded (germination proportion and colony extension rate of candidate isolates, development rate of DBM, and virulence of selected candidate EPF isolates) using five non-linear models. The suitability of each model could then be compared AIC values and r^2 so that one model could be used to describe the effect of temperature on each dependent variable. Models were fitted to data in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package Minpack.lm (version 1.2-0).

Five non-linear models were chosen which were suited to describing a bell-shaped distribution, which is often seen in distributions of biological processes over temperature. It was also important that model parameters had biological meaning. For example, the following cardinal temperatures were of particular importance, *T0* (lower temperature at which inactivation occurs), *Topt* (the optimum temperature) and *Tmax* (upper temperature at which inactivation occurs). The following five non-linear models were chosen for comparison: a fourth order polynomial model, a model proposed by Briere et al (1999), a model proposed by Logan et al. (1976), a model proposed by Taylor et al. (1981) and a model proposed by Lactin et al. (1995). Additionally, a linear regression model was used to describe the relationship between temperature and DBM development (up to the thermal optimum for development).

A linear regression model is regularly used to describe the relationship between temperature and insect development rate:

$$y = a + bT \tag{5}$$

Parameters *a* and *b* represent point at which the linear regression model crosses the vertical axis, and the slope of the linear regression model, respectively. Parameters *T* and *y* are temperature and development rate, respectively.

The Briere-1 model proposed by Briere et al. (1999) had the equation:

$$y = aT(T-T0)(Tmax-T)^{1/m} \quad (6)$$

The model is effective is describing slow increase in the dependent variable at low temperatures, before a sharp decline is observed. The model has been used a number of times to describe the thermal profile of DBM development. Parameters *T* and *y* are temperature and the dependent variable, respectively. *T0* is the lower temperature thresholds and *Tmax* is the upper temperature threshold. Parameter *a* is equational constant which allows the maximum value of the dependent variable to be adjusted. Parameter *m* is an equation constant which is set to two. *Topt* was derived using the equation provided by the author:

$$Topt = \left[2mTmax + (m+1)T0\right] + \sqrt{4m^2Tmax^2 + (m+1)^2T0^2 - 4m^2T0Tmax} \Big/ 4m + 2 \quad (7)$$

The Lactin-1 model proposed by Lactin et al. (1995) had the equation:

$$y = e^{(pT)} - e^{(pTmax-(Tmax-T/\Delta)} \quad (8)$$

The Lactin-1 model is effective in describing the rate of insect development over a large temperature range, and has been used to describe the thermal development profile DBM in the past. Parameters *T* and *y* are temperature and the dependent variable, respectively. *Tmax* is the upper developmental threshold. Parameter *p* is constant which defines the rate at the optimum temperature. Parameter Δ is the number of degrees above *T* at which temperature inhibition becomes the overriding influence. *T0* is not

estimated in this model as the curve does not intercept the horizontal axis. *Topt* was calculated by subtracting Δ from *Tmax* (Roy et al., 2002).

The Logan-1 model proposed by Logan et al. (1976) had the equation:

$$y = \Psi(e^{(pT)}-e^{(pTmax-(Tmax-T)/\Delta T)}) \quad (9)$$

The Logan-1 model has been to describe the thermal development profiles of insect development and EPF physiology. Parameters *T* and *y* are temperature and the dependent variable, respectively. Parameter Ψ represents the rate of the dependent variable at the base temperature. Parameter *p* is analogous the Q10 temperature coefficient. The Q10 temperature coefficient represents the rate of change of biological system as consequence of increasing the temperature of the system by 10^{o}C. *Tmax* in the thermal maxima, and ΔT is the number of degrees above the base temperature at which temperature inhibition occurs. *Topt* was calculated by subtracting ΔT from *Tmax* (Roy et al., 2002).

The Taylor model described by Taylor et al. (1981) had the equation:

$$y = Rm^{(-0.5[(T-Topt)/TJ2)} \quad (10)$$

The Taylor model is less suited to describing a sharp decline in rate at higher temperatures. It has been used to describe the relationship between temperature and development rate for DBM. Parameters *T* and *y* are temperature and the dependent variable, respectively. *Topt* is the optimal temperature of the dependent variable. Parameter *Rm* is the value of the dependent variable at *Topt*.

The fourth order polynomial model had the equation:

$$y = a(T)^4+b(T)^3+c(T)^2+d(T)+e \quad (11)$$

Polynomial models are based on a normal distribution, and less suited to describing a sharp decline in rate at high temperatures. The model has been used to describe DBM development over temperature. Parameters *T* and *y* are temperature and the dependent variable, respectively. Parameter *e* represents the point at which the model intercepts the horizontal axis. Parameters *b, c, d,* and *e* are the regression coefficients.

Comparison of the goodness of fit of models was completed by using r^2 values and Akaike information criterion (AIC). R^2 values are representation of how much of the variation within the data is explained by the statistical regression model. Values for r^2 values are between 0 and 1. The closer data points are to the statistical regression line, the closer to 1 the value of r^2 is. The r^2 was calculated using the following the equation:

$$r^2 = \frac{\sum(yi - ya)^2}{\sum(xi - ya)^2} \tag{12}$$

Where *yi* is equal to the observed values of the dependent variable*, ya* is the mean of the observed values, and *xi* is the value fitted by the statistical regression model.

In simple terms, an AIC value reflects the variation of the dataset explained by the statistical regression model as well as the complexity of the model. When two or more models are compared, the model with the lowest AIC is considered to be the most appropriate in explaining the data set. AIC is calculated using the following equation:

$$AIC = 2k + n\,Log(\frac{RSS}{n}) \tag{13}$$

Where *n* is the sample size, and *k* is the number of parameters of the model*. RSS* is the residual sum of squares and can be defined as the sum of squares of differences between predicted and observed values.

4.2.5.3 Estimating starting parameters for non-linear mathematical models

Non-linear statistical models like Briere-1, Lactin-1, Logan-6, and Taylor need accurate stating values for the equation-solving procedure to complete. Table 4-2 shows how starting parameters were estimated for each model parameter.

Table 4-2: Description of how starting parameters for five non-linear models.

Model	Par.	Method for obtaining starting estimate
Briere-1	*T0*	A linear regression model fitted to data up to the temperature at which highest value of the dependent variable occurs ($Topt^{est}$) , the point at which the linear regression crossed the horizontal axis was taken as the estimate for *T0* ($T0^{est}$)
	Tmax	A linear regression model was fitted to data between the optima and the highest temperature used in the study, the point at which the linear regression crossed the horizontal axis was taken as the estimate for *T0* ($Tmax^{est}$)
	a	$\frac{y}{T(T - T0^{est})\sqrt{Tmax^{est}} - T}$
Lactin-1	Δ	$Tmax^{est}$ - $Topt^{est}$
	Tmax	A linear regression model was fitted to data between the optima and the highest temperature used in the study, the point at which the linear regression crossed the horizontal axis was taken as the estimate for *Tmax* ($Tmax^{est}$)
Logan-6	Ψ	The value of the dependent variable at the lowest temperature in the experiment (Ψ^{est}).
	Tmax	A linear regression model was fitted to data between the optima and the highest temperature used in the study, the point at which the linear regression crossed the horizontal axis was taken as the estimate for *Tmax* ($Tmax^{est}$).
	ΔT	$Tmax^{est}$ - $Topt^{est}$
Taylor	*Rm*	The highest value of the dependent variable recorded (Rm^{esr})
	Topt	The temperature at which highest value of the dependent variable occurs ($Topt^{est}$).

4.2.5.4 Developing a DD models to predict rate of DBM development in the field and virulence of selected EPF isolates against groups of DBM larvae

A major aim of this study was to develop a model which could predict the proportion mortality caused in groups of DBM by selected EPF isolates in conditions of fluctuating temperature. To do this DD approach was used. DD models are regularly used to predict the rate of DBM development in the field, as well as many other crop pests.

First, a DD model was developed, using data collected using the methods from 4.2.3, to predict the rate of development of DBM in the field. The method for developing a DD model was taken from the literature (Fig. 4-4). Predictions made from this model were validated using field data.

Second, a day-degree style model was developed to predict the proportion mortality conferred by selected EPF isolates against groups of DBM larvae. Cardinal temperatures required for this analysis were taken from the best fitting model from 4.2.5.2. Predictions made from this model were validated using experimental data.

4.2.5.4.1 *Developing a DD model to predict the rate of DBM development rate in the field*

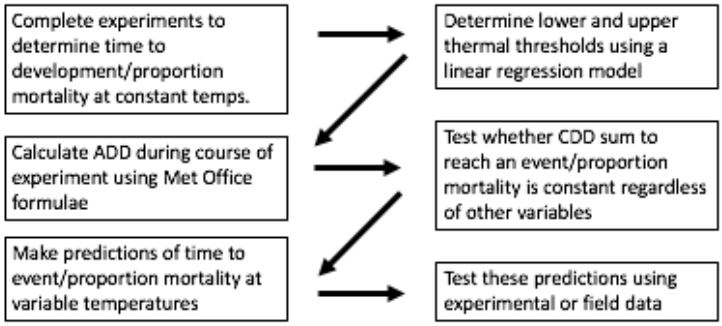


Figure 4-3: Steps taken to develop predictive DD models for DBM development and EPF virulence.

The linear model of temperature against 1/time to development (omitting data from 35^{o}C) was used to derive the lower temperature threshold (*T0*) and the thermal constant (*K*). *K* represents the number of accumulated DD necessary to achieve development. In principle, the number of accumulated DD should be constant across all various geographic locations. *K* could therefore be used to predict the time from egg lay to emergence of adult for any given location, provided temperature data were available for that area. To test this assumption, field data was taken from various sights in the UK between 1995 and 2000 (Table 4-3). Numbers of adult DBM caught in Delta pheromone traps (International Pheremone Systems, UK) were monitored weekly over the summer months. Data sets of number of DBM captured across the year in which there was "first peak" (representing a migration of DBM into the UK from mainland Europe), followed by a clear "second peak" (representing the second generation of DBM adults) were selected. Raw DBM field-catch data can be seen in Appendix II. Temperature data (comprising of daily maximum and minimum temperatures) was then used to derive the number of DD accumulated between peaks. The Met Office formulae was used to calculate accumulated DD for this period (Table 4-4). The predicted second peak was also calculated, using *K*, so that predicted and observed second peaks could be compared (Fig. 4-2).

Table 4-3: Field site location, season and weather station (from which daily maximum and minimum temperatures were taken) used to validate day degree model.

Site name (coordinates)	Season(s) used in analysis	Weather station used (miles from weather station to site)
Butterwick, Lincolshire, UK (52.9862° N, 0.0669° E)	1998	Kirton (7)
Friskney, Lincolnshire, UK (53.0763° N, 0.1804° E)	1996	Kirton (14)
Holbeach, Lincolnshire, UK (52.8040° N, 0.0134° E)	1996	Kirton (10)
Kirton, Lincolnshire, UK (52.9331° N, 0.0679° W)	1995, 1996, 2000	Kirton (<1)
Wellesbourne, Warwickshire, UK (52.1966° N, 1.5896° W)	1996	Wellesbourne (<1)

Table 4-4: Met Office equation used to calculate accumulated day degrees using upper and lower thermal thresholds (Met Office, 2017).

Temperature condition	DD value
Tmax $\leq$ *T0*	0
Tmin $\geq$ *T0*	*Tmean – T0*
Tmean $\geq$ *T0* & *Tmin* < *T0*	0.5(*Tmax – T0*) – 0.25(*T0 – T0*)
Tmean < *T0* & *Tmax* > *T0*	0.25(*Tmax – T0*)

4.2.5.4.2 Developing a DD model to predict the proportion mortality of groups of DBM larvae after being treated with selected candidate EPF isolates

A DD style approach was used to develop a predictive model of the virulence of four isolates (*B. bassiana* isolates 433.99 and 1757.15 and *M. brunneum* 275.86, 445.99 and 1760.15) against groups of DBM larvae. Mortality data of groups of DBM larvae after being treated with selected isolates and incubated at one of six temperatures were used to develop the day degree model. Analysis from 4.2.5.2, in which various non-linear models were fitted to raw percentage mortality from six days after treatment, was used to select the best fitting model. This model was used to obtain cardinal temperatures for use in the DD model. The thermal minima (*T0*) and thermal optima (*Topt*) were taken from the best fitting model and used as the lower and upper thresholds for calculation of day degrees. DD would only be accumulated if temperatures lay within these boundaries. For each temperature treatment, the number of DD accumulated, from days one to six after treatment, was calculated using the Met Office formulae (Table 4-4). Percentage mortality was then plotted against accumulated DD for each temperature. A sigmoidal regression model was then fitted to the relationship between percentage mortality and accumulated DD, so that percentage mortality could be estimated on any given day of the experiment. The sigmoidal model had the equation:

$$y \sim a/(1 + \exp(-b * (x - c))) \tag{14}$$

Parameters a,b and c represented equation constants. Parameters x and y represented accumulated DD and proportion mortality, respectively.

Calculating proportion mortality in this way is dependent on two assumptions. First, it was assumed that the same linear model could be used to calculate proportion mortality (based on accumulated DD) for each day of the experiment, that is; incorporating day after treatment as a factor should not be a significantly improvement on the linear model which does not incorporate day after treatment as a factor.

Additionally, it was assumed that a certain proportion mortality would occur at the same number of accumulated DD, regardless of the temperature. For example, at 15°C the number of accumulated day degrees at six days after treatment would be equal to the number the number of accumulated DD at three days after treatment at 30°C. Consequently, you would expect there to be the same level of mortality at six days after treatment at 15°C and at three days after treatment at 30°C.

These assumptions were tested before the DD virulence model was validated using experimental data. Proportion mortality was then predicted for three variable temperature regimes (Fig. 4-4), which follow a sigmoidal curve. Experiments were then completed to test these predictions.

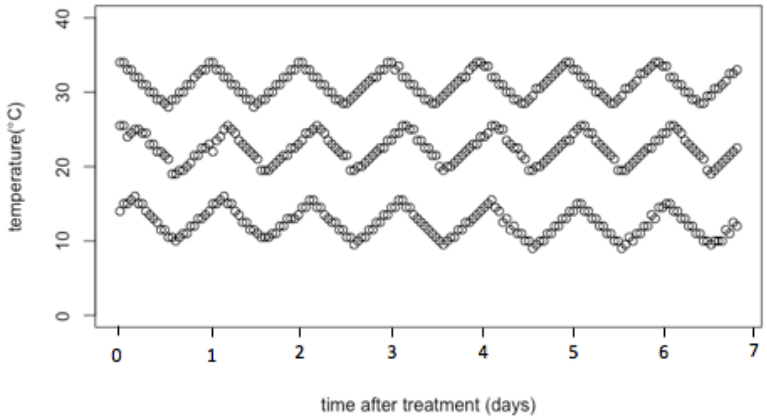


Figure 4-4: Mean temperature (recorded in real time using a Thermochron temperature sensor (OnSolution Pty Ltd, UK)) over a seven day experiment for three different temperature regimes.

4.3 Results

4.3.1 Quantifying the effect of temperature on proportion germination and colony extension rates of candidate EPF isolates

As expected there was a bell-shaped distribution of colony extension and percentage germination over temperature. These distributions were explained using five non-linear models. All models were successfully fitted to colony extension and percentage germination data for all isolates in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package Minpack.lm (version 1.2-0).

A summary table of parameters derived from the best fitting model, the Briere-1 model, for colony extension and germination experiments, can be seen in Appendix III.

4.3.1.1 Quantifying the effect of temperature on the colony extension rate of EPF isolates

Five non-linear models were fitted to colony extension rates at different temperatures for all candidate isolates. All distributions were bell shaped, and most often skewed to the left (Fig. 4-5 to 4-18).

For *B. bassiana* 433.99, 432.99, 1757.15, 1758.15, 1759.15, 11.98 and 1730.08 the best fitting models were considered to be the Briere-1 and Lactin-1 models which displayed r^2 values of between 0.76 and 0.98, and 0.79 to 0.99 for all isolates, respectively. The next best fitting model was Logan-6 which displayed r^2 values between 0.73 and 0.99. Taylor and polynomial models displayed r^2 values as low as 0.47 and 0.37 for some isolates. Briere-1 and Lactin-1 models displayed lower AIC values when compared to other models. These models had AICs in the region of -42.83 to -21.08 and -38.92 to -22.18, respectively. AIC values for polynomial, Logan-6 and Taylor models were higher is almost every case.

For *L. longisporum* 1.72 and *L. muscarium* 19.79 the Taylor model fitted the best in terms of AIC and r^2 values, probably because of the low optimal temperatures exhibited by these isolates. The Taylor model displayed r^2 values of 0.94 and 0.98, respectively. The next best fitting model was the Briere-1 model, which had r^2 values of 0.91 and 0.82. Lactin-1 and Logan-6 models had r^2 values of between 0.79 and 0.87. The Taylor model displayed the lowest AIC values, with the Briere-1 model exhibiting the second lowest (Table 4-5).

For *M. brunneum* isolates, 445.99, 275.86 and 1760.15, the Briere-1 model was considered to be the best fitting model on the basis of AIC values. Values for r^2 were relatively similar for all models, being between 0.82 and 0.91. An exception to this was the polynomial/ *M. brunneum* 1760.15 model which had an r^2 of 0.73. AIC values for the Briere-1 model were lower for *M. brunneum* 1760.15 and 445.99 when compared to all other models. The Taylor model produced a lower AIC value for 275.86, when compared to the Briere-1 model, being -27.34 and -26.64, respectively. The Taylor model was considered the second best fitting model.

For *I. fumosorosea* isolates, the polynomial and Taylor models were considered to be the best fitting, having lower AIC values and higher r^2 values when compared to the other models. The third best fitting model was considered to Briere-1, which displayed lower AIC values than the Logan-6 and Lactin-1 models (Table 4-5). Several parameters produced by models have high biological significance. Of the most important are *Topt* (produced by all non-linear models apart from the polynomial model), *Tmax* (produced by all non-linear models apart from the polynomial model) and *T0* (produced by Briere-1 and Taylor models). *Topt* is the temperature at which the highest growth rate is observed, *Tmax* is the maximum temperature at which growth can be observed, *T0* is the minimum temperature at which growth can be observed. According the Briere-1 model, *L. longisporum* and *L. muscarium* isolates displayed lower *Topt, Tmax* and *T0* temperatures when compared to other isolates. *B. bassiana*, *I. fumosorosea* and *M. brunneum* isolates displayed relatively similar *Topt, Tmax* and *T0* temperatures, being in the region of between 27 to 33^{o}C and 2 to 8^{o}C, respectively (Table 4-5).

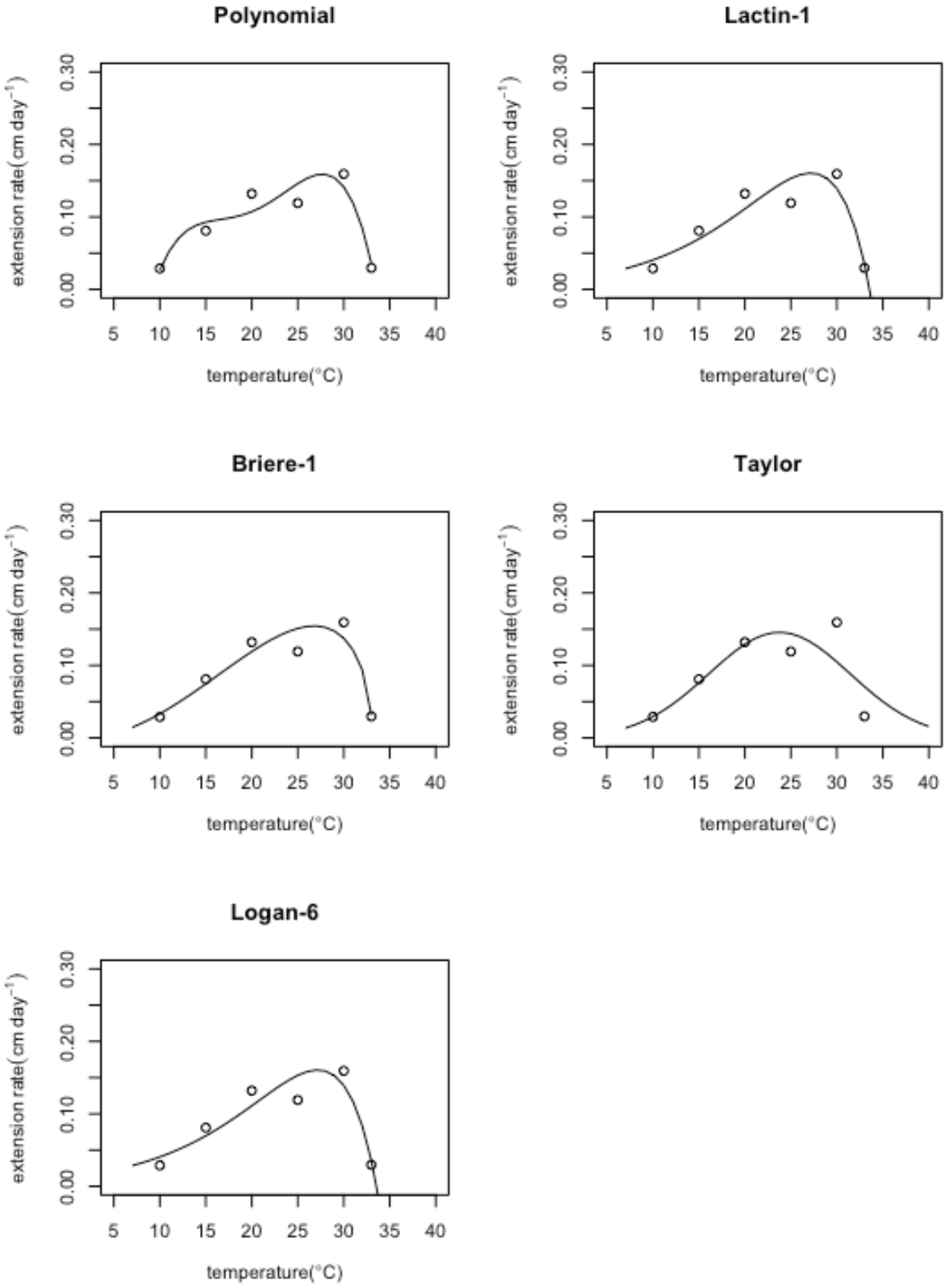


Figure 4-5: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 433.99.

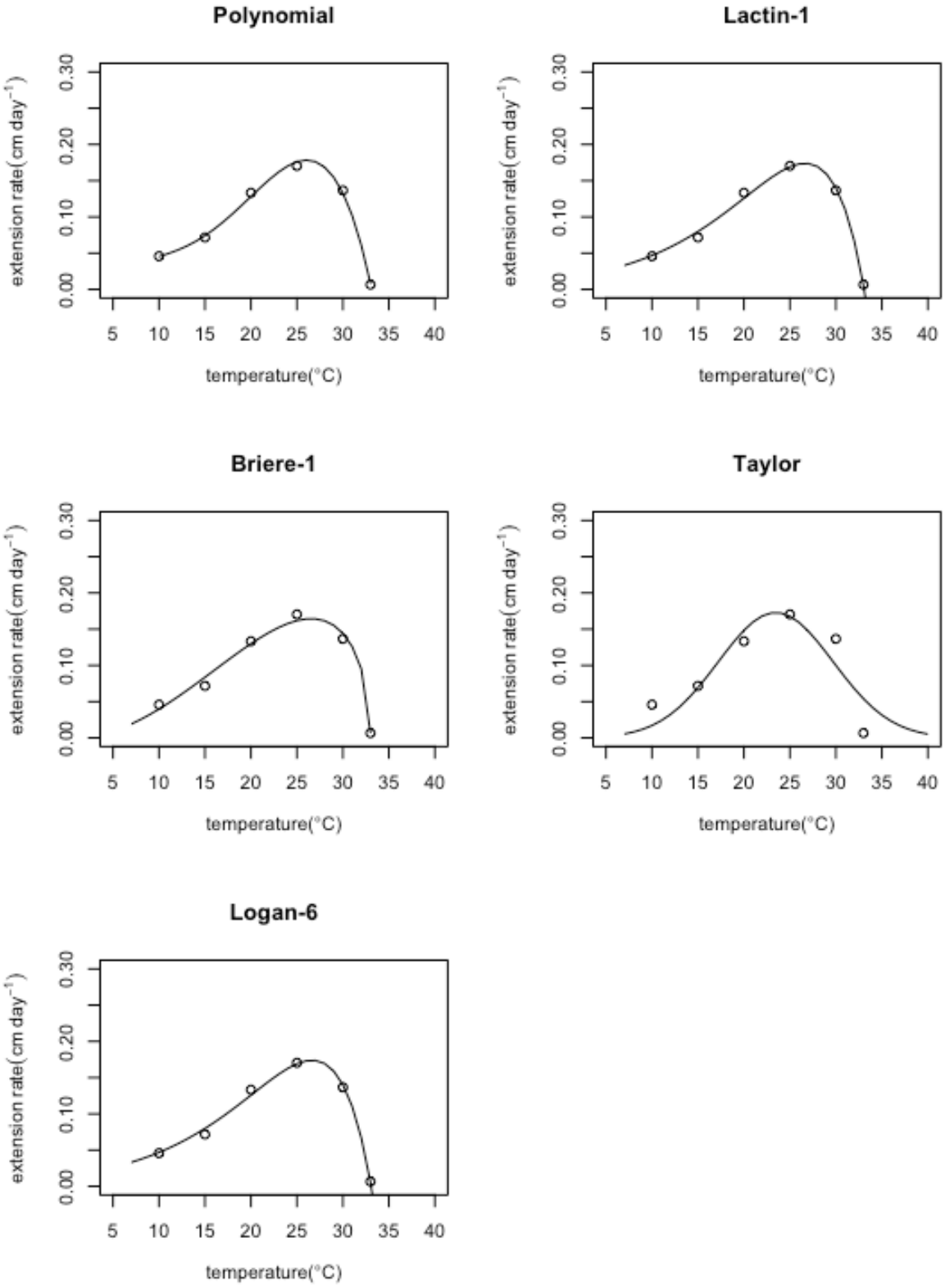


Figure 4-6: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 1757.15.

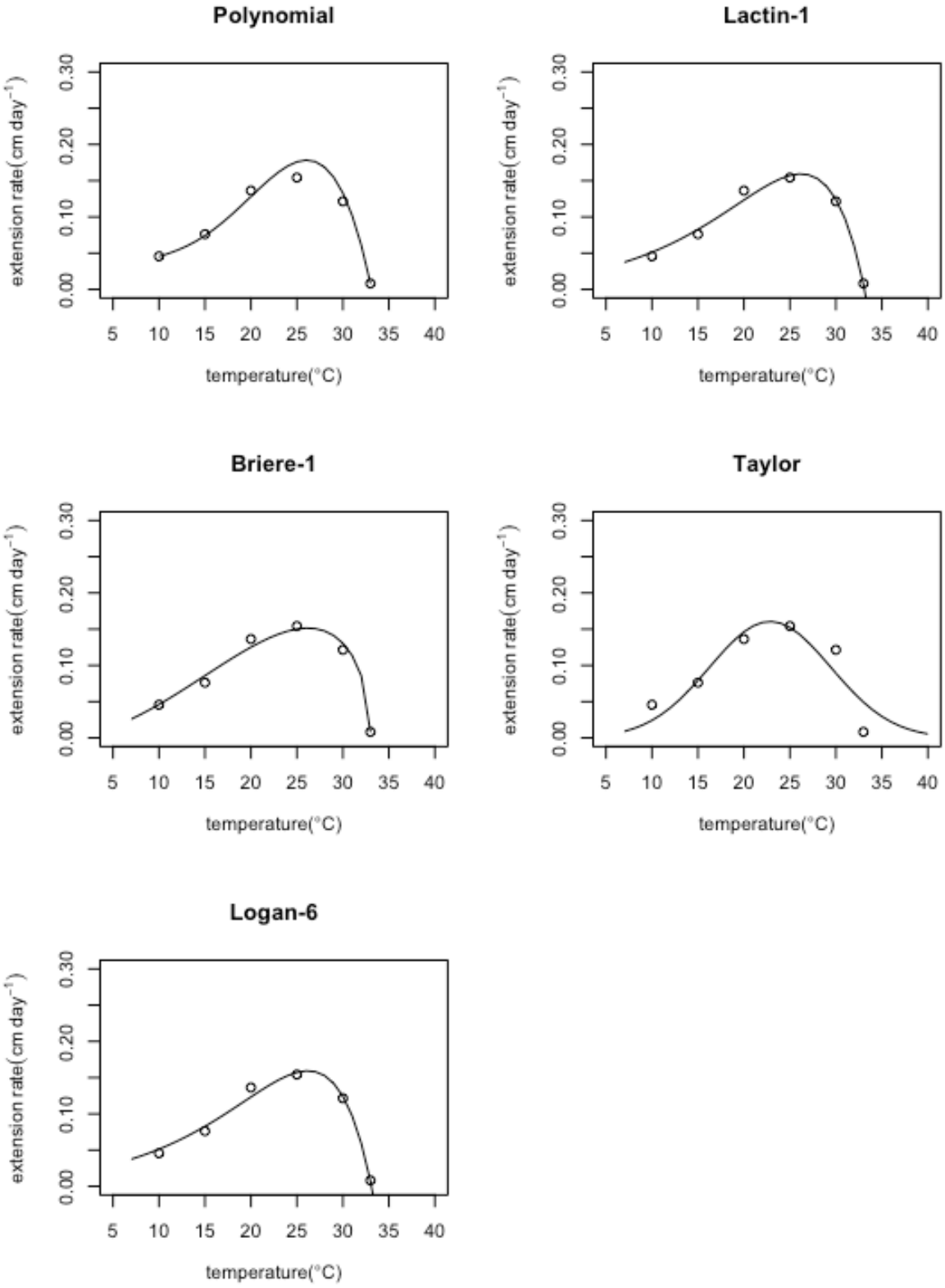


Figure 4-7: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 1758.15.

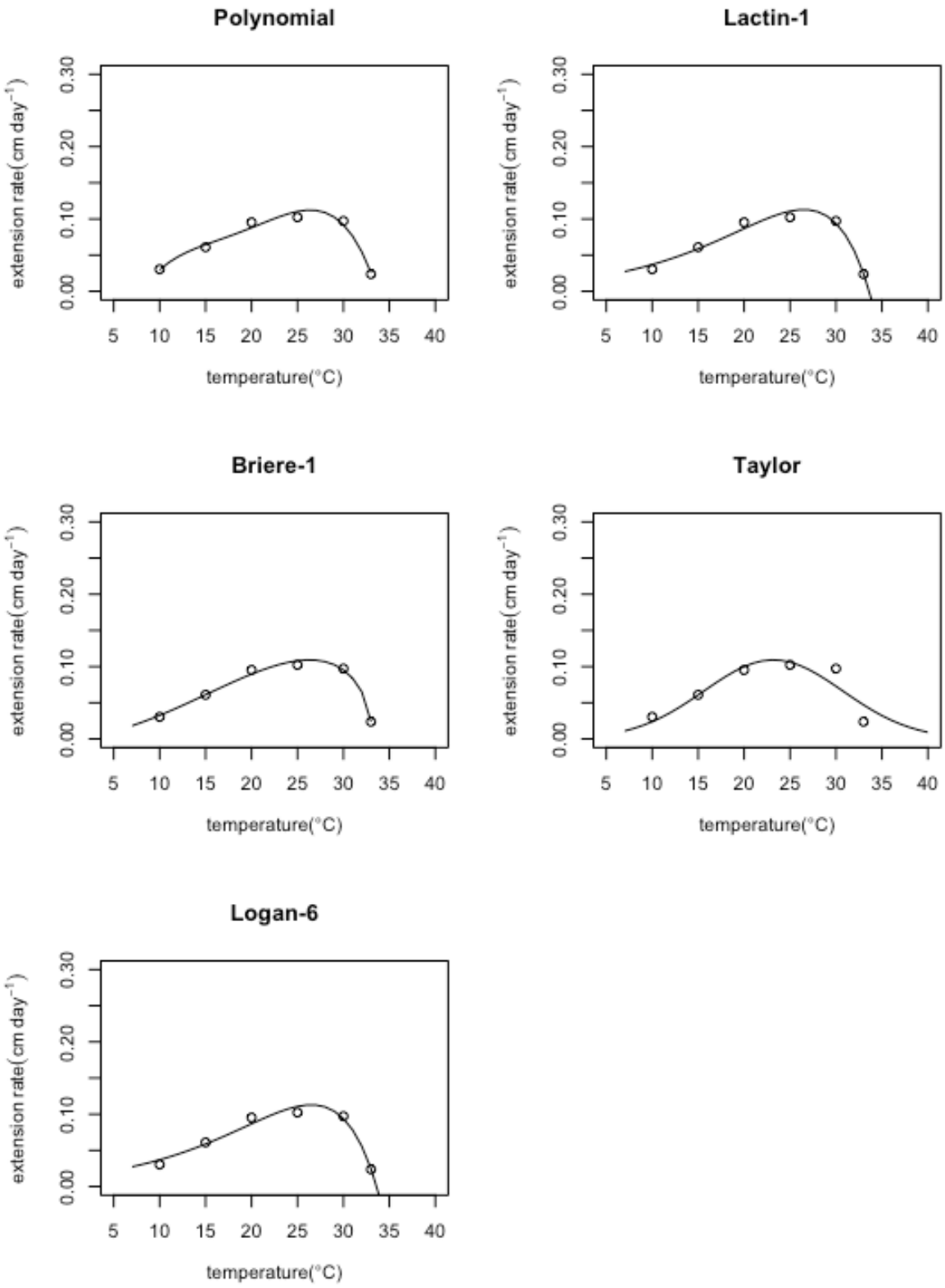


Figure 4-8: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 1759.15.

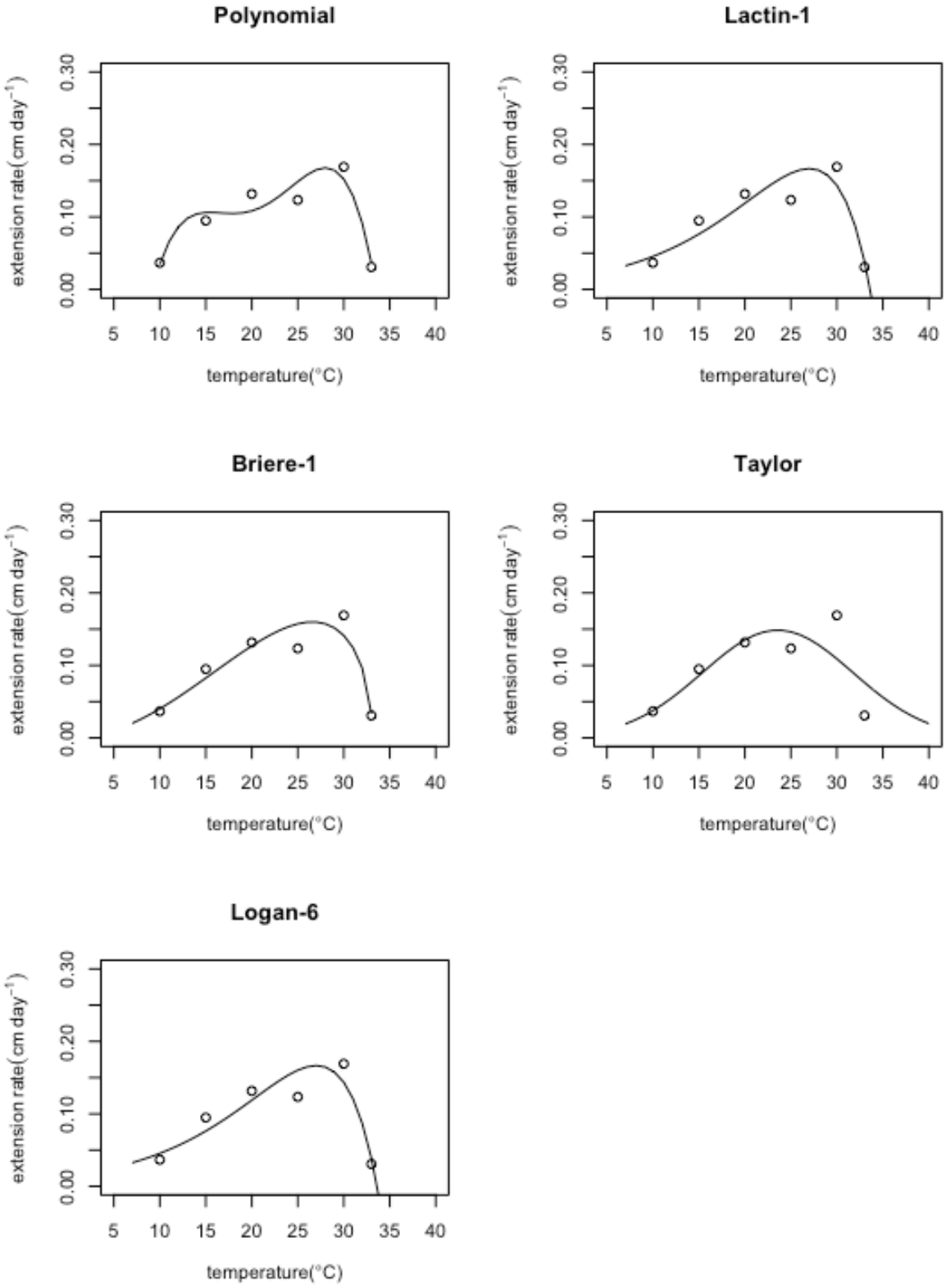


Figure 4-9: Five non-linear models fitted to mean colony extension rate (cm day-^{1}) plotted against temperatures (^{o}C) for *B. bassiana* 432.99.

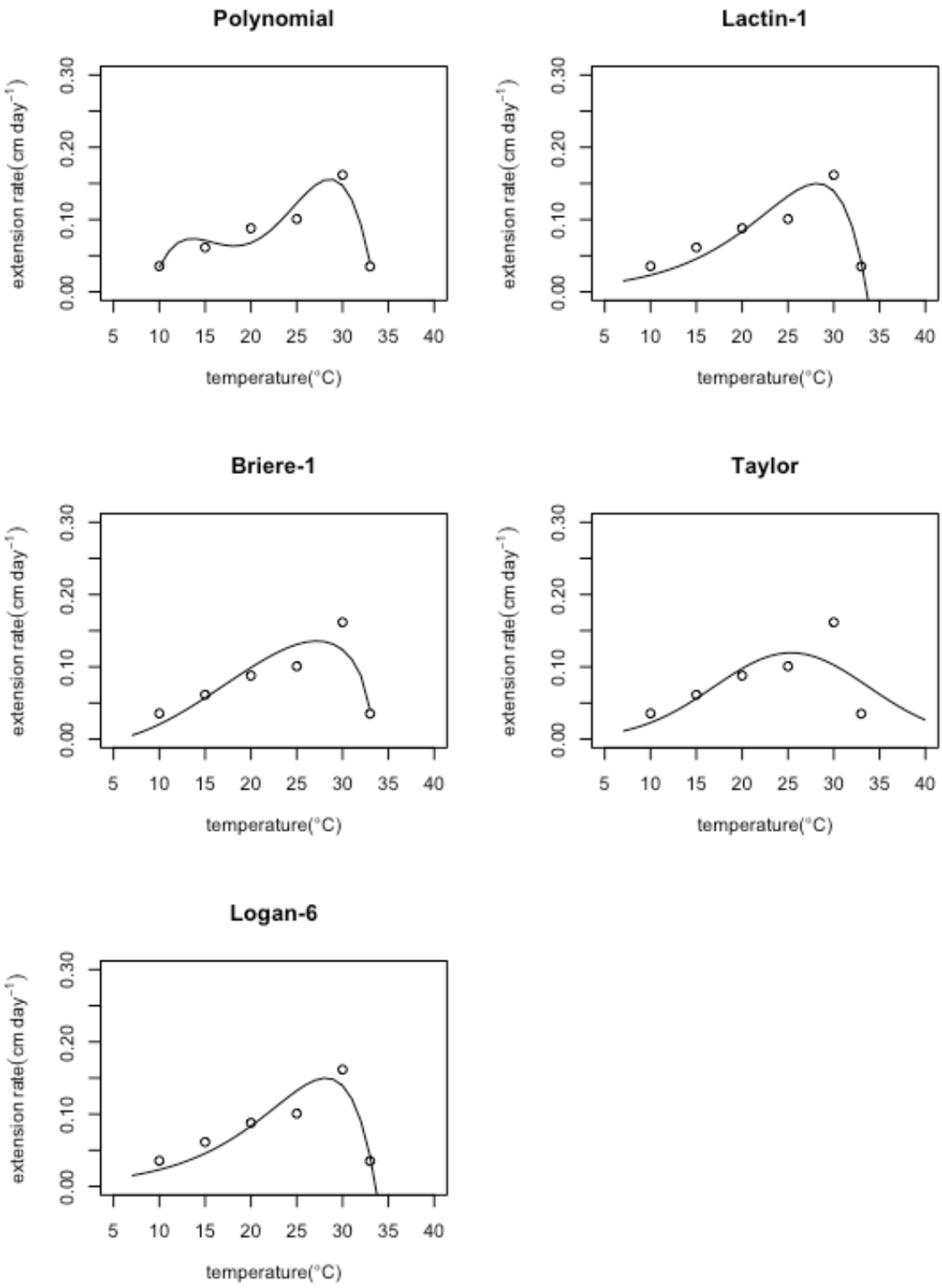


Figure 4-10: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 11.98.

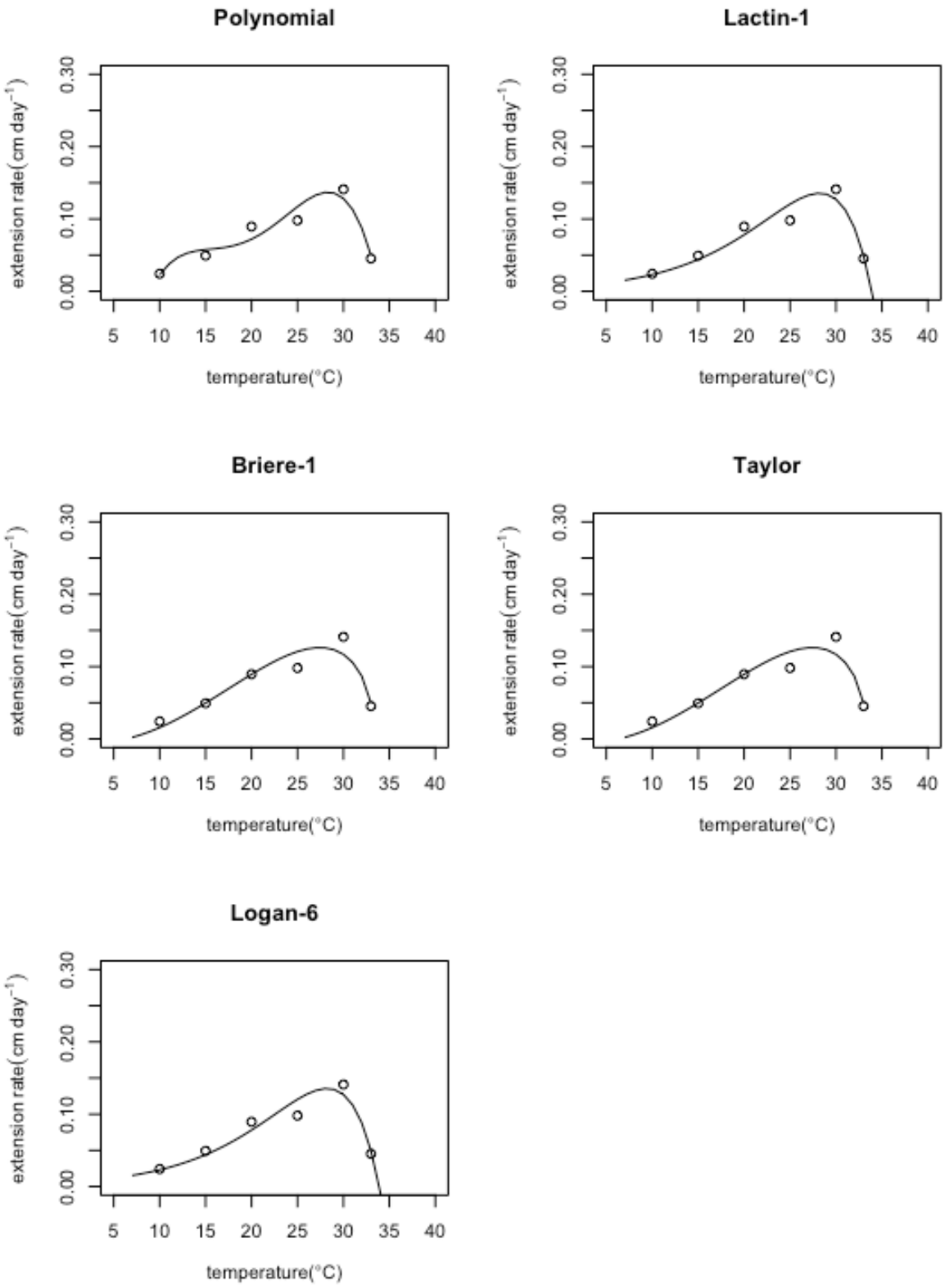


Figure 4-11: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *B. bassiana* 1730.08.

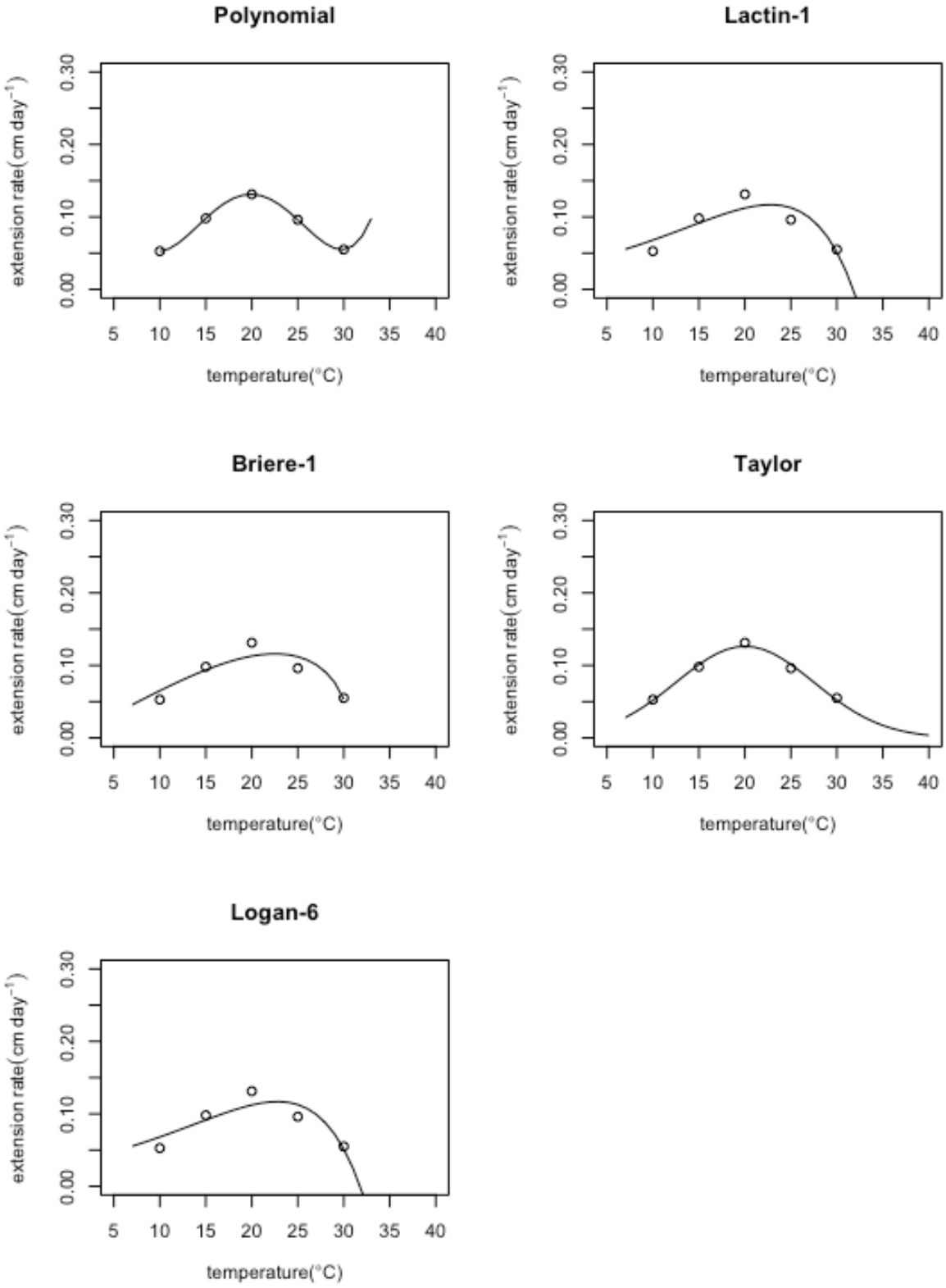


Figure 4-12: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *L. muscarium* 19.79.

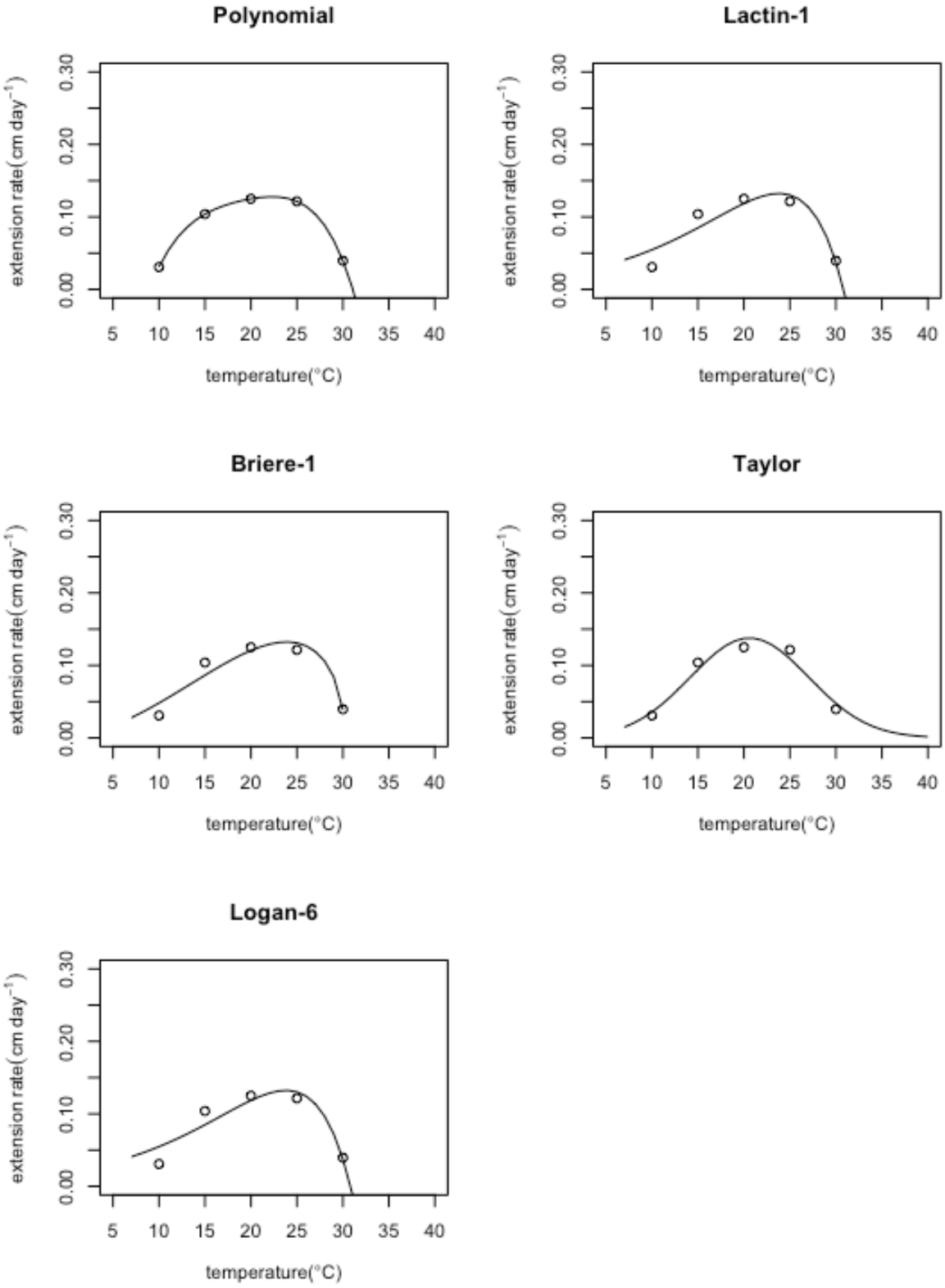


Figure 4-13: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *L. longisporum* 1.72.

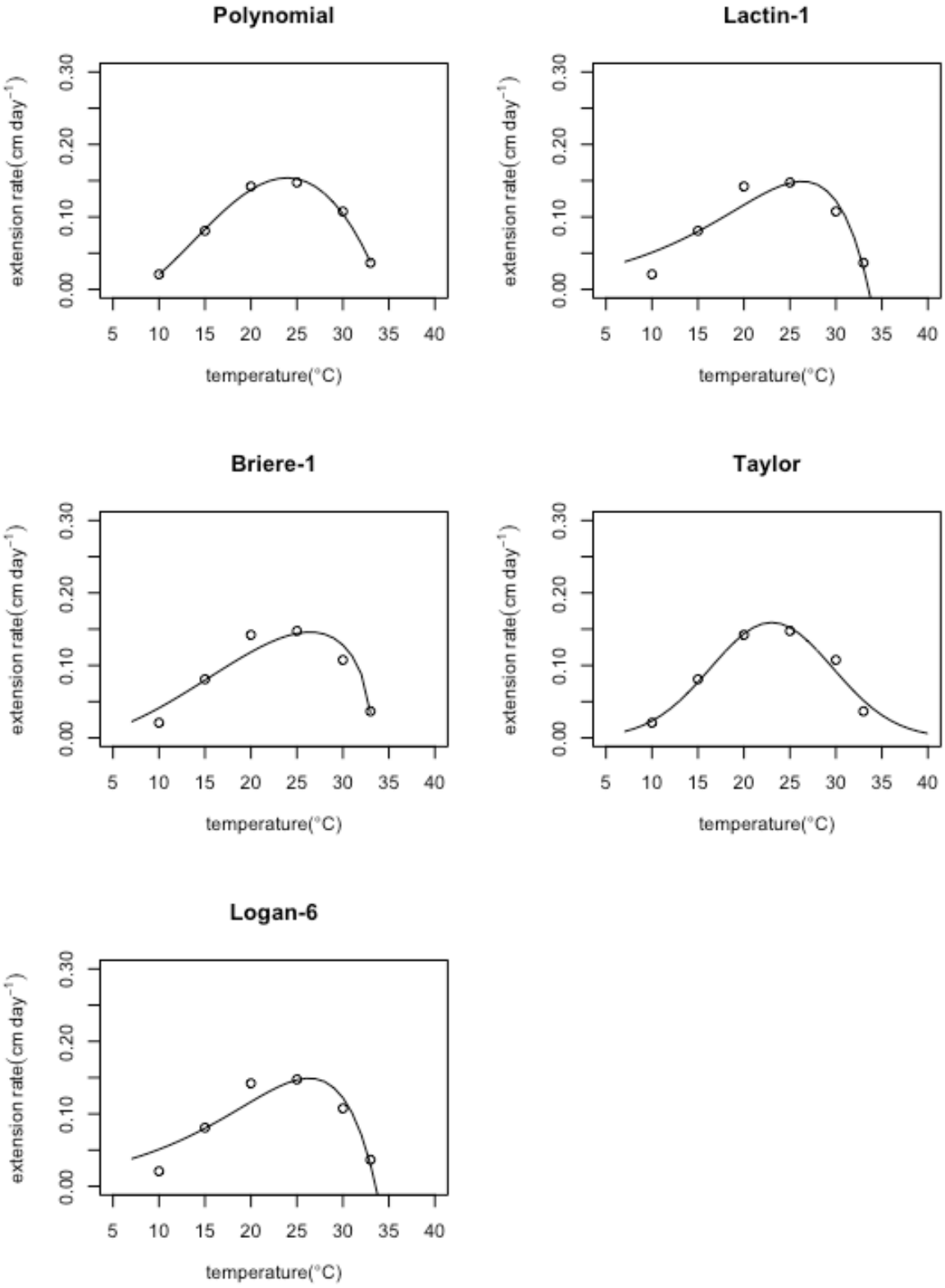


Figure 4-14: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *I. fumosorosea* 1761.15.

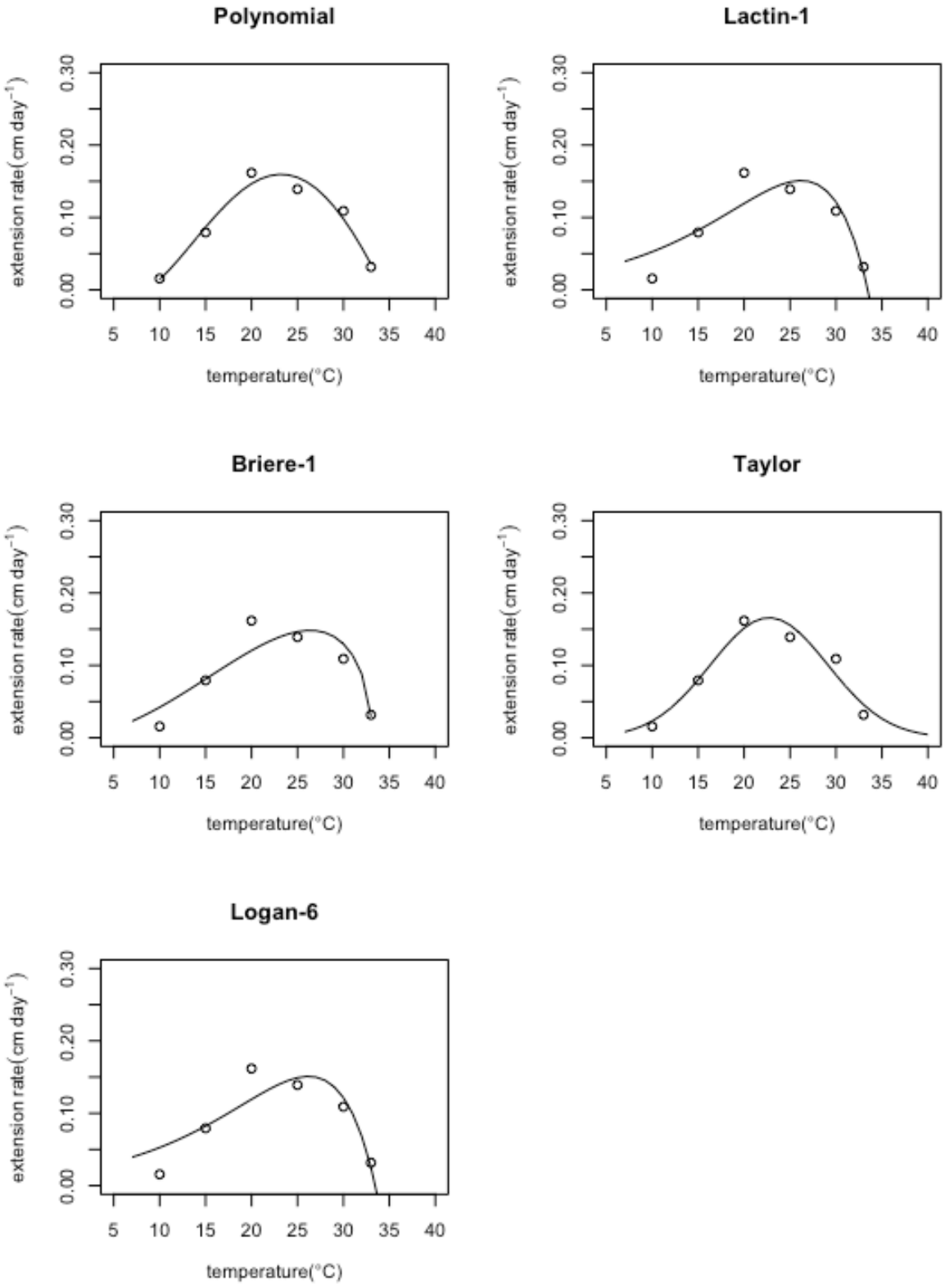


Figure 4-15: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *I. fumosorosea* 1762.15.

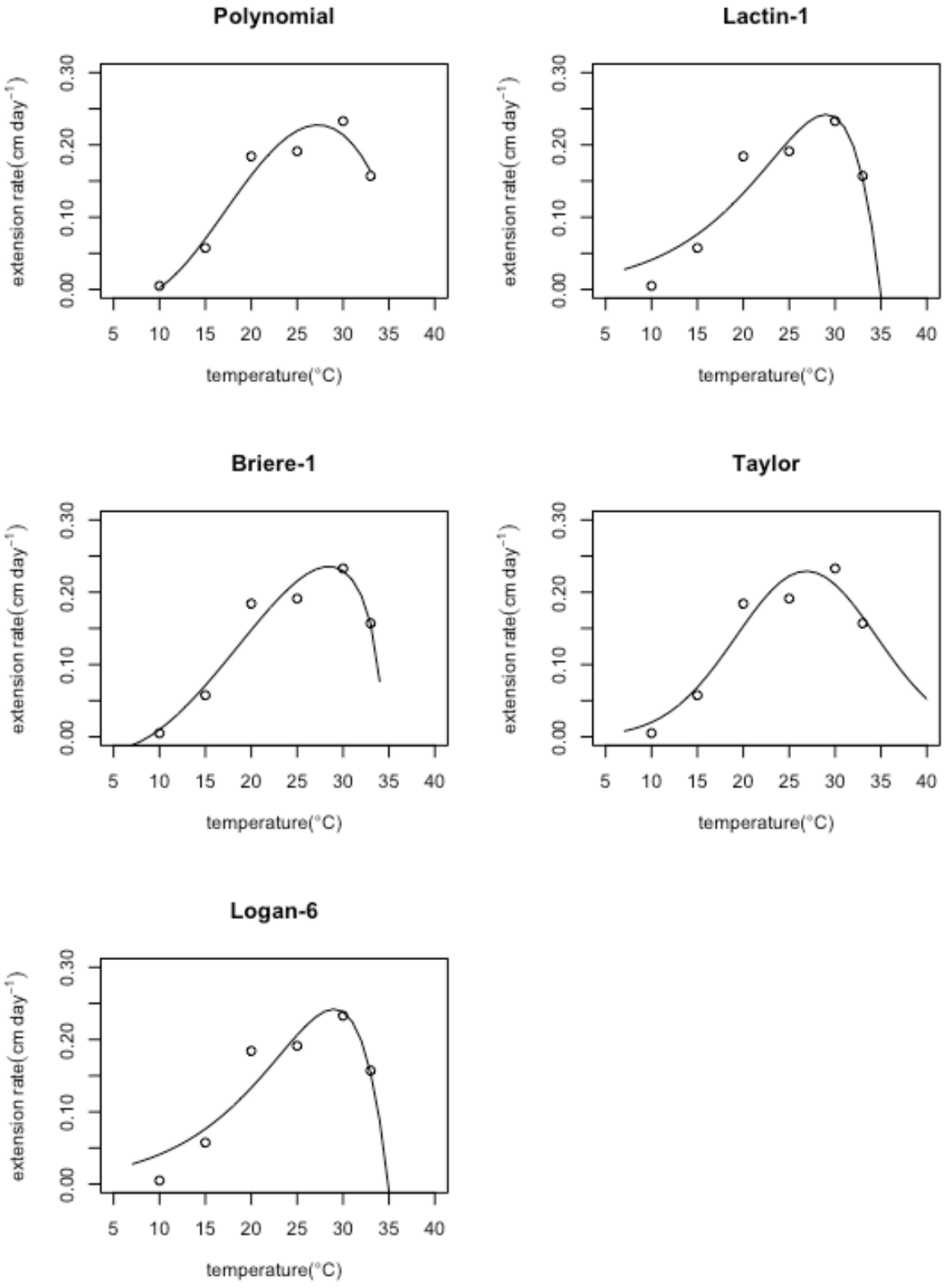


Figure 4-16: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *M. brunneum* 1760.15.

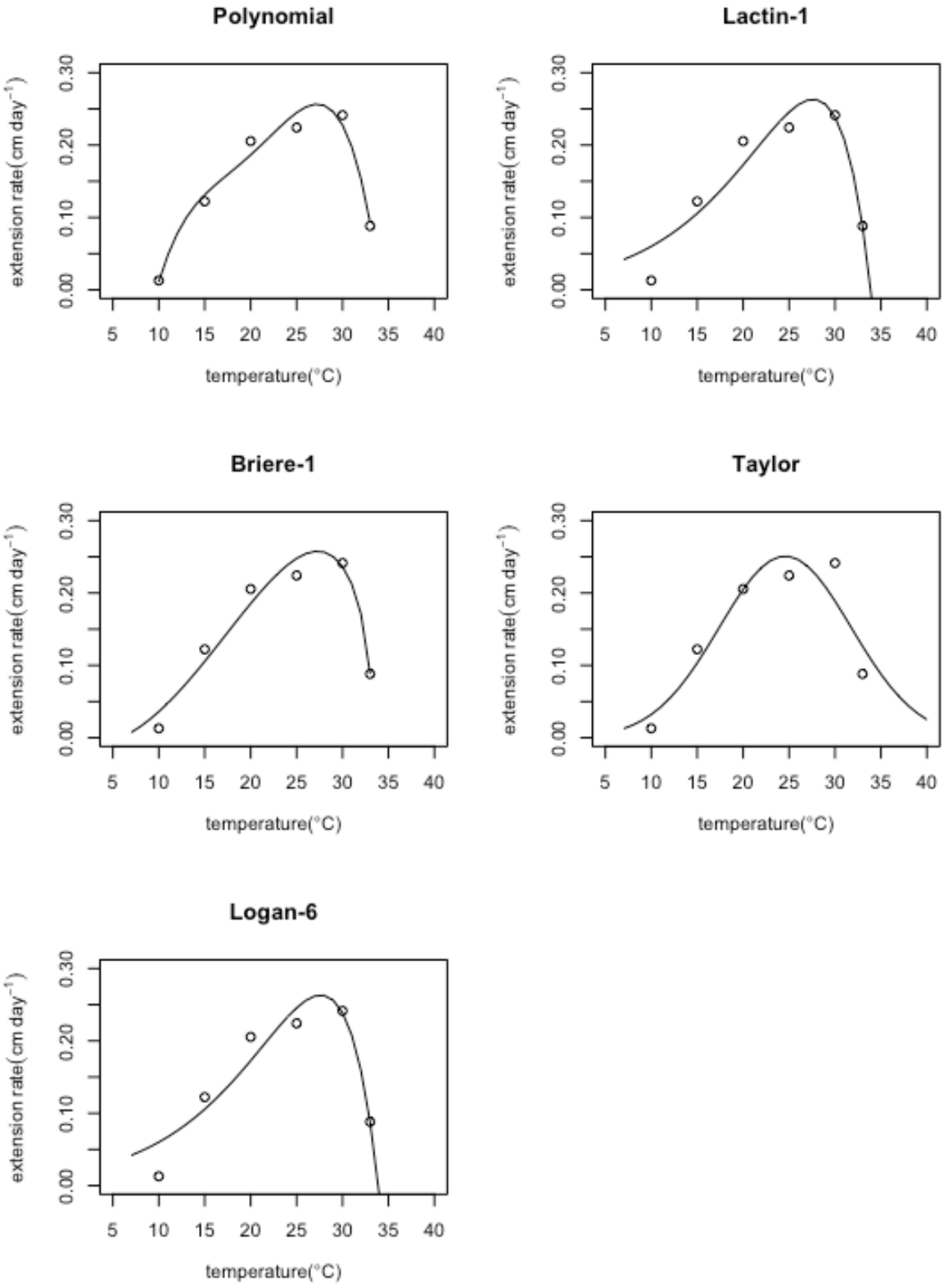


Figure 4-17: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *M. brunneum* 445.99.

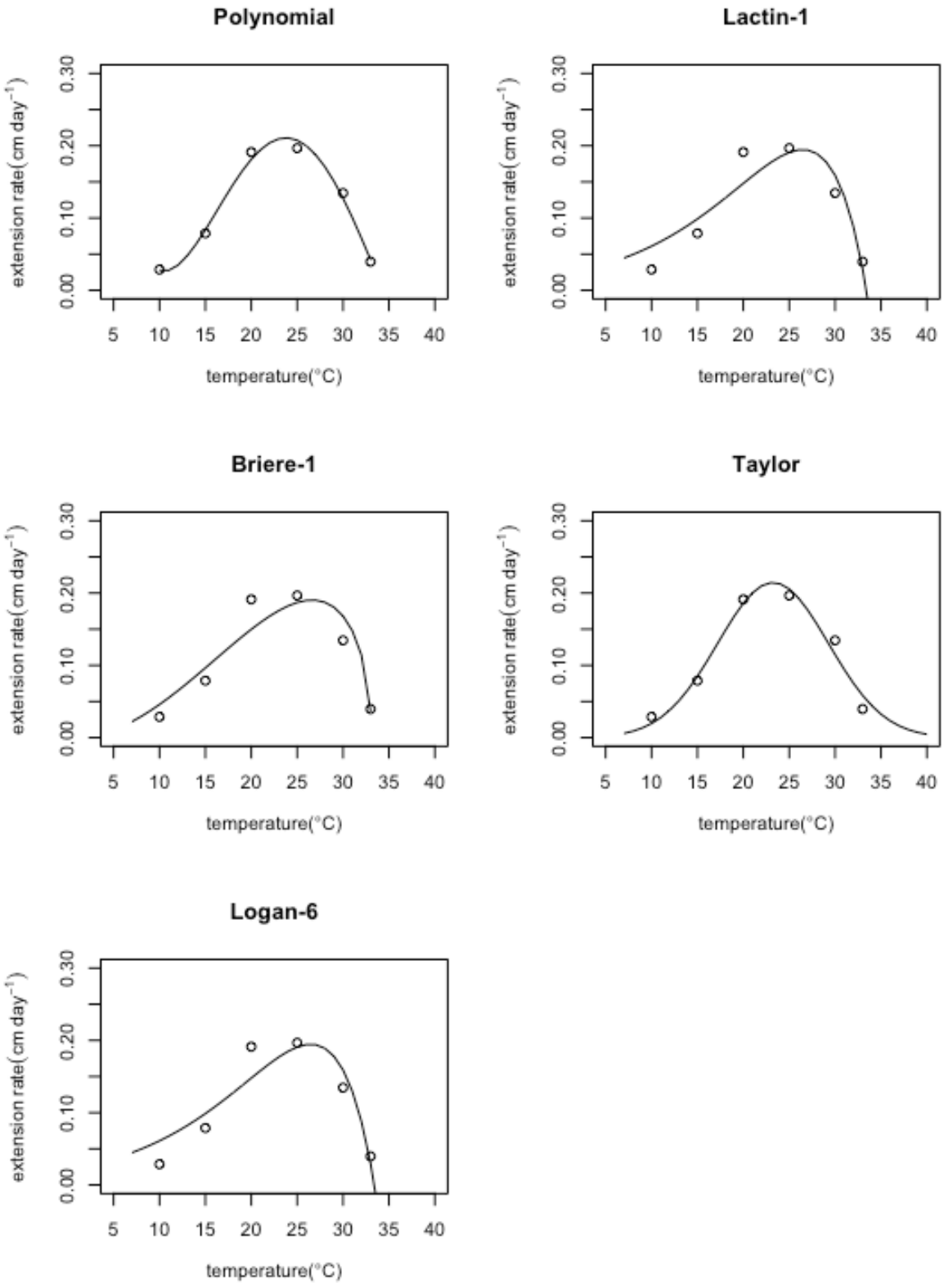


Figure 4-18: Five non-linear models fitted to mean colony extension rate (cm day^{-1}) plotted against temperatures (^{o}C) for *M brunneum* 275.86

Table 4-5: Fitted parameters, r^2 and AIC values of five non-linear models fitted to colony extension rates (cm day^{-1}) at six temperatures for 14 isolates.

		B. bassiana							*Lecanicillium* spp.		*M. brunneum*			*I. fumosorosea*	
Model	**Par.**	**1757.15**	**1758.15**	**1759.15**	**11.98**	**432.99**	**433.99**	**1730.08**	**1.72**	**19.79**	**275.86**	**445.99**	**1760.15**	**1761.15**	**1762.15**
Briere-1	***a***	9.7×10^{-5}	7.7×10^{-5}	5.6×10^{-5}	9.3×10^{-5}	9.2×10^{-5}	9.54×10^{-5}	9×10^{-5}	8.2×10^{-5}	1.3×10^{-4}	1.1×10^{-4}	1.8×10^{-4}	1.7×10^{-4}	7.75×10^{-5}	7.89×10^{-5}
	T0	1.50	-2.4	-2.10	5.46	8.86	2.77	6.38	-3.1	-0.1	1.46	5.85	8.84	-1.05	-1.1
	Tmax	33.0	33.01	33.14	33.22	33.12	33.11	33.4	30.23	26.29	33.10	33.30	34.26	33.15	33.11
	Topt	26.56	26.18	26.31	27.17	27.26	26.76	27.22	23.87	21.03	26.64	27.18	28.18	26.43	26.39
	r^2	0.98	0.98	0.99	0.76	0.86	0.88	0.87	0.91	0.82	0.87	0.96	0.94	0.90	0.83
	AIC	-33.91	-34.06	-42.83	-21.08	-22.66	-23.89	-26.14	-22.09	-21.72	-26.64	-23.65	-22.61	-25.03	-20.79
Taylor	***Rm***	0.17	0.16	0.11	0.11	0.14	0.14	0.12	0.14	0.13	0.21	0.25	0.23	0.16	0.17
	Topt	23.46	22.86	23.18	25.35	23.57	23.76	25.62	20.45	20.01	23.24	24.58	26.86	23.02	22.72
	T0	6.25	6.60	7.56	8.42	8.18	7.69	7.87	6.44	7.49	6.05	7.19	7.63	6.64	6.40
	r^2	0.76	0.80	0.80	0.47	0.58	0.64	0.67	0.94	0.98	0.96	0.86	0.93	0.96	0.93
	AIC	-17.95	-20.01	-25.99	-16.36	-16.12	-17.16	-20.62	-24.08	-33.69	-27.34	-16.80	-20.85	-31.11	-26.49
Lactin-1	***p***	0.16	0.14	0.14	0.18	0.15	0.16	0.18	0.14	0.11	0.14	0.16	0.16	0.14	0.14
	Tmax	33.07	33.11	33.62	33.63	33.62	33.57	33.89	30.82	31.76	33.41	33.89	34.91	33.59	33.48
	Topt	26.65	26.10	26.51	28.18	27.02	27.17	28.22	23.83	22.82	26.50	27.64	29.0	26.36	26.22
	Δ	6.42	6.90	7.11	5.45	6.60	6.40	5.67	6.99	8.92	6.91	6.25	5.91	7.23	7.26
	r^2	0.99	0.98	0.97	0.83	0.82	0.85	0.91	0.87	0.79	0.85	0.90	0.88	0.87	0.79
	AIC	-38.92	-35.26	-36.73	-23.11	-21.28	-22.18	-28.00	-20.14	-20.77	-18.77	-18.78	-18.13	-23.59	-19.69
Logan-6	***Ψ***	0.09	0.27	0.33	-4.6×10^{-11}	0.15	-4.6×10^{-9}	0.02	-0.27	0.30	-0.10	-0.01	-0.02	-0.04	0.05
	p	0.16	0.14	0.14	6.7×10^{-1}	0.18	5.3×10^{-1}	0.08	0.14	0.11	0.15	0.18	0.18	0.14	0.14
	Topt	26.67	26.24	26.53	32.03	27.93	31.78	32.09	23.76	22.94	26.34	26.9	28.76	26.04	26.05
	Tmax	33.07	33.11	33.62	33.3	33.33	33.28	33.38	30.82	31.76	33.41	33.87	34.90	33.58	33.48
	ΔT	6.31	6.87	7.09	1.27	5.40	1.50	1.29	7.06	8.82	7.07	6.97	6.14	7.54	7.43
	r^2	0.99	0.98	0.97	0.96	0.73	0.87	0.95	0.87	0.79	0.85	0.90	0.88	0.87	0.79
	AIC	-36.91	-33.26	-34.73	-29.13	-16.76	-21.17	-30.10	-18.14	-18.77	-16.76	-16.73	-16.12	-21.57	-17.69
Polynomial	***a***	-3.6×10^{-6}	-2.3×10^{-6}	-3.4×10^{-6}	1.25×10^{-5}	1.2×10^{-5}	-9.6×10^{-6}	-7.7×10^{-6}	-5.4×10^{-6}	7.9×10^{-6}	5.2×10^{-6}	-9.8×10^{-6}	3.9×10^{-7}	5.0×10^{-7}	1.7×10^{-6}
	b	2.1×10^{-4}	1.2×10^{-4}	2.5×10^{-4}	1×10^{-3}	9.98×10^{-4}	7.7×10^{-4}	6.0×10^{-4}	4.2×10^{-4}	-6.3×10^{-4}	-5.1×10^{-4}	7.8×10^{-4}	-9.0×10^{-5}	-7.7×10^{-5}	-1.7×10^{-4}
	c	4.0×10^{-3}	-2×10^{-3}	-6.8×10^{-2}	-2.9×10^{-2}	-3.0×10^{-2}	-2.9×10^{-1}	-1.7×10^{-2}	-0.01	0.02	$1.7\times10^{=2}$	-2.3×10^{-2}	3.9×10^{-3}	2.6×10^{-3}	5.0×10^{-3}
	d	3.5×10^{-2}	1.9×10^{-2}	8.6×10^{-2}	3.54×10^{-1}	3.8×10^{-1}	2.9×10^{-1}	2.1×10^{-1}	0.02	-0.19	-2.1×10^{-1}	$3.\times10^{-1}$	-4.5×10^{-2}	-2.0×10^{-2}	-4.5×10^{-3}
	e	-8×10^{-2}	-3.6×10^{-2}	-3.7×10^{-1}	-1.50	-1.67	-1.29	-9×10^{-1}	-0.79	0.74	8.8×10^{-1}	-1.44	1.4×10^{-1}	3.4×10^{-2}	1.1×10^{-1}
	r^2	0.98	0.95	0.87	0.45	0.46	0.37	0.50	-	-	0.94	0.87	0.73	0.98	0.80
	AIC	-38.28	-33.86	-33.99	-21.98	-20.21	-19.50	-23.73	-	-	-30.30	-22.68	-18.89	-39.76	-25.50

4.3.1.2 Quantifying the effect of temperature on the proportion germination of populations of candidate EPF conidia

Five non-linear models were fitted to the relationship between temperature and percentage germination of populations of candidate isolates, after 12hr of incubation. A bell shaped distribution was observed for all distributions (Fig. 4-19 to 4-32)

For *B. bassiana* 433.99, 432.99, 1757.15, 1758.15, 1759.15, 11.98 and 1730.08 the Briere-1 and polynomial models were considered to be the best fitting. The r^2 values of Briere-1 models, polynomial and Logan-6 models fitted to *B. bassiana* temperature/germination data were generally higher than other models. For example, Briere-1, polynomial and Logan-6 r^2 values ranged from 0.83 to 0.98, 0.78 to 0.99 and 0.78 to 0.99and respectively. Taylor and Lactin-1 r^2 values ranged from 0.51 to 0.97 and 0.52 to 0.98. The range of AIC values were relatively similar for Taylor, Briere-1, Logan-6 and Lactin-1 models being from approximately 30 to 50 across isolates. Polynomial model displayed a range AIC values from 21.12 to 48 (Table 4-6).

L. longisporum 1.72 and *L. muscarium* 19.79 had relatively low optima when compared to other isolates. Consequently, models based on normal distribution were better suited. Polynomial and Taylor models had highest r^2 values (ranging from 0.92-0.93 and 0.89-0.99, respectively). Logan-6 and Briere-1 models were the next best fitting, displaying r^2 values of 0.84 to 0.85 and 0.84 to 0.87, respectively. The Lactin-1 model displayed the lowest r^2 values of 0.56 to 0.76. In terms of AIC values, the polynomial was the best fitting model, displaying AICs between 29.79 and 49.51. All other models displayed AIC values of 50 and 60.

For *M. brunneum* isolates 445.99 ,1760.15 and 275.86 high r^2 values were observed for all models. R^2 values were between 0.88 and 0.99. AIC values were lowest for Taylor and polynomial models being between 24.91 and 53.87, and 29.21 and 31.72, respectively. AIC values for Logan-6, Breire-1 and Lactin-1 models were 40 to 55 (Table 4-6).

For *I. fumosorosea* 1761.15 and 1762.15 the Briere-1 model was the best fitting in terms of r^2 values, having a range of 0.93 to 0.94 respectively. All other models had r^2 values of below 0.90, with the exception of the Taylor/*I. fumosorosea* 1762.15 model which displayed an r^2 of 0.92. AIC values were also lowest for the Briere-1 model being between 52.80 and 53.24 respectively (Table 4-6).

Cardinal temperatures, taken from the Briere-1 model, were compared between isolates of different species. In general, *M. brunneum* isolates had higher thermal optima when compared to other isolates. For example, *M. brunneum* 445.99, 275.86 and 1760.15 had thermal optima of 29.48, 31.12 and 33.00^{o}C, respectively. All other isolates had thermal optima within the range of 26.95 to 27.61 ^{o}C. Thermal optima were relatively similar between isolates *of I. fumosorosea, B. bassiana L. longisporum* and *L. muscarium*. *L. longisporum* and *L. muscarium* isolates had lower *T0* values when compared to other isolates, being 6.80 and 5.97^{o}C for 1.72 and 19.79, respectively. All other isolates have *T0s* between 11.07 and 12.91^{o}C, with the exception of *B. bassiana* 1757.15 which had a *T0* of 7.16^{o}C (Table 4-6).

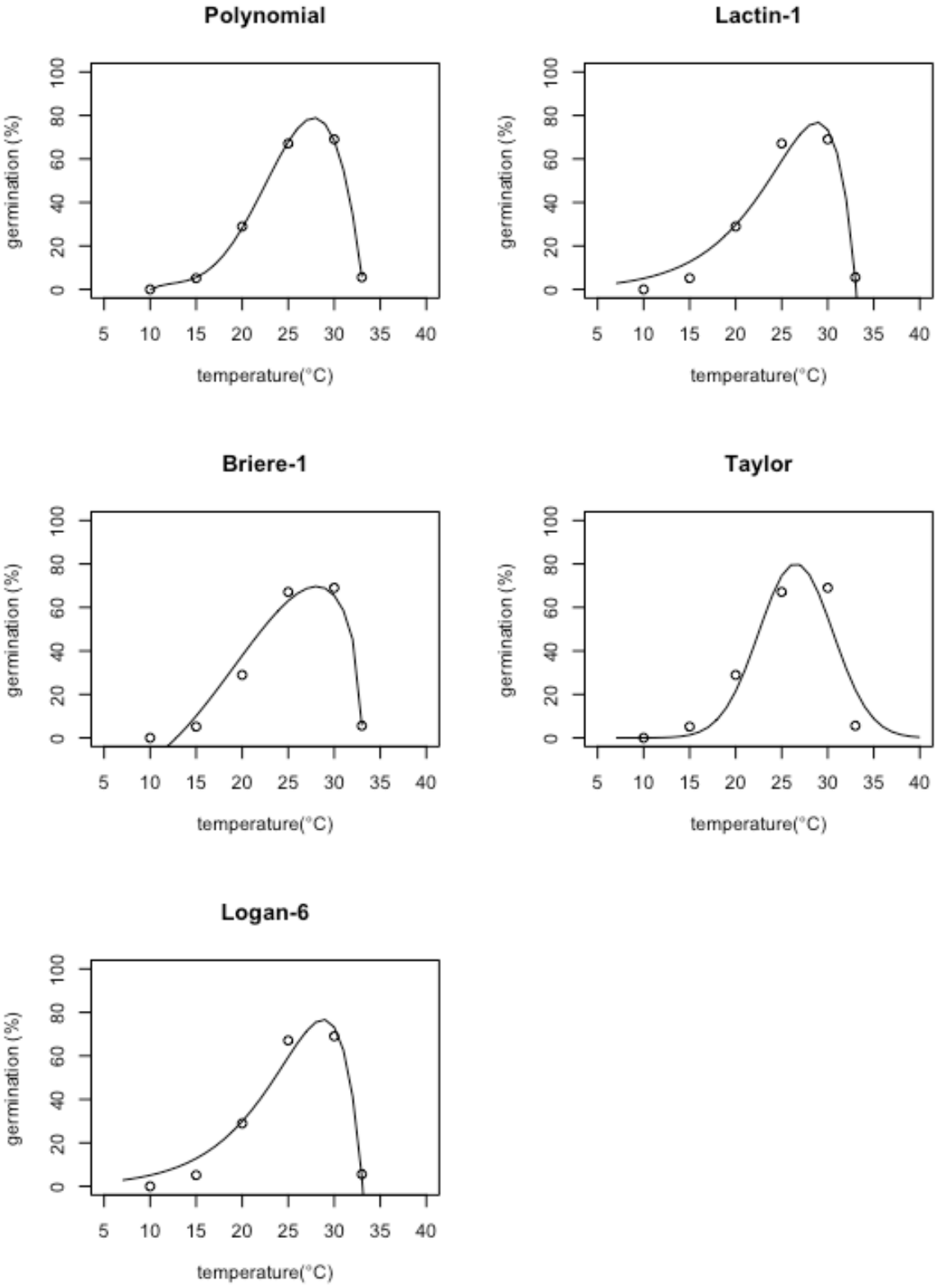


Figure 4-19: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 433.99.

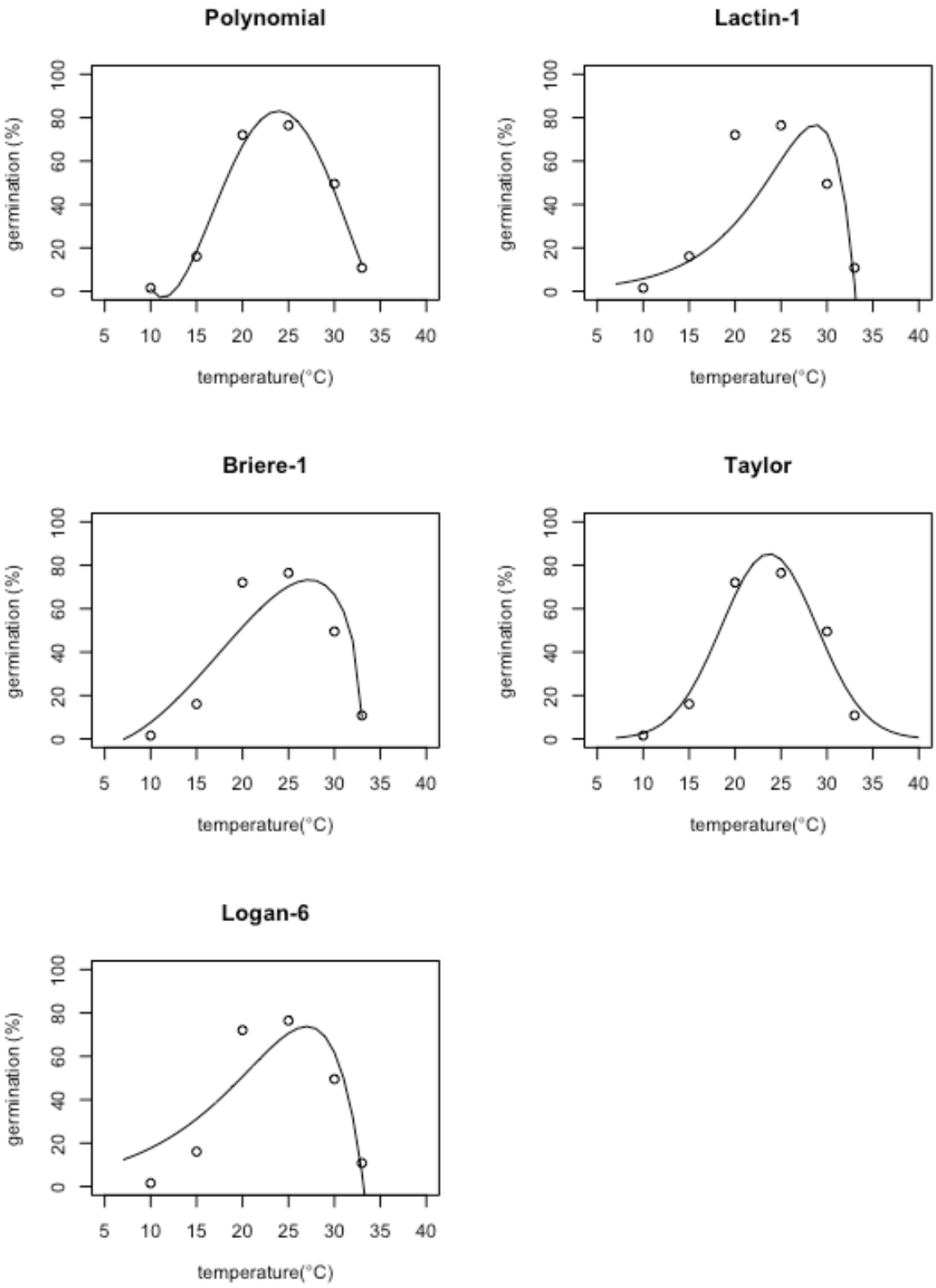


Figure 4-20: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 1757.15.

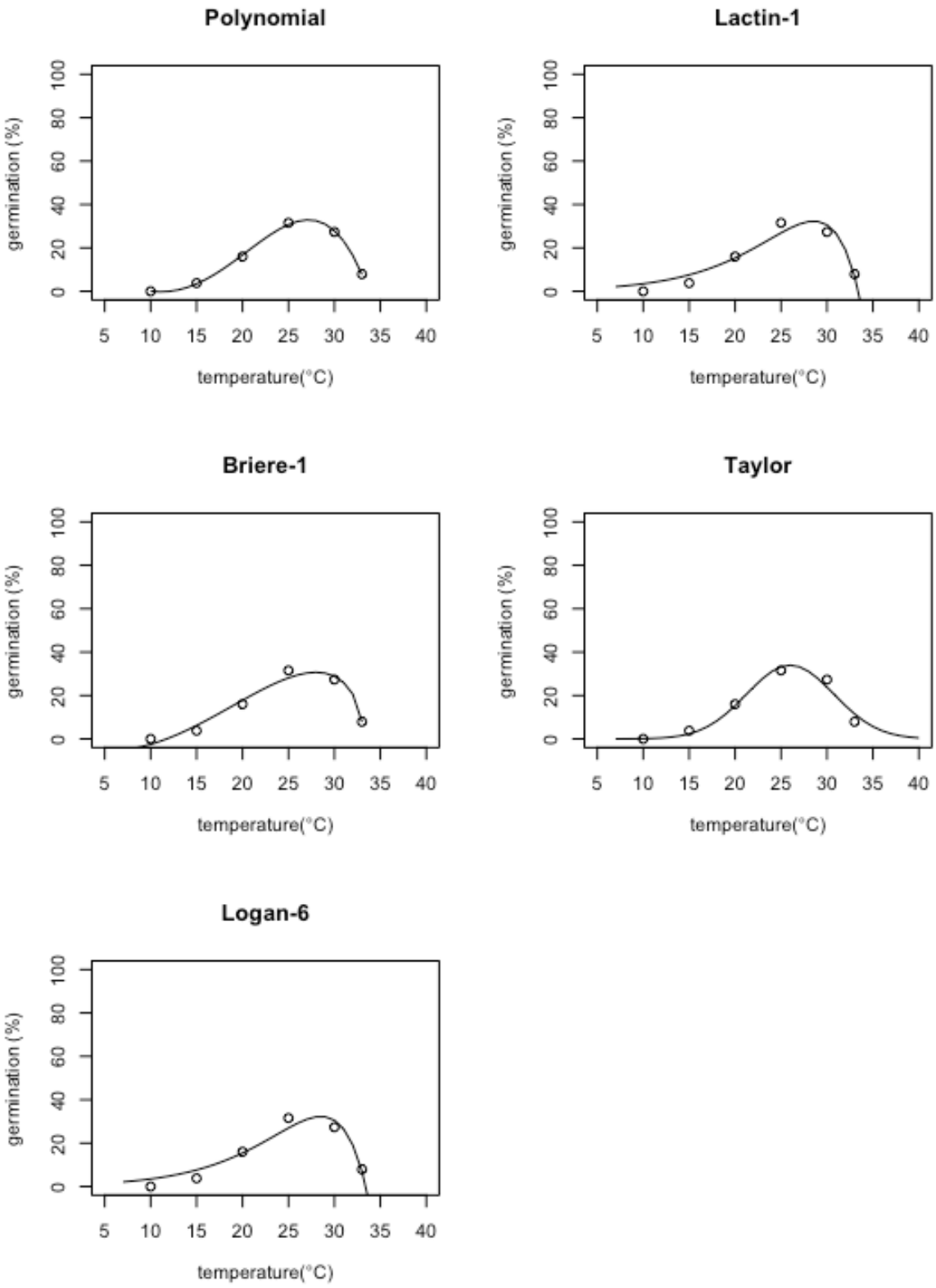


Figure 4-21: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana 1758.15*.

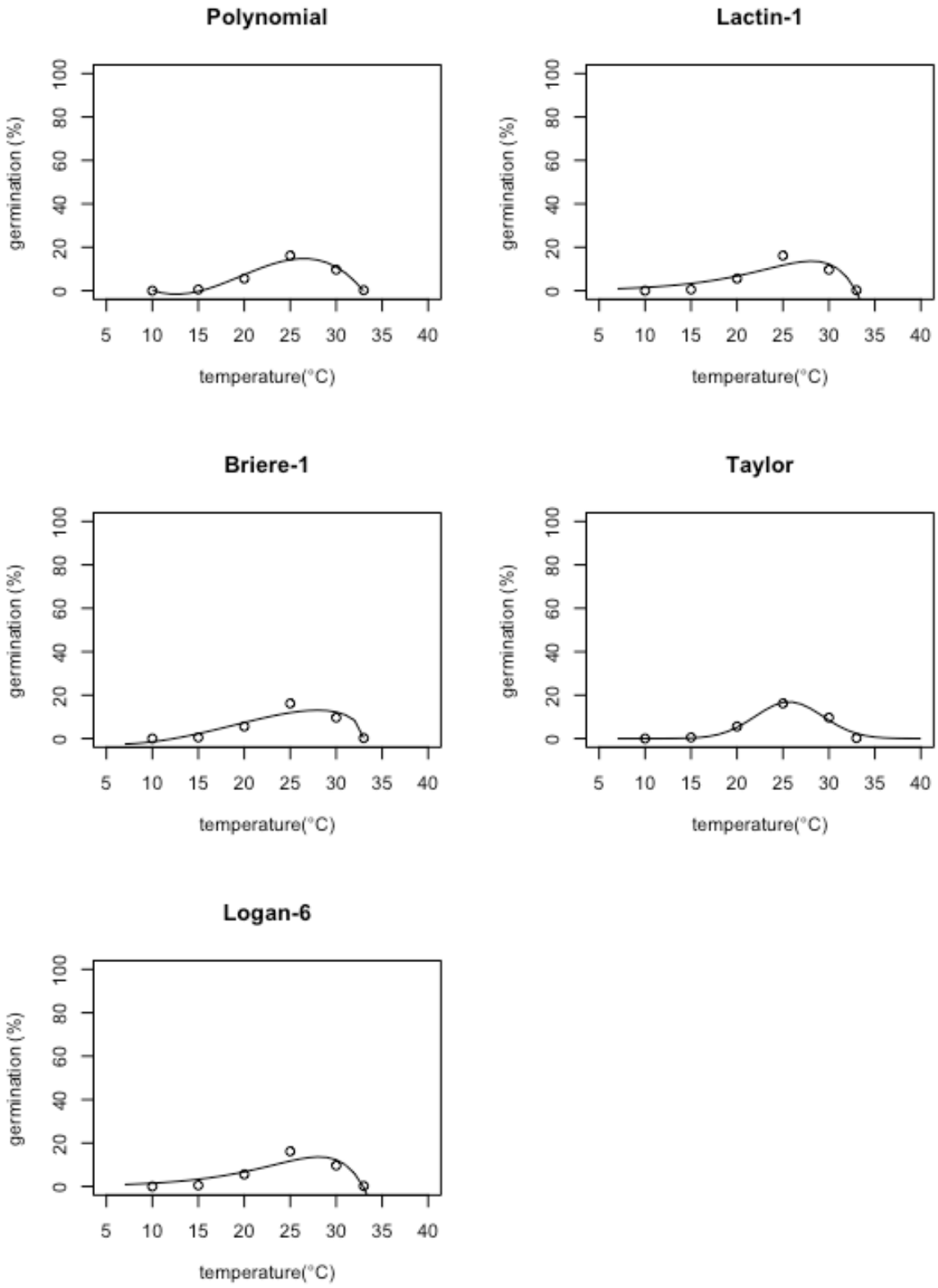


Figure 4-22: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 1759.15.

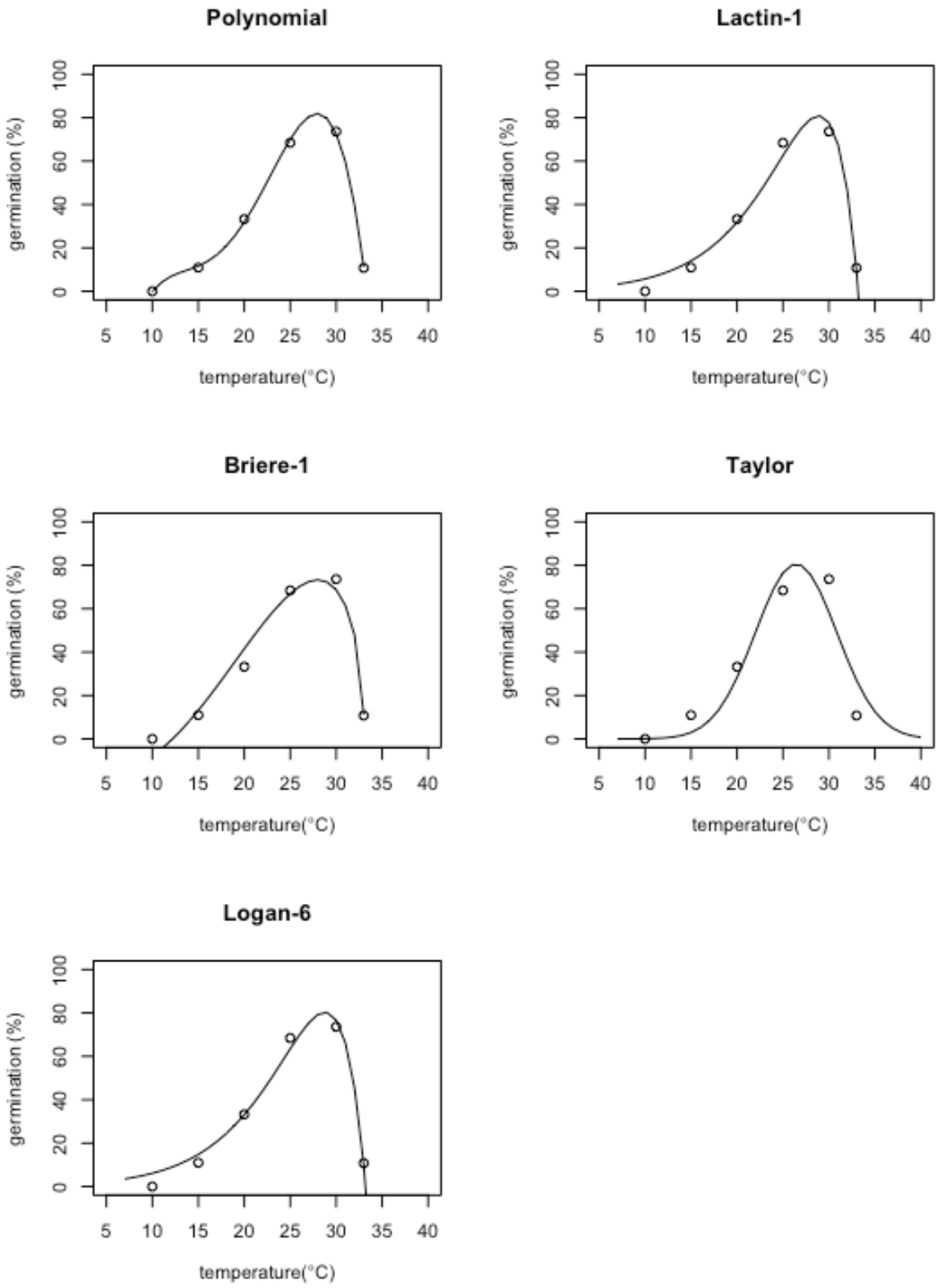


Figure 4-23: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 432.99.

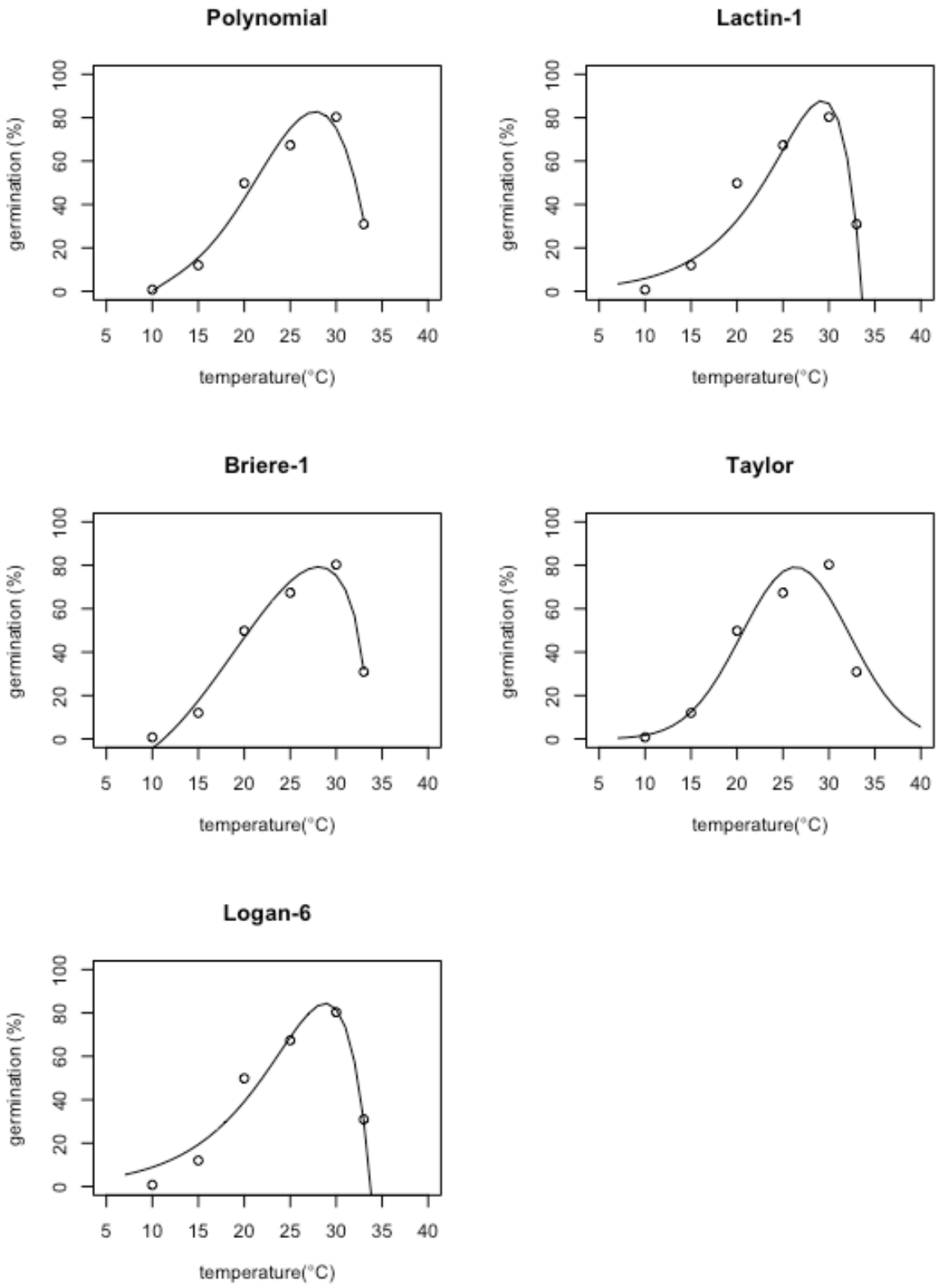


Figure 4-24: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 11.98.

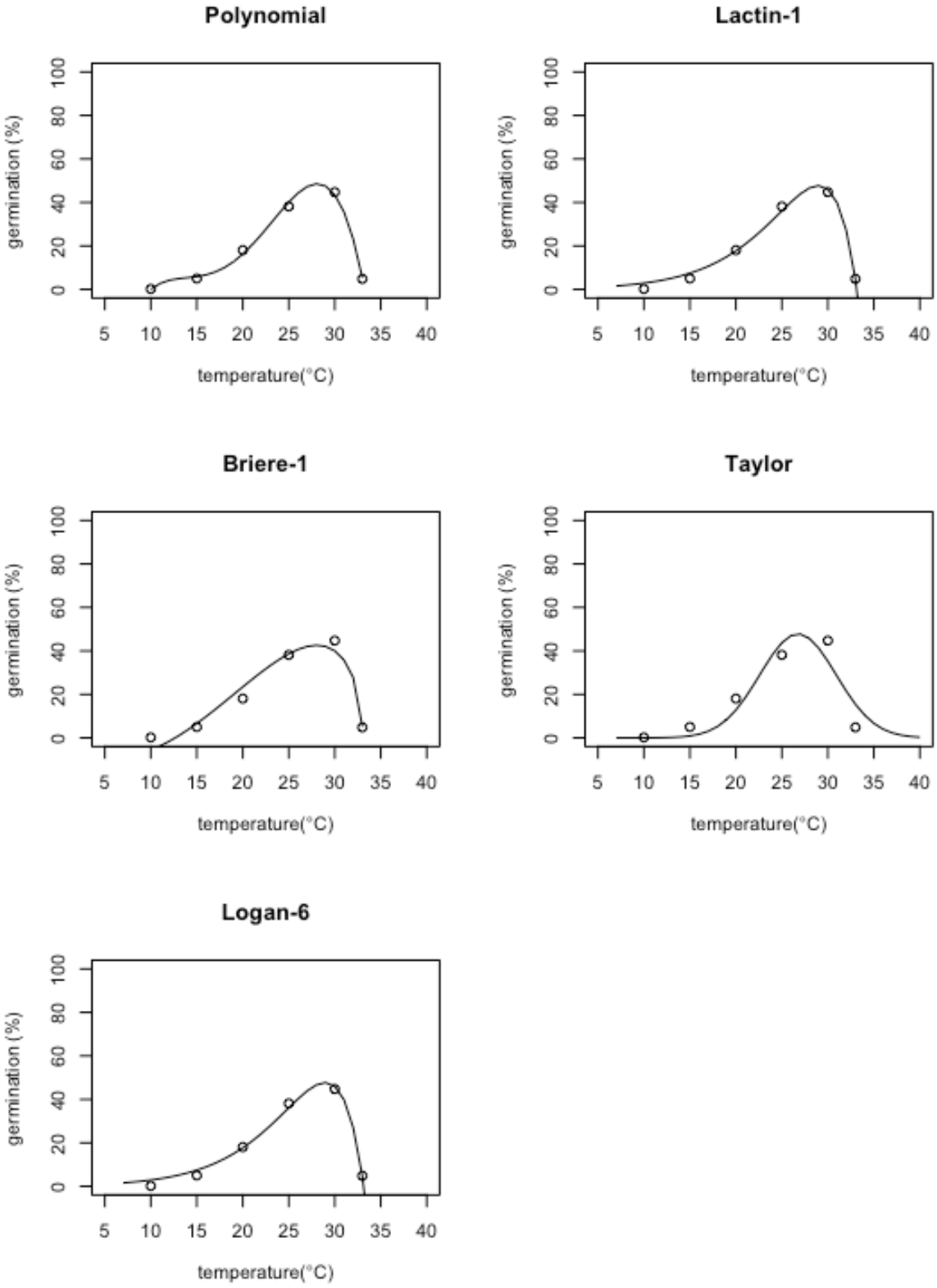


Figure 4-25: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *B. bassiana* 1730.08.

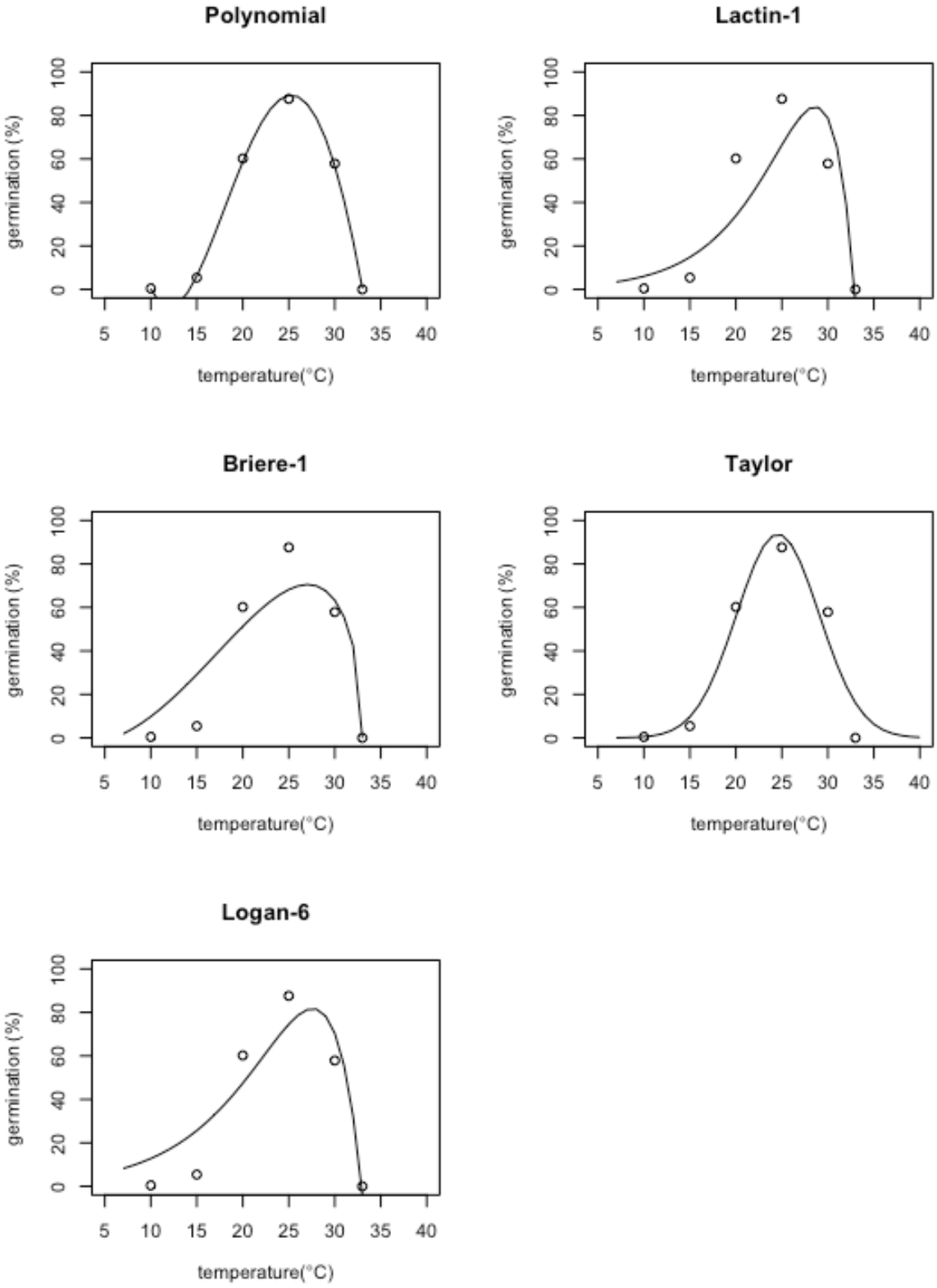


Figure 4-26: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *L. muscarium* 19.79.

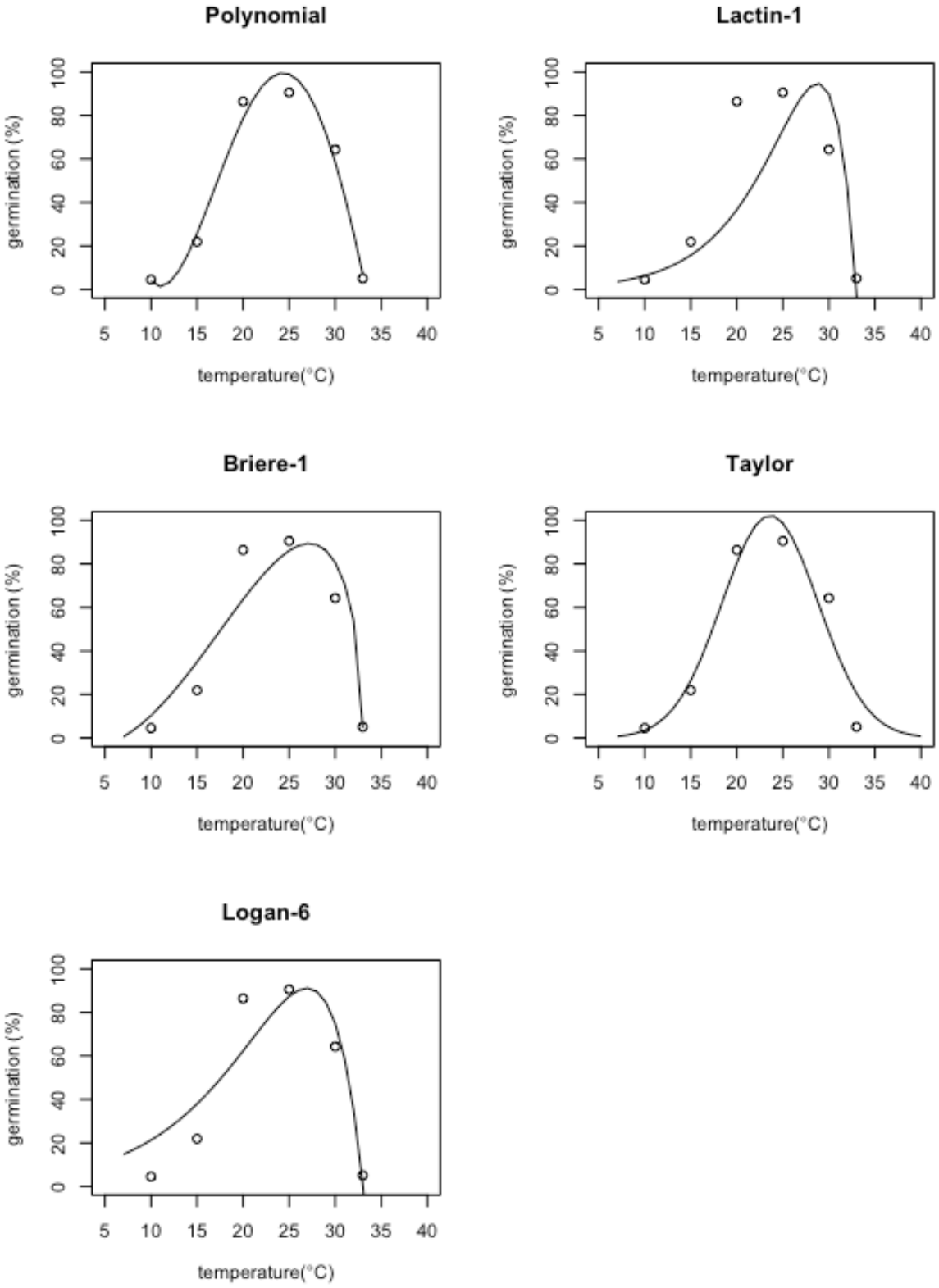


Figure 4-27: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *L. longisporum* 1.72.

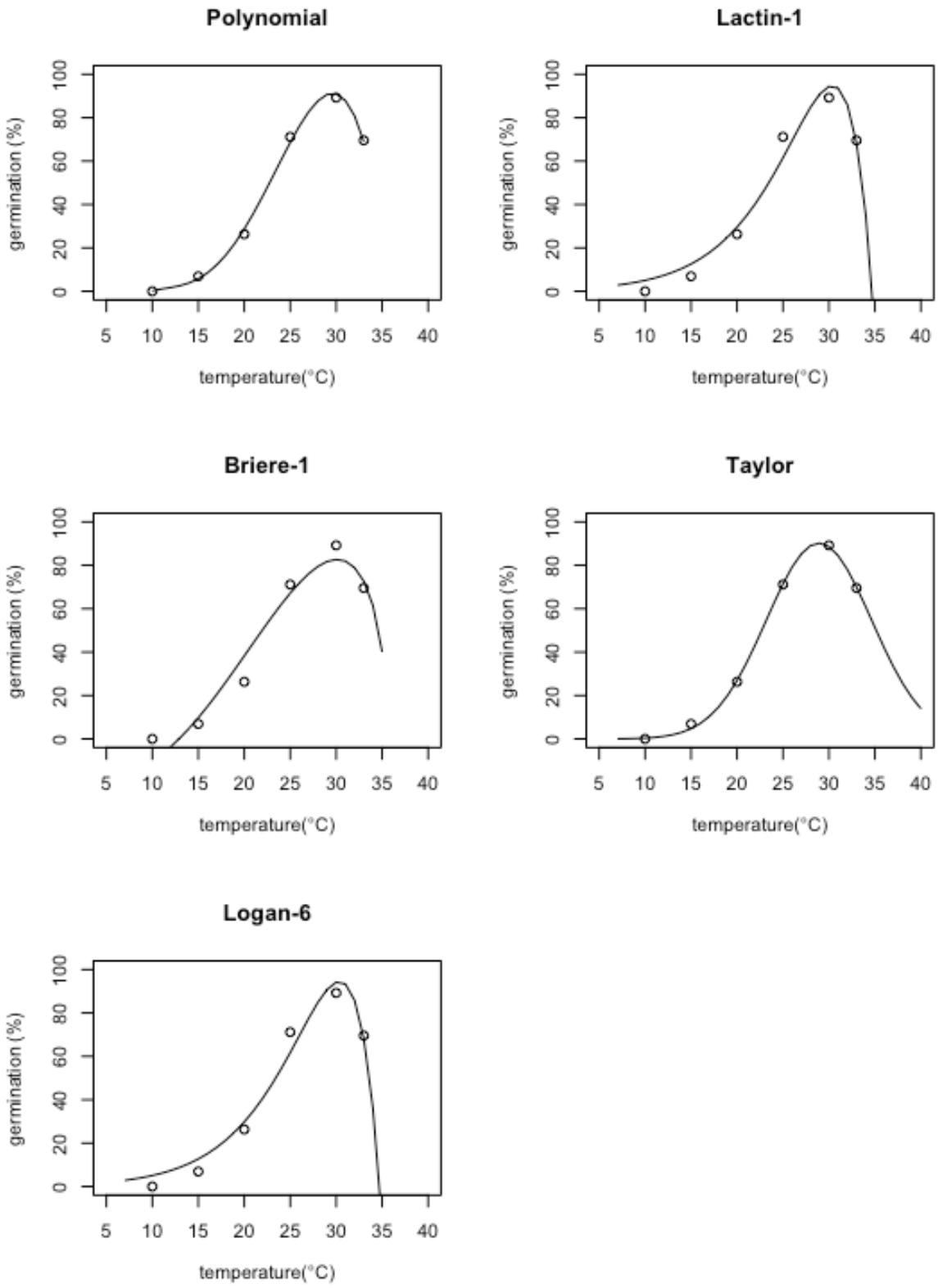


Figure 4-28: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *M. brunneum* 275.86.

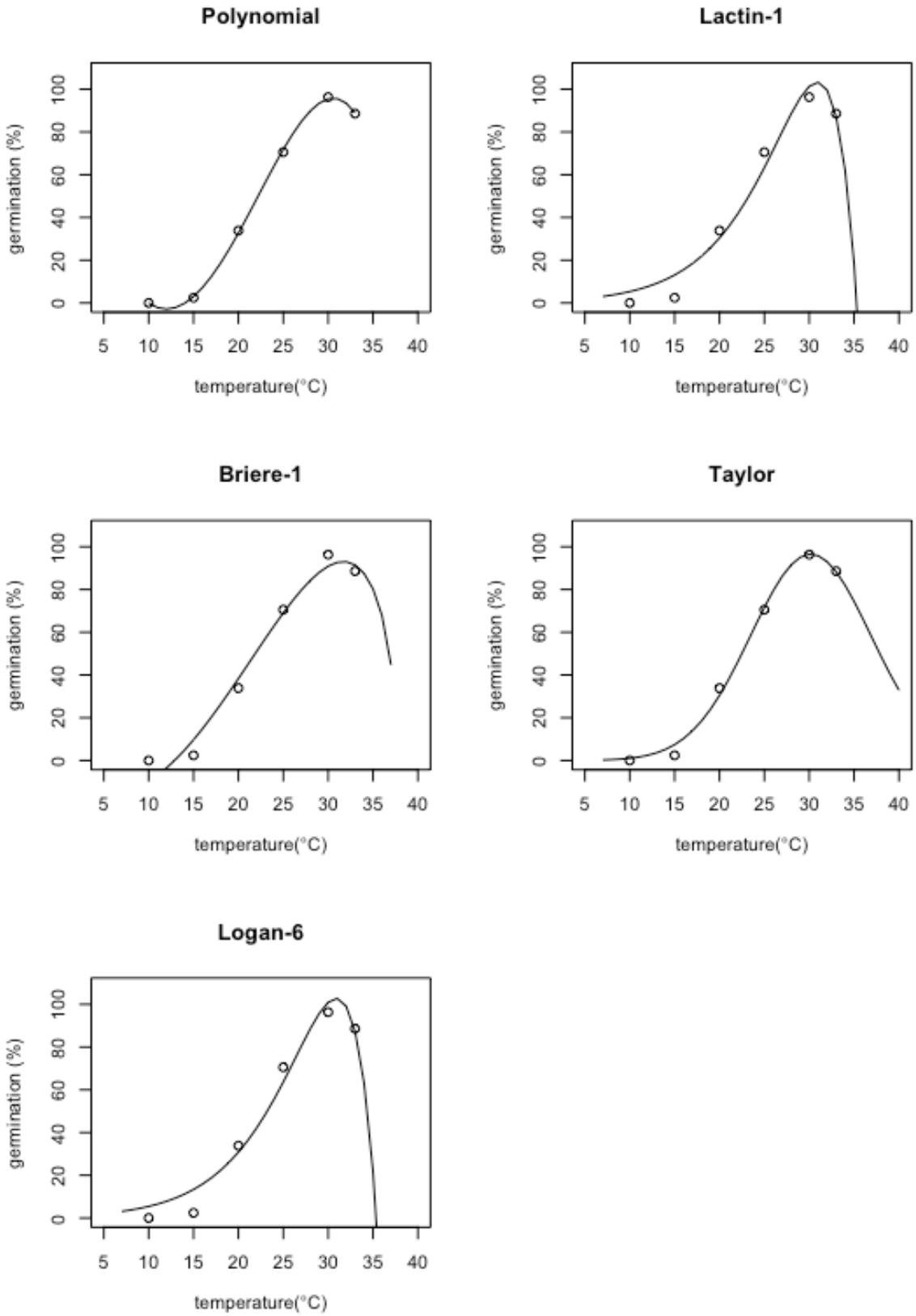


Figure 4-29: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *M. brunneum* 445.99.

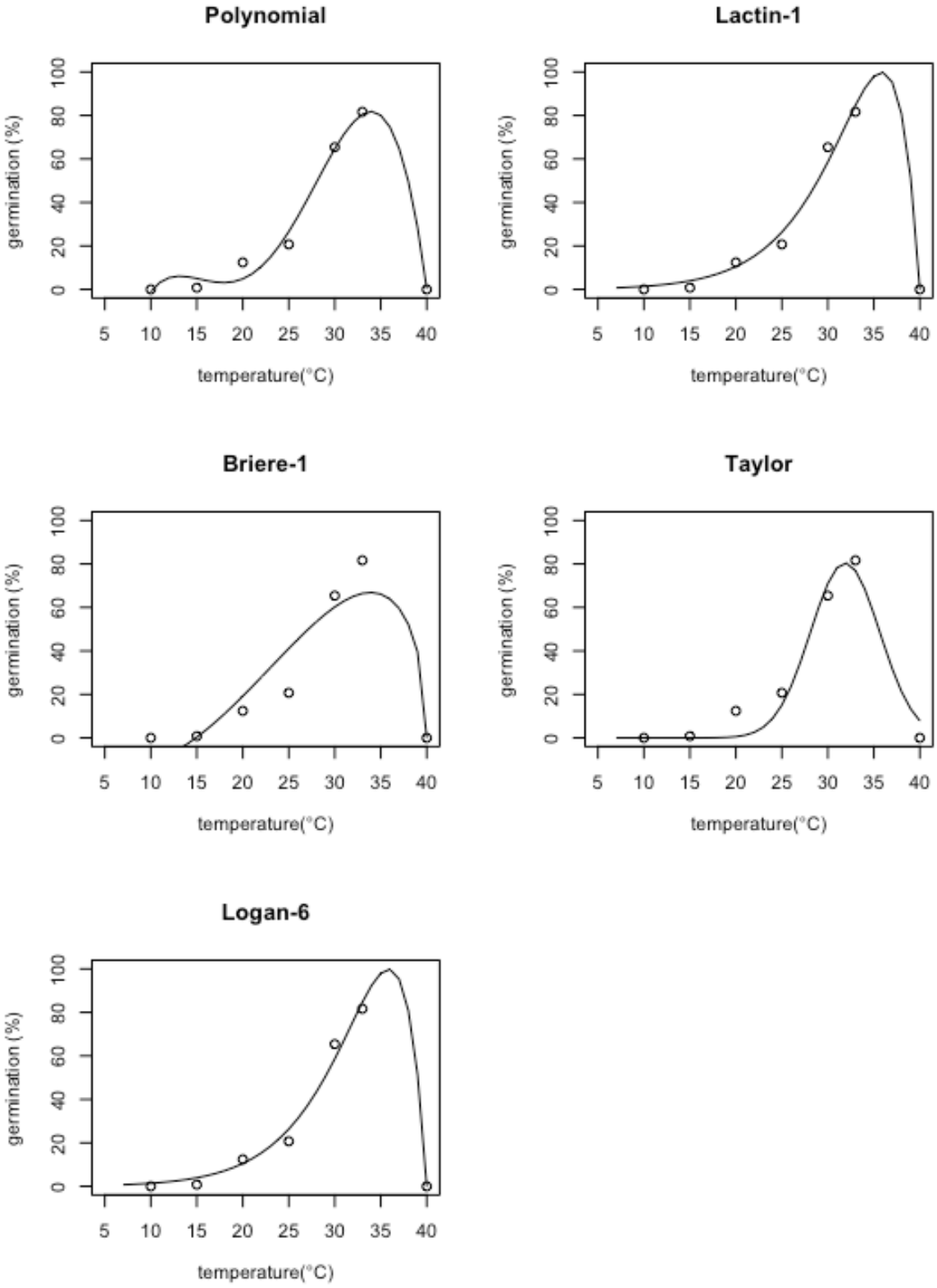


Figure 4-30: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *M. brunneum* 1760.15.

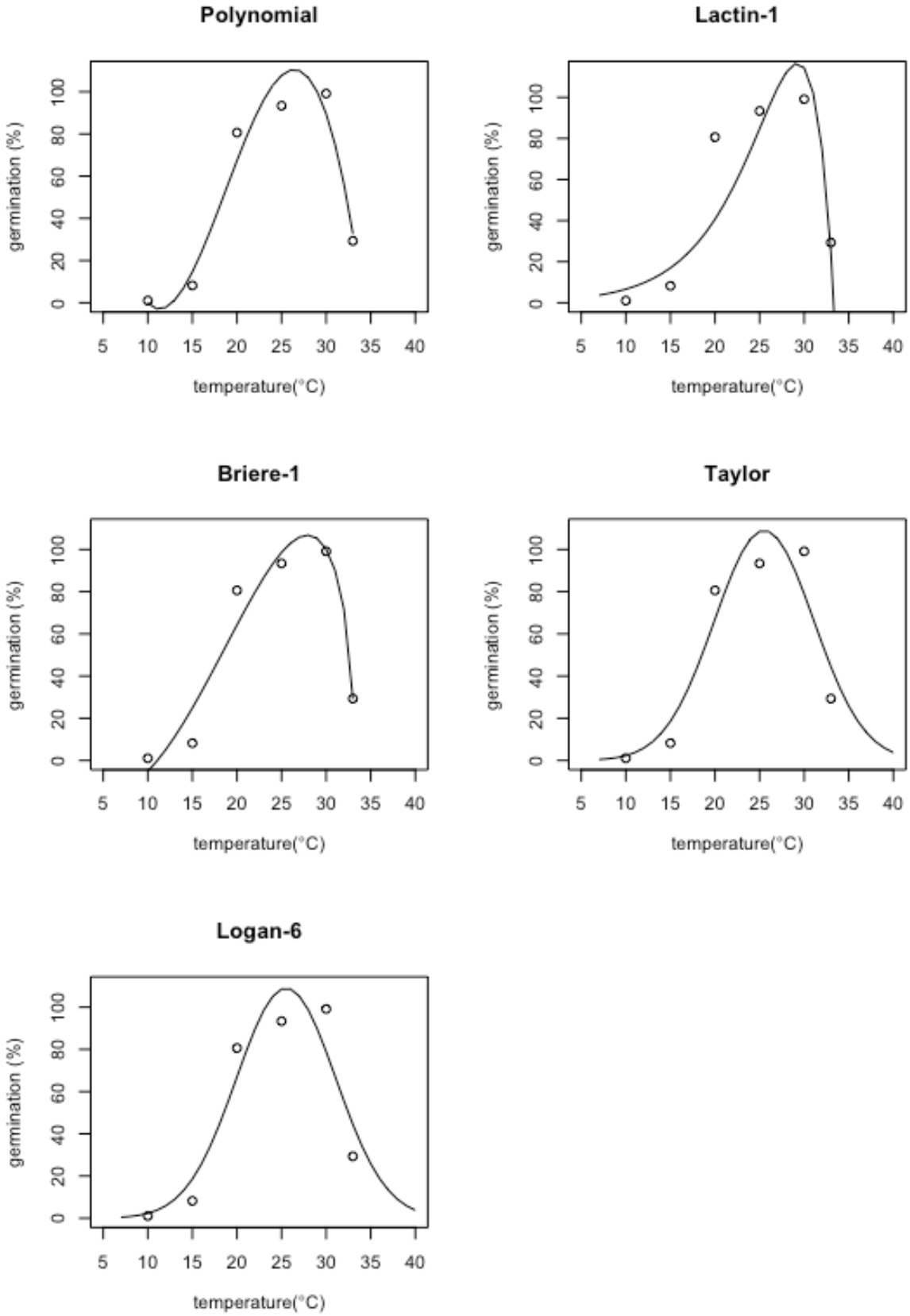


Figure 4-31: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for *I. fumosorosea* 1761.15.

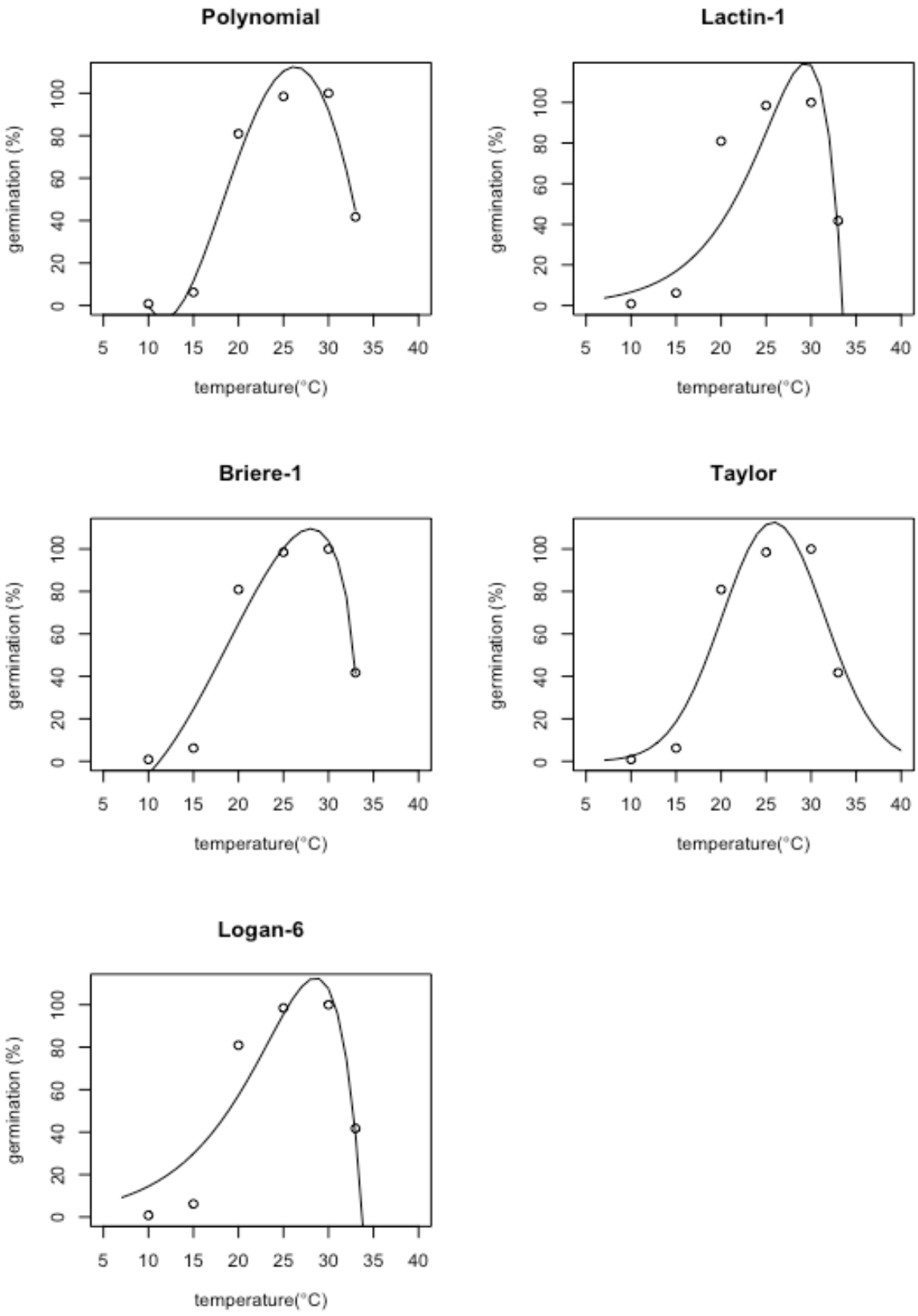


Figure 4-32: Five non-linear models fitted to mean percentage germination of populations of conidia after 12hr incubation plotted against temperatures (^{o}C) for I*. fumosorosea*1762.*15.*

Table 4-6: Fitted parameters, r^2 and AIC values of five non-linear models of percentage germination at six temperatures for 14 isolates.

		B. bassiana							*Lecanicillium* spp.		*M. brunneum*			*I. fumosorosea*	
Model	**Par.**	**1757.15**	**1758.15**	**1759.15**	**11.98**	**432.99**	**433.99**	**1730.08**	**1.72**	**19.79**	**275.86**	**445.99**	**1760.15**	**1761.15**	**1762.15**
Briere-1	***a***	0.06	0.03	0.01	0.07	0.07	0.07	0.04	0.07	0.05	0.06	0.06	0.04	0.09	0.10
	T0	7.16	11.74	12.35	11.24	12.20	12.85	12.65	6.80	5.97	12.91	12.89	12.13	11.07	11.22
	Tmax	33.04	33.14	33.00	33.37	33.04	33.01	33.02	33.00	33.0	35.58	37.61	40.00	33.16	33.32
	Topt	27.08	27.45	27.36	27.61	27.40	27.40	27.39	27.01	26.95	29.48	31.12	33.00	27.43	27.57
	r^2	0.83	0.96	0.84	0.98	0.97	0.95	0.95	0.87	0.84	0.96	0.98	0.88	0.94	0.93
	AIC	55.21	34.57	35.31	43.03	44.75	46.96	41.14	55.77	56.49	48.87	46.28	61.44	52.80	53.24
Taylor	***Rm***	85.30	33.95	16.90	79.18	80.56	80.17	47.79	102.29	93.68	90.21	96.54	80.43	108.98	112.60
	Topt	23.70	26.00	25.67	26.36	26.43	26.54	26.75	23.62	24.57	28.92	30.17	31.88	25.51	25.81
	T0	5.20	4.76	3.68	-5.90	4.44	4.02	4.15	5.23	4,48	5.73	6.70	3.76	5.59	5.70
	r^2	0.96	0.96	0.97	51.2	0.87	0.88	0.84	0.92	0.93	0.99	0.99	0.96	0.88	0.92
	AIC	46.44	34.75	25.25	51.21	53.15	52.51	48.07	52.74	51.45	24.91	36.24	53.87	56.61	54.39
Lactin-1	***p***	0.20	0.20	0.20	0.19	0.20	0.22	0.23	0.20	0.20	0.21	0.20	0.23	0.21	0.21
	Tmax	33.07	33.42	32.93	33.60	33.20	33.09	33.15	32.96	32.89	34.70	35.31	40.00	33.35	33.52
	Topt	29.48	28.91	28.24	29.9	29.4	29.16	29.08	29.35	29.21	30.70	31.31	35.9	29.79	27.96
	Δ	3.72	4.51	4.69	3.71	3.80	3.93	4.07	3.61	3.68	4.00	4.00	4.10	3.56	5.56
	r^2	0.52	0.93	0.82	0.92	0.98	0.97	0.99	0.56	0.76	0.97	0.97	0.99	0.79	0.77
	AIC	61.32	38.82	36.23	49.81	41.67	44.91	31.96	63.30	59.03	45.72	46.99	46.61	59.98	60.74
Logan-6	***Ψ***	-16.86	-2.40	-1.57	14.94	-2.15	-2.56	-0.88	-14.51	-13.42	-1.55	-1.33	0.81	37.20	24.78
	p	0.17	0.21	0.21	0.20	0.23	0.24	0.24	0.17	0.19	0.23	0.23	0.24	0.19	0.19
	Topt	26.86	28.45	28.07	28.88	28.67	28.75	28.86	26.75	27.51	30.72	30.77	35.91	28.42	28.63
	Tmax	33.20	33.42	32.93	33.76	33.21	33.09	33.15	33.02	32.91	34.72	35.38	40.00	33.52	33.77
	ΔT	6.34	4.97	4.86	4.88	4.54	4.34	4.29	6.27	5.39	4.47	4.61	4.09	5.10	5.14
	r^2	0.78	0.93	0.82	0.92	0.98	0.97	0.99	0.84	0.85	0.97	0.97	0.99	0.88	0.86
	AIC	58.17	40.76	38.23	49.10	43.02	46.81	33.94	59.11	58.11	47.64	51.29	53.66	58.88	59.63
Polynomial	***a***	0.003	-0.001	-4.1×10^{-5}	-0.002	0.004	-0.004	-0.003	0.002	0.002	0.002	0.001	-0.002	0.006	0.002
	b	-0.32	0.03	-0.009	0.13	0.31	0.26	0.21	-0.25	-0.22	0.16	0.01	0.19	-0.085	-0.19
	c	10.40	-0.21	0.63	-2.98	-7.89	-6.29	-5.54	8.66	8.34	-3.63	0.64	-5.77	4.24	7.62
	d	-132.30	-1.91	-11.14	30.51	87.67	64.44	61.89	-112.00	-117.40	35.54	-17.51	71.55	-64.66	-107.30
	e	569.40	-19.79	58.35	-120.70	-353.33	-240.00	-246.10	485.90	542.40	-124.40	103.00	-310.80	304.90	492.90
	r^2	0.93	0.99	0.78	0.84	0.99	0.99	0.97	0.89	0.99	0.99	0.99	0.95	0.73	0.82
	AIC	43.90	21.42	31.72	48.43	29.21	21.12	32.02	49.51	29.79	35.32	26.81	51.29	55.90	53.66

4.3.2 Quantifying the effect of temperature of the development rate of DBM

In general, there was a linear increase in development times, for all life stages, as temperatures increased from 12.5 to 30^{o}C. However, there were some exceptions to this; individuals spent more time as larvae and in the prepupal stage at 27.5^{o}C, when compared to 25^{o}C, this difference was considered to be significant (Anova, p=<0.05). No larvae reached the second instar of development at 35^{o}C (Table 4-7).

Table 4-7: Mean development time (days± SE) of various life stages at constant temperatures. DNC (did not complete) indicates total mortality occurred before the life stage was complete. Means followed by different letters are significantly different according to Anova, Tukey's HSD test (P< 0.05).

Temp. (^{o}C)	Egg	Larvae	Pre-pupae	Pupae	Total
12.5	9.3±0.1 a	37.5±0.5 a	3±0.4 a	19±0.2 a	68.2±0.3 a
15	9.5±0.1 a	16.9±0.2 b	2±0.2 b	10±0.3 b	38.9±0.3 a
20	5.1±0.1 b	10.6±0.1 c	0.8±0.1 c	7.9±0.1 c	24.4±0.1 a
25	3.9±0.1 c	6.6±0.2 d	0.7±0.1 c	4.4±0.2 d	15.5±0.1 b
27.5	3.9±0.1 c	8.2±0.2 e	1.3±0.2 bc	2±0.2 e	15.5±0.2 b
30	3.8 ±0.1 c	5.2±0.2 f	0.7±0.2 c	3.1±0.2 f	12.8±0.2 c
35	3.1±0.2 c	DNC	DNC	DNC	DNC
F-value	1275	1319	13.6	524.5	4823
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

When excluding development data recorded at 35^{o}C (for development of DBM from egg lay to adult) a linear increase development rate was observed between 12.5 and 30^{o}C (Fig. 4-33). A linear regression model and a second order polynomial model was compared, the latter provided no advantage in describing the data (Anova, $p>0.48$).

Five non-linear models were used to describe the effect of temperature on DBM development rate. In general, when consulting the AIC values, Lactin-1, Briere-1 and Logan-6 models described development data most adequately (Table 4-8). For example, when observing the development of DBM from egg to adult, the three afore mention models displayed r^2 values of over 0.95, and the lowest AIC values. The 4th order polynomial model did not describe data as well as Lactin-1, Briere-1 and Logan-6 models. The Taylor model was the worst fitting model for development to larvae and adult stages, but interestingly it was the best fitting model for development over the pupal stages. In general, all models fitted better to development to adult, when compared to pupal development and larval development.

T0 for the Brierie-1 model describing the development of egg to adult was 6.5^{o}C. For the Taylor model, *T0* was 6.1 ^{o}C. *Topt* was observed to be 28.6, 25.6, 29.1 and 31.7 for Briere-1, Taylor, Lactin and Logan-6 models, respectively. *Tmax* was observed to be 35^{o}C for Briere-1, Lactin and Logan-6 models.

Table 4-8: Fitted parameters of five non-linear models used to describe development to various DBM life stages over seven temperatures.

Model	Parameter	Larvae	Pupae	Adult
Linear	*a*	-0.28	-0.07	-0.03
	b	0.02	0.008	0.003
	r^2	0.54	0.88	0.98
	AIC	-2.63	-25.52	-48.19
Briere-1	*a*	1.12×10^{-4}	2.93×10^{-4}	4.58×10^{-5}
	T0	7.65	1.02	6.5
	Tmax	35	35	35.00
	Topt	26.68	28.11	28.60
	r^2	0.85	0.56	0.95
	AIC	-26.37	-3.25	-47.11
Taylor	*Rm*	0.16	0.63	0.07
	Topt	25.75	27.8	25.63
	T0	6.17	-1.98	6.08
	r^2	0.51	0.84	0.63
	AIC	-18.28	-10.55	-32.47
Lactin	*p*	0.18	0.21	0.17
	Tmax	35.02	34.97	35.01
	Topt	29.38	30.11	29.15
	Δ	5.64	4.86	5.87
	r^2	0.86	0.62	0.97
	AIC	-26.98	-4.33	-50.62
Logan-6	Ψ	-1.08×10^{-17}	2.78×10^{-1}	0.004
	p	1.08	2.05×10^{-1}	0.11
	Topt	33.78	30.11	31.69
	Tmax	35	35.00	35.00
	ΔT	1.22	4.89	3.31
	r^2	0.89	0.62	0.98
	AIC	-26.56	-2.32	-49.36
Polynomial	*a*	-1.19×10^{-5}	-2.1×10^{-5}	-3.94×10^{-6}
	b	1.04×10^{-3}	1.63×10^{-3}	3.30×10^{-4}
	c	-3.3×10^{-2}	-4.39×10^{-3}	-1.01×10^{-2}
	d	4.57×10^{-1}	5.07×10^{-1}	1.37×10^{-1}
	e	-2.28	-2.07	-6.64×10^{-1}
	r^2	0.74	0.39	0.98
	AIC	-23.47	-1.18	-46.92

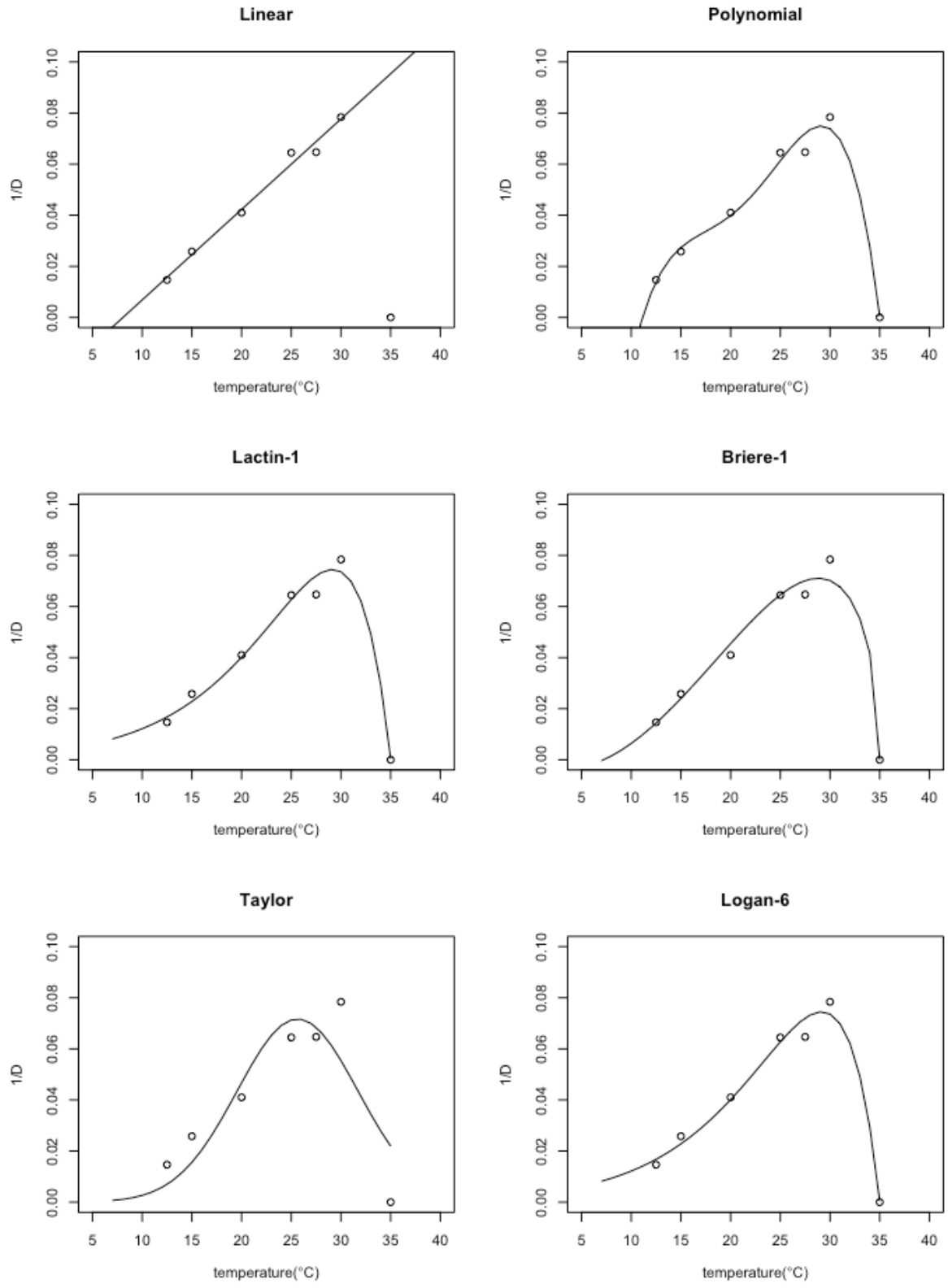


Figure 4-33: One linear and five non-linear models fitted to mean development rate (1/ time to development (days)) plotted against temperature (^{o}C).

4.3.3 Determining the effect of temperature on the virulence of selected candidate EPF isolates against groups of DBM larvae

4.3.3.1 Quantifying the effect of temperature on control mortality

Control mortality was recorded at every temperature treatment during the experiment. If there is linear relationship between control mortality and temperature it is appropriate for control mortality using an equation based on an additive assumption, such as Schneider-Orelli's formula (Puntener, 1981). During this analysis, control mortality six days after treatment was plotted against seven temperatures (Fig. 4-34). Control mortality was lowest at 10^{o}C and highest at 36.5^{o}C, with 3.3±2.3 and 61.11±20.03 % mortality being caused, respectively. A second order polynomial was not a significant improvement over a linear regression model in describing the relationship between temperature and control mortality (Anova, p=0.15) indicating the relationship between control mortality and temperature was approximately linear. The linear model displayed an r^2 value of 0.49 and differed significantly from a zero relationship (p = <0.001). The linear model can be expressed as: y = 1.74 x t + -18.40, with y being control mortality and t being temperature. The linear nature of the relationship between temperature and control mortality means that mortality data can be corrected for control mortality using an equation based on an additive assumption, such as Schneider-Orelli's formula (Puntener, 1981).

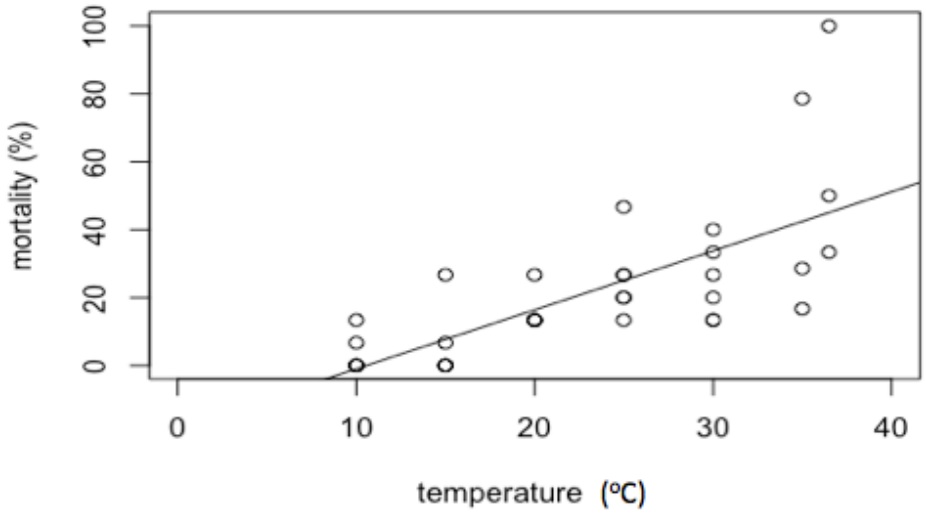


Figure 4-34: Control mortality (%) six days after treatment with 0.05% triton X-100, plotted against temperature along with a linear regression model to describe the relationship.

4.3.3.2 Quantifying the effect of temperature on corrected and un-corrected proportion mortality of groups of DBM larvae after being treated with selected candidate EPF isolates

In this instance mortality from six days after treatment was analysed. Temperature had a significant effect on mortality for every isolate and the untreated control. *B. bassiana* 1757.15 and *M. brunneum* 275.86 displayed highest mortality at either 20 or 25°C, with mortality being reduced at higher temperatures. *M. brunneum* 445.99 and 1760.15 displayed highest mortality at 35 °C. Isolate (including the control treatment) had a significant effect on mortality for all temperatures apart from 10 and 36.5°C. At 15°C, *B.bassiana* 1757.15 displayed 65±9.3% mortality, significantly higher than any other isolate. *B.bassiana* 1757.15 also displayed significantly higher mortality than all other isolates at 20 °C. At 25°C, the control was the only treatment to differ from other isolates in terms of mortality. At 30 and 35°C, *M. brunneum* 445.99 and 1760.15 had the highest mortality, the mortality of these isolates different significantly from all other treatments at 35°C.

The relationship between mortality data and temperature was examined using five non-linear models. Mortality data was analysed in two forms; raw mortality and mortality

corrected for control mortality using Schneider-Orelli's formula (Puntener, 1981). As expected there was a bell-shaped distribution of mortality over temperature for each isolate (Fig. 4-35 to 4-39)

When the mortality data was analysed in its raw form, Briere-1, Taylor and polynomial models explained the data most appropriately. AIC values ranged from 44.41-58.85, 40.98-61.50 and 41.96-63.78 for Briere-1, Taylor and polynomial models, respectively. Lactin-1 and Logan-6 AIC values ranged from 54.40-66.70 and 48.73-64.27, respectively. A similar trend was followed with r^2 values, with Briere-1, Taylor and polynomial models displaying relatively high r^2 values, when compared to Lactin-1 and Logan-6 models. When mortality was analysed after being corrected for control mortality, the best fitting models were Briere-1 and Logan-6 models. These models displayed lower AIC values and higher r^2 values when compared other models (Table 4-9).

Models based on a normal distribution (Taylor and polynomial) explained raw mortality better than corrected mortality data. Conversely, Lactin-1 and Logan-6 models displayed higher r^2 values for corrected mortality data, when compared raw mortality data. Briere-1 explained mortality adequately in both forms, consequently optima derived from this model were compared between isolates and mortality data type. Thermal optima were lower for corrected mortality when compared to raw mortality for all isolates. Similar trends were followed between isolates for both raw and corrected mortality because all isolates had the same control mortality. When looking at corrected mortality data, *B. bassiana* 1757.15 had the lowest thermal optima (24.28^{o}C), *B. bassiana* 433.99 and *M. brunneum* 275.86 displayed similar optima (28.66 and 28.94^{o}C, respectively), *M. brunneum* 445.99 and 1760.15 displayed the highest optima (30.37 and 30.21^{o}C). For corrected mortality data, *T0* was lowest for *B. bassiana* 433.99 and 1757.15 (4.62 and 2.25^{o}C). *M. brunneum* isolates displayed higher *T0*s: 8.72, 13.20 and 11.81^{o}C for *M. brunneum* 275.86, 445.99 and 1760.15, respectively.

A summary table of parameters derived from the Briere-1 model for virulence experiments can be seen in Appendix III.

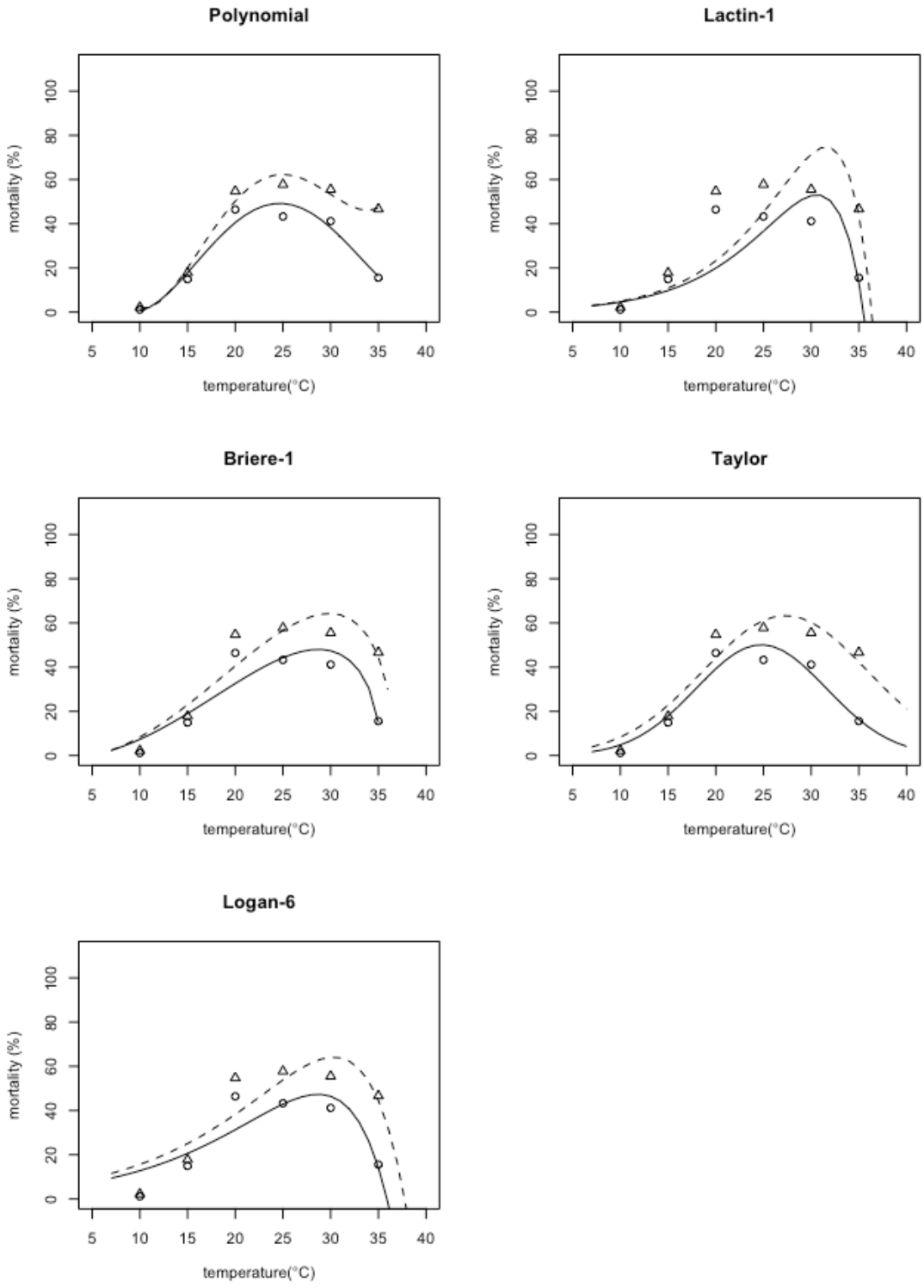


Figure 4-35: Five non-linear regression models fitted to percentage mortality six days after treatment by *B. bassiana* 433.99 at six temperatures. Non-corrected and corrected mortality are represented by Δ and ○ symbols, respectively. Regression lines fitted to non-corrected and corrected mortality data are represented by broken and solid lines, respectively.

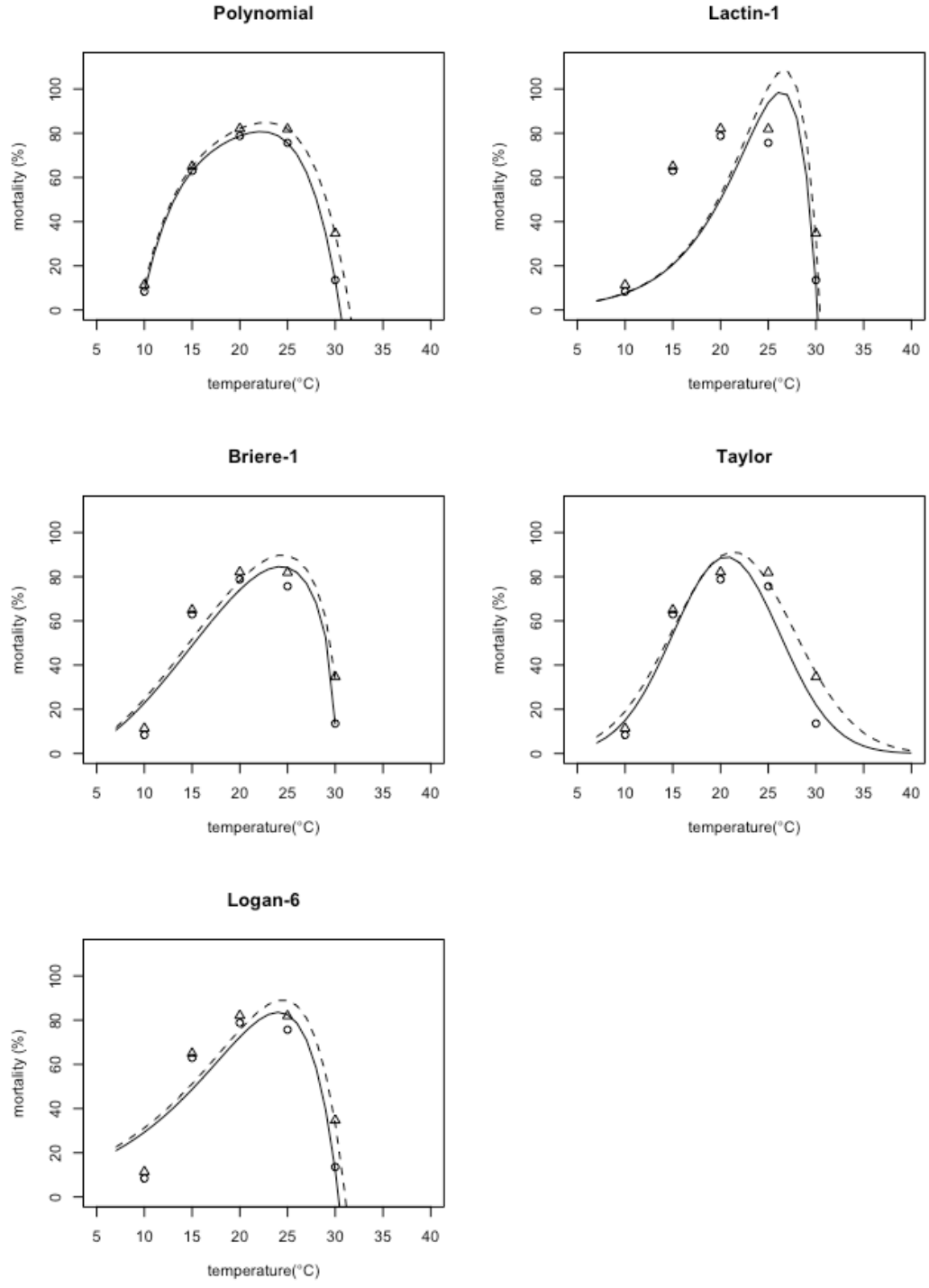


Figure 4-36: Five non-linear regression models fitted to percentage mortality six days after treatment by *B. bassiana* 1757.15 at six temperatures. Non-corrected and corrected mortality are represented by Δ and ○ symbols, respectively. Regression lines fitted to non-corrected and corrected mortality data are represented by broken and solid lines, respectively.

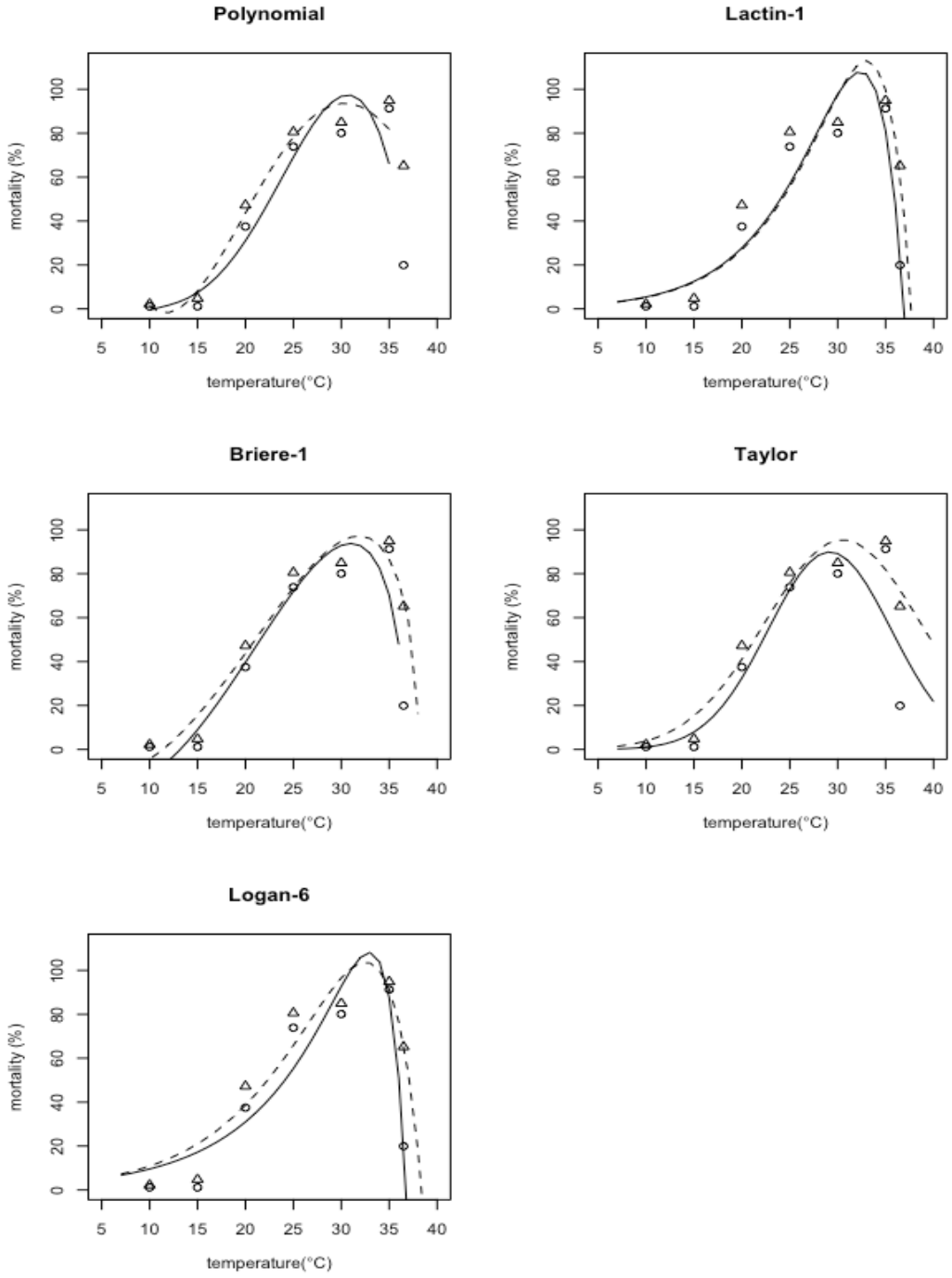


Figure 4-37: Five non-linear regression models fitted to percentage mortality six days after treatment by *M. brunneum* 445.99 at six temperatures. Non-corrected and corrected mortality are represented by Δ and ○ symbols, respectively. Regression lines fitted to non-corrected and corrected mortality data are represented by broken and solid lines, respectively.

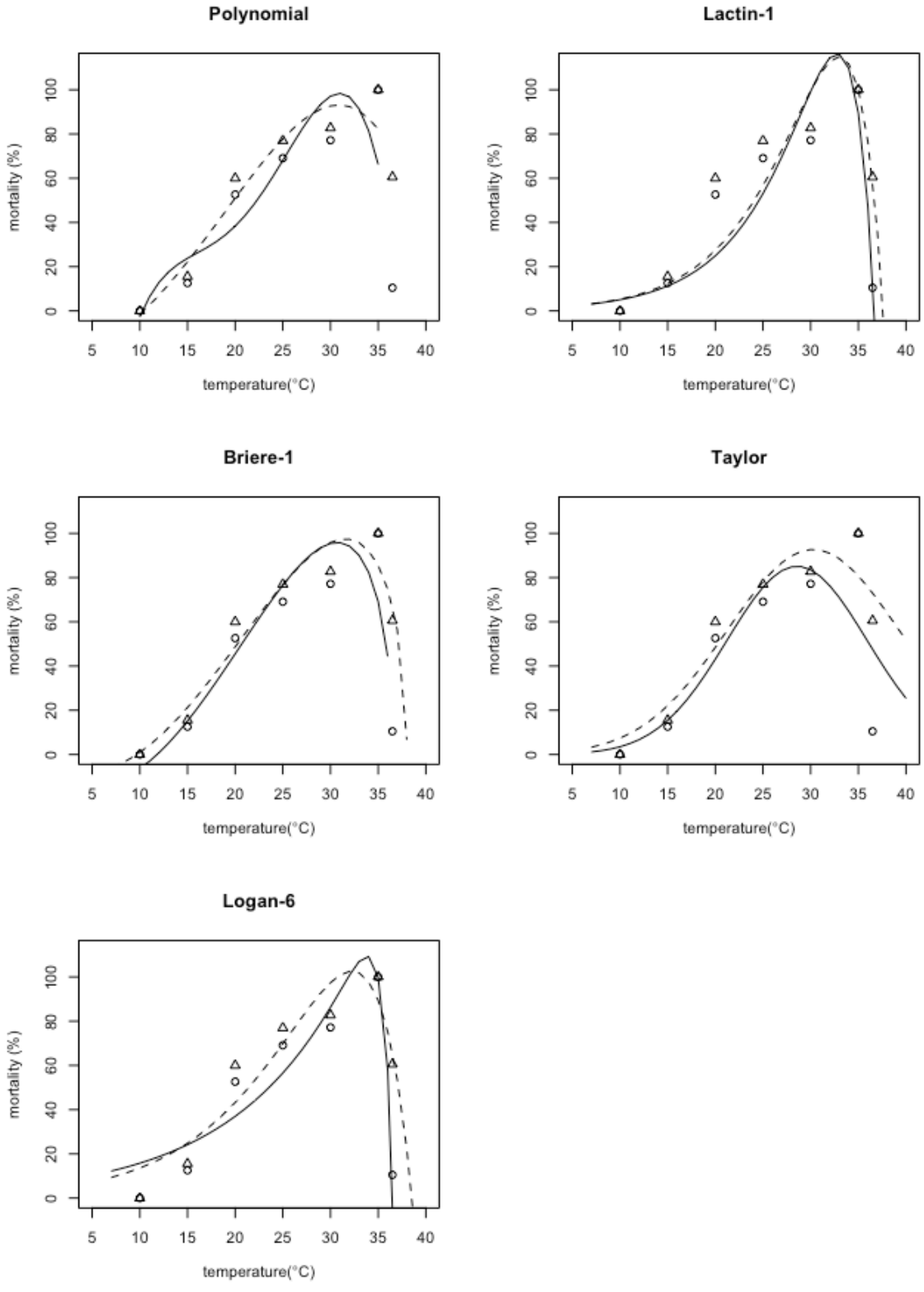


Figure 4-38: Five non-linear regression models fitted to percentage mortality six days after treatment by *M. brunneum* 1760.15 at six temperatures. Non-corrected and corrected mortality are represented by Δ and ○ symbols, respectively. Regression lines fitted to non-corrected and corrected mortality data are represented by broken and solid lines, respectivel

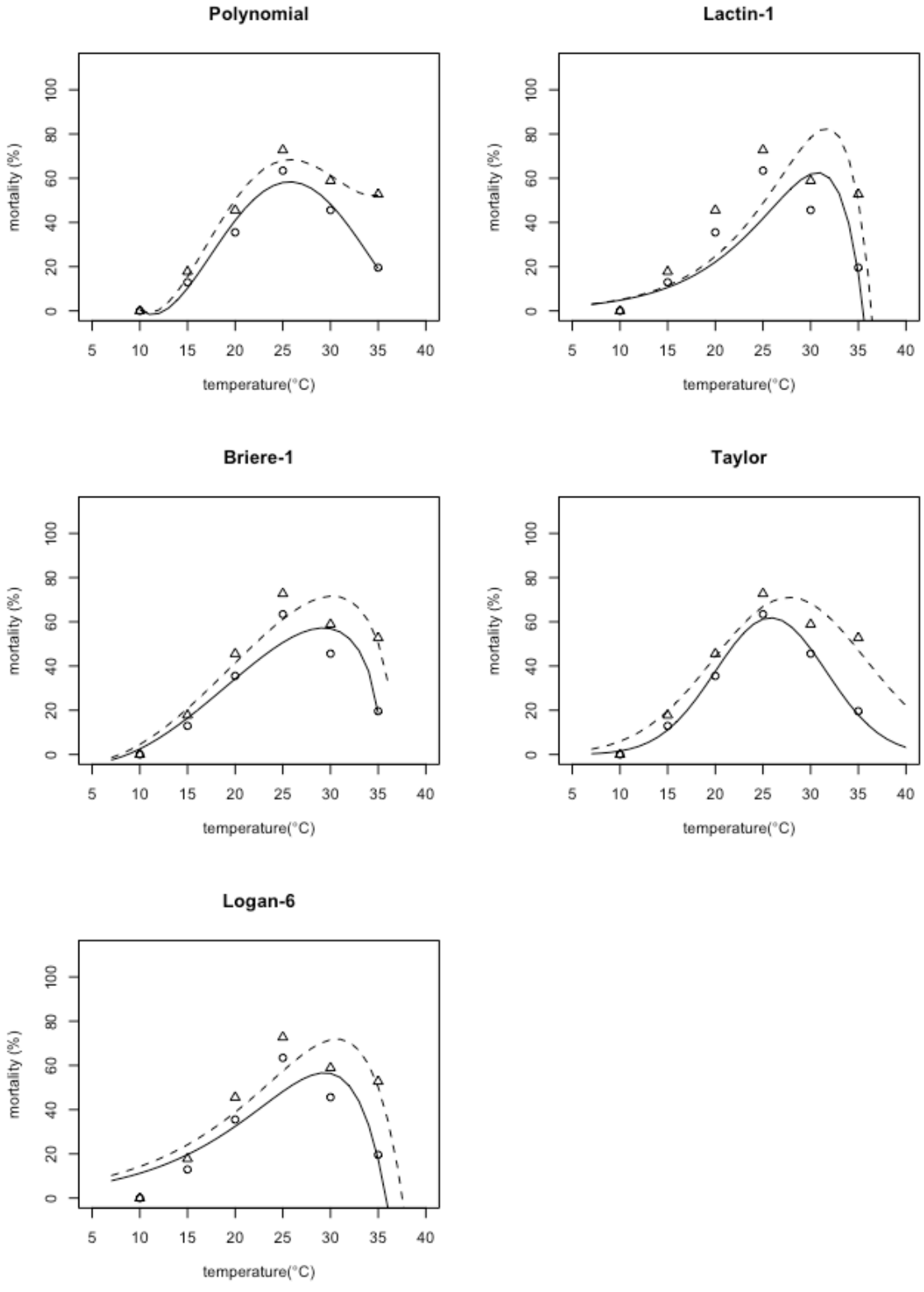


Figure 4-39: Five non-linear regression models fitted to percentage mortality six days after treatment by *M. brunneum* 275.86 at six temperatures. Non-corrected and corrected mortality are represented by Δ and ○ symbols, respectively. Regression lines fitted to non-corrected and corrected mortality data are represented by broken and solid lines, respectively.

Table 4-9: Fitted parameters of five non-linear models used to describe raw and corrected mortality (%) six days after treatment at various temperatures of groups of DBM larvae treated by one of five EPF isolates.

		uncorrected mortality					corrected mortality				
		B. bassiana		*M. brunneum*			*B. bassiana*		*M. brunneum*		
Model	**Parameter**	**433.99**	**1757.15**	**275.86**	**445.99**	**1760.15**	**433.99**	**1757.15**	**275.86**	**445.99**	**1760.15**
Briere-1	***a***	0.03	0.07	0.04	0.06	0.06	0.03	0.07	0.04	0.07	0.07
	T0	5.17	1.80	7.91	11.39	9.68	4.62	2.25	8.72	13.20	11.81
	Tmax	36.64	30.36	36.61	38.07	38.01	35.27	30.06	35.27	36.67	36.56
	Topt	29.80	24.47	30.00	31.40	31.25	28.66	24.28	28.94	30.37	30.21
	r^2	0.87	0.89	0.91	0.95	0.93	0.84	0.89	0.88	0.90	0.84
	AIC	49.40	44.41	49.38	56.18	58.85	48.22	45.24	48.75	61.57	65.22
Taylor	***Rm***	63.31	91.20	71.05	95.45	92.70	50.11	88.99	61.83	89.91	85.11
	Topt	27.30	21.33	27.8	30.51	30.27	24.78	20.61	25.81	6.43	7.35
	T0	8.60	6.40	7.98	8.09	9.01	6.82	5.61	5.83	29.17	28.57
	r^2	0.92	0.94	0.95	0.94	0.89	0.92	0.92	0.99	0.78	0.63
	AIC	46.80	40.98	45.96	57.80	61.50	43.74	43.95	33.06	67.04	71.02
Lactin-1	***p***	0.18	0.22	0.18	0.18	0.18	0.17	0.22	0.18	0.18	0.16
	Tmax	36.37	30.47	36.50	37.70	37.60	35.51	30.17	35.53	36.91	36.69
	Topt	32.32	27.24	32.49	33.69	33.64	31.36	26.92	31.44	33.17	34.06
	Δ	4.05	3.23	4.01	4.01	3.96	4.15	3.25	4.09	3.74	2.63
	r^2	0.46	0.17	0.60	0.85	0.77	0.48	0.37	0.65	0.89	0.81
	AIC	58.03	54.50	58.05	64.42	66.70	55.24	54.10	55.40	62.33	66.19
Logan-6	Ψ	56.53	155.90	42.00	25.31	25.82	51.34	93.24	37.03	2.93	6.74
	p	0.13	0.15	0.14	0.17	0.16	0.14	0.16	0.15	0.12	0.09
	Topt	30.39	24.56	30.77	32.57	32.33	28.75	24.1	29.59	34.73	35.55
	Tmax	37.76	31.06	37.57	38.40	38.55	35.91	30.36	35.84	36.78	36.58
	ΔT	7.37	6.50	6.80	5.83	6.22	7.16	6.26	6.39	2.05	1.03
	r^2	0.78	0.82	0.81	0.91	0.88	0.76	0.84	0.80	0.90	0.90
	AIC	54.62	48.73	55.50	62.82	64.27	52.59	49.10	53.95	63.79	63.61
Polynomial	***a***	0.002	-0.003	0.001	0.001	-0.0002	0.002	-0.004	0.001	-0.002	-0.003
	b	-0.17	0.25	-0.19	-0.09	0.007	0.15	0.30	-0.14	0.11	0.24
	c	5.23	-7.65	6.14	3.63	0.11	4.77	-9.01	4.96	-2.39	-6.99
	d	-62.93	108.10	-77.29	-52.48	0.74	63.92	123.90	-65.20	22.79	90.36
	e	255.85	-525.50	327.47	246.10	-24.27	298.40	-593.70	286.15	-81.56	-416.90
	r^2	0.91	0.89	0.92	0.88	0.75	0.76	0.87	0.86	0.57	0.23
	AIC	41.96	-	42.49	59.05	63.78	45.10	-	44.00	68.21	72.44

4.3.4 Examining the relationship between thermal thresholds for colony extension, germination and virulence of EPF isolates

Cardinal temperatures estimated for colony extension, proportion germination and virulence of EPF estimated by the Briere-1 model were compared for the same isolates. The germination thermal optima were plotted against colony extension optima for each isolate. This was repeated for *T0*. For the five isolates, which were included in temperature virulence experiments, virulence optima were plotted against colony extension and germination optima. These plots were also completed for *T0* values. Linear regression models were used to assess the relationship between cardinal temperatures for colony extension, germination and virulence for isolates.

For the plot in which colony extension optima were plotted against germination, the linear regression model did not differ significant from a zero relationship, indicating that there is no relationship between the two variables (Fig. 4-40). For the plot in which virulence optima were plotted against germination optima, the relationship did not differ significantly from zero (Fig. 4-40). The relationship between colony extension optima and virulence also did not display a significant relationship. The linear regression models for optima of virulence vs. germination, virulence vs. colony extension and colony extension vs. germination returned p-values of 0.13, 0.28 and 0.1, respectively (Table 4-10).

Similarly, linear regression models describing the relation between *T0*s for colony extension and germination, germination and virulence and colony extension and virulence did not differ significantly from a zero relationship (Fig. 4-41). The linear regression models for *T0*s of virulence vs. germination, virulence vs. colony extension and colony extension vs. germination returned p-values of 0.22, 0.14 and 0.13, respectively (Table 4-11).

Table 4-10: Parameters of linear regression models describing the relationship between *Topts* of germination, colony extension and virulence.

Model	P-value	*a*	*b*	r^2	AIC
Germination vs colony extension	0.10	0.46	16.16	0.14	57.90
Germination vs virulence	0.13	0.77	7.61	0.44	23.89
Colony extension vs virulence	0.28	0.16	22.38	0.16	12.73

Table 4-11: Parameters of linear regression models describing the relationship between *T0*s of germination, colony extension and virulence.

Model	P-value	*a*	*b*	r^2	AIC
Germination vs colony extension	0.14	0.52	-0.11	0.42	26.56
Germination vs virulence	0.22	0.35	8.68	0.26	25.28
Colony extension vs virulence	0.13	0.25	10.39	0.11	66.62

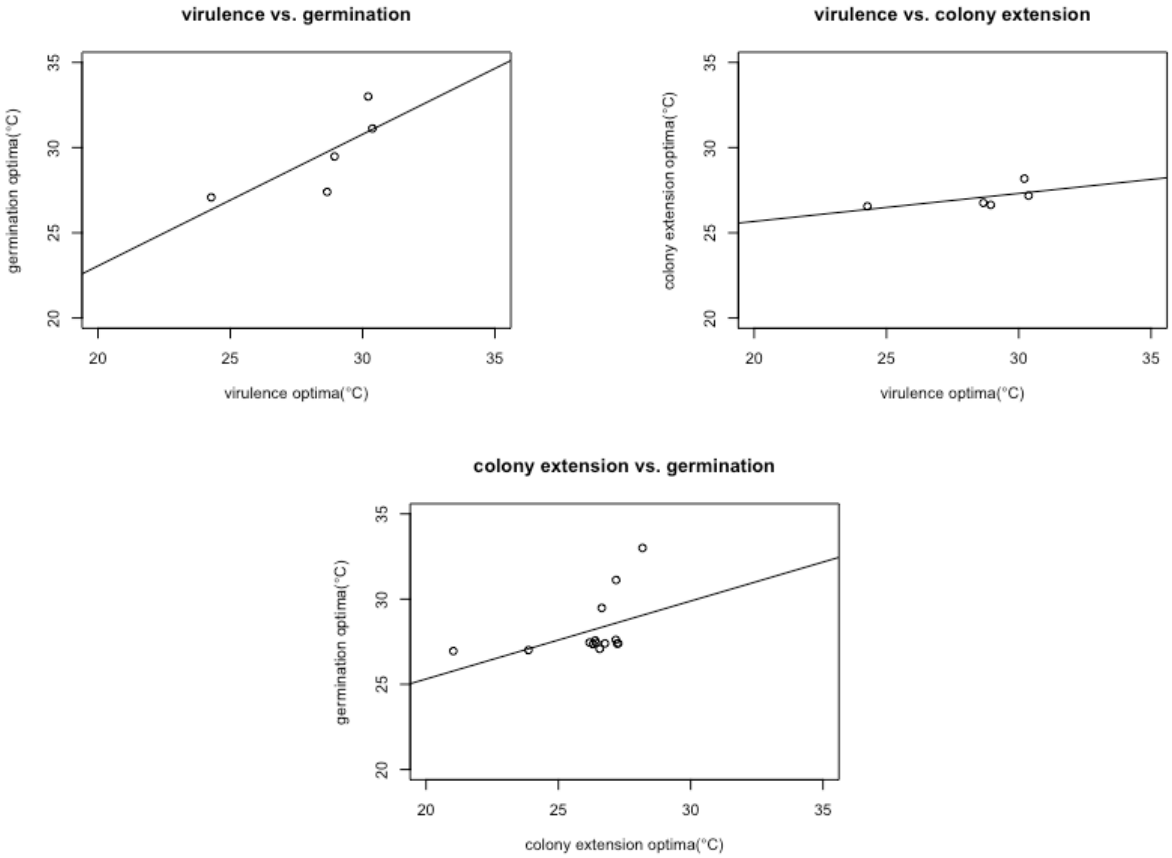


Figure 4-40: Optima (defined by Briere-1 model) for virulence plotted against germination optima, virulence optima plotted against colony extension optima and colony extension optima plotted against germination optima. Linear regression models are included for each plot.

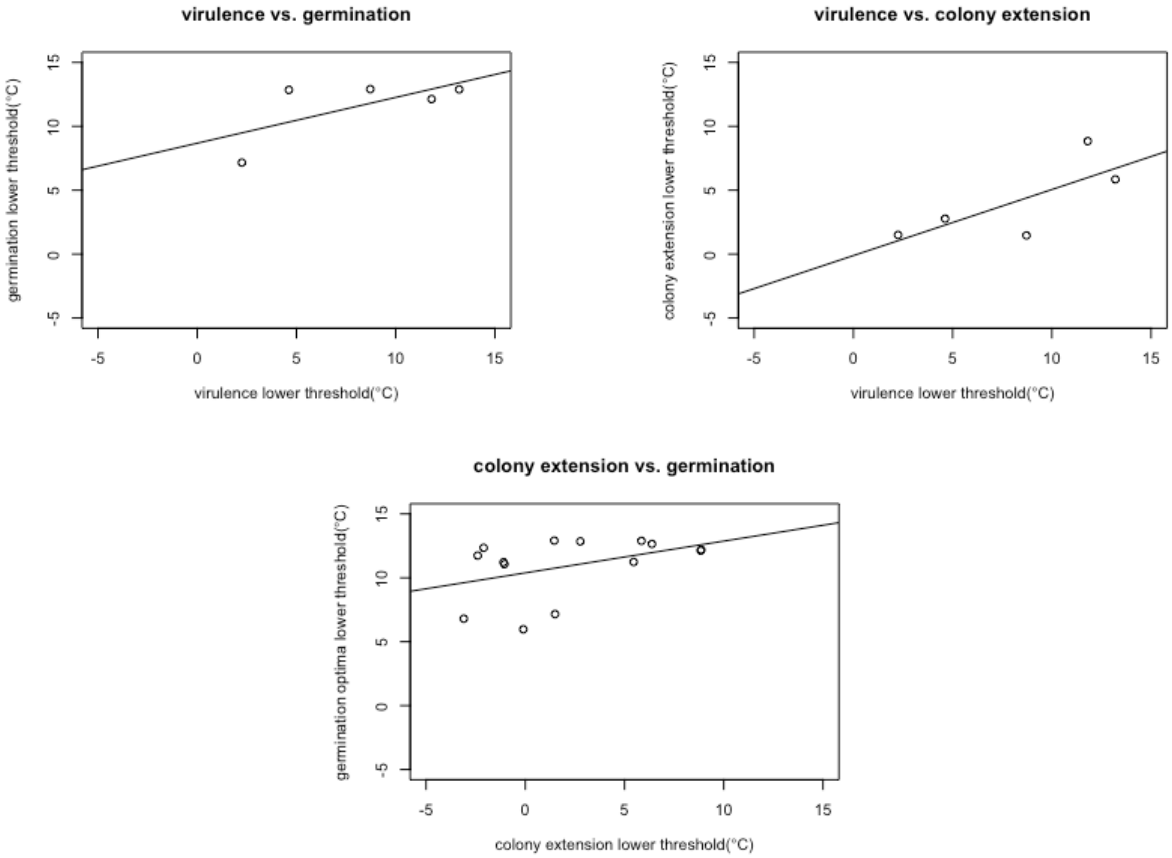


Figure 4-41: *T0* (defined by Briere-1 model) for virulence plotted against germination *T0*, virulence *T0* plotted against colony extension *T0* and colony extension *T0* plotted against germination *T0*. linear regression models are included for each plot.

4.3.5 Examining the relationship between virulence, colony extension rates and proportion germination across a range of temperatures

Data for colony extension, proportion germination and proportion mortality recorded at specific temperatures were paired for each isolate and plotted against each other to assess whether there was correlation between these data across temperature. When looking at the relationship between proportion germination and colony extension rate, data for five isolates were available. When looking at the relationship between colony extension rate and proportion germination across several temperatures, data for 14 isolates were available.

For scatter plots explaining all three relationships it was found that there was a positive correlation which differed significantly from a zero relationship (Table 4-12; Fig, 4-42). This indicates that, across the temperature range tested, virulence can be predicted by colony extension rate and germination.

Table 4-12: Various parameters of linear models describing the relationship between proportion germination, colony extension and proportion mortality.

Model	P-value	*a*	*b*	r^2	AIC
Germination vs colony extension	<0.001	0.46	384.7	0.37	797.0
Germination vs virulence	<0.001	0.70	12.2	0.57	271.02
Colony extension vs virulence	<0.001	0.89	228.7	0.16	12.73

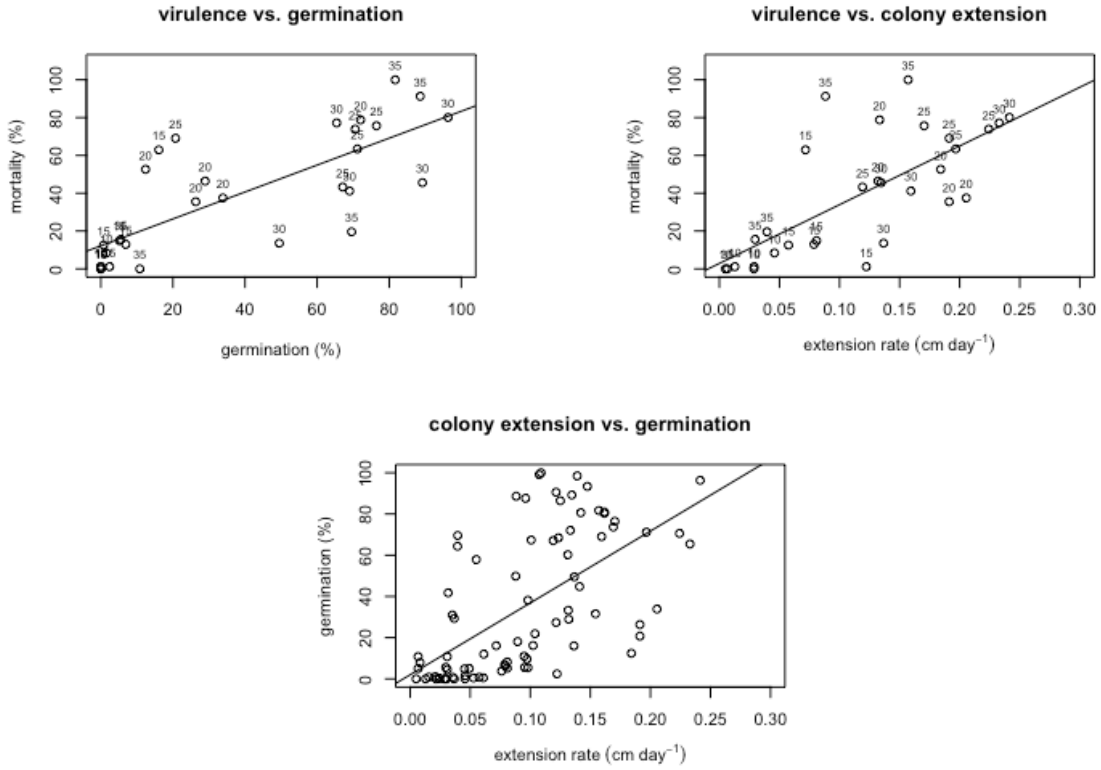


Figure 4-42: Mean proportion germination plotted against mean proportion mortality for six temperatures, mean colony extension rate (cm day^{-1}) plotted against proportion mortality, and mean colony extension rate (cm day^{-1}) plotted against mean proportion germination. Labels indicating temperature are included for “virulence vs germination” and “virulence vs colony extension” plots. A linear model is included for each plot.

4.3.6 Developing DD models to predict rate of DBM development in the field and virulence of selected EPF isolates against groups of DBM larvae

4.3.6.1 Developing a DD model to predict the rate development of DBM in the field

The relationship between temperature and development from egg lay to adult was linear between 12.5 and 30^{o}C. A linear regression model (defined as y = -0.029 + 0.0036*x) was fitted to this relationship which displayed an r^2 value of 0.98. This model established that, according to experimental data, the lower thermal threshold (*T0*) was 8.06^{o}C and 277.78 accumulated DD were required for DBM to develop from egg lay to adult.

A Linear regression model was used to describe the relationship between days to development and cumulative DD (using field data Table 4-3), and it was confirmed that there was constant DD sum regardless of how many days it took for the insects to develop (linear regression, p-value = 0.17) (Figure 4-43).

Two sources of data were used to validate the DD model:

i. Daily max and min field temperature
ii. Field data of DBM caught over the course of the summer months

Field temperatures (i) were used to calculate the number of DD accumulated. The starting point of DD accumulation was the day at which the first peak in DBM was observed (from the DBM field data (i)). The day at which 277.78 DD was accumulated was taken as the prediction for the emergence of the second generation of DBM adults. The observed date of the second peak was then compared to the predicted date of the second peak. The minimum difference between observed and predicted second peaks was four days, which occurred at two sites. The maximum difference was 10 days which occurred at one site. The mean absolute difference between observed and predicted second peaks was 6.62 days (Table 4-13).

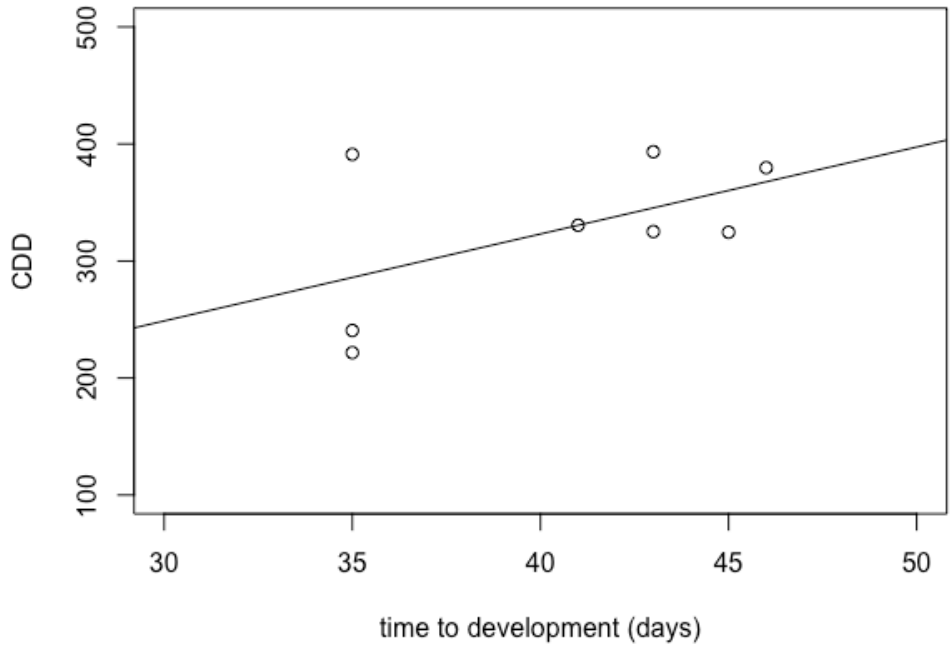


Figure 4-43: Time taken for DBM to develop from egg to adult (days) plotted against cumulative DD (CDD) to development for eight field sites, along with a linear regression model

Table 4-13: Comparison between dates of peaks predicted using DD model, and observed dates for peaks for eight sites.

Site	Year	Observed time to second peak (days)	Predicted time to second peak (days)	Difference between observed and predicted (days)
Kirton	1995	35	26	9
Kirton	1996	43	39	4
Wellesbourne	1996	46	37	9
Butterwick	1996	44	34	10
Friskney	1996	45	41	4
Holbeach	1996	35	41	6
Butterwick	1998	41	35	6
Kirton	2000	35	40	5
			Mean total difference	**6.62**

4.3.6.2 Developing a DD model to predict the virulence of EPF isolates against groups of DBM larvae at fluctuating temperatures

To calculate accumulated DD over a specific period using field temperatures it is necessary to know the upper and lower thermal thresholds of the EPF, in terms of virulence. The best fitting model, based on uncorrected control mortality, from section 4.3.3 was used. Briere-1 was found to be the best fitting model, a table summarising the parameters obtained using this model for germination, colony extension and virulence experiments can be seen in Appendix III. *T0* and *Topt* from the Briere-1 were used as the lower and upper thermal thresholds respectively, these values can be found in Table 4-13. For *B. bassiana* 1757.15, because replicate three of the experiment was excluded from the analysis here (for reasons explained below) a separate Breire-1 model was fitted to the mean of percentage mortality values from repetition one and two of the

experiment. This model displayed *Topt, T0, Tmax* and *a* parameters of 25.12, 10.67, 30.37 and 0.11 respectively. AIC and r^2 values were 48.65 and 0.84, respectively.

Table 4-14: Lower and upper thermal thresholds (^{o}C) used in day-degree model to predict proportion mortality caused by groups of four isolates. Lower and upper thresholds are *T0* and *Topt* taken from Briere-1 model from Table 4-9.

Isolate	Lower temperature threshold (^{o}C)	Upper temperature threshold(^{o}C)
B. bassiana 433.99	5.17	29.80
B. bassiana 1757.15	10.30	24.91
M. brunneum 445.99	11.39	31.40
M. brunneum 1760.15	9.68	31.25

It was next determined whether a certain percentage mortality occurred a constant accumulated DD sum, regardless of the temperature the accumulated DD were accumulated over. Ideally, any given percentage mortality would occur at the same DD sum, regardless of the temperature the DD were accumulated at. However, in this instance at least four data points were required to fit a linear model. So, the maximum percentage mortality value that could be used was 15%. For each isolate, the number of accumulated DD at which 15% mortality occurred was plotted against temperature (Fig. 4-43). Temperatures over the upper threshold were omitted with the exception of *B. bassiana* 433.99 and *B. bassiana* 1757.15 where 30 and 25^{o}C were included, to insure there were at least four data points in the analysis. The linear regression model did not differ significantly from zero relationship for any of the isolates (p=>0.05). This indicates that 15% mortality occurs at the same accumulated DD sum, regardless of the temperature at which the DD are accumulated.

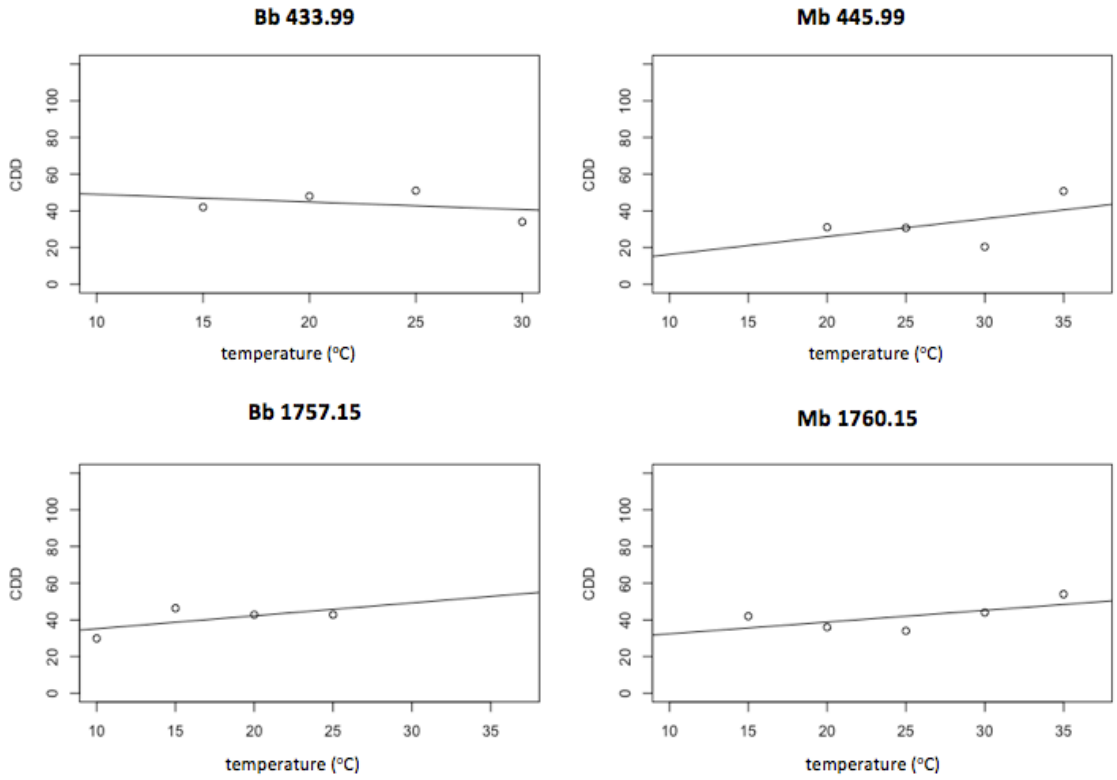


Figure 4-44: The number of accumulated DD at which 15% mortality was reached in groups of DBM larvae treated with one for four EPF isolates, for various temperatures. A linear regression model is included for each isolate.

To determine proportion mortality from a certain day degree sum, it would be necessary to use a statistical regression model to explain the relationship temperature and mortality. But first it had to be determined whether a separate linear model was required for each day of the experiment. For each isolate, two linear models were compared, one incorporating day as a factor, and one in which pooled all days of the experiment. For *B. bassiana* 433.99, *M. brunneum* 445.99 and *M. brunneum* 1760.15 incorporating day as a factor did not significantly improve the linear model (Anova, p= >0.05). Consequently, one equation could be used to describe the relationship between proportion mortality and accumulated DD for these isolates. For *B. bassiana* 1757.15, the linear model incorporating day as a factor was a significant improvement over the linear model not including day as a factor (Anova, p=<0.05). It was observed that there was abnormally low mortality at low temperatures in replicate three of the experiment, so the analysis was repeated for *B. bassiana* 1757.15 omitting the third repetition of the experiment. After removing replicate three, the linear model incorporating day as a

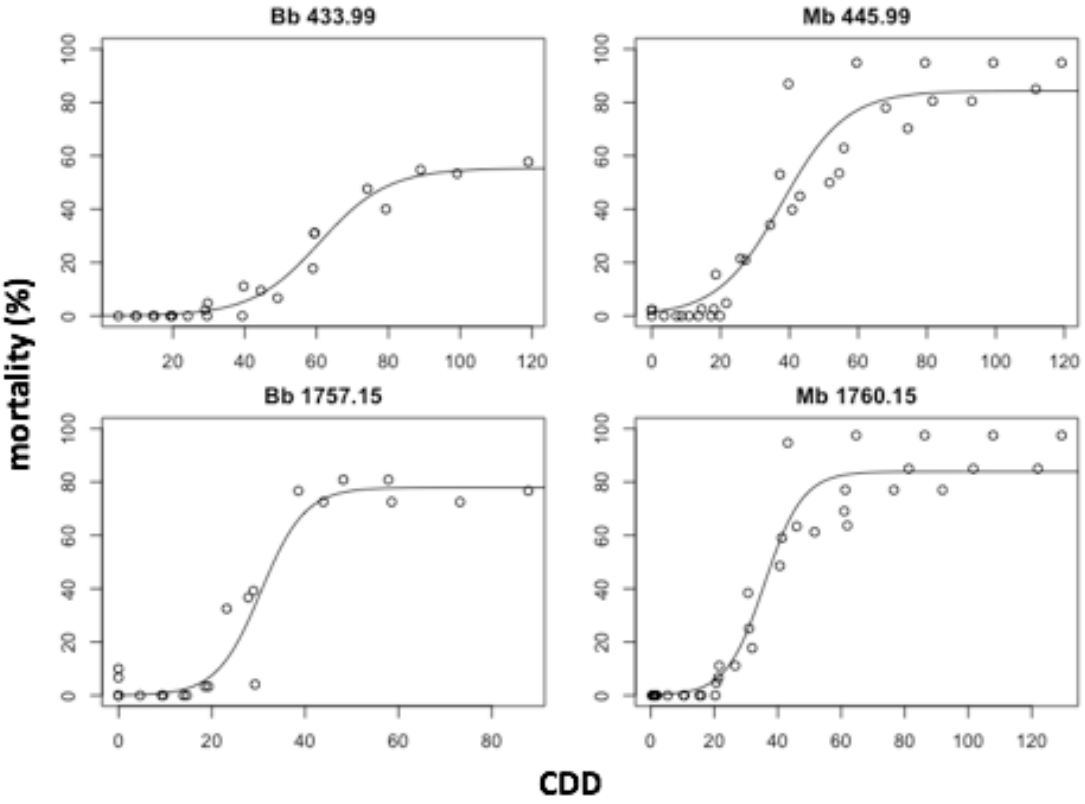
factor was not a significant improvement over the linear model including day as a factor (Anova, p=>0.05). For each isolate, the same equation was used for each day of the experiment to describe the relationship temperature and proportion mortality (Table 4-14).

After plotting proportion mortality against cumulative DDs it was observed the relationship followed a clear sigmoidal relationship (Fig. 4-44). After fitting sigmoidal and linear models to the relationship between cumulative DD and percentage mortality for the four isolates, it was observed that AIC values were lower and r^2 values were higher for the sigmoidal when compared to the linear model (Table 4-14). This indicated that the sigmoidal model should be used to calculate the percentage mortality from cumulative DDs. Due to its better fit when compared to the linear regression model, predictions were made for the three variable temperature regimes using the sigmoidal model, for four isolates.

Table 4-15: Parameters for sigmoidal and linear regression models, describing the relationship between proportion mortality and CDD, for four isolates. For sigmoidal models, parameters a, b and c represent the maximum y-value, the scaling factor and the equation constant, respectively. For linear models, parameters a and b represent the slope and intercept, respectively.

	Sigmoidal					**Linear**			
Isolate	*a*	*b*	*c*	AIC	R^2	a	b	AIC	R^2
B. bassiana 433.99	55.30	0.11	60.95	137.87	0.97	-12.14	0.63	164.44	0.89
B. bassiana 1757.15	77.87	0.21	30.54	178.82	0.93	-2.14	1.18	205.26	0.77
M. brunneum 445.99	84.30	0.02	37.77	280.41	0.91	-1.37	0.98	299.66	0.84
M. brunneum 1760.15	83.86	0.16	35.85	271.17	0.94	-0.25	0.95	310.33	0.80

Figure 4-45: Mean mortality (%) of groups of DBM larvae plotted against CDD for four isolates, along with sigmoidal model for each isolate. Bb and Mb refer to *B. bassiana* and *M. brunneum* isolates, respectively.



The effect of *M. brunneum* 445.99 and 1760.15 on the survival of groups of DBM larvae were tested at all three variable temperature regimes, because of the broad thermal range these EPF exhibited. *B. bassiana* 433.99 was tested at 10-16 and 19-25^{o}C, but omitted from the 29-35^{o}C treatment because the isolate displayed an optimum of 29.8 ^{o}C. *B. bassiana* displayed an optimum of below 25^{o}C and so was omitted from the 19-25^{o}C and 29-25^{o}C experiments. The mean real-time temperatures for all three variable temperature regimes can be seen in Fig. 4-4.

Control mortality was 9.7±0.3, 3.7±3.7 and 13.9±5.6% at seven days after treatment for temperature regimes 9-15, 19-25 and 29-35^{p}C, respectively. Generally, mortality was highest at the 29-35^{p}C regime and lowest at 9-15^{p}C across all isolates. At 29-35^{p}C over 90% insect mortality occurred after day three for *M. brunneum* 445.99 and 1760.15. At 19-25^{p}C, *B. bassiana* 433.99 and *M. brunneum* 1760.15 conferred between 50 and 60% mortality by seven days after treatment. *M. brunneum* 445.99 conferred approximately 75% mortality by this point. At 9-15^{p}C, *B. bassiana* 1757.15 conferred the highest mortality, displaying approximately 50% mortality by seven days after treatment. All other isolates conferred under 20% mortality.

The sigmoidal DD model was used to calculate the number of larvae expected to die on each day of the experiment by using the formulae from Table 4-14 to derive proportion mortality from accumulated DD value. Expected counts of dead larvae were calculated by multiplying the proportion mortality derived from the sigmoidal DD model by the total number of larvae in the larvae in the experiment. Comparisons of expected and observed proportion mortality for each of the four EPF isolates, from one to six days after treatment can be seen in Fig. 4-46.

As Expected numbers of dead larvae were below 5 in over 20% of cases for each isolate and temperature regime, Fisher's exact test was used to test the hypothesis that there was no difference in counts of dead larvae between predicted and expected values.

For groups of larvae treated with *B. bassiana* 433.99 at 10-16 and 19-25^{o}C temperature regime, there was no difference between expected and observed values (Fisher's exact test, P=>0.05). For groups of larvae treated with *B. bassiana* 1757.15 treated at 10-16^{o}C, there was no difference between expected and observed numbers of dead larvae (Fisher's exact test, P=0.40). For groups of larvae treated with *M. brunneum* 445.99, there was no difference between expected and observed numbers of dead larvae on each day of the experiment for all three temperature regimes (Fisher's exact test, P=>0.05). Similarly, there was no difference between expected and observed values for dead larvae treated with *M. brunneum* 1760.15 for all temperature regimes (Fisher's exact test, P=>0.05). Predictions were generally more accurate at 19-25^{o}C when

compared to 10-16 and 29-35°C (Fig. 4-46). Mortality was generally higher for observed mortality when compared to expected mortality at 29-35°C. For example, for *M. brunneum* 445.99 and 1760.15 predictions and observed mortality (%) for six days after treatment was 86.1 and 100, and 85.7 and 91.6. For 10-16 °C, predictions of percentage mortality were lower than observed mortality for all isolates, by six days after treatment (Fig. 4-46). In conclusion, the sigmoidal provided accurate descriptions of percentage mortality for all isolates and temperature regimes. Predictions were generally more accurate at 10-16 and 19-25°C when compared to 29-35°C.

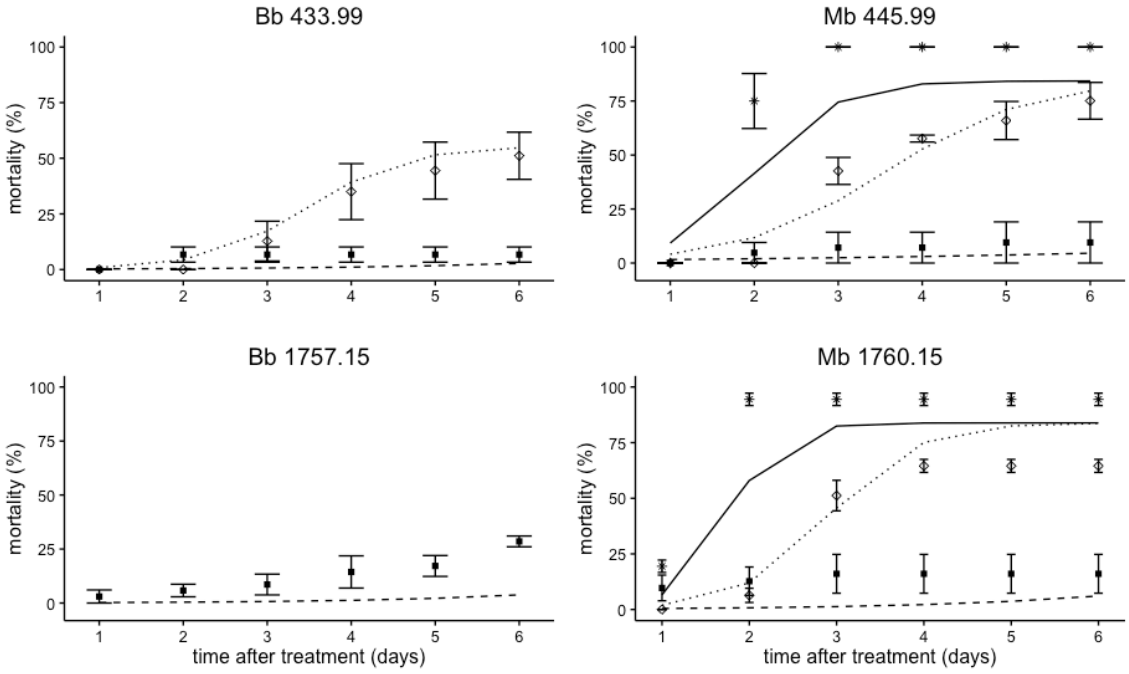


Figure 4-46: Mean percentage mortality (±SE) of groups of DBM larvae at one of three fluctuating temperature regimes for four isolates. Temperature regimes 10-16, 19-25, 29-35°C are represented by■, ◇,and ∗, respectively. Predictions for percentage mortality at the three variable temperature regimes are shown by lines. Dotted, longdashed and solid lines represent predictions for 10-16, 19-25 and 29-35°C, respectively.

4.4 Discussion

The current investigation can be split into two sequential stages:

i. **The suitability of five non-linear mathematical models in describing the effect of temperature on the following processes was assessed:**
    - EPF physiology (colony extension of EPF isolates and percentage germination of populations of EPF conidia)
    - Development rate of DBM
    - Virulence of EPF isolates against DBM

i. **The best fitting model to describe interactions from (i) was determined to be the Briere-1 model, cardinal temperatures from this model were used for the following ends:**
    - To assess whether the thermal optima for EPF physiology could be used predict the thermal optima of EPF virulence
    - To compare and match the thermal profiles of virulence of EPF isolates and DBM development in order to suggest the most suitable EPF isolates to be used in DBM biocontrol
    - To develop a DD model (based on data collected on the effect of temperature on virulence of EPF isolates from (i)) to predict the virulence of EPF isolates against groups of DBM larvae at fluctuating field "field realistic" temperatures

4.4.1 Analysis of interactions between pest, pathogen and environment using non-linear models

The aim of this part of the investigation was to evaluate the suitability of five non-linear models for the purpose of describing the effect of temperature on EPF growth, DBM development and EPF virulence. Once one model had been determined to be suitable

to describe all these interactions, thermal profiles of EPF growth of isolates, DBM development and EPF virulence could then be appropriately compared.

EPF thermal physiology investigations, comprising of colony extension and germination experiments, were completed for all isolates. The majority of bell-shaped distributions were skewed to the left, with the exception of some isolates with lower thermal optima, such as *L. longisporum* 1.72 and *L. muscarium* 19.79. An investigation completed by Smits et al. (2003), in which colony extension rates of five EPF isolates over several temperatures were described using non-linear mathematical models, yielded similar left-skewed distributions. In the current study, Logan-6 and Lactin-1 models tended to better fit to data that was skewed more to the left. Conversely, Taylor and polynomial models were best at explaining more normally distributed data. The Briere-1 model displayed relatively high r^2 values for both left skewed and normally distributed data, and so is the most appropriate model to be used in this scenario. Similarly, Smits et al. (2003) compared the observed thermal colony extension optima of five isolates with those derived from non-linear mathematical models and found Briere-1 and Lactin-1 to provide the most accurate estimations.

Germination optima were generally higher when compared to colony extension optima, *B. bassiana, I.fumosorosea*, *L. longisporum* and *L. muscarium* isolates had germination optima of ~27^{o}C. *M. brunneum* isolates displayed germination optima of over 30^{o}C, this is in line with reports of *M. anisopliae* isolates germinating at up to 37^{o}C (Walstad et al., 1970). There was less variation between colony extension optima when compared to germination optima, with colony extension optima being in the region of 25-28^{o}C for *M. brunneum, I.fumosorosea* and *B. bassiana* isolates. This was similar to the optima reported for *M. anisopliae* and *B. bassiana* isolates in a colony extension study by (Smits et al., 2003). *L. longisporum* 1.72 and *L. muscarium* 19.79 displayed lower colony extension optima (23.87 and 21.03^{o}C, respectively). The latter is comparable to the optimimum of 22.9^{o}C found by Davidson et al. (2003) for *L. muscarium* 19.79, although this optimum was derived using a non-linear model not used in this study.

For DBM development Briere-1, Lactin-1 and Logan-6 models proved to be the best fit, as they are designed to model biological responses to temperature which are skewed to the left. These models were also shown to fit well to DBM development in other studies (Golizadeh et al., 2007, Marchioro and Foerster, 2011). However, it is not possible to determine the lower threshold temperature using Lactin-1 and Logan-6 models because the horizontal axis is not crossed. The lower threshold temperature is important in terms of biocontrol and pest forecasting as it represents the minimum temperature at which development can occur. Consequently, Briere-1 is more suitable for modeling DBM development even though Lactin-1 and Logan-6 models displayed slightly higher r^2 values.

DBM developed to adulthood between 12.5 and 30^{o}C, this temperature range is similar across the majority of studies on DBM development. At 20^{o}C it took 24.4±0.1 days for DBM to develop from egg lay to adult. This is comparable to the figures of 24.3±0.18 and 21.4-23.5 days reported by Golizadeh et al. (2007) and Marchioro and Foerster (2011), respectively. The optima for DBM development was 28.6^{o}C (using the Briere-1 model), this is similar to other reports of temperature dependent DBM development in the literature which generally determine the development optima to be in the region of 27-30^{o}C.

To determine the effect of temperature on the virulence of fungal isolates it was necessary to separate mortality caused from EPF infection from mortality caused from temperature stress alone. If there is no interaction between mortality caused by temperature stress and mortality caused by EPF infection, the proportion mortality caused from EPF infection could be determined by correcting for control mortality using an equation based on an assumption of additivity, such as Schneider-Orelli's formula (Puntener, 1981).

It was observed that control mortality increased with temperature; at 10^{o}C mortality was ~3% and at 35 and 36.5^{o}C mortality was ~50 and ~65%, respectively. This is similar to a report in the literature of larvae undergoing approximately 50% mortality after being placed at 35^{o}C at the beginning of the larval stage of development (Ngowi et al.,

2017). It was determined that this increase in control mortality was linear, therefore it can be assumed that there is additive interaction between temperature stress and EPF infection stress (*personal communication*). It was therefore appropriate to use a correction equation based on additive assumption, to separate mortality caused from EPF infection from mortality as a result of temperature stress. Scneiders-Orelli's formulae was used to correct for control mortality.

The relationship between temperature and virulence followed a bell-shaped distribution skewed to the left for all isolates. A similar bell-shaped distribution in mortality was found when *B. bassiana*, at a concentration of $1x10^8$ conidia ml^{-1}, was applied to groups of housefly larvae, although this relationship was not described using mathematical models (Mishra et al., 2015). In the current study, Briere-1 was the only model to maintain a high r^2 for all isolates. Two *M. brunneum* isolates displayed relatively high virulence optima. *B. bassiana* 433.99 displayed highest mortality at 25-30^oC, this is in agreement with a study by Vandenberg et al. (1998) in which this isolate was applied to second instar DBM larvae using the same application technique as used in the current study. However, mortality was higher at 25^oC in the Vandenberg et al. (1998) study compared to the current study, being 72±9% in the former and 57.8±12% in the latter. In the current study, *B. bassiana* 433.99 displayed a thermal optimum of 28-30^oC depending on the mathematical model used. This is in agreement with Mishra et al. (2015), where the virulence optima of *B. bassiana* was reported to be 30^oC when applied to groups of housefly larvae.

To appropriately compare cardinal temperatures of EPF growth, EPF germination, EPF virulence and DBM development of different isolates it is necessary to identify one model that is able to explain all of the said relationships. We suggest that the Briere-1 model can be used to do this. As explained in the previous paragraphs, this model fits well to data with both low and high thermal optima. It also has an advantage over other models in that it reports useful biological parameters such as the minimum threshold temperature, the maximum threshold temperature and optimum temperature.

The comparison of thermal optima had two applications in this study. First, for the biocontrol of a pest such as DBM, it is vital to match the thermal profile of the insect to the thermal profile of the EPF biopesticide. This is because DBM is likely to cause the most crop damage at temperatures close to the insect's development optimum, as there will be a high number of individual larvae. Self-evidently, an EPF isolate with virulence optima similar to that of the DBM development optima would be most useful. This is an important because initial screens of EPF isolates are often completed at a fixed temperature (normally 20 or 25^{o}C) which is separate to that of the development optima (Thomas and Blanford, 2003).

In the initial screen of candidate isolates completed in Chapter three, *B. bassiana* 1757.15 was observed as being highly virulent. In fact, it was far more virulent than *M. brunneum* 1760.15 at 20^{o}C. But after thermal profiling of EPF virulence it was found that *M. brunneum* 1760.15 was far more active at the optimal DBM development temperature. This isolate would therefore be more appropriate for use as biocontrol control product, when compared to *B. bassiana* 1757.15 (Fig. 3-47).

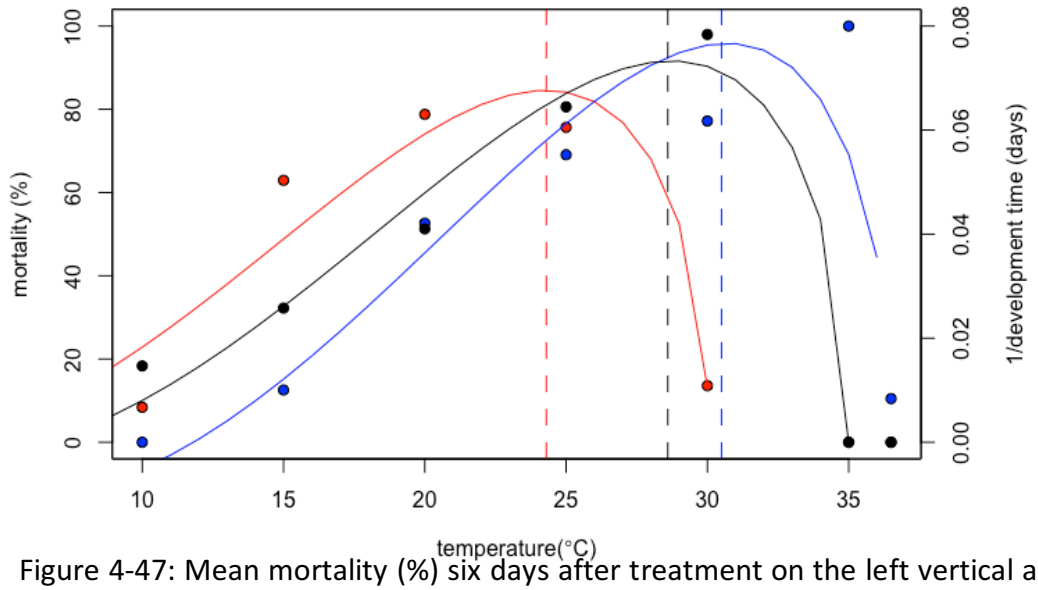


Figure 4-47: Mean mortality (%) six days after treatment on the left vertical axis plotted against temperature (°C), along with a fitted Briere-1 models, for *B. bassiana* 1757.15 (red data points and lines) and *M. brunneum* 1760.15 (blue data points and lines). DBM development rate (1/ development time (days)) from egg lay to adult was also plotted on the right horizontal axis against temperature (°c), along with a fitted briere-1 model (represented by black data points and lines). Red and blue broken horizontal lines represent virulence optima for *B. bassiana* 1757.15 and *M. brunneum* 1760.15, respectively. The development optimum is represented by the broken black line.

Using one model to define thermal thresholds for fungi and EPF virulence makes it possible to assess the relationship between thermal optima for EPF physiology and EPF virulence. There have been many incidences in the literature of determining the effect of temperature on either EPF germination or EPF colony extension, with the expectation of assessing the isolates for use with in biocontrol system. However, there has been no attempt to directly compare the relationship between EPF physiology optima and EPF virulence optima. It is often assumed that because EPF isolates grow or germinate well at high temperatures they will confer high mortality at high temperatures. For example, Davidson et al. (2003) completed an extensive study in which a cohort of EPF isolates were tested for suitability for use with in an IPM system to control *Varoea Destructor*, which live at high temperatures. The colony extension rates of isolates were tested over

a range of temperatures, and those with high thermal optima were suggested as being candidates to control the pest.

Here, after fitting linear regression models, we found that there was no significant correlation between the following parameters:

i. Thermal optima for EPF colony extension and thermal optima for EPF germination
ii. Thermal optima for EPF virulence and thermal optima for EPF colony extension
iii. Thermal optima for EPF virulence and thermal optima for EPF germination

Consequently, it was concluded that virulence optima of EPF isolates cannot be predicted by thermal optima for either colony extension or germination of EPF isolates. The interaction between the pathogen (EPF), host (DBM) and environment (temperature) can be described as genotype x genotype x environment (GxGxE). It may not be possible to predict virulence based on the far simpler genotype x environment (GxE) interaction (such as determining EPF growth over a range of temperatures).

However, it was also noted that because of the lack of data points, the statistical test associated with the linear model lacked power, which made it likely that a non-significant relationship would be found. Keeping this in mind, further analysis was completed in which colony extension rates, proportion germination and proportion mortality measured at different temperatures were plotted against each other. It was found that there were significant positive correlations between:

i. Colony extension rate and proportion germination
ii. Colony extension rate and proportion mortality
iii. Proportion germination and proportion germination

This indicates that, although it may not be possible to predict cardinal temperatures using this approach, each of these variables can be used to predict the other over the temperature range tested. This is in line with a review of the effect of temperature on virulence of fungal isolates Thomas and Blanford (2003) which suggested that although

determining the effect of temperature on each component of the GxGxE interaction (e.g. GxE interactions) can provide useful insights in to how virulence will be effected by temperature, direct experimentation is still necessary to fully understand how virulence changes with temperature. It is suggested that this is especially important when there is a distinct difference between thermal profiles of the pathogen (in this case, EPF) and the pest (in this case, DBM).

In this study, we found that EPF isolates with high thermal optima for EPF physiology, tended to have high thermal optima for EPF virulence. For example, *M. brunneum* 445.99 and 1760.15 displayed optima of ~30^{o}C for EPF growth and virulence. *B. bassiana* 1757.15 displayed an EPF growth optimum of ~27^{o}C, but much lower virulence optimum of ~24^{o}C. These differences can be explained by taking the thermal profile of the insect in to account. The thermal optima of DBM development is ~28^{o}C, meaning that *M. brunneum* 445.99 and 1760.15 are active at a temperature at which DBM may be more susceptible to infection. Although DBM immunity is an under explored area, it is known enzymes such as proteases are associated with the innate immune response (Eum et al., 2007). It is possible these enzymes would become less effective as temperature stress rises, making it easier for fungi to proliferate with in the hemolymph and cause insect mortality. Conversely, fungi which have similar thermal optima to DBM would encounter more resistance from the host immune system.

4.4.2 Day degree analysis of DBM development and fungal virulence

Although it is known that the response of insect development to temperature is non-linear in nature, linear regression can be useful in describing this relationship at permissive temperatures, that is; temperatures up to the optima for development. Here, a linear regression model explained the response of DBM development rate well between 12.5 and 30^{o}C. This was used to determine, *T0* (lower threshold temperature) and *k* (CDD required for development). The values for *T0* and *k* found here (8.06 ^{o}C and 277.78 DD, respectively) were in agreement with the numerous of studies of DBM development. For example, Harcourt (1954) reported *T0* and *k* to be 7.3 ^{o}C and 283 DD,

respectively. Most other studies report similar figures (Golizadeh et al., 2007, Marchioro and Foerster, 2011).

After developing a DD model, it was necessary to validate it using field data. In a similar methodology to study by Baker et al. (1982) DD accumulations based on a *T0* value were used to predict peaks of adult abundance in the field, this prediction was then compared to the observed peak (by using field data). The main difference between the current study and Baker et al. (1982) was that the latter study used a fixed start date, from which DD were accumulated. This was possible because DBM were known to over winter in the US. However, it is known that DBM do not over winter in the UK, infestations usually occur through migration of the moth from mainland Europe (Chapman et al., 2002). In the current study, it was decided the start date for DD accumulation would be the date at which a first peak in adult DBM numbers was observed in the field.

Field data of DBM abundance was obtained for years between 1995 and 2000 for five sites across the UK. Of these, 12 datasets displayed a first generation peak followed by a clear second generation peak. Temperature data was only available for eight of these sites. The DD model was accurate in predicting the date of the second peak in the field. There was a mean absolute difference of 6.6 days between observed and expected peaks with minimum difference of four days and maximum difference of 10 days. This similar to the study by Baker et al. (1982) in which maximum and minimum differences between predicted and observed peaks were six and one days, respectively.

The differences seen between expected and observed peaks may be due to potential inaccuracies in temperature data used in this study. The weather stations used to provide temperature data were up to 14 miles away from the field study site, meaning temperature experienced by developing DBM may be different to temperature used in the analysis. It was shown in a previous study that small variations in temperatures used in analysis can affect predictions of development time by up to 87% (Mohandass, 2001). Other meteorological factors may also have an impact on the development of DBM, for example; humidity or wind speed.

The fact that a DD model can be used to accurately predict the development, and emergence of DBM in the field has important implications for crop protection in terms of timing of insecticide applications and cultural control techniques. This is especially relevant in countries in which DBM are able to overwinter. However, it has long been known that accurate predictions can be made on the development on DBM using DD models.

One of the main problems with use of EPF in pest control is the unpredictability of their performance when applied in the field. Seemingly promising isolates in the laboratory can be taken into the field and be substantially less effective (Thomas and Blanford, 2003). This unpredictability has proven to be a significant barrier to uptake of EPF as biocontrol products in the field (Lacey et al., 2001). There have been reports in the literature of predictions of temperature-related phonological events based on constant temperatures proving to be inaccurate when applied to fluctuating temperature regimes (Geden, 1997). Because of this, it is important to determine whether a DD style model, based on data recorded at constant temperatures, can be used to predict virulence at fluctuating "field realistic" temperatures. To the best of this author's knowledge, there has no attempt in the literature to predict fungal virulence based on a DD style model.

The best fitting model established in the first part of this study was used to develop a DD model used to predict proportion mortality at fluctuating temperature regimes. DD were only accumulated between *T0* and *Topt*, as defined by the Briere-1 model. It was found that the relationship between DD and proportion mortality of groups of DBM larvae followed a sigmoidal relationship. Consequently, a sigmoidal model was used to describe this relationship. The relationships between proportion mortality for *M. brunneum* 1760.15 and *M. brunnuem* 445.99 were not significantly different, presumably because both isolates displayed similar virulence and are well suited to higher temperatures. However, *B. bassiana* 1757.15 and *B. bassiana* 433.99 displayed sigmoidal models which were significantly different, most likely because of the relatively high virulence of the former isolate. Because only two isolates shared statistically similar relationships, isolates were not pooled for analysis. Predictions were made at three

variable temperature regimes for proportion mortality caused by each isolate. *M. brunneum* 445.99 and 1760.15 displayed thermal virulence optima of over 30^{o}C and were determined to be high temperature isolates, and so were included in all three temperature regimes. *B. bassiana* 433.99 was considered to suited to lower and mid-range temperatures and so was not included in the high fluctuating temperature regime. Because *B. bassiana* 1757.15 had a relatively low thermal optimum, it was only included in the low variable temperature regime. The predictions made here was observed to be relatively accurate according to a Fisher's exact test. However, predictions were far more accurate for the 10-16 and 19-25^{o}C, when compared to the 29-35^{o}C regime.

These results indicate that is possible to predict mortality at variable temperatures using a sigmoidal DD model based on experiments completed at constant temperatures. A similar conclusion was drawn by Burgess and Griffin (1967) who found that colony extension rates of fungi experiencing fluctuating temperatures could be predicted from the colony extension rates of fungi at constant temperatures. The use of this model may help reduce the discrepancy often observed between isolates' performance in the laboratory and in the field. However, it is important to note that the experiments completed here were in controlled laboratory conditions. Humidity was constantly over 90% during experiments and only early second instars were used. In the field, EPF would be used to control a mixed age population of insects. As explained in the introduction, the shedding of conidia through moulting may provide another source of variation. Consequently, validation of the sigmoidal DD using semi-field or field experiments would be the next logical step in this research.

4.4.3 Summary

i. The suitability of five non-linear models for describing the following interactions was assessed:
    - The effect of temperature on proportion germination of groups of EPF conidia.
    - The effect of temperature on colony extension of EPF isolates.
    - The effect of temperature on the development rate of DBM.
    - The effect of temperature on the virulence of selected EPF isolates.

i. The Briere-1 model was suggested as the most appropriate model to describe the interactions from (i).

ii. Cardinal temperatures from the Briere-1 model were used to develop a DD model which was based on mortality data collected at constant temperatures. This model was used to make predictions on proportion mortality of groups of DBM larvae treated by one of four selected isolates in conditions of fluctuating temperatures.

iii. Predictions made were compared with experimental data. There was no difference between predicted and observed proportions according to a Fisher's exact test.

5 Quantifying the effect of co-application of *B. bassiana* with low-concentration commercially available insecticides on the survival of groups of DBM larvae

5.1 Introduction

The injudicious use of synthetic insecticides since the 1960s has resulted in increased selection pressure on DBM populations (Sarfraz et al., 2005). DBM has now shown resistance to all classes of commercially available insecticides (Talekar and Shelton, 1993, Sarfraz et al., 2005). The problem is particularly acute in South East Asia. For example, in Malaysia, 90% of farmers use synthetic insecticides to control DBM (Mumford and Mazlan, 2005). Overuse of synthetic insecticides may also cause a crash in natural enemy populations (Furlong et al., 2004), which could contribute to the prevalence of DBM as a pest (Sarfraz et al., 2005). As insecticide-only approaches have consistently failed, it is the prevailing opinion of experts that an integrated approach should be used to control DBM (Sarfraz et al., 2005). IPM uses many complementary pest control elements, including EPF biopesticides, to achieve control (Grzywacz et al., 2010). EPF biopesticides are known to take longer to cause insect mortality when compared to synthetic insecticides (Lacey et al., 2001). For this reason, it is likely that EPF biopesticides and synthetic insecticides would be applied together within an IPM system (Nian et al., 2015). EPF biopesticides are considered to have great potential for use with in IPM systems, but as of yet there are only a small number of EPF biopesticides which are used against DBM (Furlong et al., 2013). Part of the reason for this is a general lack of understanding of how different elements of the IPM system work together (Grzywacz et al., 2010). This principle can also be applied to IPM systems which combine EPF biopesticides and synthetic insecticides against other pests.

The co-application of low-concentration insecticides with EPF biopesticides has been relatively well studied in insect pests. In general this is considered a good strategy to reduce the volume of synthetic insecticides applied, whilst maintaining a high level of control, resulting in an economic and environmental saving amongst many other benefits (Butt and Ansari, 2011) (Table 5-1). For example, co-application of imidacloprid

at 1% of the recommended rate with *B. bassiana* provided the same level of control against black vine weevil as applying imidacloprid at 100% of the recommended rate (Shah et al., 2007). Additionally, the use of EPF biopesticides has the potential to resolve the increasing problem of resistant DBM populations. For example, in mosquitoes it was found that treatment of pyrethroid resistant individuals with EPF biopesticides reduced the expression of pyrethroid resistance in those individuals (Farenhorst et al., 2009).

The greatest number of EPF biopesticides have *B. bassiana* as the active ingredient (Chandler, 2017), of these EPF biopesticides, the product BotaniGard is the most widely used. BotaniGard, based on *B. bassiana* 433.99, has consistently shown to provide control against a wide range of pests including DBM, wireworms, whitefly and spider mites (Wraight et al., 2010, Reddy et al., 2014, Ayalew, 2016, Ullah and Lim, 2015). Wraight et. al. (2010) reported that the BotaniGard isolate caused 95% mortality in goups of DBM larvae six days after treatment when applied at a concentration of 142 conidia mm^{-2}. In the current research *B. bassiana* 433.99 caused a higher level of mortality in groupd of DBM larvae when compared to other commercially available EPF biopesticides.

In this study, we have used the BotaniGard isolate, *B. bassiana* 433.99, as an exemplar EPF biopesticide to elucidate the effects of co-application of commercially available insecticides with an EPF biopesticide to control groups of DBM larvae. When coapplied, control products can interact in two ways. In an antagonistic interaction one insecticide may inhibit the function of the other, resulting in a lower level of mortality than the aggregate mortality if the both insecticides were applied separately. In a synergistic interaction, a higher level of mortality than the aggregate mortality if both insecticides were applied separately would be observed. An additive relationship indicates there is no interaction between the two insecticides. In this case, the mortality level observed would be the same as aggregate mortality if the both insecticides were applied separately (Nian et al., 2015).

To give an indication of how co-application effects the efficacy of an EPF biopesticide and a commercially available insecticide when controlling insect pests, the physiology

of the EPF isolate can be assessed on media containing the insecticide. For example, Neves et al. (2001) reported that three neonicotinoid insecticides did not negatively affect the germination, colony area or number of conidia produced by a *B. bassiana* isolate. Experiments have also been completed in which the effect of co-application of two biocontrol products has been determined (Table 5-1). For example, in one field study that was replicated over three seasons, *B. bassiana* was applied alone and in combination with *Bacillus thuringiensis* (Bt). It was found in each combined treatment almost total insect mortality was observed, the authors described the interaction between both treatments synergistic at a low level (Wraight and Ramos, 2005). There have also been reports of synthetic insecticides (such as Imidacloprid and phloxine B) displaying synergism when applied with EPF formulations against insect pests (Ye et al., 2005, Kim et al., 2010). Additionally, there has been research on the compatibility of Bt with an *I.fumosorosea* EPF isolate, with promising results that indicate that a synergistic interaction is present (Nian et al., 2015). However, there have been limited studies in to how co-application effects the efficacy of insecticides when controlling DBM and the resulting knowledge gaps have prevented EPF biopesticides from being widely used in field.

The great majority of studies into the effects of co-application of insecticides are focused on characterising the interaction between the control products tested. However, it also important in terms of IPM to elucidate the mechanisms behind these interactions. Although mechanisms are suggested in some research, there has been little progress into elucidating these mechanisms. For example, Nian et al (2010) suggested that the observed synergism between Bt and *B. bassiana* in controling groups of DBM was due to Bt increasing the length of the intermoult period and allowing conidia more time to penetrate the insect cuticle and cause a systemic infection. It has also been suggested that some synthetic insecticides increase the movement of insect pests, resulting in more conidia being picked up,leading to a faster speed of kill or overall percentage mortality (Roditakis et al., 2000, Shah et al., 2007). For example, it was reported that aphids (*Myzus persicae)* increased their movement when applied with a sub-lethal concentration of imidacloprid which resulted in more EPF conidia being picked up

(Roditakis et al., 2000). This increase in movement is likely to be due to insects searching for better feeding sites.

The mechanism of synergism is likely to be highly related to the mode of action of the insecticide that is being co-applied with *B. bassiana*. Modes of action vary between insecticides of different classes (IRAC, 2017). For example, the soil borne bacterium Bt produces protein crystals during spore production known as Cry endotoxins. Cry endotoxins are ingested as an inactive, non-toxic, precursor. Shortly after ingestion, the Cry endotoxin undergoes solubilisation before being processed into its active form. The Cry endotoxin then crosses the peritrophic matrix (the semi-permeable membrane surrounding the insect gut) and binds to adheren receptors. This interaction results in the activation of a cell death pathway, which leads to the formation of pores in the insect gut wall which results in insect death (Gill et al., 1992). Synthetic insecticides tend to have neurotoxic properties. Neonicotinoids work by binding to nicotinic acetylcholine receptors, which are present in the insect nervous system. This causes over stimulation of the receptors which quickly leads to insect paralysis and death (Matsuda et al., 2001). Azadirachtin, a liminoid secondary metabolite, is the active ingredient present in neem oil based insecticides. The active compound has a strong anti-feedant effect in Lepidopteran species. Azadirachtin interacts with chemoreceptors in the insect mouthparts to block receptor cells that normally stimulate feeding. Azadirachtin is also known to consistently interfere with growth and moulting of insects (Mordue and Nisbet, 2000). Mode of actions of insecticides against DBM have generally been well studied. Despite this, it is not known how mode of actions effects the type outcome that is observed when two different insecticides are applied together.

Over the past 50 years, DBM has presented resistance to every major class of insecticide (Sarfraz et al., 2005). DBM was the first insect to display resistance to Bt Cry toxins. This resistance is now considered to be wide spread with resistant populations in Central America, China, India, Malaysia, the United States, Taiwan and Thailand (Perez and Shelton, 1997, Gong et al., 2010, Wang et al., 2006, Mohan and Gujar, 2007, Iqbal et al., 1996, Liu et al., 1996, Kao and Cheng, 2001, Imai and Mori, 1999). DBM which are resistance to pyrethroids are also widespread. Resistant populations are now present in

Australia, Brazil, China, India, Japan, Malaysia, New Zealand, Nicaragua, Pakistan, Philippines, South Africa, South Korea and the United States (Furlong et al., 2013). Resistance to neonicotinoid insecticides is far less widespread, with resistant populations only being observed in Malaysia (Furlong et al., 2013, Sayyed and Crickmore, 2007). Co-application of EPF biopesticides with commercially available insecticides has the potential to reduce selection pressure on DBM populations, and reduce the prevalence of insecticide resistance. In the absence of an available insecticide resistant DBM population to be used in this study, the use of low concentration insecticides (resulting in low level insect mortality) can be seen as an alternative to applying high concentration insecticides to a resistant DBM population (which would also result in low level mortality).

Although there are EPF biopesticides available on the market for the control of insect pests, these are rarely used for the management of DBM infestations (Furlong et al., 2013). To expedite the uptake of EPF biopesticides in the field, there must be greater understanding of how different elements of the IPM system work together (Grzywacz et al., 2010). The co-application of insecticides with EPF biopesticides to control insect pests has the potential to provide the following benefits to growers, the environment and consumers (Butt and Ansari, 2011):

i. More consistent control
ii. Economic savings, as insecticide concentrations required to achieve control may be drastically reduced
iii. Reduced exposure of growers to potentially harmful insecticides
iv. Reduced pesticide residues in produce
v. Reduced environmental contamination

The overall aim of this study was to determine the effect of co-application of an exemplar EPF biopesticide with low concentration commercially available insecticides when applied against groups of DBM larvae. Mortality data from experiments was used to determine the presence or absence of interactions between the two control products,

and to determine whether co-application resulted in a reduction in the *B. bassiana* LC50. In addition, information was collected on the effect of insecticides on the colony extension rate and germination of *B. bassiana* 433.99 and the mortality of groups of DBM larvae. It is hoped that this study will provide valuable information for developing IPM system to control DBM. In addition, the procedure used here can be applied to IPM systems involving other EPF biopesticides and insect pests. The component objectives were as follows:

i. The effect of candidate commercially available insecticides on the following was determined:
    - Physiology (colony extension and germination) of *B. bassiana* 433.99.
    - Survival of groups of DBM larvae.
ii. Based on information from (i), plus information from the literature, three insecticides were selected to undergo co-application experiments with *B. bassiana* 433.99
iii. Concentration response experiments were completed to establish sub-lethal concentrations of insecticides from (ii)
iv. A concentration range of *B. bassiana* was then applied with and without the fixed sub lethal dose of insecticide from (iv) to groups of DBM larvae
v. Analysis was then completed to quantify the effect of co-application on the survival of groups of DBM larvae

Table 5-1: Examples of EPF and insecticides which have been shown to interact synergistically when controlling various pests (adapted from Butt and Ansari, 2011)

Insecticide	EPF	Insect	Number of papers	References
Neonicotinoid	*B. bassiana* *M. anisopliae*	Burrower bug, Weevil, Colorado potato beetle, BVW, Mosquito, Aphid	10	(Jaramillo et al., 2005, Feng et al., 2004, Quintela and McCoy, 1998, Furlong and Groden, 2001, Shah et al., 2007, Steinkraus and Tugwell, 1997, Boucias et al., 1996, Paula et al., 2011, Roditakis et al., 2000, Ye et al., 2005)
Neem oil	*B. bassiana* *M. anisopliae*	Fall army worm, BVW, Mosquito	3	(Gomes et al., 2015)
Fipronil	*M. anisopliae*	BVW	1	(Shah et al., 2007)
Organophosphate	*M. anisopliae*	Cockroach	1	(Pachamuthu and Kamble, 2000)
Spinosad	*M. anisopliae*	Wireworm	1	(Ericsson et al., 2007)
Pyrethroid	*M. anisopliae*	Mosquito	1	(Farenhorst et al., 2010)
Bt	*I. fumosorosea*	DBM, Colorado potato beetle	2	(Nian et al., 2015, Wraight and Ramos, 2005)

5.2 Materials and methods

5.2.1 Quantifying the effect of 16 commercially available insecticides, applied at the recommended rate, on mortality of groups of second instar DBM larvae

The aim of this experiment was to determine the effect of commercially available insecticides on the mortality of groups of DBM larvae. A group of 16 commercially available insecticides were used to undergo experimentation (Table 5-2), suspensions were prepared in a Class II Safety Cabinet. Insecticides were diluted to their respective recommended field concentrations with 0.05% Triton X-100 (Table 5-2). Aliquots (100µl) of the insecticide suspension were pipetted on to both sides of a 2 cm diameter leaf disc (Skywalker variety obtained from Elsoms Seeds Ltd, Lincoln, UK) and spread evenly over the leaf surface using a L-shaped hockey stick spreader (Fisher Scientific). The leaf disc was left to dry for approximately one hour on 5% water agar contained in a 9cm petri dish (Merck). A control leaf disc was prepared, by spreading 100µl of 0.05% Triton X-100 on a 2cm diameter leaf disc.

DBM were reared as described in 2.1. A group of fixed age second instar larvae was prepared using the procedure described in 2.1. Groups of 10-15 larvae were transferred to filter paper contained within a 9cm petri dish (Merck). To prevent excessive movement of DBM larvae, they were sprayed lightly with reverse osmosis water using a handheld sprayer until the filter paper was damp.

The treated leaf discs were then placed in the petri dish containing larvae using plastic disposable forceps which were discarded between handling each insecticide treated leaf disc. Petri dishes were then sealed using Parafilm (VWR International, UK) and kept at 20^{o}C (16:8 LD). After 24hr, larvae were transferred to a 6 cm diameter untreated leaf disc kept on 5% water agar, contained within a 9 cm petri dish (which was modified for aeration by cutting three 1.5cm diameter holes in the lid and covering them with gauze to prevent larval escape).

Numbers of living and dead larvae were recorded at 1, 6, 12, 24, 48, 72, 96, 120 and 144hr after treatment. Larvae were considered dead if they did not move after being agitated with a fine paint brush. Cadavers were removed from the experiment. Leaf discs were changed every 3-4 days.

The toxicity of all 16 insecticides was assessed in one block, for each insecticide, the mortality of 10-15 larvae in a Petri dish was monitored. Three repetitions of the experiment were completed, with each repetition occurring a different time point, so that the mortality of 30-45 larvae were assessed for each insecticide (3 Petri dishes x 10-15 individuals).

Active	Trade name	Supplier	Class	RFR	Volume /mass in 10ml
acetamiprid	Gazelle	Certis Europe	neonicotinoid	375g/300L	0.01g
chlorpyrifos	Dursban	Dow Agrosciences	organophosphate	30g/1L	0.003g
cyantraniliprole	Coregan	DuPoint	anthranilic diamides	175ml/300L	5.8 µL
deltamethrin	Decis	Bayer	pyrethroid	300ml/300L	10.0 µL
diflubenzuron	Dimilin flo	Certis Europe	benzimide	200ml/500L	4.0 µL
indoxacarb	Steward	DuPoint	oxadiazine	650ml/300L	21.7 µL
lambda-cyhalothrin	Hallmark Zeon	Syngenta	pyrethroid	100ml/300L	3.3 µL
maltodextrin	Majestik	Certis Europe	–	25ml/1L	250.0 µL
pyrethrins	Pyrerhrin 5ec	Agropharm ltd	pyrethrin	20ml/5L	40 µL
spinosad	Tracer	Dow Agrosciences	spinosad	200ml/300L	6.7 µL
spirotetrama	Movento	Bayer	ketoenols	500ml/300L	16.7 µL
thiacloprid	Calypso	Bayer	neonicotinoid	375ml/300L	12.5 µL
thiamethoxam	Actara	Syngenta	neonicotinoid	100g/300L	0.003g
azadirachin	Neemazal	Trifolio-M	–	500ml/100L	50.0g
emamectin	Affirm	Syngenta	avermectin	300ml/300L	10.0ml
Bt	Dipel DF	Interfarm	gut disrupter	50-100g/100L	0.01g

Table 5-2: Insecticides used in experiments, including active ingredient, trade name, supplier, class (according to IRAC), recommended field rate (RFR) and volume/mass mixed with 10ml of 0.05% Triton X-100.

5.2.2 Determining the effect of candidate insecticides on the colony extension rate and germination of *B. bassiana* 433.99

SDA was mixed and autoclaved using the procedure described in 2.2. Media was then allowed to cool until “hand hot”. The volume/mass of insecticide required to make the suspension up to the field recommended dose, was then added (Table 5-2). The SDA/insecticide solution was then stirred using a magnetic stirrer to ensure even distribution of the insecticide. The SDA/insecticide solution was then poured into 9cm

petri dishes (Merck). Plates were stored at 4°C in darkness. These SDA/insecticide plates were then used in both colony extension and germination experiments.

5.2.2.1 Determining the effect of candidate insecticides on the proportion germination of populations of B. bassiana 433.99 conidia

The effect of 16 insecticides on the germination of populations of *B. bassiana* 433.99 conidia was quantified. Germination of the *B. bassiana* 433.99 was assessed using the procedure described in 2.6.2. Experiments were carried out at 20°C using all candidate insecticides. All insecticides were included in one block. Three repetitions of the experiment were completed.

5.2.2.2 Determining the effect of candidate insecticides on the colony extension rate of B. bassiana 433.99

The effect of 15 insecticides on the colony extension rate of *B. bassiana* 433.99 was quantified. Colony extension rate of *B. bassiana* 433.99 was assessed using the procedure described in 2.6.1. Experiments were carried out at 20°C using all candidate insecticides. All insecticides used in the experiment were included in one block. Three replicates of the experiment were completed. Dipel DF was not included in this experiment.

5.2.3 Determining the effect of concentration of selected insecticides of on the survival of groups of DBM larvae and time taken for individual DBM larvae to develop between from second to third instar

Due to limits in the number of DBM that could be produced, three candidate insecticides were selected to go forward to the next stages of experimentation. The rationale for inclusion of each insecticide can be seen in the discussion.

The effect of concentration was determined for two dependent variables:

i. Mortality of groups of DBM larvae
ii. Time taken for larvae to develop from second to third instar

Three candidate insecticides were selected to undergo concentration response experiments: Dipel DF, Neemazal and Calypso. A group of fixed aged second instar larvae (n=12-15 per treatment) were treated with desired concentration of insecticide using the procedure described in 5.2.1. Insecticides were diluted in 0.05% Triton X-100 to obtain the desired concentrations. Preliminary investigations were used to find the minimum insecticide concentration which caused 100% mortality (the highest concentration used here) and highest concentration which conferred mortality which was not significantly different from the control (the lowest concentration used here), intermediate concentrations were evenly distributed on a Log scale. The concentrations of the insecticides in terms of grams of active ingredient per ml of 0.05% Trition X-100 were as follows:

- Calypso: 2×10^{-7}, 10^{-6}, 6×10^{-6} and 3×10^{-5} plus an untreated control
- Neemazal: 4×10^{-7}, 4×10^{-6}, 10^{-5}, 2×10^{-5} plus an untreated control
- Dipel DF: 1×10^{-9}, 1×10^{-8}, 1×10^{-7} and 1×10^{-6} g ml^{-1} plus an untreated control

Numbers of living and dead larvae were recorded every day after treatment for seven days. Larvae were considered dead if they did not move after being agitated with a fine paint brush. Time taken for larvae to develop from second to third instar was also recorded for DBM individuals. Transition to the third instar was indicated by a change in head capsule size of larvae, visible darkened cuticle, and the presence of the moulted cuticle on the leaf surface. Each insecticide concentration response was completed as a separate block. Each block was repeated three times.

5.2.4 Experiments to determine the effect of co-application of *B. bassiana* 433.99 with low concentrations of selected insecticides on the survival of groups of DBM larvae

The following protocol was used for experiments using Calypso, Dipel DF and Neemazal. The concentration response experiment was used to determine the LC40 of selected insecticides. Two experiments were completed per insecticide, in the first a fixed LC40 dose of insecticide was applied with and without a concentration range of *B. bassiana* 433.99 made up of 5.0, 5.5, 6.0, 6.5 and 7.0 log10[conidia ml^{-1}] (Table 5-3). In the second experiment a fixed dose of a 10-fold dilution of the LD40 dose was applied with and without and concentration range of *B. bassiana* 433.99 made up of 5.0, 5.5, 6.0, 6.5 and 7.0 log10[conidia ml^{-1}] (Table 5-2). Each experiment was repeated three times (Fig. 5-1).

For the insecticide only treatment, the desired concentration (either the LC40 or a 10-fold dilution of the LC40) was applied to a leaf disc and used to treat larvae using the protocol described in 5.2.1. For the *B. bassiana* 433.99 only treatment, the application procedure described in 2.4 was used to treat larvae (Fig. 5-1).

For the co-application treatment of insecticide and *B. bassiana* 433.99, the procedures described 2.4 and 5.2.1 were combined: groups of 10-15 larvae were treated using a Potter tower (Potter, 1952)(as described in 2.4), instead of an untreated leaf disc being added to the petri dish containing the larvae post spray, a leaf disc treated with the desired concentration of insecticide was added for 24hr. This was then replaced with a with an untreated leaf disc kept on water agar in a 9cm petri dish, as in the procedure described in 2.4 (Fig. 5-1).

Petri dishes containing larvae were kept at 20^{o}C (16:8 LD) for seven days after treatment. Numbers of living and dead larvae were counted every 24 hours for the duration of the experiment. Experiments involving different insecticides were completed separately. Three replicates of the experiments were completed.

Table 5-3:Description of treatments used in experiments to quantify the effect of co-application of insecticides and *B. bassiana* on survival of groups of DBM larvae. Two experiments were completed per commercial insecticide, one using the LC40 and one using a 10-fold dilution of the LC40.

	Experiment 1			**Experiment 2**	
Treatment	***B. bassiana* concentration (log10[conidia ml^{-1}])**	**Insecticide concentration**	**Treatment**	***B. bassiana* concentration (log10[conidia ml^{-1}])**	**Insecticide concentration**
B. bassiana only	5.0	0	*B. bassiana* only	5.0	0
	5.5	0		5.5	0
	6.0	0		6.0	0
	6.5	0		6.5	0
	7.0	0		7.0	0
B. bassiana/ insecticide co-application	5.0	LC40	*B. bassiana*/ insecticide co-application	5.0	LC40/10
	5.5	LC40		5.5	LC40/10
	6.0	LC40		6.0	LC40/10
	6.5	LC40		6.5	LC40/10
	7.0	LC40		7.0	LC40/10
insecticide only	0	LC40	insecticide only	0	LC40/10
	0	LC40		0	LC40/10
control	0	0	control	0	0
	0	0		0	0
	0	0		0	0

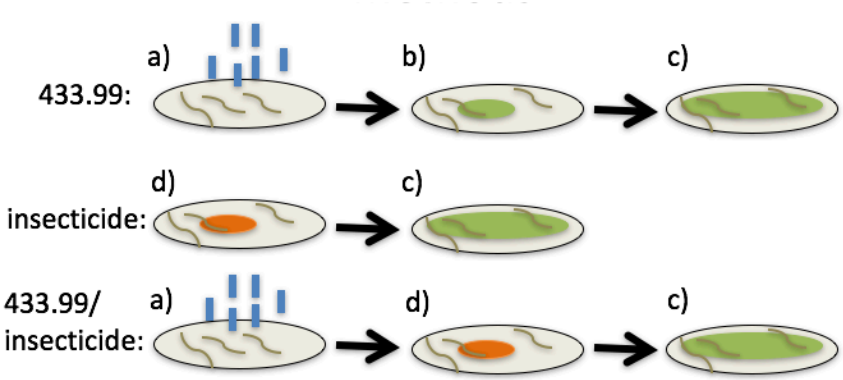


Figure 5-1: Illustration of how treatments were applied to groups of larvae. **a)** Groups of larvae were sprayed with *B. bassiana* 433.99 using a Potter tower. **b)** A 3cm leaf disc was added to the petri dish for 24hr. **c)** After 24hr larvae were transferred to a clean 6cm leaf disc on agar, and mortality monitored for seven days. **d)** An insecticide treated 3cm leaf disc was added to a sealed petri dish containing groups of larvae for 24hr. A corresponding control was completed for each application method, using 0.05% Triton X-100 instead of *B. bassiana* 433.99/insecticide

5.2.5 Statistical analysis

For colony extension experiments, for each time point the diameter of the mycelial mass was calculated by taking the mean of the two diameter measurements. This figure was then halved to get the radius of the mycelial mass. The radial rate was calculated by plotting mycelial mass radius against time for each EPF isolate. A linear regression model was used to obtain the radial rate. Anovas were completed to obtain significant differences between radial rates of *B. bassiana* 433.99 grown on different insecticides.

To compare the means of colony extension rates and germination percentages of *B. bassiana* 433.99 under different insecticide conditions Anovas of arcsine square-route transformed data were completed in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc). Before Anovas were completed data was tested for a normal distribution using the Q-Q plot function. Specific differences between isolates were identified by using Tukey's HSD.

Analysis of survival data was conducted in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package DRC (version 3.0-1) separately for each insecticide concentration-mortality response experiment using the procedure described in 2.7.

The same analysis for characterising responses as being antagonistic, additive or synergistic was completed as was used in Nian et al. (2015) in Microsoft Excel (version 15.38 (170815)). Mortality data was analysed from days four, five and six of the experiment. A chi squared test was completed to compare expected and observed values. The hypothesis being tested was that there was no interaction between *B. bassiana* 433.99 and a selected insecticide when applied to group of DBM larvae. Because separate chi squared tests were completed for separate days and concentrations, no assumptions were made on the distribution of concentration-mortality response of insecticides against DBM. The suitability of chi squared in comparison with other techniques for analysing treatment interactions is discussed in

detail in *7.3 Directions for Future Research*. Expected values were calculated using the following formulae:

$$Pe = P0 + (1 - P0)\,(P1) + (1 - P0)\,(1 - P1)\,(P2) \tag{15}$$

With *Pe* being the expected proportion mortality from application of insecticide and *B. bassiana*, *P0* being the control proportion mortality, *P1* being the proportion mortality from *B. bassiana* alone and *P2* being proportion mortality from insecticide alone. *Pe* was multiplied by the total number of insects in a treatment to calculate expected dead and alive larvae on different days of the experiment. Chi squared values were generated using the following formulae:

$$Chi\ squared\ value = \frac{(L0 - Le)^2}{Le} + \frac{(D0 - De)^2}{De} \tag{16}$$

With *L0* being the observed number of living larvae, *Le* being the expected number of living larvae, *D0* being the observed number of dead larvae and *De* being the expected number of dead larvae. After completing the analysis, a chi squared value was generated. The chi squared value associated with a 95% confidence level (p=0.05) at one degrees of freedom was 3.84 (Turner, 2014). If chi squared was below 3.84 an additive relationship was indicated. If chi squared was above 3.84 and observed mortality was above expected mortality, a synergistic relationship was indicated. If chi squared was above 3.84 and observed mortality was lower than expected mortality, an antagonistic relationship was indicated (Nian et al., 2015).

The LC50s of *B.bassiana* 433.99 applied with and without a fixed dose of insecticide were estimated using probit analysis (SPSS, Version 24, 2016) with 95% confidence limits (Richardson, 1960). Percentage mortality data, from each concentration of *B. bassiana* 433.99 used, plus the untreated control was used from six days after treatment. Non-

over lapping 95% confidence limit error bars were considered to indicate significant differences between LC50s (Krzywinski and Altman, 2013).

5.3 Results

5.3.1 Quantifying the effect of 16 commercially available insecticides, applied at the recommended field rate, on mortality of groups of second instar DBM larvae

Before Anovas were completed, percentage mortality data was arcsine square-route transformed. Mortality data was analysed at 1, 12, 72 and 120hr after treatment (Fig. 5-2). Data was corrected for control mortality using Schnieders orriellas formulae (Puntener, 1981), control mortality was consistently below 10% across all time points and replicates. Mortality increased significantly with time for all isolates apart from Dimilin Flo, Majestick and Movento (Anova, P=<0.001). Mortality varied significantly with insecticide at all time points (Anova, P=<0.001). At 1hr after treatment Decis and Tracer caused 100% mortality, which was significantly higher than all other isolates (Tukey's HSD, P=<0.05). At 12hr after treatment six insecticides caused over 80% mortality, these were Steward, Affirm, Pyrethrin 5EC, Decis, Dursban and Tracer. At 72hr after treatment, the majority of insecticides caused over 75% mortality. The exceptions to this were Gazzelle, Neemazal, Movento, Majestik and Dimilin Flo, which all caused under 50% mortality. By 120hr after treatment, Dimilin Flo, Majestik and Movento caused below 5% mortality. Significantly lower than all other isolates (Tukey's HSD, P=<0.05)(Fig. 5-2).

Mortality also varied at all time points according to insecticide classification (Anova, P=<0.001). By 120hr after treatment pyrethroids/pyrethrins and neonicotinoids caused 94.1±11.9 and 72.4±28.25% mortality, respectively (Tukey's HSD, P=<0.05). Insecticides with a non-specified class according to IRAC , benzimide and ketenols caused 9.4±12.7, 0.0±0.0 and 2.8±4.8% mortality after 120hr, significantly less than other insecticide classes (Tukey's HSD, P=<0.05).

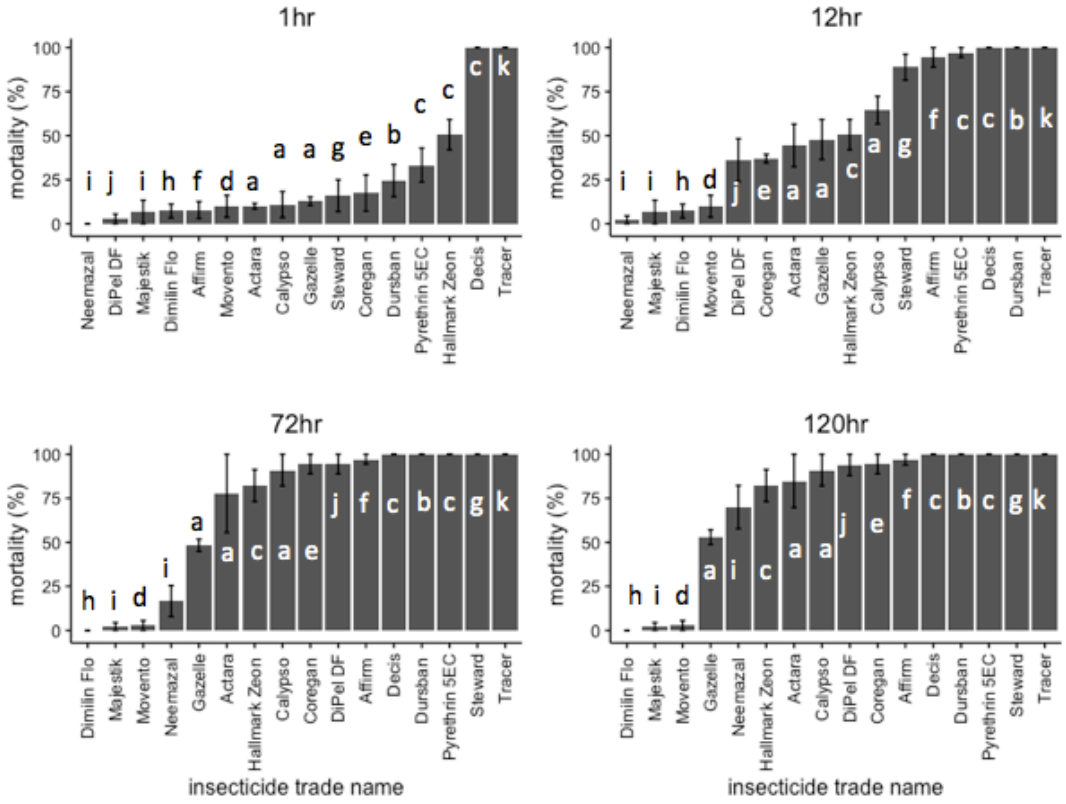


Figure 5-2: Mean mortality (±SE) of groups of DBM larvae after being treated with one of 16 candidate insecticides 1, 12, 72 and 120hr after treatment. Class of insecticide is coded by letter:

a) neonicotinoid
b) organophosphate
c) pyrethrin/pyrethroid
d) ketoenol
e) anthranilic diamides
f) avermectin
g) oxadiazine
h) benzimide
i) non-specified mode of action (according to IRAC)
j) Bt (insect gut disrupter)
k) spinosad

5.3.2 Determining the effect of candidate insecticides on the proportion germination of populations of *B. bassiana* 433.99 conidia

Percentage germination data was arcsine transformed before Anovas were completed. Percentage germination varied significantly with insecticide used (Anova, P=<0.001) (Fig. 5-3). After 12hr, 28.9±3.6% germination was observed when *B. bassiana* 433.99 was grown on SDA, 29.6±3.9% germination was observed for *B. bassiana* 433.99 grown on Neemazal. There was no significant difference between the germination percentages of SDA (control) and Neemazal (Tukey's HSD, P=>0.05). Germination on a number other insecticides was lower than that of the control; Movento, Tracer, Actara, Coregan, Affirm, Calypso, Hallmark Zeon, Steward, Dimilin displayed 17.0 to 28.9% germination, but this did not differ significantly from the control (Tukey's HSD, P=>0.05). Germination on Pyrethrin 5EC, Majestik, Decis, Dipel DF, Gazzelle, Dursban and Movento was significantly lower than the SDA control (Tukey's HSD, P=>0.05). Of these insecticides, Pyrethrin, Majestik and Decis displayed the highest level of inhibition, with 0.2±0.3, 1.0±0.9, 1.6±1.5% germination of *B. bassiana* 433.99 conidia being observed on these insecticides, respectively.

Percentage germination also varied significantly with class of insecticide used (Anova, P= 0.02). *B. bassiana* 433.99 germinated less when grown on media containing pyrethroid insecticides, when compared to the SDA control (Tukey's HSD test, P=0.02). There was no difference in germination between any of the other classes of insecticide (Tukey's HSD test, >0.05).

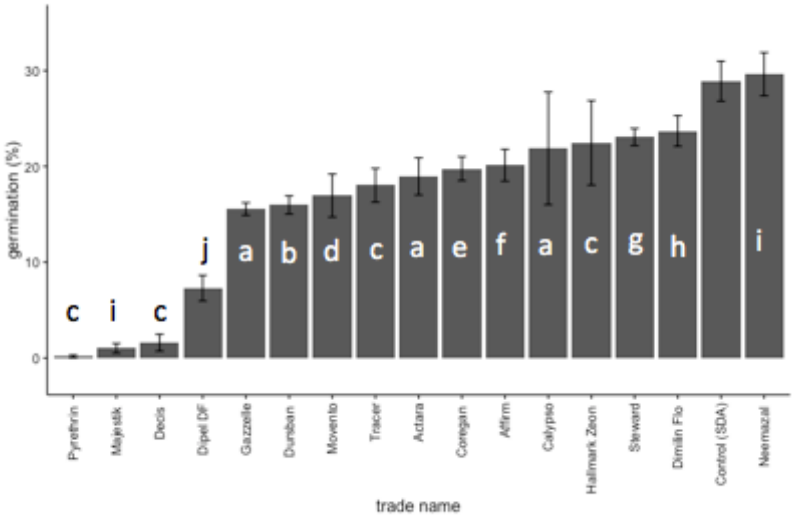


Figure 5-3:Germination (%±SE) of populations of *B.bassiana* 433.99 conidia on media containing one of 16 insecticides at the field recommended rate. Insecticide class is coded by letter:

a) neonicotinoid
b) organophosphate
c) pyrethrin/pyrethroid
d) ketoenol
e) anthranilic diamide
f) avermectin
g) oxadiazine
h) benzimide
i) non-specified mode of action (according to IRAC)
j) Bt (insect gut disrupter)
k) spinosad

5.3.3 Determining the effect of candidate insecticides on the colony extension rate of *B. bassiana* 433.99

Type of insecticide had a significant effect on colony extension rate of *B. bassiana* 433.99 (Anova, P=<0.001) (Fig. 5-4). *B. bassiana* grown on SDA (control) had a colony extension rate of 0.14±0.01 cm day^{-1}. Higher colony extension rates were observed when *B. bassiana* 433.99 was grown on five other insecticides, but this difference wasn't significantly different from the control (Tukey's HSD, P=>0.05). These insecticides were Steward, Dimilin Flo, Affirm, Calypso and Hallmark Zeon which displayed rates of between 0.14 - 0.15 cm day^{-1}. Of the insecticides on which colony extension rates were lower than the control, Actara, Coregan, Decis, Tracer, Majestik, Neemazal, and Dursban did not cause a significant inhibition of growth rate *in B. bassiana* (Tukey's HSD, P=>0.05). *B. bassiana* grown on Pyrethrin, Gazzelle and Movento, displayed colony extension rates of 0.05 – 0.09 cm day^{-1} which reflected a significant inhibition of colony extension rate (Tukey's HSD, P=<0.05). Colony extension rate did not vary with class of insecticide (Anova, P=0.5).

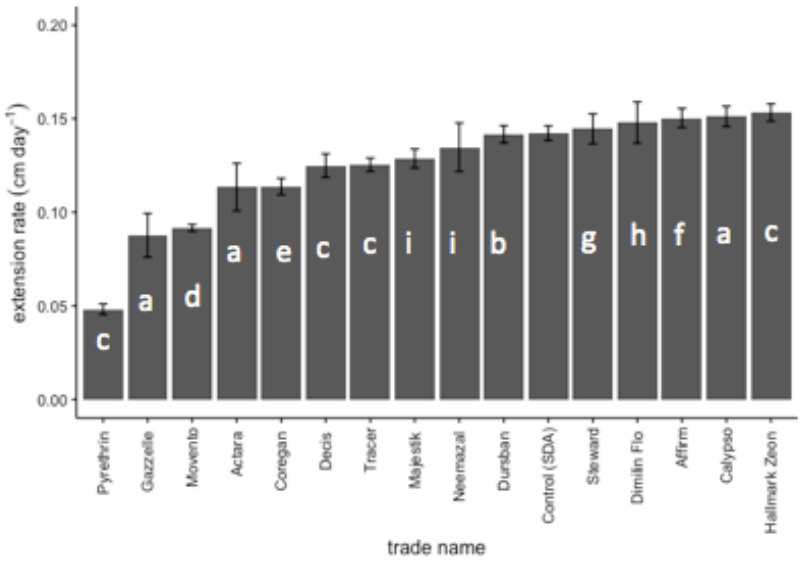


Figure 5-4: Colony extension rate (cm day^{-1} ±SE) of *B. bassiana* on one of 15 insecticides for 20-21 days at 20°C. Insecticide class is coded by letter:

a) neonicotinoid
b) organophosphate
c) pyrethrin/pyrethroid
d) ketoenol
e) anthranilic diamides
f) avermectin
g) oxadiazine
h) benzimide
i) non-specified mode of action (according to IRAC)
j) Bt (insect gut disrupter)
k) spinosad

5.3.4 Determining the effect of concentration of selected insecticides on the survival of groups of DBM larvae and time taken for individual DBM larvae to develop between second and third instar

The effect of insecticide concentration on two dependant variables was quantified. Mortality of groups of second instar DBM larvae was recorded. Additionally, the time between second and third instar of individual DBM larvae was measured.

5.3.4.1 Quantifying the effect of concentration of Dipel DF, Neemazal and Calypso on mortality of groups of DBM larvae

In this instance, mortality from six days after treatment was used to calculate the approximate concentration at which 40% mortality occurs, to be used in subsequent experiments. A clear concentration response was observed for each insecticide (Fig. 5-5). At the highest concentration over 80% mortality was observed for all insecticides. Control mortality was below 5% for experiments involving Dipel DF and Neemazal. For the Calypso concentration response, control mortality was 19.1%. For all insecticides 2,3 and 4-parameter log-logistic and Weibull 1 and 2 models were compared for goodness of fit. A 2-parameter log-logistic model was the best fitting model for both Dipel DF and Neemazal mortality concentration responses (lack-of-fit test, P= 0.39 and 0.20, respectively) (Table 5-4). For the Calypso concentration response, a 4-parameter log logistic model was the only model to adequately describe the relationship between concentration and mortality (lack-of-fit test, P= 0.07) (Table 5-4). The aforementioned models were used to determine LC40s for each insecticide. LC40s of $4.4x10^{-8}$ $\pm1.6x10^{-8}$, $4.3x10^{-6}$ $\pm8.6x10^{-7}$, $3.1x10^{-6}$ $\pm3x10^{-6}$ g ml^{-1} were determined for Dipel DF, Neemazal and Calypso. These concentrations were determined to be 0.004, 0.002 and 0.007% of the recommended field rate.

Table 5-4: Best fitting models for insecticide concentration response experiments for Dipel DF, Neemazal and Calypso. LL2 = two parameter log-logistic model, LL4 = four parameter log-logsitic model. P-values from the lack-of-fit test are included, a P-value of over 0.05 indicates the model represents the data appropriately. The LC50 parameter of the model is also included.

Insecticide trade name	**Model**	**P-value**	**LC50 (g ml^{-1})**
Dipel DF	LL2	0.39	8.9×10^{-8}
Neemazal	LL2	0.20	1.0×10^{-6}
Calypso	LL4	0.07	3.4×10^{-5}

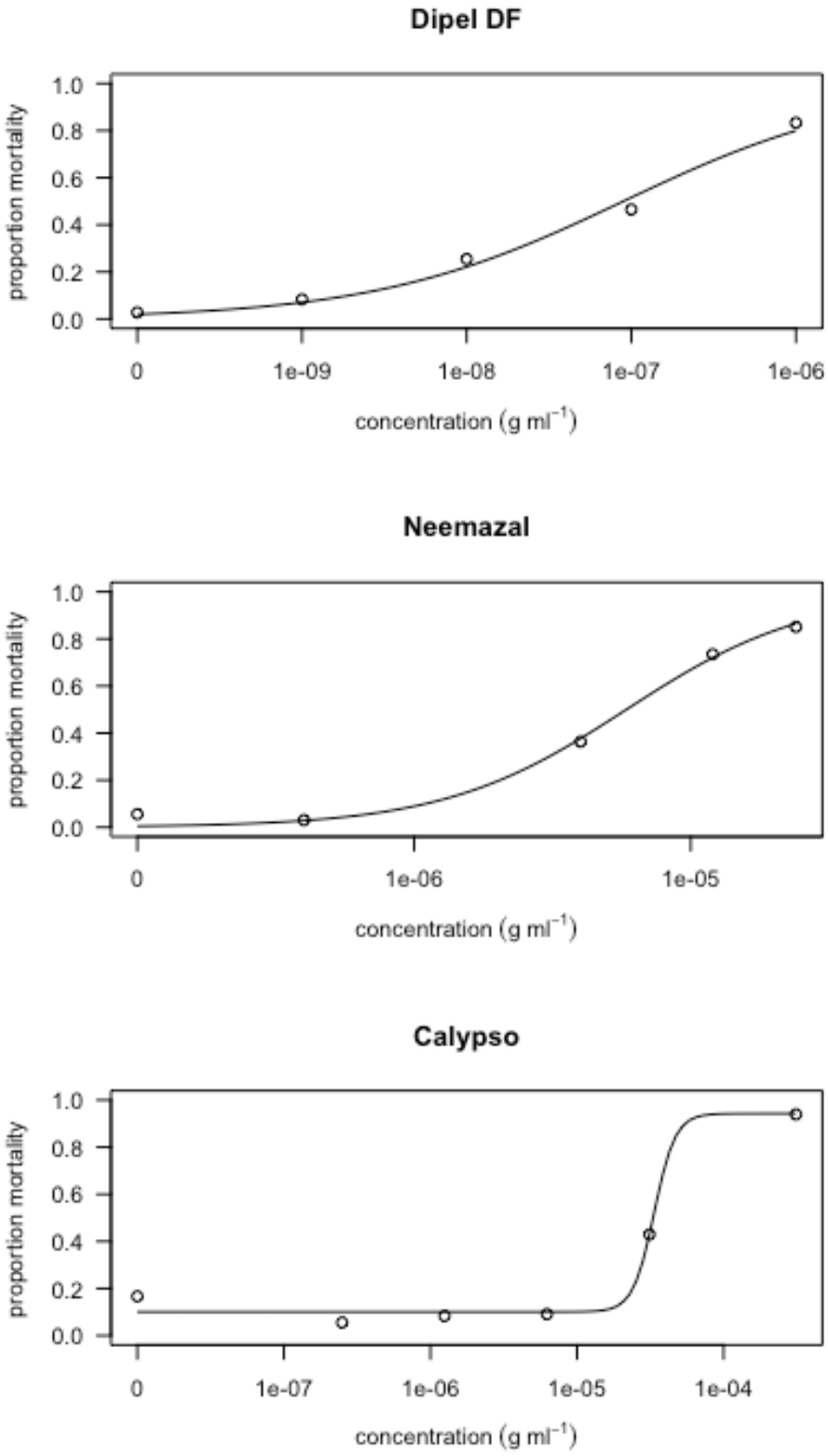


Figure 5-5: Mean proportion mortality of groups of DBM larvae at various doses of insecticide (g ml^{-1}) six days after treatment for Dipel DF, Neemazal and Calypso. A 2-parameter log-logistic model was included for Dipel DF and Neemazal dose response curves. A 4-paramter log-logistic model is included for Calypso.

5.3.4.2 *Quantifying the concentration effect of Dipel DF, Neemazal and Calypso on time taken for DBM larvae to develop from second to third instar*

For Dipel DF, Neemazal and Calypso length of second instar varied significantly with concentration of insecticide (Anova, P=>0.001).

For Dipel DF, mean time between second and third instar increased at every increase in concentration. Time between second and third instar was not significantly different between 0 and $5x10^{-9}$ (Tukey's HSD test =>0.05). At concentrations $5x10^{-8}$,$5x10^{-8}$,$5x10^{-8}$,$5x10^{-8}$ g ml^{-1} larvae took significantly longer to develop from second to third instar than the control. Maximum and minimum mean development time was 4.5±0.17 and 3.07±0.09 days at the highest concentration and the control, respectively.

For Neemazal, there was no significant difference between time between second and third instar between the control and $4x10^{-7}$ g ml^{-1}. Doses of $4x10^{-6}$, $1x10^{-5}$ and $2x10^{-5}$ g ml^{-1} conferred development times which were significantly longer than the control (Tukey's HSD test =<0.05). Maximum and minimum mean development (±SE) times were 4.61±0.70 and 3.35±0.60 days for the highest and lowest dose of Neemazal, respectively (Fig. 5-6).

For Calypso, there was no difference between the control and $2x10^{-7}$ g ml^{-l} in terms of time to development. Concentrations of $1x10^{-6}$, $6x10^{-6}$ and $3x10^{-5}$ g ml^{-1} conferred significantly longer development times than the control. A concentration of $3x10^{-5}$ g ml^{-1} conferred a significantly longer development time than all other concentrations. Maximum and minimum development times were 4.68±0.89 and 2.94±0.49 days for $2x10^{-7}$ g ml^{-l} and $3x10^{-5}$ g ml^{-1}, respectively (Fig. 5-6).

Polynomial regressions were also used to explain the relationship between concentration of insecticide active and time between second and third instar (Fig. 5-6,Table 5-5). For all three insecticides, a second order polynomial was used to describe the relationship between insecticide concentration and time between second and third

instar. After comparison with a linear model using an Anova, a second order polynomial was deemed to fit to the data significantly better when compared to a linear regression model. P-values were 0.003, 0.03 and 0.03 for Anovas comparing second order polynomials to linear regressions for Dipel DF, Neemazal and Calypso, respectively. This indicated that second order polynomial models should be used to explain the relationship.

Table 5-5: Fitted parameters of second order polynomial models fitted to the relationship between time for DBM larvae to develop from second and third instar and concentration of insecticide. P-value is also included, a P-value of below 0.05 indicates the relationship is significantly different from zero. The r^2 indicates what proportion of the variation with in the data set is explained by the model.

Insecticide	**P-value**	r^2	***a***	***b***	***c***
Dipel DF	<0.001	0.60	-1.30	1.80	3.87
Neemazal	0.005	0.52	-1.00	1.70	3.98
Calypso	<0.001	0.87	-0.58	2.35	3.56

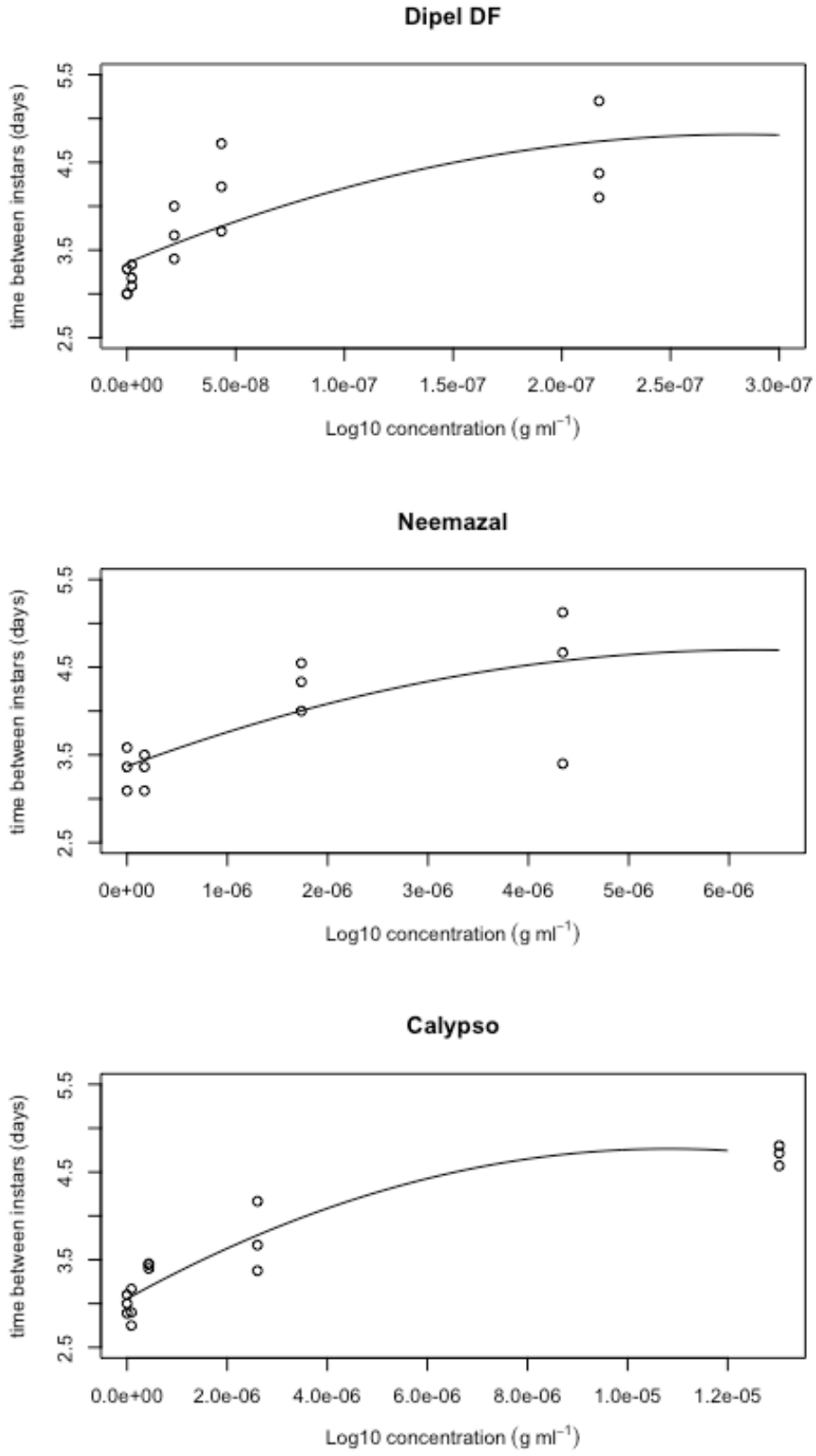


Figure 5-6: Concentration of insecticide applied (g ml^{-1}) plotted against time taken for larvae to develop from second to third instar (days) for Dipel DF, Neemazal and Calypso. A second order polynomial is included for each insecticide (parameters can be seen in Table 5-6).

5.3.5 Experiments to quantify the effect of co-application of *B. bassiana* 433.99 with low concentrations of selected insecticides on the survival of groups of DBM larvae

5.3.5.1 Quantifying the effect of co-application of B. bassiana *433.99 with low concentrations of Dipel DF on the survival of groups of DBM larvae*

Across both Dipel DF/*B. bassiana 433.99* co-application experiments, a concentration response was observed when *B. bassiana* was applied alone and in combination with Dipel DF. For the Dipel DF LC40 experiment, insect mortality increased when *B. bassiana* was applied with Dipel DF, this was true of every dose of *B. bassiana* including the untreated control (Table 5-7). In the same experiment, evidence of synergism was found at five and six days after treatment, but not at four days after treatment. On day five, synergism was found at the two highest doses of B*. bassiana.* For $3.16x10^6$ conidia ml^{-1}, 65.8 and 27.5% mortality was observed, five days after treatment, for co-application and lone *B. bassiana* treatments, respectively. At day six, synergism was observed at doses $3.16x10^5$, $3.16x10^6$ and $1x10^7$ conidida ml^{-1}. For the remaining time points and doses, additivity was observed. Interactions were characterised on 15 occasions. Of these, 10 additive interactions and five synergistic interactions (Table 2).

For the Dipel DF LD40/10 experiment, insect mortality generally increased if *B. bassiana* was applied in combination with DIpel DF (Table 5-8). There were some exceptions to this, for example; on day four at $3.16x10^6$ and $1x10^7$ conidida ml^{-1} mortality was lower when *B. bassiana* was applied with Dipel DF. On day four at $3.16x10^6$ and $1x10^7$ conidia ml^{-1}, an antagonistic interaction was observed. Here, at $1x10^7$ conidia ml^{-1}, 42.9 and 54.6% was observed for lone *B. bassiana* and co-application treatments, respectively. Of the 15 interactions characterised, 13 were characterised as being additive.

Probit analysis was used to estimate the *B. bassiana* LC50 when applied with and with a fixed concentration of Bt at LC40. Non-over lapping 95% confidence error bars indicated

that the *B.bassiana* LC50 was considered to be significantly lower when applied with Bt (LC40), when compared to *B.bassiana* applied on its own (Table 5-9).

The same analysis was used to estimate the *B. bassiana* LC50 when applied with and with a fixed concentration of Bt at a 10-fold dilution of the LD40. As 95% confidence error bars over lapped between insecticide only and co-application treatments, the LC50 was no considered to be significantly different (Table 5-10).

Table 5-6: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Dipel DF at LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		**Observed mortality (%)**			**Expected mortality (%)**			**Chi squared value**			**Response**		
Dipel (g ml^{-1})	*Bb* (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$4x10^{-8}$	$1x10^{5}$	15.8	18.4	18.4	8.7	12.4	13.7	2.4	1.3	0.7	A	A	A
$4x10^{-8}$	$3.16x10^{5}$	16.7	25.0	27.8	10.8	14.4	15.6	1.3	3.3	4.0	A	A	S
$4x10^{-8}$	$1x10^{6}$	15.8	23.7	28.9	15.4	25.4	30.8	0.0	0.1	0.1	A	A	A
$4x10^{-8}$	$3.16x10^{6}$	31.6	65.8	68.4	20.4	34.7	51.9	2.9	16.3	4.1	A	S	S
$4x10^{-8}$	$1x10^{7}$	43.9	87.8	92.7	31.0	66.2	68.6	3.2	8.6	11.1	A	S	S
0	$1x10^{5}$	0.0	2.8	2.8									
0	$3.16x10^{5}$	2.4	4.9	4.9									
0	$1x10^{6}$	7.3	17.2	22.0									
0	$3.16x10^{6}$	12.9	27.5	45.9									
0	$1x10^{7}$	24.5	62.5	64.6									
$4x10^{-8}$	0	5.0	7.0										
0	0	3.8	3.8										

Table 5-7: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Dipel DF at a 10-fold dilution of LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		**Observed mortality (%)**			**Expected mortality (%)**			**Chi squared value**			**Response**		
Dipel (g ml^{-1})	*Bb* (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$4x10^{-8}$	$1x10^{5}$	18.2	20.5	20.5	24.3	24.3	24.3	0.9	0.4	0.4	A	A	A
$4x10^{-8}$	$3.16x10^{5}$	14.6	19.5	26.8	24.2	28.5	32.2	2.0	1.6	0.5	A	A	A
$4x10^{-8}$	$1x10^{6}$	22.7	38.6	47.7	28.3	36.0	47.1	0.7	0.1	0.0	A	A	A
$4x10^{-8}$	$3.16x10^{6}$	28.9	48.9	55.6	46.2	51.7	61.0	5.4	0.1	0.6	An	A	A
$4x10^{-8}$	$1x10^{7}$	42.9	66.7	71.4	62.1	73.5	81.2	6.6	1.0	2.6	An	A	A
0	$1x10^{5}$	9.4	9.4	9.4									
0	$3.16x10^{5}$	9.2	14.4	18.8									
0	$1x10^{6}$	14.1	23.3	36.7									
0	$3.16x10^{6}$	35.6	42.2	53.3									
0	$1x10^{7}$	54.6	68.3	77.5									
$4x10^{-8}$	0	13.0	13.0	13.0									
0	0	4.0	4.0	4.0									

Table 5-8: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Dipel DF at LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50 (conidia ml^{-1})	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$6.32x10^6$	$4.96x10^6$	$8.18x10^6$
LC40	$3.18x10^6$	$2.02x10^6$	$4.54x10^6$

Table 5-9: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Dipel DF at a 10-fold dilution of LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50 (conidia ml^{-1})	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$5.36x10^6$	$3.95x10^6$	$7.28x10^6$
LC40/10	$4.39x10^6$	$3.09x10^6$	$6.09x10^6$

5.3.5.2 Quantifying the effect of co-application of B. bassiana *433.99 with low concentrations of Neemazal on the survival of groups of DBM larvae*

For both Neemazal/*B. bassiana* co-application experiments a concentration-mortality response was observed for both *B.bassiana* only and combination treatments. In the LC40 experiment, insect mortality increased at every dose of *B. bassiana* if applied in combination with Neemazal (Table 5-10). At $1x10^7$ conidia ml^{-1} a synergistic interaction between *B. bassiana* and Neemazal was observed at four, five and six days after treatment. For example, on day six, 94.9 and 72.2% mortality was observed for duel and *B. bassiana* only treatments, respectively. Synergistic interactions were also found at $3.16x10^7$ conidia ml^{-1} on days five and six after treatment, and at $3.16x10^5$ conidia ml^{-1} on day six. Of the 15 interactions characterised, six were synergistic and nine were additive (Table 5-10).

For the interaction involving a 10-fold dilution of the Neemazal LD40, mortality was similar in both the duel and lone *B. bassiana* treatments, in some cases mortality was lower in duel treatments (Table 5-11). Of the 15 interactions characterised, all 15 were found to be additive. There was no evidence of synergism or antagonism (Table 5-11).

Probit analysis was used to compare *B. bassiana* LC50s between EPF only and co-application treatments. For experiments involving Neemazal LC40 and the 10-fold dilution of the LC40, error bars representing 95% confidence intervals of the *B. bassiana* overlapped indicating there was no significant difference in *B. bassiana* LC50 when applied with and without a fixed concentration of insecticide (Table 5-13,Table 5-14).

Table 5-10: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Neemazal at LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		Observed mortality (%)			Expected mortality (%)			Chi squared value			Response		
Neem (g ml^{-1})	Bb (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$4.3x10^{-6}$	$1x10^{5}$	5.3	27.7	45.7	16.8	29.8	38.1	3.8	0.0	1.1	A	A	A
$4.3x10^{-6}$	$3.16x10^{5}$	8.3	41.2	57.7	19.4	31.8	40.2	0.2	1.3	4.2	A	A	S
$4.3x10^{-6}$	$1x10^{6}$	5.3	54.9	63.2	16.8	46.3	59.5	0.6	0.9	0.1	A	A	A
$4.3x10^{-6}$	$3.16x10^{6}$	36.8	77.8	77.8	44.4	49.7	59.7	1.0	11.3	4.9	A	S	S
$4.3x10^{-6}$	$1x10^{7}$	52.8	89.7	94.9	58.5	75.3	81.3	6.0	3.9	4.3	S	S	S
0	$1x10^{5}$	5.3	5.3	7.9									
0	$3.16x10^{5}$	8.3	8.1	10.9									
0	$1x10^{6}$	5.3	27.6	39.7									
0	$3.16x10^{6}$	36.8	32.3	40.0									
0	$1x10^{7}$	52.8	66.7	72.2									
$4.3x10^{-6}$	0	11.2	20.9	28.4									
0	0	1.5	6.2	6.2									

Table 5-11: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Neemazal at a 10-fold dilution of the LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		Observed mortality (%)			Expected mortality (%)			Chi squared value			Response		
Neem (g ml^{-1})	Bb (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$4.3x10^{-7}$	$1x10^{5}$	3.0	15.2	30.3	15.5	24.8	28.4	3.8	1.7	0.1	A	A	A
$4.3x10^{-7}$	$3.16x10^{5}$	8.1	13.5	18.9	5.0	15.0	18.6	0.8	0.1	0.0	A	A	A
$4.3x10^{-7}$	$1x10^{6}$	5.4	13.5	21.6	9.9	17.2	28.4	0.8	0.4	0.8	A	A	A
$4.3x10^{-7}$	$3.16x10^{6}$	18.9	35.1	48.6	27.6	41.3	41.8	1.4	0.6	0.7	A	A	A
$4.3x10^{-7}$	$1x10^{7}$	44.4	63.9	72.2	54.4	70.4	78.0	1.4	0.7	0.7	A	A	A
0	$1x10^{5}$	11.0	20.7	23.5									
0	$3.16x10^{5}$	0.0	10.3	13.1									
0	$1x10^{6}$	5.2	12.7	23.6									
0	$3.16x10^{6}$	23.7	38.1	37.9									
0	$1x10^{7}$	52.0	68.8	76.5									
$4.3x10^{-7}$	0	5.0	5.0	6.4									
0	0	0.0	0.0	0.0									

Table 5-12: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Neemazal at LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$4.40x10^6$	$2.29x10^6$	$7.98x10^6$
LC40	$1.99x10^6$	$-3.24x10^5$	$4.39x10^6$

Table 5-13: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Neemazal at a 10-fold dilution of LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$5.99x10^6$	$4.07x10^6$	$8.84x10^6$
LC40/10	$5.39x10^6$	$3.60x10^6$	$8.05x10^6$

5.3.5.3 Quantifying the effect of co-application of B. bassiana *433.99 with low concentrations of Calypso on the survival of groups of DBM larvae*

A concentration-mortality response was observed for both co-application and lone *B. bassiana* 433.99 treatments. For the LC40 experiment there was a general increase in insect mortality if *B. bassiana* 433.99 was applied with Calypso (Table 5-15). Synergistic interactions were observed at $1x10^7$ conidia ml^{-1} between *B. bassiana* 433.99 and Calypso on days four, five and six after treatment. For example, on day five, 100.0 and 59.2% mortality was observed for co-application and lone *B. bassiana* 433.99 treatments, respectively. Synergistic interactions were also observed at $3.16x10^6$ conidia ml^{-1} on days five and six. Of the 15 interactions characterised, five were synergistic and 10 were additive (Table 5-15).

For the co-application experiment involving a 10-fold dilution of the Calypso LC40, a combination of *B. bassiana* 433.99 and Calypso resulted in an increase in insect mortality (Fig. 5-15). For $3.16x10^6$ conidia ml^{-1}, synergistic interactions were observed on four, five and six days after treatment. On day six, 73.7 and 84.4% mortality was observed for lone *B. bassiana* and combination treatments, respectively. Synergistic interactions were also observed at $3.16x10^5$ conidia ml^{-1} on days five and six after treatment. Of the 15 interactions characterised, 10 were found to additive and five were found to be synergistic (Table 5-16).

Probit analysis was completed to compare *B. bassiana* 433.99 LC50s between insecticide and non-insecticide treatments. In the experiment involving a concentration range of *B. bassiana* 433.99 and Calypso at LC40, non-over lapping 95% confidence interval error bars indicated that *B. bassiana* 433.99 has a significantly lower LC50 when applied with Calypso at LC40, when compared to being applied on its own (Table 5-17).

Non-over lapping 95% confidence interval error bars indicated the *B. bassiana* LC50 was significantly lower if applied with Calypso at a 10-fold dilution of the LC40 (Table 5-18).

Table 5-14: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Calypso at LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		**Observed mortality (%)**			**Expected mortality (%)**			**Chi squared value**			**Response**		
Calypso (g ml^{-1})	*Bb* (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$3.1x10^{-6}$	$1x10^{5}$	38.5	43.6	46.2	34.8	37.8	39.3	0.2	0.6	0.8	A	A	A
$3.1x10^{-6}$	$3.16x10^{5}$	40.5	56.8	64.9	42.6	47.3	49.5	0.1	1.3	3.5	A	A	A
$3.1x10^{-6}$	$1x10^{6}$	36.1	55.6	55.6	48.4	50.9	57.7	2.2	0.3	0.1	A	A	A
$3.1x10^{-6}$	$3.16x10^{6}$	61.5	82.1	92.3	56.9	60.8	68.2	0.3	7.4	10.5	A	S	S
$3.1x10^{-6}$	$1x10^{7}$	87.5	100.0	100.0	72.7	73.2	83.9	4.4	14.7	7.7	S	S	S
0	$1x10^{5}$	2.8	5.3	7.7									
0	$3.16x10^{5}$	14.3	19.9	23.2									
0	$1x10^{6}$	23.0	25.4	35.7									
0	$3.16x10^{6}$	35.6	40.4	51.6									
0	$1x10^{7}$	59.2	59.2	75.5									
$3.1x10^{-6}$	0	29.0	29.0	29.0									
0	0	5.6	5.6	7.4									

Table 5-15: Chi squared analysis to determine the effect of co-application of *B. bassiana* with Calypso at a 10-fold dilution of LC40 on the survival of groups of DBM larvae on days four, five and six after treatment. For responses: A = additive, S = synergistic and An = Antagonistic.

Concentrations		**Observed mortality (%)**			**Expected mortality (%)**			**Chi squared value**			**Response**		
Calypso (g ml^{-1})	*Bb* (conidia ml^{-1})	4d	5d	6d	4d	5d	6d	4d	5d	6d	4d	5d	6d
$3.1x10^{-7}$	$1x10^{5}$	10.0	12.5	20.0	10.6	16.4	21.0	0.0	0.4	0.5	A	A	A
$3.1x10^{-7}$	$3.16x10^{5}$	25.6	30.8	33.3	9.9	18.2	22.1	10.9	4.1	15.1	S	S	S
$3.1x10^{-7}$	$1x10^{6}$	17.1	31.7	41.5	18.0	25.8	34.7	0.0	0.7	0.8	A	A	A
$3.1x10^{-7}$	$3.16x10^{6}$	60.5	65.8	73.7	17.7	27.5	33.5	47.9	28.0	75.9	S	S	S
$3.1x10^{-7}$	$1x10^{7}$	76.9	82.1	92.3	59.9	79.7	86.2	4.7	0.1	4.8	S	A	S
0	$1x10^{5}$	8.3	8.3	11.1									
0	$3.16x10^{5}$	7.5	10.3	12.4									
0	$1x10^{6}$	15.9	18.7	26.6									
0	$3.16x10^{6}$	15.6	20.4	25.2									
0	$1x10^{7}$	58.9	77.8	84.4									
$3.1x10^{-7}$	0	2.5	6.0	7.5									
0	0	0.0	3.0	3.9									

Table 5-16: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Calypso at LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$4.73x10^6$	$3.43x10^6$	$6.65x10^6$
LC40	$1.40x10^5$	$-1.21x10^6$	$1.30x10^6$

Table 5-17: The LC50 (conidia ml^{-1}) of *B. bassiana* 433.99 against groups of DBM larvae when applied with and without Calypso at a 10-fold dilution of LC40. LC50s were estimate using probit regression (SPSS). Upper and lower bound 95% confidence intervals are included.

Concentration of insecticide	LC50	Lower bound 95% confidence interval	Upper bound 95% confidence interval
0	$5.60x10^6$	$4.26x10^6$	$7.43x10^6$
LC40/10	$2.87x10^6$	$1.74x10^6$	$4.21x10^6$

5.4 Discussion

As expected, it was found that a wide range of insecticides were toxic to DBM larvae. The mortality found in response to organophosphates, pyrethroids and the Bt based Dipel DF were in agreement with a study completed by (Odhiambo, 2005). Neonicotinoids and avermectins are also well known to cause quick mortality in non-insecticide resistant populations (Sarfraz et al., 2005). Some insecticides were observed not to confer insect mortality, for example Movento conferred a similar mortality to the control; this can be explained by its systemic mode of action which would not necessarily by suited to a leaf dip bioassay (Nauen et al., 2007). Majestick, an insecticide based on maltodextrin, conferred no significant mortality against DBM, but there has been no previous investigation into the toxicity of this insecticide against DBM. Neemazal, based on azadirachtin, was found to cause significant insect mortality, but only after six days, this in line with literature citing the slow but effective action of neem oil extracts (Schmutterer, 1992).

To assess the suitability of candidate insecticides for use with in an IPM system with EPF, the germination and colony extension rate of *B.bassiana* 433.99 was recorded whilst on SDA media containing candidate insecticides. This is an approach which has been used before in the literature (Olmert and Kenneth, 1974, Neves et al., 2001). In the current study, insecticides were more likely to inhibit the germination of *B. bassiana 433.99*, when compared to the colony extension rate, there are many possible reasons for this. Germination and mycelial growth are separate processes with very different characteristics; germination involves the enzymatic break down of the conidial cell wall and the generation of significant mechanical force for the germ tube to penetrate and extend from the conidia, whereas mycelial growth involves a separate set of enzymes, and a suite of cellular apparatus, devoted to the synthesis of the cell wall at the tip of the hyphae (Deacon, 2005). Compared to mycelial growth, germination requires more energy to proceed and, therefore, may be more effected by an insecticide.

A group of insecticides including Neemazal and Calypso were found to have no significant effect on the physiology of *B. bassiana* 433.99 *in vitro*. A similar study, Neves

et al. (2001) found imidacloprid, a closely related compound to the Calypso active thiacloprid, conferred no significant inhibition of fungal colony extension rate. In addition, a study by Gomes et al. (2015) also showed that neem oil had no effect on germination or vegetative growth of *M. anisopliae.* Although, it should be noted that, in the current study, when Neemazal and SDA were mixed, neem oil tended to clump together in certain areas, this may have had an impact on the bioassay, with *B. bassiana* encountering non-uniform concentrations of neem oil throughout the experiment.

Before experiments were completed to quantify the effect of co-application of *B. bassiana* with insecticides, three insecticides were chosen to be included the experiments. Insecticides were chosen based on three factors:

i. High toxicity to DBM larvae
ii. No inhibition of EPF colony extension rate and germination
iii. Evidence in the literature to suggest that the insecticide would be compatible with an EPF biopesticide when controlling insect pests

The three insecticides that were chosen were Dipel DF, Calypso and Neemazal. Through the screen of insecticides, it was found that each insecticide was highly toxic to groups of DBM larvae. By 144hr after treatment, Dipel DF, Calypso and Neemazal conferred 94, 91 and 70% mortality, respectively. Additionally, Neemazal and Calypso caused no significant inhibition of *B. bassiana* 433.99 colony extension rate or germination. This can be seen as indication that an antagonistic interactions between the EPF and the insecticides are less likely to occur (Anderson and Roberts, 1983). This is because insect mortality is highly dependent on conidial germination on the insect cuticle (Hajek and St. Leger, 1994). Dipel DF was found to inhibit germination of *B. bassiana* 433.99, but was chosen because of evidence in the literature which suggested that EPF biopesticides and Bt interact synergistically with EPF biopesticides when applied to groups of DBM larvae (Nian et al., 2015). It was also advantageous to the study that the three insecticides chosen had separate modes of action, this allows the opportunity to elucidate whether mode of action effects the observed response after co-application.

The influence of mode of action on the interaction between insecticides and EPF biopesticides when applied to insect pests has not previously been investigated.

It has been reported that the active ingredients which make up Dipel DF, Calypso and Neemazal are able to increase either speed of kill or overall percentage mortality of insect pests when applied with an EPF biopesticides. For example, it was found that if the aphid species *Myzus persicae* was treated with *B. bassiana* and a sub-lethal concentration of imidacloprid at a concentration of 0.1-0.5 ug ml^{-1} there was significant enhancement of fungal action, reflected in an increased speed of kill. In the same study, The LC50 of *B. bassiana* was reduced by up to 100 times by combining the EPF biopesticide with sublethal concentrations of imidacloprid (Ye et al., 2005). Consequently, an imidacloprid-EPF treatment was suggested as an alternative control strategy for control of *Myzus persicae.* Nian et al. (2015) completed experiments to establish whether an *I. fumosorosea* EPF isolate interacted with a sub-lethal concentration of Bt when applied to second instar DBM larvae. It was found, through chi squared analysis, that there was a predominantly synergistic interaction between Bt and *I. fumosorosea*. For example, at a concentration of 5 x 10^5 conidia ml^{-1} *I. fumosorosea* conferred 69% and 84% mortality when applied without and with a sub-lethal concentration of Bt, respectively. Neem oil was found to increase to efficacy of an *M. anisopliae* isolate when treating *Aedes aegypti* larvae (Gomes et al., 2015). Mortalities of 78 and 36% were reported when *M. anisopliae* was applied with and without a low concentration of neem oil. The authors also found co-application of neem oil and *M. anisopliae* resulted an increase speed of kill, as well as overall mortality percentages (Gomes et al., 2015).

Before co-application experiments could be completed, a concentration-mortality response experiment was completed to determine the LC40 for each insecticide. A clear concentration response was found for each insecticide. In the literature, concentration-mortality response experiments have not previously been completed for thiacloprid or azadirachtin based insecticides. However, Odhiambo (2005) completed a concentration-mortality response experiment in which a Bt insectice was applied to groups of DBM larvae and reported the LC95 to be approxmatley 200 mg/L.

In the concentration-response analysis, Calypso displayed a far steeper slope when compared to Dipel DF and Neemazal. The slope represents the variation in susceptibility of the group of DBM larvae to the insecticide in question. This suggests that DBM had a more uniform susceptibility to thiacloprid (the active ingredient of Calypso) when compared to Dipel DF and Neemazal. Thiacloprid acts on contact with the insect cuticle, and therefore has a more direct mode of action when compared to Dipel DF and Neemazal, which may result in a faster speed of kill (Gill et al., 1992, Mordue and Nisbet, 2000). Dipel DF and Neemazal insecticides are reliant on consumption of leaf material (treated with insecticide) to be effective, leaf consumption rates vary between individual DBM larvae depending on various factors such as age with in the instar (Harcourt, 1957). This could contribute to non-uniformity of response observed in groups of larvae treated with Dipel DF and Neemazal, when compared to Calypso.

Through this series of experiments, it was found that application of *B. bassiana* 433.99 with the three candidate insecticides used most often results in a higher level of mortality in groups of DBM larvae. The most commonly found response between insecticide and *B. bassiana* 433.99 was additivity. Synergy was also found, mostly at high concentrations of EPF and insecticide, the exception to this being the synergistic interactions found between Calypso and *B. bassiana* at a 10-fold dilution of the LC40.

Dipel DF was found to be compatible with *B. bassiana*. The LC50 of *B.bassiana* when applied with Dipel DF at LC40 was lower than when applied on its own. The use of Dipel DF at a LD40/10 dose did not have a significant effect on mortality. Additionally, Dipel DF was found to act synergistically only at the LC40 dose of insecticide, at high doses of *B.bassiana* on days five and six after treatment. No evidence of synergism was found in the experiment using the lower dose of Dipel DF. 30 chi squared analyses were competed, but because these analyses were completed on data from consecutive days, only three independent interactions were assessed. Overall, across all Dipel DF experiments, 5 of 30 chi squared analyses were found to be synergistic, 2 were antagonistic, and 23 were additive. Nian et al. (2015) completed a study with same experimental design, in which a concentration range of EPF was applied with and

without a fixed dose of Bt. Like in the current study, two doses of Bt were used, one causing approximately 20% mortality and the other causing approximately 10% mortality. Far more synergism was found in the study by Nian et al. (2015) when compared to the current study. Of the 30 chi squared analyses , 19 were found to be synergistic. No evidence of antagonism was found. By day six it was found that all interactions were synergistic regardless of the dose of Bt or EPF. However, there were some differences in experimental design between Nian et al. (2015) and the current study. In the Nian et al. (2015) study, treatment consisted of immersing larvae in a suspension of Bt and EPF biopesticide for periods of 10s. In the current study, EPF biopesticide was applied using a Potter tower sprayer, and Bt was applied by spreading Dipel DF on a leaf disc. The method of treatment employed by Nian et al. (2015) may not be suitable for Bt application as it is dependant of ingestion by larvae. This may explain why higher concentrations of Bt were required to cause mortality in the Nian et al. (2015) study, when compared the current study.

Neemazal was found to interact with *B. bassiana* either synergistically or additively depending on the doses of EPF and Neemazal, and the time after treatment. In the Neemazal LC40 experiment synergy was more likely to be observed at high doses of *B. bassiana.* For example, at $1x10^7$ conidia ml^{-1} of EPF plus the LC40 of Neemazal synergy was observed on days four, five and six after treatment. But for $3.16 x10^6$ conidia ml^{-1} plus the LC40 of Neemazal synergy was observed on days five and six after treatment. No evidence of synergy was found in the experiment involving the lower dose of Neemazal. Previous study has found that neem oil and *B. bassiana* are compatible. For example, it was found that a dual application of neem oil and *B. bassiana* significantly reduced the survival rate of mosquito larvae (Gomes et al., 2015). In the current study, it was also found that applying *B. bassiana* 433.99 with Neemazal at LC40 reduced the LC50 of *B. bassiana*, although it should be noted that 95% confidence error bars overlapped between *B. bassiana* that was co-applied and *B. bassiana* applied on its own. Applying *B. bassiana* with at 10-fold dilution of the LC40 had no effect on the LC50 of *B. bassiana.*

Of the three insecticides tested, Calypso provided the most evidence of synergism. Synergistic interactions were found at both the LC40 and lower concentrations of Calypso. At the LC40 concentration of Calypso, synergistic interactions were more likely to be found at high concentrations of *B. bassiana*. Ye et al., (2005) found that the co-application of EPF biopesticide with a neonicotinoid insecticide can significantly reduce the LC50 of *B. bassiana*. Considering the extent of reduction of the LC50, this can probably be considered a synergistic interaction and is consistent with the current research. In the current research, when applied with Calypso at LC40, the *B. bassiana* LC50 was significantly reduced from 4.7×10^6 to 1.4×10^5 conidia ml^{-1}. A significant reduction in *B. bassiana* LC50 was also observed when the EPF biopesticide was applied with a 10-fold dilution of the LC40 of Calypso. The results from the current study are generally in agreement with the findings reported by Ye et al. (2005). However, there are some differences between the two studies. Ye et al. (2005) was completed on aphid species and used a different class of neonicotinoid.

Further analysis was also completed (which can be seen in *Appendix IX*) to assess the interaction between selected insecticides and *B. bassiana*. In this analysis, a 2^{nd} order polynomial model was fitted to mortality data across *B. bassiana* concentrations with and without a commercially available insecticide. The presence of a synergistic interactions can be inferred by noting the deviation between both polynomial curves. This analysis can be used to give an indication of synergism (Raymond et al., 2006). The analysis completed here produced similar results to the chi squared analysis completed earlier in this chapter. At higher doses of Neemazal and Dipel DF, synergistic interactions were indicated. At both Calypso doses, evidence of synergism was present.

Other than simply reporting the presence or absence of certain interactions, it is important to investigate the mechanism behind these responses. Two mechanisms for synergistic interactions between Bt and EPF were proposed by Nian et al., (2015). Firstly, the infection of EPF may increase the susceptibility of DBM to the other pathogens. Furlong and Groden (2001) reported synergy between sub-lethal concentrations of immidacloprid and a *B. bassiana* isolate when applied to second instar Colorado potato beetle larvae. This interaction was attributed to sub-lethal insecticide concentrations

causing a reduction in larval feeding, leading to starvation stress which in turn rendered the larvae more susceptible to the EPF. Furlong and Groden (2001) further supported this hypothesis by starving larvae for 24hr before application of *B. bassiana*; a similar effect was observed. In the current study, it was observed that larvae treated with insecticide as well as *B. bassiana* 433.99 consumed less leaf area when compared to larvae treated with *B. bassiana* 433.99 only (**personal observation**). Therefore, starvation stress could be contributing factor to the synergy observed.

Secondly, it has long been known that shedding of fungal conidia during the moult from one instar to the next can be an effective route to avoid infection for DBM larvae (Vandenberg et al., 1998b). Additionally, it has been found that Bt can arrest the development of potato beetle larvae (Cloutier and Jean, 1998, Costa et al., 2000). Self-evidently, if larvae take longer to develop to the third instar of development after treatment of a sub-lethal concentration of insecticide, conidia would have more time to penetrate through the cuticle and cause a systemic infection (Nian et al., 2005). It was found in this study that all three insecticides inhibited development of DBM larvae from second to third instar. For example, certain doses of Bt were found to increase length of the second instar by approximately 1 to 1.5 days compared to the control, giving conidia more time to penetrate the cuticle. It was also found there was a concentration response of DBM development rate to Bt. This would explain why no synergy was found in the experiment involving the lower concentration of Bt. This same concept can be applied to any of the insecticides used here, as all insecticides were shown to clearly arrest development. To the best of this author's knowledge this is the first time the sub-lethal effects of commercially available insecticides against DBM, in terms of development, have been investigated.

When studying the combination of products to achieve insect control, it should be assessed whether their coapplication has any influence on the development of resistance in the insect population. IPM strategies rely on the premise that if many different control products are used, each of which has a separate mode of action, insects will be less likely to evolve resistance during a series of applications (Sarfraz at al, 2005). In fact, when assessing the effect of strain diversity on the evolution of

resistance in DBM larvae, Raymond et al. (2013) found that mixed infections of Bt did not result in increased resistance in DBM populations. However, research on antibiotic resistance in a clinical setting has shown that when synergy occurs between two drugs, selection pressure is increased dramatically, which may result in development of bacterial resistance (Torella et al., 2010). The use of synergistic interactions should, therefore, be treated with caution in IPM strategies.

Studies into the interaction between synthetics and biological insecticides are invaluable when it comes planning the application of such control products. For example, if it is known that a synthetic severely inhibits the action of a biological, they should be sprayed at different times and, if possible, in different areas. The mechanism behind a certain interaction may also dictate the spraying schedule of insecticides; for example, if a synthetic has been shown to increase the length of the intermoult period, a biological such as *B. bassiana* (which is yields better control with longer intermoult periods) should be sprayed in quick succession directly after the synthetic to maximise the potential gains brought about by the longer intermoult period.

Here it was demonstrated that, on the whole, the insecticides tested are compatible with *B. bassiana* for control of DBM. The combination of these treatments usually results in an increase in insect mortality. Additionally, synergy was observed at high concentrations of EPF and insecticide for all insecticides. Calypso was the only insecticide to interact synergistically with *B. bassiana* at both low and high concentrations. Synergistic interactions provide an opportunity to achieve a high level of control, whilst making an economic saving, although, such interactions may increase the risk of development of resistance.

5.4.1 Summary

i. Results indicate that Calypso, Dipel DF and Neemazal are compatible with *B. bassiana*, and can be used in an IPM system to control DBM.

- The response after applying one of three commercially available insecticides (Calypso, Dipel DF and Neemazal) with a concentration range of *B. bassiana* against groups of DBM larvae was mostly additive.
- Some evidence of synergism was found at high concentrations of *B. bassiana.*

ii. All three insecticides were shown to arrest development; this could provide conidia more time to penetrate the cuticle before moult into the third instar occurs which we propose could be a mechanism for the observed synergism.

6 Investigating the effect of age of DBM larvae on their susceptibility to EPF

6.1 Introduction

As insecticide only control strategies of DBM have failed to be sustainable, relevant experts believe the use of IPM is the way forward in controlling DBM populations (Sarfraz et al., 2005, Furlong et al., 2013). EPF have great potential for DBM control in an IPM system, with certain species (such as *B. bassiana*) being shown to cause significant DBM control both in the laboratory (Wraight et al., 2010) and when applied as a foliar spray (Vandenberg et al., 1998b). EPF may also have good compatibility with other elements of the IPM system. For example, Nian et al., (2015) showed that a predominantly synergistic interaction between was found between a Bt based insecticide and an *I. fumosorosea* isolate. A similar conclusion was found in the current research project, with *B. bassiana* being compatible with Bt when applied to groups of DBM larvae.

Despite the undoubted potential EPF hold as biocontrol control products against DBM, there are several barriers that must be overcome before EPF biopesticides can be used widely in the field. From the point of view of growers, perhaps the issue of greatest importance is the inconsistent performance by EPF in the field (Thomas and Blanford, 2003, Lacey et al., 2001).

There are several factors which contribute to this observed variation. One factor – which has not been investigated in detail for EPF biopesticides – is the effect of the age structure of an insect population on EPF biopesticide efficacy. Insect mortality caused by EPF is a relatively lengthy process when compared to the instantaneous death caused by most synthetic insecticides (Kumar and Chapman, 1983, Lacey et al., 2001). Conidia cause infection by attaching to the insect cuticle (Hajek and St. Leger, 1994, Xia et al., 2013), and then take approximately 60hr to germinate, break though the insect cuticle and cause a systemic infection (Xia et al., 2013) (Xia et al., 2013). As the infection of EPF

advances, DBM larvae are developing from one instar to the next. A change of instar involves the moulting of the larvae cuticle – known as ecdysis (Golizadeh et al., 2007). The larval cuticle is a rigid structure, so it must be shed for larvae to increase in size. At 20^{o}C larvae take approximately three days to move through each larval instar.

It was found by Vandenberg et al. (1998) that DBM larvae treated later in the instar were far less susceptible to infection by EPF conidia. The authors hypothesised that larvae avoid infection by shedding EPF conidia during moult. It was also found that EPF conidia were present on the cuticular exuvae shed after moult, which further supported the authors' hypothesis. This concept is well known in other larval insects. For example, it was found that the moulting process can remove EPF conidia from Colorado potato beetle larvae and allow the insect to avoid infection (Vey and Fargues, 1977). The process of removal of EPF inoculum during moult can reduce the efficacy of EPF biopesticides when applied against a mixed age population of larvae, or lead to inconsistent results.

This problem is compounded because the majority of initial screens of fungal isolates are completed against fixed aged cultures of DBM (Wraight et al., 2010, Huang et al., 2010). This means that problems of reduced EPF biopesticides efficacy, caused by mixed age larvae populations, may not become apparent until expensive field testing is completed.

However, direct application is not the only means by which larvae become infected with EPF conidia. Secondary pick-up occurs when larvae move across a leaf surface treated with EPF conidia, during this process conidia attach to the insect cuticle and may cause infection. Studies have shown that DBM larvae are able to acquire a lethal EPF infection through secondary pick-up (Cui et al., 2014). In the preliminary investigations into bioassay protocols (section 3.2.2) in this PhD project, it was found that significant mortality of populations of DBM larvae resulted from exposing larvae to a leaf surface treated with *B.bassiana* EPF conidia.

In a field situation, secondary pick-up may be an important means of causing infection against DBM larvae. After a foliar application, a certain proportion of DBM larvae would most likely shed conidia, as they transition from one instar to the next. These insects are likely to become infected by a repeated spray application or secondary pick-up of EPF conidia from foliage. The propensity of EPF to cause infection through secondary pick-up should therefore be assessed as part of the initial assessment of EPF isolates.

The aims of this study were as follows. First, it was investigated whether DBM larvae shed conidia during moult to avoid infection. Secondly, work was done to investigate whether secondary pick-up of EPF conidia from treated leaves could cause infection in late instar DBM larvae. Thirdly, a concentration response experiment was completed in which DBM larvae are exposed to leaf material treated with EPF at a range of concentrations. Finally, the propensity of EPF conidia of a range of different isolates to persist on leaf material, after being applied, was investigated.

The component objectives were as follows:

i. Determine whether time between treatment of DBM larvae *B. bassiana* 433.99 and moult has an effect on the susceptibility of groups of DBM larvae to infection by EPF conidia
ii. Determine the effectiveness of secondary pick-up of *B. bassiana* 433.99 conidia in causing infection of groups of late and early instar DBM larvae
iii. Record the number of *B. bassiana* 433.99 conidia acquired by larvae after being exposed to leaf material treated with a range of EPF concentrations
iv. Determine whether time between treatment of the leaf surface with one of three candidate EPF isolates (*B. bassiana* 433.99, *B. bassiana* 1757.15 and *M. brunneum* 1760.15) and exposure to DBM larvae has an effect on the mortality of groups of DBM larvae

6.2 Materials and methods

6.2.1 Determining the effect of the length of the inter-moult period on the susceptibility of DBM to EPF infection

Groups of larvae were treated using the "larval spray" application technique described in 2.4 in which controlled concentrations of EPF conidia were applied to fixed aged populations of DBM larvae using a Potter tower. *B. bassiana* 433.99 was used in this experiment. 4ml of *B. bassiana* 433.99 was applied at a fixed dose of $1x10^7$ conidia ml^{-1}. Mortality was monitored daily for seven days. Groups of larvae of several different ages were used to determine whether time between application and moult had an effect of larval susceptibility.

Fixed age cultures of larvae were set up using the procedure described in 2.1 so that groups of larvae were available on the same day. Five different age groups were used, larvae were selected to be included in these age groups based on the number of days after eclusion. The following ages groups were used: early second instar (eight days after eclusion), late second instar (10 days after eclusion), early third instar (11 days after eclusion), late third instar (14 days after eclusion) and early fourth instar (16 days after eclusion). Data from the larval development experiment completed in chapter two was used to provide times at which to expect the desired instar to emerge. Newly emerged larvae from each instar were identified by the darkened cuticle and the presence of the exuvia on the leaf surface. Late instar larvae were identified by their visibly lighter cuticle. 10-15 DBM larvae were included in each age group. For each treated age group of larvae, an untreated control group was also completed. The control treatment consisted of treatment of larvae with 0.05% Triton X-100 using the Potter tower. Each of the five groups of known-age larvae (plus the corresponding controls) were bioassayed at the same time, forming one block. Each block was repeated on three separate occasions.

For each treated age group of larvae, an untreated control groups was also completed. The control treatment consisted of treatment of larvae with 0.05% Triton X-100 using the Potter tower.

After EPF treatment larvae were maintained as described in 2.4 at 20^{o}C (16:8 LD) and mortality was assessed every 24hr.

6.2.2 Quantifying mortality and effective dose of EPF conidia received by early and late second instar DBM larvae conferred by three application techniques

Groups of early and late second instar larvae (eight and 11 days after eclusion, respectively) were treated with a fixed dose of *B. bassiana* 433.99 using one of three application techniques. *B. bassiana* 433.99 was applied at 1x10^{8} conidia ml^{-1}. These application techniques can be described as "larval spray", "leaf spray" and a combination of leaf application and leaf spray techniques. For the larval spray technique, the procedure described in 2.4 was used, 4ml of *B. bassiana* 433.99 was applied to groups of larvae using the Potter tower.

For the leaf spray method, 3cm diameter leaf discs (cauliflower, variety Skywalker, Elsons, UK) were sprayed with a 4ml solution of the desired concentration of EPF conidia using a hand-held sprayer before being left to air dry on damp filter paper for 10 minutes at room temperature. Groups of 10-15 early second instar larvae were then treated by using a fine paint brush to transfer larvae to surface of the treated leaf disc, which was contained in a 9cm petri dish (Merck) with a lid modified for aeration containing water agar (14g/L, Sigma). Leaf discs were replaced after four days with identically treated leaf discs. Mortality was monitored daily for seven days. Larvae were kept at 20^{O}C (16:8 LD).

For the combination treatment (leaf spray plus larval spray), larvae were sprayed using the procedure described in 2.4. Larvae were then transferred directly on to a leaf disc, which had been sprayed with EPF conidia as described previously. Leaf discs were

replaced after four days with similarly identically treated leaf discs. Mortality was monitored daily for seven days. Larvae were kept at 20^{O}C (16:8 LD).

Each replicate of the experiment consisted of six treatments (two insect ages x three application techniques), plus a corresponding untreated control for each treatment. Each treatment comprised of two groups of 10-15 larvae maintained in separate petri dishes. One group was used for mortality assessment, the other was used to quantify deposition of EPF conidia on larvae. The number of conidia deposited on larvae was estimated at 0, 24 and 48hr after inoculation.

For larval spray, leaf application and combination treatment conidia present on larvae at 0, 24 and 48hr after treatment were quantified. Four larvae were removed from the experiment at the said time points and suspended in 1ml of 0.05% Triton X-100 contained in a 1.5ml Eppendorf tube. Suspensions were then diluted before aliquots of 100 µl were plated on Genera specific media as described in 2.5.1. Plates were left in darkness at 20°C for six days, before colony forming units were counted. Conidia per larvae were then calculated retrospectively.

All treatments were completed at the same time, forming one block of the experiment. This block was repeated on three separate occasions.

6.2.3 Examining the concentration-mortality response of B. bassiana 433.99 on groups of DBM larvae using the leaf spray application technique

Groups of 10-15 early second larvae (8d after eclusion) were prepared using the procedure described in 2.1. Whole plants (*Brassica oleracea,* var: Skywalker, Elsoms Seeds Ltd, Lincoln, UK) were treated with *B. bassiana* 433.99 conidia suspensions using an adapted version of the leaf application method, described above. Plants were produced using the procedure described in 2.1. *B. bassiana* 433.99 suspensions were adjusted to $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1} in 0.05% Triton X-100 using the procedure described in 2.3, an untreated control was made up of 0.05% Triton X-

100. Semi-mature cauliflower plants (Skywalker, Elsoms Ltd, UK), approximately six weeks in age and at the seven leaf stage, were sprayed with 10ml of the desired concentration of *B. bassiana* 433.99 using a hand-held sprayer until run-off was observed. Plants were then left to air dry at 20^{o}C for approximately 1hr. A 6cm diameter leaf disc was then cut from the plant and kept on water agar (14g/L, Sigma) contained within a 9cm petri dish, with a lid modified for aeration. The remainder of the plant was then kept in an incubator for the next seven days (20^{o}C 16:8 LD). A group of 10-15 early second instar were transferred to the leaf disc using a fine paint brush, which was contained within a 9cm petri dish, and sealed using Parafilm. Petri dishes containing larvae were kept at 20^{o}C (16:8 LD) for the duration of the experiment. Mortality was monitored every 24hr for the duration of the experiment.

For each replicate of the experiment, this procedure was completed two times for each dose applied. The first petri dish was used to monitor mortality; the second repeat was used to monitor conidia present on larvae. To do this, four larvae were removed from the experiment at 3, 24, 48, 96, 144 and 192hr after treatment and suspended in 1ml of 0.05% Triton X-100 contained in a 2ml Eppendorf. The number of conidia present on the cuticle of each larvae was estimated using the procedure described in 2.5.1.

Additionally, conidia present on whole plants were determined at 0, 3, 24, 48, 96, 144 and 192hr after treatment. For each time point, three replicate 0.7cm leaf discs were cut from three separate leaves from the same plant using a cork borer. Leaf discs were suspended in 1ml of 0.05% Triton X-100 contained in a 2ml Eppidorff. The number of conidia present on the surface of each leaf disc was estimated using the procedure described in 2.5.2.

All concentrations of *B. bassiana* 433.99, plus an untreated control were assessed simultaneously. The experiment was repeated three times.

6.2.4 Determining the effect of length of time between leaf treatment and exposure of larvae to treated leaf on EPF virulence, for selected EPF isolates

The experiment was completed using three isolates, *M. brunneum* 1760.15, *B. bassiana* 433.99 and *B. bassiana* 1757.15, plus an untreated control consisting of Triton X-100. These isolates were applied at a fixed concentration of $1x10^8$ conidia ml^{-1} using the protocol described in 6.2.3, with the exception that whole plants were left at 20°C (16:8 LD) after being treated for various time intervals before being exposed to larvae, and watered when necessary. Time intervals of 0, 24, 48, 96 and 144hr were used. Plants were treated in such a way that plants left for 0, 24, 48, 96 and 144hr were available on the same day, so that the same cohort of early second instar larvae could be treated at the same time using all treatments. Mortality was assessed daily for the duration of the seven day experiment. Leaf discs were replaced using material from the original treated plant after five days. Larvae were kept at 20°C (16:8 LD).

Each repetition consisted of 20 treatments: five time intervals for each of the three isolates, plus five time intervals for the untreated control. Three replicates of the experiment were completed.

6.2.5 Statistical analysis

Percentage mortality was corrected for control mortality using Schneider-Orelli's formula (Puntener, 1981). Before Anovas were completed, percentage mortality data was arcsine-sine of the squareroot transformed. Before Anovas were completed, data was tested for normality through visual observvation of a residual-residual plots. Differences between specific treatments were identified by using Tukey's HSD test. Probit analysis was used to determine the LC50 of EPF against groups of DBM larvae.

6.3 Results

6.3.1 Determining the effect of the length of the inter-moult period on the susceptibility of DBM to EPF infection

In this instance mortality data were taken from six days after treatment and corrected for control mortality using Schneider-Orelli's formula (Puntener, 1981). Age of larvae significantly affected mortality conferred (Anova, P=<0.001). Late instar larvae consistently displayed consistently low mortality: late second and late third instar larvae had mortality of 15.0±7.5 and 7.9±1.3% by six days after treatment, respectively. There were no significant differences between these two values (T-test, P=>0.05). Early instar larvae generally had a higher level of mortality: early second, early third and early fourth instar larvae displayed mortality of 70.0±19.8, 90.3±10.9, 81.5±17.0%, there was no significant differences between these values (Tukey's HSD, P=>0.05). Mortality was significantly higher in larvae treated early in the instar compared to larvae treated late in the instar (Tukey HSD, P=<0.05) (Fig. 6-2).

Corrected mortality was also plotted against time before moult. A linear regression model was fitted to this data which had the following equation:

$$\mathrm{y} = -33.6 + \mathrm{x}\ 33.9$$

A positive correlation was observed, and the relationship was significantly different from zero (linear regression, P=<0.001). The linear regression model had an r^2 value of 0.70. This suggested that the longer the time between treatment and moult, the greater the mortality will be (Fig. 6-1).

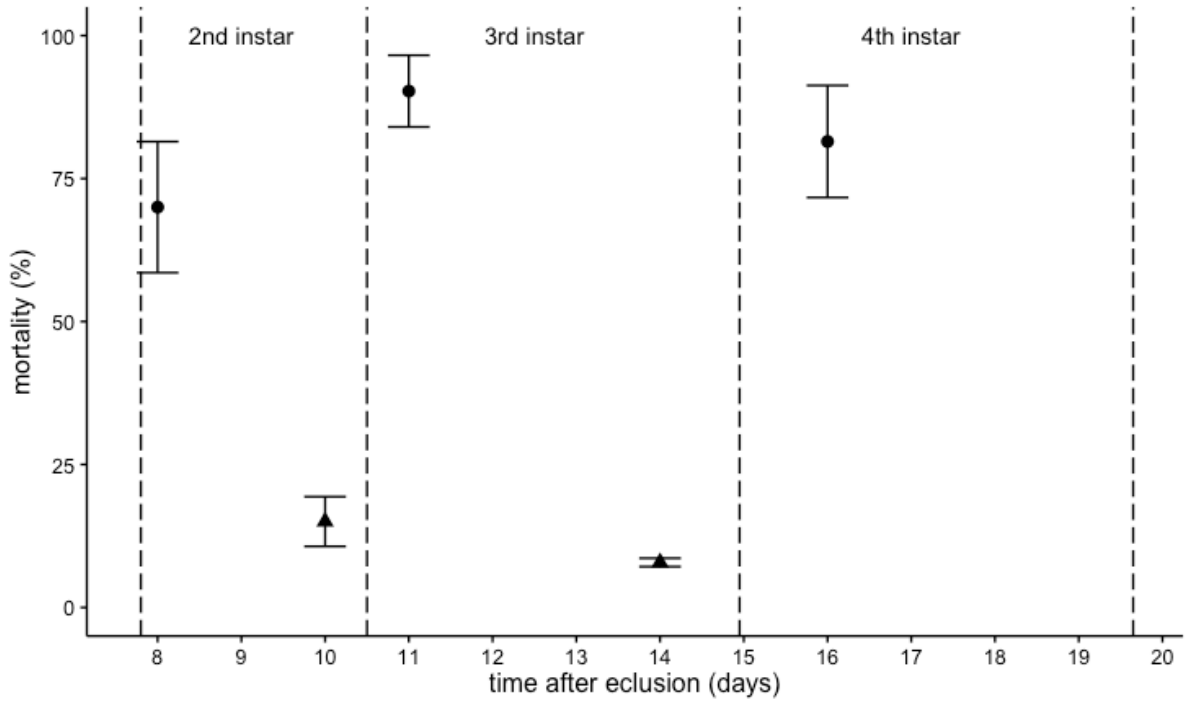


Figure 6-2: Corrected mortality (%±SE) six days after treatment for groups of DBM larvae of different ages (days). Larvae taken from early and late in their respective instars are represented by circular and triangular data points respectively. Broken vertical lines represent the transition from one instar to the next.

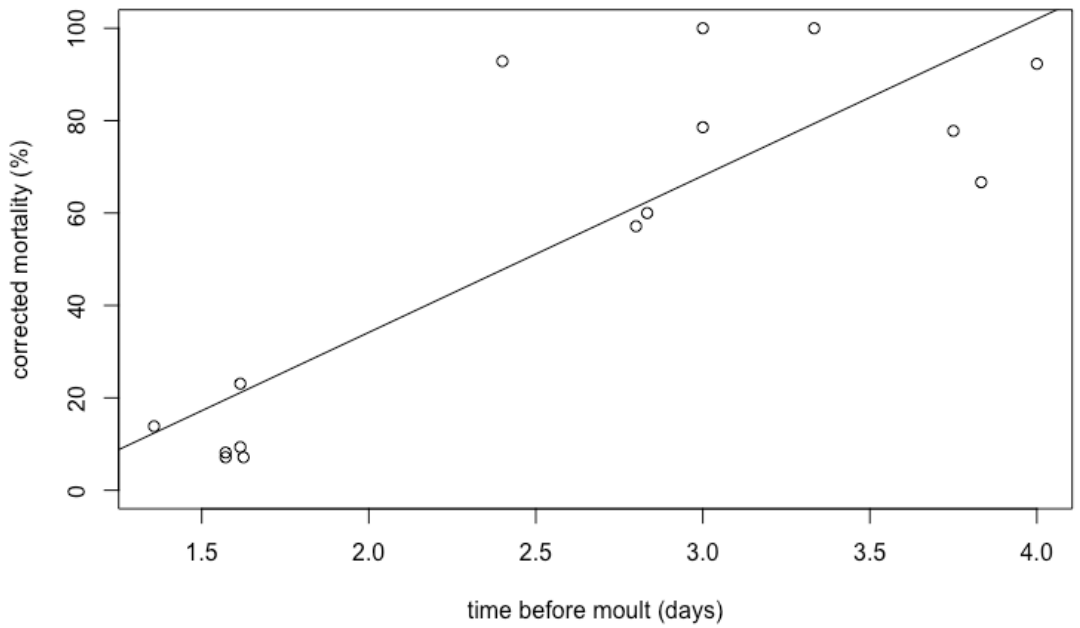


Figure 6-1: Corrected mortality (%) six days after treatment plotted against time elapsed between treatment and moult in the next instar (days). A linear regression model is also included.

6.3.2 Quantifying mortality and effective dose of EPF conidia received by early and late second instar DBM larvae conferred by three application techniques

Mortality from six days after treatment was corrected for control mortality using Schneider-Orelli's formula. Control mortality was consistently below 10% for all treatments, with the exception of the control treatment for late instar larvae treated using the larval spray technique which displayed 10.9% mortality after six days. Mortality was significantly affected by treatment (Anova, P= 0.002). Application method had no significant effect on the mortality of second instar (8d after oclusion) larvae (Tukey HSD, P=>0.05). Mortality was 47.5±25.8, 85.4±12.6 and 73.6±23.3% for groups of larvae treated early in the second instar using leaf spray, larvae spray and combination treatments, respectively. For the larval spray application proportion mortality of groups of late instar larvae was lower than in groups of early second instar (0 and 85.4±12.6%, respectively) (Tukey HSD <0.01). Mortality was significantly lower in larvae treated late in the second instar using the larvae spray technique when compared late instar larvae treated using the leaf spray technique (Tukey HSD <0.05). There was no significant difference between late second instar larvae treated by larval spray and the combination treatment (larval spray and leaf spray) (Tukey HSD, P=>0.05). Mortality was 62.4±20.6, 0.0±0.0 and 22.2±28.5% for groups of larvae treated late in the second instar using leaf spray, larvae spray and combination treatments, respectively. For the larval spray and combination treatments, significantly higher mortality was observed in larvae treated early in the second instar, when compared to larvae treated late in the second instar (Tukey HSD, P=<0.05). For the leaf spray application technique, there was no significant difference in mortality between late second and early second instar larvae (Tukey HSD, P=>0.05) (Fig. 6-3).

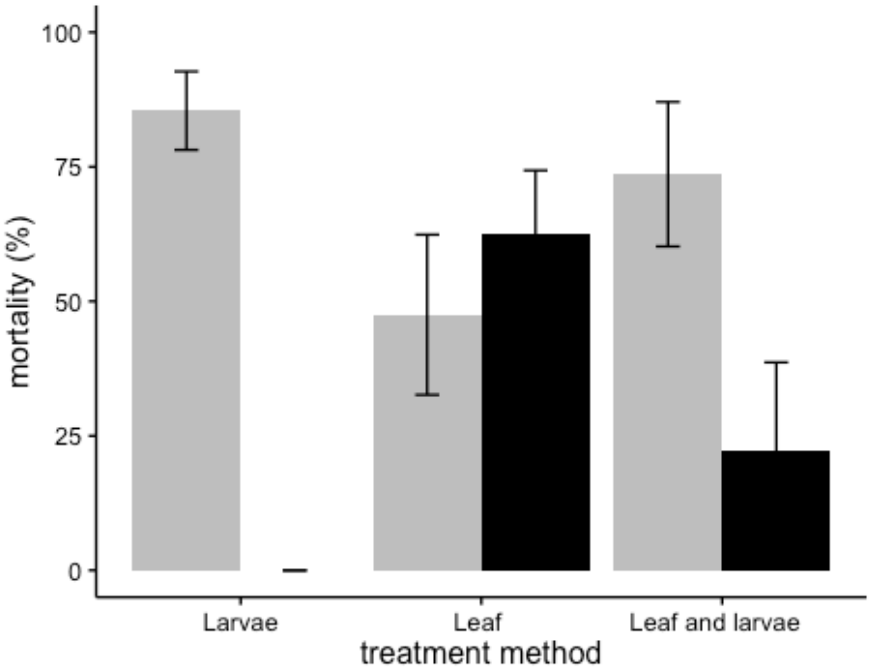


Figure 6-3: Mean corrected mortality of groups of larvae (%±SE) six days after treatment with a fixed dose of *B. bassiana* using one of three application techniques: a combination of leaf spray and larval spray, larval spray and leaf spray. Grey and black bars represent early and late second instar larvae, respectively.

For control treatments, conidia present per larvae was consistently below 60 conidia larvae^{-1} across all treatments and time points.

Spores present on larvae (Log10[conidia larvae^{-1}]) were significantly affected by treatment at 1hr after treatment (Anova, P=0.002). Larvae treated using the leaf spray technique had significantly fewer spores when compared to larvae that had been treated using the spray or combination technique (Tukey HSD, P=<0.05). There was no significant difference in conidia present on larvae between early and late second instar larvae at each treatment (Tukey HSD, P=>0.05) (Fig. 6-4).

At 24hr after treatment the late second instar larvae had all transitioned to the third instar and, in doing so, shed their cuticle. All larvae treated early in the second instar were in the second instar at 24hr after treatment. At this time point, conidia present on larvae were significantly affected by treatment (Anova, P=0.002). There was no

significant difference in conidia present on larvae between early and late second instar larvae treated using leaf spray and combination techniques. In these treatments, dose received by larvae ranged from 794 to 2,500 conidia larvae^{-1}. For the larvae spray treatment, there was a significantly fewer number of conidia present on larvae treated late in the second instar, when compared to larvae treated early in the second in the second instar (Tukey HSD, P=<0.05). Conidia present on larvae was 794±25 and 25±3 conidia larvae^{-1} for early and late second instar larvae treated using larval spray, respectively (Fig. 6-4).

At 48hr after treatment, all larvae treated early in the second instar still remained in the second instar. Treatment significantly affected conidia present on larvae (Anova, P=0.006). There was no significant difference in numbers of conidia present on larvae between larvae treated by leaf spray and combination treatments (across both early and late second instar larvae) (Tukey HSD, P=>0.05). For these treatments conidia present on larvae ranged from 1000 to 1995 conidia larvae^{-1}. There was no significant difference in the number of conidia present on larvae between early and late second instar larvae treated using the larvae spray technique (Tukey HSD, P=>0.05). The number of conidia present was 158±25 and 39±50 conidia larvae^{-1} for early and late second instar larvae treated with larvae spray technique, respectively. Larvae treated using larval spray had significantly fewer conidia present on their cuticle, when compared to leaf dip and combination treatments (Fig. 6-4).

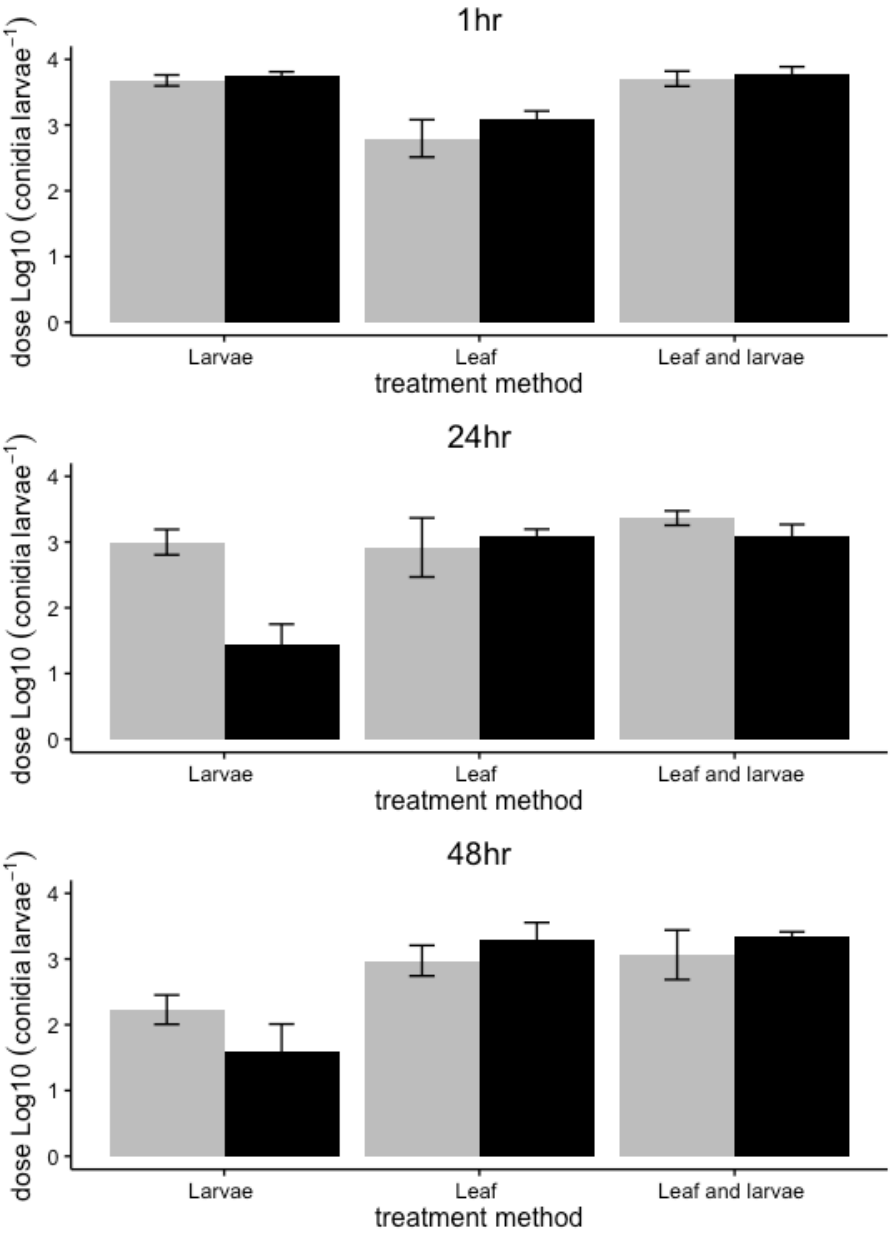


Figure 6-4: Conidia present on larvae (Log10[conidia larvae^{-1}] ±SE) after being treated with a fixed dose of *B. bassiana* using one of three application techniques: leaf spray, larval spray and a combination of leaf dip and larval spray. Grouped by time after treatment (1, 24 and 48hr). Grey and black bars represent early and late second instar larvae, respectively.

6.3.3 Quantifying mortality, conidia picked up per DBM larvae and concentration of conidia on leaf discs in response to *B. bassiana* 433.99 applied at a range of EPF concentrations using the leaf dip technique

From days three to eight, mortality of groups of DBM larvae was significantly affected by concentration (Anova, P=<0.001). By eight days after treatment, larval mortality after being treated by $1x10^8$ and $1x10^7$ conidia ml^{-1} was 89.4±7.5 and 58.4±8.5%. There was no significant difference between mortality at $1x10^8$ and $1x10^7$ conidia ml^{-1} , but mortality at $1x10^8$ conidia ml^{-1} was significantly higher than all other doses (Tukey HSD). Control mortality after eight days was zero. There was no significant difference between control mortality and mortality at $1x10^4$, $1x10^5$ and $1x10^6$ conidia ml^{-1}. After eight days, mortality was 17.1±13.1, 13.9±10.0, 23.5±11.6% for $1x10^4$, $1x10^5$ and $1x10^6$ conidia ml^{-1} treatments, respectively (Fig. 6-5).

Probit analysis was then completed to determine the concentration of EPF required to cause 50% mortality in groups of DBM larvae by six days after treatment using the maximum likelihood method. The best fitting model indicated that the LC50 was $5.5x10^7$ (95% confidence interval: $3.7x10^7$ – $9.1x10^7$ conidia ml^{-1}). By fitting linear a regression model to the relationship between concentration (conidia ml^{-1}), and dose received by larvae (conidia $larvae^{-1}$), it was determined that the LC50 was approximately 3,801 conidia $larvae^{-1}$.

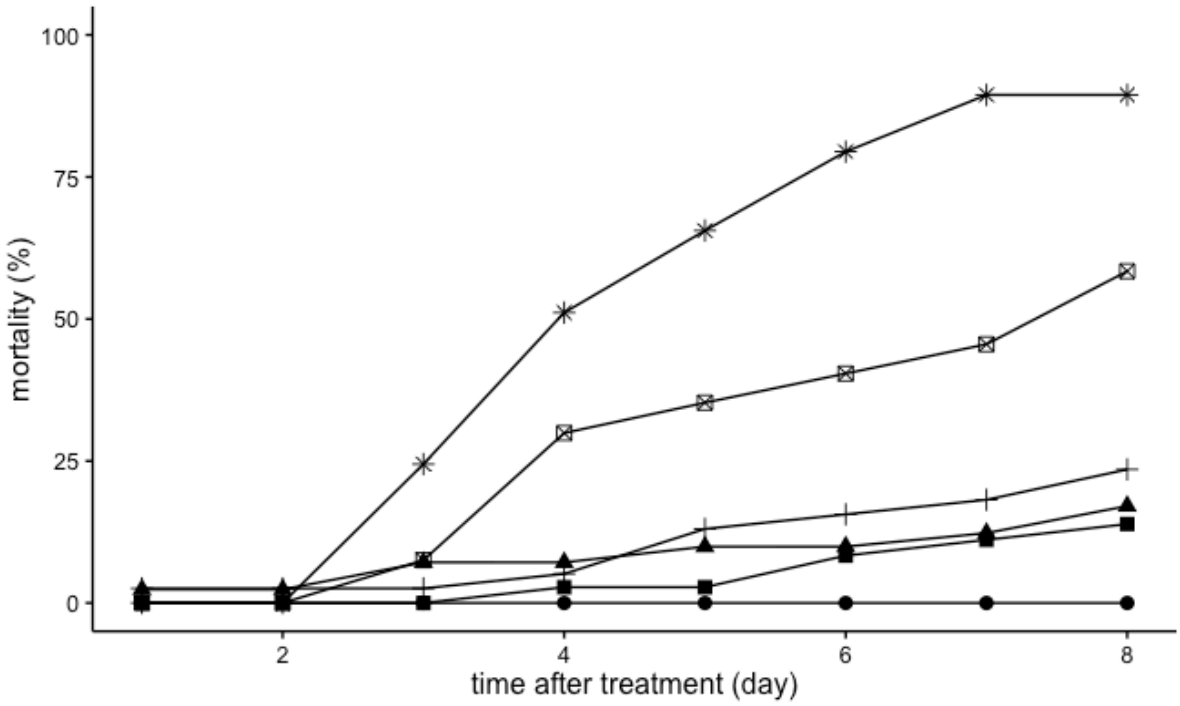


Figure 6-5: Mean mortality (%) of groups of DBM larvae over a seven day long experiment after being treated with one of five doses of *B. bassiana*, plus an untreated control. Concentrations of EPF conidia can be identified as follows: control (●), 10^4 (▲), 10^5 (■), 10^6 (+), 10^7 (☒) and 10^8 (✱)

In general conidia present on the leaf surface decreased over the course of the eight-day experiment (Fig. 6-6). Separate linear regression models were used to describe the relationship between time after treatment and concentration of conidia on the leaf surface, for each concentration of conidia applied to the leaf surface. For the control and $1x10^4$ conidia ml^{-1} treatments linear regression models were not significantly different from a zero relationship (linear regression, P=>0.05). For the control treatment there were 198±101 and 141±114 conidia cm^{-2} on leaves at 0 and 192hr after treatment, respectively. For the $1x10^4$ conidia ml^{-1} , $1.3x10^3$±441 and 967±850 conidia cm^{-2} were present on leaves at 0 and 192hr after treatment, respectively. For $1x10^5$, $1x10^6$ and $1x10^8$ conidia ml^{-1} treatments, there was a significant negative relationship between conidia present on leaves and time after treatment (linear regression, P=<0.05). For the $1x10^5$ conidia ml^{-1} , $2.4x10^3$±716 and 759±715 conidia cm^{-2} were present on leaves at 0 and 192hr after treatment, respectively. For the $1x10^6$ conidia ml^{-1} , $8.4x10^3$±$2.1x10^3$ and

$2.8x10^3 \pm 1.6x10^3$ conidia cm^{-2} were present on leaves at 0 and 192hr after treatment, respectively. For the $1x10^8$ conidia ml^{-1} , $6.9x10^5 \pm 2.1x10^5$ and $2.8x10^5 \pm 3.6x10^4$ conidia cm^{-2} were present on leaves at 0 and 192hr after treatment, respectively. For the $1x10^7$ conidia ml^{-1}, there was a negative correlation between conidia present on leaves and time after treatment, but this was not considered to be significantly different from a zero relationship (linear regression, P=0.13). For the $1x10^7$ conidia ml^{-1} treatment, $2.2x10^5 \pm 5.4x10^4$ and $8.8x10^4 \pm 3.4x10^4$ conidia cm^{-2} were present on leaves at 0 and 192hr after treatment, respectively.

At 192hr after treatment, conidia present on leaves was significantly affected by treatment (Anova, P=<0.001). There were a significantly higher number of conidia on leaves after being treated with $1x10^8$ conidia ml^{-1}, when compared to control, $1x10^4$, $1x10^5$ and $1x10^6$ conidia ml^{-1} treatments (Tukey's HSD, P=<0.05). There was no significant difference between conidia present on leaf discs between $1x10^7$ and $1x10^8$ conidia ml^{-1} treatments (Tukey's HSD, P=>0.05). Additionally, there was no significant difference in conidia present on leaves between control, $1x10^4$, $1x10^5$ and $1x10^6$ treatments (Tukey's HSD, P=>0.05).

The number of conidia present on larvae increased over the course of the eight day experiment (Fig. 6-6). Linear regression models were fitted to the relationship between time after treatment and conidia present on larvae, for each concentration of conidia applied to the leaf surface. A significant positive correlation was observed between conidia present on larvae and time after treatment for $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ conidia ml^{-1} treatments (linear regression, P=<0.05). Although there was an increase in conidia present on larvae over time for control and $1x10^8$ conidia ml^{-1} treatments, this relationship was not significantly different from zero (linear regression, P=>0.05). Between 3 and 192hr, conidia present on larvae increased from 38.3±18.3 to $1.0x10^3 \pm 820.3$ conidia $larvae^{-1}$ for the $1x10^4$ conidia ml^{-1} treatment. Between the same time points, an increase of 110.0±37.7 to $3.3x10^3 \pm 885.2$ and 245.0±93.1 to $8.2x10^3 x 4.7x10^3$ conidia $larvae^{-1}$ for $1x10^5$ and $1x10^6$ conidia ml^{-1} treatments, respectively. For $1x10^7$ and $1x10^8$ conidia ml^{-1} treatments, increases of $1.3x10^3 \pm 292.6$ to

$6.7x10^3 \pm 1.8x10^3$ and $1.0x10^4$ to $2.0x10^4 \pm 1.6x10^4$ conidia $larvae^{-1}$ between 3 and 192hr after treatment.

At 192hr after treatment, concentration of EPF conidia applied did not significantly affect conidia picked up by larvae (Anova, P=0.06). This was assumed to be because of the large degree of variation in numbers of conidia picked up by larvae.

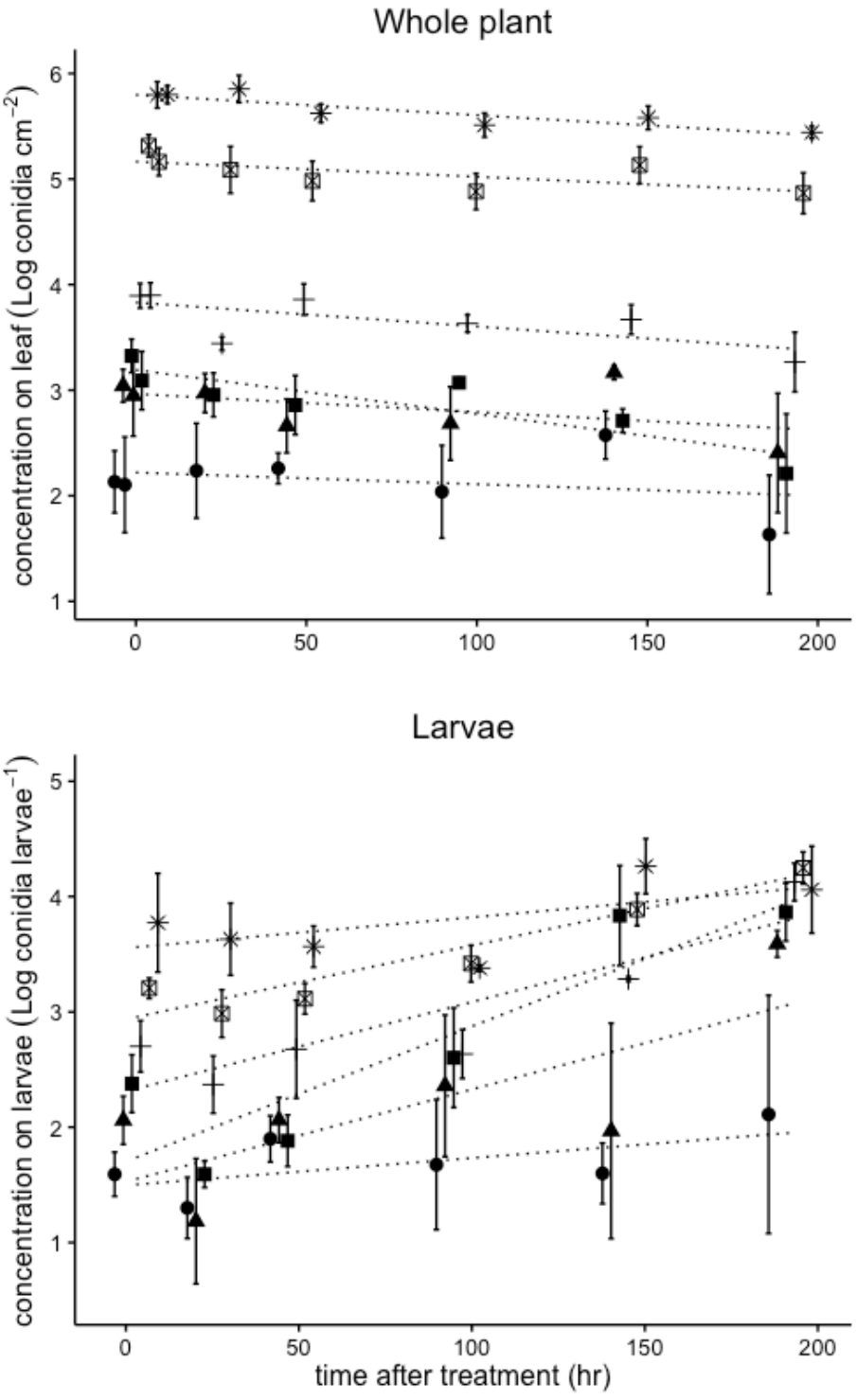


Figure 6-6: Concentration of conidia (Log10[conidia larvae^{-1}] ±SE for larvae and Log10[conidia cm^{-2} ±SE for whole plants) at various hrs after treatment along with linear regression models. Concentrations of EPF conidia can be identified as follows: control (•), 10^4 (▲), 10^5 (■), 10^6 (+), 10^7 (☒) and 10^8 (✱).

Table 6-1: Concentration received by leaf surface (conidia cm^{-2}) 0hr after treatment and larvae (conidia $larvae^{-1}$) 3hr after treatment after being treated with range of doses of *B. bassiana* (Log10[(conidia ml^{-1})]).

	concentration received	
Dose applied Log10[(conidia ml^{-1})]	**conidia cm^{-2}**	**conidia $larvae^{-1}$**
Control	198±101	38±18
4.0	$1.3x10^3$±441	110±38
5.0	$2.4x10^3$±716	245±93
6.0	$8.4x10^3$±$2.1x10^3$	515±250
7.0	$2.2x10^5$±$5.4x10^4$	$1.3x10^3$±293
8.0	$6.9x10^5$ ±$2.1x10^5$	$1.0x10^4$±$7.1x10^3$

6.3.4 Determining how time between leaf treatment and exposure of larvae to the treated leaf affects fungal virulence for a range of insect virulent fungi

Control mortality was abnormally high in the third rep of the 0hr interval treatment, being 54.5%. For replicates one and two, control mortality was zero and 8.3%. For all other treatments control mortality was consistently below 10%. Mortality six days after treatment was not significantly affected by time between treatment and exposure to larvae for any isolate (Anova, P=>0.05). However, significant differences in mortality were observed between isolates for 24, 48, 96 and 144hr interval treatments (Anova, P=<0.05). *B. bassiana* 1757.15 generally caused higher mortality than the control treatment and other isolates (Tukey's HSD test, P=<0.05) (Table 6-2) .

Linear models were also fitted to the relationship between mortality and time between application and exposure to larvae. For each treatment (all isolates plus the untreated control) the linear regression was not significantly different from a zero relationship (linear regression model, P=>0.05), indicating that time interval between application and exposure does not have an impact on mortality caused against groups of DBM larvae (Fig. 6-7).

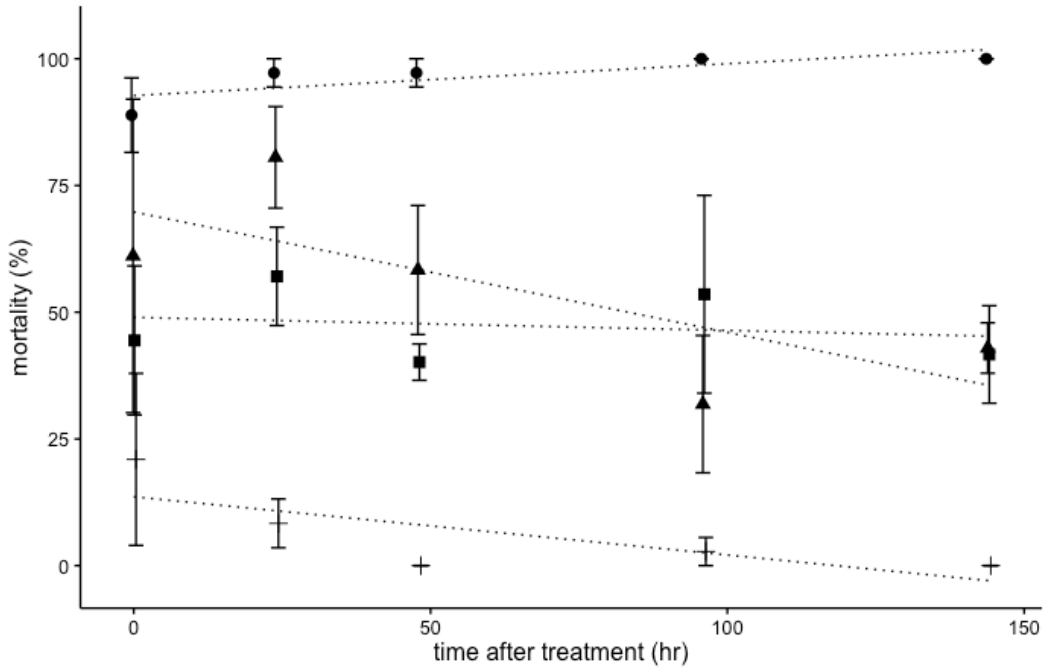


Figure 6-7: Mortality (%±SE) six days after treatment after being treated with a fixed dose of one of three EPF isolates, plus an untreated control plotted against time between treatment of leaf surface and exposure to larvae (hr). Linear regression models are also included for each treatment. Treatments can be identified as follows: control (+), *B. bassiana* 1757.15 (●), *B. bassiana* 433.99 (■) and *M. brunneum* 1760.15 (▲).

Table 6-2: Mortality (%±SE) six days after treatment of groups of DBM larvae after being treated with one of three isolates plus an untreated control which were left for varying amounts time (hr) between leaf treatment and exposure to larvae. Significant differences between mortality are also displayed, different lower case letters with in the same column indicate significant differences are present between different time intervals, whereas different uppercase letters in the same row indicate significant differences between isolates (Anova, Tukey's HSD).

	WCC isolate number					
Time interval (hr)	**433.99**	**1757.15**	**1760.15**	**C**	**F-value**	**P-value**
0	44.4±14.7 a A	88.9±7.3 a A	61.1±30.9 a A	21.0±17.0 a A	2.2	0.2
24	57.1±9.7 a C	97.2±2.8 a B	80.6±10.0 a BC	8.3±4.8 a A	26.5	<0.001
48	40.2±3.6 a DE	97.2±2.8 a C	58.3±12.7 a BE	0.0±0.0 a A	35.7	<0.001
96	53.5±19.5 a AC	100.0±0.0 a BC	31.9±13.5 a A	2.8±2.8 a A	11.7	0.003
144	41.7±9.6 a DE	100.0±0.0 a C	42.9±5.0 a BE	0.0±0.0 a A	57.5	<0.001
F-value	0.4	1.5	1.2	1.2		
P-value	0.8	0.3	0.4	0.4		

6.4 Discussion

It has been suggested that time between exposure to spores and moult can have a clear effect on susceptibility of DBM larvae to EPF. Vandenberg et. al. (1998) found that larvae taken from late in the second and third instar survived for a significantly longer period of time after inoculation when compared to larvae taken from early in these instars. The same authors also determined that spores were readily shed along with the moulted cuticle as larvae transitioned from one instar to the next, this was identified as the means by which larvae taken from late in an instar avoid infection. The length of the inter-moult period has also identified to be related to susceptibility to infection in other insects (Vey and Fargues, 1977). In the current investigation, a similar conclusion was drawn. It was found that larvae taken from early from second, third and fourth instars had significantly higher mortality after six days when compared to larvae taken from late in these instars. A significant relationship was also found between proportion mortality and time between treatment and moult; larvae displayed higher mortality if treated earlier in the instar.

Next, it was assessed whether secondary pick-up could mitigate moulting as an avoidance mechanism in larvae. The results were found to be in agreement with those described by Vandenberg et al. (1998). After being treated using the larval spray technique, there were significantly fewer conidia present on larvae after moult when compared to larvae before moult indicating that conidia are readily shed during moult. This could be further confirmed by quantifying or visualising conidia present on the moulted cuticle, however, due to the cuticle's delicate nature, conidia should be observed by using either confocal microscopy or scanning electron microscopy, rather than estimation of colony forming units as was used in this investigation. As expected, larvae treated early in the instar displayed higher mortality when compared to larvae treated late in the instar after the larval spray technique was used.

If treated late in the instar, more EPF conidia were present on the larval cuticle if treated with leaf spray or combination treatments, when compared to the larval spray treatment. Presumably because conidia were readily picked up by larvae from the leaf

disc after moult had occurred. It was expected that an increase in conidia present on larvae would result in higher mortality in larvae taken from late in the instar. However, this was not observed to be consistently the case. Larvae taken from late in the instar, and treated using the leaf spray technique, displayed significantly higher mortality when compared to larvae of the same age which were treated using the larval spray technique. Mortality of larvae taken from late in the instar treated using the combination treatment was the same as larvae of the same age treated using the larval spray technique. As there was no clear increase in mortality through leaf spray technique, it may be that secondary pick-up is less effective when compared to larval spray, in terms of causing a systemic infection.

To further investigate secondary pick-up as a route to infection, a leaf spray concentration-mortality response was completed using *B. bassiana* 433.99. There are many examples in the literature of bioassays against DBM being completed using the leaf spray technique (Batta, 2013, Cui et al., 2014, Huang et al., 2010). A clear concentration-mortality response was found after completing the experiment, but when compared to the larval spray application for the same isolate, the LC50 was far higher ($5.5x10^6$ and $3.6 x10^7$ conidia ml^{-1} for larval spray and leaf spray, respectively). The LC50 for the leaf spray and larval spray experiments in terms of conidia present on larval was determined to be approximately 75 and 3,801 conidia $larvae^{-1}$, respectively. Indicating that the incidental pick-up of conidia is less effective in causing infection when compared to conidia being sprayed directly on the cuticle surface. A possible reason for this may be that conidia are able to penetrate the cuticle only if they are applied to the insect cuticle directly after moult. Conidia picked up by feeding larvae after a certain time after moult would not be destined to penetrate the cuticle, and may be reliant on causing infection through other means (for example, penetration through the insect mouth parts); these conidia would have less of an effect on virulence. Consequently, you would expect to see far higher numbers of conidia required to cause mortality through pick up, when compared to a larva which was sprayed directly after moult.

In general, when compared to previous research that employed the leaf spray technique, higher concentrations of conidia were required to cause significant insect

mortality in the current study. Huang et al. (2010) reported an LC50 of $1.1x10^5$ conidia ml^{-1} for an *I. fumosorosea* isolate five days after treatment of second instar DBM larvae. Additionally, Batta et al. (2013) reported DBM mortality of 86.7% in response treatment by a *M. anisopliae* strain at $5x10^7$ conidia ml^{-1}. In comparison, a concentration of $1x10^7$ conidia ml^{-1} in the current study caused approximately 50% mortality. However, it should be noted that the studies used for comparison here used different isolates, and varying methodologies.

In the current study, a decrease in the concentration of conidia present on leaves was observed after the time of treatment. In a similar study by Inglis et al. (1993), *B. bassiana* conidia were applied to crested wheatgrass and alfalfa at a concentration of $5x10^8$ conidia ml^{-1}. Immediately after application, conidia were present on leaves at a concentration of between 5.1×10^3 and 1.2×10^5 conidia cm^{-2}, the number of viable conidia was reduced by 75% by four days after treatment (Inglis et al., 1993). Kouassi et al. (2003) reported that after application of a *B. bassiana* suspension to lettuce and celery leaves, the concentration of conidia present on leaves was reduced by approximately 60-95% depending on the variety of plant used (Kouassi et al., 2003). In comparison, in the current study, conidia were present at a concentration of $6.9x10^5 \pm 2.1x10^5$ on leaves after being applied at $1x10^8$ conidia ml^{-1}, by four days after treatment conidial populations were reduced by 50%. The lower conidial survival in the Inglis et al. (1993) and Kouassi et al. (2003) studies was likely an artefact of the experiments being completed in field conditions. Precipitation, UV exposure and wind are all known to negatively the effect the persistence of EPF conidia on leaves (Goettel et al., 2001). In the current study, it is likely that a significant proportion of the drop off in conidia viability can be accounted for by conidia dropping off the leaf surface soon after spray, and conidia germinating with in the first 48h after spray.

In the field, EPF biopesticides would be sprayed at intervals of up to a week, so it is important to understand how virulence is affected by the length of time conidia are left standing on a leaf before coming in to contact with larvae. The most suitable EPF isolates would be those that maintain their virulence for long periods time on leaf discs. Here, we found a slight, but not statistically significant, drop off in virulence of EPF conidia

after they were left standing of leaf discs before introduction of larvae for increasing periods of time. After applying *B. bassiana* conidia at a fixed dose to celery and lettuce plants, Kouassi et al. (2003) exposed treated leaves that had been left to stand for periods of one to 26 days to groups of tarnished plant bugs (*Lygus lineolaris*); they found mortality varied from 76-92 and 66-75% for zero day old and 26 day old treated leaves, respectively. Similar to the current study, this indicates that *B. bassiana* conidia are able to maintain their virulence whilst on the leaf surface. A similar study was completed by Brobyn et al (1985), it was found that *Erynia neophidis* conidia were able to cause mortality against aphids if left on leaves for up to 14 days. In this study, virulence of *E. neophidis* conidia decreased linearly over the course of the experiment (Brobyn et al., 1985). Batta (2013) investigated the effect on EPF virulence of time between application of *M. anisopliae* conidia to leaves, and exposure of leaves to DBM larvae. It was found that if larvae were introduced to leaves treated with a *M. anisopliae* isolate (at 5 $x10^7$ conidia ml^{-1}) directly after treatment, 86.7% mortality was observed. Whereas if larvae were introduced one week after leaf treatment, mortality dropped to 45%. This represented a more significant loss of virulence, when compared to the current study. It should be noted that a higher dose of conidia was used in the current study when compared to Batta (2013), which may have masked a drop off in virulence over time. In general, studies into the persistence of EPF conidia on leaves show that conidia can maintain virulence for at least 10-14 days. When compared to most other studies, a higher level of virulence was maintained in the current experiment after EPF conidia were left standing on leaves. This may be due to the current experiment being completed under controlled conditions, whereas other experiments described here were completed under field conditions, which are known to reduce the persistence of EPF in the environment.

Here, we confirmed that the length of the inter-moult period has a clear effect on the susceptibility of larvae to EPF conidia. Larvae treated later in the instar are more likely shed conidia along with the moulted skin and avoid infection. However, it is not clear whether secondary pick-up from a treated leaf disc mitigates shedding as mechanism for larvae to avoid infection. This is important in the context of biocontrol because in the field, a mixed population of larvae would be present. After treatment, some of these

larvae would avoid infection through moulting. When taking into account the data from current study, it is not clear whether these larvae would become infected through secondary pick-up.

However, there are other strategies that could be used to mitigate the effect of moulting on fungal efficacy. Insect growth regulators (IGRs) could be used to reduce the development rate of the insect, which would allow fungal conidia a longer time to penetrate the cuticle. Certain classes of IGR have been shown to have this effect by inhibiting the synthesis of chitin, an important structural component of the insect's exoskeleton (Hirose, 2010). Juvenile hormone insecticides have also been shown to disrupt insect moulting by interfering with the hormone signalling pathways associated with ecdysis (Wyatt, 1996). Either of these two insecticides could be used to optimise the use of EPF in the field.

The use of staggered fungal sprays could also be used to increase the chances of insect mortality occurring. Using the literature, and experiments completed in this study, the length of the intermoult period could be estimated based on the average temperature of a particular area. If, for example, the intermoult period is estimated to be 3.5 days, a spray every three days would maximise the number of larvae who get treated early in the instar and, therefore, increase the insect mortality caused.

It was also found that if mortality of groups of larvae is to occur through secondary pick-up, far higher numbers of conidia are required when compared to the number of conidia required to cause mortality through direct spray. Additionally, we also found that the virulence of a subset of isolates is not affected by time left standing on the leaf surface (up to six days) when treated at relatively high doses. This has implications in biocontrol, because it is likely that conidia will rest on leaf surface before being picked up by larvae.

6.4.1 Summary

i. Time between treatment of a *B. bassiana* EPF and insect moult was found to have a significant effect on the susceptibility of DBM larvae to infection.
ii. In line with previous literature, larvae which were treated late in the instar were found to shed EPF conidia in the moult to the next instar, and in doing so reduced the likelihood of a systemic infection occurring.
iii. Secondary pick-up on EPF conidia were found to cause significant larval mortality at high concentrations.
iv. EPF conidia were able to maintain virulence whilst standing on the leaf surface for up to six days.

7 Discussion

7.1 Conclusions

Synthetic insecticide-only strategies have consistently failed to provide control for Brassica growers against DBM infestations. In fact, the injudicious use of broad spectrum synthetic insecticides has promoted the development of resistance in DBM populations (Talekar and Shelton, 1993). There are now populations of DBM which are resistant to every class of synthetic insecticide (Furlong et al., 2013). Additionally, synthetic insecticide only applications have caused a reduction in natural enemies of DBM, which has further contributed to the prevalence of DBM as a pest (Furlong et al., 2013). It is now the opinion of most experts that IPM is the most viable alternative strategy to control DBM (Grzywacz et al., 2010, Lim, 1992, Furlong et al., 2013, Sarfraz et al., 2005). EPF have considerable potential to be used with in an IPM system (Chandler, 2017). Several EPF biopesticides are available on the market which are effective against Lepidopteran pests and hundreds more EPF isolates have displayed virulence against insect pests in the laboratory (Chi, 2007). Additionally, EPF biopesticides can be brought to market far more rapidly and cheaply when compared to synthetic insecticides (Marrone, 2011). However, there has been little uptake of EPF biopesticides to control DBM by Brassica growers. This is mainly due to the variability of performance of EPF biopesticides from one application to the next (Lacey et al., 2001). Understanding and addressing the sources of variability represents the main challenge to be overcome before EPF biopesticides can be regularly used by growers to control DBM. By examining the literature, several areas were chosen for study in the current project – which have been understudied in the past – that contribute to variability of EPF performance in the field. First, a cohort of EPF isolates were screened against DBM so that a sub-set of isolates could be selected for use in the remainder of the project. Then, the effect of fluctuating temperatures on EPF virulence was investigated. The effect of co-application of EPF biopesticides with synthetic insecticides on the survival of DBM larvae was then determined. Finally, the influence of the age of DBM on their susceptibility to EPF biopesticides was examined. There are considerable knowledge gaps in the literature, concerning the areas studied here. It is hoped that addressing these sources of EPF

biopesticide variability will expedite the use of EPF biopesticides, with in an IPM system, to control DBM.

It was important to develop a reliable and robust bioassay protocol to assess the virulence of EPF isolates against DBM larvae. When developing the bioassay procedure, several problems were encountered, the most important of which was variability of susceptibility of groups of DBM larvae from one bioassay to the next. Through examining the literature, and our own observations, it was determined that larvae had to be treated with EPF directly after moult to allow conidia enough time to penetrate the cuticle and cause a systemic infection (Vandenberg et al., 1998b). Consequently, only early second instar larvae were used in experiments. Two bioassay procedures were assessed for suitability: a leaf spray method in which DBM larvae were exposed treated leaf material, and a larval spray method in which DBM larvae were sprayed directly with EPF conidia. The latter method was chosen because it was easier to determine the dose of EPF conidia received by the larvae. In addition, the larval spray method caused lower control mortality.

The larval spray method was used to assess the virulence of 14 candidate isolates against groups of DBM larvae. The cohort of 14 isolates included commercially available EPF biopesticide products, EPF isolates known to be highly virulent against DBM and novel EPF isolates. The purpose of the screen of 14 isolates was to select a subset of EPF isolates to use during the remainder of the project. Overall proportion mortality (corrected for control mortality) and LT50s were used to assess the virulence of each isolate. *B. bassiana* 433.99, *B. bassiana* 1757.15, *M. brunneum* 275.86, *M. brunneum* 445.99 and *M. brunneum* 1760.15 were selected to undergo further experimentation. *B. bassiana* 1757.15 out performed other isolates causing over 95% mortality by six days after treatment. When completing experiments it is important to use commercial biopesticide EPF products so that results can more applicable to the field. *B. bassiana* 433.99, *M. brunneum* 275.86 and *M. brunneum* 445.99 are active ingredients in the EPF biopesiticde commercial products BotaniGard, Met-52 and Bio-Blast, respectively. These three isolates caused intermediate mortality (between 40 and 60% by six days after treatment). *M. brunneum* 1760.15 was included because it caused significant

mortality against DBM larvae, but interestingly, displayed a relatively low germination percentage of approximately 40%. We hypothesised that *M. brunneum* 1760.15 may be more virulent against DBM at temperatures more suited to its physiology. After completing concentration-mortality response experiments for the sub-set of isolates, it was found that *B. bassiana* 1757.15 required the lowest number of conidia per insect to cause 50% mortality in groups of DBM larvae, which was in agreement with a study completed by Wraight et al. (2010).

The effect of temperature on virulence can be described as a GxGxE interaction (Thomas and Blanford, 2003). Temperature affects the growth and germination of EPF isolates, and immune response of DBM. Because it is such a complex interaction it is often hard to predict EPF virulence at different temperatures. It has been suggested that temperature is the most important cause of EPF isolate variability in the field (Thomas and Blanford, 2003). Understanding the interaction of temperature and a physiological response poses several challenges. Most importantly, such an interaction generally has a left skewed distribution which means models based on a normal distribution tend to underestimate thermal optima (Golizadeh et al., 2007, Smits et al., 2003, Davidson et al., 2003). Several non-linear models have been proposed to counter this problem. This include the Briere-1 model, Lactin-1 and Logan-6 models, which are all designed to describe a slow increase in response as temperature rises, followed by a rapid decline (Briere et al., 1999, Lactin et al., 1995, Logan et al., 1976). Polynomial models and Taylor models are less well suited to describing such distributions, but were included in the analysis because they have been used in the literature to describe the effect of temperature on DBM development (Golizadeh et al., 2007, Marchioro and Foerster, 2011). The five non-linear models were assessed for describing data collected on the following interactions:

i. The effect of temperature on EPF physiology (colony extension and proportion germination of EPF conidia)
ii. The effect of temperature on DBM development
iii. The effect of temperature on virulence of EPF isolates against DBM

Across all three interactions the Briere-1 model was determined to be the most appropriate model as it maintained low AIC values and high r^2 values. Additionally, the Briere-1 model contains parameters that are biologically relevant. For example: *T0* (the minimum temperature at which a response occurs), *Topt* (the optimum temperature of the response) and *Tmax* (the maximum temperature at which a response occurs). *T0* was not could not be estimated Lactin-1 and Logan-6 models because the curve does not cross the horizontal axis. *Tmax* could not be estimated by the Taylor model and the polynomial model contained little biological relevance.

Once the Briere-1 model had been selected to describe all three interactions, cardinal temperatures (taken from the Briere-1 model) of EPF physiology, DBM development and EPF virulence could be appropriately compared.

It is often assumed that the thermal profile of an EPF isolate's physiology *in vitro* is an indication of the EPF isolate virulence profile (Davidson et al., 2003). For example, if an EPF isolate has a high temperature colony extension optimum, it may be assumed that the same isolate would have a high temperature virulence optimum. To assess the extent to which this assumption is true, linear regression models were fitted to the following relationships between thermal optima (taken from the Briere-1 model):

i. Germination *Topt* against colony extension *Top*t
ii. Germination *Topt* against EPF virulence *Topt*
iii. Colony extension *Topt* against EPF virulence *Topt*

The following relationships between thermal minima were also assessed using linear regression models (taken from the Briere-1 model):

i. Germination *T0* against colony extension *T0*
ii. Germination *T0* against EPF virulence *T*0
iii. Colony extension *T0* against EPF virulence *T0*

None of the linear regressions fitted to these relationships were significantly different from a zero relationship. This suggests that the thermal profile of EPF virulence can not be predicted from the thermal virulence profile of EPF physiology. This is may be due to the complexity of the GxGxE interaction (Thomas and Blanford, 2003).

However, it was also noted that statistical tests associated with the aforementioned linear regressions lacked statistical power, making it unlikely that significant relationship would be found. Because of this, a separate analysis was completed in which germination, colony extension and mortality data for separate temperatures were plotted against each other. It was found that, over the temperature range tested, the three variables could be used to predict each other.

It is common practice the assess the physiology of EPF *in vitro*, before selecting EPF isolates that are suited to the temperature range of a particular insect pest (Davidson et al., 2003). This analysis has shown, although it may not be able to predict cardinal temperatures in this way, determining the thermal profiles of EPF physiology can still provide useful information. For example, in the current experiment, *M. brunneum* 445.99 and 1760.15 displayed relatively high germination optima, colony extension optima and virulence optima. Similar to the findings in this study, Thomas and Blanford (2003) suggest that determining the thermal profiles of EPF physiology provides useful information, but to fully understand the virulence profile of an isolate, virulence must be directly assessed over the entire thermal range of the insect pest and the EPF isolate.

A common problem concerning the development of EPF biopesticide products is that seemingly promising EPF isolates do not perform when they are taken from the laboratory to the field (Thomas and Blanford, 2003). A major reason for this is that screens of EPF isolates are often completed at constant temperatures, normally 20 or 25^{o}C. To better understand how EPF isolates will perform in the field it is vital to compare the thermal profiles of both the EPF isolate and DBM development. The use of one model to describe the effect of temperature of DBM development and EPF virulence allows this comparison to be made. For an EPF biopesticide to be a success it should share the same thermal profile as the pest it is designed to control, for two reasons:

i. The insect pest is likely to cause the most crop damage at its thermal optimum, if the EPF biopesticide shares this thermal optimum it would be effective at temperatures at which DBM is most prevalent
ii. If the insect pest and EPF biopesticide share the same thermal profile it is easier to predict the virulence of EPF isolates at fluctuating temperatures

To illustrate this point, the thermal virulence profiles of *B. bassiana* 1757.15 and *M. brunneum* 1760.15 were compared with the thermal profile of DBM development. It was found that *M. brunneum* 1760.15 better matched the thermal profile of DBM development when compared to *B.bassiana* 1757.15. Consequently, *M. brunneum* 1760.15 would be better candidate to be a EPF biopesticide to control DBM. Despite this, in the initial screen of candidate EPF isolates against DBM larvae, *B.bassiana* 1757.15 far outperformed *M. brunneum* 1760.15. This further reinforces the point that thermal profiles of both pest and pathogen must be fully understood before EPF isolates are taken from the laboratory to the field.

Growers lack confidence in EPF biopesticides because of the variability in efficacy often seen from one application to the next in field (Lacey et al., 2001). To address this, we used cardinal temperatures from the Briere-1 model to develop a DD model designed to predict proportion mortality of DBM larvae after being treated with EPF isolates. DD models have long been used to predict the development of insect pests in the field; for example, the time to emergence of the cabbage aphid on canola can be accurately predicted using this method(Nematollahi et al., 2016). DD models can also be used to predict the infectivity of plant pathogens; for example, a model was developed to inform grape growers when to apply to fungicide to powdery mildew infestations, the concentration of airborne inoculum was estimated using the accumulation of DDs (Carisse et al., 2009). In a DD model, when the temperature is within a predefined range, DD accumulate which are associated with a physiological process (Bryant et al., 1998).

To confirm this principle, we developed a DD model using data from the experiment in which the effect of temperature on DBM development was determined. The lower

thermal threshold, and the number of DD required for insects develop from egg to adult was determined. The DD model was validated using field data in which a clear first peak in DBM adult numbers (representing the initial migration of DBM to the UK) was followed by a clear second peak (representing the emergence of the second generation of adult DBM). The DD model developed was able to predict the second peak of DBM adults with a degree of accuracy similar to that seem in literature previously (Baker et al., 1982) .

Because temperature is such an important determinant of EPF growth and germination and DBM development rate(Hallman and Denlinger, 1998, Davidson et al., 2003), we proposed the accumulation of heat units over time (DDs) could provide useful information for prediction of EPF virulence over fluctuating temperatures. Using a similar procedure to develop a DD model for insect development, a DD model was developed to predict the virulence of *B. bassiana* 433.99 and 1757.15 and *M. brunneum* 1760.15 and 445.99 against groups of DBM larvae at fluctuating temperatures. Mortality data from the experiments in which survival of groups of DBM larvae were monitored after being treated with EPF isolates at a range of constant temperatures were used to develop the DD model. Four separate DD models were developed, on for each EPF isolate. The temperature range over which DD were accumulated were taken as *T0* and *Topt* estimated from the Briere-1 model. A DD calculator was then developed which could predict proportion mortality of DBM larvae conferred by each isolate at any given number of accumulated DDs, from one to six days after treatment.

Predictions of proportion mortality were made (from one to six days after treatment) for three fluctuating temperature regimes, which followed a sinusoidal pattern as would be seen in the field. The three regimes fluctuated between 10 and 16, 19 and 25 and 29 and 35^{o}C. Predictions of proportion mortality were only made if the fluctuating temperature regime lay below the thermal optima of the EPF isolate. After the predictions had been made, experiments were completed in which groups of DBM larvae were treated with each EPF isolate and incubated at the fluctuating temperature regimes. Experimental values of proportion mortality were then compared with values

estimated using the DD models. Predictions for the low and intermediate temperature regimes were generally accurate, with the majority of predictions falling with in the standard error bars of the experimental data points. Predictions were less accurate for the highest temperature regime, with predictions of generally lying outside the standard error bars of the experimental data points. The less accurate predictions in the highest temperature regimes may be because the entire thermal range of this regime lies above the thermal optimum for DBM development (28^{o}C). DBM larvae may be more susceptible EPF infection at temperatures over the optimum as thermal stress reduces the effectiveness of the immune response, which could result in a faster speed of kill and higher overall proportion mortality (Neven, 2000).

There has been debate in the literature on whether prediction of physiological response at fluctuating temperature can be made using data collected at constant temperatures (Bale, 1999). Here we have demonstrated that a DD model approach can be used to accurately predict virulence of EPF isolate at fluctuating temperatures, provided the temperature regime lies with in the lower and upper (*T0* and *Topt*, respectively) range of both DBM development and EPF virulence. The conclusions drawn here are similar to those drawn by Burgess and Griffin (1967) who found that fungal growth rate at fluctuating temperatures could be predicted from fungal growth rates at constant temperatures. To make these predictions the authors utilised the assumption that fungal growth at anyone temperature is unaffected by the fungi's previous temperature experience. In effect, this means there is no lag time in fungal growth when the temperature environment changes (provided the change in temperature is relatively small). This helps explain why we were able to develop an accurate predictive model of EPF virulence at fluctuating temperatures.

It is likely that EPF biopesticides would be applied with in an IPM strategy that also includes synthetic insecticides. These types of IPM systems generally work as a threshold based system: the EPF biopesticide would be applied regularly throughout the growing season, when the pest density exceeds a certain level, synthetic insecticides would be applied with the EPF biopesticide to reduce the pest density to below the threshold (Lim, 1992). At this point the EPF biopesticide would applied on its own again. This type of

system may be particularly suited to the UK because there are typically only two generations of DBM per year, which means only two synthetic insecticide applications may be necessary.

There has been little uptake of IPM systems which include EPF biopesticides by growers to control DBM populations. This is partially due to the lack of understanding of the how EPF interacts with other elements of the IPM system. Although there is generally good knowledge of individual elements of the IPM system, a more holistic approach should be applied to research (Lim, 1992). Here, we assessed the effect of co-application of EPF biopesticides with commercially available synthetic insecticides on the survival of groups of DBM larvae. The response from co-application was characterised being synergistic, antaogistic or additive. Synergistic interactions are valuable for IPM, they allow a reduced volume of synthetic insecticide to be applied, which can result in a financial and environmental saving (Butt and Ansari, 2011).

A cohort of 16 commercially available synthetic insecticides were chosen for experimentation. First, the following experiments were completed using the cohort of insecticides:

i. Determining the survival of groups of DBM larvae after being treated with candidate insecticides
ii. Determining the effect of candidate insecticides on proportion germination and colony extension rate of *B. bassiana* 433.99

Three insecticides were then chosen to be included in co-application experiments with *B. bassiana* 433.99. The criteria for selection were:

i. High toxicity to DBM larvae
ii. Little effect on the colony extension rate and germination of EPF isolates
iii. Evidence in the literature to suggest the insecticide is compatible with EPF biopesticides when controlling DBM populations

It was important that the chosen insecticides were active against DBM (criteria i) and non-fungicidal so the process of EPF infection was not distrupted (ii). Based on these criteria, Dipel DF (a Bt based insecticide), Calypso (a thiacloprid based insecticide) and Neemazal (an azadirachtin based insecticide) were selected. The insecticides chosen had three separate modes of action: Bt produces endotoxins which disrupt the insect gut and cause insect death, azadirachtin effects the development, moulting and feeding habits of insects and thiacloprid causes death by over stimulating the insect nervous system (Gill et al., 1992, Matsuda et al., 2001, Mordue and Nisbet, 2000). Bt and azadirachtin based insecticides require ingestion to be effective, whereas thiacloprid based insecticides do not. It was found that there was less variation of the DBM larval population to Calypso, when compared to Dipel DF and Neemazal. It was presumed this was because of the neonicotinoid's more direct mode of action.

Low concentrations of Dipel DF, Calypso and Neemazal were applied with and without a concentration range of *B. bassiana* 433.99 to groups of DBM larvae. The most common response observed after co-application was additive, although some synergy was observed at high concentrations of EPF and synthetic insecticide. Of the three insecticides, Calypso displayed the most evidence of synergism. This was presumed to be due to its more direct mode of action when compared to Dipel DF and Neemazal. Results here indicate that each of the insecticides is compatible with *B. bassiana* 433.99 when co-applied. Additionally, if applied at the correct concentration, synergy may occur between the two control products which may result significant environmental and financial savings (Butt and Ansari, 2011).

It should be noted that if synthetic insecticides are applied at a low concentration – for example, in a cocktail containing other synthetic insecticides – there may be an increased risk of the development of DBM resistance (Grzywacz et al., 2010). However, in this case, the two separate modes of action of the synthetic insecticide and the EPF biopesticide may help to prevent the development of a resistant DBM population as individuals are less likely to evolve resistance mechanisms to both modes of action (Phillips et al., 1989). Additionally, if other IPM system elements are incorporated – for

example, parasitoids of DBM – the development of resistance to synthetic insecticides would be further forestalled (Kfir, 2005).

It was also found that each of the synthetic insecticides effected the development rate of DBM larvae when applied at sub-lethal concentrations. In general, sublethal insecticide concentrations were able to increase time taken for larvae to develop from second to third instar by approximately one day when compared to an untreated control. This was proposed as a potential mechanism for the synergism observed as the increased time taken to reach the third instar would allow the EPF conidia more time to penetrate the insect cuticle and cause a systemic infection. Additionally, development rate was clearly dependant on insecticide concentration which may explain why no synergy was observed when a low concentration was co-applied with *B. bassiana* 433.99.

The results here were generally in agreement with the literature on EPF-insecticide interactions. Co-application of Bt, azadirachtin and neonicotinoid based insecticides with EPF has been shown to increase speed of and overall proportion mortalities of various insect pests (Nian et al., 2015, Gomes et al., 2015, Ye et al., 2005). Additionally, Nian et al. (2015) suggested that synergy between EPF and low concentration insecticides may be due to larval development being arrested by the insecticide. After further investigation – in which the effect of insecticides on the rate of insect development was determined – we found evidence to support this hypothesis.

Screens of candidate isolates against DBM are most commonly completed against fixed age larval populations, in doing so, the effect of the age structure of the insect populations of susceptibility to EPF infection is disregarded. It has been reported that time between EPF conidia treatment and ecdysis has a significant effect on susceptibility of DBM larvae to infection (Vandenberg et al., 1998b). A similar conclusion was drawn in the current research; larvae taken from early their respective instar were far more susceptible to EPF conidia when compared to larvae treated late in the instar. The number of conidia present on the insect cuticle before and after larval were monitored. Results indicated that larvae shed conidia along with the cuticle, as they moulted from

one instar to the next. This mechanism of avoiding EPF infection in larval insect has been suggested in the past in Colorado potato beetle larvae and DBM (Vey and Fargues, 1977, Vandenberg et al., 1998a). Consequently, if DBM larvae are treated with a direct spray, it is likely the age structure of the DBM larval population would have significant effect on susceptibility to EPF infection.

However, in a foliar application, EPF conidia would be applied to the leaf surface as well directly to the insect cuticle. DBM larvae may then acquire further EPF conidia through secondary pick-up. Research on has shown that secondary pick-up can be an important route to infection for insect pests (Roditakis et al., 2000, Batta, 2013, Cui et al., 2014, Huang et al., 2010). We found that a far higher number of conidia were required to cause 50% mortality in groups of DBM larvae for leaf spray (relying on secondary pickup for inoculation of insects with EPF) when compared to direct spray. Despite this, at a relatively high concentration ($1x10^8$ conidia ml^{-1}) of *B. bassiana* 433.99 conidia, consistently high mortality of groups of DBM larvae was achieved.

In the field, it is likely that EPF biopesticides would be applied at intervals of up to a week. Ideally, an EPF would continue to cause infection during this period. The persistence of three isolates was assessed when applied at a relatively high dose ($1x10^8$ conidia ml^{-1}). In general, a slight decline in virulence was observed as EPF isolates were left to stand on leaves before being exposed to larvae for periods of one to six days. However, this decline was not considered significant different from a zero relationship. The results indicate that when EPF biopesticides are applied at a relatively high concentration, they maintain virulence whilst standing on the leaf surface. The results found here were in general agreement with those found in the literature. It is reported that EPF conidia can maintain their virulence fo at least 10 days (and in some cases, up to 26 days) in the field against a range of insect pests (Brobyn et al., 1985, Batta, 2013, Kouassi et al., 2003).

7.2 Summary of conclusions

7.2.1 Initial assessment of EPF virulence against DBM larvae

i) The following three bioassay procedures were assessed:

- Direct larval spray
- Leaf spray
- Larval immersion

ii) Direct larval spray was used to assess the virulence of 14 candidate EPF isolates against DBM:

- EPF isolates with virulence ranging from low to high were found
- *B. bassiana* 433.99 and 1757.15 and *M. brunneum* 275.86, 445.99 and 1760.15 were chosen to be included in further experimentation

7.2.2 Development of DD model to predict isolate virulence

i) The effect of temperature on the following was determined experimentally:

- EPF physiology (colony extension and germination)
- DBM development
- EPF virulence against DBM larvae

ii) After assessing the suitability of five non-linear models in describing the interactions from (i), the Briere-1 model was proposed as being the most appropriate.

iii) Cardinal temperatures from the Briere-1 model were used to:

- Match the thermal profiles of EPF virulence and DBM development to suggest the EPF isolates most suited to be used in a DBM biocontrol system
- Asses the relationship between cardinal temperatures of colony extension, germination and virulence of EPF isolates
- Develop a DD model that could accurately predict the virulence of EPF isolates against groups of DBM larvae at fluctuating, “field realistic”, temperatures

7.2.3 Determining the effect of co-application on commercially available insecticides with *B. bassiana* 433.99

i) The effect of 16 candidate insecticides on the following was assessed experimentally:

- The survival of groups of DBM larvae
- The physiology (colony extension and germination) of *B. bassiana* 433.99

ii) Based on (i), and evidence from the literature, the insecticides Calypso, Dipel DF and Neemazal were chosen to undergo co-application experiments with *B. bassiana* 433.99:

- The most common response observed was additive
- There was some evidence of synergism at high doses of EPF and insecticide
- The ability of low concentration insecticides to arrest DBM larval development was suggested as a possible mechanism for synergism

7.2.4 Investigating the effect of age of DBM larvae on their susceptibility to EPF

i) Time between treatment of EPF and insect moult was investigated:

- Larvae treated later in the instar were less susceptible to EPF infection
- Late instar larvae were found to shed conidia, along with the moulted cuticle, as they transitioned from one instar to the next

ii) Secondary pick-up of EPF conidia was investigated using the "leaf spray" bioassay technique:

- At high concentrations, secondary pick-up caused significant mortality against groups of DBM larvae
- EPF conidia (at high concentrations) were able to maintain virulence whilst standing on the leaf surface for up to six days

7.3 Directions for future research

An important aim of this research project was to develop a model to predict the virulence of EPF isolates against DBM at fluctuating temperatures. Here, a DD model was developed that was able to do this well under laboratory conditions. This provided a proof of concept that, in principle, virulence of EPF isolates can be estimated by calculating the number of DD accumulated over a period of time. However, similar experiments should be completed on other cultures of DBM to ensure that virulence of EPF can be predicted in the same way against different DBM biotypes.

The DD model developed had two major limitations:

i. It was unable to predict EPF isolate virulence when temperatures exceeded the thermal optima of isolate virulence
ii. It was developed using data collected in the laboratory, so may not be directly applicable to growers

The DD model developed in this study was only able to predict isolate virulence at temperatures up to the thermal optima of EPF isolate virulence. This is because a major assumption necessary to develop a DD model was broken at temperatures over the optima: proportion mortality did not occur a constant DD sum when temperatures exceeded the thermal optima for isolate virulence. In certain climate zones, it is likely that temperatures will exceed the EPF virulence optima. For example, the thermal optima of *B. bassiana* 433.99 is approximately 29^{o}C, temperatures in Thailand (where DBM is a significant problem) regularly exceed 35^{o}C. It is likely that the complexity of the GxGxE interaction becomes more extreme over the EPF virulence thermal optima as the both the pathogenicity and growth of the EPF isolate and the immune response of the DBM larvae is inhibited. To further develop the DD model, more experimentation should be completed in which EPF virulence is assessed at high temperatures to address why proportion mortality does not occur at a constant DD sum. The DD model should then be extended to include two equations: one for estimating mortality below the EPF virulence optima, and one for estimating the mortality above the EPF virulence optima.

In practice, there many more factors other than temperature that effect EPF efficacy. During the study is was found that the age structure of the insect population also has a major effect of the efficacy of EPF biopesticides. The DD model was based on bioassay data completed on early second instar larvae, and so disregards insect age as a factor which may influence susceptibility. It was also found that secondary pick-up can cause significant infection of DBM larvae if applied at a high enough concentration. Again, this was disregarded, as the DD was completed based on bioassay data completed using the larval spray technique. The next logical phase of research would be to develop a DD model isolate virulence based on data collected in semi-field conditions in which foliar sprays are used to treat mixed-age populations of larvae. The protocol for developing the DD model would be similar to that used in Chapter 4. This DD model would help growers design and implement their IPM systems.

However, the influence of the age of DBM larvae on the efficacy of DBM was shown to be highly significant. Consequently, this may reduce the accuracy of a model that just takes temperature into account when making estimations. Depending on the accuracy of the DD model in a semi-field environment, a separate model may have to be developed that predicts the efficacy of an EPF biopesticide based on the age structure of an insect population. For example, EPF conidia could be applied to DBM larvae of varying ages and a regression model fitted to the relationship between larval and EPF efficacy. In a semi-field trial, the age of DBM larvae in could be determined in a small subset of the study area so that the efficacy of the EPF biopesticide could be estimated. In practice, this model would have to be integrated with the DD model for predicting EPF virulence. In the past models have been developed which incorporate the age structure of populations. For example, Thompson (1975) was able to use the age structure of a population of, along with other factors, to predict the outcome of a predation interaction between of *Daphnia magna* and *Ischnura elegans*.

Apart from age of insects and temperature, many other important factors affecting EPF virulence exist. Humidity of the environment also has a significant effect on virulence of EPF isolates (Mishra et al., 2015). Consequent, any predictive model of virulence would

benefit from using humidity as a predictor. Humidity of seeds, along with temperature has long been used to accurately predict the germination of seeds using similar methods employed in the current study to develop the EPF virulence DD model (Bradford, 2002). In such seed germination models, hydrothermal time is used to measure the progress towards germination and is based on a minimum temperature and water potential that seeds can germinate at (Allen, 2003). This method has been used to accurately predict the germination of tomato, courgette, rye grass, carrot and onion seeds (Atashi et al., 2015, Rowse and Finch-Savage, 2003, Bradford and Cheng, 1999, Meyer et al., 2007). In principle, the same approach could be used to use humidity, as well as temperature, to predict the virulence of EPF isolates.

In the current study, we found a predominantly additive response when commercially available synthetic insecticides were co-applied with *B. bassiana* 433.99. This indicates that the application of insecticides would not affect the efficacy of the EPF biopesticide. This is useful when it comes to predicting the virulence of EPF biopesticides, as the application of synthetic insecticides would not have to be incorporated into the model. Although the most common response in the current study was additive, there was also limited evidence of synergism occurring between some insecticides at and certain concentrations of EPF. The extent to which synergism occurred in the current study was assessed by applying synthetic insecticide at a fixed concentration with and with a concentration of *B. bassiana* 433.99. Chi squared analysis was used to provide a threshold of effect over which in was assumed synergy between the two control products occurred. However, it has been suggested that this threshold based approach is not particularly robust in estimating the response after co-application of treatments (Nieuwenhuis et al., 2011). For example, in one experiment the mortality caused by two treatments may just cross the threshold required for a synergistic interaction to be indicated (chi squared = 3.75, P = 0.049). In another experiment, two treatments may just fall short of the threshold value (chi squared = 3.73, P = 0.051), indicating that an additive response occurred (Fig. 7-1). According to the analysis completed here, the interaction between the two treatments is fundamentally different, but this might not necessarily be the case (Foucquier and Guedj, 2015).

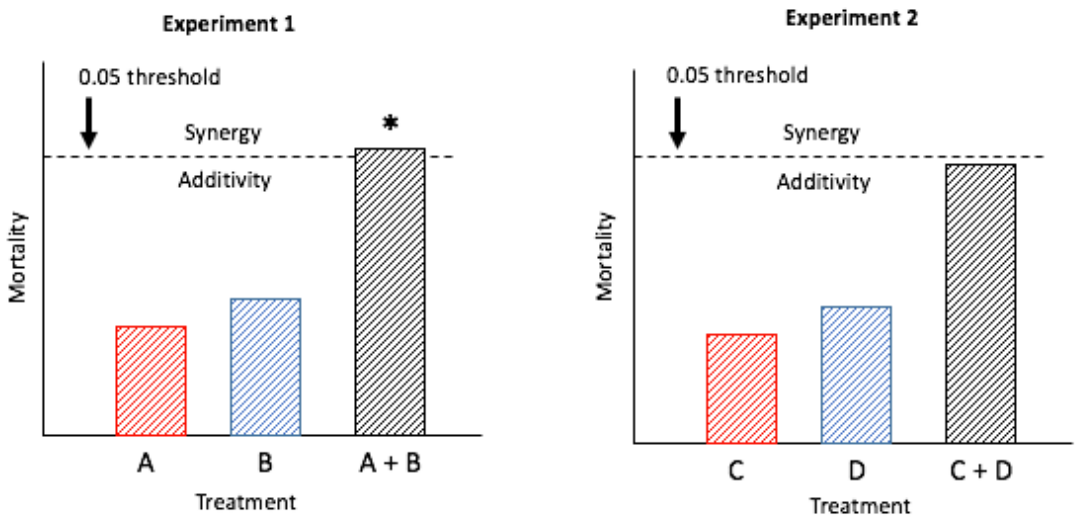


Figure 7-1: Threshold based analysis of two treatment combinations. In experiment one, the mortality caused by combining treatments A and B is slightly above the 5% significance threshold, meaning a synergistic interaction has occurred. In experiment two, the mortality caused by combining treatments C and D is slightly below the 5% significance threshold, meaning an additive response has occurred.

In future study, isobologram analysis would provide a more accurate way of defining the response after co-application of treatments (Tallarida, 2006). In the isobole approach, relative potencies of each of the two coapplied treatments are plotted together so that additivity and synergy can be easily observed (Tallarida, 2006). However, such analysis requires a far higher number of insects per experiment when compared to threshold based analysis. Due to time constraints, it was not possible to produce the number of insects required for isobologram analysis in the current study. In isobologram analysis, the relative concentrations of the two treatments required to cause a specific effect on the group of DBM larvae (for example, the concentration required to cause 50% mortality) are plotted against one another (Fig. 7-2). A straight line from between point A (insecticide LC50) and point B (EPF LC50) is indicative of simple additivity occurring between the two treatments. Data points above the line of additivity indicates that there is evidence of antagonism between the two control products. Data points below the line of additivity indicate that synergism between the two control products has occurred (Foucquier and Guedj, 2015).

Once the effect of co-application has been more robustly characterised, the effect of insecticide application on the efficacy of EPF biopesticides can be predicted more accurately. This would be a great help for growers deciding which concentrations and volumes of EPF biopesticides to apply in the field.

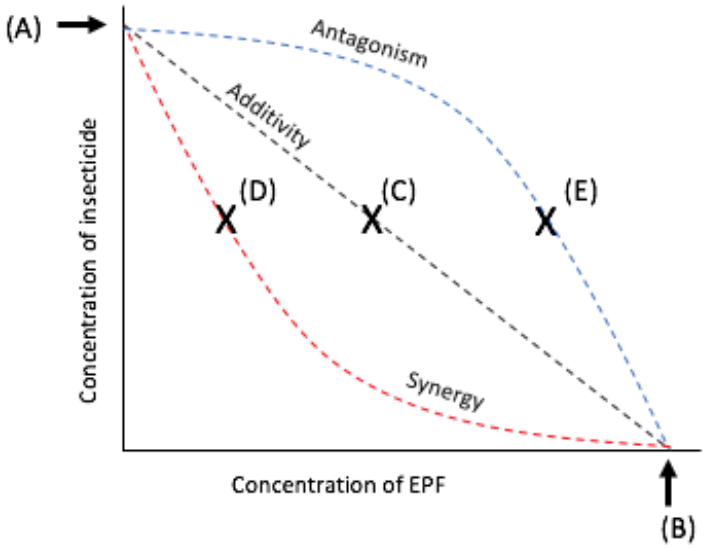


Figure 7-2: Example of an isobologram. The relative concentrations of insecticide and EPF required to 50% mortality are plotted against one another. Points A and B represent the LC50 of insecticide and EPF, respectively. The broken black line between these two points the line of additivity. For example, at point C, half of LC50 concentration of both the insecticide and EPF results in 50% mortality of DBM. The red broken line represents the line of synergism. For example, at point D, a reduced concentration of EPF is required to cause 50% mortality of DBM. The blue broken line represents the line of antagonism. For example, at point E, an increased concentration of EPF is required to cause 50% mortality.

7.4 Concluding remarks

EPF are useful biocontrol agents of insect pests. At the moment, the biopesticides industry is undergoing a rapid expansion, and an increasing number of proprietary EPF biopesticide products are reaching the market. The successful deployment of these products in the field will depend largely on whether farmers and growers employ IPM as the basis for their crop protection. This is highly likely in economically developed countries, where government policies are promoting the use of IPM and reduced risk pest management tools. The challenges for implementing IPM might be greater for resource-poor countries, but even here there is both a demand and a potential for much greater use of EPF and other biopesticides, driven largely by their safety and low cost of development. However, it is still the case that many growers lack confidence in EPF biopesticides, which require greater levels of knowledge and have a lower efficacy than fully effective conventional chemical pesticides. In the case of DBM, IPM can forestall the development of resistance evolving in DBM populations to synthetic insecticides, making them more viable in the long-term. EPF biopesticides have potential to be used against DBM as part of an IPM program, but in order for this to be successful, it is important that their use is based on detailed knowledge of (i) how EPF biopesticide performance is affected by environmental conditions, and (ii) how EPF biopesticides interact with other IPM tools. These issues have not been widely investigated with EPF and DBM, and it is hoped that the research presented here provides new knowledge that can underpin the deployment of EPF as useful components of IPM for DBM. The development of a DD model to predict the effect of fluctuating temperatures on fungal virulence has potential to be a useful tool in implementing EPF biopesticides in the field. In principle, it could allow growers to build their IPM systems around the predicted efficacy of EPF biopesticides, which could also include using EPF biopesticides in combination with applications of synthetic chemical pesticides. However, the DD model should be validated in the field and be developed further to take account of other factors, such as humidity and insect population age structure. This needs to be coupled with training for agronomists, growers and others so that EPF biopesticides can be used in the most effective way in IPM.

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Appendix I

Reagents were obtained from Sigma (UK) unless otherwise stated.

I.1 Media

a) Media used to maintain leaf discs during DBM bioassays

14g Water agar technical no. 3 (Fisher Scientific, UK)

1000ml Reverse osmosis water

Media was autoclaved at 121^{o}C for 20 minutes in a Prestige Medical 2100 Classic. The solution was stirred using a magnetic bead before being poured.

a) Media used to maintain EPF cultures

58g Sabouroud dextrose agar (SDA) (Fisher Scientific, UK)

900ml Reverse osmosis water

Media was autoclaved at 121^{o}C for 20 minutes in a Prestige Medical 2100 Classic. The solution was stirred using a magnetic bead before being poured.

a) *B. bassiana* specific media

58g	Sabouroud dextrose agar (SDA) (Fisher Scientific, UK)
900ml	Reverse osmosis water
58µl	Dodine

SDA and reverse osmosis water were autoclaved at 121^{o}C for 20 minutes in a Prestige Medical 2100 Classic before being left to cool until hand hot, dodine was then added. The solution was stirred using a magnetic bead before being poured.

c) *M. brunneum* specific media

39g	PDA
1g	Yeast extract
0.5g	Chloramphenicol
0.25g	Cycloheximide
0.004g	Thiabendazole
0.01g	Rose Bengal
1000ml	Reverse osmosis water

Media was autoclaved at 121^{o}C for 20 minutes before the solution was stirred using a magnetic bead and poured.

Appendix II

Field data was collected as part of the AHDB funded LINK Brassica project (Collier, 1998), and was kindly provided by Dr Rosemary Collier of the Warwick Crop Centre, University of Warwick, UK.

DBM adults were collected in a Delta Trap: a triangular house hung in the cropping area, with a sticky insert that contains a pheromone dispenser. There were 25 Delta Traps per plot. For each day, total number of adults captured was divided by the total number of Delta Traps to calculate the number of adults per trap per day.

II.1 Kirton, Lincolnshire, UK

a) 1995

Day of year	Number of adults per trap
151	0.52
157	0.37
163	0.13
170	0.23
177	1.17
184	11.94
191	2.34
198	4.29
205	2.74
212	4.11
220	17.60
226	22.80
233	2.00
241	1.00
247	0.53
254	0.46
261	0.60
268	0.14
275	0.03
282	0.03
289	0.03
296	0.09
303	0.00
310	0.00
317	0.03

b) 1996

Day of year	Number of adults per trap
130	3.16
136	1.67
143	0.37
150	0.89
155	4.92
162	17.71
169	25.54
176	7.17
185	4.71
190	4.04
197	2.31
204	12.46
212	19.08
218	9.93
225	5.69
232	5.37
240	6.03
246	1.90
253	0.77
260	0.14
267	0.09
274	0.00
281	0.00
288	0.00
296	0.00
303	0.00
310	0.00

c) 2000

Day of year	Number of adults per trap
145	0.00
153	0.00
160	1.29
167	1.71
174	6.71
181	0.50
187	3.75
195	4.43
202	4.43
209	12.14
216	4.71
223	4.00
229	18.43
236	5.86
243	3.86
250	1.71
257	2.88
265	0.14
272	0.14
279	0.00
286	0.00
292	0.00
300	0.00
307	0.00

II.2 Butterwick, Lincolnshire, UK

a) 1998

Day of year	Number of adults per trap
131	0.00
134	0.00
138	0.00
141	0.75
146	3.00
149	1.20
152	0.67
155	0.67
159	6.00
162	2.25
166	2.00
169	1.00
173	1.00
176	14.75
180	29.00
183	11.00
187	17.33
190	15.75
194	9.33
197	6.00
201	6.67
204	2.25
208	3.00
211	1.75
215	9.00
218	1.25
222	16.67
225	1.75
229	5.00
232	4.50
236	2.33
239	1.25
244	2.67
247	0.20
250	0.00
253	0.33
257	0.00
260	0.00
264	0.33
268	0.00
271	0.00
274	0.00
280	0.00
287	0.17
294	0.00
301	0.00

308	0.00
289	0.00

II.3 Friskney, Lincolnshire, UK

a) 1996

Day of year	Number of adults per trap
123	1.33
128	0.00
131	0.33
134	5.67
137	0.00
141	0.25
144	4.33
149	1.40
152	0.67
156	1.50
159	8.00
163	14.25
166	78.00
170	50.50
173	30.67
177	24.00
180	20.33
184	18.25
187	17.33
191	18.50
194	18.33
198	25.50
201	8.33
204	11.33
207	36.33
211	50.25
214	50.00
218	12.25
221	2.67
225	7.00
228	2.67
232	6.25
235	14.33
240	2.80
242	0.00
246	1.00

II.4 Holbeach, Lincolnshire, UK

a) 1996

Day of year	Number of adults per trap
123	0.22
128	0.00
131	0.00
134	0.00
137	0.33
141	0.00
144	0.33
149	0.00
152	0.00
156	0.00
159	3.67
163	45.50
166	18.00
170	14.00
173	23.00
177	6.25
180	7.33
184	1.25
187	2.67
191	3.50
194	2.67
198	15.25
201	27.33
204	33.33
207	26.00
211	10.50
214	12.00
218	31.75
221	21.67
225	8.00
228	2.33
232	4.50
235	10.33
240	0.40
242	0.50
246	1.25

II.5 Wellesbourne, Warwickshire, UK

a) 1996

Day of year	Number of adults per trap
145	0.25
151	1.27
157	1.60
166	28.29
172	11.00
176	7.45
185	4.56
192	4.71
199	4.91
206	3.46
212	7.00
219	3.69
226	2.23
233	1.09
241	0.55
250	0.42
254	0.05
261	0.00
268	0.00
275	0.00
282	0.00
289	0.00
297	0.00
304	0.00

Appendix III

Fitted Briere-1 model parameters, r^2 and AIC values for colony extension rates (cm day^{-1}), proportion germination and corrected and uncorrected percentage mortality data, at six temperatures for various isolates.

		B. bassiana							*Lecanicillium* spp.		*M. brunneum*			*I.fumosorosea*	
Model	**Par.**	**1757.15**	**1758.15**	**1759.15**	**11.98**	**432.99**	**433.99**	**1730.08**	**1.72**	**19.79**	**275.86**	**445.99**	**1760.15**	**1761.15**	**1762.15**
Germination	***a***	0.06	0.03	0.01	0.07	0.07	0.07	0.04	0.07	0.05	0.06	0.06	0.04	0.09	0.10
	T0	7.16	11.74	12.35	11.24	12.20	12.85	12.65	6.80	5.97	12.91	12.89	12.13	11.07	11.22
	Tmax	33.04	33.14	33.00	33.37	33.04	33.01	33.02	33.00	33.0	35.58	37.61	40.00	33.16	33.32
	Topt	27.08	27.45	27.36	27.61	27.40	27.40	27.39	27.01	26.95	29.48	31.12	33.00	27.43	27.57
	r^2	0.83	0.96	0.84	0.98	0.97	0.95	0.95	0.87	0.84	0.96	0.98	0.88	0.94	0.93
	AIC	55.21	34.57	35.31	43.03	44.75	46.96	41.14	55.77	56.49	48.87	46.28	61.44	52.80	53.24
Colony extension	***a***	$9.7x10^{-5}$	$7.7x10^{-5}$	$5.6x10^{-5}$	$9x10^{-5}$	$9x10^{-5}$	$9.5x10^{-5}$	$9x10^{-5}$	$8x10^{-5}$	$1.3x10^{-4}$	$1x10^{-4}$	$2x10^{-4}$	$1.7x10^{-4}$	$7.7x10^{-5}$	$7.9x10^{-5}$
	T0	1.50	-2.4	-2.10	5.46	8.86	2.77	6.38	-3.1	-0.1	1.46	5.85	8.84	-1.05	-1.1
	Tmax	33.0	33.01	33.14	33.22	33.12	33.11	33.4	30.23	26.29	33.10	33.30	34.26	33.15	33.11
	Topt	26.56	26.18	26.31	27.17	27.26	26.76	27.22	23.87	21.03	26.64	27.18	28.18	26.43	26.39
	r^2	0.98	0.98	0.99	0.76	0.86	0.88	0.87	0.91	0.82	0.87	0.96	0.94	0.90	0.83
	AIC	-33.91	-34.06	-42.83	-21.1	-22.66	-23.89	-26.14	-22.09	-21.72	-26.64	-23.65	-22.61	-25.03	-20.79
Virulence (corrected)	***a***	0.07	-	-	-	-	0.03	-	-	-	0.04	0.07	0.07	-	-
	T0	2.25	-	-	-	-	4.62	-	-	-	8.72	13.20	11.81	-	-
	Tmax	30.06	-	-	-	-	35.27	-	-	-	35.27	36.67	36.56	-	-
	Topt	24.28	-	-	-	-	28.66	-	-	-	28.94	30.37	30.21	-	-
	r^2	0.89	-	-	-	-	0.84	-	-	-	0.88	0.90	0.84	-	-
	AIC	45.24	-	-	-	-	48.22	-	-	-	48.75	61.57	65.22	-	-
Virulence (non-corrected)	***a***	0.07	-	-	-	-	0.03	-	-	-	0.04	0.06	0.06	-	-
	T0	1.80	-	-	-	-	5.17	-	-	-	7.91	11.39	9.68	-	-
	Tmax	30.36	-	-	-	-	36.64	-	-	-	36.61	38.07	38.01	-	-
	Topt	24.47	-	-	-	-	29.80	-	-	-	30.00	31.40	31.25	-	-
	r^2	0.89	-	-	-	-	0.87	-	-	-	0.91	0.95	0.93	-	-
	AIC	44.41	-	-	-	-	49.40	-	-	-	49.38	56.18	58.85	-	-

Appendix IV

Analysis was completed to assess the interaction between a *B. bassiana* and three commercially insecticides. Two parameter polynomial models were fitted to the relationship between concentration and percentage mortality for lone *B. bassiana* and combination treatments.

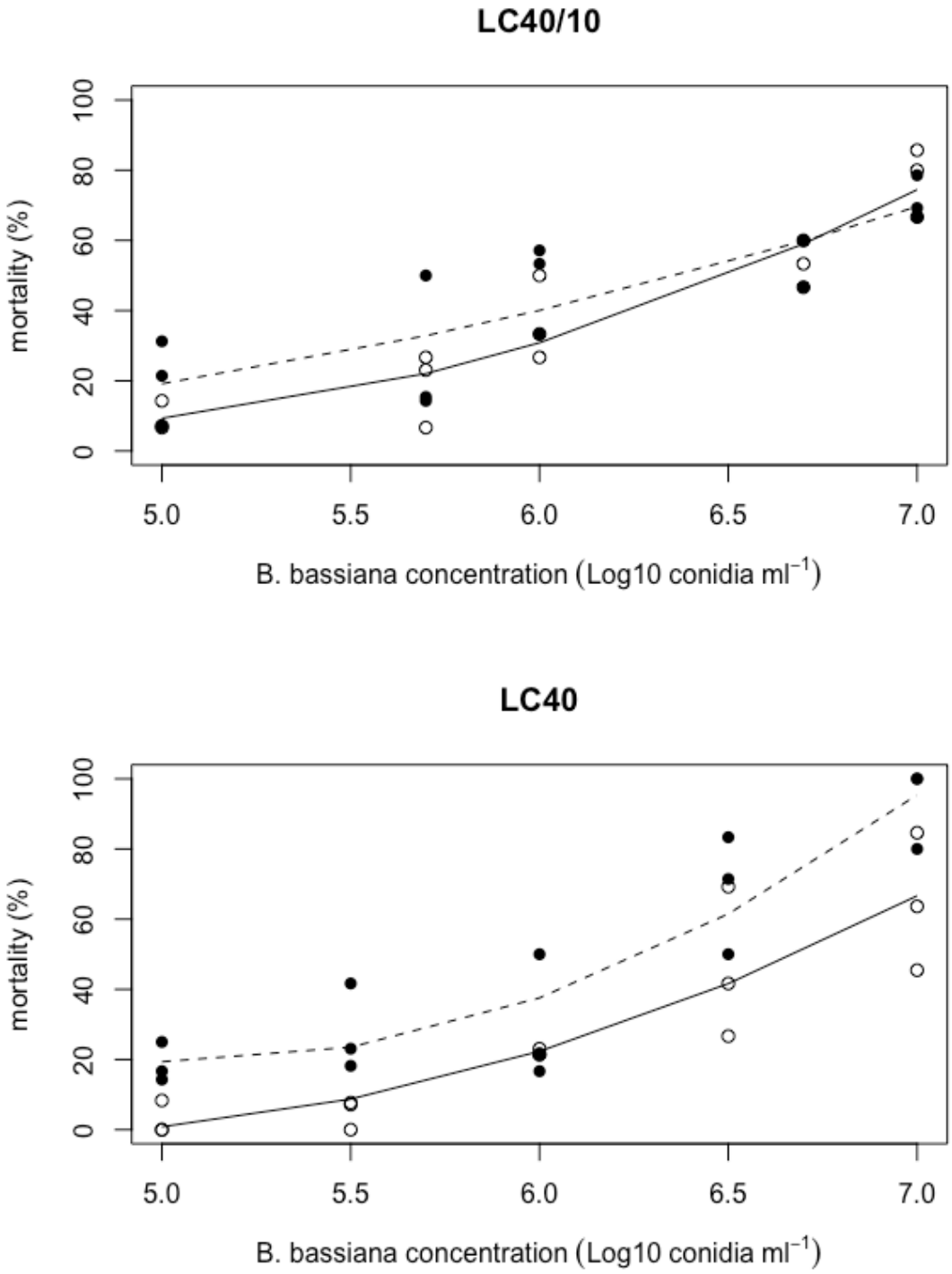

Figure A4-1: Mortality of groups of DBM larvae, from six days of treatment, after being treated with a concentration range of *B. bassiana* with and without a fixed dose of Dipel DF insecticide at either LC40 or a 10-fold dilution of LC50. Two parameter polynomial models are included. Open and filled markers represent treatment by *B. bassiana* only and combination treatments, respectively. Solid and dashed lines represent polynomial models fitted to *B. bassiana* only and combination treatments, respectively.

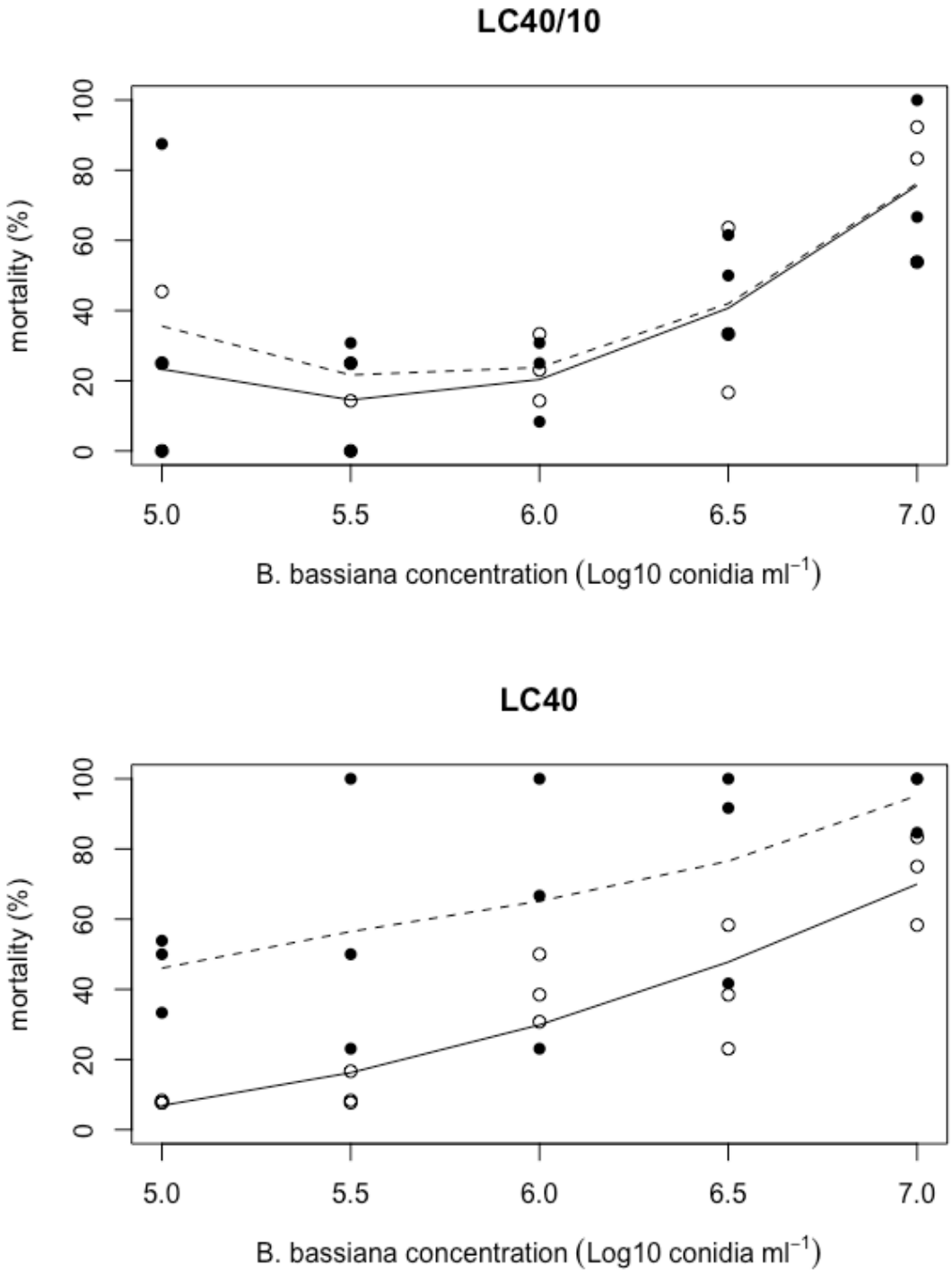

Figure A4-2: Mortality of groups of DBM larvae, from six days of treatment, after being treated with a concentration range of *B. bassiana* with and without a fixed dose of Neemazal insecticide at either LC40 or a 10-fold dilution of LC50. Two parameter polynomial models are included. Open and filled markers represent treatment by *B. bassiana* only and combination treatments, respectively. Solid and dashed lines represent polynomial models fitted to *B. bassiana* only and combination treatments, respectively.

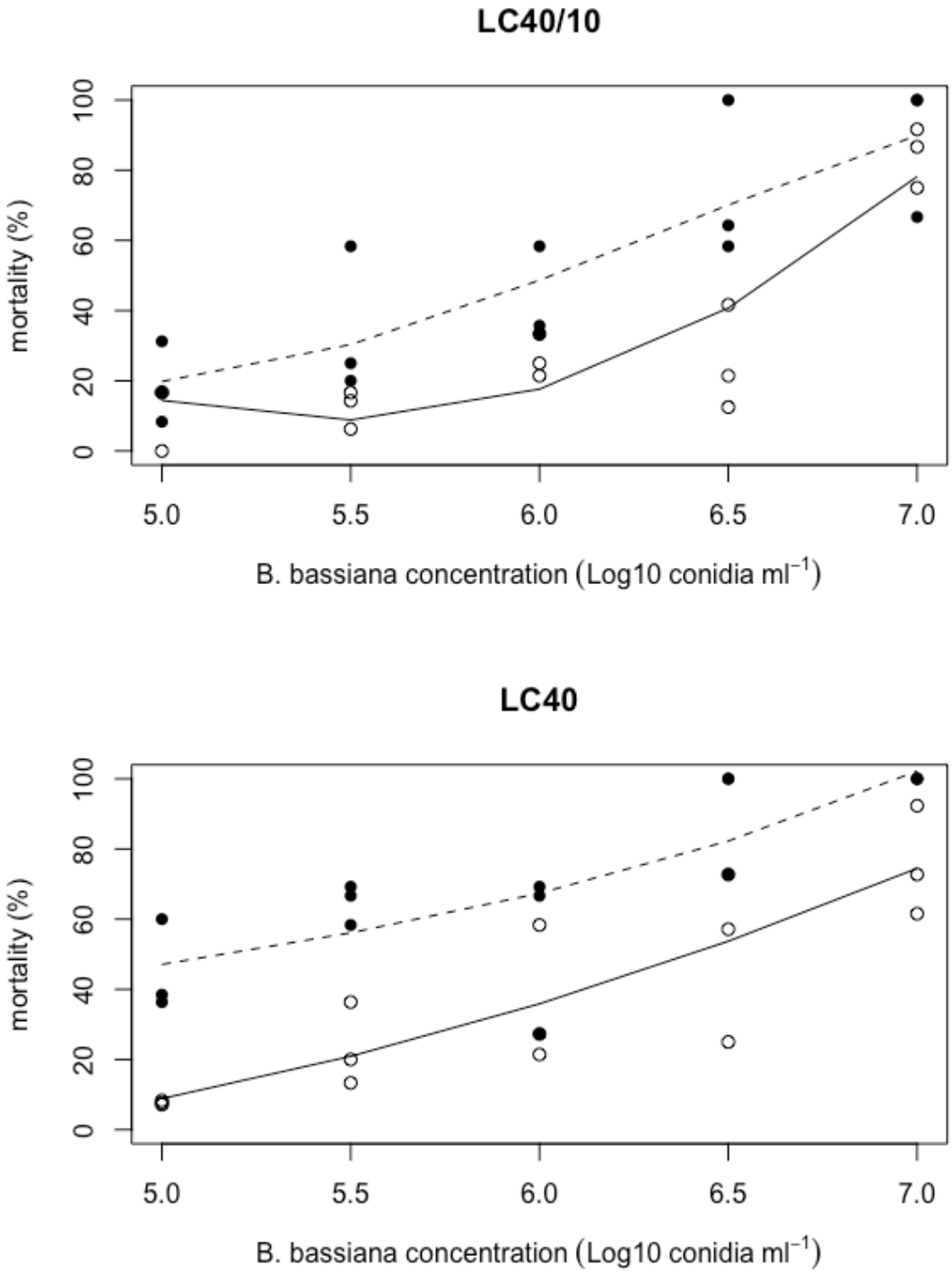

Figure A4-3: Mortality of groups of DBM larvae, from six days of treatment, after being treated with a concentration range of *B. bassiana* with and without a fixed dose of Calypso insecticide at either LC40 or a 10-fold dilution of LC50. Two parameter polynomial models are included. Open and filled markers represent treatment by *B. bassiana* only and combination treatments, respectively. Solid and dashed lines represent polynomial models fitted to *B. bassiana* only and combination treatments, respectively.