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Multiple Stressor Effects in Biological Pest Control;
Improving Efficacy in Challenging Environments

By

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Abbreviations

AIC	Akaike information criterion
ANOVA	Analysis of Variance
BPYV	Beet pseudo yellows virus
Bt	<i>Bacillus thuringiensis</i>
DDT	Dichlorodiphenyltrichloroethane
EFSA	European Food Safety Authority
EPF	Entomopathogenic fungi
FAO	Food and Agriculture Organization
GLM	Generalized linear model
IGR	Insect Growth Regulator
IPM	Integrated Pest Management
LC	Lethal Concentration
LT	Lethal Time
UV	Ultraviolet
PPP	Plant protection products
RR	Recommended application Rate
SDA	Sabouraud dextrose agar
SPaV	Strawberry pallidosis associated virus
T0	Thermal minima
TICV	Tomato infectious chlorosis virus
Tmax	Thermal maxima
ToCV	Tomato chlorosis virus
Topt	Thermal optima
UKCEH	United Kingdom Centre for Ecology and Hydrology
USDA	United States Department of Agriculture

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Summary

Greenhouse whitefly (*Trialeurodes vaporariorum*) are a major global pest, causing direct damage to >850 plant species and transmitting viral plant diseases. Management of *T. vaporariorum* is increasingly difficult because of widespread pesticide resistance. Many greenhouse growers rely on biological control agents to maintain *T. vaporariorum*. However, biological control agents are slow acting and variable in their efficacy, resulting in subsequent application of chemical insecticides when pest populations become unmanageable. There is an increasing drive to use integrated, multiple pest control strategies, limiting chemical application and increasing the use of sustainable alternatives. Entomopathogenic fungi (EPF) have the potential to be used as one of these alternative control methods, but EPF efficacy is highly variable between applications. Co-application of a chemical insecticide with an entomopathogenic fungus can result in improved pest control, assist resistance management, target multiple pest species and increase the range of environmental conditions over which control is effective. Combining chemical and biological pesticides has the potential to result in both positive and negative interactions, so it is important to understand how mixture components interact. Positive interactions result in synergism, whilst negative interactions cause antagonism and a reduction in pest control.

In this thesis, EPF with potential to control *T. vaporariorum* were identified through a series of laboratory *in vitro* and *in vivo* tests. Co-application of a mixture of selected EPF and the chemical insecticide spiromesifen were evaluated in laboratory based bioassays. Using an ecotoxicological MixTox model, complex interactions between the EPF and insecticide were described; depending on the EPF and the concentrations applied, mixtures resulted in additivity, synergism or antagonism. The types of interactions were influenced by temperature and applications of a low concentration of spiromesifen with EPF resulted in additive mortality of *T. vaporariorum* in greenhouse trials. By understanding of the complex interactions between components of integrated pest management strategies, optimised approaches can be developed to control pests.

1 Literature review

Declaration

This PhD thesis is presented in accordance with the Guide to Examinations for Higher Degrees by Research provided by the Graduate School, University of Warwick. All research and writing was conducted by myself unless otherwise stated. Parts of this thesis have been published, though additional information and explanation is provided within this thesis. This thesis has not been submitted for a degree at another Univeristy.

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1.1 Introduction

The global population has quadrupled within the last century and is expected to reach 9.1 billion by 2050 (FAO, 2018). Food production must increase by 70% in order to support a population of this size but the United Nations Food and Agriculture Organization (FAO) estimates that global arable land per capita is predicted to be 0.2 ha in 2050, less than half of that in 1960 (FAO, 2018). So, optimisation of current land use, sustainably increasing productivity and avoiding waste is key to ensuring future food security (Popp *et al.*, 2012).

Protected cultivation of horticultural crops was developed to protect plants from adverse abiotic conditions, allowing year-round production of high quality products. Yield from greenhouses can be up to 15 times more than field grown crops per hectare (Padmanabhan *et al.*, 2016).

There are estimated to be 405,000 ha of greenhouses spread across the world (Duque-Acevedo *et al.*, 2020). Though, within the greenhouse, several pests and diseases contribute to crop loss.

On average, 35% of global crop production is lost to pests (Oerke, 2005) One of the most destructive greenhouse pests is the greenhouse whitefly (*Trialeurodes vaporariorum*).

Trialeurodes vaporariorum is a cosmopolitan and highly polyphagous pest species of significant economic importance. Damage to ornamental and crop plants is caused by feeding nymphs and adults, extracting nutrients from the plant and reducing plant productivity (Osborne and Landa, 1992). Most importantly, *T. vaporariorum* are vectors of plant viruses, capable of reducing crop yields dramatically, and in some cases by 100% (Saurabh *et al.*, 2021).

Prophylactic application of synthetic chemicals has been used for crop protection but this is no longer an acceptable method of *T. vaporariorum* control for several reasons;

1. Broad spectrum insecticides have detrimental impacts on non-target organisms and the environment.
2. Excessive application of these chemicals often results in pest resurgence, secondary pest outbreaks and pest resistance to chemical active ingredients.
3. Increasing consumer and retailer demands for chemical residue free products that require a longer interval between spray applications and harvest.
4. Reduced availability of plant protection products and stricter restrictions on introducing new active ingredients meaning that currently effective active ingredients should be protected against resistance developing in target pests.

Consequently, there is increased pressure to develop effective and sustainable pest management. This is recognised in legislation such as the EC Directive on Sustainable Use of Pesticides

2009/128/EC which aims to prioritise non-chemical alternatives over chemical control of insect pests (European Commission, 2009). Experts agree that future crop protection should be based on Integrated Pest Management (IPM) which is the use of a combination of plant protection strategies and monitoring of pest and natural enemy populations to maintain the pest population below a size that would cause economic injury to crops (Dent, 1991). IPM programmes for *T. vaporariorum* have been implemented for 40 years, but these programmes are still heavily reliant on synthetic chemicals upon the breakdown of pest control (Parrella *et al.*, 1999).

Entomopathogenic (insect pathogenic) fungi (EPF) are considered to have great potential for *T. vaporariorum* control, especially within the greenhouse system where environmental conditions are more predictable than in field grown crops. EPF are attractive as control agents because of their host selectivity, their lack of toxic residues left on crops and their low development costs compared to conventional pesticides (Hajek and Eienberg, 2004). In addition, EPF commercially sold for plant protection can be applied to crops using farmers' existing foliar spray equipment (Chandler *et al.*, 2011). However, EPF have a relatively slow rate of kill compared to synthetic insecticides. In addition, EPF are known to be highly variable in efficacy due to their susceptibility to unfavourable environmental conditions (Lacey *et al.*, 2001). Most EPF are not suitable as stand-alone treatments because they are less efficacious than their synthetic chemical counterparts. However, they can be used alongside other control measures and provide substantial and sustainable pest control (Chandler *et al.*, 2011).

In order to improve the uptake of EPF within an IPM programme, consistency in efficacy must be improved. One approach that is receiving increasing attention in crop protection research is the co-application of microbial pathogens with chemical insecticides (Falcon, 1971). Positive interactions between co-applied mixture components can be exploited to increase mortality of the target pest, speed up the rate of kill or increase susceptibility of resistant pest populations. Indeed, there is evidence in the literature of interactions between EPF and some chemical insecticides against whitefly causing increased mortality and faster speed of kill (Islam, Castle and Ren, 2010; Zou *et al.*, 2014; Ali *et al.*, 2017). Mixture applications can also reduce resistance selection pressure due to the different modes of action used by microbial and chemical insecticides. Positive interactions between an EPF and a chemical insecticide could maintain pests below economic injury level whilst concurrently reducing the amount of active synthetic chemical ingredient needed, providing a more sustainable approach to pesticide usage.

Another factor to consider when developing an integrated approach for pest control is the target environment, which will influence both EPF and insecticide efficacy. Adverse environmental conditions have been shown to negatively impact the level of pest control caused by EPF (Jaronski, 2009) and chemical insecticides (Glunt *et al.*, 2014). Under greenhouse conditions, humidity is often maintained above 60% to maximise productivity of crops (Barker, 1990; Shamshiri *et al.*, 2018) but this also provides optimal humidity for EPF to infect their hosts. However, temperature variation in the greenhouse can be considerable (Shamshiri *et al.*, 2018). Temperature has a significant effect on EPF infectivity, influencing the growth and germination of fungal conidia (asexually produced fungal spores) whilst simultaneously affecting the susceptibility of insect hosts by altering their growth and development rates and as well as their immune response (Ratte, 1984; Jaronski, 2009). Most studies into the disease producing power of EPF have been conducted under constant temperature regimes, but in order to identify successful IPM approaches, experiments should emulate the target crop system as closely as possible. Few studies have investigated EPF efficacy under oscillating temperature conditions (Feng *et al.*, 1999; Inglis *et al.*, 1999; Fargues and Luz, 2000), despite this being a major factor influencing success of EPF. Additionally, temperature variation has been shown to alter the susceptibility of pests to chemical insecticides (Boina *et al.*, 2009). It is not currently known how temperature can influence the interactions between co-applied EPF and chemical control agents. But, as temperature has a significant effect on the efficacy of EPF and chemical insecticides applied independently, it is likely that interactions could be affected by the temperature oscillations that occur under greenhouse conditions.

A basic understanding of the relationship between different components used within an IPM system is required in order to both understand and exploit potential positive interactions. Thus, identification of negative (antagonistic) interactions is equally important in order to avoid situations that may result in a breakdown of pest control. The success of an EPF within an IPM programme also depends on its compatibility with other essential plant protection products (PPPs) such as herbicides and fungicides. Throughout the pest management literature, there is a focus on experiments involving one element of the IPM system rather than interactions of multiple components. Just a few recent examples of this are the independent applications of parasitoids (Walia *et al.*, 2021), predators (Leman *et al.*, 2018) or physical control methods such as short wavelength ultra violet light (UV-C) (Leskey *et al.*, 2021) in the control of *T. vaporariorum*. In addition, there is a lack of experiments conducted under conditions emulating greenhouse environmental conditions or within a greenhouse environment. As a result, uptake of

many IPM approaches by growers is minimal, due to a disconnect between experimental and actual crop production systems (Grasswitz, 2019). In a study by Buurma and van der Velden (2017), another impediment to improved IPM uptake by growers was identified to be a lack of incentive focussed research. For example, the main motivation to apply IPM strategies by Dutch greenhouse growers were demonstrable increased plant health or reduced crop loss.

The use of mixtures of EPF and chemical insecticides have shown potential for significantly improving pest control efficacy (Feng *et al.*, 2004; Farenhorst *et al.*, 2010; Meyling *et al.*, 2018; Santos *et al.*, 2018b). However, interactions between EPF and chemicals cannot be generalised as incompatible mixtures can result in reduced pest control (Bitsadze *et al.*, 2013). Therefore, in order to exploit positive interactions between mixture components, the factors determining interaction outcomes must be fully understood. During this project, investigations into the relationship between EPF and a synthetic insecticide were conducted using an ecotoxicological approach. The effect of temperature variation on the outcome of mixture applications were determined in laboratory and greenhouse based trials. These experiments were conducted with the aim of improving EPF efficacy by increasing our knowledge on the response of EPF to temperature stress and compatibility with other elements of the IPM system.

1.2 *Trialeurodes vaporariorum* as pests

1.2.1 Damage caused by *Trialeurodes vaporariorum*

The greenhouse whitefly (*Trialeurodes vaporariorum*) is a major insect pest causing substantial damage to >850 plant species, including greenhouse ornamental and agricultural crops (CABI, 2021). Hosts of economic importance include; tomato, watermelon, cucumber, pumpkin, rose, poinsettia, tobacco, green beans, lettuce, strawberry, potato, sweet potato, and courgette (Mound and Halsey, 1978). *Trialeurodes vaporariorum* is capable of damaging host plants in three primary ways. Firstly, individuals feed on the plant, sucking sap from the phloem, reducing yields and killing the plant if infested with a large *T. vaporariorum* population. Secondly, *T. vaporariorum* excrete honeydew to rid themselves of excess amino acids and carbohydrates from the plant sap. Adults secrete more honeydew than nymphal stages (Hong and Rumei, 1993). Honey dew covers the leaves and fruit of host plants, supporting the growth of saprophytic fungi such as *Penicillium* sp. and *Cladosporium* sp. (Perring *et al.*, 2018). Whilst these fungi do not infect the plant, the ‘sooty mould’ which covers the leaves can hinder photosynthesis by preventing light reaching the leaf surface, plus fruit and marketable plants must be cleaned before sale. This can cause up to 5% reduction in yield of tomato crops (Johnson *et al.*, 1992). Finally, the most economically damaging effects of *T. vaporariorum* are caused by the vectoring of plant viruses. *T. vaporariorum* is capable of vectoring four viruses, all within the genus Crinivirus (Jones, 2003). These viruses are known as Beet pseudo yellows virus (BPYV), Strawberry pallidosis associated virus (SPaV), Tomato chlorosis virus (ToCV) and Tomato infectious chlorosis virus (TICV) (Wintermantel, 2004). These diseases cause stunted growth, reduced fruit yield and early senescence of plants. *Trialeurodes vaporariorum* vectored viruses are predicted to cost the global agricultural economy more than \$1 billion a year (Gonzalez *et al.*, 1992; Legg *et al.*, 2006; Aregbesola *et al.*, 2019). As mentioned earlier, a combination of the direct and indirect effects of feeding *T. vaporariorum* on crops and ornamentals can result in up to 100% yield loss (Saurabh *et al.*, 2021).

1.2.2 Life cycle of *Trialeurodes vaporariorum*

Trialeurodes vaporariorum has six life stages; the egg, four nymphal stages and the adult. Development time is highly dependent on temperature; for example, individuals can take 21.87 days to develop from egg to adult at 28°C compared to 46.71 days at 15°C on tomato plants (Gamarra *et al.*, 2016). Optimal temperatures for reproductive output are considered to be between 20 and 25°C (Xie *et al.*, 2011; Gamarra *et al.*, 2020), though humidity and host plant also have a significant effect on rate of development and fecundity of *T. vaporariorum* (Capinera *et al.*, 2008; Lee *et al.*, 2010).

Female *T. vaporariorum* lay eggs on the abaxial surface of the uppermost leaves on the host plant. The female inserts her piercing mouthpart (stylet) into the leaf, and moves her body around in a circular motion, laying eggs in a circular pattern. Individual females have been recorded to lay up to 534 eggs in a lifetime (Lloyd, 1922). *Trialeurodes vaporariorum* have a haplodiploidy reproductive system meaning that diploid females are produced from fertilised eggs and haploid males are produced asexually by parthenogenesis (Galis and Alphen, 2020). Females are generally distinguishable from males by their larger size.

Initially, eggs appear yellow but darken to brown with time before first instar nymphs emerge. The first instar are the only mobile nymphal stage and are called ‘crawlers’ as they move short distances across the leaf surface until a suitable location to insert their piercing mouthpart (stylet) is found. Once in position, nymphs remain with their stylet inserted into the leaf throughout second, third and fourth instar moults (Nechols and Tauber, 1977) and these nymphal stages may feed on plant sap for up to 24 hours a day (Hong and Rumei, 1993). Second and third instars are only distinguishable by size, as they are both transparent and oval in shape but third instar nymphs are slightly larger (0.54 x 0.33mm) compared to second instar nymphs (0.38 x 0.23mm). Whereas, the fourth instar, also known as the pupa, is opaque and is characterised by the presence of visible red eyes (Gamarra *et al.*, 2016). Wax rods stand upwards from the dorsal surface and the body is more elevated from the surface of the leaf compared to previous instars (Hill, 1969). Moulting occurs between each instar and sloughed skin (exuviae) fall from the leaf once shed.

Once adults emerge from the pupal stage, they are able to fly within a few hours. Initially, their wings are transparent but wax from the lateral abdominal glands is spread over the wings by the hind legs, giving the characteristic dusty appearance. Following this, adults migrate to newly emerged leaves at the top of the plant where they aggregate to feed, mate and oviposit (figure 1.1). A complex courtship ritual is performed whereby males attempt to impress females

through vibrational signals, a sequence of drumming on the female thorax using antennae and fluttering of the males' wings (Fattoruso *et al.*, 2021). Oviposition of fertilised eggs occurs a few hours after copulation (Las, 1980). Females continue to lay eggs for the remainder of their lifespan, which can be up to 4 weeks depending on environmental conditions and host plant (Las, 1980).

Trialeurodes vaporariorum adults disperse upon disturbance of the plant and are capable of flying up to 2m a day. Movement between fields is assisted by wind and migration between regions, countries and continents is caused by the transportation of infested plant material (Gamarra *et al.*, 2016).



Figure 1.1.1 An aggregation of *Trialeurodes vaporariorum* adults on the abaxial surface of the most recently emerged aubergine leaf (*Solanum melongena*), found at the top of the plant.

1.2.3 Geographic distribution of *Trialeurodes vaporariorum*

Initially, *T. vaporariorum* was described as *Aleurodes vaporariorum* by Westwood in 1856 after being sampled from greenhouse grown tomato crops in Europe (Quaintance, 1900). However, shortly after, Cockerell (1902) reported that the species originated from Brazil. It is now understood that the transport of Orchidaceae from Mexico to the UK allowed the spread of the invasive pest throughout the European continent (Mound and Halsey, 1978). Currently, *T. vaporariorum* is found on every continent except Antarctica, as shown in figure 1.2. Though *T. vaporariorum* is associated with warmer climates, the pest has been able to successfully colonise most regions due to its extensive host range and the worldwide distribution of greenhouse habitats providing opportunity to shelter from extreme cold temperatures on greenhouse grown crops (Perring *et al.*, 2018). In addition, the haplodiploid mode of reproduction and high reproductive output mean that large populations can quickly develop from a single female *T. vaporariorum* (van Lenteren and Noldus, 1990).

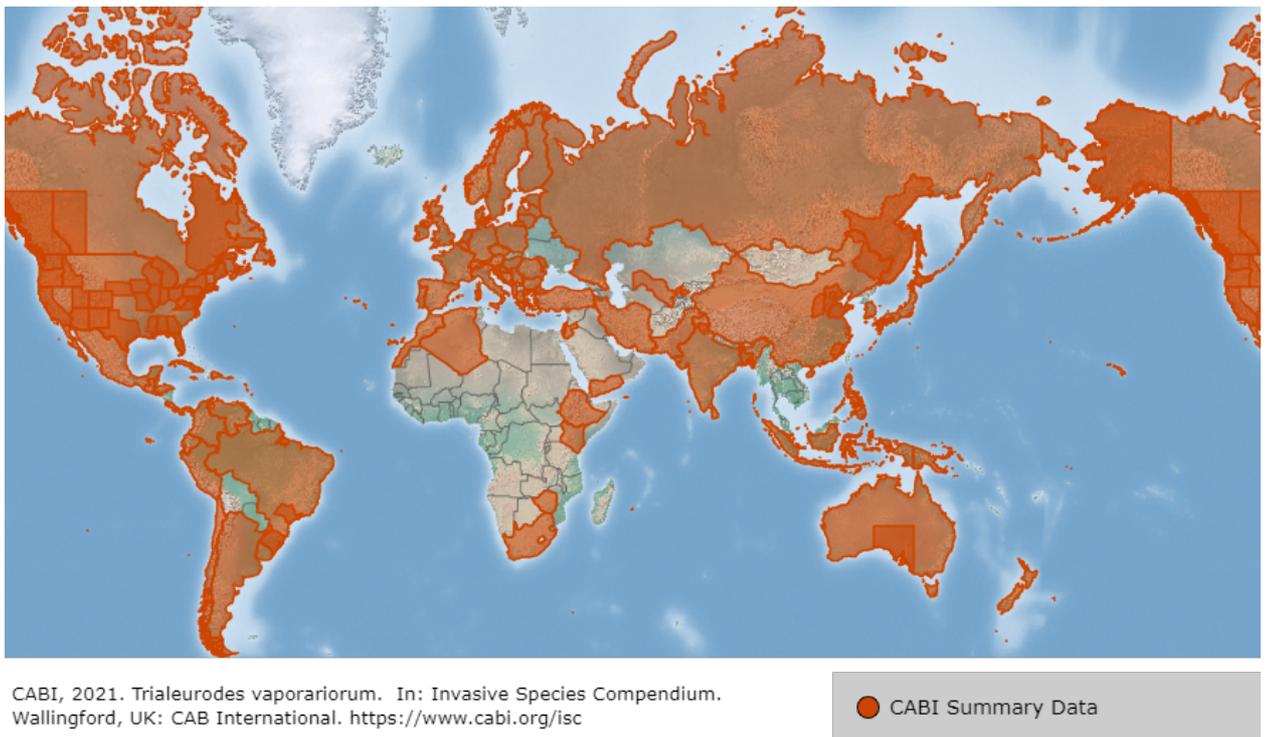


Figure 1.1.2. Current distribution of *Trialeurodes vaporariorum* according to records deemed reliable by the Centre for Agriculture and Bioscience International (CABI). Areas highlighted in orange identify reported cases of *T. vaporariorum*.

1.2.4 Insecticide resistance of *Trialeurodes vaporariorum*

The difficulties of resistant populations developing as well as pressures to reduce pesticide inputs to crop production, has strengthened the development of alternative approaches through integrated pest management (IPM) strategies. In this project, the potential of the chemical insecticide spiromesifen, used as part of an integrated approach against *T. vaporariorum*, was investigated.

Historically, growers were more reliant on the use of broad spectrum chemical insecticides for *T. vaporariorum* control compared to current integrated approaches. In early years, this initially involved the fumigation of greenhouses using hydrocyanic acid gas (Speyer and Owen, 1926) or tetrachloroethane (Parker, 1928). Following this, *T. vaporariorum* was controlled using Dichlorodiphenyltrichloroethane (DDT) and nicotine fumigation before organophosphates were introduced in the 1960s (Richardson, 1943; Smith *et al.*, 1947; Condrón *et al.*, 1962). An emphasis on ‘alternative methods’ of control emerged as a result of reduced efficacy of “older” insecticide groups such as organophosphates and pyrethroids (Gorman *et al.*, 2002). In addition to the development of resistant *T. vaporariorum* populations, these broad spectrum pesticides also removed natural enemies resulting in more severe pest resurgences (Hill *et al.*, 2017). As a result, there was a high reliance on neonicotinoids upon their release onto the market in 1991, as this group of insecticides were known to be more selective due to their systemic mode of action and therefore targeted the pest and allowed natural enemies to persist (Jeschke and Nauen, 2008). However, by 2004-2005, *T. vaporariorum* populations resistant to neonicotinoids had been reported in the UK, the Netherlands and the US (Gorman *et al.*, 2007; Bi and Toscano, 2007a). Since then, neonicotinoid resistance has become widespread throughout global populations of whitefly (Karatolos *et al.*, 2010; Pappas *et al.*, 2013; Ovčarenko *et al.*, 2014; Bass *et al.*, 2015) as well as the significant risk posed to bees through consumption of pollen and nectar (Lundin *et al.*, 2015) and secondary exposure of natural enemies through consumption of intoxicated pests (Prabhaker *et al.*, 2011).

Resistance is defined as “a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species” according to the Insecticide Resistance Action Committee (IRAC) (Elbert *et al.*, 2007). Resistance arises from randomly occurring genetic mutations which protect individuals against the pesticide being applied and

these beneficial mutations, being inherited by offspring of surviving individuals. *T. vaporariorum* populations can quickly develop resistance to chemical insecticides because females reproduce by parthenogenesis, producing several genetically identical haploid males, ensuring that beneficial mutations are inherited. Additionally, the greenhouse environment is confined, reducing the periodic immigration of susceptible individuals, as seen opposed to pests of field grown crops where there is regular ingress of individuals into populations (Karatolos, 2011). The rate of development of resistance in a population is directly related to the selection pressure imposed upon the pest, whereby 'the greater the use of insecticides, the greater the selection for insecticide resistance and the faster insects become resistant' (Sternberg and Thomas 2018).

Insecticide resistance occurs by several mechanisms, the most common being; in-sensitivity as a result of target site mutations preventing the binding of the insecticide to the target site or detoxification of insecticides by increased enzyme activity (Karatolos, 2011). Other mechanisms include penetration resistance, slowing the absorption of chemical through the insect cuticle and behavioural resistance whereby the insect actively avoids toxins in the environment. In *T. vaporariorum*, both types of mechanisms of resistance have been observed. Resistance to pyrethroid insecticides and DDT have been attributed to three amino acid substitutions in the para-type voltage-gated sodium channel which is the target site of these insecticides. The pest has also developed resistance to more recently developed novel insecticides such as pyriproxyfen and spiromesifen (Karatolos , *et al.*, 2012a; Karatolos *et al.*, 2012b), which are used in experiments in this project. The active ingredient, pyriproxyfen (4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether), mimics juvenile hormone; an important modulator of ecdysteroids, a class of hormone which causes moulting in insects (Hiruma and Kaneko, 2013). Throughout insect development, juvenile hormone is synthesised, secreted into the hemolymph and degraded at important stages of development. Whitefly synthesise high levels of the hormone during the first, second and third instar before reducing levels in the final instar, to activate ecdysteroid synthesis and metamorphosis (Gelman *et al.*, 2007). As a result, addition of the juvenile hormone analogue, through foliar application of pyriproxyfen, egg hatching and development from larval to adult stages is prevented and adult females are sterilised (Ishaaya *et al.*, 1994).

Resistance to pyriproxyfen is associated with an over-expression of a single gene causing increased activity of cytochrome-P450 dependent monooxygenases (P450s), a group of detoxifying enzymes (Karatolos *et al.*, 2012a).

Comparatively, spiromesifen works by inhibiting acetyl-CoA-carboxylase, a lipid metabolism enzyme and reducing total lipid production (Nauen *et al.*, 2005). Insect processes such as growth and moulting require increased levels of metabolism. The insect fat body serves not only as an important energy source during these periods, but also synthesises most of the hemolymph proteins and metabolites required (Arrese and Soulages, 2010). This insecticide is considered safe to use in conjunction with other biological control agents in protected crops, such as *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) and *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae) because of its systemic mode of action. Due to its high efficacy and low non target effects, spiromesifen has been incorporated into many crop protection programmes, especially in protected crops in Spain (Bielza *et al.*, 2019). However, growers have recently noticed a decrease in efficacy of the insecticide and confirmed populations of resistant *B. tabaci* have been reported (Bielza *et al.*, 2019). In *T. vaporariorum* populations, resistance to spiromesifen was determined to be mediated through a mutation at the target site of acetyl-coenzyme A carboxylase (ACCase) enzyme, which moderates lipid biosynthesis. Though the level of resistance found throughout the UK and Europe was determined to not reduce the suitability of spiromesifen to control *T. vaporariorum* populations at the time of the study (Karatolos *et al.*, 2012b).

1.3 Integrating pest management strategies in greenhouse horticulture

Greenhouse growers have long been incentivised to avoid an insecticide-only approach for *T. vaporariorum* control (Kogan, 1998). Instead, IPM is encouraged by many experts, which combines different control approaches with decision support tools, maintaining pests below the economic action threshold (the pest density beyond which chemical intervention would be required to avoid economic loss of the crop) and minimising pesticides use. These systems focus on the prevention, early detection and control of pest species. In greenhouse IPM systems, the most effective programmes involve applications of arthropod predators and parasitoids to suppress pest populations and prevent crop damage (van Lenteren, 2000), as part of an IPM 'pyramid' approach (Stenberg, 2017). Under this system, ecology based PPPs such as natural enemies and biopesticides are co-applied, using multiple knock-down strategies to manage insect pests. Biopesticides are control agents reliant on microbial organisms or natural products such as biochemicals or semio-chemicals. For example, secondary metabolites produced by plants or insect sex pheromones (Chandler *et al.*, 2011). Often, IPM compatible pesticides are still applied,

but are used as supplementary treatments, acting as a second line of defence should pest populations increase beyond an economic damage threshold when natural enemies are no longer able to control them (Jacobson *et al.*, 2001).

IPM practitioners in greenhouse crops are increasingly reliant on the applications of biopesticides based on entomopathogenic bacteria (predominantly *Bacillus thuringiensis*), viruses, fungi and nematodes (Wraight *et al.* 2017). Biopesticides are often able to be applied using conventional pesticide application techniques. However, unlike synthetic pesticides they are non-toxic, have a short pre-harvest and re-entry interval and have the potential to provide sustained pest control by continuing to spread within the host population, infecting subsequent generations (Lacey *et al.*, 2015). However, compared to fully effective synthetic chemical pesticides, microbial control agents can be slower acting, less efficacious, and more expensive to purchase (Glare *et al.*, 2012). Additionally, further training is required to ensure suitable storage, application and integration with other control methods, relying on the provision of training by extension services (Barzman *et al.*, 2015).

The increasing uptake of microbial control agents by growers is reflected in the number of biopesticide products coming onto the market. The biopesticide market (including microbial control agents and natural predators) was valued at \$4 billion in 2016, which had increased from \$0.68 billion in 2005 (Zaki *et al.*, 2020). This was partly due to legislation introduced by the EU Sustainable Use Directive on pesticides (2009/128/EC), which aimed to prioritise non-chemical alternatives over chemical control of insect pests as part of IPM (European Parliament, 2009). Additionally, implementation of the (EC) 1107/2009 regulation concerning the placement of PPPs onto the market (European Commission, 2009) led to the removal of >200 substances from the European market since 2010 (Zaki *et al.*, 2020), leaving space for biopesticides to replace the synthetic chemicals which had been removed. Equally, chemical companies must invest in alternatives such as biopesticides in order to prevent or delay resistance development to the synthetic active ingredients in chemical products remaining on the market. Whilst biopesticides represented just 3.6% of the \$56 billion pesticide market in 2018, the registered compound annual growth rate was 14-32% (IBMA, 2020). It is predicted that further development of IPM programs is likely to increase uptake of biopesticides (Leahy *et al.*, 2014).

1.3.1 Integrated pest management in control of *Trialeurodes vaporariorum*

Biological control of *T. vaporariorum* has been used successfully by progressive growers of protected environment crops in the UK and the Netherlands for over 40 years (Parrella *et al.*, 1999). The most popular approach is the inundative introduction of parasitoids and predators which prevent crop damage by maintaining *T. vaporariorum* populations below the economic action threshold. Inundative control of *T. vaporariorum* by parasitoids belonging to the genus *Encarsia* has been available commercially since 1926 (van Lenteren, 2011); the parasitic wasps, *Encarsia formosa* and *Eretmocerus eremicus* are now sold by biological control companies such as Fargro©, Koppert© and Bioline©.

There are approximately 75 species of *T. vaporariorum* predators described (van Lenteren *et al.*, 1996) belonging to five insect orders: Coleoptera, Heteroptera, Diptera, Neuroptera and Hemiptera (Gerling, 1992). Although, the predatory mites *Amblyseius swirskii* and *A. montdorensis*, the predatory beetle *Delphastus catalinae* and the mirid *Macrolophus pygmaeus* are predominantly the only predators sold for biological control of *T. vaporariorum* in Europe (Leman *et al.*, 2018). The introduction of *Macrolophus pygmaeus* into the United States has been prevented because this predator will also feed on plant tissue when *T. vaporariorum* numbers are low (Castañé *et al.*, 2011).

In this system, *T. vaporariorum* natural enemies may be combined with biopesticides, and deployment of a number of monitoring and preventative measures, in order to reduce pest pressure below an economic damage threshold (Capinera, 2001; Ehler, 2006).

Biopesticides are microorganisms, plant extracts or semio-chemicals that are used against pests in plant protection (Gwynn, 2014). The most effective pathogens against *T. vaporariorum* are principally the entomopathogenic fungi (insect killing fungi; EPF). Entomopathogenic fungi infect the insect through direct penetration of the cuticle (Evans and Hywel-Jones, 1997).

Bacteria and viruses are not ingested by *T. vaporariorum* as their mouthparts remain inside host plant tissues whilst they feed but rare infections through existing wounds can sometimes occur (van Lenteren *et al.*, 1996). The development of EPF as biopesticides, or 'mycoinsecticides', has been successful in greenhouse, horticultural, orchard and arable field crops (Gold *et al.*, 2001; Kivett *et al.*, 2015; Nyasani *et al.*, 2015; Arthurs and Bruck, 2016; Javal *et al.*, 2019). The most commonly used approach for whitefly control using EPF involves the inundative introduction of large numbers of infective conidia (Faria and Wraight, 2001) in a similar application manner to conventional insecticides; indeed, many EPF from the order Hypocreales are easily mass reared on artificial culture media and have been formulated into commercially sold water dispersible powders which should be targeted at the abaxial leaf surface (Chandler, 2016).

Mycoinsecticides (fungus based biopesticides), are ideal candidates for control of *T. vaporariorum* because there are little to no residues left on the crop, EPF are often compatible with other natural enemies and the risk of resistance developing to the EPF is very low because death of the host is caused by multiple modes of action (Chandler *et al.*, 2011). EPF can be used successfully as a preventative measure, alongside the use of beneficial invertebrates (Fransen and van Lenteren, 1993; Jeong *et al.*, 2005). Alternatively, EPF can be used as a second line of defence when predators and parasitoids fail to provide consistent pest management as environmental conditions change during a growing season. Several EPF are commercially available in Europe to target *T. vaporariorum*, such as *Beauveria* spp. (Naturalis®, Fargro; Botanigard®, Certis;) *Cordyceps* spp. (PreFeRal®, BioBest; Nofly®, Futureco Bioscience) and *Akanthomyces muscarium* (Mycotal®, Koppert Biological systems).

1.3.2 Entomopathogenic fungi taxonomy

The Fungi Kingdom holds approximately 100,000 described species of eukaryotic organisms which are defined to the kingdom by the presence of chitin and glucan filled cell walls (Chandler, 2017). Entomopathogenic fungi (EPF) are those which cause infection and disease in insects as well as other closely related arthropods, including ticks, mites and spiders. There are estimated to be 750 species of EPF found within five of the eight fungal phyla (Araujo and Hughes, 2016). There are also EPF found within the phylum oomycote, within the Kingdom Chromista (previously classed as Fungi) (Shah and Pell, 2003), as shown in Figure 1.3.

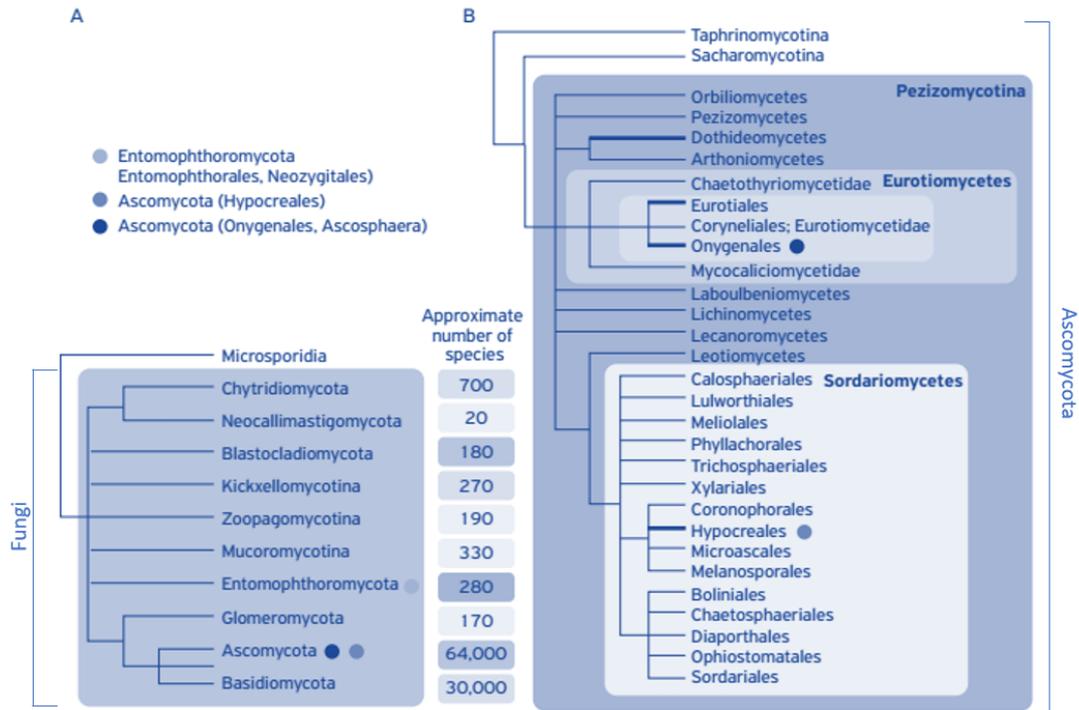


Figure 1.3 Taxonomic tree of entomopathogenic fungi. (A) depicts the Kingdom Fungi, with numbers representing approximate species numbers within each (sub) phylum. Shading in represents relative proportions of known species of entomopathogens (highest proportion = darker shading). Branches with the most common and well-known entomopathogens are indicated by blue dots (darker= greatest number of species). In (B), further classification within Ascomycota is shown. Thick lines represent orders with insect pathogenic fungi and light grey, black and dots mark those with the most known insect pathogens: Hypocreales (blue, for example, *Cordyceps*, *Metarhizium* and *Beauveria*) and Onygenales (dark blue, *Ascospaera*). Adapted from Mora *et al.* 2017.

The Chytridiomycota contains a significant number of parasites of other fungi, plants, animals, rotifers and tardigrades (Money, 2016). In comparison, very few species in this division cause diseases in insects. Most species from the Chytridiomycota phylum are aquatic and as such, produce zoospores which are asexual spores with a single posterior flagellum, used to respond to chemical gradients and locate their host (Araujo and Hughes, 2016). The Chytridiomycota is the only phylum within the kingdom to have motile cells or zoospores. The most notable genus for EPF is the Coelomycetes which contains over 70 species of Dipteran pathogens (Scholte *et al.*, 2004).

Microsporidia were originally thought to be basic eukaryotic cells but we now know that they are specialised Fungi, with reduced genome size (Williams and Keeling, 2011). Microsporidia are obligate intracellular pathogens which insert their protoplasts into host cells using a

projectile tube originating from the spore (Keeling and Fast, 2002). There are 339 EPF species in this group, the most well-known are those pathogenic to beneficial insects such as *Nosema ceranae* which infects honeybees (Higes *et al.*, 2009).

One of the most important EPF groups is the Entomophthoromycota and in particular, the order Entomophthorales. Fungi in this group have positively photo-trophic conidiophores which produce asexual spores (conidia). These conidia are forcibly discharged to increase dispersal by hydrostatic pressure both in the conidium and the structures that produce them (conidiophores) (Hajek, 1999). If the conidia land on an unsuitable host, they are capable of producing secondary and in some cases, tertiary conidia (Araújo and Hughes, 2016). Important genera for infecting insect pests include *Entomophthora* (aphids), *Zoophthora* (aphids, caterpillars and beetles) and *Entomophaga* (grasshoppers and caterpillars). Species in these genera are capable of causing natural epizootics, often resulting in 100% mortality of the insect population (Federici, 1999). For example, *Entomophaga maimaiga* is known to naturally infect larvae of the gypsy moth (*Lymantria dispar*) and cause epizootics throughout populations in North America every year (Hajek, 1999). Insect death is commonly caused by tissue damage during colonisation of the host and rarely due to toxins produced by the EPF (Humber, 1984). However, potential for these EPF species to be developed into commercially sold myco-insecticide is limited. Host specificity of these EPF make mass production of conidia extremely difficult. *In vitro* production is possible in supplemented media, but these approaches are not practical or economical for mass production. In addition, conidia are viable for only a short period after production (Hajek, 1997).

Ascomycota and Basidiomycota form the subkingdom Dikarya or the higher fungi. EPF belonging to Basidiomycota produce sexual basidiospores which are forcibly ejected (Araújo and Hughes, 2016). EPF within this division are found within the genera *Septobasidium* and *Uredinella* (order= Septobasidiales) and are exclusively parasites of scale insects.

The largest phyla within the Fungi Kingdom is Ascomycota which holds 64,000 described species. Most EPF are within the order Hypocreales (subphylum Pezizomycota, class Sordariomycetes) and are generally considered opportunistic pathogens, infecting a range of insect orders (Shah and Pell, 2003). EPF within the subphylum Pezizomycota have a complicated life cycle with an anamorph (asexual) and teleomorph (sexual) state. For EPF in this order, the asexual state usually occurs before the sexual state and asexual spores are

produced on a raised hyphal structure for increased dispersal between hosts (Mora, Castilho and Fraga, 2017). In some cases, the teleomorph state is rarely produced and for this reason, the anamorph state was formerly used for taxonomic classification of fungi (Mora, Castilho and Fraga, 2017). The anamorphic states of Ascomycota fungi are the most commonly used EPF applied as inundative biological control (Hesketh *et al.*, 2010). Until recently, the different states of each species were considered to be separate species and some are still referred to by different scientific names (Chandler, 2017). However, a new rule in effect since January 2012, known as the ‘one fungus, one name’ rule has resulted in reclassifying all species under their teleomorph name only (Taylor, 2011). A few exceptions have been made for significantly important species which are still named by their anamorph state such as *Beauveria bassiana* and *Metarhizium* species. Within the Hypocrealean order, there are three families; Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae. All species within Cordycipitaceae are EPF, though there are several species within Ophiocordycipitaceae and Clavicipitaceae which do not infect insects and are associated with plants or animals (Chandler, 2017).

In this study, all EPF used are from either the Hypocrealean family Clavicipitaceae or Cordycipitaceae, namely: *Beauveria* spp., *Metarhizium* spp., *Cordyceps* spp. and from the *Akanthomyces* genus.

- *Beauveria bassiana* is characterised by the formation of dense white conidia and fascicles of hyphae on the host cuticle after infection. *Beauveria bassiana* conidia are ovoid and formed on individual toothed (denticulate) apical extensions (rachis) (Humber, 2012).
- *Metarhizium* species (including *Metarhizium brunneum* and *Metarhizium anisopliae*) have white hyphae which branch out to produce long chains of cylindrical green conidia (Humber, 2012).
- *Cordyceps* species *Cordyceps farinosa*, *Cordyceps javanica* and *Cordyceps fumosorosea* (formerly known as *Isaria* species, and before that, *Paecilomyces* (Taylor, 2011) produce chains of cream/grey or smoky pink fusiform conidia on white conidiogenous cells (phialides) which are arranged in whorls.
- Entomopathogenic fungi within the genus *Akanthomyces* (formerly classified as *Lecanocillium* and *Verticillium*) produce white or cream short ovoid conidia. Depending on the species, conidia are borne in slime balls found on phialides that are arranged in whorls, pairs or singly (Humber, 2012).

The hydrophobic properties of conidia produced by *Beauveria*, *Metarhizium* and *Cordyceps* species is due to the presence of hydrophobins (cysteine rich proteins) in the cell wall and are characteristic of aerial conidia. In comparison, *Akanthomyces* EPF produce hydrophilic conidia (Inglis *et al.*, 2001) which do not become airborne but are easily suspended in water and dispersed within greenhouse crops for pest control (Bruck *et al.*, 2007).

1.3.3 Infection and colonisation process of entomopathogenic fungi

Hypocrealean EPF spread when susceptible hosts directly come into contact with sporulating cadavers or in-directly, through airborne conidia or contact with those which have been deposited on soil and vegetation. Once conidia have made contact with the host, the first stage of infection is the adhesion of conidia to the host exoskeleton. Fast adhesion and germination of conidia are important characteristics of highly virulent EPF (Qu and Wang, 2018). Virulence is defined as the disease producing power of an organism (Shapiro-Ilan *et al.*, 2005).

The insect cuticle is inhospitable to EPF conidia, with low water and nutritional content and with secretions of antifungal lipids and glandular secretions to prevent fungal growth. For example *Tribolium castaneum* prevents infection by *B. bassiana* by secreting benzoquinones onto the cuticle (Pedrini *et al.*, 2015). In response to these challenges, EPF secrete mucus to increase their stability and aid in adhesion to the cuticle surface (Boucias *et al.*, 1988). Environmental conditions are extremely critical during this time. If conditions such as temperature and humidity are favourable and other chemical and physical requirements are available, the conidia will swell during imbibition of water. The spore germinates, forming a germ tube, if there is a source of carbon available (Dillon and Charnley, 1990). An appressorium may form on the end of a differentiated germ tube. The appressorium is a swollen structure which aids in penetration of the cuticle. Penetrative hyphae or an infection peg emerge from the appressorium (Inglis *et al.*, 2001). At this stage, many enzymes are secreted by the fungus to break down the insect cuticle and release nutrients. Cuticle degrading enzymes and physical pressure by the appressorium and hyphae are used to penetrate the exoskeleton. Proteases, chitinases and lipases are used to degrade the protein, chitin matrix which form the insect cuticle layers (Mora *et al.*, 2017). Following the detection of pathogen cells, the pro-phenoloxidase pathway is activated as part of the insect immune response (González-Santoyo and Córdoba-Aguilar, 2012). Melanin deposits will form around invading structures to prevent further entry;

darker spots within the cuticle are often visible in many insect species and indicate the early stages of infection by EPF. Immature insects may also evade infection by ecdysis, shedding their old cuticle and the EPF before the fungal conidia are able to penetrate the new cuticle underneath (Ortiz-Urquiza and Keyhani, 2013).

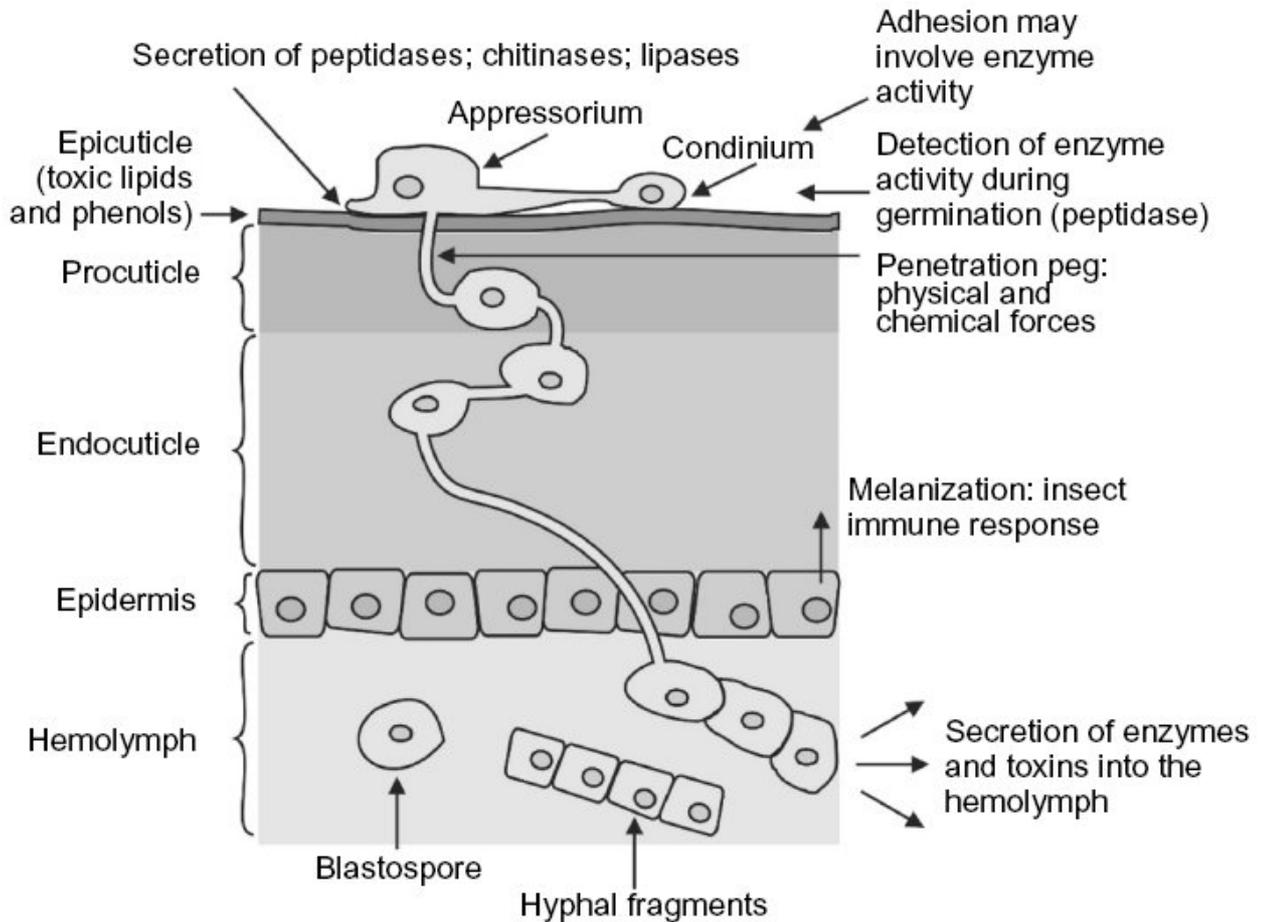


Figure 1.1.4 Summary of the infection process of an entomopathogenic fungus on an insect host, taken from (Samuels *et al.*, 2015).

If EPF hyphae successfully reach the haemocoel of their host, the insect hemostatic response is activated immediately and clotting agents attempt to reduce hemolymph leakage (Vilmos and Kurucz, 1998). Hemocytes are cells involved in the insect immune response of which there are three types of hemocyte. These are responsible for phagocytosis of invading small microbials, the melanisation reaction to prevent further invasion, and encapsulation of invading objects too large to be removed by phagocytes (Lemaitre and Hoffmann, 2007). To overcome detection by the insect immune response, Hypocrealean EPF switch from hyphal growth to produce small blastospores which rapidly disperse within the hemolymph (Chandler, 2017). Some species are

also able to suppress the hemocyte response to allow further spread of the infection (Hou and Chang, 1985). Invasion of the haemocoel also activates the insect systemic immune response. Unlike the cellular response, the systemic immune response is slow and is initiated several hours after infection; the systemic response is the last attempt by the host to stop pathogens infecting the insect. The systemic response initiates a cascade of reactions through three pathways. The Toll pathway is primarily responsible for the production of antimicrobial peptides (AMPs) following fungal infection. The other pathways, the immune deficiency (Imd) and Janus kinase/signal transduction and activator of transcription (JAK/STAT) pathways, are also responsible for AMP production and communication between the pathways ensures a different and appropriate response to infections caused by different pathogens (Chandler, 2017). EPF have evolved mechanisms to avoid detection by the systemic immune response, though currently these are not fully understood. It is likely that the switch from hyphal growth to hyphal bodies/ blastospores aids in avoiding detection of β -glucans, which are polysaccharides present in the cell walls of fungi that are recognised by insect hemocytes (Stączek *et al.*, 2020). In addition, the polysaccharide α -1,3-glucan, may act as a shield of the β -1,3-glucan layer, preventing detection by the insect immune response (Rappleye *et al.*, 2007).

Once the insect immune responses have been overcome, EPF hyphae proliferate throughout the insect body and death can be caused by organ function being prevented due to fungal colonisation, physical damage, starvation, poisoning by EPF metabolites or water loss (Chandler, 2017). Time to death varies depending on several factors including virulence of the EPF, environmental conditions and the number of conidia infecting the insect. A stressed host as a result of overcrowding, nutritionally inappropriate food sources, starvation, infection from another pathogen or the presence of chemicals can be more susceptible to EPF infection (Hajek and St. Leger, 1994; Hesketh *et al.*, 2010; Scheepmaker and Butt, 2010). Most EPF infections result in host death in 3-20 days, but there may be a reduction in host feeding as early as 24 hours after infection, which is an often overlooked benefit of using EPF for biological control (Hajek and St. Leger, 1994). Several EPF produce toxic secondary metabolites which have insecticidal properties. For example, the hexadepsipeptide Beauvericin is known to increase cell membrane permeability to ions, causing cytolysis and cell death (Grove and Pople, 1980). It is known to be produced by *Beauveria bassiana*, *Cordyceps fumosorosea* and *Cordyceps farinosa* (Wang and Xu, 2012). Some Metarhizium species are known to produce cyclic depsipeptide toxins, known as destruxins (Wang, Hu and Hu, 2019). The destruxin produced by *Metarhizium*

anisopliae was found to affect Ca²⁺ channels in muscle membranes of the tobacco hornworm (*Manduca sexta*), causing contraction and then paralysis (Samuels *et al.*, 1988).

After host death, the EPF grows outwards from the insect cuticle and, under desirable conditions, will sporulate to spread to another host. Unlike Entomophthorales, Hypocrealean fungi are not actively discharged from their host, so they rely on wind and the movement of host insects amongst sporulating cadavers to cause secondary infections (Nielsen and Wraight, 2009).

1.3.4 Entomopathogenic fungi as mycoinsecticides

Entomopathogenic fungi can be used in augmentation, classic and conservation biological control, though the most popular approach is inundative biological control using Hypocrealean fungi (Chandler, 2017). There have been over 170 EPF biopesticides developed since the 1960s, mostly using species belonging to *Metarhizium*, *Beauveria*, *Cordyceps* (was *Isaria*) and *Akanthomyces* (was *Lecanicillium*), for use in greenhouse and field crops (Faria and Wraight, 2007). The most desirable characteristics of an EPF used for inundative biological control are high virulence against target pests, the ability to produce stable propagules which can be easily mass produced, withstanding the application process and showing high efficacy in varied environmental conditions (Jackson *et al.*, 2010). It is recommended that 2×10^{12} - 5×10^{13} conidia ha⁻¹ are applied for effective pest control (Jaronski, 2009).

Hypocrealean conidia are easily mass produced and can be stable i.e. retain infectivity for years if formulated and stored correctly (Moore *et al.*, 1996). Therefore, development of mycopesticides has focused on Hypocrealean fungi. Current registered mycoinsecticides in Europe are shown in Table 1.1

Entomophthorales EPF are difficult to rear outside of the insect host and are impossible to apply through spray applications, so their use has been limited to conservation biological control (Pell, 2007). In this approach, changes are made to crop protection practices to encourage the activity of EPF that naturally occur in the environment. Under these circumstances, local pest populations can quickly be reduced to zero after an epizootic. For example, the EPF *Zoophthora radicans* regularly eliminates local population outbreaks of the diamond back moth (*Plutella xylostella*) in the Phillipines (Riethmacher and Kranz, 1994).

Table 1.1 Mycoinsecticides registered for use within Europe.

Fungal strain	Product	Manufacturer	Target
<i>Beauveria bassiana</i> GHA	BotaniGard	Bioworks Inc.	Whitefly, aphids and thrips
<i>Beauveria bassiana</i> ATCC74040	Naturalis-L	Fargro	Whiteflies and Thrips
<i>Cordyceps fumosorosea</i> Wize Apopka 97	PreFeRal	BioBest	Whiteflies
<i>Cordyceps fumosorosea</i> Wize FE9901	Nofly	Futureco Bioscience	Whiteflies
<i>Akanthomyces muscarium</i> Petch Ve6	Mycotal	Koppert Biological systems (Netherlands)	Whiteflies
<i>Metarhizium brunneum</i> Petch Bipesco5	Taerain	Fargro	Whiteflies and Thrips
<i>Metarhizium brunneum</i> Petch F52	Met52	Fargro	Aphids, thrips, mites, whitefly, vine weevils
<i>Purpureocillium lilacinus</i> (Thom) Samson 251	BioAct	Bayer	Root-knot nematodes

Whitefly are not susceptible to microbial infections via ingestion as they feed by inserting their needle-like stylets into the host plant phloem. All stages of whitefly can be infected by EPF, including the egg and the adult. The most susceptible stages of whitefly to EPF infection are the first, second and third instars (Osborne and Landa, 1992). If eggs are not infected by EPF, emerging first instars can be susceptible to the EPF conidia covering the egg (Fransen and van Lenteren, 1993). Whitefly adults can also be infected by EPF and more easily pick up conidia whilst moving on the plant rather than being targeted by spray applications (Mascarin *et al.*, 2013a).

Aside from death of the host, EPF can cause sublethal effects in whitefly populations with studies showing reduced egg laying, deformation of offspring and reduced survivorship of later generations (Torrado-León *et al.*, 2006). Several studies have shown that predators and parasitoids will avoid whitefly infected with EPF (Meeke, 2001; Labbé *et al.*, 2009), reducing intraguild interactions of the fungus with other organisms beneficial for whitefly control. Development of heritable resistance towards EPF has not been reported in whitefly, despite multiple generations of selection and continued exposure through biopesticide use (Gao *et al.*, 2017). This is likely due to the multi-modal route of infection of EPF involving combinations of different enzymes, toxins, secondary metabolites and methods to evade the host immune

response, as previously described. However, insects can develop resistance to EPF through increased melanisation, phenoloxidase activity, inhibiting EPF enzyme production and increasing AMP synthesis (Wilson *et al.*, 2001; Zhao *et al.*, 2012; Dubovskiy *et al.*, 2013). For example, *Galleria melonella* larvae were selected for *B. bassiana* resistance across 25 generations in a study by (Dubovskiy *et al.*, 2013) resulting in ~10% increased survivorship of larvae exposed to the fungus by differential expression of genes coding for one AMP and four supposed stress management factors. However, the development of this resistance was at the cost of fecundity. The study also found that the resistance was not effective against other EPF such as *M. anisopliae*.

Arguably, one of the most successful EPF biopesticides for whitefly control is based on the *Akanthomyces muscarium* strain (Ve6) (formerly *Verticillium* before *Lecanicillium lecanii*) sold as Mycotal® (Koppert Biological Systems ©). The EPF was reclassified as a separate species from *A. longisporum*, another mycoinsecticide, previously sold as Vertalec® (Koppert Biological Systems ©) which targeted aphids. Though, several studies have shown that *A. muscarium* is often not the most virulent when compared to other candidate EPF (Nakai & Lacey, 2017), including the findings in the current study (Chapter 3). One of the major factors leading to the success of *A. lecanii* as a biopesticide was the integration of academic research during the development of the product. Host virulence and growth studies were conducted in many screenings, whilst the effect of environmental conditions on control efficacy, methods of production and storage of infective propagules, comparisons of potency relative to chemical standards and the compatibility of the EPF with other natural enemies, synthetic chemicals and fungicides were studied in detail (Hall, 1981). The *Akanthomyces muscarium* strain Ve6 continues to be used as an industry standard today and is included in most studies as a comparator. As a result, a substantial amount of in depth knowledge into the biology and compatibility of this EPF with other IPM elements has been collected. A similar approach was taken to successfully develop the mycoinsecticide Botanigard® (Certis ©) against aphids, thrips, spider mite and whitefly using *B. bassiana* strain GHA.

1.3.5 The potential of mixtures for pest control

Effective pest control strategies employing an IPM approach should follow the conceptual framework outlined by Stenberg (2017). Under this concept, a holistic approach is taken to synergistically combine physical, ecological and chemical methods of pest control in a series of tiers, implemented in response to incremental changes in pest populations. The first tier in the IPM pyramid involves a number of farming practices which prevent pest infestations. These include sanitation of the greenhouse, planting resistant plant cultivars, using optical filters and increasing the presence of natural enemies in the system, amongst other cultural, mechanical and physical methods (Stenberg, 2017). Following this, another tier in the IPM pyramid involves compatible ecologically based approaches, implemented to manage pest numbers. This includes the application of environmentally friendly PPP's such as biopesticides and botanicals. Should pest populations cause significant damage after the application of the first two tiers of the IPM pyramid, synthetic chemicals may be applied as a final attempt to protect the crop. However, there is potential for an intermediate step between tiers two and three which could exploit interactions between synthetic chemicals and ecologically based control agents, such as biopesticides, by applying a mixture of the two approaches. Including an additional tier in the IPM pyramid could be a strategy to reduce the amount of synthetic active ingredients applied, whilst maintaining control of the target pest, resulting in economic and environmental benefits (Ali *et al.*, 2017). The pyramidal IPM conceptual framework and the proposed additional tier can be seen in figure 1.5.

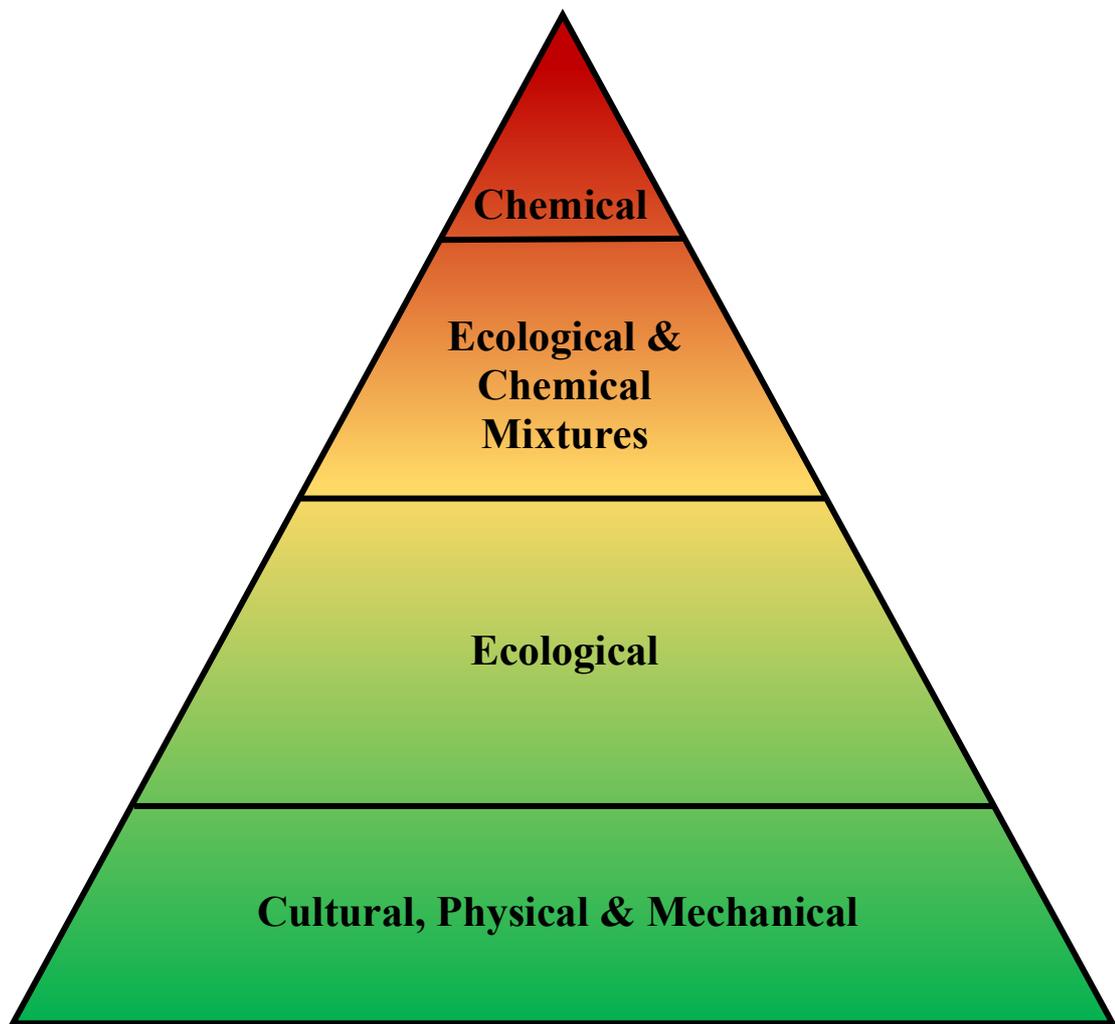


Figure 1. 1.5. The IPM pyramid described by (Stenberg, 2017), adapted to include an additional tier. Each tier describes pest control strategies of increasing potency which are implemented in response to increasing severity of pest infestations on the plant. The first consists of abiotic methods which can be conducted throughout plant production, followed by a tier of ecological approaches. The proposed additional tier involves the co-application of compatible synthetic pesticides and biological control methods. The final tier is the application of chemical insecticides which are only used if pest populations are not managed by more basal IPM approaches.

Synergistic interactions between synthetic chemicals and biopesticides such as EPF can provide higher levels of pest control than separately applied individual approaches. For example, combinations of *Metarhizium anisopliae* and sub-lethal concentrations of the neonicotinoid thiamethoxam resulted in increased mortality of the rice stalk stink bug (*Tibraca limbativentris*) in field trials in Brazil (Quintela *et al.*, 2013). One of the main disadvantages to biopesticides is the relatively slow rate of kill compared to their synthetic counterparts. However, combinations of EPF and low levels of synthetic chemicals have resulted in increased rate of kill as well as higher overall mortality of target pests. For example, Yui *et al.* (2016) observed that mortality of subterranean termites (*Coptotermes curvignathus*) was faster following the application of *M. anisopliae* and sub-lethal doses of fipronil, reducing the median lethal time (LT₅₀) from 6.5 days when using the EPF alone, to 4.9 days as a mixture.

In the case of *T. vaporariorum*, populations have developed resistance to all major insecticide groups (Insecticide Resistance Action Committee (IRAC), 2019), but there is evidence that co-application of EPF with synthetic insecticides can stop the expression of insecticide resistance in the pest population (Farenhorst *et al.*, 2010) and reduce resistance acquisition in pest populations (Ambethgar, 2009). Combined applications of microbial and chemical pesticides may also allow growers to target more than one pest at one time or ensure pest control across a wider range of environmental conditions (Das, 2014).

Several studies have demonstrated the potential benefits of a combination of an EPF and a chemical insecticide to control whitefly species.

Control of *B. tabaci* by *Akanthomyces muscarium* was improved by application of the chemical insecticide matrine on cotton plants (Ali *et al.*, 2017). Synergism occurred between a range of combinations of the EPF and matrine. This synergism was attributed to a reduction in the enzyme acetylcholinesterase as a result of matrine and the EPF secondary metabolite, bassianolide, binding to the target site of acetylcholinesterase. In another study, low rate applications of imidacloprid combined with *B. bassiana* resulted in increased infection rate and mortality of *T. vaporariorum* (Feng *et al.*, 2004). Additionally, *B. tabaci* infested leaves of Chinese hibiscus (*Hibiscus sinensis*) treated with four different concentrations of spirotetramat, acetamiprid, imidacloprid or thiamexotham with conidial suspensions of *Cordyceps* (previously *Isaria*) *fumosorosea* at a ratio of 1:1 resulted in increased *B. tabaci* mortality compared to pesticides applied alone (Zou *et al.*, 2014). Such studies indicate a significant potential for combinations to be used in IPM for control of *T. vaporariorum*.

In principle, combinations of biological and synthetic pesticides could provide more efficient control of pests, but this needs to be based on knowledge about the nature of interactions. There would be no benefit in combining a biopesticide with a fully effective chemical insecticide i.e. one that kills all target pests. But, in practice, no chemical insecticide provides 100% efficacy and therefore there is room for improvement through a combination of insecticides (Nansen and Ridsdill-Smith, 2013). As mentioned previously, there are several additional benefits to application of a mixture of control agents, including; resistance management, targeting more than one pest or maintaining control over a range of environmental conditions. Therefore, to uncover the potential of mixtures for pest control, the types of interactions occurring between control agents must first be understood and quantified.

In order to determine which, if any, interaction is occurring between co-applied control strategies, the expected outcome of a mixture must first be calculated. To calculate the expected outcome of a mixture, outcomes of each mixture component when applied alone must be combined (Roell *et al.*, 2017). However, this is not just a sum of the mortalities individually caused by each agent. Instead, expected mortality is often calculated using one of two of the most successful approaches, Loewe additivity or Bliss independence, described in detail in Chapter 5. Once the expected outcome of the mixture is known, interactions resulting in deviation from the expected mortality can be determined.

There are four potential outcomes of a mixture (Table 1.2). Additivity in a mixture of two or more components occurs when the observed combined effect is no different to the expected combined effect calculated from the single compounds using reference models for non-interaction (M. J. Jonker *et al.*, 2005). Reference models for non-interaction are based on non-interaction equations relating to concentration addition or independent action, which are explained in detail in Chapter 5. Concentration addition equations are used to predict the outcome of mixtures involving control agents with the same mode of action, whereas independent action determines the expected outcome when mixture components have different modes of action (Loewe, 1928; Bliss, 1939). Synergism or antagonism can only be identified by deviation from additivity and the definitions of these outcomes should not vary dependant on field of research or aims of the stakeholder. For example, ecotoxicologists and organisations such as EFSA (European Food Safety Authority) use terminology referring to the effect of mixtures on non-target organisms in order to influence regulation of PPPs and other chemicals

released into the environment. From this perspective, synergism results in an undesirable increase in toxicity. Comparatively, in crop science, the motivation is to improve pest control through additive or synergistic mixture outcomes. It is important to apply strict definitions for types of mixture interactions and avoid biased descriptors such as positive or negative for synergism or antagonism to avoid confusion between stakeholders.

Antagonism occurs when there is an interaction between components in a mixture, leading the mixture to cause less mortality than expected (Schäfer and Piggott, 2018). Alternatively, synergism and potentiation result in higher mortality than expected for mixtures (Schäfer & Piggott, 2018). Synergism or potentiation is the most ideal outcome for mixtures applied in pest control, causing a greater decrease in the pest population without increasing the amount of active ingredients applied. However, there is a distinct difference between synergism and potentiation. The term synergism is used to describe the outcome of a combination of two insecticidal agents such as a microbial pathogen and a chemical insecticide, whereas potentiation describes the combination of an insecticidal agent with a non-lethal agent. For example, if we consider agent A is lethal when used on its own whilst agent B has a non-lethal effect on the pest. Potentiation has occurred if the combination of agent A and B results in a joint lethal effect that is greater than that of agent A. A well-known example of potentiation has been identified in honeybees exposed to demethylation inhibiting (DMI) fungicides. Honeybees are not killed by DMI exposure, but their P450 enzymes are inhibited. The inhibition of these enzymes reduces the ability of the insect to metabolise toxicants, such as chemical pesticides applied to crops. Chemical pesticides such as acetamiprid and spinetoram are more toxic to honeybees when exposed at the same time as DMI fungicides (Pilling and Jepson, 1993; Iwasa *et al.*, 2004).

Table 1.2 Potential outcomes resultant from combined applications of two or more pest control agents.

Term	Definition
Additivity	Two or more pest control agents causing the same effect as predicted by non-interaction equations relating to concentration addition or independent action.
Antagonism	Two or more pest control agents causing a smaller effect than predicted.
Synergism	Two or more pest control agents causing a larger effect than predicted.
Potentiation	The use of one or more pest control agents mixed with a component that causes no mortality on its own, causing a larger effect than predicted.

The success of an EPF within an IPM programme also depends on its compatibility with crop disease management, for example the application of fungicides, which are more likely to negatively impact EPFs than other groups of PPPs (Er and Gökçe, 2004). One of the major plant diseases for greenhouse crops is powdery mildew, first described in 300BC by Theophrastus and still a worldwide problem now (Linde and Shishkoff, 2003). This disease is caused by the obligate biotrophic plant pathogens, powdery mildew fungi (Phylum: Ascomycota). These fungi infect a wide range of crops, causing reduced photosynthesis when mycelia densely cover leaves, infection of flowers and fruit resulting in yield loss and ultimately necrosis and defoliation of the plant (Hacquard, 2014). Periodic application of fungicides is essential to prevent development of plant diseases but some fungicides can severely impact the growth, germination and infectivity of EPF (Roberti *et al.*, 2017). For example, *B. bassiana* is inhibited in *in vitro* growth experiments and in the field for control of Colorado potato beetle by the fungicide mancozeb (Clark, 1982; Jaros-Su *et al.*, 1999), but increasing the dose of *B. bassiana* can make the impact of mancozeb less detectable (Jaros-Su *et al.*, 1999). However, other fungicides have no impact on EPF and some can enhance growth and sporulation (Tkaczuk *et al.*, 2015; Fiedler and Sosnowska, 2017). It has been shown that the variability in compatibility is due to certain chemical groups not targeting EPF and the natural resistance of the EPF to some active ingredients. In a study by Roberti *et al.* (2017), nine commercially sold fungicides were combined in a tank mixture with *B. bassiana* against *T. vaporariorum* on Zucchini plants. Seven of the fungicides had little to no effect on *B. bassiana* growth *in vitro* and the efficacy of the EPF to control whitefly in the field was not compromised when combined with either of these fungicides. On the other hand, fungicides Signum ® and Switch ® inhibited fungal growth and reduced whiteflies succumbing to fungal infection by 91.2% and 87.4% respectively (Roberti *et al.*, 2017). However, the fungicides Signum and Switch contain active ingredients (pyraclostrobin, boscalid, cyprodinil and fludioxonil) which inhibit enzyme activity essential for spore germination and survival (Roberti *et al.*, 2017).

Though the application of multiple pest control strategies is the cornerstone of IPM, the interactions of different elements within an IPM are poorly understood. There has been too much focus on the research of individual elements of the IPM system, rather than a holistic approach (Dara, 2019). It is essential to study the interactions of each component of the IPM system in order to predict and understand crop protection outcomes. Determination of interactions between mixture components is complicated because the type of interactions occurring can vary depending on the concentration of each component (Feng *et al.*, 2004), the

ratio of components (Zou *et al.*, 2014) and the type of application; sequential or simultaneous. In addition, interactions between mixture components may be influenced by biotic factors such as varying susceptibility of different insect stages (Wari *et al.*, 2020) and interactions between the target pest and the host plant (Ocampo-Hernández *et al.*, 2019).

Abiotic factors are also likely to affect the types of interactions between EPF and chemical insecticides and we are only starting to understand the complexity involved in mixture interactions. In a study by Inglis *et al.* (1997), grasshoppers consuming lettuce treated with co-applied *Metarhizium flavoviride* and *B. bassiana*, were equally likely to be killed by either EPF at 25°C, but *B. bassiana* had a competitive advantage at lower temperatures (13-25°C) and *M. flavoviride* caused higher mortality at 25-30°C (Inglis *et al.*, 1997). The disparate temperature profiles of each EPF resulted in an increase in mortality of grasshoppers treated with the mixture compared to that caused by a single EPF at higher temperatures, and similar mortality values at lower temperatures (Inglis *et al.*, 1997). Although, the benefits of this combination were shown to be minimal when temperatures were oscillated to imitate fluctuations which occur in the field (Inglis *et al.*, 1999).

It is unknown whether the temperature profile of an EPF, determined by constant temperature experiments, could determine the interactions between co-applied EPF and chemical insecticides when exposed to oscillating temperatures such as those observed in the greenhouse.

1.4 Aims and Objectives

The aim of this project was to increase our understanding of interactions between EPF and other aspects of the IPM system to improve efficacy in control of *T. vaporariorum*. There is some evidence that interactions between components of the IPM system, such as EPF and synthetic insecticides can result in synergism. Therefore, this project aims to develop techniques and experimental protocols to identify the relationship between multiple elements in an IPM system and determine how environmental stress can influence interaction outcomes. A selection criteria based on the virulence of EPF as well as their thermal profiles and compatibility with chemical pesticides were used to identify isolates with potential as biopesticides in an IPM system against *T. vaporariorum*. The steps taken to collect essential information for this thesis are outlined in figure 1.6 and the aims described above were achieved through the following objectives in each chapter;

Chapter 3: Develop a bioassay to accurately determine the effects of applications of candidate insecticides, fungicides and EPF on *T. vaporariorum* mortality.

Chapter 4: Predict cardinal temperatures for EPF candidates based on growth and germination data using non-linear mathematical models. Quantify the effect of temperature on the *in vitro* sporulation of EPF isolates and identify *in vitro* interactions between EPF and synthetic chemical insecticides or fungicides. This was necessary to select EPF isolates to be used in further experiments.

Chapter 5: Quantify the effect of co-application of EPF isolates with the synthetic insecticide, spiromesifen, and determine the effect that an oscillating temperature regime has on these interactions.

Chapter 6: Assess the ability of EPF and synthetic insecticide combinations to control *T. vaporariorum* in greenhouse trials.

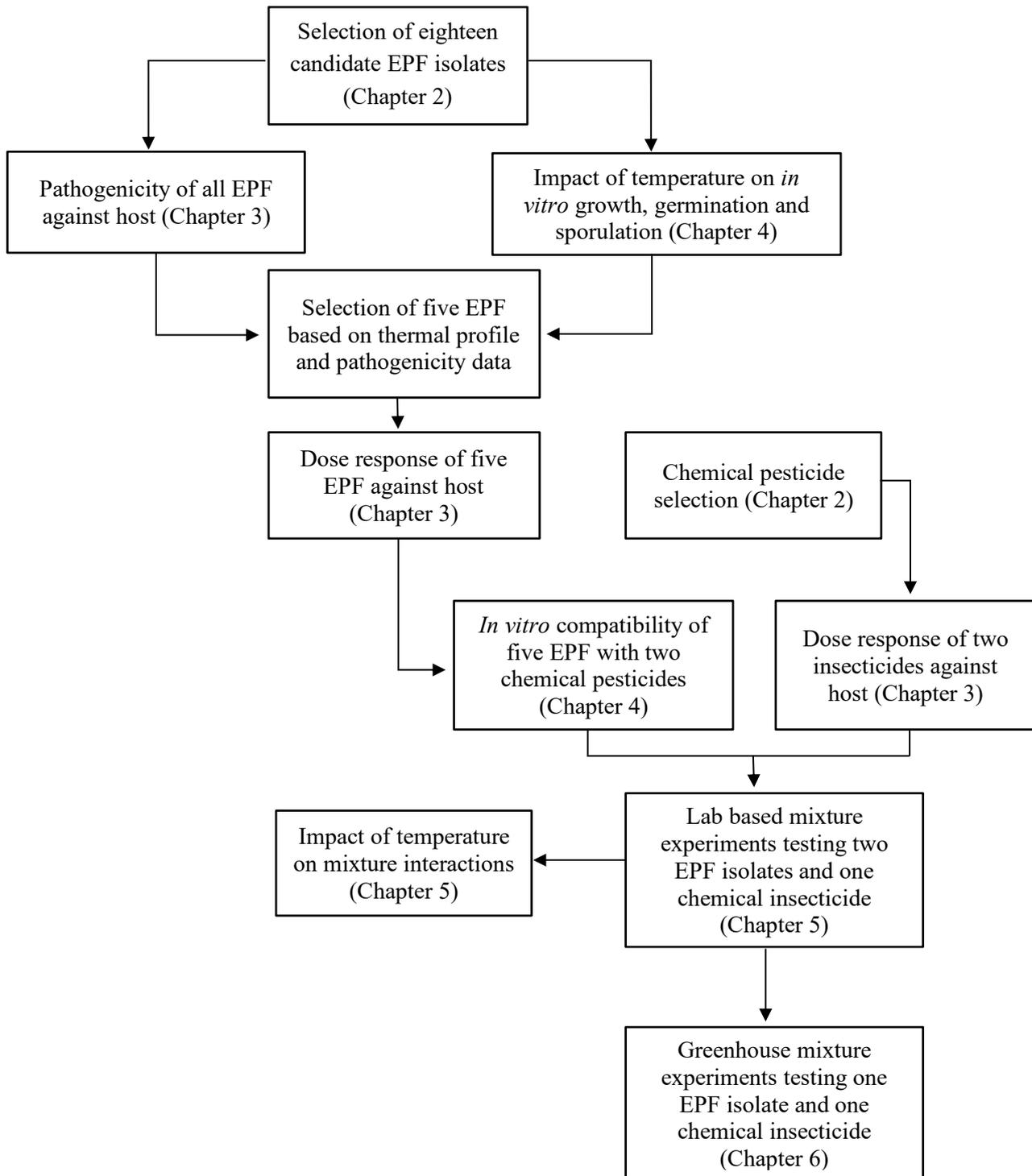


Figure 1.6 Stepwise approach taken to develop techniques and experimental protocols to identify the relationship between multiple elements in an IPM system and determine how environmental stress can influence interaction outcomes of mixtures used in control of *Trialeurodes vaporariorum*.

2 General materials and methods

2.1 Rearing *Trialeurodes vaporariorum*.

A stock culture of *Trialeurodes vaporariorum* was obtained from a colony held at the University of Warwick (UK) which originated from a natural population found in Evesham, UK in 2018. Stock *T. vaporariorum* were subsequently maintained on small aubergine plants (*Solanum melongena* var. Paris; Ramiro Arnedo, Spain) in 60x60x60cm nylon and Perspex insect rearing tents (BugDorm-2[®] insect rearing tent, Watkins & Doncaster, UK). The *T. vaporariorum* cultures were maintained at 24 ± 0.5 °C under a light: dark 16:8h photoperiod. Every 14 days, fresh un-infested plants were added to the rearing tents and dead plants were removed. Each rearing tent contained at least four aubergine plants at any one time and *T. vaporariorum* were moved to fresh plants by placing clean plants amongst the heavily infested older plants. *Trialeurodes vaporariorum* nymphs are unable to move on the leaf surface until emergence as adults so to encourage *T. vaporariorum* adults to actively move to fresh plants, adults were disturbed by gently shaking leaves on infested plants.

2.2 Plant cultures

Aubergine seedlings used for bioassays were established from seeds sown individually in small plastic pots (4.5x4.5x5.5cm) in 50g of compost (John Innes Seed and Cutting compost) and were kept pesticide free. A specific variety of aubergine was used for rearing insects and for all experimental work based on the successful use of the same plants by BASF in greenhouse trials in Utrera, Spain (*Solanum melongena* var. Paris; Ramiro Arnedo, Spain). Plants were grown in a greenhouse under a light: dark 16:8h photoperiod with supplemented overhead lighting to ensure a minimum light intensity of $300 \mu\text{mol}/\text{m}^2/\text{s}$. Temperature in the greenhouse was maintained at 25 ± 3 °C during daylight hours and 15 ± 2 °C at night. Seedlings were approximately 7 weeks old and at the 2-3 true leaf stage when they were used in bioassays. Every 2 weeks, at least 50 seeds were sown and placed in trays inside insect rearing tents to prevent pests attacking the stock culture of aubergine. Stock plants were always watered or planted before any work involving *T. vaporariorum* was conducted on a day-to-day basis. Despite this, some pest species managed to enter the greenhouse and feed on the aubergine. Clean aubergine plants were never exposed to chemical pesticides but in the case of an infestation of aphids, thrips or spider mite, plants were dipped in Savona (a mixture of natural fatty acids; Koppert, Berkel-Rodenrijs, The Netherlands). Sciarid flies were removed by treating the soil of aubergine plants with Nemasys[®] (nematode treatment for fruit and vegetable protection, BASF, Ludwigshafen,

Germany). Plants treated with Savona or Nemasys were not used in bioassays or to supplement the *T.vaporariorum* stocks until at least 8 weeks after application.

2.3 Fungal cultures

2.3.1 Collection

Fungal isolates were obtained from several sources (Table 2.1). The USDA (United States Department of Agriculture) Agricultural Research Service collection of entomopathogenic fungal cultures supplied eleven isolates. Five isolates were from the University of Warwick and a commercial sample of *Beauveria bassiana* isolate PPRI5339 was supplied by the commercial company BASF. The remaining isolate was provided from the UK Centre for Ecology & Hydrology, Wallingford, UK culture collection. Isolates were selected from origins of varied climates and were originally isolated from a number of different host species including, *Bemisia tabaci*, *T. vaporariorum* and Hemiptera: Aphididae. Re-isolations of fungi purchased from commercially available biological control products were also used (see Table 2.1). Samples from the ARSEF database were sent as either fungal isolates growing on Sabouraud dextrose agar (SDA) or lyophilised stocks in glass vials. For lyophilised stocks, 500 µl of sterile distilled water was pipetted into each vial and left for several minutes. The contents of each vial was then spread onto ¼ strength SDA (diluted by adding sterile deionised water). A weak medium was chosen because the fungi had been lyophilised with milk powder and therefore required less nutrient content for sporulation. Samples were spread onto 10mL Sabouraud Dextrose Agar (SDA; 65g/ 1L deionised water) in 90mm Plastic triple vented Petri dishes.

Table 2.1 Identification of Entomopathogenic fungi used in bioassays against third instar greenhouse whitefly (*Trialeurodes vaporariorum*).

ATCC ID/ Name	Species	Strain	Host ^a	Origin ^a
ATCC 5278	<i>Beauveria bassiana</i>		<i>Bemisia tabaci</i>	Vermont, USA
ATCC 6920	<i>Beauveria bassiana</i>		<i>Trialeurodes vaporariorum</i>	Canada
ATCC 6921	<i>Beauveria bassiana</i>		<i>Trialeurodes vaporariorum</i>	Canada
ATCC 9451	<i>Beauveria bassiana</i>		<i>Trialeurodes vaporariorum</i>	Kazakhstan
Botanigard	<i>Beauveria bassiana</i>	GHA	<i>Diabrotica undecimpunctata</i>	Product
GHA	<i>Beauveria bassiana</i>	GHA	<i>Diabrotica undecimpunctata</i>	Oregon, USA
PPRI5339	<i>Beauveria bassiana</i>	PPRI 5339	<i>Conchyloctenia punctata</i>	Product
ATCC 7477	<i>Cordyceps javanica</i>		<i>Trialeurodes vaporariorum</i>	Argentina
ATCC 4412	<i>Cordyceps farinosa</i>		<i>Trialeurodes vaporariorum</i>	Malaysia
ATCC 2658	<i>Cordyceps fumosorosea</i>		<i>Trialeurodes vaporariorum</i>	Florida, USA
PFR	<i>Cordyceps fumosorosea</i>	Apopka 97	Aphididae sp.	USA
ATCC 4205	<i>Cordyceps fumosorosea</i>		<i>Trialeurodes vaporariorum</i>	Malaysia
ATCC 4060	<i>Akanthomyces lecanii</i>		<i>Trialeurodes vaporariorum</i>	Malaysia
ATCC 972	<i>Akanthomyces lecanii</i>		<i>Trialeurodes vaporariorum</i>	Poland
ATCC 6544	<i>Akanthomyces lecanii</i>		<i>Trialeurodes vaporariorum</i>	UK
Vertalec	<i>Akanthomyces muscarium</i>	Ve6	N/A	Product
Met 52	<i>Metarhizium brunneum</i>	F52	N/A	Product
Bioblast	<i>Metarhizium anisopliae</i>	ESC1	N/A	Product

^a Host and origin: Information taken from the ARSEF database.

2.3.2 Storage

Handling of fungal isolates was performed under sterile conditions in a laminar flow hood. All equipment and solutions were sterilised in an autoclave prior to use. Fungal isolates from commercial products were re-isolated by spreading the product pellets/powder across a selective SDA in Petri dishes using sterile plastic spreaders. Selective SDA was prepared by allowing the agar to cool to approximately 40°C before adding Dodine (0.1g in 1mL deionised water), a fungicide and the antibiotic, 500µl chloramphenicol (prepared as 1g/10mL 95% ethanol) to liquid SDA. Subcultures were then taken from the selective agar and spread onto standard SDA.

All Petri dishes were sealed with Parafilm[®] and then incubated in the dark at 25°C for 1 week. After this incubation period, three plugs were taken from each Petri dish using a sterile 'cork' borer (7mm diameter) and stored in 1mL aqueous glycerol (10% by volume) to prevent the formation of ice crystals in the biological material during freezing. Several plugs were taken of each entomopathogenic fungus (EPF) to create a stock of stored isolates. The fungal isolates were stored in cryovials (1.2mL, Thermo Scientific[™] Nalgene[™]) at 4°C overnight to allow the glycerol solution to be absorbed into fungal cells. Cryovials were then stored at -80°C as first or second subcultures only, to prevent attenuation of virulence through repeated sub-culturing.

2.3.3 Harvesting conidia and preparing suspensions

Fungal isolates were removed from the -80°C freezer and left to defrost at room temperature for approximately 30 minutes. Following this, all procedures were conducted under a sterile laminar flow hood. The contents of each cryovial was mixed using a sterile plastic pestle before being spread onto 10mL SDA within a 90mm Petri dish using the same pestle. The content of each cryovial was spread across three separate Petri dishes, approximately 0.3mL in each. Plates were sealed with Parafilm[®] and incubated at 25°C for 14 days in the dark. To prepare conidial suspensions for bioassays, conidia were removed by agitating the surface of the dish using a sterile pestle and approximately 3mL of Tween 80[®] (0.03% v/v). The suspension was then filtered by pouring the liquid onto double folded sterile muslin cheese cloth and allowed to drip through into a sterile 25mL tube placed below. This process successfully removed all mycelia and culture debris in the suspension. Vials containing conidial suspensions were then agitated for two minutes by shaking vigorously on a vortex mixer. Concentrations of the resultant stock conidia suspensions were estimated by counting in an Improved Brightline Neubauer haemocytometer (x400 magnification) and subsequently diluted in sterile 0.03% Tween 80[®] to give the desired final concentration.

Conidial suspensions were kept on ice during this process to prevent germination. Generally, collecting conidia from 6 Petri dishes of the same isolate resulted in 20mL suspensions containing 1×10^8 conidia mL^{-1} . However, there were variations in the number of conidia counted due to differences in isolate characteristics, such as conidia production and varying difficulty in suspending conidia. Conidia suspensions were kept on ice in an insulated container at 4°C in the dark for no more than 24 hours before being used in experiments. On the same day as experimental set up, samples of suspension were observed under the microscope in a haemocytometer to check whether conidia had germinated. No conidia germinated overnight whilst following this procedure.

2.3.4 Spraying treatments

All treatments of fungi, insecticides, fungicides or a mixture of these were applied using a mini spray tower which was built and calibrated at the UK Centre for Ecology and Hydrology (Wallingford, UK) using methods described by Spence et al. (2020). All treatments had a volume of 1mL and were applied at 138 kPa. Spray applications were performed under sterile conditions within a fume hood. The spray tower was cleaned between applications by running ethanol (70%) through the artist spray gun, followed by 0.03% Tween 80. The spray tower cylinder and fume hood surface were cleaned using surface disinfectant (Rely+On™ Virkon®) and wiped dry. Further details on design and calibration of the spray tower can be found in chapter 3.

2.3.5 *In vitro* assessment of germination

Several experiments involved the quantification of germinated EPF conidia. Suspensions containing EPF conidia were diluted to 1×10^6 conidia mL^{-1} before $4\mu\text{l}$ of each suspension was placed in the centre of a 90mm Petri dish containing 10mL SDA. A small cross was drawn on the underside of the Petri dish so that conidia could be easily found at a later time. Several germination experiments were conducted during the project, incubated at several temperatures and running for varied amounts of time depending on the isolate being tested. The germination process was stopped by placing one droplet of lactophenol cotton blue on top of the germinating conidia which kills the fungus, preventing further development and stains chitin in the cell walls of conidia to improve visibility under the microscope. Plates were stored in the fridge (4°C) until conidia were counted. The number of germinated conidia was counted in random fields of view using a microscope (x400 magnification). Conidia were counted as being germinated when the length of a germ tube was at least as long as the width of the conidium that it originated from (Figure 2.1). At least 300 conidia were counted per plate.



Figure 2.1 An example of a random field of view of SDA at 400x magnification showing germinated and un-germinated conidia of an EPF stained with 10% lactophenol cotton blue.

2.3.6 *In vitro* assessment of growth

Isolates were removed from the -80 freezer, thawed, spread onto 10mL SDA and stored at 25°C following the methods in section 2.3.3. After 3 days, plugs of unsporulated mycelia were taken from the plates using a sterile metal cork borer (6mm diameter) and placed upside down in the centre of 90mm Petri dish containing 22mL SDA so the mycelia were in direct contact with the SDA. Plates were sealed with Parafilm® and placed in the dark in an incubator for the duration of the experiment. Radial growth was measured every 48 hours using electronic callipers (SparkFun TOL-10997 6 inch digital callipers) along two cardinal diameters which were drawn onto the base of the Petri dish (Figure 2.2). Fungal growth was measured for 24 days or until the fungus had grown to the limits of the Petri dish.

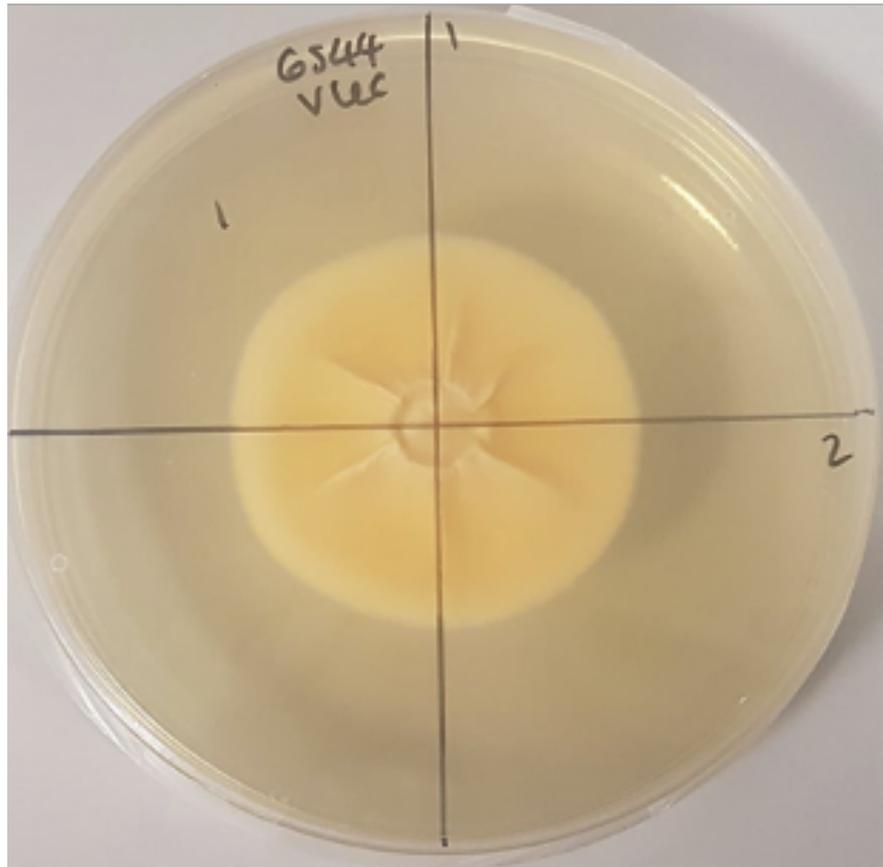


Figure 2.2 Photograph showing the underside of a Petri dish with an x-y axis drawn to aid in measuring rate of growth of entomopathogenic fungi.

2.4 Rationale for selecting control agents

2.4.1 Rationale for selection of insecticides

Chemical pesticides and fungicides used in this study were chosen based on product popularity with UK and Spanish vegetable greenhouse growers and the potential for compatibility with EPF based on anecdotal evidence. Insight was provided by industrial partners and independent advisors. One of the pesticides used in experiments was Admiral advance® (10 EC: 100g/L pyriproxyfen, Sumitomo). This pesticide is a water-based insect growth regulator sold for the control of *Bemisia tabaci* and *Trialeurodes vaporariorum* on cotton, cucurbits, sweet potatoes and fruiting vegetables.

The second chemical pesticide chosen for experimentation was Oberon® (spiromesifen 240g/L; SC 240 Bayer) which contains the active ingredient spiromesifen. This insecticide is sold for control of whitefly and mites on field and protected crops and prevents moulting and further development of immature stages.

Presently, chemical fungicides are routinely applied to most greenhouse crops and can provide high efficacy of disease removal and prevention, although such frequent applications provide a strong selection pressure for resistance development (Hahn, 2014). In

this study, three topical fungicides which are regularly used against powdery mildew in greenhouse crops were selected. Namely, Luna Sensation® (250 g/L (25 % w/v) fluopyram and 250 g/L (25 % w/v) trifloxystrobin, Bayer), Kumulus®(80% w/w sulphur, BASF) and Takumi® SC (100g/l cyflufenamid, Certis).

2.4.2 Preparing insecticide and fungicide solutions

All insecticide and fungicide solutions were prepared in a Class II safety cabinet in sterile glass Duran® bottles. In all experiments, active ingredients of the chemical pesticides, spiromesifen and pyriproxyfen, were diluted in a stock solution equivalent to the recommended application rate as stated in the manufacturer's instructions or double this concentration for mixture bioassays. Recommended application rates and the equivalent dilutions used in preparation of laboratory stocks can be seen in Table 2.2. All chemicals diluted in sterile deionised water were prepared on the same day that the experiment was performed and kept at room temperature until being used.

In bioassays investigating the effects of mixtures, stock solutions were made at double the recommended application rate. As such, once the treatment is mixed in a 1:1 ratio, the final solution is equal to the recommended application rate.

For experiments investigating *in vitro* interactions of EPF and synthetic chemicals, agar preparations were made by autoclaving SDA (32.5g/500mL) in 500mL glass Duran® bottles (121°C, 15PSI). Once the agar had cooled to 40°C, the same volume of solution for each insecticide and fungicide was added to the agar. Spiked agar was thoroughly mixed before 10mL was poured into individual 90mm diameter Petri dishes and stored at 4°C overnight. All Petri dishes prepared in this way were used in experiments no later than 24 hours after being poured.

Table 2.2 Chemical insecticides and fungicides used in *in vitro* and *in vivo* experiments. Recommended application rates were taken from the label as advised for the crop most similar to the experimental system.

Commercial name	Active ingredient	Company	Target crop	Recommended application rate (per hectare)	Equivalent dilution for laboratory stocks (product in sterile water)
Oberon®	Spiromesifen 240g/L	Bayer	fruiting vegetables	75mL in 100L water	75µl Oberon in 100mL
Admiral®	Pyriproxyfen	Sumitomo Chemical	fruiting vegetables	500mL in 100L	500µl in 100mL
Takumi ® SC	100 g/l Cyflufenami d	Certis	fruiting vegetables	0.15L in 500L	150 µl in 500mL
Kumulus®	80% w/w sulphur	BASF	blackcurrants and strawberries	20g in 10L	0.01g in 500mL
Luna Sensation®	Fluopyram 250 g/L Trifloxystrob in 250 g/L	Bayer	strawberries	0.8L in 300L	1.33 mL in 500mL

2.4.3 Selection process for isolates included in each experiment

Throughout this study, a series of laboratory experiments were conducted to identify EPF candidates with potential to control *T. vaporariorum* under greenhouse conditions in combination with a chemical insecticide. Isolates included in each experiment were chosen based on several selection criteria.

The selection criteria applied to the EPF were related to pathogenicity and virulence, speed of kill, sensitivity to abiotic factors as well as the compatibility of EPF with chemicals used in IPM. In addition, the ease of rearing isolates on artificial media and the number of conidia produced across a range of temperatures was used as a proxy to mass production. The isolates included in each experiment are indicated in figure 2.3. The selection rationale for isolates included in further experiments are discussed in further detail in each chapter, following the results of each experiment. Initially, eighteen isolates were chosen originating from a range of climates with reported pathogenicity against *T. vaporariorum*. Inclusion of isolates in germination assays was based on their *in vitro* growth temperature optima and limits, ensuring

that there was a mix of isolates that could grow at high or low temperatures, or across a broad range of temperatures. The ability of the isolates to produce large quantities of conidia was also a selection factor. Following this, Pareto Dominance rankings (chapter 3) were used to determine isolates which caused the highest mortality as well as demonstrating fast growth and germination rates across a range of incubation temperatures. Those ranked highest based on these criteria were included in dose response bioassays (chapter 3). *In vitro* compatibility of five EPF isolates with two chemical insecticides are described in chapter 4. These isolates were chosen based on the suitability of lethal concentrations determined from dose response curves and consistency in efficacy across bioassays. Next, *in vivo* interactions between spiromesifen and two EPF isolates were investigated in laboratory based experiments. These EPF isolates were selected based successful combinations in the *in vitro* compatibility tests as well as ensuring that temperature profiles of the EPF were relatively dissimilar. Dissimilar temperature profiles were chosen to determine whether mixture interactions under variable temperature regimes were influenced by characteristics of the EPF identified in chapter 4. Finally, greenhouse mixture application trials were conducted with *Cordyceps farinosa* and spiromesifen based on the interesting and complex interactions discovered in laboratory based mixture experiments (chapter 5).

Chapter 3 & 4
In vitro growth and sporulation assays
 Pathogenicity bioassays

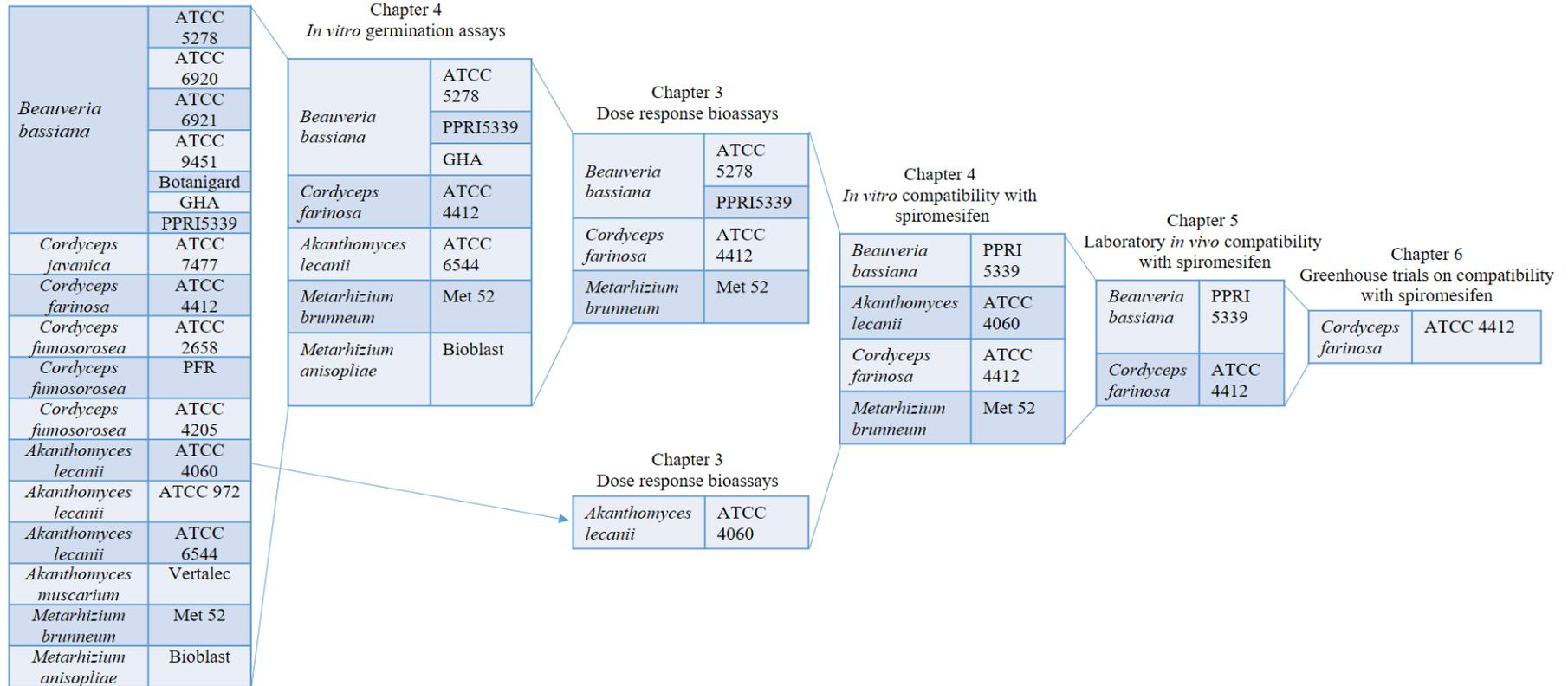


Figure 2.3 Selection process for isolates of entomopathogenic fungi included in experiments throughout this study. Some experiments in chapter 3 and 4 did not follow a chronological order, therefore chapter numbers do not always increase across the flow chart.

2.5 Laboratory bioassay methods

2.5.1 Preparing third instar *Trialeurodes vaporariorum* nymphs

A method was developed to ensure that nymphs were constrained to an area of the leaf that received uniform conidia deposition during spray applications. Methods to determine spray deposition across the leaf surface are described in detail in chapter 4. Clip cages (similar to those used to confine aphids individually to plant leaves (MacGillivray and Anderson, 1957) were designed using truncated plastic pots (base diameter of 40mm and top diameter of 50mm; 25mL frosted container from Ashwood[®], Derby, UK; Figure 2.4). A 15mm diameter circle was cut into the centre of the base of each plastic pot to allow a limited area of the leaf to be exposed for *T. vaporariorum* adults to oviposit i.e. corresponding to the central target area identified in sprayer calibration experiments. The top of the pot was covered with fine nylon mesh (0.5 x 0.5mm) and secured using hot glue applied by a glue gun (Tacklife 20W hot glue gun GGO20AC). Metal hair clips were bent and glued to the side of each pot and to 30mm diameter plastic acetate discs (0.3mm thickness). The acetate disc held the cage flat to the ventral leaf surface and ensured the cage was as ‘light-weight’ as possible to minimise damage to leaf surface tissues. *Trialeurodes vaporariorum* adults were taken from the stock culture using a pooter (Watkins & Doncaster; Pyrex glass pooter E709) and gently blown into the clip cage pots by blowing into the pooter. Each clip cage, containing 10-20 male and female adult *T. vaporariorum*, was attached to the youngest available leaf on 7 week old aubergine plants. Individual plants were contained within ventilated 0.9 L transparent plastic pots (dimensions: height 14.3 cm; width (rim, base) 9.4 cm, 6.7 cm with a circle of nylon mesh for ventilation added to the lid and sealed with formalin; diameter 3.4cm) as shown in figure 2.5. Pots were maintained for 14 hours at 24°C under a 16:8h light: dark photoperiod and after this period the adult *T. vaporariorum* were removed using a pooter and gently placed back into the stock culture by placing the disassembled pooter inside the rearing tent and allowing adults to fly out. Eggs laid by *T. vaporariorum* adults on experimental plants were left to develop on individual plants *in situ* inside the ventilated plastic pots for approximately 16 days until mostly third instar occupied the leaf. Once plants were ready for use in the experiments, the number of nymphs on each leaf were counted using a clicker counter and a 40x magnification stereo microscope. Plants were grouped so that each treatment had a similar number of nymphs spread across the replicates. For example, most bioassays had at least 100 nymphs per treatment but did not have an equal number of nymphs on each replicate leaf for each treatment. Some second instar whitefly were included in the bioassays to avoid the faster developing nymphs becoming late third instar before treatment.



Figure 2.4 Clip cages used to expose adult *Trialeurodes vaporariorum* to a limited area on the leaf surface. Clip cages were made from truncated plastic pots, acetate discs, metal hair clips and fine nylon mesh.



Figure 2.5 Transparent plastic pots (0.9L) used to contain individually treated aubergine plants during laboratory based bioassays. Dimensions: height 14.3 cm; width (rim, base) 9.4 cm, 6.7 cm with a circle of nylon mesh for ventilation added to the lid and sealed with formalin; diameter 3.4cm.

2.5.2 Assessment of Bioassays

Throughout the duration of each bioassay, the instar, emergence and mortality of *T. vaporariorum* was recorded every 48 hours for a total of 14 days following spray applications.

During each assessment, individual trays were removed from the CT rooms and treated leaves were observed under a 40 x magnification stereo microscope. For each replicate, the time of assessment, nymphal instar, emergence and mortality of *T. vaporariorum* was recorded. *T. vaporariorum* instar was determined by size and the presence or absence of red eyes; fourth instar nymphs can be readily identified by the red eyes of the developing adult which are visible through the integument. Nymphs infected with an entomopathogenic fungus appeared pink, brown or white depending on the species of fungus applied, whereas dead nymphs resulting from handling or other unknown causes appeared orange and desiccated. If cadavers were infected with an entomopathogenic fungus, hyphal growth emerged from the cadavers several days after death. Mortality caused by pesticide application resulted in brown, sometimes black cadavers flattened to the surface of the leaf. Dead nymphs were not removed from the leaf surface, as the desiccated cadavers would be difficult to remove, causing leaf damage and secondary infection of healthy nymphs from sporulating cadavers was unlikely due to their immobility. Occasionally, whitefly adults died during emergence from their puparium. These were included in the dataset and were not categorised as succumbing to the pathogen. Cumulative mortality and emergence were checked on the final day of assessments by counting the total number of cadavers and empty pupal cases which remained on the leaf.

2.5.3 Correcting for control mortality

During laboratory and greenhouse scale experiments, it is common for a certain number of whitefly to die during the bioassay due to causes other than the chemical or fungus applied to the plant. In order to determine the number of deaths caused by the substances applied during bioassays, the ‘background’ mortality was accounted for. This was done using Schneider-Orelli’s equation (Schneider-Orelli, 1947);

$$\text{Corrected mortality (\%)} = \left(\frac{a-b}{100-b} \right) * 100 \quad \text{Equation 1}$$

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

Abbotts formulae is also a common adjustment for control mortality, however, Abbot’s formulae uses the number of individuals rather than proportion or percentage data (Abbott,

1925). In these experiments the number of *T. vaporariorum* individuals in the control and treatment groups were not the same. Therefore, Schneider-Orelli's formulae was preferred (Puntener, 1981).

For treatments resulting in a negative value after this adjustment, mortality was set as 0. This only occurred when mortality was lower than in the control group. For analysis of mortality over time and MixTox analysis, uncorrected data were used.

2.5.4 Modelling dose response data

Several dose response bioassays were conducted during this project. Analysis of concentration or dose response bioassays allows the estimation of lethal concentrations (LC), or concentrations that result in an expected proportion mortality of *T. vaporariorum*. The effect of both concentration applied and dose received on *T. vaporariorum* mortality was analysed for each EPF and concentration applied for chemical pesticides using the DRC package in RStudio.

Following the protocol for analysis of binomial data in the DRC package (analysis of dose-response curves), five common models were tested for suitability: three log-logistic models and two Weibull models. The generalized log-logistic model has the following equation:

$$f(x, (b, c, d, e)) = c + \frac{d-c}{1+\exp(\exp(b(\log \log(x) - \log \log(e))))} \quad \text{Equation 2}$$

Where b is the slope of the dose-response curve, c and d represent the upper and lower limits of the response, and e is the LC_{50} . (Figure 2.6A).

The Weibull models are also commonly used to fit dose response data and can account for any asymmetry in the data (Cockerton, 2013).

The Weibull-1 model:

$$f(x, (b, c, d, e)) = c + (d - c)\exp(-\exp(b(\log(x) - \log(e)))) \quad \text{Equation 3}$$

The Weibull-2 model:

$$f(x, (b, c, d, e)) = c + (d - c)(1 - \exp(-\exp(b(\log(x) - \log(e)))) \quad \text{Equation 4}$$

Where b is the slope of the curve, c and d are the lower and upper limits of the model, and e represents the LC_{50} (Figure 2.6B).

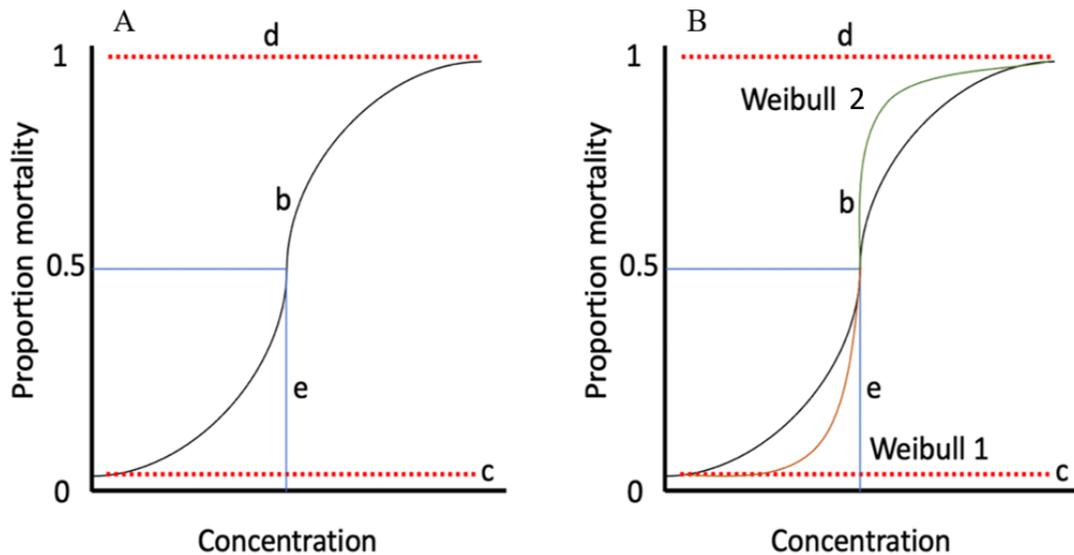


Figure 2.6 A) An example of a four parameter log logistic model, where b is the slope of the dose-response curve, c and d represent the upper and lower limits of the response, and e is the LC_{50} . B) Weibull models following equations involving the same four parameters, where b is the slope of the curve, c and d are the lower and upper limits of the model, and e represents the LC_{50} . The Weibull- 1 model has a steep slope towards the lower asymptote, whereas the Weibull- 2 model has a steep slope towards the upper asymptote.

Firstly, data was fitted to a two-parameter log logistic model which specifies that c is set to one and d is set to zero. Then, these parameters were altered in order to create the three and four parameter models. In a three-parameter log logistic model, c is set to 0. In a four-parameter log-logistic model, parameters are estimated from the experimental data.

A goodness of fit test based on Pearson's Chi-squared test was used to determine the most appropriate model. A non-significant P-value (>0.05) indicates no deviation from the theoretical model and therefore no lack-of-fit. The higher the P-value above 0.05, the better the fit of the model. If more than one model showed a significant fit, the model showing the highest P-value was used (Crawley, 2013. p516, p549).

Once the most appropriate model was fitted for each bioassay, differences between treatments were determined through model simplification, for example, reducing from separate dose response curves for different EPF isolates to one model fitting all EPF isolates. Models with undifferentiated slope and LC_{50} values (i.e. with the same and/or slope and LC_{50}) were fitted to the data and compared to the original differentiated models. Nested models were compared using an ANOVA F test, whereby the most simplified model was chosen if the fit was better, or equally as good as the more complex model. If a non-significant P- value was produced, there was no significant difference in the fit of the complex model compared to the simpler model, so the simple model was chosen. A significant value indicated that additional parameters in the complex model improved the fit of the model to the data. There were

significant differences between the dose responses of different isolates if the undifferentiated model did not successfully describe the data. Significant differences between bioassays conducted at different time points were tested by comparing model fit of a recurring EPF isolate used in each group (a 'standard' isolate used in each assay to allow comparability between assays), following the same procedure as described above.

Once the best fit model was determined, model values were extracted to determine different LC values. This process was also used to analyse mortality over time, whereby concentration was replaced with time in order to calculate LT_{50} values (time to 50% mortality after application of treatments).

3 Development and validation of a standardised bioassay to screen entomopathogenic fungi against *Trialeurodes vaporariorum*

3.1 Introduction

The greenhouse whitefly (*Trialeurodes vaporariorum*) is a highly polyphagous homopteran pest of several host plant species (Capinera, 2001) and causes extensive pest damage to crops globally. The species originates from tropical or subtropical America, (Mound and Halsey, 1978) but has become established in all continents except Antarctica (De Vis *et al.*, 2002). Direct plant damage is caused by adults and nymphs feeding on phloem, while indirect damage occurs through secretion of honeydew which supports growth of sooty moulds (*Cladosphaerospermum* spp.) that reduce plant photosynthesis, leading to stunted growth (van Lenteren and Noldus, 1990). However, the main problems occur through transmission of *Criniviruses* (positive sense single-stranded RNA viruses vectored exclusively by whitefly) (Wintermantel, 2004). Crop damage caused by *T. vaporariorum* vectored viruses is estimated to cost the global agricultural economy more than \$US1 billion a year (Gonzalez *et al.*, 1992; Legg *et al.*, 2006; Aregbesola *et al.*, 2019).

Management of *T. vaporariorum* using synthetic chemical pesticides is made difficult because of widespread pesticide resistance. Resistance has been documented to all major pesticide groups including pyrethroids, organophosphates, carbamates, neonicotinoids, as well as the insect growth regulator buprofezin (Gorman *et al.*, 2002; Bi and Toscano, 2007a; Jeschke and Nauen, 2008; Insecticide Resistance Action Committee (IRAC), 2019). Biological control of *T. vaporariorum* has been used successfully by growers of protected crops in the UK and the Netherlands for over 40 years (Gorman *et al.*, 2002). The most popular approach is the inundative application of parasitoids (mainly *Encarsia formosa* and *Eretmocerus eremicus*) and predators (such as mites (e.g. *Amblyseius swirskii*, *Amblyseius montdorensis*), beetles (e.g. *Delphastus catalinae*) and mirids (e.g. *Macrolophus pygmaeus*)) (van Lenteren *et al.*, 1996; Gorman *et al.*, 2002). There are occasions when whitefly populations can outstrip the ability of predators and parasitoids to control them, and under these circumstances, conventional pesticides are applied as a supplemental treatment. However, given the problems with pesticide resistance, there is a need for alternative interventions as part of a more sustainable, integrated pest management approach. Biopesticides (increasingly referred to as bioprotectants) are microorganisms, plant extracts or semio-chemicals that are used against pests in plant protection (Gwynn, 2014). In 2018, biopesticides represented 3.6% of the \$56 billion pesticide market, with a compound annual growth rate of 14-32% (IBMA, 2020). The number of registered biopesticides in Europe is increasing which is largely due to legislation introduced by the EU Sustainable Use

Directive on pesticides (2009/128/EC) which aims to prioritise non-chemical alternatives over chemical control of insect pests as part of IPM (European Commission, 2009). The research and development of biopesticides costs significantly less than synthetic pesticides, but production costs of microbes are higher which often results in a higher purchase price for biopesticides compared to synthetic pesticides in order to recover costs (Glare *et al.*, 2012). However, it is possible to avoid development of un-profitable biopesticide products by considering production costs early in the research and development process (Ravensberg, 2011).

The most effective microbial pathogens against *T. vaporariorum* are entomopathogenic fungi (insect killing fungi; EPF) which infect the insect through direct penetration of the cuticle (Evans and Hywel-Jones, 1997). Bacteria and viruses are not considered an option as they must be ingested by whitefly (whose mouthparts remain inside host plant tissues whilst they feed) although rare infections through existing wounds can sometimes occur (van Lenteren *et al.*, 1996). The development of EPF as bioprotectants, or ‘mycoinsecticides’, has been successful in greenhouse, horticultural, orchard and arable field crops (Gold *et al.*, 2001; Kivett, Cloyd and Bello, 2015; Nyasani *et al.*, 2015; Arthurs and Bruck, 2016; Javal *et al.*, 2019). The most commonly used approach for whitefly control using EPF involves the inundative application of large numbers of infective conidia (Faria and Wraight, 2001); indeed, many anamorphic (= asexual) EPF from the order Hypocreales are easily mass reared on culture media and an increasing number have been developed as proprietary bioprotectants against a range of pest species (Chandler, 2017). The risk of resistance developing to EPF is reported to be very low due to their multiple modes of action, (Kraaijeveld and Godfray, 2008; Dubovskiy *et al.*, 2013) there are little to no residues left on the crop and EPF are often compatible with other natural enemies (Chandler *et al.*, 2011). EPF can be successfully used in IPM as a preventative measure, alongside the use of beneficial invertebrates (Fransen and van Lenteren, 1993; Jeong *et al.*, 2005) including as a second line of defence to supplement the use of predators and parasitoids. Several EPF are commercially available in Europe to target *T. vaporariorum*, such as *Beauveria* spp. (Naturalis, Intrachem Bio Italia; Botanigard, LAM International Corp.) *Cordyceps* (= *Isaria*) *fumosorosea* (PreFeRal, SePRO Corporation; Nofly, Natural Industries, Inc.) and *Akanthomyces* (= *Lecanicillium*) *muscarium* (Mycotal, Koppert Biological systems).

Determining pathogenicity of EPF to demonstrate the potential to control *T. vaporariorum* is a fundamentally important step in the development of EPFs as biological control agents, particularly at the screening stages. To ensure consistent results, testing potential biological

insecticides in the laboratory requires a method to deliver reproducible doses of test substances.

Female *T. vaporariorum* lay eggs on the leaf surface. After eclosion, first instar nymphs ‘crawl’ until they find a suitable location for feeding (Smith, 2009). Nymphs then remain in this feeding location until they emerge as adults. The most susceptible stages of *T. vaporariorum* to EPF are the first, second and third nymphal instars (Osborne and Landa, 1992) but, due to their immobility, nymphs will only become infected by conidia that are sprayed directly onto their cuticle and secondary acquisition of conidia only occurs by the adult walking across the plant surface (Mascarin *et al.*, 2013a).

There are two approaches commonly used to apply a known concentration of an EPF to *T. vaporariorum* nymphs in a bioassay; dipping the target in a suspension of conidia for a known length of time (Negasi *et al.*, 1998) or spraying the target with a known volume of suspension (Wraight *et al.*, 1998; Meekes *et al.*, 2002). Dipping target leaves in a fungal suspension provides 100% contact between *T. vaporariorum* and the EPF conidia but it is difficult to provide a reproducible dose because of run-off from the target (Butt and Goettel, 2009). Spray applications in the laboratory are often performed using an air assisted Potter tower (Burkard Manufacturing Co Ltd) or similar spraying equipment to provide uniform coverage of the target area (Meekes *et al.*, 2000; Meekes, 2001; Er and Gökçe, 2004; Mantzoukas *et al.*, 2013; Fazeli-Dinan, Talaei-Hassanloui and Goettel, 2016) and whilst this is highly efficient, the equipment is often expensive and therefore inaccessible to some researchers. A cheap alternative sprayer for laboratory work has been suggested by Mascarin *et al.* (Mascarin *et al.*, 2013a; Mascarin *et al.*, 2013b) based on a portable artist airbrush that could readily be used for testing chemical toxicity and EPF effectiveness. This has subsequently been used to determine virulence of EPF against several pests, including the silverleaf whitefly *Bemisia tabaci* and the carmine spider mite *Tetranychus cinnabarinus* (Mascarin *et al.*, 2013a; Topuz *et al.*, 2016) in detached leaf bioassays.

Previously reported EPF bioassays against *T. vaporariorum* have most often used the leaf disc method (Quesada-Moraga *et al.*, 2006; Mascarin *et al.*, 2013b; Oreste *et al.*, 2016; Seyed *et al.*, 2017) or detached leaves (Mascarin *et al.*, 2013b) to expose whitefly to fungal conidia. Nutritional reserves of detached leaves are limited and the duration of these bioassays can be constrained due to plant senescence. Variation in plant quality plays a role in insect development and growth, causing variation in susceptibility of insects to infection by EPF (Cory and Ericsson, 2010). The amount and type of plant volatiles produced in response to damage are different depending on whether detached leaves or whole plants are

used (Jansen *et al.*, 2009). Therefore, results from bioassays using detached or cut leaves should be cautiously interpreted. By using whole plants, changes at the microclimatic scale are reduced and bioassay results are less likely to be altered by plant surface chemistry (Elliot *et al.*, 2000).

The leaf-dip bioassay is also a common method used to test the efficacy of pesticides in laboratory bioassays with sucking insects that are not easily removed from the leaf (Butt and Goettel, 2009). This method ensures exposure of every target nymph to the insecticide and is useful as a quick and cheap screening process for bioprotectants. However, it can be difficult to determine dose due to variation in duration of immersion and the gradual removal of conidia when multiple leaves are sequentially dipped into a stock suspension (Ekesi and Maniania, 2007). Mortality caused by surfactants such as Tween 80 and Triton-X have been greater in immersion/leaf-dip bioassays compared to spray applications and the potential for nymphs to drown can also be higher following the leaf-dip method (Ferron, 1985; Wraight *et al.*, 2000; Hornbostel *et al.*, 2005).

In this study, an integrated bioassay approach that combines a clip cage for housing treated whitefly nymphs on leaves of intact plants (designed as an important component of the bioassay set up) was developed. When the clip cages were used alongside the sprayer, reproducible exposure concentrations to a known target area were provided in whole plant assays. This bioassay approach was validated by testing the pathogenicity of 18 EPF isolates in whole plant assays, from four genera of hypocrealean fungi (*Beauveria*, *Cordyceps*, *Akanthomyces* and *Metarhizium*) to third instar *T. vaporariorum*. The bioassay developed has standardised set-up which allows for comparisons between results from different experiments.

The overall aim of this study was to screen a range of hypocrealean EPF isolates for their virulence against *T. vaporariorum* nymphs, and to identify virulent isolates for further study. A bioassay was developed to conduct pathogenicity tests of each EPF isolate. Following selection of a subset of EPF isolates from a pool of candidates based on their pathogenicity and speed of kill, the bioassay was used again to conduct dose response assays for the EPF in addition to two insecticides. The insecticidal effect of three commonly used fungicides was also determined. Lethal concentrations of the EPF and insecticides were calculated for use in further experiments. The component objectives were as follows:

- i) Develop a bioassay method which provides reproducible exposure concentrations to a target area following the application of EPF isolates, insecticides or fungicides.
- ii) Use the bioassay to identify virulent EPF isolates against *T. vaporariorum* nymphs.
- iii) Determine the effect of two insecticides and three fungicides on the mortality of *T. vaporariorum* nymphs.
- iv) Conduct dose response assays for a select number of EPF isolates and two insecticides.
- v) Calculate LC values for insecticides and EPF isolates for use in further experiments.

3.2 Material and Methods

3.2.1 Sprayer design

The mini spray tower was built at the UK Centre for Ecology & Hydrology (Wallingford, UK) and was based on the design of Mascarin *et al.* (2013a) with some modifications. A gravity fed universal dual action airbrush (Spraycraft SP60) was attached to a 3D printed removable lid and placed on top of an acrylic cylinder (width = 115mm, height = 240mm) to form the spray tower. The cylinder was held 10mm from the laboratory bench by mounting on removable feet (Figure 3.1). The airbrush sprayer was powered by a mini air brush compressor (Sealey, Model no. AB900.V3). All parts of the mini spray tower can be disassembled to be decontaminated. The sprayer was designed to fit a 90mm Petri dish within the spray target area (10387 mm² area base).

3.2.2 Ensuring uniform coverage across the target spray area

To calibrate the spray tower, the relationship between the volume of spray applied and the volume received in the target spray area at a range of spray pressures was investigated. A volume of 1mL of deionised water was sprayed onto the base of an upturned empty 90mm Petri dish at a range of pressures, namely 103.4, 137.9, 172.4, 206.8 kPa. Five replicate sprays were applied to each of five separate dishes for each pressure and the weight of the Petri dish was measured before and after each spray. The size of water droplets across the Petri dish at each pressure was also compared by visual inspection (Figure 3.1).

Preliminary experiments indicated that the spray reaching the outer edge of the Petri dish was not uniform. A 1mL sample of 0.1% stock solution (0.5g/500mL) of green dye (FastColours, E142 Green S) was sprayed onto 90mm filter paper which visually indicated variation in deposition across the target area. Therefore, potential differential deposition of spray across the centre and toward the edges of the target spray area was investigated by spraying five 13mm diameter circular glass cover slips, arranged across 45mm in the formation shown in Figure 3.1c, to cover the whole target area (1590 mm²). A 1mL sample of 0.1% stock solution (0.5g/500mL) of green dye was sprayed onto the coverslips at 103.4, 137.9, 172.4, 206.8 kPa and left to dry for 2 hours. Individual coverslips were then submerged in 2mL of deionised water in 50mL tubes (Falcon 50 mL conical centrifuge tube, Fischer Scientific) and agitated on a vortex mixer for 2 minutes. A 200µl aliquot was taken from each coverslip wash and loaded into a 96 well plate (Nunclon™ Delta 96-well Microwell™ plates, Thermo Scientific) and light absorbency at 634nm of the solution was determined using a spectrophotometer (BioTek®, Cytation 5). Each treatment had three

replicate sprays (i.e. 15 coverslips per treatment) and the whole experiment was repeated three times to give a total of 45 test solutions at each of the three spray pressures. A standard calibration curve was created by measuring light absorbency for known concentrations of green dye that had been sprayed onto coverslips using the spray tower and this was used to estimate the concentration of green dye on each cover slip.

3.2.3 Calibration to determine conidia deposition

Tests were also performed to calibrate the sprayer for conidia deposition on the target area when an EPF was applied using the methods described above. Conidial deposition of all EPF isolates used in pathogenicity bioassays were investigated for applications of 1×10^7 conidia mL^{-1} . Individual 22 x 22 mm square glass cover slips were placed in the centre of 90mm diameter plastic Petri dishes and sprayed at 138kPa, based on analysis of the previous results, with 1mL of conidia suspension. Each suspension was sprayed onto three replicate cover slips in three separate applications. Sprayed coverslips were immediately placed individually in 1mL 0.03% Tween 80[®] in 50mL tubes and agitated on a vortex mixer for 2 minutes to dislodge conidia into suspension. Serial dilutions were made from 40 μl aliquots taken from each suspension, ensuring that each sample contained a low enough number of conidia for enumeration. Diluted suspensions were spread evenly across individual Petri dishes (90mm diameter) containing 10 mL SDA. Dishes were then sealed with Parafilm[®]M and incubated in the dark at 25°C for 4-5 days. After that time, the number of colony forming units (CFUs) were counted and used to calculate the number of conidia received per mm^2 on each coverslip. By allowing conidia to germinate, this calibration method ensures that only infective viable conidia applied to the target area are counted. This approach was used as direct enumeration of conidia from suspension washes was not viable due to low counts under the haemocytometer.

3.2.4 Determining Pathogenicity of entomopathogenic fungi isolates towards *Trialeurodes vaporariorum*

Fungal pathogenicity of 18 different isolates was assessed in bioassays using third instar *T. vaporariorum* on *Solanum melongena* plants (results presented in Section 2.3.1.). Isolates were divided into three groups containing different isolates that were used as treatments in bioassays with experiments conducted three days apart in a randomised block design. Applications of the *B. bassiana* isolate PPRI5339 and the negative control of 0.03% Tween 80[®] (untreated nymphs) were replicated in every group as a standard for comparison. A single leaf per plant was infested with nymphs as described in section 2.5.1 using clip cages

(40mm base diameter, 30mm top diameter, 25mL frosted containers) (Figure 3.1) and three replicate separate plants were used in each treatment. Due to variability between numbers of eggs laid, this resulted in a range of between 51-146 nymphs per treatment. The number of nymphs was counted on each leaf prior to assays and assigned to treatments to evenly distribute the total number of nymphs overall within a treatment and reduce variability as much as possible. One millilitre of 1×10^7 conidia mL^{-1} suspension was sprayed onto the third instar nymphs on the abaxial leaf surface using the spray tower at 138kPa. *T. vaporariorum* instar was determined by methods described in 2.5.1.

3.2.5 Dose response bioassays

Isolates for further experimentation were chosen based on high mortality in pathogenicity testing, fast rate of kill, high conidia production and suitability of the temperature profile (see below). Decisions were informed by Pareto dominance ranking whereby each isolate is ranked based on pathogenicity which was determined by total mortality after application of 1×10^7 conidia mL^{-1} , time to 50% mortality in pathogenicity bioassays (section 3.4.3), fastest growth rate and greatest conidial production when incubated at nine temperatures (*in vitro* results are presented in chapter 4). Isolates were ranked 1-18 for each characteristic, with the highest scoring isolate being numbered as one. Then isolates were given a value indicating whether they were ranked equal to or lower (dominated) compared to all other isolates ((Barwell, Isaac and Kunin, 2015); Pareto dominance table (Appendix I). Though the effect of temperature on the germination of *A. lecanii* (ATCC 4060) conidia was not investigated, this isolate was also included in further experiments because of the high mortality caused by application of 1×10^7 conidia mL^{-1} as shown in section 3.4.3 and the high conidia production in chapter 4.

A dose response bioassay was performed with the isolates *Beauveria bassiana* (PPRI5339), *B. bassiana* (ATCC 5278), *Cordyceps farinosa* (ATCC 4412), *Akanthomyces lecanii* (ATCC 4060) and *Metarhizium brunneum* (Met 52). All isolates could not be used in a bioassay at the same time so bioassays were performed in three groups, including *B. bassiana* (ATCC 5278) in each group as a standard isolate as well as a negative control of 0.03% Tween 80. The standard isolate was chosen because of the consistency of proportion mortality results between replicates when this isolate was used in the pathogenicity bioassay presented in section 3.2.4. The groups were as follows:

Group 1 = *Beauveria bassiana* isolates PPRI5339 and ATCC 5278.

Group 2 = *Cordyceps farinosa* (ARSEF 4412), *Akanthomyces lecanii* (ATCC 4060) and *B. bassiana* (ATCC 5278)

Group 3 = *Metarhizium brunneum* (ATCC 52), the standard and another repeat of *Beauveria bassiana* (PPRI5339).

Isolate *Beauveria bassiana* (PPRI5339) was included in Group 3 because of unexpected mortality results in Group 1 (i.e., the first dose response bioassay). For each bioassay, early third instar *T. vaporariorum* were sprayed with 1mL of 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 conidia mL^{-1} at 138kPa. However, it was not possible to obtain a suspension of 1×10^9 conidia mL^{-1} for isolate *B. bassiana* 5278, so a reduced number of treatments was applied. Each treatment was sprayed onto three replicate leaves on separate plants holding at least 100 nymphs per leaf. The number of conidia received by target nymphs in each treatment was also determined using the same methods as described in section 3.2.3. Additional dilutions were made to adjust for applications of higher concentrations to ensure that CFUs could be counted. Treated nymphs were maintained as described in section 2.5.1. Mortality was observed every 48 hours as described in section 2.5.2.

A concentration response bioassay was performed for the chemical pesticides Admiral and Oberon, in addition to the fungicides Takumi SC®, Kumulus® and Luna® Sensation. The standard EPF isolate (*B. bassiana* ATCC 5278) was again used in this bioassay at a single concentration of 1×10^7 conidia mL^{-1} alongside a negative control treatment of 0.03% Tween 80. Recommended application concentrations were prepared by mixing 30 μL Takumi SC, 400mg Kumulus or 266 μL Luna sensation into 100mL sterilised deionised water. Dose response treatments were prepared by making stock solutions of recommended application concentrations of Admiral and Oberon, as described in section 2.4.1. Serial dilutions of the stock solutions were made to produce treatment solutions equal to 10, 25, 50 and 70% of the recommended application concentration. Early third instar *T. vaporariorum* were sprayed with 1mL of each treatment. Each treatment was replicated three times using separate leaves on different plants, with at least 200 nymphs per leaf. A larger number of nymphs per leaf was used than in the previous bioassay as a greater number of eggs were laid by *T. vaporariorum* on this occasion. The number of conidia received by target nymphs in each EPF treatment was calculated using methods described in 3.2.3. Treated nymphs were maintained as described in section 2.5.1. Mortality was observed every 48 hours as described in section 2.5.2.

3.2.6 Determining the effect of synthetic insecticides and fungicides on *Trialeurodes vaporariorum* mortality

Another dose response bioassay using Admiral and Oberon was conducted because observed mortality was high for all concentrations applied in the previous bioassay. Early third instar *T. vaporariorum* nymphs were treated with recommended application concentrations of Admiral and Oberon, prepared using the methods described in section 2.4.1 and four dilutions of these stock solutions. Each treatment was applied as 1mL aliquots onto one leaf holding at least 100 *T. vaporariorum* nymphs. Admiral was applied at a range of concentrations equal to 0.01, 0.1, 1, 10 and 100% of the recommended application concentration. Oberon was applied at concentrations equal to 0.1, 1, 10, 25 and 100% of the recommended application concentration. The negative control treatment was an application of 1mL sterilised water. Each treatment was replicated on three leaves on separate plants. Nymphs were maintained in conditions described in section 2.5.1. Mortality was observed every 48 hours using methods described in section 2.5.2.

3.2.7 Determining the relationship between concentration of conidia suspension applied and conidia dose received at the target spray area

The relationship between the concentration of conidia suspensions of EPF applied and the dose received by the target leaf was determined using the methods described in section 3.2.3. For each isolate used in the dose response bioassays, additional treatments were prepared to apply to coverslips in between leaf treatments. For each EPF isolate, coverslips were sprayed with 1mL of 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 conidia mL^{-1} , except *B. bassiana* 5278 as there were too few conidia produced when harvested from plates to test the highest concentration. Each treatment was replicated three times. Conidia per unit area were determined by methods described in section 3.2.3. The relationship between the concentration of EPF solution applied and the dose received by the target area was investigated through a series of linear and polynomial models.

3.2.8 Statistical analysis

Data to assess the relationship between the volume of spray applied and the volume received in the target spray area at a range of spray pressures were subjected to Analysis of variance (ANOVA) followed by Tukey's range test. Deposition of green dye on each cover slip after 1mL application to assess differential deposition of spray across target spray areas was normalised using a \log_{10} transformation and subjected to an ANOVA followed by Tukey's range test. Differences in the number of conidia received on each cover slip in each

replicated pathogenicity experiment was determined by conducting ANOVAs. The relationship between conidia received and mortality was determined by fitting Generalised Linear Models (GLMs) to mortality observed and dose received with Poisson or quasi-Poisson link functions (depending on overdispersion of the data). The interaction between genus, dose and mortality was tested and comparisons between GLMs were made by Chi squared comparisons.

Similarly, data for conidia deposition after application of a range of concentrations were transformed using a natural logarithm. Data was modelled as a linear regression as well as a second, third, and fourth order polynomial regression and the fit of each model was compared using ANOVAs. The relationship between nymph dispersion and the number of nymphs per leaf was analysed using a Pearson's correlation coefficient.

Data for conidia dose and total proportion mortality at the end of the bioassay data could not be normalised and so were analysed using the Kruskal Wallis test followed by a post hoc Dunn test. Control mortality was corrected for using Schneider-Orelli's formula (Equation 1) (Puntener, 1981). The relationship between conidia dose received in each treatment and total nymph mortality was assessed using a Pearson correlation. A non-parametric survival analysis was conducted by creating Kaplan-Meier curves for each treatment. Differences in mortality caused by each treatment were analysed using a log-rank test, followed by multiple pairwise log-rank comparisons. Multiple pairwise comparisons of isolates causes an increase in overall type 1 error, so Bonferroni adjustments were applied to the p-value. Then, a Cox proportional hazards model was created to estimate the mean increase in risk of mortality of *T. vaporariorum* in treated groups compared to the control. The package 'survival' in R was used to conduct the Kaplan Meier curves and the Cox proportional hazard model. (Therneau and Grambsch, 2000).

Concentration response curves were analysed using methods described in section 2.5.4. Calculation of LT_{50} values was done by fitting a GLM with quasi-Poisson link for each concentration of each isolate tested followed by predictions using the MASS package in R (Venables and Ripley, 2002).

3.3 Results

3.3.1 Determining spray coverage of applied solutions over target area

The volume of water deposited across each 90mm Petri dish was significantly different depending on the application pressure, which ranged from 103 – 207 kPa (df=3, F=5.3, $p < 0.001$) as shown in Table 3.1. Average deposition was similar across the range of pressures tested except at 103 and 172 kPa where significantly higher deposition was observed at the lowest application pressure (t value = 12.66, $p = 0.007$; Table 3.1). At the highest pressure setting (207 kPa) the spray gun was observed ‘spitting’ instead of producing a uniform mist. Visual inspection of water droplets on Petri dish lids indicated that a higher volume of water was received in the central area compared to the outer edge of each dish and water droplet size increased with application pressure above 138 kPa (Figure 3.1b).

The volume of green dye applied to each coverslip arranged within the central 45mm of Petri dishes to assess the pattern of deposition within the spray target area was not significantly different in each replicate experiment (df=2, $\chi^2 = 0.75$, $p = 0.69$, Kruskal Wallis test), therefore data were combined for analysis. The mean volume of green dye received by each coverslip on the Petri dish differed significantly when applied with 172 kPa, giving an average deposition of $114 \mu\text{l mm}^{-2} \pm 30$ (df=4, $\chi^2 = 30.36$, $p < 0.001$). In comparison, coverslip position did not affect the mean volume of green dye received at 103 kPa ($130 \mu\text{l mm}^{-2} \pm 16$; df=2, F=0.047, $p = 0.96$) or 138 kPa ($110 \mu\text{l mm}^{-2} \pm 4$; df=2, F=0.48, $p = 0.622$). However, droplet size was visibly more uniform at 138kPa compared to 103kPa so a pressure of 138kPa was used for further experimentation.

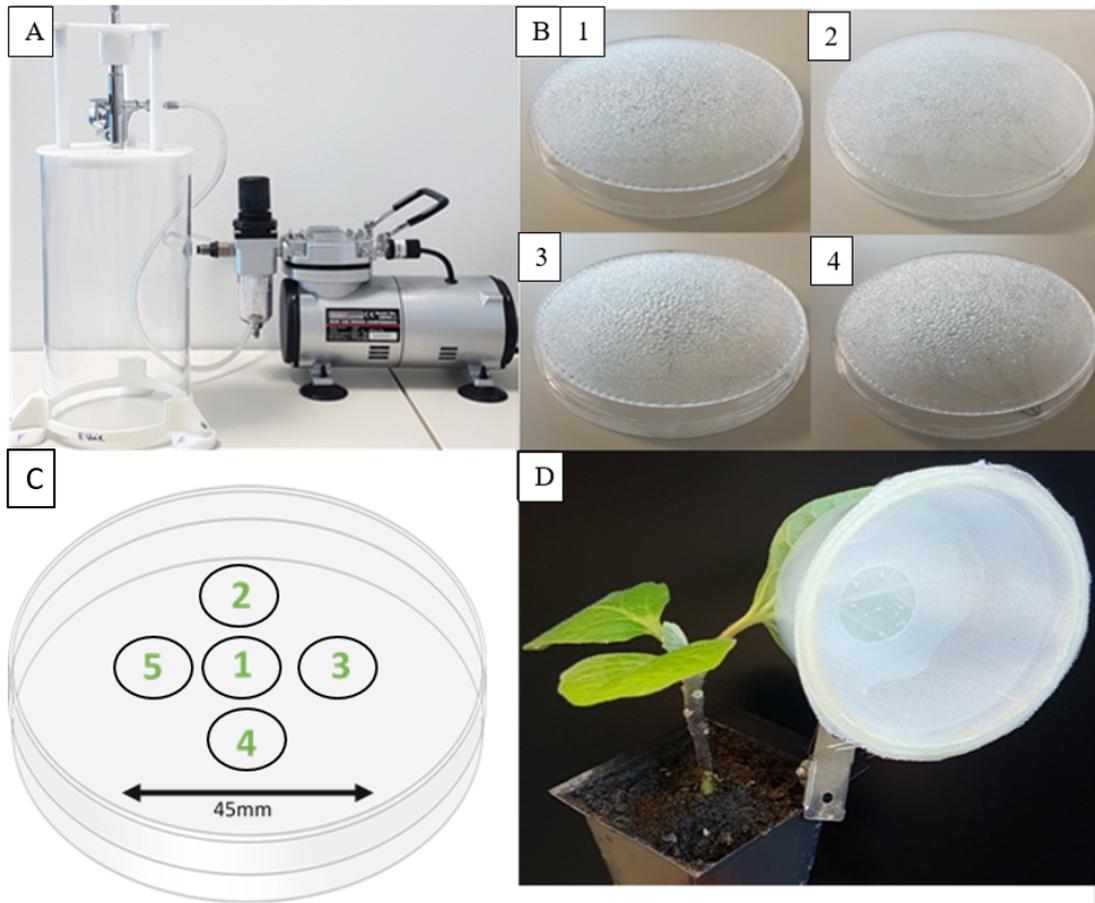


Figure 3.1 A) The spray tower consisting of a gravity fed universal dual action airbrush (Spraycraft SP60) attached to a removable lid and acrylic cylinder (width = 115mm, height = 240mm). The spray tower was connected to a mini air brush compressor (Sealey, Model no. AB900.V3); B) Visible differences in water droplet size and variation across the surface of 90mm diameter Petri dishes when sprayed with 1mL of deionised water at 1.) 103.2 kPa, 2.) 137.9 kPa, 3.) 172.4 kPa and 4.) 206.8 kPa; C) Arrangement of 13mm diameter cover slips used to calibrate the volume of liquid deposited across the surface of a 90mm diameter Petri dish; D) Clip cages used to contain whitefly adults on a small known area of the abaxial leaf surface. Clip cages are made using truncated plastic pots, fine nylon mesh, acetate and metal hair clips.

Table 3.1 Volume of liquid deposited on 90mm Petri dish lids after spraying with 1mL of deionised water at pressures ranging 103- 207 kPa and the estimated field application volume that this would represent. Means not followed by the same letter are significantly different as indicated by 95% confidence limits.

Pressure (kPa)	Total volume deposited (mL ± sd)	Volume deposited per unit area (mL mm ⁻²)	L/ha ^a
103	0.42 a± 0.018	0.067 ± 0.003	666.93
138	0.39 ab ± 0.021	0.062 ± 0.003	617.13
172	0.38 b ± 0.023	0.059 ± 0.004	590.29
207	0.39 ab ± 0.017	0.061 ± 0.003	611.63

^a Based on 1 hectare = 1 x 10¹⁰ mm²

3.3.2 Calibration to determine conidia deposition

Throughout the pathogenicity bioassay experiments, *B. bassiana* isolate PPRI5339 was used as a common isolate in each of the three groups to compare consistency between experiments. Conidia deposition for *B. bassiana* isolate PPRI5339 ranged from 1.01x10⁵-1.55x10⁵ mm⁻² and was not significantly different between the groups of bioassays (df=2, F=1.903, p=0.24). Therefore, data across all assays were combined in a single analysis. Conidia deposition varied for each isolate (df=19, F=4.93, p=<0.001) and ranged from 189-332 conidia mm⁻² or equivalent to 1.9–3.3 x10¹² conidia ha⁻¹ (Table 3.2). Conidia deposition was significantly different when isolates were grouped by genus (df=3, F=4.098, p=0.01). A post hoc Tukey showed that conidia deposition for *Beauveria* spp. was not significantly different from *Cordyceps* spp. (p=0.70) and *Metarhizium* spp. (p=0.16). However, the average number of conidia deposited by *Akanthomyces* spp. isolates was significantly lower than *Metarhizium* spp. isolates (p=0.008; Table 3.3). Despite there being a small variation between conidia received per coverslip when grouped by fungal genus, and hence a variation in dose received by nymphs, this did not translate to a significant effect on *T. vaporariorum* mortality after treatment. There was no significant difference in mortality when isolates were grouped by each genus (*Beauveria* t=1.44, p=0.15; *Cordyceps* t=0.73, p=0.46; *Akanthomyces* t=1.16, p=0.25, *Metarhizium* t=0.097, p=0.92) and there was no significant interaction between genus and dose influencing mortality ($\chi^2= 31911$, df=53, p=0.67).

Table 3.2 Average conidial deposition after 1mL of 1×10^7 conidia mL^{-1} of 18 different entomopathogenic fungal isolates was sprayed onto a 22x22mm square coverslip using the spray tower at 137.9 kPa.

ATCC ID/ Name	Species	Deposition (conidia per mm^2) Mean \pm SE	Equivalent field deposition (conidia ha^{-1}) ^a
ATCC 5278	<i>Beauveria bassiana</i>	250 \pm 44.46	2.5×10^{12}
ATCC 6920	<i>Beauveria bassiana</i>	231 \pm 12.99	2.3×10^{12}
ATCC 6921	<i>Beauveria bassiana</i>	260 \pm 38.30	2.6×10^{12}
ATCC 9451	<i>Beauveria bassiana</i>	204 \pm 31.63	2.04×10^{12}
Botanigard	<i>Beauveria bassiana</i>	303 \pm 27.75	3.03×10^{12}
GHA	<i>Beauveria bassiana</i>	332 \pm 21.10	3.32×10^{12}
PPRI5339	<i>Beauveria bassiana</i>	257 \pm 44.38	2.57×10^{12}
ATCC 7477	<i>Cordyceps javanica</i>	297 \pm 42.46	2.97×10^{12}
ATCC 4412	<i>Cordyceps farinosa</i>	189 \pm 42.41	1.89×10^{12}
ATCC 2658	<i>Cordyceps fumosorosea</i>	250 \pm 61.19	2.49×10^{12}
PFR	<i>Cordyceps fumosorosea</i>	203 \pm 21.50	2.03×10^{12}
ATCC 4205	<i>Cordyceps fumosorosea</i>	301 \pm 11.38	3.01×10^{12}
ATCC 4060	<i>Akanthomyces lecanii</i>	199 \pm 29.32	1.99×10^{12}
ATCC 972	<i>Akanthomyces lecanii</i>	198 \pm 35.31	1.98×10^{12}
ATCC 6544	<i>Akanthomyces lecanii</i>	257 \pm 10.75	2.57×10^{12}
Vertalec	<i>Akanthomyces muscarium</i>	205 \pm 28.45	2.05×10^{12}
Met 52	<i>Metarhizium brunneum</i>	291 \pm 18.60	2.91×10^{12}
Bioblast	<i>Metarhizium anisopliae</i>	303 \pm 17.57	3.03×10^{12}

^a Field deposition calculated based number of conidia per 1 hectare ($\text{ha} = 1 \times 10^{10} \text{mm}^2$)

Table 3.3 Average dose received after 1mL of 1×10^7 conidia mL^{-1} of 18 different entomopathogenic fungi was sprayed onto a 22x22mm square coverslip using the spray tower at 138 kPa.

Genus	Average dose received after spray application (conidia mm^{-2})
<i>Beauveria</i> spp.	262 ^{ab} \pm 49
<i>Cordyceps</i> spp.	248 ^{ac} \pm 58
<i>Akanthomyces</i> spp.	216 ^c \pm 33
<i>Metarhizium</i> spp.	297 ^b \pm 17

3.3.3 Determining pathogenicity of entomopathogenic fungi isolates towards *Trialeurodes vaporariorum*

To ensure that a uniform dose of conidia was applied to whitefly nymphs in the bioassay, ovipositing adult *T. vaporariorum* were restricted to a 7.5mm radius circle (area = 177mm²) on the centre of the abaxial leaf surface to ensure eggs were oviposited within this area. The number of eggs oviposited per leaf ranged from a minimum of 8 to a maximum of 97 with an average of 40 ± 23 eggs per leaf. On average, 91% of eggs hatched successfully and nymphs were subsequently counted when they reached the third instar stage, at which point, they were dispersed across an average area of 373.76 ± 5 mm². There was no correlation between number of eggs laid and area of leaf occupied by nymphs as there was no relationship between total number of eggs and area ($t=1.46$, $df = 25$, $p=0.16$, Pearson's).

Total proportion mortality caused by the replicated *B. bassiana* PPRI5339 isolate in each group did not differ significantly ($df=2$, $F= 0.82$, $p=0.49$), so bioassay mortality results were combined in a single analysis. When different fungal isolates were sprayed onto whitefly nymphs at the same concentration (1×10^7 conidia mL⁻¹), there were small differences in dose received by target *T. vaporariorum* (measured as number of conidia per mm² on coverslips) (Figure 3.2). However, there was no correlation between dose received and total proportion mortality of whitefly nymphs observed for all *Beauveria* spp. ($df=23$, $t=-0.48$, $p=0.64$), *Cordyceps* spp. ($df=13$, $t=-1.4$, $p=0.17$), *Akanthomyces* spp. ($df=9$, $t=-1.0$, $p=0.35$) or *Metarhizium* spp. isolates ($df=4$, $t=0.39$, $p=0.72$). Thus, natural variation in dose of conidia received (as measured using coverslips) when conidia were applied at a constant concentration, did not translate into differences in mortality.

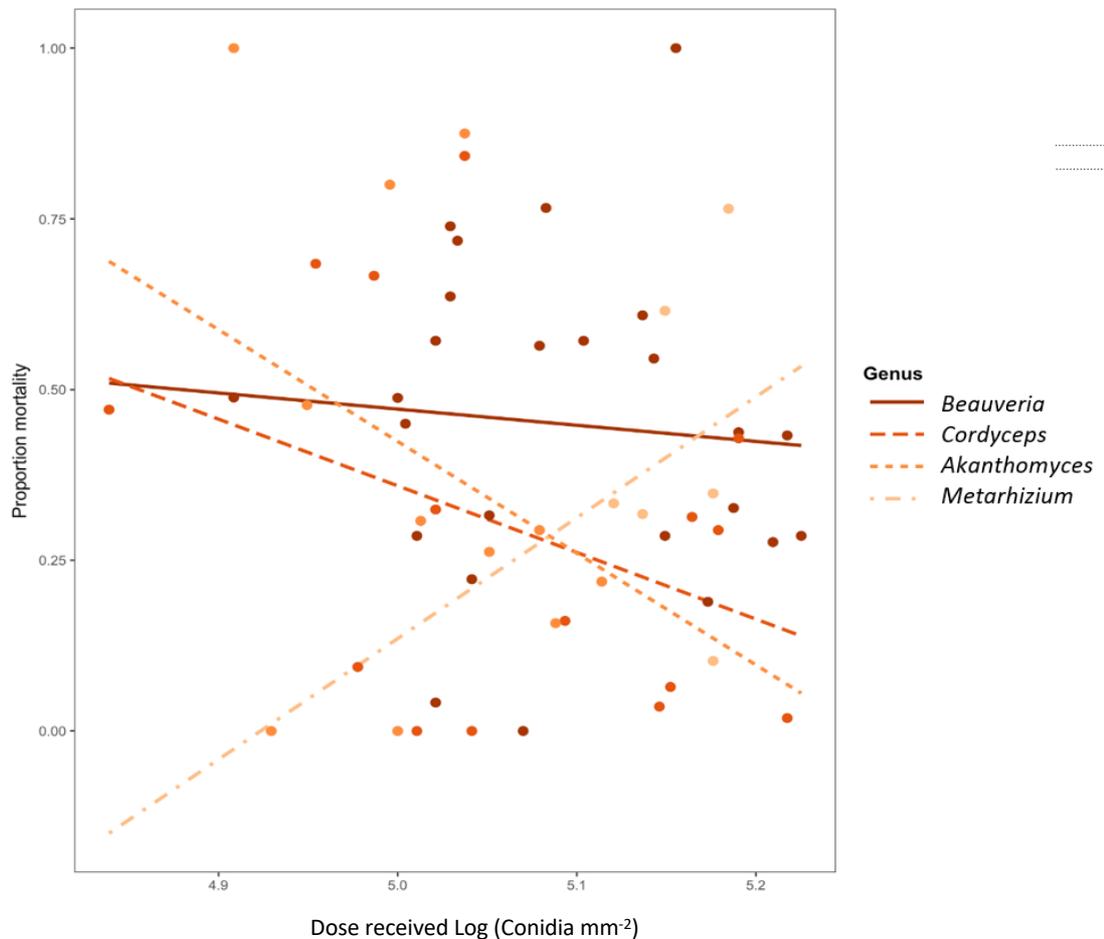


Figure 3.2 Average proportion mortality of *Trialeurodes vaporariorum* after application of 1×10^7 conidia mL^{-1} and the variation observed in dose received per unit area for 18 EPF isolates grouped by Genus. No significant relationship between dose received and proportion mortality for each Genus was detected.

Fungal induced mortality and control mortality were observed as early as 2 days after leaves were sprayed with all treatments. All entomopathogenic fungi applied were pathogenic to *T. vaporariorum*, as shown by total proportion mortality (Figure 3.3) and Kaplan Meier survival curves (Figure 3.4), which differed significantly from the control, irrespective of the species or isolate applied (Kaplan-Meier log Rank: $\text{df} = 18$, $\chi^2 = 437$, $p < 0.001$; Kruskal Wallis test $\text{df} = 18$, $\chi^2 = 44.50$, $p < 0.001$). After 14 days, total mortality ranged between $8.1 \pm 7.3\%$ to $89 \pm 10\%$ depending on the isolate applied. The average mortality in the control group (treated with 0.03% Tween 80) was $3.2 \pm 6.3\%$, with all surviving nymphs emerging as adults. Isolate 4060 of *A. lecanii*, 9451, 5278 and 6921 of *B. bassiana*, and 4412 of *C. farinosa* were more pathogenic to *T. vaporariorum* nymphs ($\geq 60\%$) than all other isolates and overall, isolate 4060 caused the greatest mortality in *T. vaporariorum* nymphs (89%) which was >50 times higher than control mortality (Cox proportional hazards: 95% CI: 27.6-118). The Kaplan Meier curve estimated for this isolate was significantly different to all

other isolates except *C. farinosa* 4412 ($p=0.053$), *B. bassiana* 6921 ($p=1.00$) and *B. bassiana* 9451 ($p=0.21$).

The Kaplan Meier survival analysis indicated there were significant differences in mortality caused by isolates within the genera *Beauveria* ($p<0.0001$), *Cordyceps* (*C. farinosa*, *C. fumosorosea*, *C. javanica*) ($p<0.0001$) and *Akanthomyces* ($p<0.0001$). There was no significant difference in survival of *T. vaporariorum* when treated with *M. brunneum* (Met 52) or *M. anisopliae* (Bioblast) ($p= 0.37$), as shown in Figure 3.4.

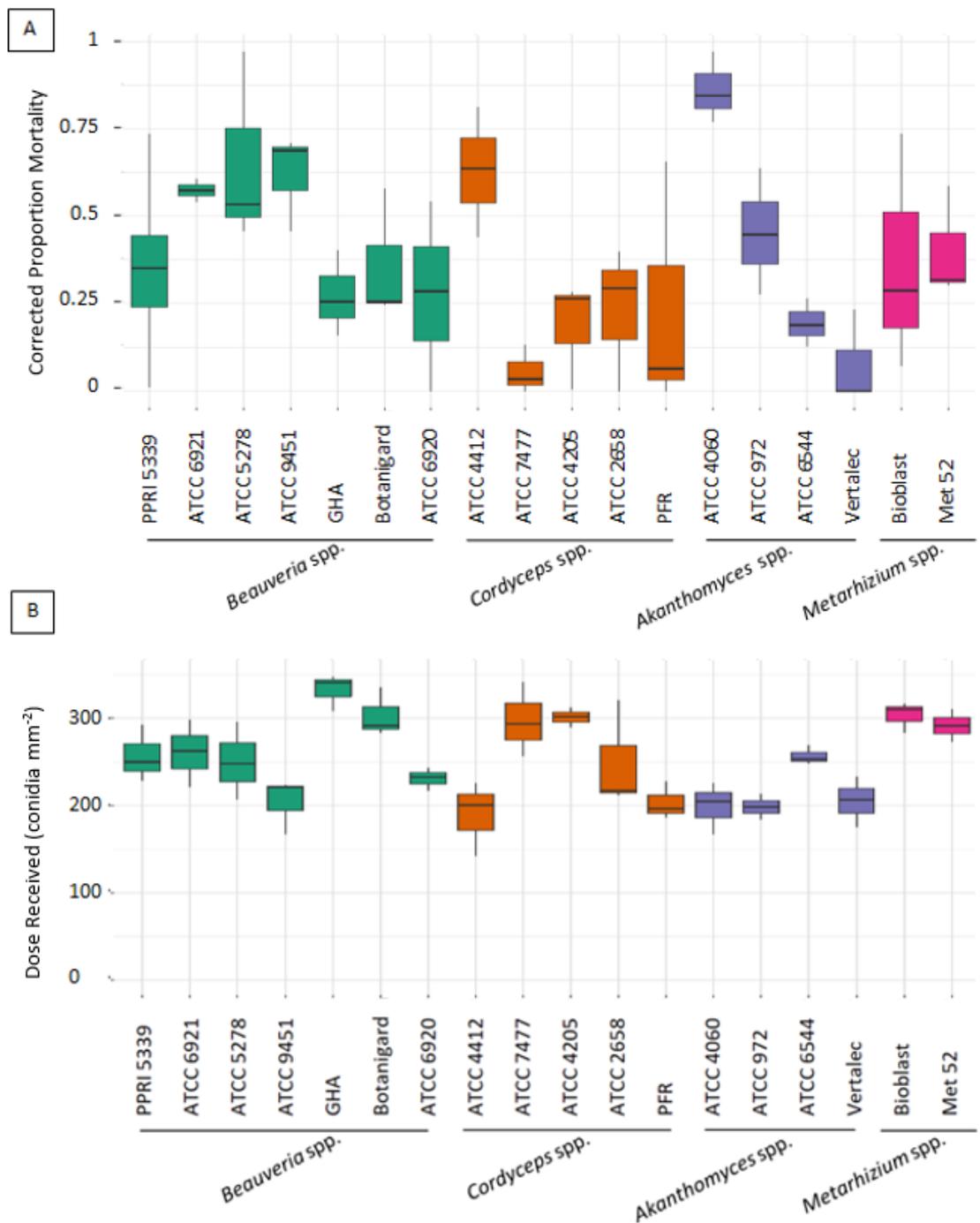


Figure 3.3 A.) Average percent mortality of third instar greenhouse whitefly (*Trialeurodes vaporariorum*) nymphs 14 days after application of 1mL of 1×10^7 conidia mL^{-1} of 18 different entomopathogenic fungal isolates applied at 138 kPa. Error bars indicate standard deviation. Control mortality (3%) was corrected for using Schneider-Orelli's formula: corrected mortality (%) = $(a-b / 100-b) * 100$. Where a is the percentage mortality data from the treated group and b is the percentage mortality from control group. B.) Dose received per mm^2 of 22x22mm coverslips in the centre of a Petri dish sprayed with 1mL of 1×10^7 conidia mL^{-1} of 18 different entomopathogenic fungal isolates. Error bars indicate standard deviation.

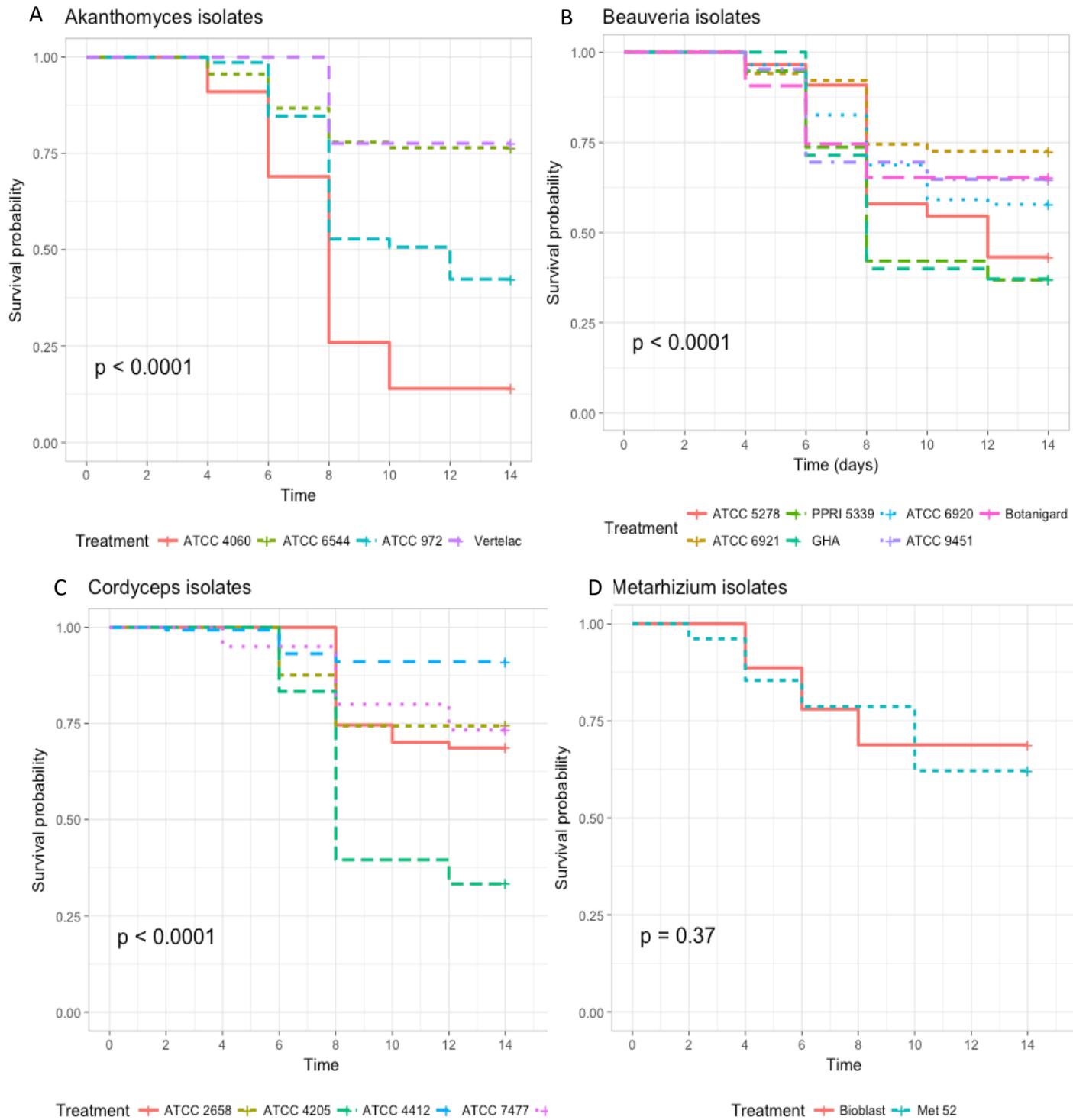


Figure 3.4 Kaplan- Meier survival curves for *Trialeurodes vaporariorum* nymphs treated with 1×10^7 conidia mL^{-1} of 18 different entomopathogenic fungi, separated by genus into A) *Akanthomyces* isolates, B) *Beauveria* isolates, C) *Cordyceps* isolates and D) *Metarhizium* isolates. P values indicate differences in survival rate 14 days after *T. vaporariorum* were exposed to different isolates of the same genus using log rank tests for overall comparison with the highest survival rate used as a reference. Survival was corrected for control mortality at each time point using Schneider–Orelli's formula.

3.3.4 Dose response bioassays of EPF isolates targeting *Trialeurodes vaporariorum*

In the Pareto dominance rankings (Appendix I), there were six EPF isolates which scored 155 or less and were therefore ranked higher than the remaining 18 EPF isolates across the characteristics tested. These were *M. brunneum* (Met 52), *C. farinosa* ATCC 4412, *B. bassiana* ATCC 5278, *A. lecanii* ATCC 4060, *M. anisopliae* (Bioblast) and *B. bassiana* PPRI 5339. The highest scoring (lowest ranking) EPF isolate was *A. lecanii* ATCC 972 which was outperformed by most of the other EPF isolates in previous experiments. Both *Metarhizium* spp. isolates were isolated from commercially sold products. *Metarhizium brunneum* (Met 52) and *M. anisopliae* (Bioblast) showed similar results in temperature response in *in vitro* experiments, however *M. brunneum* caused a higher proportion mortality than *M. anisopliae* so only *M. brunneum* was used in further experiments.

Akanthomyces lecanii ATCC 4060 showed one of the fastest growth rates at 10°C. Though the effect of temperature on the germination of *A. lecanii* ATCC 4060 conidia was not investigated, this isolate was also included in further experiments because it induced the highest proportion mortality of all isolates following the application of 1×10^7 conidia mL⁻¹ and produced significantly more conidia than other isolates tested (Chapter 4).

The selection process resulted in EPF being taken forward which were predicted to perform well at high temperatures (*M. brunneum* (Met52) and *B. bassiana* ATCC 5278), lower temperatures (*A. lecanii* ATCC 4060) or across a wide range of temperatures (*C. farinosa* ATCC 4412 and *B. bassiana* PPRI 5339) compared to other isolates tested according to *in vitro* temperature profiling experiments.

The dose response curves for the standard isolate *B. bassiana* ATCC 5278 used in groups one, two and three of the bioassays were fitted to a two, three and four parameter log-logistic regression as well as a Weibull-1 and Weibull-2 model and compared using a lack of fit test. The four parameter log-logistic model provided the best fit and highest p value (p=0.88) so was used for further testing. Two ANOVAs were used to determine whether there were significant differences between the LC₅₀ and slope of dose response for *B. bassiana* ATCC 5278 between replicate bioassays; P values of these ANOVAs were 0.0023 and 0.0035 respectively. This showed that the model fit was worse if these parameters were constrained and there were significant differences between the slope and LC₅₀ for each bioassay when using concentration of conidia applied against mortality observed.

The same analysis was conducted for conidia mm⁻² against mortality. The four parameter log logistic model was also the best fitting for the conidia mm⁻² dose response curve (p=0.79).

Similarly, there were significant differences in the slope and LC_{50} calculated for each bioassay for *B. bassiana* ATCC 5278 ($p=0.004$, $p=0.008$). Therefore, variability in virulence of this isolate between bioassays meant that assays could not be combined for a single analysis.

In the first dose response bioassay (Group 1), total mortality ranged from $28 \pm 22\%$ to $84 \pm 19\%$ dependant on the concentration and isolate applied. Control mortality was 19% which was corrected for in the concentration response analysis. Isolate *B. bassiana* ATCC 5278 followed the expected dose response with increasing mortality resulting from increasing concentration of conidia applied, except for the concentration 1×10^6 conidia mL^{-1} , where mortality was lower than expected (Figure 3.5). However, average mortality of *T. vaporariorum* did not follow a linear relationship with increasing concentration of isolate *B. bassiana* PPRI 5339. Mortality caused by this isolate was highly variable and could not be predicted based on concentration of conidia applied. The first mortalities of *T. vaporariorum* were noted on day four for both isolates in the assay (Figure 3.6). Dose response curves for concentration applied (conidia mL^{-1}) of each isolate and calculation of LC values was done using a four parameter log-logistic model. First, a two, three and four parameter log logistic model and Weibull-1 and Weibull-2 models were fitted to all EPF isolate dose responses. The four parameter log logistic model fitted significantly better than others tested according to a lack of fit test ($p=0.81$). Following this, two ANOVAs were used to determine the significance of fixing the LC_{50} or slopes of the dose response curves for each isolate. The model with identical slopes for each isolate described the data equally as well as the free model, therefore the constrained model was used to describe the dose responses of *B. bassiana* ATCC 5278 and PPRI5339 ($p=0.53$). The LC_{50} values were significantly different for these isolates ($p=0.04$).

The same process was followed for dose response curves of mortality against dose received (conidia mm^{-2}) for isolates in bioassay one. For this analysis, the two parameter log-logistic model fitted significantly better than the other models according to a lack of fit test ($p=0.17$). Comparisons of simplified models using ANOVA demonstrated that the data was described significantly better by the model with a shared slope ($p=1$) but with separate LC_{50} values for each isolate ($p=0.023$, Figure 3.5).

Calculated LT_{50} values (Table 3.5) were variable for *B. bassiana* isolates in the first run of the bioassay. Generally, as concentration applied increased, the predicted time to 50% mortality decreased, with the exception of 1×10^5 conidia mL^{-1} for isolate PPRI 5339 which induced mortality faster than 1×10^6 - 1×10^8 conidia mL^{-1} . The application of 1×10^6 conidia

mL⁻¹ for isolate ATCC 5278 resulted in a slower rate of mortality than application of the two lower concentrations.

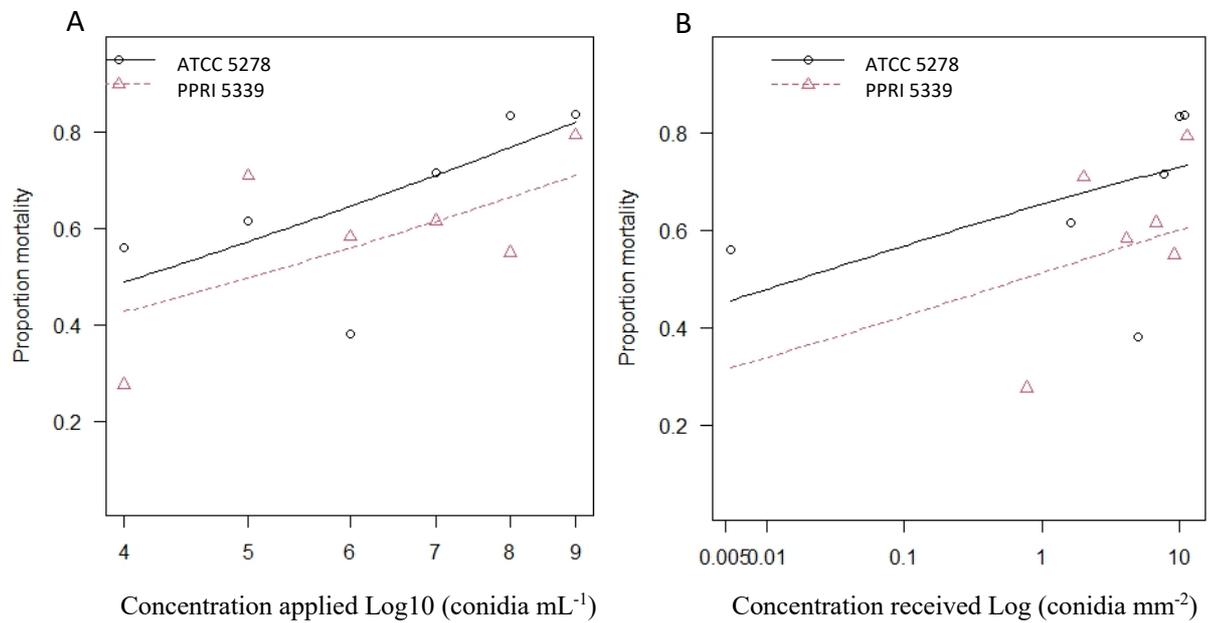


Figure 3.5 A.) Proportion mortality of *Trialeurodes vaporariorum* treated with two entomopathogenic fungi isolates (*Beauveria bassiana* isolates PPRI5339 and ATCC 5278) at a range of application concentrations in the first dose response bioassay (Group 1, see methods section 3.2.5). B.) Conidia received per unit area and the resultant mortality observed in *T. vaporariorum* nymphs.

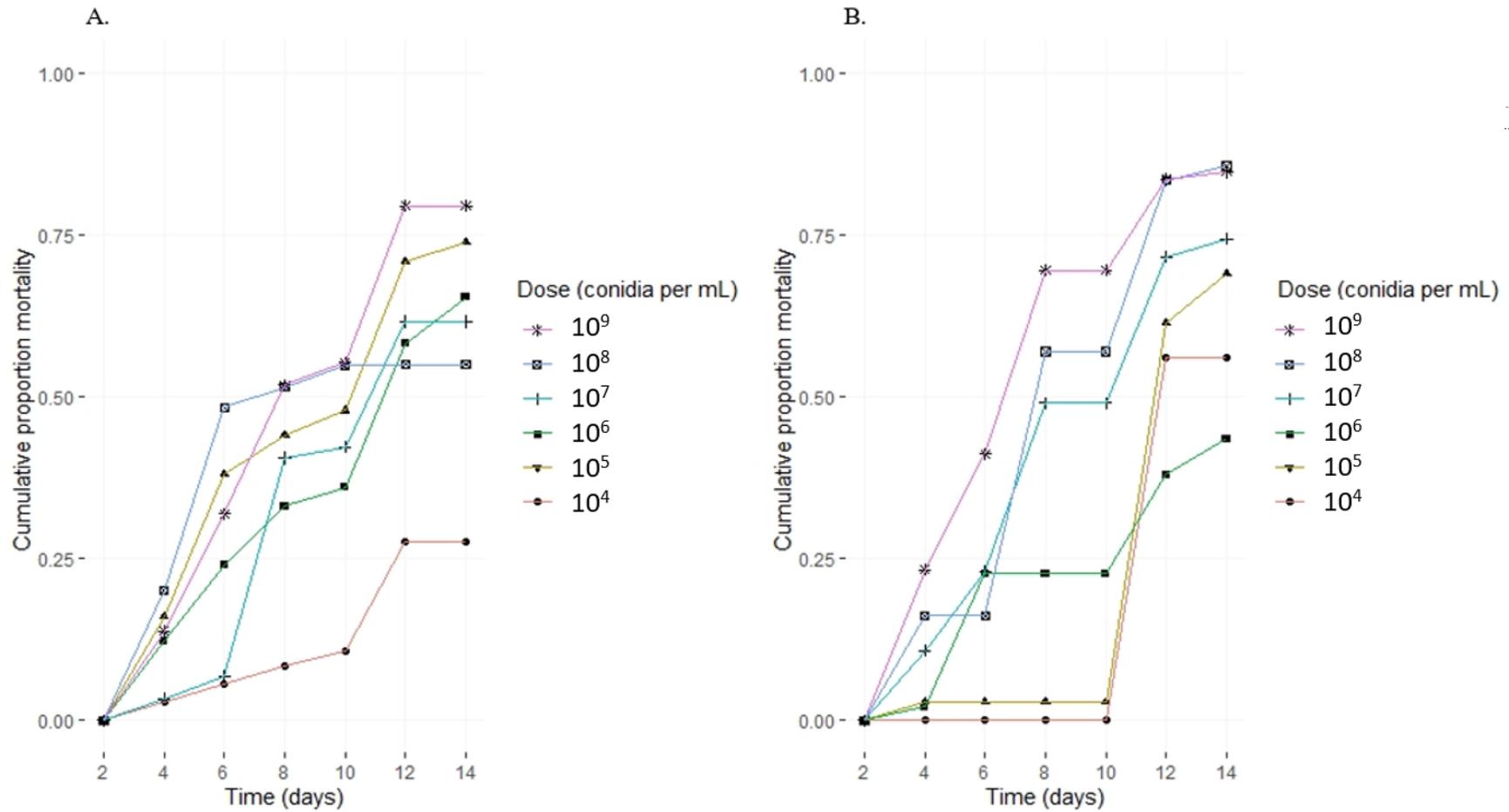


Figure 3.6 Cumulative mortality over 14 days of *Trialeurodes vaporariorum* after application of A.) *Beauveria bassiana* (PRI 5339) and B.) *B. bassiana* (ATCC 5278) at six different application concentrations.

Table 3.4 Calculated LT₅₀ values for *Beauveria bassiana* isolates tested in the first concentration response bioassay against *Trialeurodes vaporariorum* at concentrations ranging 1x10⁴- 1x10⁹ conidia mL⁻¹.

Isolate	Concentration	LT ₅₀ (days)	SE of LT ₅₀
<i>B. bassiana</i> (PPRI 5339)	1x10 ⁴	16.59	5.1
	1x10 ⁵	9.52	1.74
	1x10 ⁶	11.24	2.04
	1x10 ⁷	11.22	1.67
	1x10 ⁸	10.23	2.68
	1x10 ⁹	8.82	1.95
<i>B. bassiana</i> (ATCC 5278)	1x10 ⁴	12.96	1.29
	1x10 ⁵	12.40	1.15
	1x10 ⁶	14.30	4.53
	1x10 ⁷	9.73	1.56
	1x10 ⁸	8.76	1.31
	1x10 ⁹	7.49	1.43

Bioassays of group two involved the application of *B. bassiana* ATCC 5278, *A. lecanii* ATCC 4060 and *C. farinosa* ATCC 4412. The four parameter model described the data significantly better than all other models tested ($p=0.99$, Figure 3.7). The dose response curves were significantly different as shown by improved fit of the logistic model when LC₅₀ and slopes were estimated for each isolate compared to the constrained models (0.0036 , <0.001). Proportion mortality increased with increasing concentration of EPF applied for isolate *A. lecanii* ATCC 4060 and *C. farinosa* ATCC 4412. However, mortality of *T. vaporariorum* treated with *B. bassiana* ATCC 5278 did not differ significantly according to concentration applied and ranged from $53 \pm 19\%$ to $63 \pm 28\%$. Similar to results from bioassay one, mortality results for *T. vaporariorum* against dose (conidia mm⁻²) was best described using a two parameter log logistic model ($p=0.12$, Figure 3.7). For this model, LT₅₀ and slope parameters were significantly different for each isolate applied ($p<0.001$, 0.006). The first mortalities of *T. vaporariorum* were also observed by day four of this assay. Control mortality only reached a maximum of 6.7% by the end of the bioassay.

Calculated LT₅₀ values for isolates tested in the second run of the concentration response bioassay decreased with increasing concentration for each isolate (Table 3.5). The one exception was the standard isolate *B. bassiana* ATCC 5278 which demonstrated a similar rate of kill for all concentrations 1x10⁴-1x10⁷ conidia mL⁻¹ (Figure 3.8). Standard error in predicted LT values was highest for the lowest application concentrations. This was due to 50% mortality not being caused by the end of the duration of the bioassay, therefore predictions are based on extrapolation of the fitted model.

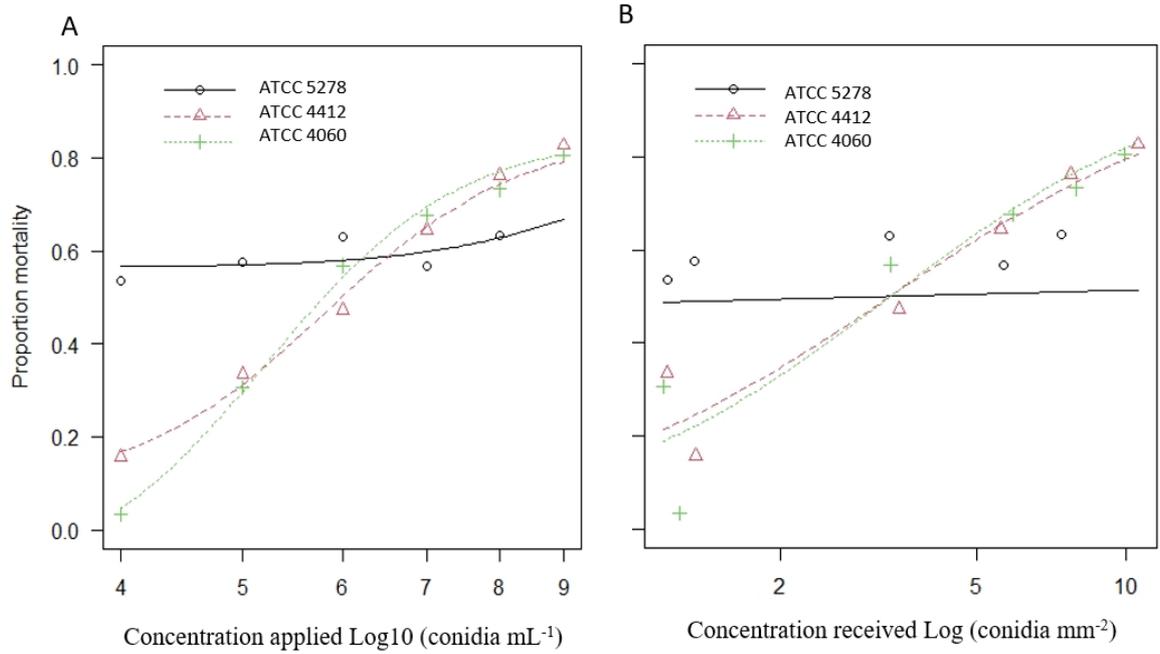


Figure 3.7 A.) Proportion mortality of *Trialeurodes vaporariorum* treated with three entomopathogenic fungi isolates at a range of application concentrations in the second concentration response bioassay. B.) Concentration received per unit area after application of each EPF and the resultant mortality observed in *T. vaporariorum* nymphs. Isolates tested were *Beauveria bassiana* (ATCC 5278), *Cordyceps farinosa* (ATCC 4412) and *Akanthomyces lecanii* (ATCC 4060).

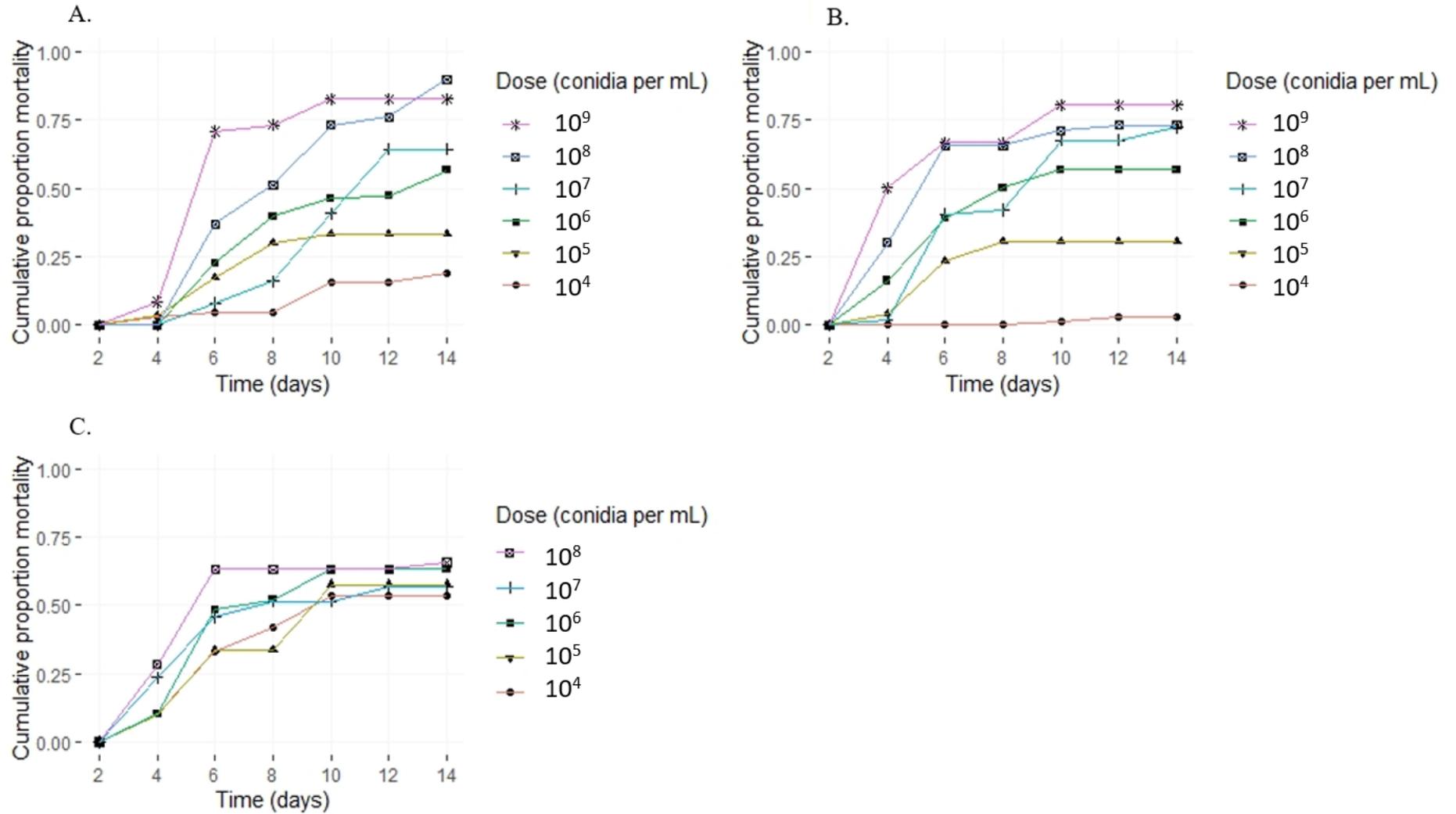


Figure 3.8 Cumulative mortality over 14 days of *Trialeurodes vaporariorum* after application of A.) *Cordyceps farinosa* (ATCC 4412), B.) *Akanthomyces lecanii* (ATCC 4060) and C.) *Beauveria bassiana* (ATCC 5278) at five and six different application concentrations.

Table 3.5 Calculated LT₅₀ values for entomopathogenic fungi isolates tested in the second concentration response bioassay against *Trialeurodes vaporariorum* at concentrations ranging 1x10⁴- 1x10⁹ conidia mL⁻¹.

Isolate	Concentration	LT ₅₀ (days)	SE of LT ₅₀
<i>Cordyceps farinosa</i> (ATCC 4412)	1x10 ⁴	19.39	9.5
	1x10 ⁵	15.14	4.88
	1x10 ⁶	11.70	2.15
	1x10 ⁷	11.49	1.42
	1x10 ⁸	8.47	1.22
	1x10 ⁹	6.84	1.39
<i>Akanthomyces lecanii</i> (ATCC 4060)	1x10 ⁴	21.06	19.9
	1x10 ⁵	16.25	6.57
	1x10 ⁶	10.27	2.35
	1x10 ⁷	9.37	1.55
	1x10 ⁸	7.17	1.92
	1x10 ⁹	5.96	1.92
<i>Beauveria bassiana</i> (ATCC 5278)	1x10 ⁴	11.13	2.43
	1x10 ⁵	10.80	2.13
	1x10 ⁶	9.39	1.94
	1x10 ⁷	10.21	2.66
	1x10 ⁸	8.10	2.25

The final block of isolates (Group 3) used in dose response bioassays were *M. brunneum* (Met 52), *B. bassiana* PPRI5339 and *B. bassiana* ATCC 5278. Control mortality was 4.04% in this bioassay. The response of *T. vaporariorum* to concentration of EPF applied was best described by a four parameter log logistic model (p=0.84, Figure 3.9). There were significant differences between the LC₅₀ of each isolate used in this assay (p<0.001). The slope of each dose response curve was not significantly different (p=0.072). The response of *T. vaporariorum* to EPF isolates when enumerated as conidia received (mm⁻²) was best described by a two parameter log logistic model (p=0.53). The most complex model with individual estimates for LC₅₀ and slope for each EPF isolate was a significantly better fit than more simplified models (p=0.0021, p<0.001).

In this bioassay, an additional dose response bioassay was conducted for *B. bassiana* PPRI 5339 due to variability in the first experiment. Following the same procedure as for *B. bassiana* ATCC 5278, comparisons between the bioassays were made by fixing parameters of a four parameter log logistic regression which was determined to be the best fitting model compared to a two or three parameter model and the Weibull-1 or Weibull-2 model (p=0.87, Figure 3.9). There were no significant differences in the fit of the model following the fixation of LC₅₀ or slope between bioassays with this isolate (p=0.91).

In the third run of the concentration response bioassay, LT_{50} values decreased with increasing concentration of all isolates tested (Figure 3.10). However, predictions beyond the scope of the dataset were common for LT_{50} values for the lowest concentrations applied, resulting in large standard error margins (Table 3.6). The most accurate LT_{50} predictions were calculated for concentrations higher than 1×10^7 conidia mL^{-1} . The LC values for each isolate in each bioassay were determined from the best fitting model and are shown in Table 3.7.

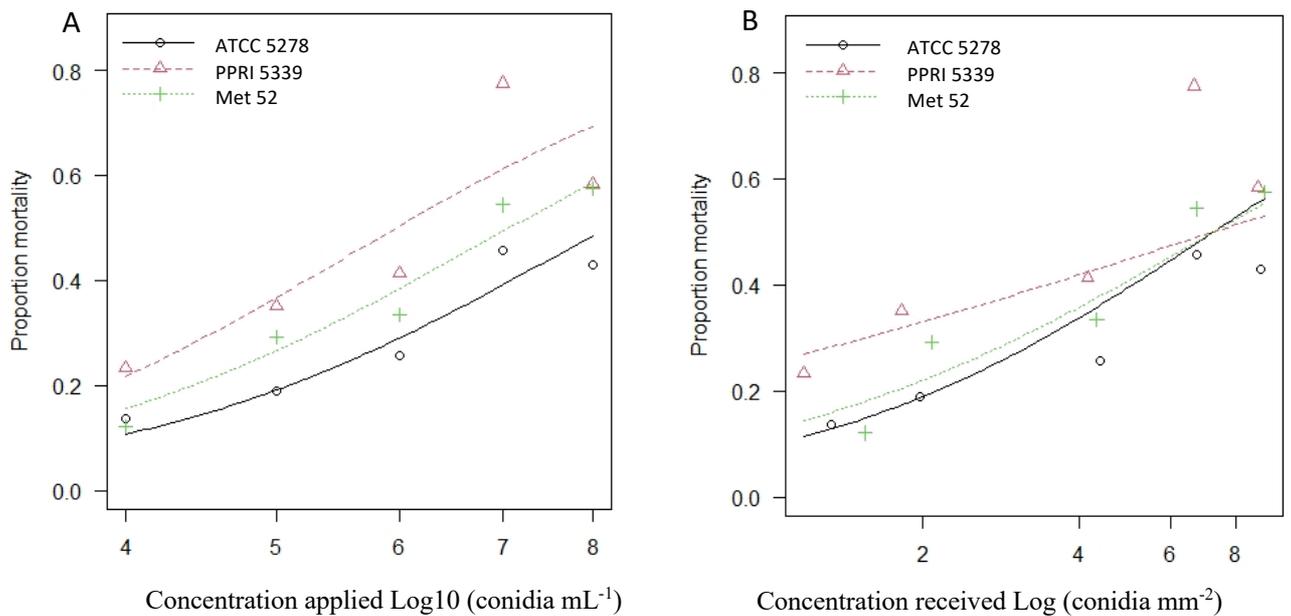


Figure 3.9 A.) Proportion mortality of *Trialeurodes vaporariorum* treated with three entomopathogenic fungi isolates at a range of application concentrations in the third concentration response bioassay. B.) Concentration received per unit area after application of each EPF and the resultant mortality observed in *T. vaporariorum* nymphs. Isolates tested were *Beauveria bassiana* (ATCC 5278 & PPRI 5339) and *Metarhizium brunneum* (Met 52).

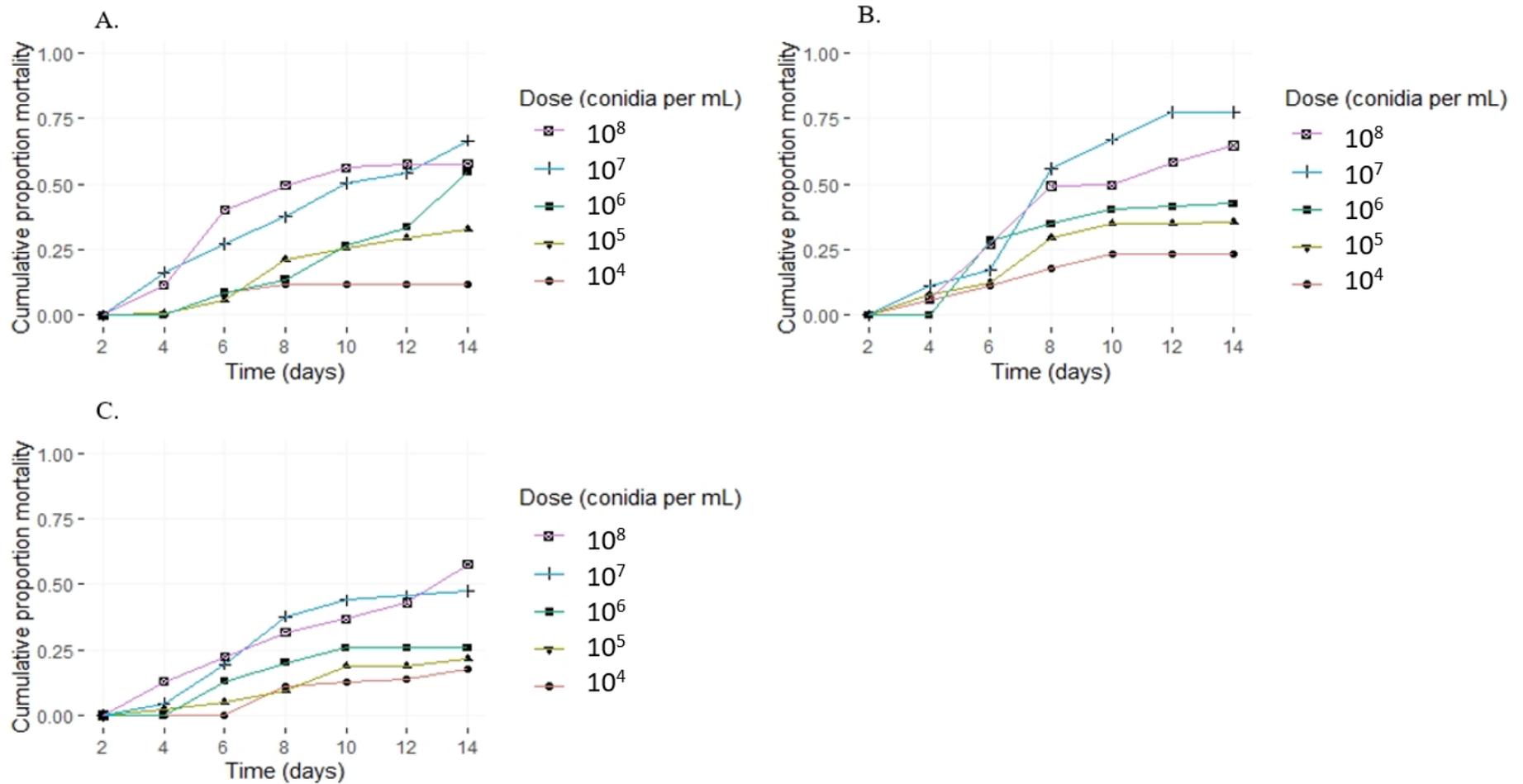


Figure 3.10 Cumulative mortality over 14 days of *Trialeurodes vaporariorum* after application of A.) *Metarhizium brunneum* (Met 52), B.) *Beauveria bassiana* (PPRI 5339) and C.) *B. bassiana* (ATCC 5278) at five different application concentrations.

Table 3.6 Calculated LT₅₀ values for entomopathogenic fungi isolates tested in the third concentration response bioassay against *Trialeurodes vaporariorum* at concentrations ranging 1x10⁴- 1x10⁹ conidia mL⁻¹.

Isolate	Concentration	LT ₅₀ (days)	SE of LT ₅₀
<i>Metarhizium brunneum</i> (Met 52)	1x10 ⁴	23.78	23.65
	1x10 ⁵	15.36	4.14
	1x10 ⁶	13.37	2.17
	1x10 ⁷	10.69	2.07
	1x10 ⁸	10.27	2.22
<i>B. bassiana</i> (PPRI 5339)	1x10 ⁴	18.61	9.11
	1x10 ⁵	14.85	4.57
	1x10 ⁶	13.23	3.33
	1x10 ⁷	8.97	1.38
	1x10 ⁸	10.50	2.23
<i>B. bassiana</i> (ATCC 5278)	1x10 ⁴	18.52	7.79
	1x10 ⁵	18.27	7.68
	1x10 ⁶	16.97	7.84
	1x10 ⁷	12.51	2.72
	1x10 ⁸	12.42	2.77

Table 3.7 LC₅₀ ± SE for five entomopathogenic fungi isolates measured in two units (concentration applied and dose received) against third instar *Trialeurodes vaporariorum* nymphs in a bioassay completed over 14 days.

Species	Isolate	Bioassay group	LC ₅₀ ± SE : Concentration applied (conidia mL ⁻¹)	LD ₅₀ ± SE: Dose received (conidia mm ⁻²)
<i>B. bassiana</i>	ATCC 5278	1	1.02 x10 ⁶ ± 5.5 x10 ⁶	1911 ± 1854
		2	8.5 x10 ⁶ ± 1.3x10 ⁶	1418 ± 1346
		3	1.1 x10 ⁸ ± 2.7x10 ⁸	2788 ± 3377
	PPRI 5339	1	1.05 x10 ⁵ ± 6.8 x10 ⁵	42 ± 104
	PPRI 5339	3	1.46 x10 ⁶ ± 3.7x10 ⁶	124 ± 285
<i>A. lecanii</i>	ATCC 4060	2	2.1 x10 ⁵ ± 4.43 x10 ⁵	84 ± 132
<i>C. farinosa</i>	ATCC 4412	2	7.41 x10 ⁵ ± 4.05 x10 ⁵	40 ± 64
<i>M. brunneum</i>	Met 52	3	2.5x10 ⁶ ± 6.3 x10 ⁶	251 ± 636

3.3.5 Determining the effect of synthetic insecticides and fungicides on *Trialeurodes vaporariorum* mortality

Application of the recommended application rate (RR) of each insecticide resulted in over 80% mortality. Pyriproxyfen was more toxic to *T. vaporariorum* than Oberon and as a result, mortality observed in the first insecticide bioassay ranged from 80-99% mortality compared to 45-85% mortality. The second bioassay involved further dilutions of both insecticides, though an additional tenfold dilution of pyriproxyfen was required to achieve a concentration response curve. Control mortality was 16% and 1.5% in each insecticide bioassay. There were no significant differences between the mortality observed following application of 0.1x RR spiromesifen in each bioassay ($df=1$, $F=6.37$, $p=0.065$) or 0.1xRR Admiral ($df=1$, $F=0$, $p=0.992$). Therefore, mortality caused by the insecticides in different bioassays were combined for further analysis. A clear concentration response was observed for both insecticides tested (Figure 3.11). Analysis of the response curves was conducted using the DRC package in R, following the same procedure as for EPF isolates. The four parameter logistic model provided the best fit for the data ($p=0.92$) and there were significant differences between slope ($p<0.001$) and LC_{50} ($p<0.001$) parameters for each insecticide applied. Again, mortality was observed as early as four days after treatment. Predicted LC values are shown in table 3.8.

Application of fungicides Takumi-SC, Luna Sensation and Kumulus caused significant mortality in *T. vaporariorum* which ranged from $35\pm 21\%$ for Kumulus to $80\pm 17\%$ for Luna Sensation. Due to high variation between replicates, there were no significant differences between mortality for treatments for the different fungicides ($df=2$, $F=1.84$, $p=0.24$) as shown in figure 3.12. Luna sensation was noted to cause black pigmentation in *T. vaporariorum* nymphs, as shown in Figure 3.13.

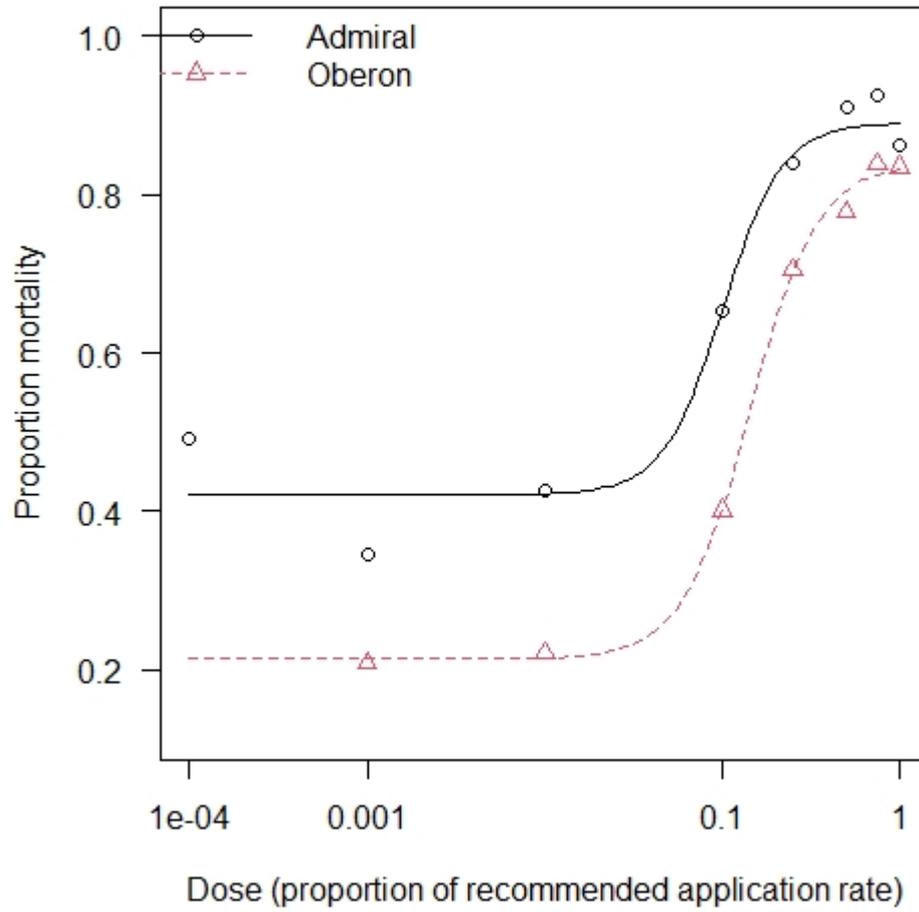


Figure 3.11 Proportion mortality of *Trialeurodes vaporariorum* treated with two insecticides (Admiral; AI= pyriproxyfen, Oberon; AI= spiromesifen) at a range of application concentrations in a concentration response bioassay.

Table 3.8 Calculated LC values for two insecticides against *Trialeurodes vaporariorum* following the application of recommended application rates (RAR) and serial dilutions of RAR during 14 day bioassays.

Insecticide	LC value	Estimate \pm SE	Amount of AI (mg mL ⁻¹)
Admiral AI= Pyriproxyfen	15	0.05 \pm 0.038	2.5
	30	0.07 \pm 0.027	3.5
	50	0.10 \pm 0.02	5
	80	0.17 \pm 0.098	8.5
Oberon AI= Spiromesifen	15	0.067 \pm 0.023	0.04
	30	0.099 \pm 0.022	0.2
	50	0.14 \pm 0.029	1.2
	80	0.26 \pm 0.088	17.7

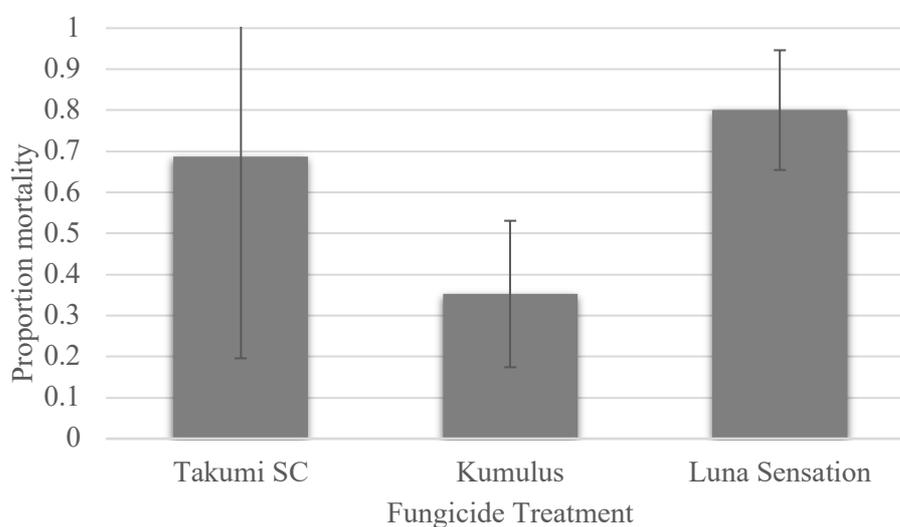


Figure 3.12 Average proportion mortality of third instar *Trialeurodes vaporariorum* after 14 days following the application of recommended application rates of three fungicides; Takumi SC, Kumulus and Luna Sensation.



Figure 3.13 Photograph showing a healthy fourth instar *Trialeurodes vaporariorum* (right) and a dead *T. vaporariorum* nymph (left) following treatment with the fungicide Luna Sensation.

3.3.6 Determining the relationship between concentration of conidia suspension applied and conidia dose received at the target spray area

Initial analysis was to determine whether there were significant differences between the numbers of conidia received per unit area for each isolate at each concentration applied. The number of conidia received on the target area between assays was not significantly different for each concentration applied for the standard isolate (*B. bassiana* ATCC 5278; $df=2$, $F=3.81$ $p=0.051$), so data for the different fungal isolates were combined for further analysis.

Dose received ranged from 2 ± 1 conidia mm^{-2} at an application concentration of 1×10^4 conidia mL^{-1} to 82369 ± 7734 conidia mm^{-2} at an application concentration of 1×10^9 conidia mL^{-1} . Concentration received in the target area increased with increasing concentration applied, though this relationship was not linear. This data was modelled as a linear regression as well as a second-, third- and fourth-order polynomial regression and the fit of each model was compared using ANOVAs. The relationship between concentration applied and concentration received was significantly described by a second-order polynomial ($R^2=0.97$, $AIC=23.64$, $F_{6, 118}=743.1$, $p<0.001$; Figure 3.14). The fit of the model was significantly improved by fitting a line for each EPF isolate ($p<0.001$). The conidial deposition of *M. brunneum* (Met 52) was significantly higher for application concentrations 1×10^5 -

1×10^7 conidia mL^{-1} compared to *C. farinosa* (ATCC 4412, $p=0.026$) and *A. lecanii* (ATCC4060, $p=0.011$). There were no other significant differences between conidia received and concentration applied for all isolates.

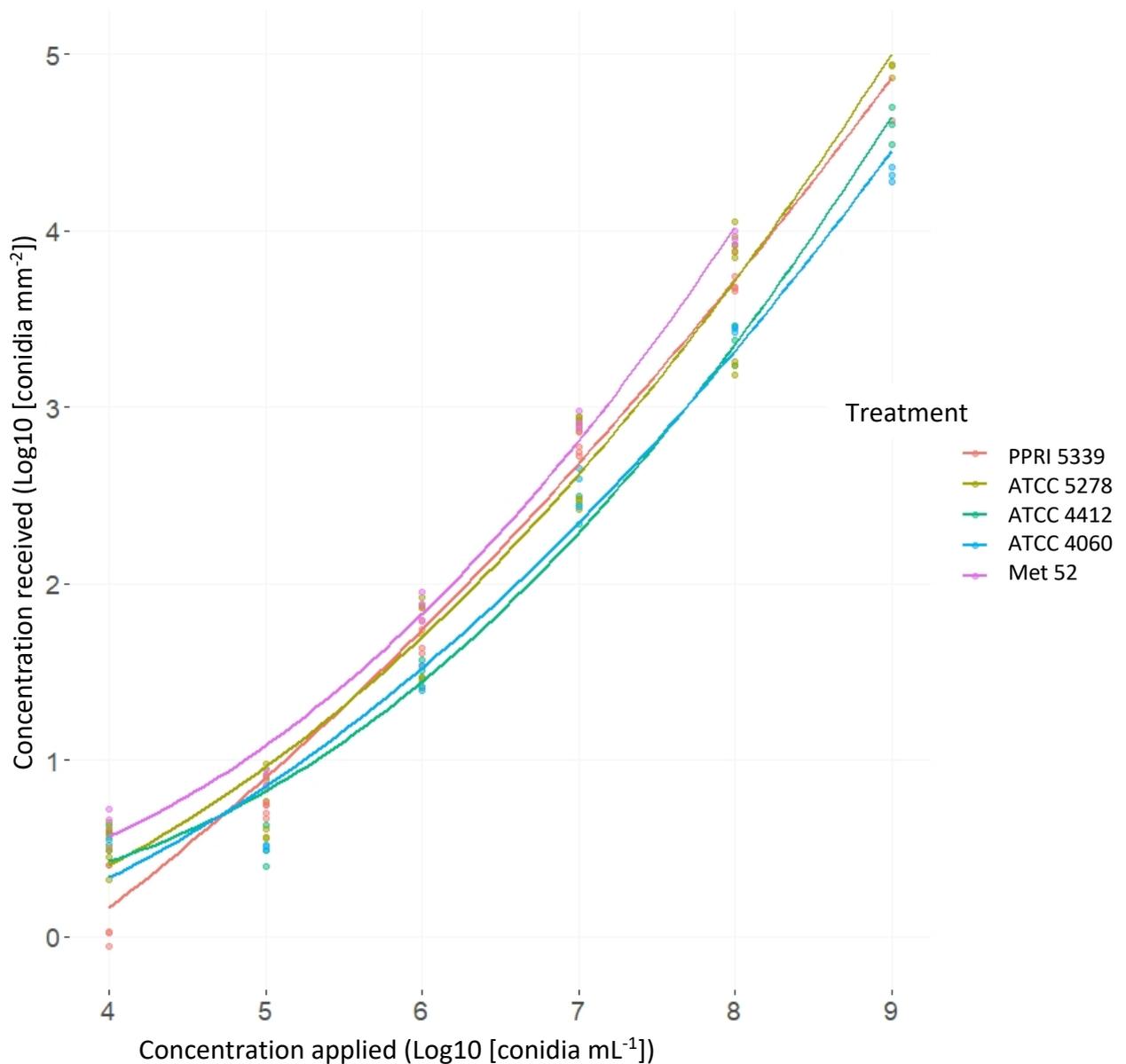


Figure 3.14 Concentration of entomopathogenic fungal isolate conidia deposited on a 22×22 mm square coverslip (Log_{10} [conidia mm^{-2}]), pooled across replicate experiments, after being applied at a range of concentrations (Log_{10} [conidia mL^{-1}]) and 138 kPa. The five isolates tested were *Beauveria bassiana* (PPRI5339 & ATCC 5278), *Cordyceps farinosa* (ATCC 4412), *Akanthomyces lecanii* (ATCC 4060) and *Metarhizium brunneum* (Met 52). The data is fitted to a second order polynomial.

3.4 Discussion

There were a number of technical issues that were overcome whilst developing the bioassays design. Initially, *T. vaporariorum* were cultured on *Solanum lycopersicum* (var. moneymaker) and individual leaves were selected for use in bioassays. Detached leaves were sustained in pots containing a plant nutrient solution that allowed the leaf to produce roots *in situ* (Figure 3.15). However, the numbers of *T. vaporariorum* nymphs produced after allowing adults to lay eggs using the clip cage method described in 2.5.1 were very low. Increasing the amount of time for egg laying by *T. vaporariorum* adults resulted in a population of whitefly nymphs of different ages. Because different life stages of *T. vaporariorum* vary in their susceptibility to EPF (Osborne and Landa, 1992), this would be likely to increase the amount of variation observed in dose response, and time to response, bioassays. Newly moulted nymphs are more vulnerable to infection, whereas those preparing to moult are likely to shed conidia as well as the old cuticle before EPF are able to penetrate the host (Butt and Goettel, 2000).



Figure 3.15 Individual *Solanum lycopersicum* leaf supported by sponge submerged in fertiliser solution in small truncated plastic pot with a lid to reduce evaporation.

During a visit to BASF Utrera (Spain) testing facility, *S. melongena* (var. F1 Paris) was noted to be a proven successful host plant for mass rearing *Bemisia tabaci*. Therefore, suitability of the plant for rearing the *T. vaporariorum* culture and for use in experiments was tested upon return to the UK, alongside several varieties of *S. lycopersicum* and cucumber (*Cucumis sativus*). The *T. vaporariorum* population size markedly increased on *S. melongena*. However, detached *S. melongena* leaves were unable to root and desiccated before reaching the end of the duration of the bioassays. Instead, the bioassay was re-designed to allow one leaf on a small plant to be sprayed in whole plant bioassays.

The sprayer delivered a uniform spray volume to the central 1590 mm² of the spray area. Restricting *T. vaporariorum* adults to a single leaf using the clip cage allowed egg laying and subsequent developing nymphs to be successfully contained in a known area to ensure they received a uniform cover of spray. By introducing a known number of adults, there was an added advantage that the cages allowed some control over the resultant replicate sample size of nymphs. Several clip cages have been developed in the past, many of them following the same basic design by MacGillivray & Anderson (1957) and some varying in shape to allow for *T. vaporariorum* natural behaviours (Muñiz, 2001). First instar *T. vaporariorum* nymphs crawl across the leaf surface before securing themselves at a preferred site where they remain, until emerging as adults (Capinera *et al.*, 2008); there is a lack of information on the ability of clip cages to restrict *T. vaporariorum* nymphs to a known area once the cage and *T. vaporariorum* adults inside have been removed. This information is required for sufficient calibration of the spray tower. In the current study, *T. vaporariorum* nymphs migrated to cover a maximum area of 380 mm² but never onto the upper leaf surface. By restricting egg laying to 177 mm², third instar nymph distribution was within the central 1590 mm² which received uniform coverage at 138 kPa, despite the migration of first instar nymphs by several mm.

The pressure used during application of pesticides affects the applied volume, size of droplets and uniformity across the spraying area. In comparison to work by Mascarin *et al.* (2013b), in the current study, a known volume of solution was sprayed during calibration and subsequent bioassays instead of spraying based on duration of time (seconds) which can introduce variation due to human error (as found in preliminary observations in this study). A spray pressure of 138 kPa was the selected setting, based on the percentage volume of spray reaching the target, and uniformity of spray across the target area. As application pressure was increased, the volume of liquid reaching the target area decreased, similar to findings by Liu & Stansly (1995) when using the Potter spray tower. However, unlike the calibration of the Potter spray tower, the spray received was more uniform at lower application pressures. This was likely due to the spray gun ‘spitting’ at higher pressures, resulting in uneven application of the solution. It is also possible that the higher pressure spray rebounded from the surface once hitting the target area. The portable spray tower designed by Mascarin *et al.* (2013a) delivered 150 ± 34 conidia mm⁻² following an application of 4mL 1×10^7 conidia mL⁻¹ of three different EPF at 69 kPa. Another study which used a similar spray tower set-up consisting of an artist spray gun attached to an acrylic cylinder conducted by Erdos *et al.* (2021) resulted in 615 conidia mm⁻² following the application of 400µL of *Akanthomyces muscarius* at 83kPa at 4×10^6 conidia mL⁻¹. In comparison, our tower delivered an average of 256 ± 31 conidia mm⁻² after a 1mL

application of 1×10^7 conidia mL^{-1} of 18 different fungal isolates. This variation demonstrates the importance of calibration of sprayers under different laboratory conditions.

Inundative control of whitefly using EPF in greenhouse or field crops requires at least 10^{12} – 10^{14} conidia per hectare (Jaronski, 2009). Conidia received per unit area using the calibrated spray tower are equivalent to the concentration applied to field crops and therefore mortality results from this laboratory experiment might be more representative of field efficacy than leaf-dip bioassays (although spray cover in the field will be more patchy due to leaf shielding and efficacy is strongly influenced by prevailing environmental conditions).

In this study, the spray tower was used to apply EPF onto target *T. vaporariorum* nymphs in pathogenicity bioassays testing at a single concentration of 1×10^7 conidia mL^{-1} . Steinhaus and Martignoni (1970) define pathogenicity as the potential ability to produce disease. In the field of invertebrate pathology, pathogenicity is often confused with the term virulence and vice versa. As discussed by Shapiro-Ilan *et al.* (2005), virulence and pathogenicity are mistakenly used interchangeably though virulence is defined as the disease producing power of a pathogen rather than the ability to cause disease. All isolates tested were capable of infecting *T. vaporariorum* nymphs. However, there was large variation in the total mortality of nymphs, indicating that isolates taken from different hosts and geographical locations have varied pathogenicity toward *T. vaporariorum* nymphs. *Beauveria bassiana* isolate GHA originates from the coleopteran *Diabrotica undecimpunctata* and *C. fumosorosea* isolate PFR from Aphididae, yet they infected a higher proportion of whitefly nymphs than some other isolates originating from the target species *T. vaporariorum*. Several other studies have found that pathogenic isolates may not originate from the target species, (Leatherdale, 1970; Bugeme *et al.*, 2008) which is well known for anamorphic EPF belonging to the order Hypocreales as they generally have an extensive host range (Faria and Wraight, 2007), infecting multiple species across several taxonomic orders (Brodeur, 2012). This characteristic is expected from EPF isolated from commercial products because their selection was based on high virulence toward a broad range of pest species. Of the eighteen fungal isolates used in the pathogenicity bioassays against *T. vaporariorum*, *A. lecanii* isolate 4060 caused the highest overall mortality.

Following pathogenicity screening, concentration-response bioassays were conducted to determine the virulence of a select number of isolates. Concentration response bioassays allow the calculation of lethal concentrations (for example, LC_{50}) which can be used as a measure of virulence, defined as the disease producing power of a pathogen (Steinhaus and Martignoni, 1970; Shapiro-Ilan *et al.*, 2005). These experiments involved five EPF isolates

selected based on temperature profiles (described in chapter 4) and pathogenicity results. *Akanthomyces lecanii* (ATCC 4060) was included due to the high rate and overall mortality observed in pathogenicity bioassays. This isolate also produced a large number conidia which is desirable for biocontrol agents for ease of mass production. Isolate *C. farinosa* ATCC 4412 was chosen for further experimentation based on its ability to cause high mortality of *T. vaporariorum* and fast *in vitro* growth and germination across a range of temperatures. *Beauveria bassiana* (PPRI 5339) was selected based on high conidia production across a range of temperatures. Isolates from commercially sold products were also included for comparison; *B. bassiana* ATCC 5278 is the active ingredient for Naturalis and caused high mortality of *T. vaporariorum* whilst the active ingredient from Met52 (*M. brunneum*) was chosen as it was predicted to have a high temperature optimum and may cause contrasting results compared to other isolates in later experiments involving temperature.

The most virulent isolate was found to be *B. bassiana* PPRI 5339 in assay one, as it was calculated to have the lowest LC₅₀ value at 1.05 x 10⁵ conidia mL⁻¹. It is difficult to make comparisons between EPF virulence bioassays in general against *T. vaporariorum* due to the range of application techniques, different quantification methods for dose and studies targeting different nymphal stages. However, the range of LC₅₀ values observed in this study were comparable to lethal concentrations calculated in studies involving virulent Hypocrealean fungal isolates against *T. vaporariorum* (Drummon *et al.*, 1987; Avery *et al.*, 2015).

There was variation in the response of *T. vaporariorum* following the application of *B. bassiana* PPRI 5339 and the standard isolate *B. bassiana* ATCC 5278 in replicate bioassays. Considerably high control mortality (>30%) was also observed for the first few preliminary bioassays. It was determined that this was due to *T. vaporariorum* nymphs drowning in the treatment droplets which did not evaporate before being placed into the plastic containers. In order to overcome this, all treated plants were left at room temperature to dry before being placed into the plastic containers and control mortality significantly reduced after this adjustment. However, the variability in response of *T. vaporariorum* to *B. bassiana* (PPRI 5339) and ATCC 5278 remained an issue.

This variation was determined to be because of the inherent variation observed in insect bioassays due to multiple interactions between biological and abiotic factors which can not be controlled. As a result, direct comparisons between isolates used in different assays could not be made with a level of confidence.

The shape of dose response curves were varied for each isolate. For most isolates (e.g. *C. farinosa* 4412, *A. lecanii* 4060), increasing the concentration of conidia applied resulted in significantly higher *T. vaporariorum* mortality. However, increasing the concentration of *B. bassiana* ATCC 5278 had little effect on the response of *T. vaporariorum* in assay one and two in particular and LC₅₀ values were different for each repeat of the same isolate. Though regression slopes of dose-mortality curves are usually low, increases in dose at the scale shown in this study should result in commensurate increases in efficacy as infection of hosts is proportional to the number of conidia reaching the target for hypocrealean fungi (Jaronski, 2009). The relationship between concentration applied and dose received was consistent between bioassays which highlights the bioassay system as providing a standard approach which could be used by other researchers. Comparisons between LC and LD values showed that the number of conidia needed to cause 50% mortality was not as markedly different when comparing dose between bioassays instead of concentration applied for the standard isolate used in all dose response bioassays (*B. bassiana* ATCC 5278). This was also true for *B. bassiana* PPRI 5339 which was tested in two of the three dose response bioassays. However, the variation in response of *T. vaporariorum* to increasing concentration of *B. bassiana* (ATCC 5278) in different bioassays was likely caused by factors other than the spray application of conidia, as dose was consistent between experiments for the different concentrations applied.

Differences in virulence of isolates is determined by several factors. Conidia which germinate faster are able to evade undesirable environmental conditions and penetrate the insect cuticle before *T. vaporariorum* develop to the next developmental instar, moulting the exoskeleton and the EPF conidia (Drummon *et al.*, 1987). Highly virulent EPF will produce target specific effectors such as enzymes to break down the insect cuticle and allow host colonisation as well as secondary metabolites which can aid in reducing host immune response (Chandler, 2017). Different strains of EPF have evolved genes which differ in their molecular mechanisms determining virulence as a result of co-evolution with the target host and overcoming defence strategies at each stage of the invasion and infection process (Butt *et al.*, 2016).

Increasing the concentration of conidial suspensions resulted in higher mortality as well as faster rate of infection of the host. The accuracy of determining time of death in these bioassays is low. Firstly, mortality observations were only recorded every 48 hours, so differences in rate of kill between isolates, or between concentrations of the same isolate would not be detected if less than two days. The 48 hour time point was chosen as a trade-off between being able to apply the number of treatments tested rather than reducing the

number of treatments and conducting 24 hour observations. Secondly, determining the time of death of *T. vaporariorum* nymphs is difficult as they are sedentary. In this study, dead nymphs were identified by a change in colour or a lack of ‘plumpness’ which often later resulted in hyphal growth from the cadaver. However, it is possible that nymphs died prior to obvious changes in appearance. Other studies have estimated the number of alive nymphs by measuring honeydew droplets (Cameron *et al.*, 2014) which may be a more accurate method to determine exact time of death. Using this approach though, does not allow for large numbers of individuals in each treatment, resulting in higher variability in results. Additionally, this method relies on *T. vaporariorum* feeding, which will be effected following the application of EPF or insecticides.

Again, the use of LT_{50} and LC_{50} values are only indicative of the comparable virulence of pathogens tested under the same experimental conditions against the same host population. Robertson *et al.* (2017) described these values as neither biological constants nor measurements. For this reason, results may be used to give an indication of virulence of pathogens but direct comparisons between experiments which may involve different host plants, stages of insect development and abiotic conditions should be made with caution. Although, these estimations are useful when screening for desirable characteristics in a collection of potential biopesticides.

Mortality caused by EPF is also affected by methods used to handle and apply the pathogen (Khan *et al.*, 2012). The number of conidia reaching the target nymphs in the pathogenicity bioassays ranged from 189 to 332 conidia mm^{-2} . The effect of conidial deposition on mortality could not be individually determined for each isolate because replicate conidia deposition was collected separately from leaf treatments, therefore only estimates of average conidial deposition for each treatment could be provided, using the same stock conidial suspensions applied to the hosts. However, comparisons between conidial deposition and mortality could be made when isolates were grouped by genera.

For the pathogenicity bioassay, conidial suspensions were applied at one concentration (1×10^7 conidia mL^{-1}). Despite the small variation in the number of conidia received by the target area at this application concentration, there was no significant effect of conidial deposition on *T. vaporariorum* mortality. In the concentration-response bioassays, mortality increased with increasing concentration though there was no relationship between variation in conidial deposition within each application concentration and observed mortality across EPF used. However, in both experiments, application of *Akanthomyces* isolates resulted in significantly fewer conidia deposited onto the target area than *Metarhizium* isolates. There

was no difference in conidial deposition of *Akanthomyces* isolates and *Beauveria* or *Cordyceps* isolates, therefore these differences are unlikely to have been caused by differences in conidial suspensions caused by the hydrophobicity of *Beauveria*, *Cordyceps* and *Metarhizium* conidia isolates compared to the hydrophilic conidia of *Akanthomyces* isolates. Small variation in conidia number could be attributed to counting errors in the haemocytometer and differences in percentage germination of conidia from different species. Conidia may have also attached to the inside of pipette tips and tubes during dilution and enumeration.

The calibration method demonstrated in this study uses an effective way to quantify dose and accounted for varied conidia viability by counting colony forming units after conidia are sprayed. It is possible that suspensions of *A. lecanii* contained a higher proportion of ungerminated conidia because proportion germination of suspensions applied was not tested. In addition, CFU counts may underestimate actual concentrations as conlonies may overlap, but direct enumeration of suspensions after spray was not possible due to low conidial counts under the haemocytometer.

The spray tower is equally capable of uniformly applying synthetic chemical insecticides and fungicides. Recommended application rates of pyriproxyfen resulted in 79% mortality which was comparable to 79% mortality observed by Hosseini and Pourmirza (2010) after whole *Nicotiana sylvestris* plants infested with *T. vaporariorum* were sprayed with the same recommended application rate. However, application of spiromesifen resulted in significantly lower mortality than in a previous study by Bi and Toscano (2007b) where a 30 fold dilution resulted in 100% mortality of third instar *T. vaporariorum*. Whereas, in the current study, application of the recommended application rate of spiromesifen resulted in 82% mortality. These differences could be observed due to the high control mortality (>30%) observed by Bi and Toscano (2007b) which was not accounted for. It is also possible that there is a small level of resistance in the *T. vaporariorum* population used in this study because their exposure to pesticides is unknown prior to collection in 2017.

Few studies have investigated the direct insecticidal effect of topically applied fungicides against *T. vaporariorum*. Throughout the literature, there is a focus to determine the effect of fungicides on the efficacy of EPF to control *T. vaporariorum* (Jaros-Su *et al.*, 1999; Avery *et al.*, 2013; Roberti *et al.*, 2017). However, in a field study by Van De Veire (2004), the potential of two fungicides to function as insecticides towards *T. vaporariorum* was observed incidentally whilst determining the effect of these fungicides on *Cordyceps fumosorosea* (Apopka Strain 97). In a subsequent study, 100% mortality of first instar

nymphs was observed following the application of recommended application rates of the fungicides mancozeb and maneb whilst having no effect on the parasitic wasp *Encarsia formosa* (Van De Veire et al 2004b). Although the mechanisms behind the insecticidal effect of the fungicide were not determined, it could be inferred that the product formulation was not responsible for this effect after testing other fungicides which caused no pest mortality. In this study, significant *T. vaporariorum* mortality was observed following the application of Kumulus, Luna Sensation and Takumi-SC. Determining the interaction of these fungicides with other pest control management strategies would suggest whether there is potential for improved *T. vaporariorum* control whilst also protecting the crop from foliar diseases.

In this study, a simplistic and affordable spray tower was developed in order to provide a standardised method of delivering doses of entomopathogenic fungi which can be enumerated for the target area. Several calibration methods were conducted to ensure uniform delivery of suspensions across a known target area. The spray tower is versatile and equally capable of applying chemicals to the target area, allowing for direct comparisons between chemical and fungal induced mortalities in target insects.

3.4.1 Summary

- i) A standardised bioassay method was developed using an affordable, small-scale and reproducible spray tower design. The standardised system ensured the delivery of conidia to a target area proportional to the application concentration across a range concentrations (1×10^4 - 1×10^9 conidia mL⁻¹).
- ii) This method was used to screen several candidate EPF isolates as well as two insecticides and three fungicides against *T. vaporariorum* nymphs.
- iii) LC values were determined for selected EPF isolates and insecticides for use in further experiments.

4 Assessing the response of entomopathogenic fungi to environmental stress

4.1 Introduction

Whilst it is recognised that the important characteristics of pathogenicity or virulence of EPF isolates must be considered when choosing an EPF for control of a pest, there are multiple other characteristics that will influence efficacy and reliability of control. Environmental abiotic factors are well known to strongly influence the persistence of microbial entomopathogens and their virulence towards target pests. In order to select successful EPF for integration into IPM, Yeo *et al.*, (2003) for example proposed that a biologically rational approach should be taken to identify specific isolates; isolates are selected in a tiered approach which considers not only their innate virulence but also the ability to achieve control over the range of conditions that EPF are exposed to post-application. Conidia production is another important characteristic for a biological control agent (Muñiz-Paredes *et al.*, 2017); the EPF must be able to produce large amounts of conidia to enable mass production for a formulated product. The production of large numbers of conidia on insect cadavers is also a desirable characteristic as an indicator of secondary cycling, which enables further infection of the population following the initial application of the EPF and leads to a degree of self-sustaining pest control, i.e. without the need for additional applications of EPF (Thomas *et al.*, 1995). Though, this quality is difficult to predict through *in vitro* experiments, as nutritional requirements for conidial production differ for different species of EPF (Kamp and Bidochka, 2002). For EPF isolates, humidity, temperature and solar radiation (UV) are particularly important in influencing the rate of within-host growth, germination, conidia production and persistence of conidia within the environment (Jaronski, 2009). Each of these factors play a role in the different life cycle stages of the host; adhesion to the cuticle, penetration of the host, establishment within the host, host death and transmission. The sensitivity of EPF to abiotic factors varies depending on the fungal species and isolate being tested so it is important to screen potential biological control agents under conditions similar to those that they will be used in. As well as variable abiotic conditions, it is likely that EPF used for biological control will be directly or indirectly exposed to chemicals such as fungicides and insecticides which can also have detrimental impacts on the efficacy of EPF. As part of the selection criteria of a control agent in IPM, compatibility with other commonly used control agents such as insecticides and fungicides should be considered.

Exposure to short periods of high intensity ultraviolet radiation can kill EPF conidia or cause delays in germination (Braga *et al.*, 2001). However, targeting applications to the

abaxial leaf surface where pests such as *T. vaporariorum* reside, can provide shade allowing EPF conidia to persist longer in the environment (Jaronski, 2009). High humidity is the most important requirement for germination of EPF conidia (Lord, 2005). A minimum of 93% relative humidity is required for germination of EPF conidia, with rate of germination increasing as relative humidity increases towards saturation (Lacey, 2017). However, in practical use, because of the boundary layer and microclimate effects within the crop canopy, infection can often occur in greenhouses in ambient humidity conditions much lower than 93% (Wraight *et al.*, 2000). Optimal relative humidity for aubergine and tomato production ranges from 60-90% (Barker, 1990; Shamshiri *et al.*, 2018). Under these conditions, sufficient water should be available to EPF to allow germination of conidia, as variation in relative humidity within this range is not reflected in the boundary surface layer (Lord, 2005). This is reflected in Fargro© recommendations for use of Naturalis-L® (*Beauveria bassiana*), which states that relative humidity is not a limiting factor in the use of Naturalis-L®. Their product is described to work under ‘desert-like’ conditions due to the microclimate maintained 2mm under the leaf surface, where most target insects reside (Fargro, 2021). Despite this, the product label recommends relative humidity to be maintained at at-least 60% for effective use of the EPF.

Temperature is one of the most important factors limiting the success of EPF in host pathogenesis (Fargues *et al.*, 1997). Entomopathogenic fungi are exposed to temperature variation as a result of natural abiotic conditions or through host behaviours, for example, behavioural fever is common in some EPF hosts whereby the host will bask in sunlight in order to elevate body temperature to reduce or stop further infection by the EPF (Elliot *et al.*, 2002; Clancy *et al.*, 2018). Within the greenhouse, mycoinsecticides can be exposed to temperatures ranging 10-35°C in the UK and this range is even greater for greenhouses in Mediterranean climates. Most hyphomycetes have an optimal temperature between 20°C and 25°C for infection, though infections do occur outside of this temperature range at a slower rate (Inglis *et al.*, 2001). Different EPF isolates from the same species show significant differences in their responses to temperature, but there is no simple relationship between the thermal biology of an isolate and its provenance (i.e. host or geographic origin). Some studies have shown that EPF isolated from locations with different climatic conditions have thermal tolerances which reflect their place of origin (Roberts and Campbell, 1976; Vidal *et al.*, 1997), while other studies indicate that the differences in thermal profiles of EPF are related to their host, rather than the geographical location that they were originally isolated from. For example, Fargues *et al.* (1997) found no correlation between temperature profile and geoclimatic origin of sixty-five *B. bassiana* isolates. Of these isolates, it was noted that those isolated from grasshopper species had a higher temperature threshold compared to

EPF isolated from hosts that do not perform behavioural fever. In another study, infection of the mealworm beetle (*Tenebrio molitor*) at 12°C or 32°C was not different for *B. bassiana* isolates originating from tropical or arctic climates despite *in vitro* experiments suggesting that those isolates had significantly different temperature profiles (Seid *et al.*, 2019).

Nevertheless, studies have demonstrated that taking temperature conditions into consideration when selecting EPF for biological control can allow well suited EPF isolates to be exploited under temperature extremes for example mortality of black vine weevil (*Otiorhynchus sulcatus*) caused by *Beauveria pseudobassiana* at 6°C (Klingen *et al.*, 2015) and *Beauveria bassiana* infecting house fly *Musca domestica* L. at 45 °C (Mishra, Kumar and Malik, 2015).

In order to categorise differences between thermal profiles of isolates, several mathematical models have been used to analyse the effect of temperature on the physiology of EPF. These models can often be used to predict workable thermal ranges and optima. The physiological response of EPF across a range of temperatures results in an asymmetrical bell-shaped distribution (Cohen and Yarwood, 1952). Typically, there will be no growth or germination of EPF below a minimum temperature threshold, the physiological response increases with temperature, reaching a maximum at the thermal optima before a steep decline to the thermal maximum. The development of insects at different temperatures follows the same distribution and models are often successfully used to describe EPF physiology and insect development interchangeably. Given that the physiological response of EPF and insects are similar, a successful strategy for selection of EPF as control agents is to match the thermal biology of the EPF to its target host. As such, the growth rate of the EPF and the insect will be similar despite exposure to oscillating temperature conditions, and the insect will not be able to evade infection by growing and reproducing under temperature conditions where the EPF is unable to infect.

Models developed to describe insect or EPF response to temperature range from simple linear regressions (Cohen and Yarwood, 1952), to polynomial models (Mihaan and Aghajani, 2016), normal models (Taylor, 1981) and non-linear models. Several non-linear models have been used to describe the relationship between temperature and EPF physiology (Mihaan & Aghajani, 2016; Davidson *et al.*, 2003; Keen & Smits, 1989). For example, Smits *et al.* (2003) compared the effectiveness of five non-linear models to describe the relationship between temperature and colony extension for five EPF isolates. In this study, the Briere model (Briere *et al.*, 1999) was highlighted to be particularly successful at accurately describing the relationship by consistently producing high R² values and low standard errors for each isolate.

Determining the compatibility of potential control agents to be applied simultaneously or consecutively onto a crop is an important aspect of an IPM programme. A common method to determine whether EPF would be compatible for co-application or exposure to residues of a chemical is to assess how continued exposure affects the physiology of the EPF on media spiked with the chemical.

A recent study by Sain *et al.* (2019), investigated the compatibility of ten EPF including isolates of *M. anisopliae*, *B. bassiana* and *Cordyceps javanica* with nine synthetic and three botanical insecticides. Growth and conidial production of EPF in spiked agar containing 100%, 50% or 0% recommended field application rates of each insecticide was observed. Compatibility of several EPF with botanical oils such as neem and pongamia oil was observed as well as high compatibility with insect growth regulators such as pyriproxyfen and spiromesifen. Growth and conidia production were significantly higher at full or half doses of spiromesifen for several EPF compared to control exposures, with growth increasing by 169% and conidia production by 25%. This was significantly higher than that for EPF exposed to the botanical oils. Germination of *Akanthomyces muscarium* (previously known as *Lecanicillium muscarium*) conidia was suppressed by imidacloprid, teflubenzuron, buprofezin and nicotine after being exposed to recommended dose rates for 24 hours (Cuthbertson *et al.*, 2005). However, application of the EPF on verbena plants covered in pesticides residues had no impact on *B. tabaci* mortality and co-application of *A. muscarium* with imidacloprid caused increased silverleaf whitefly (*Bemisia tabaci*) mortality compared to control agents applied singly (Cuthbertson *et al.*, 2005).

The interactions observed in *in vitro* laboratory based experiments do not always translate to results found *in vivo*. A comparison of the mortality of *Galleria melonella* L. larvae caused by soil dwelling EPF, for example *B. bassiana* and *M. flavoviride* after being exposed to herbicides, fungicides and insecticides showed that the application of some chemicals had detrimental effects on the ability of EPF to infect larvae. The negative interaction of benomyl and *B. bassiana* was confirmed by reduced growth of the EPF when grown in agar containing recommended concentrations of the insecticide. However, this study also identified many combinations of EPF and chemical that showed contradictory outcomes depending on lab or field-based interactions. For example, triadimefon applied to the soil resulted in the highest overall mortality of *G. melonella*, despite causing reduced *in vitro* growth of *B. bassiana*. Comparatively, aldicarb stimulated *B. bassiana* *in vitro* growth but had no impact on mortality of *G. melonella* when applied to soil samples (Mietkiewski *et al.*, 1997). EPF grown in agar under laboratory conditions are provided with the necessary nutrients and optimum conditions for germination, growth and sporulation but this may also

affect their tolerance to pesticides (Mietkiewski *et al.*, 1997). What is clear is that it is difficult to predict the compatibility of EPF and chemicals used in the field or greenhouse based on laboratory experiments because there are multiple biotic and abiotic factors directly and indirectly affecting the interaction. In the greenhouse, the pesticide and the EPF may be spatially separated. For example, EPF may no longer be exposed to active ingredients of chemical pesticides once inside the insect host. The degree of separation will also be affected by methods of application; whether applied as a tank mix or in separate sprays or if the pesticide is systemic compared to contact acting. However, active ingredients may interact once inside the host, either directly or mediated through changes in the hosts toxicokinetics.

The overall aim of this study was to determine the optimal temperatures for a number of EPF isolates for their germination, growth and sporulation as well as determining the impact of stressors such as temperature extremes and the presence of synthetic chemical insecticides and fungicides. Isolates were selected for further experimentation based on their response to temperature and chemical stressors with the intention that these *in vitro* experiments could be used as predictors of performance of EPF efficacy as biological control agents when exposed to similar conditions *in vivo*. The component objectives were as follows:

- i. Quantify the effect of a range of temperatures on the *in vitro* growth, germination and conidia production of EPF.
- ii. Predict the thermal optima for growth and germination of EPF using non-linear models to obtain information for the selection of EPF isolates for *T. vaporariorum* control.
- iii. Quantify the effect of an insecticide and several fungicides on the *in vitro* growth and germination of EPF.

4.2 Materials and Methods

4.2.1 Quantifying the effect of temperature on growth rate of fungal isolates

Growth rate of fungal isolates was determined using methods described in section 2.3.6. Growth rate was assessed for all isolates described in Table 2.1 (section 2.3.1). Replicate plates of each isolate were incubated in the dark at a range of constant temperatures (10, 15, 18, 20, 23, 25, 27, 30, 32°C). Due to the large number of isolates being tested, the 20 isolates were randomly split into 4 groups. Group one, two and three contained *B. bassiana* PPRI 5339 and groups one, two and four contained *B. bassiana* GHA; these isolates were used as reference isolates in repeated tests whilst the remainder of the isolates in each group were tested once. Two reference isolates were used in the first two groups as a contingency in case of contamination or issues in the production of mycelial plugs. This was conducted due to the size of the experiment and amount of time required to set up each repeat in time. Once two groups had been tested, only one reference isolate was used in further experiments as techniques for experimental setup had been perfected. Four replicates for each isolate were tested at each temperature resulting in 216 plates per group (except the last group which had 180 plates) and a total of 828 plates overall.

4.2.2 Quantifying the effect of temperature on *in vitro* conidial production of fungal isolates

The effect of temperature on *in vitro* conidial production per unit area was investigated by taking 8mm diameter plugs from plates of all 20 fungal isolates after 28 days growth in the dark on 22mL SDA at one of 9 temperatures ranging from 10-32°C (as for 4.2.1.1). Each plug was taken from the highly sporulating area near to the leading edge of fungal growth. Four replicate plugs were taken from separate replicate plates of each isolate at each of the 9 temperatures. The plugs were placed in 1mL of aqueous glycerol (10% per volume) and stored at -20°C until conidia could be enumerated. The solution used to store plugs was determined in preliminary experiments using *B. bassiana* (GHA) (Appendix II). A month later, vials were thawed at room temperature before their contents were ground using a sterile pestle and the suspension filtered through double-folded sterile muslin cloth. The concentration of each solution was estimated by making serial dilutions of the stock and counting conidia using a haemocytometer under x400 using an Olympus BH2 microscope. Conidial production per unit area was determined based on the estimated concentration of the original suspension and the area of the sample taken.

4.2.3 Quantifying the effect of temperature on *in vitro* germination rate of fungal isolates.

Isolates with the fastest growth rates ($\text{mm}^2 \text{ day}^{-1}$) at each temperature were identified from the results of experiments in section 4.2.1.1. Seven isolates were selected based on those found to grow consistently fast at each temperature or those with growth rates faster than other EPFs tested at temperature extremes (10-32°C). The geographical origin and genus of the isolates selected were also considered in order to ensure a mixture of these factors to be used in germination experiments.

Preliminary experiments using *B. bassiana* (GHA) were conducted to establish time of conidial germination at temperatures ranging from 20-30°C using methods described in section 2.3.5. This experiment indicated the maximum number of isolates and plates that could be completed in one day. For this reason, the seven chosen isolates were grouped into two groups of four with *B. bassiana* PPRI 5339 repeated in each of the groups as a reference species. Proportion germination of conidia was assessed for seven isolates across a range of temperatures (10, 15, 18, 20, 25, 30, 32°C) and time points (6-48 hours). This was replicated three times per isolate per temperature resulting in 600 plates in each group.

4.2.4 Quantifying the effect of the presence of insecticides on germination of fungal isolates

Two methods were used to test the affect of the insecticide spiromesifen on the germination rate of selected fungal isolates in *in vitro* experiments. The selection rationale for Oberon ® (AI: spiromesifen) is shown in section 2.4. Spiromesifen was combined with four species of EPF; *Beauveria bassiana* (PPRI 5339), *Metarhizium brunneum* (Met 52), *Akanthomyces lecanii* (ATCC 4060) and *Cordyceps farinosa* (ATCC 4412). In the first method to test the effect of spiromesifen on fungal germination, conidial suspensions for each isolate were collected and diluted to 2×10^6 conidia mL^{-1} using methods described in section 2.3.3. Based on dose response bioassays, lethal concentrations (LC) were calculated for spiromesifen against third instar *T. vaporariorum* as described in section 2.5.4.

An insecticide stock solution equal to double the recommended field rates of Oberon was made by adding 150 μl to 100mL deionised and sterilised water. Dilutions of the stock solution were made to create four concentrations equivalent to double the LC₈₀, LC₅₀, LC₃₀ and LC₁₅ of Oberon against *T. vaporariorum*. Each solution was mixed in equal parts with 500 μl of conidial suspensions of each isolate, resulting in vials containing 1mL mixtures of an Oberon solution and conidial suspension. Each mixture was vortexed for 2 minutes

before 100µl of each treatment was placed onto the centre of a Petri dish containing 10mL SDA. Control treatments were prepared by mixing 500µl of each conidial suspension with 500µl deionised and sterilised water. Each Petri dish was stored in the dark in an incubator with a constant temperature of 25°C. Petri dishes were removed from the incubator at two time points; 13 and 24 hours. At this time, a small drop of lactophenol and cotton blue was placed onto the centre of the dish before each dish was stored in the fridge (4°C) until conidia were counted no later than after 7 days. The proportion of germinated conidia were counted on each dish using methods previously described in section 2.3.5.

The second method used to determine the effect of spiromesifen on germination of fungal isolates involved the exposure of fungi to spiked SDA. Sabouraud dextrose agar was autoclaved and left to cool to 40°C before 250mL of SDA was added to 0.4, 2.25, 12.1 and 184.7µl of RR of Oberon®, resulting in concentrations equal to the LC₁₅, LC₃₀, LC₅₀ and LC₈₀ based on mortality results from a dose response bioassay against third instar *T.vaporariorum*. Treated and untreated SDA was poured into 90mm diameter Petri dishes in 10mL aliquots. Conidial suspensions for each fungal isolate were prepared using methods described in 2.3.3 and diluted to 1 x10⁶ conidia mL⁻¹ before 4µl of each suspension was placed in the centre of the Petri dish. Each treatment had three replicates, resulting in 15 Petri dishes per fungal isolate, except *Cordyceps farinosa* ATCC 4412 which had two time points and therefore 30 Petri dishes. Petri dishes were stored in the dark at 25°C in a constant temperature incubator. Germination was stopped by fixing with lactophenol with cotton blue at 8 hours for *M. brunneum*, 12 hours for *A. lecanii*, 18 hours for *B. bassiana* and both 18 and 13 hours for *C. farinosa*. Timings were determined in preliminary experiments as detailed in Appendix III.

4.2.5 Quantifying the effect of the presence of insecticides on *in vitro* growth of fungal isolates.

The effect of Oberon on the growth of four fungal isolates was investigated by placing inverted 6mm diameter plugs of *B. bassiana* PPRI 5339, *M. brunneum* (Met 52), *A. lecanii* ATCC 4060 and *C. farinosa* ATCC 4412 in the centre of a 90mm diameter Petri dish. Fungal plugs were taken from Petri dishes prepared using the methods described in 2.3.6. Each experimental Petri dish contained 22mL of spiked SDA, prepared as detailed in section 4.2.2.1. Each fungus had three replicates in each treatment including a negative control, resulting in 15 Petri dishes per fungus and 60 Petri dishes in the experiment. All Petri dishes

were kept in the dark at a constant temperature of 25°C in an incubator. Radial growth was measured every 48 hours using methods described in 2.3.6 for 17 days.

4.2.6 Quantifying the effect of fungicides and temperature on the *in vitro* growth of an entomopathogenic fungus

Interactions between *B. bassiana* isolate PPRI5339 and three chemical fungicides were investigated in *in vitro* experiments. Sabouraud dextrose agar was made by mixing 32.5g premade mixture with 500mL deionised water and autoclaved (121°C, 15PSI). Once the SDA had cooled to 40°C, each glass Duran containing SDA was spiked with a known volume of different fungicides. The highest concentration of fungicide used was based on field application rates which were 150µl of Takumi SC (100g/l Cyflufenamid, Certis UK), 20mg of Kumulus (sulphur, BASF) and 1330µl of Luna Sensation (250 g/L fluopyram + 250 g/L trifloxystrobin, Bayer) in 500mL SDA. Dilutions of the stock solution of each fungicide were made, resulting in final concentrations of 0.5, 0.25, 0.1 and 0.001 of the recommended field application rate. Petri dishes were filled with 22mL of spiked SDA and stored at 4°C overnight. Growth experiments were conducted for *B. bassiana* on the spiked agar using methods described in 2.3.6. Each plate was stored in the dark in an incubator set at a constant temperature of either 10, 25 or 32°C. Each fungicide concentration and temperature treatment had three replicates resulting in 53 Petri dishes per fungicide, 159 Petri dishes in total, including negative controls for each treatment combination.

4.2.7 Statistical analysis

For growth data, average colony width was calculated using the two cardinal growth measurements, giving an average radial colony extension for each fungal replicate at each temperature. The linear phase of growth was found visually by plotting average radial colony extension against time and was determined to be from day 4 to day 16 for all isolates by modelling colony size over time. For each isolate, average radial colony extension for each replicate at each temperature was modelled against time for days 4 to 16 in a GLM linear regression using the 'glm' function in R studio (version 4.0.0 2020/04/24). The radial growth rate was extracted from the slope of the regression. Differences between growth rate at each temperature and between isolates was tested by fitting a linear regression as well as a fourth-order polynomial (quadratic) regression. Quadratic models were fit with isolate and genus as explanatory variables and the fit of each model was compared using ANOVAs.

Conidia production per unit area was calculated by scaling haemocytometer counts to conidia mL⁻¹ and converting to conidia mm⁻² by dividing by the area of the plug taken from

the fungal surface (51mm²). Count data of conidia per unit area were not normally distributed. Data were normalised by natural log transformation (+1) and significant differences between conidia production at each temperature for *B. bassiana* standard isolates (PPR 5339 and GHA) were tested by linear regressions. Differences between models were determined by ANOVA. Differences between genera were determined by ANOVA and post-hoc Tukey multiple pairwise comparisons.

Origins of EPF were classified according to the Köppen–Geiger climate classification system (Kottek et al., 2006) which groups climates according to temperature patterns and seasonal precipitation (Table 4.1). Isolates for which origin was unknown were not included in the analysis. Significant differences in growth rate and conidia production across all tested temperatures for isolates grouped by the Köppen–Geiger climate classification system was determined by two-way ANOVA.

Table 4.1 Origin of entomopathogenic fungal isolates based on the Köppen–Geiger climate classification. (Kottek et al., 2006)

Isolate	Köppen–Geiger class
<i>Beauveria bassiana</i> ATCC 6921	Humid continental climate
<i>Beauveria bassiana</i> ATCC 6920	Humid continental climate
<i>Beauveria bassiana</i> ATCC 5278	Humid continental climate
<i>Beauveria bassiana</i> ATCC 9451	Humid continental climate
<i>Cordyceps javanica</i> ATCC 7477	Humid subtropical climate
<i>Cordyceps fumosorosea</i> ATCC 2658	Humid subtropical climate
<i>Cordyceps fumorosea</i> ATCC 4205	Tropical rainforest climate
<i>Cordyceps farinosa</i> ATCC 4412	Tropical rainforest climate
<i>Akanthomyces lecanii</i> ATCC 4060	Tropical rainforest climate
<i>Akanthomyces lecanii</i> ATCC 972	Oceanic climate
<i>Akanthomyces lecanii</i> ATCC 6544	Oceanic climate
<i>Metarhizium anisopliae</i> Bioblast	Hot semi-arid climate
<i>Metarhizium brunneum</i> Met 52	Hot semi-arid climate

Significant differences between germination data for *B.bassiana* PPRI5339 in group one and two were tested by ANOVA. A probit analysis was conducted on germination data of each isolate at each temperature in order to determine time to 50% germination (GT₅₀). The natural logarithm of the GT₅₀ was calculated to transform the data. Differences between germination rate at each temperature and between isolates were tested by fitting a linear regression as well as a second-, third- and fourth-order polynomial regression and the fit of each model was compared using ANOVAs. Quadratic models were fit with isolate and genus as explanatory variables and the fit of each model was compared using ANOVAs. Data for all isolates were grouped and models were compared to test for significant differences between isolates. The relationship between predicted growth and germination optima was investigated by extracting predicted optima from the best fit non-linear models and fitting to a linear regression.

Differences between germination rates of EPF isolates exposed to spiromesifen prior to application on SDA were tested by non-parametric methods unless stated otherwise. The data could not be normalised by transformation so a Kruskal-Wallis test was performed followed by pairwise comparisons using a post hoc Dunn test with p values adjusted by the Benjamini & Hochberg method (Haynes, 2013). Differences between germination rates of EPF isolates on agar spiked with spiromesifen was determined by ANOVA. Two-way ANOVAs were conducted for EPF tested under different treatments with multiple time points (e.g. *C. farinosa* ATCC 4412).

Analysis of growth data after EPF were exposed to SDA spiked with spiromesifen or one of three fungicides was also conducted using non- parametric analysis. The average growth rate for each EPF isolate was calculated from day 3 onwards for the insecticide experiment and from day 4 onwards for the fungicide experiment. The radial growth rate was calculated using the same methods described for experiments investigating the effect of temperature on growth. Kruskal-Wallis analysis was performed followed by pairwise comparisons using a post hoc Dunn test with p values adjusted by the Benjamini & Hochberg method (Haynes, 2013). A quadratic regression was fit to temperature and growth rate data for the EPFs grown on fungicide spiked agar in order to show an interaction plot.

4.2.8 Modelling temperature profiles of entomopathogenic fungi to predict temperature optima and limits.

The relationship between temperature and *in vitro* growth or germination was described using five non-linear models, adapted from the methods described by Perry (2017) to describe the effect of temperature on development of diamondback moth *Plutella xylostella* (Lepidoptera; Plutellidae). The relationship between development of arthropods and temperature shows a similar distribution as other biological processes, including the response of fungi to temperature.

Proportion of germinated conidia after 16 hours was chosen as the time point to determine differences between isolates because a good range of proportion of germinated conidia was seen across temperatures for each isolate, but it was early enough that 100% of conidia had not germinated. Earlier time points had too few conidia germinated for analysis.

Each non-linear model was best suited to data with a bell-shaped distribution, which was found for all dependant variables in response to increasing temperature. Several of the following equations have been used to describe EPF physiology. Analysis was conducted in RStudio using the Minpack.lm package (version 1.2-0).

The first non-linear model was a fourth order polynomial model with the following equation:

$$y = a(T)^4 + b(T)^3 + c(T)^2 + d(T) + e \quad \text{Equation 5}$$

Where y is equal to the dependant variable and T is temperature. Parameter e is the intercept of the x axis and b, c, d , and e are all regression coefficients. The polynomial models are better suited for data with normal, symmetrical distribution. Therefore, the model does not fit response variables which are skewed and have a sharp decline at temperatures above the optimum.

The second model fitted was the Briere-1 model (Briere *et al.*, 1999), which has the following equation:

$$y = aT(T - T_0)(T_{max} - T)^{1/m} \quad \text{Equation 6}$$

Where y is the dependant variable. T refers to the independent variable, temperature. T_0 and T_{max} are the lower and upper temperature thresholds, respectively. Parameter m was set to two and is an equation constant. Parameter a is also an equation constant which allows

adjustment of the highest value of the dependant variable. Unlike the polynomial model, the Briere-1 model is effective at describing data with a slow increase towards the optimum before a sharp decline. The temperature optimum for each fungal isolate could be determined using the following equation:

$$T_{opt} = [2mT_{max} + (m + 1)T_0] + \frac{\sqrt{4m^2 T_{max}^2 + (m+1)^2 T_0^2 - 4m^2 T_0 T_{max}}}{4m+2} \quad \text{Equation 7}$$

The Lactin-1 model is effective at describing changes in a dependant variable over a wide range of temperatures. It has the following equation:

$$y = e^{(pT)} - e^{(pT_{max} - (T_{max} - \frac{T}{\Delta}))} \quad \text{Equation 8}$$

Again, parameters y and T represent the dependant variable and temperature, respectively. T_{max} is the upper temperature threshold. As the model does not intercept the x-axis, T_0 is not calculated. Parameter p is an equation constant which determines the dependant variable at the optimum temperature. Parameter Δ is the number of degrees above T where temperature becomes the overriding influence on y . Temperature optimum can be calculated by subtracting Δ from T_{max} (Lactin *et al.*, 1995).

The Logan-6 model, proposed by Logan *et al.* (1976) had the following equation;

$$y = \varphi (e^{(pT)} - e^{(pT_{max} - \frac{T_{max} - T}{\Delta T})}) \quad \text{Equation 9}$$

Parameters y and T describe the dependant variable and temperature. Parameter φ is the value of the dependant variable at the base temperature. Parameter p is a coefficient that represents the rate of change as a result of increasing the temperature by 10°C. T_{max} is the maximum temperature that the system can withstand. ΔT is the number of degrees above the base temperature where temperature inhibition starts to occur. Temperature optimum can be calculated by subtracting ΔT from T_{max} .

Finally, the last model used was the Taylor model (Taylor, 1981). This model is less effective at describing sharp declines in the dependant variable at high temperatures. The Taylor model has the following equation:

$$y = Rm(-0.5[\frac{T - T_{opt}}{T}]^2) \quad \text{Equation 10}$$

Parameters y and T are the same as the previous equations, the dependant variable and temperature, respectively. T_{opt} is the optimum temperature for the dependant variable. R_m is the value of the dependant variable at T_{opt} .

Once raw data had been fit to each non-linear model, comparisons of fit were made by comparing r^2 values and Akaike information criterion (AIC values). R^2 values can be used as a determination of how much variation within the data is described by the model fit. Values are produced between 0-1, with a value closer to 1 indicating that the data points are close to the regression line. When comparing models using AIC values, the lowest AIC value indicates a better fit. An AIC value estimates the amount of information lost by the fit of the model whilst also taking into account the complexity of the model. The AIC deals with the trade-off between fitting a model which describes the data best and the simplicity of the model, where simple models are preferred.

4.2.9 Estimating starting parameters for non-linear models

In order to accurately fit the five non-linear models described in section 4.2.3.1, some values needed to be calculated in order for the equations to be completed. Starting parameters were calculated for the Briere-1 model (Briere et al., 1999), Lactin-1 model (Lactin et al., 1995), Logan-6 model (Logan et al., 1976) and the Taylor model (Taylor, 1981). Table 4.2 demonstrates the calculations performed to determine parameters for each model.

Table 4.2 Description of calculations performed to determine starting parameters for five non-linear models used to model the response of entomopathogenic fungi growth and germination to temperature. Models used included; the Briere-1 model (Briere et al., 1999), Lactin-1 model (Lactin et al., 1995), Logan-6 model (Logan et al., 1976) and the Taylor model (Taylor, 1981).

Model	Parameter	Method
Briere-1	T_0	A linear regression was fitted to data to the temperature at which the highest value of the dependant variable occurred. T_0 was determined as the temperature at which the linear regression crossed the x-axis.
	T_{max}	A linear regression was fitted to data to the temperature from the optima to the highest temperature used in the study. The temperature at which the linear regression crossed the x-axis was determined as the T_{max} .
	a	$\frac{y}{T(T - T_0)\sqrt{T_{max} - T}}$
Lactin-1	ΔT	$T_{max} - T_{opt}$
	T_{max}	A linear regression was fitted to data to the temperature from the optima to the highest temperature used in the study. The temperature at which the linear regression crossed the x-axis was determined as the T_{max} .
Logan-6	ϕ	The value of the dependant variable at the lowest temperature in the study.
	T_{max}	A linear regression was fitted to data to the temperature from the optima to the highest temperature used in the study. The temperature at which the linear regression crossed the x-axis was determined as the T_{max} .
	ΔT	$T_{max} - T_{opt}$
Taylor	R_m	The highest value of the dependant variable.
	T_{opt}	The temperature at which the highest value of the dependant variable was recorded.

4.4 Results

4.4.1 Quantifying the effect of temperature on *in vitro* growth rate of fungal isolates

There was no significant difference between the average growth rates for *B. bassiana* isolate PPRI 5339 used as a reference isolate in group one, two and three of *in vitro* growth experiments ($X^2=0.027$, $df=2$, $p=0.9862$). There was also no significant difference between average growth rate calculated for *B. bassiana* GHA for group two and four. However, growth rate for *B. bassiana* (GHA) used in group one was significantly different to both group two and four ($p=0.005$ and $p=0.013$ respectively). However, group one was kept in the analysis because the other reference isolate was consistent between all groups, as was GHA for group 2 and 4. Growth rates extracted from linear regressions were not significantly different whether group one was included or not.

Average growth rate against temperature produced a bell-shaped curve for each isolate and each isolate produced growth rate distributions which skewed to the left. Temperature had a significant effect on the growth rate of each isolate ($F_{2,158}=78.43$, $R^2=0.49$, $p<0.001$). There were significant interspecific differences between the responses of fungal isolates to temperature as indicated by improved fit of the GLM when modelling isolates independently ($F_{19,141}=19.25$, $R^2=0.72$, $p<0.001$; ANOVA $F=6.66$, $df=141$, $p<0.001$). Growth rate was highest for each isolate at temperatures between 18°C and 27°C and lowest for all isolates at temperatures above or below this range. *Metarhizium* isolates showed a significantly different response in growth rate to temperature than other genera ($p=0.003$) Figure 4.1. At the highest temperature, 32°C, *M. anisopliae* (Bioblast) had the fastest growth rate at 2.4mm day⁻¹ and *Akanthomyces* isolates, *A. muscarium* (ATCC 972) and *A. lecanii* (ATCC 6544) had the slowest growth rates with 0.49 and 0.53mm day⁻¹ respectively. Comparatively, *Akanthomyces* isolates, *A. lecanii* ATCC 6544 and 4060 had the fastest growth rates at 10°C, whereas *M. anisopliae* (Bioblast) had the slowest growth rate with 0.03mm day⁻¹. Interspecific differences in growth rate were exaggerated between temperatures of 23°C and 27°C, where growth rate optima occurred for each isolate. Average growth rate for each isolate across all temperatures are shown in Appendix III.

Average radial colony extension for each isolate was successfully fitted against temperature using five non-linear models. When isolates were grouped according to their Köppen–

Geiger climate classification, there was no significant relationship between class of isolates and growth rate with temperature ($F=2.06$, $df=5$, $p=0.071$).

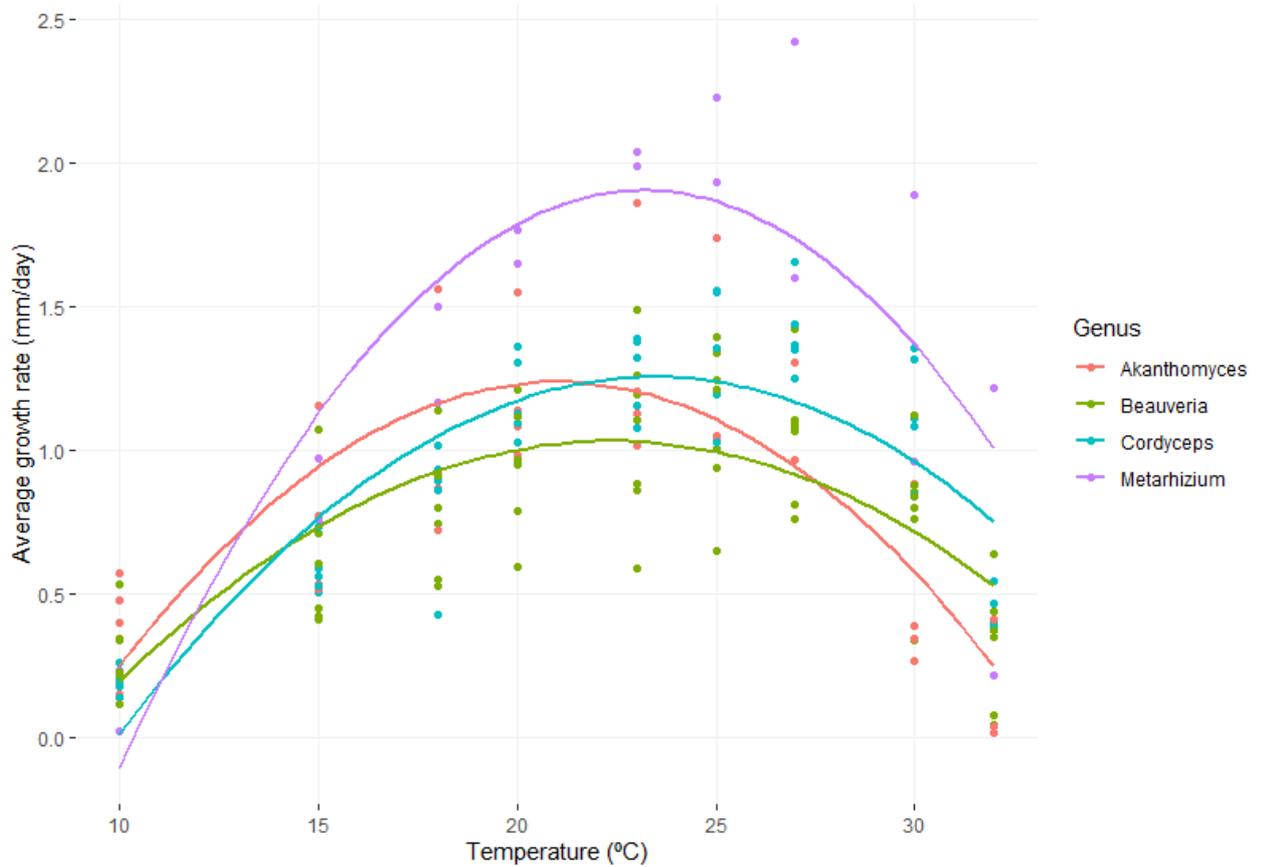


Figure 4.1 Average growth rate for the linear phase of growth of 18 fungal isolates at nine incubation temperatures. This plot shows the results of a quadratic linear regression, with each line modelling response by each Genera.

4.4.2 Modelling *in vitro* growth data of fungal isolates using non-linear models to predict temperature optima and limits.

The best fitting models for all *B. bassiana* isolates (PPRI 5339, Botanigard, GHA and ATCC; 6921, 6920, 5278, 9451) was considered to be the polynomial model which had the highest R^2 values and lowest AIC values compared to other non-linear models. The polynomial model fitted to each *B. bassiana* isolate growth rate data displayed R^2 values ranging from 0.82 to 0.99 and AIC values -25.83 to -6.14. The next best fitting model was the Logan-6 model which provided R^2 values of 0.88 to 0.99 and AIC values as low as -20.99 to 28.12 (Table 4.3).

Cordyceps isolates displayed temperature profiles which were best described by the Briere-1 model. The Briere-1 model showed R^2 values of 0.84 to 0.99 and low AIC values (-22.75 to -2.29). The next best fitting model was the Lactin-1 model which displayed similar R^2 and AIC values, ranging from 0.85 to 0.95 and -9.81 to -2.85 respectively.

Akanthomyces isolates were best described by the polynomial model which produced R^2 values ranging from 0.81 to 0.94 and AIC values ranging from -8.78 to 8.94. The Lactin-1 model also described the data well and displayed R^2 values in the range of 0.79 to 0.89 and AIC values of -4.24 to 16.21.

For *Metarhizium* isolates (Bioblast and Met 52), the Taylor model displayed the best fit of the data when comparing R^2 and AIC values. The Taylor model produced R^2 values as high as 0.95 and 0.90 as well as AIC values as low as 0.094 and 4.21. The next best fitting model was the Logan-6 model, though R^2 and AIC values indicated that every model described the data well.

Four of the five non-linear models estimated parameters for each EPF isolate. These parameters are biologically significant and could be used to infer workable conditions of EPF when used as biological control agents. The lowest temperature that an isolate can withstand whilst growing is shown as T_0 . The highest temperature that an isolate could withstand whilst growing is shown as T_{max} . Most importantly, the temperature at which the highest rate of growth was shown is shown as T_{opt} . All models except the polynomial model predicted T_{opt} . The T_{max} was predicted by Briere-1, Lactin-1 and Logan-6 models. T_0 is predicted by the Briere-1 and Taylor model. There were no intergeneric differences between temperature parameters of each fungal genus but differences were seen between individual isolates. The Briere-1, Logan-6 and Lactin-1 model produced the highest R^2 values when the bell shaped curve was skewed left. Whereas, more normally distributed curves were described best by polynomial and Taylor models.

Table 4.3 Fitted parameters, r^2 and AIC values for five non-linear models for growth rate of eighteen fungal isolates incubated at nine different temperatures.

Par.	<i>B. bassiana</i>							<i>Cordyceps</i>					<i>Akanthomyces</i>				<i>Metarhizium</i>	
	5339	6921	GHA	6920	5278	Botan-igard	9451	7477	2658	4205	4412	PFR	4060	Vertalec	972	6544	Bioblast	Met52
Briere-1																		
<i>a</i>	8.81e-4	7.2 e-4	6.40e-4	8.13e-4	7.86e-4	7.81e-4	3.1 e-4	1.41e-3	8.73e-4	1.085e-3	1.5 e-3	8.81e-4	3.43e-4	6.80e-4	6.10 e-4	2.30 e-4	1.86e-03	1.2e-03
<i>T0</i>	5.98	-7.32	-0.17	0.44	5.5	6.32	-6.79	8.13	4.068	6.227	5.2	5.98	22.4	0.78	-21.5	-74.05	7.42	4.55
<i>Tmax</i>	32.59	30.91	32.20	32.40	33.02	32.36	32.02	32.12	32.21	32.23	32.23	32.59	32.08	32.32	32.00	32.00	32.63	32.02
<i>Topt</i>	26.74	24.09	25.74	25.96	27.028	26.61	25.018	26.65	26.21	26.48	26.36	26.75	29.29	25.93	24.11	22.78	26.96	26.11
<i>r²</i>	0.83	0.95	0.95	0.968	0.93	0.96	0.86	0.976	0.95	0.99	0.97	0.84	0.78	0.87	0.77	0.78	0.97	0.86
<i>AIC</i>	-2.29	-12.64	-17.78	-12.45	-11.06	-18.66	-12.03	-11.93	-12.05	-22.75	-15.26	-2.29	1.53	-4.83	15.02	11.40	-5.23	6.95
Taylor																		
<i>Rm</i>	1.23	1.397	1.105	1.39	1.07	0.98	0.68	1.63	1.26	1.4	1.5	1.16	1.21	1.16	1.12	1.86	2.29	1.94
<i>Topt</i>	22.83	22.24	23.71	23.07	25.52	24.81	22.34	24.35	23.74	24.13	24.01	25.09	21.96	23.15	22.03	21.10	24.99	23.51
<i>T0</i>	6.77	8.52	7.38	6.12	8.14	6.87	6.89	5.98	6.51	6.4	6.81	7.16	6.57	6.80	5.51	5.87	6.99	5.16
<i>r²</i>	0.93	0.86	0.86	0.75	0.88	0.84	0.69	0.88	0.91	0.93	0.88	0.77	0.76	0.93	0.81	0.88	0.95	0.90
<i>AIC</i>	-11.21	-4.05	-7.63	5.83	-6.64	-5.67	-4.75	2.50	-6.28	-6.89	0.13	0.89	2.26	-11.02	0.40	5.57	0.094	4.21
Lactin-1																		
<i>p</i>	0.13	0.127	0.14	0.16	0.16	0.17	0.14	0.17	0.15	0.16	0.15	0.16	0.12	0.138	0.13	0.11	0.16	0.16
<i>Tmax</i>	32.73	31.9	33.06	32.74	32.73	32.89	32.16	32.48	32.68	32.73	32.79	33.21	32.18	33.02	31.71	31.65	33.29	32.09
<i>Topt</i>	25.59	24.58	26.22	25.8	26.74	27.08	25.2	26.96	26.39	26.74	26.66	27.24	24.56	26.018	24.41	23.85	27.35	26.07
\otimes	7.14	7.32	6.84	6.24	5.99	5.81	6.96	5.52	6.29	5.99	6.13	5.97	7.62	7.002	7.30	7.80	5.94	6.02
<i>r²</i>	0.93	0.94	0.97	0.99	0.92	0.97	0.90	0.95	0.94	0.95	0.93	0.85	0.87	0.81	0.89	0.88	0.91	0.89
<i>AIC</i>	-10.34	-10.6	-20.69	-28.86	-9.81	-23.05	-15.03	-4.4	-9.64	-9.81	-5.65	-2.85	-3.19	-1.64	-4.24	5.39	4.73	5.1

		<i>B. bassiana</i>						<i>Cordyceps</i>					<i>Akanthomyces</i>				<i>Metarhizium</i>		
		5339	6921	GHA	6920	5278	Botan-igard	9451	7477	2658	4205	4412	PFR	4060	Vertalec	972	6544	Bioblast	Met52
Logan-6	ψ	-0.031	-0.004	-0.0018	0.0401	0.21	-0.0012	-0.01	-0.014	-0.0005	-0.035	-0.26	-0.067	-0.069	-0.016	-0.02	-0.27	-0.069	-0.08
	p	0.16	0.22	0.23	0.014	0.14	0.24	0.17	0.2	0.26	0.19	0.16	0.21	0.15	0.18	0.17	0.13	0.15	0.17
	$Topt$	23.85	19.82	22.42	29.57	27.73	24.74	23.43	29.57	22.55	25.92	26.37	25.68	22.9	24.09	22.09	22.38	22.9	25.42
	$Tmax$	32.70	31.69	32.93	32.06	33.82	32.82	32.15	32.47	32.57	32.71	32.79	33.16	32.17	32.96	31.71	31.64	32.17	32.09
	$\otimes T$	8.85	11.87	10.51	2.49	6.09	8.08	8.72	6.34	10.02	6.79	6.42	7.48	9.27	8.87	9.62	9.26	9.27	6.67
	r^2	0.92	0.93	0.96	0.99	0.88	0.98	0.90	0.94	0.918	0.95	0.93	0.85	0.87	0.81	0.88	0.88	0.96	0.89
	AIC	-7.88	-7.81	-16.33	28.12	-5.4	-20.99	-12.99	-2.23	-4.83	-7.58	-3.64	-0.83	-1.05	0.68	-1.84	7.46	-1.05	7.17
Polynomial	a	3.78	-8.646	0.31	-4.33	-3.89	-3.54	-3.064	-2.92	-2.084	-2.63	-6.12	-1.64	4.67	2.22	7.91	8.81	-8.25	3.95
	b	-0.87	2.052	0.016	1.12	0.84	0.89	0.74	-0.69	0.46	0.57	1.36	0.47	-0.96	-0.61	-1.71	-2.04	1.69	-0.84
	c	0.067	-0.1645	-0.0057	-0.099	-0.063	-0.077	-0.061	-0.0062	-0.037	-0.047	-0.107	-0.047	0.073	0.057	0.13	0.17	-0.13	0.59
	d	-0.0019	0.0059	0.000057	0.0039	0.0023	0.0029	0.0023	0.0027	0.0016	0.0019	0.0039	0.0019	-0.002	-0.0018	-0.0039	-0.0056	0.0046	-0.0012
	e	1.78e-5	-8.6 e-4	-1.28e-5	-5.72 e-5	-2.99 e-5	-4.17 e-5	-3.86 e-5	-4.27 e-5	-2.48 e-5	-5.0 e-4	-5.38 e-5	-3.16 e-5	1.76e-5	1.90e-5	4.042e-5	5.99e-5	-6.18e-5	1.24e-6
	r^2	0.97	0.97	0.95	0.99	0.84	0.94	0.82	0.93	0.95	0.98	0.93	0.69	0.81	0.89	0.91	0.94	0.98	0.89
	AIC	-20.23	-13.48	-19.56	-25.83	-6.14	-16.70	-11.82	-3.89	-14.41	-21.08	-6.57	1.35	-2.27	-8.78	-8.52	-2.22	-12.83	2.29

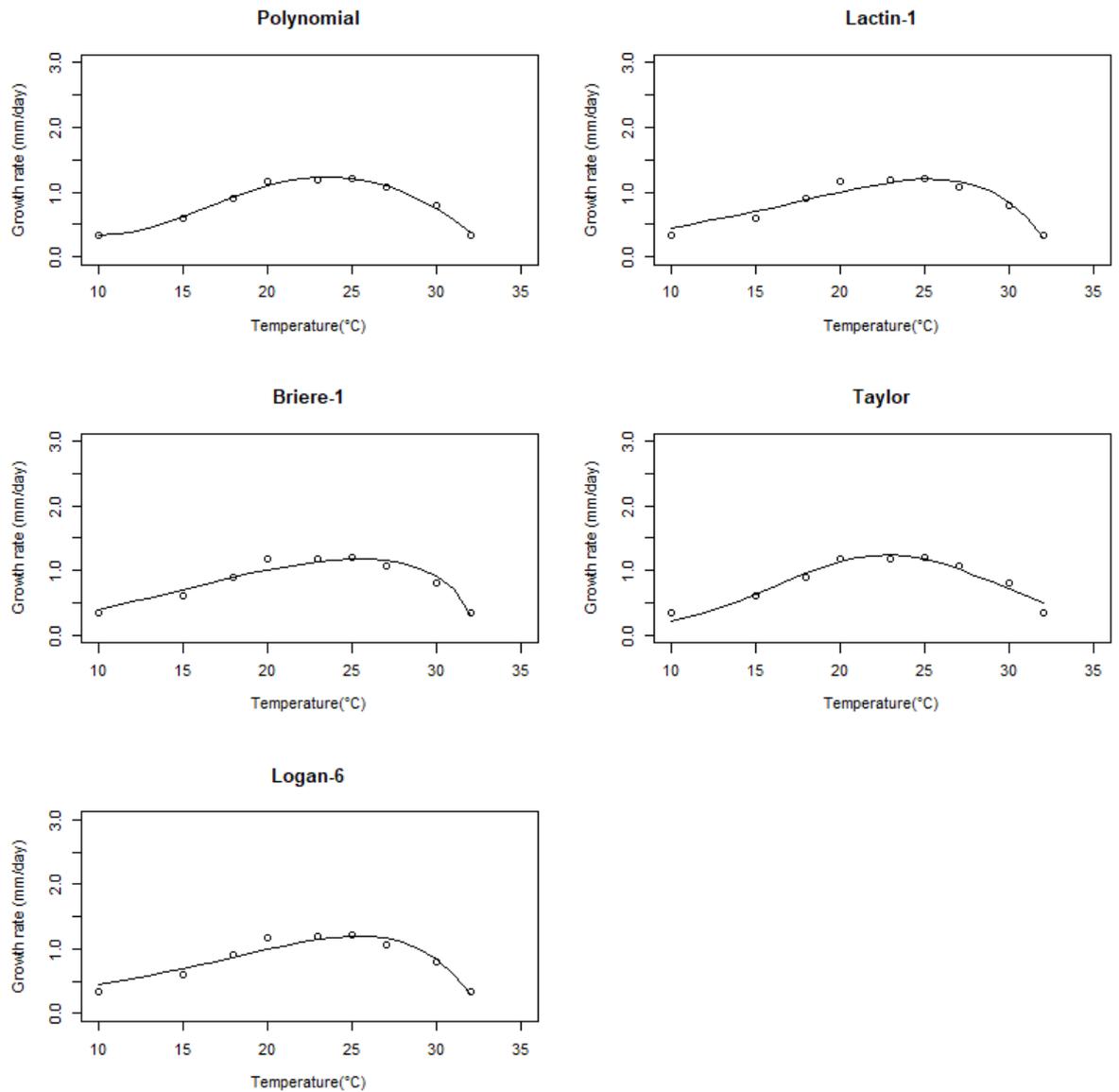


Figure 4.2 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* PPRI 5339.

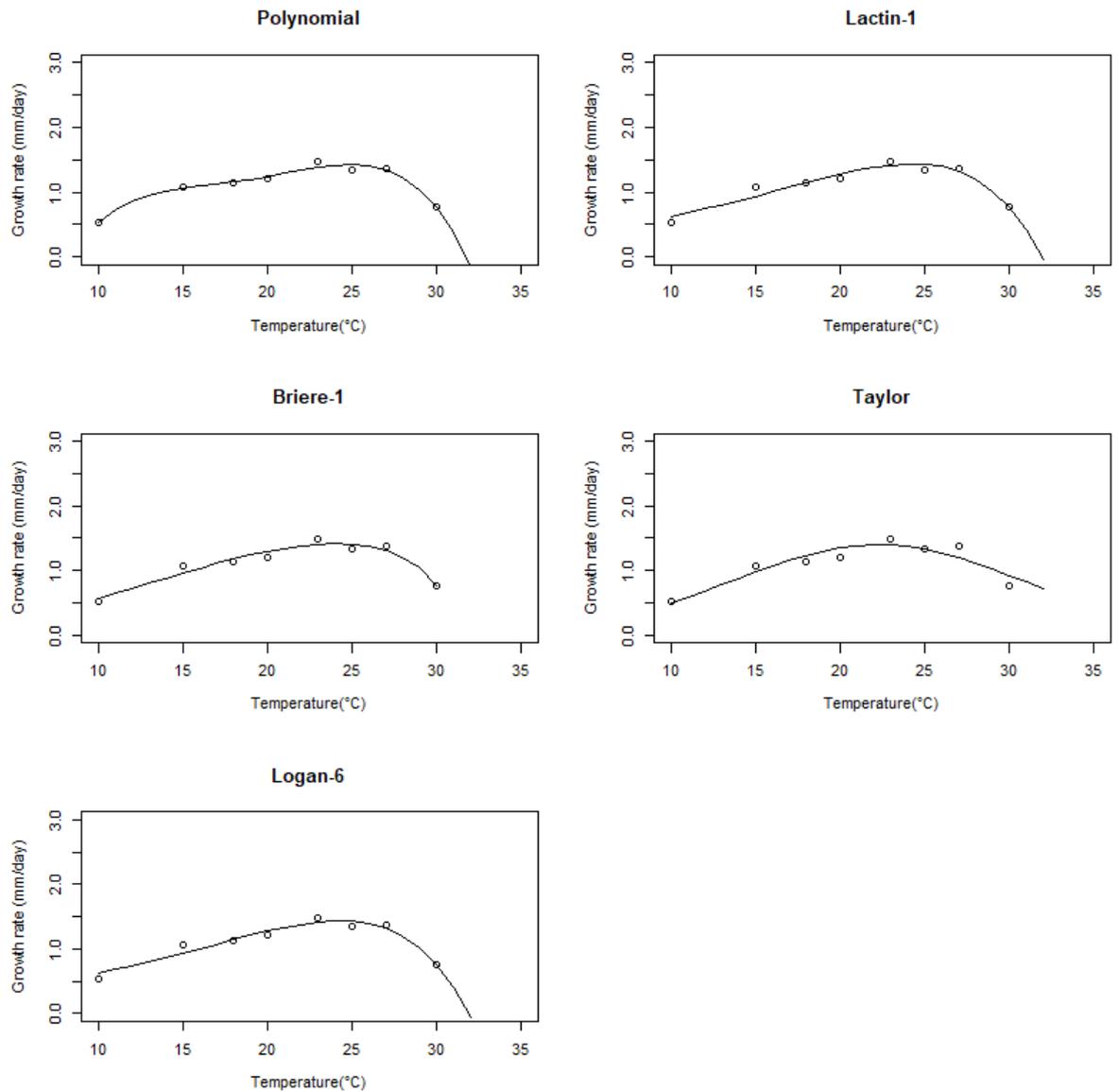


Figure 4.3 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* ATCC 6921.

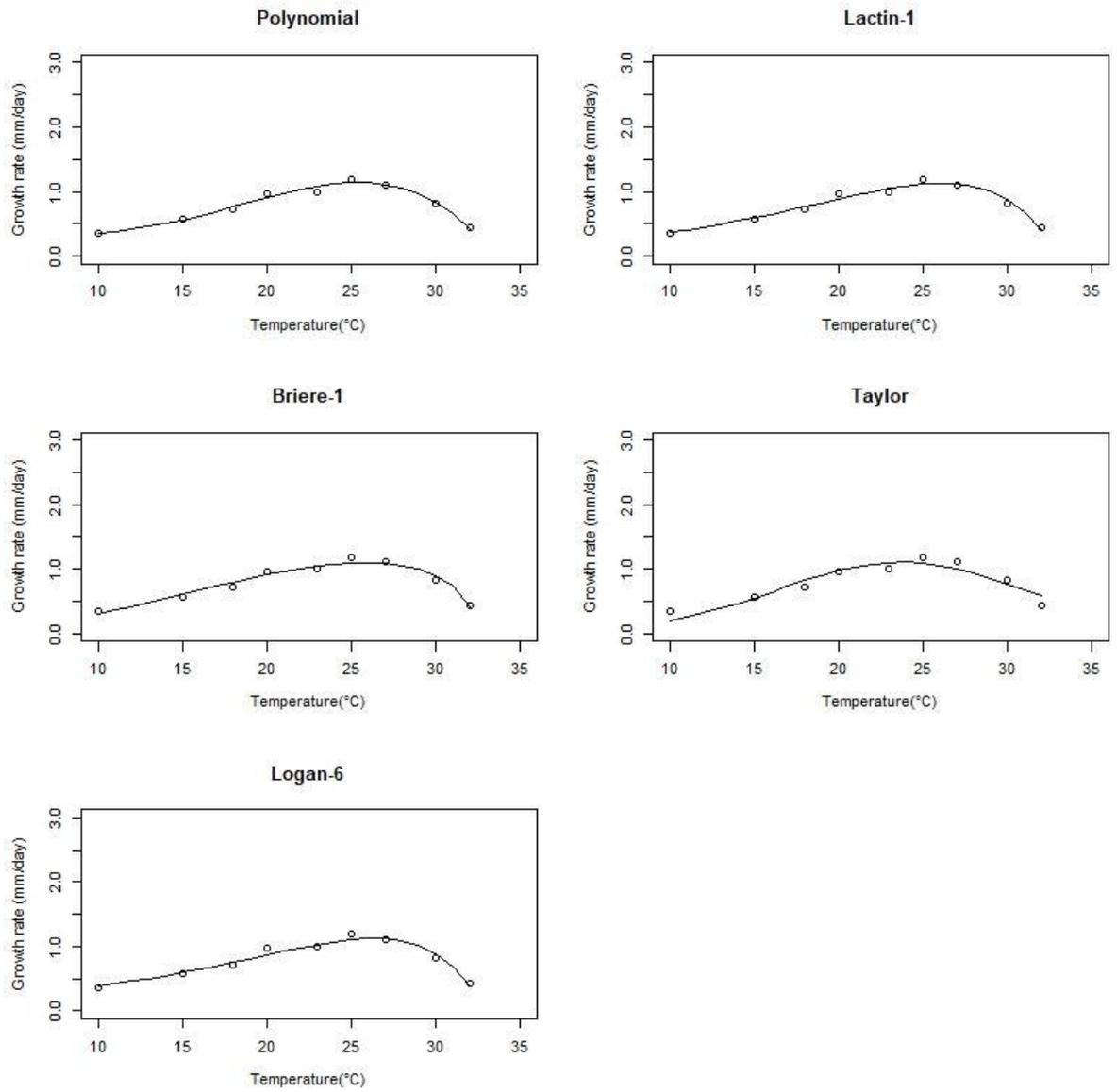


Figure 4.4 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* GHA.

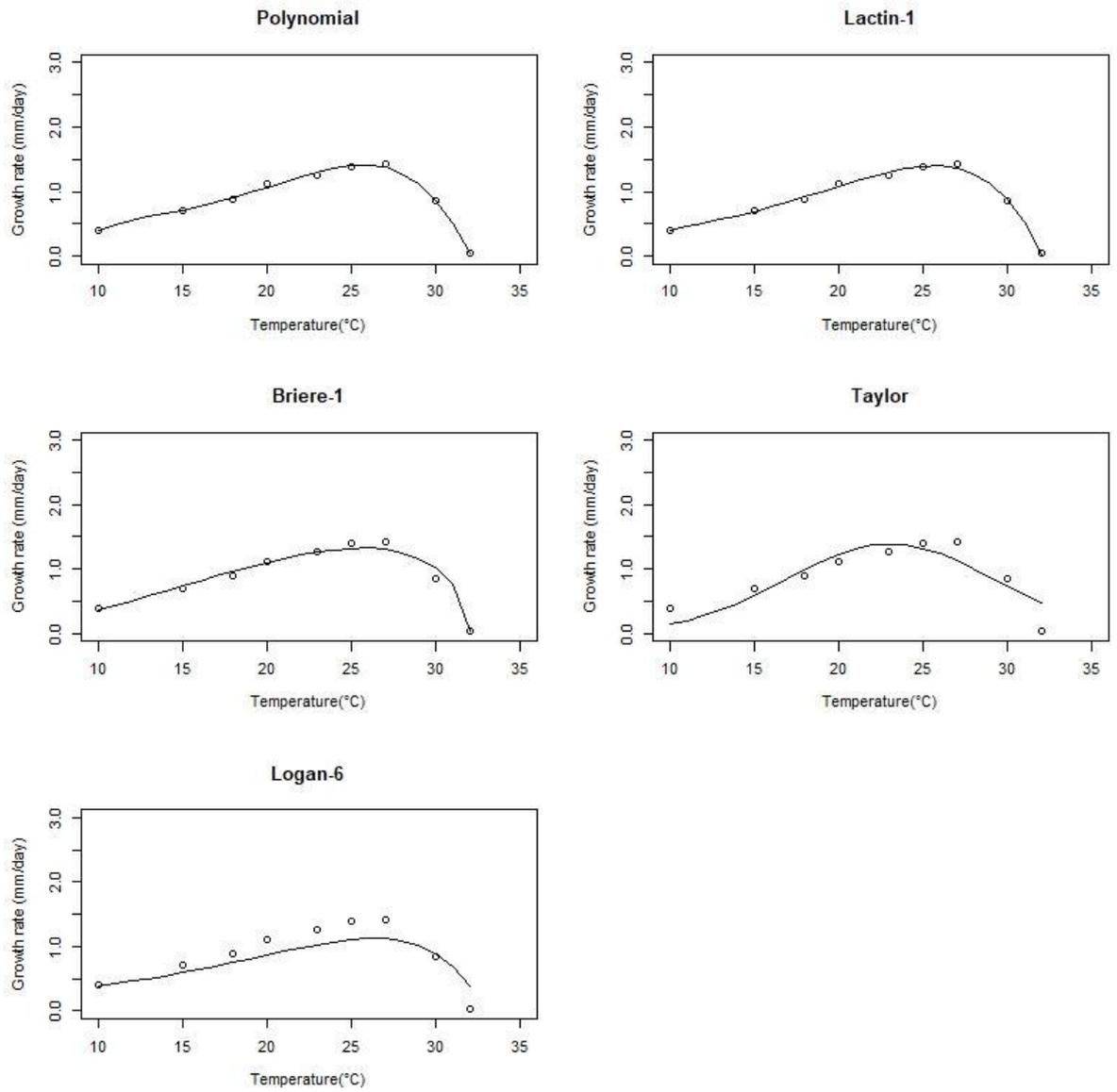


Figure 4.5 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* ATCC 6920.

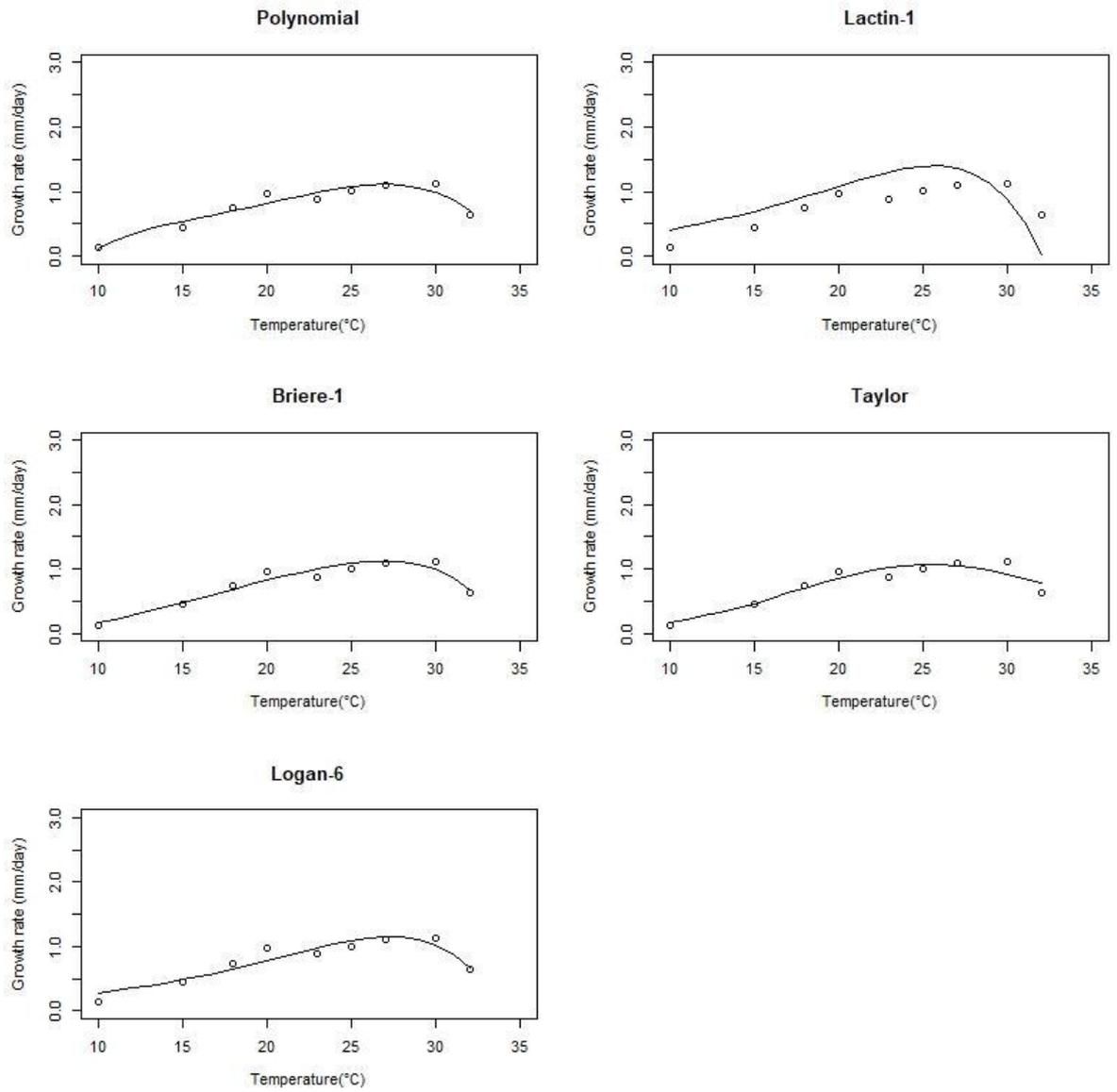


Figure 4.6 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* PPRI 5278.

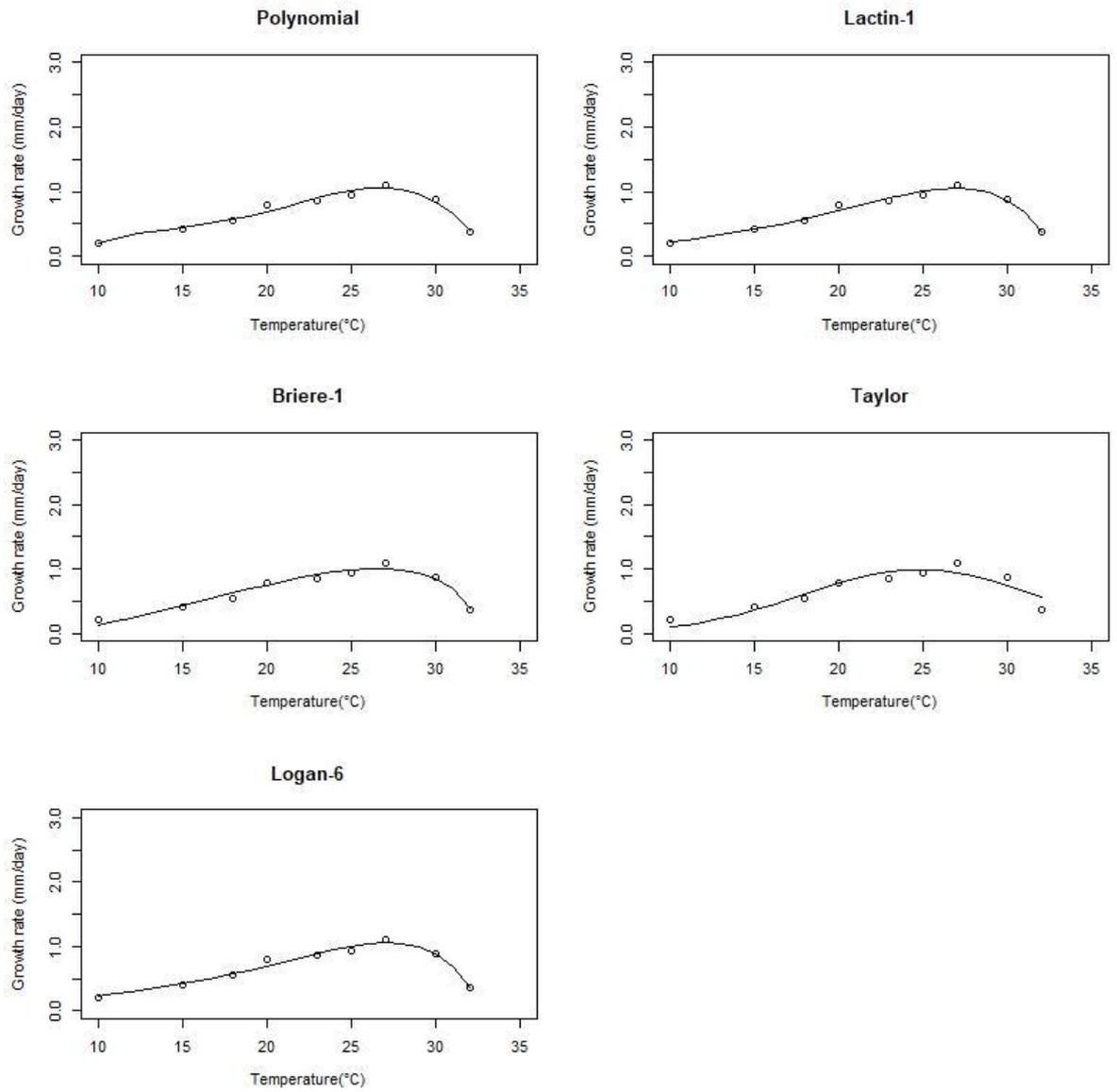


Figure 4.7 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* Botanigard.

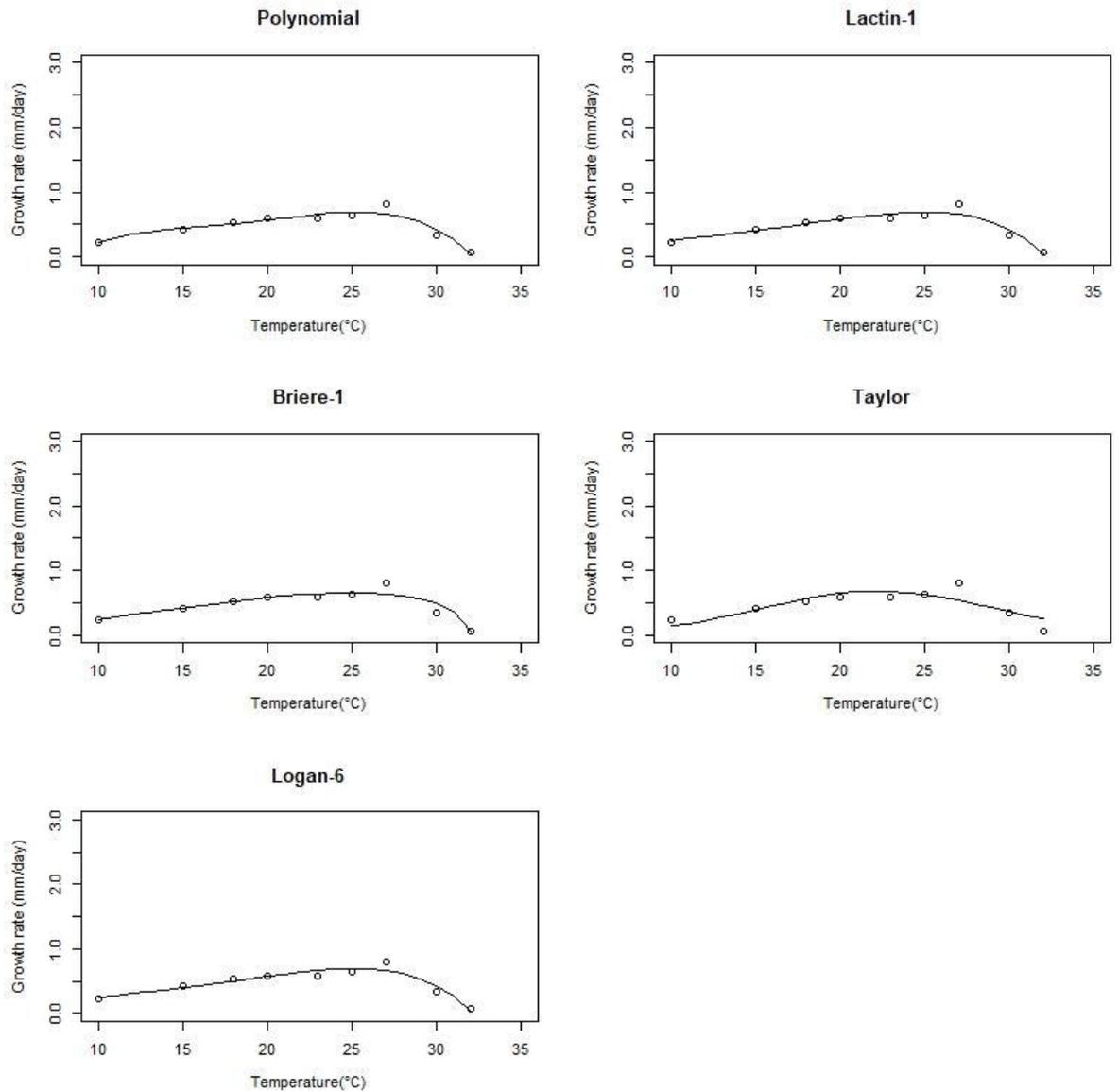


Figure 4.8 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Beauveria bassiana* ATCC 9451.

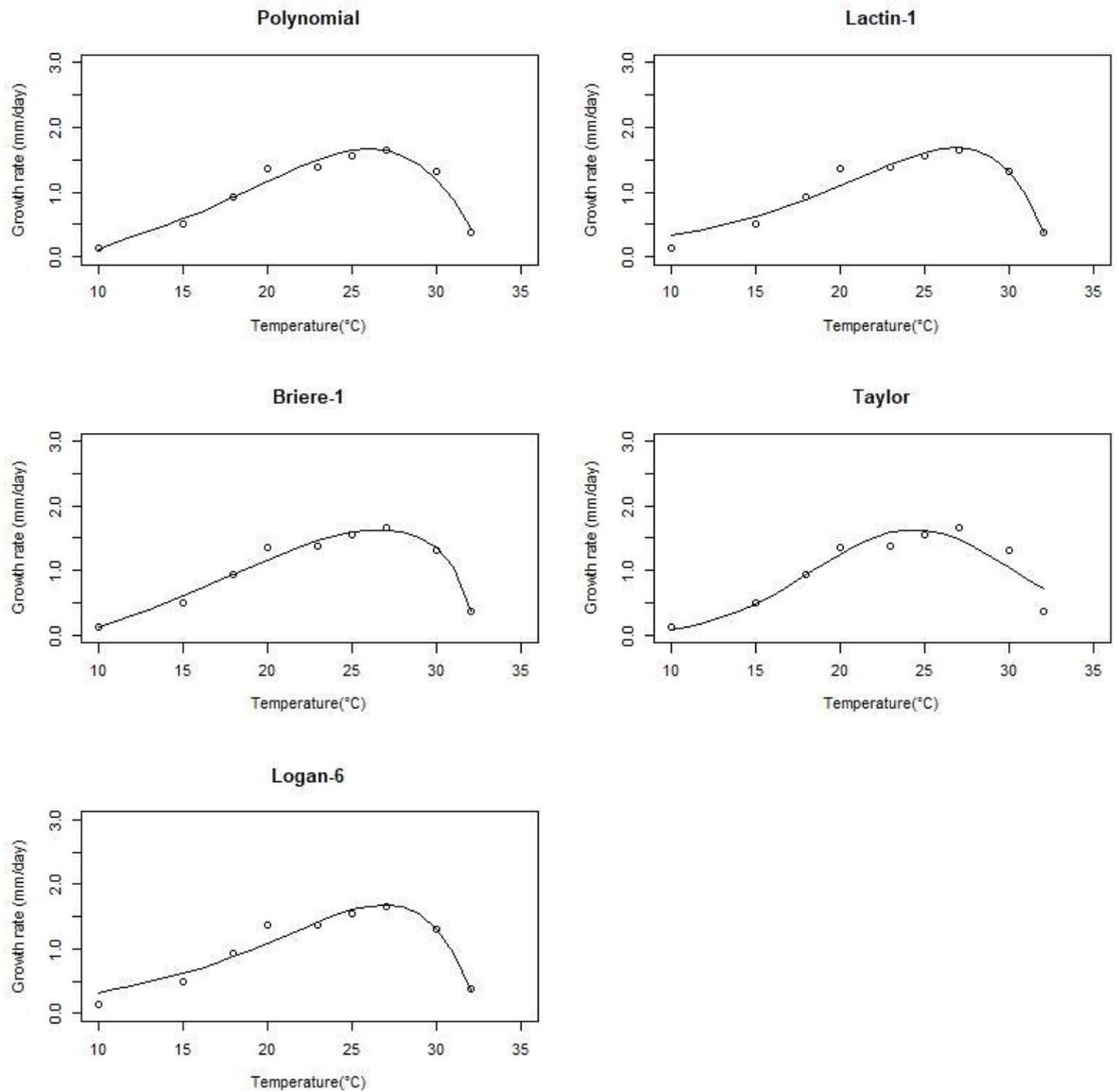


Figure 4.9 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Cordyceps javanica* ATCC 7477.

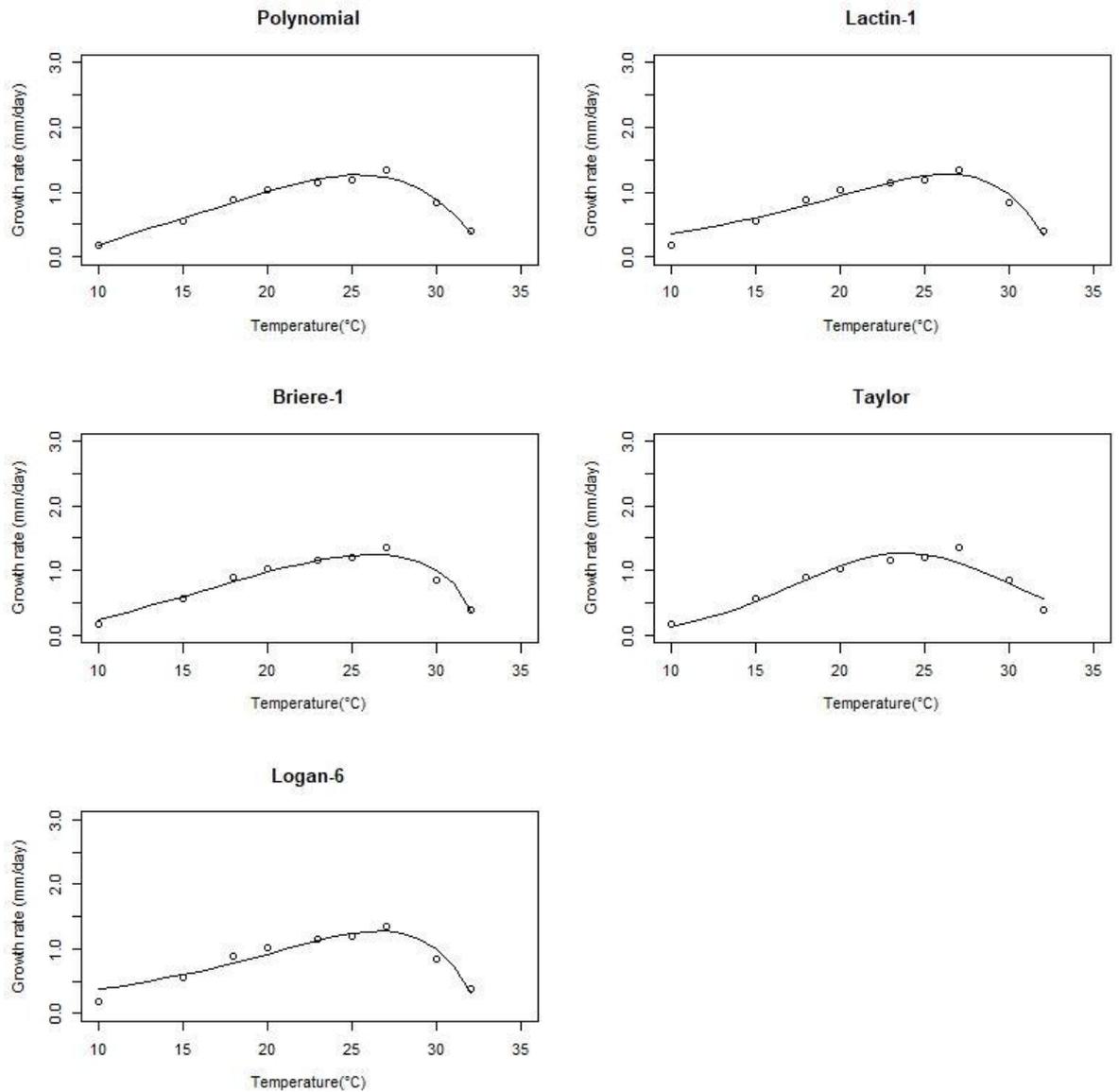


Figure 4.10 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Cordyceps fumosorosea* ATCC 2658.

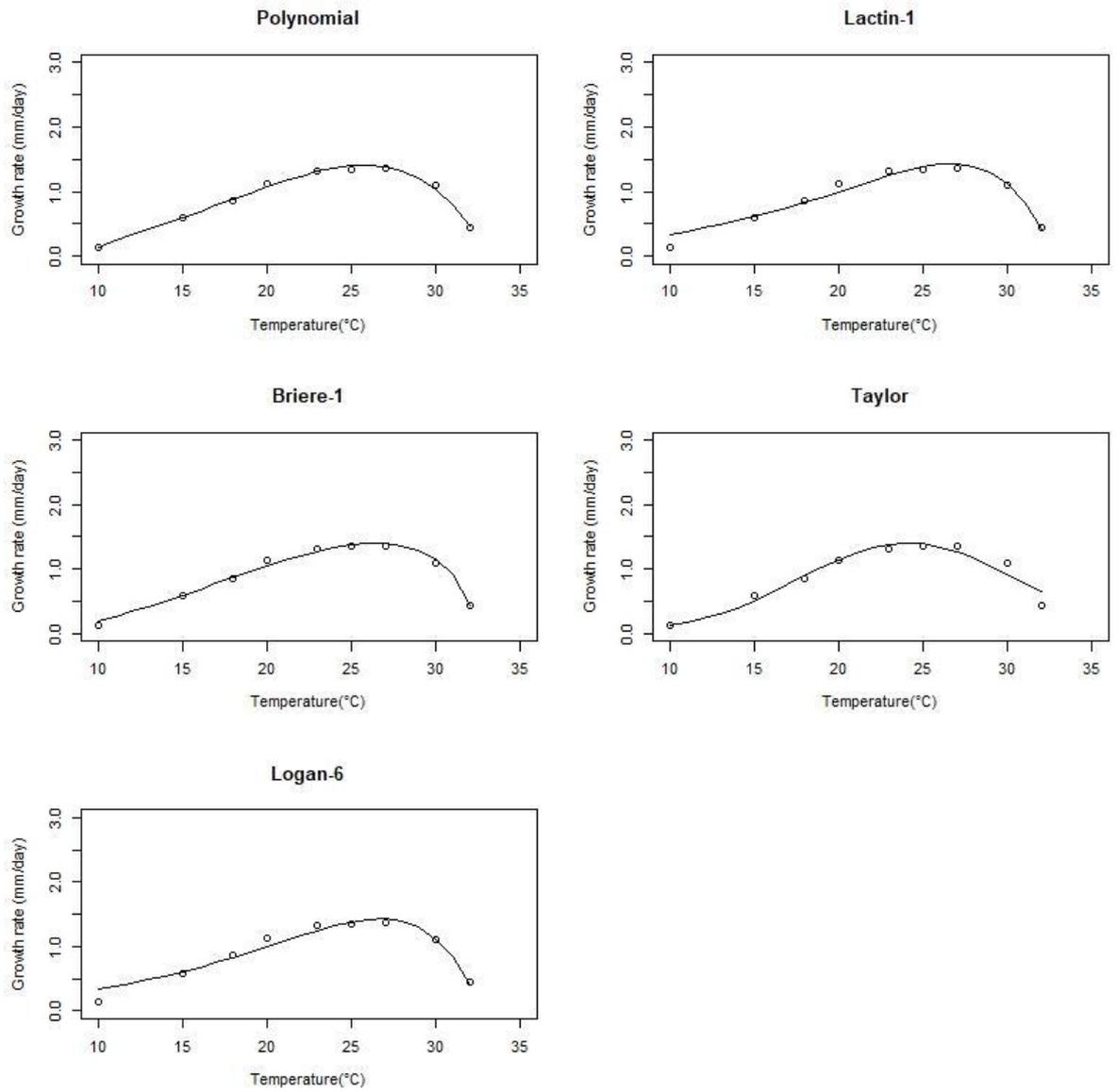


Figure 4.11 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Cordyceps fumosorosea* ATCC 4205.

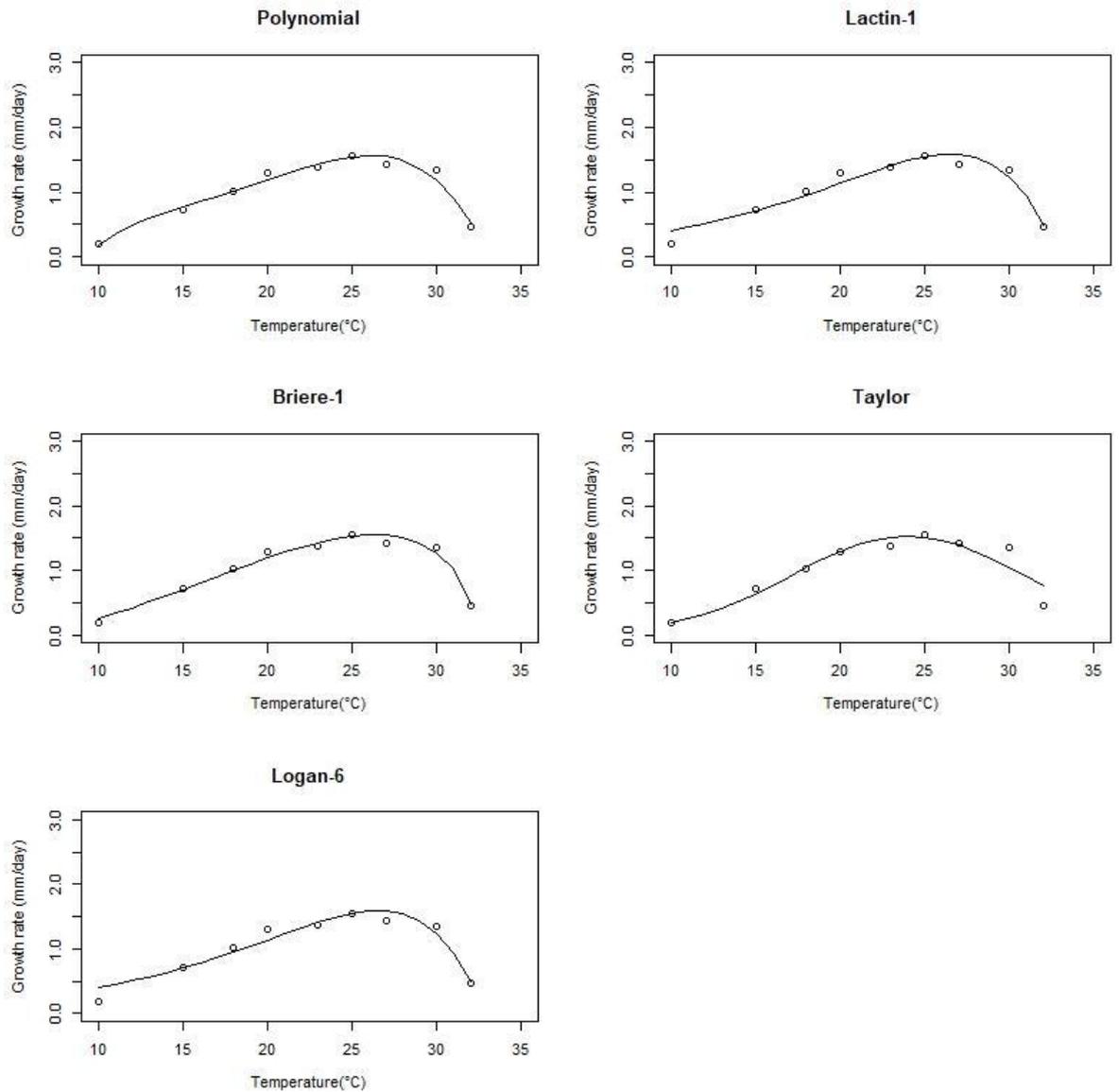


Figure 4.12 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Cordyceps farinosa* ATCC 4412.

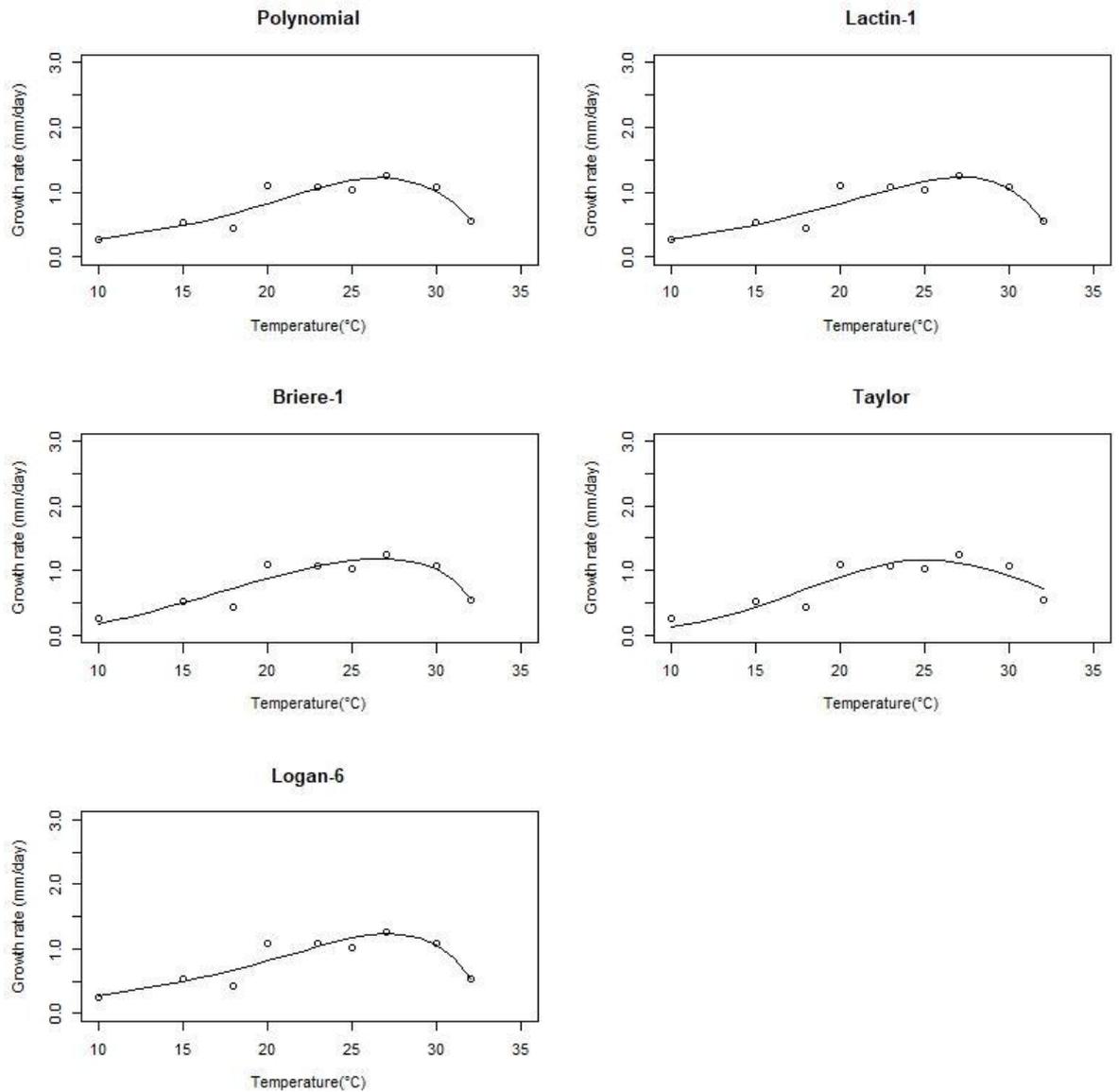


Figure 4.13 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Cordyceps fumosorosea* PFR.

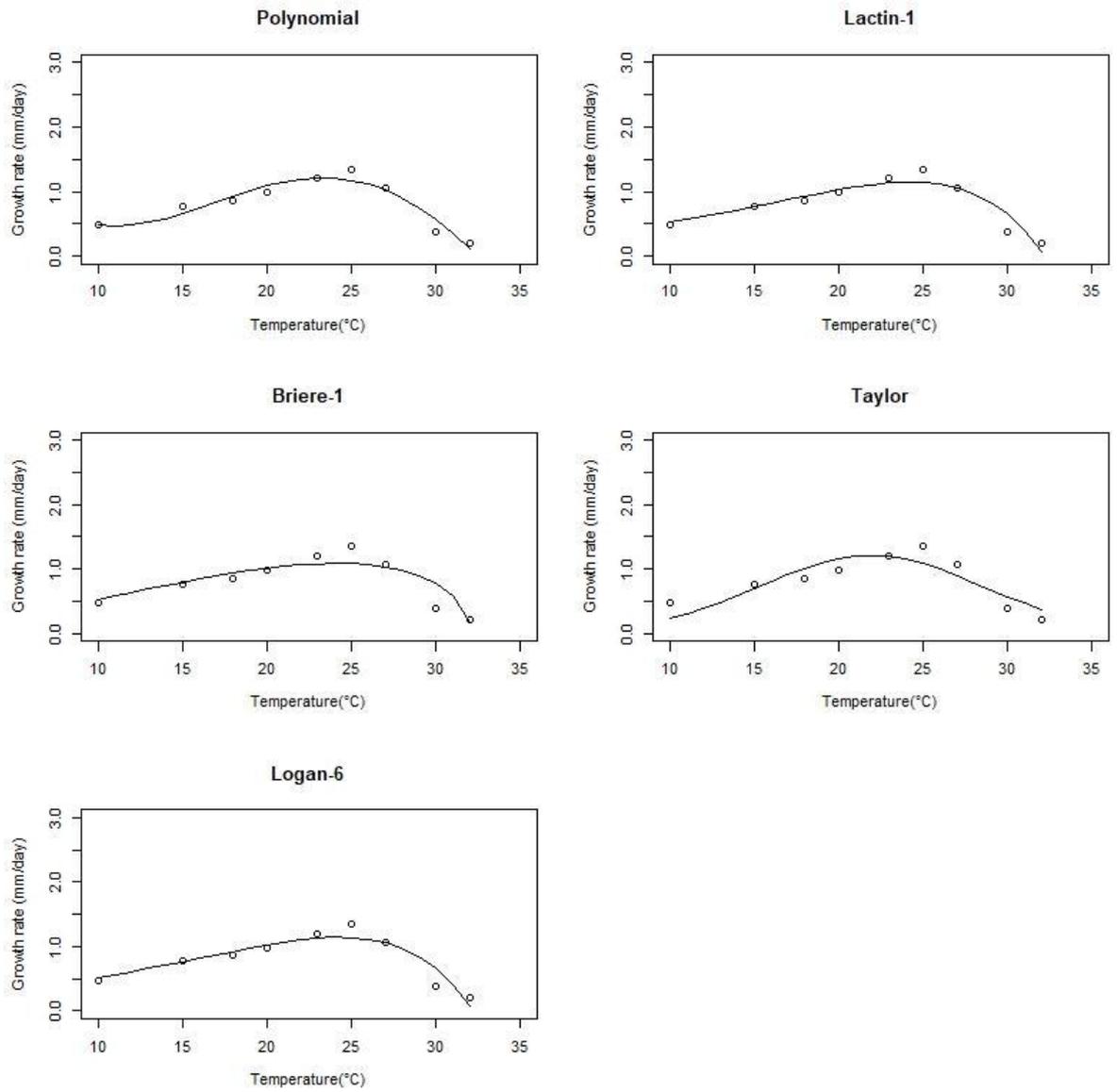


Figure 4.14 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Akanthomyces lecanii* ATCC 4060.

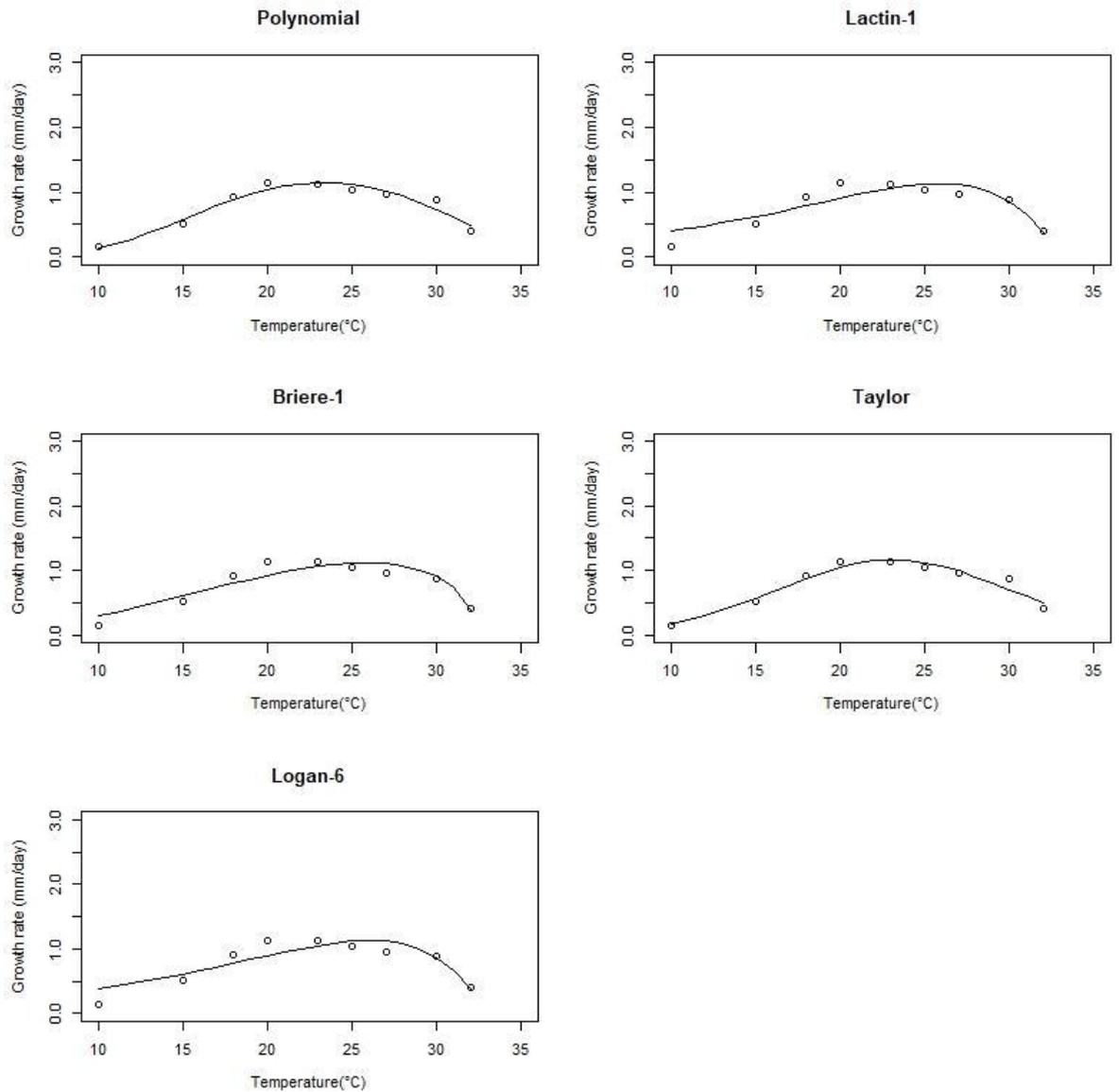


Figure 4.15 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Akanthomyces lecanii* Vertalec.

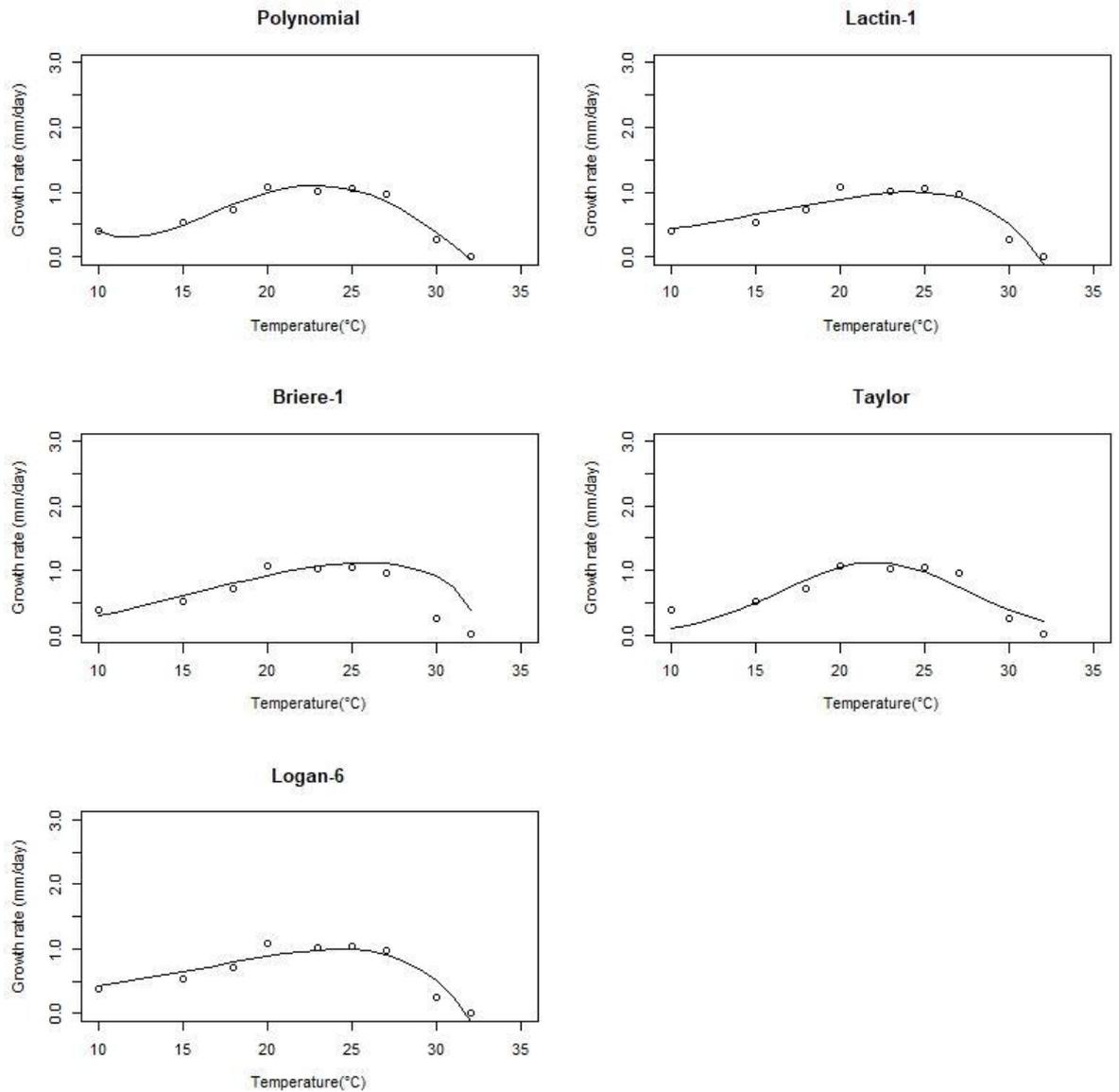


Figure 4.16 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Akanthomyces lecanii* ATCC 972.

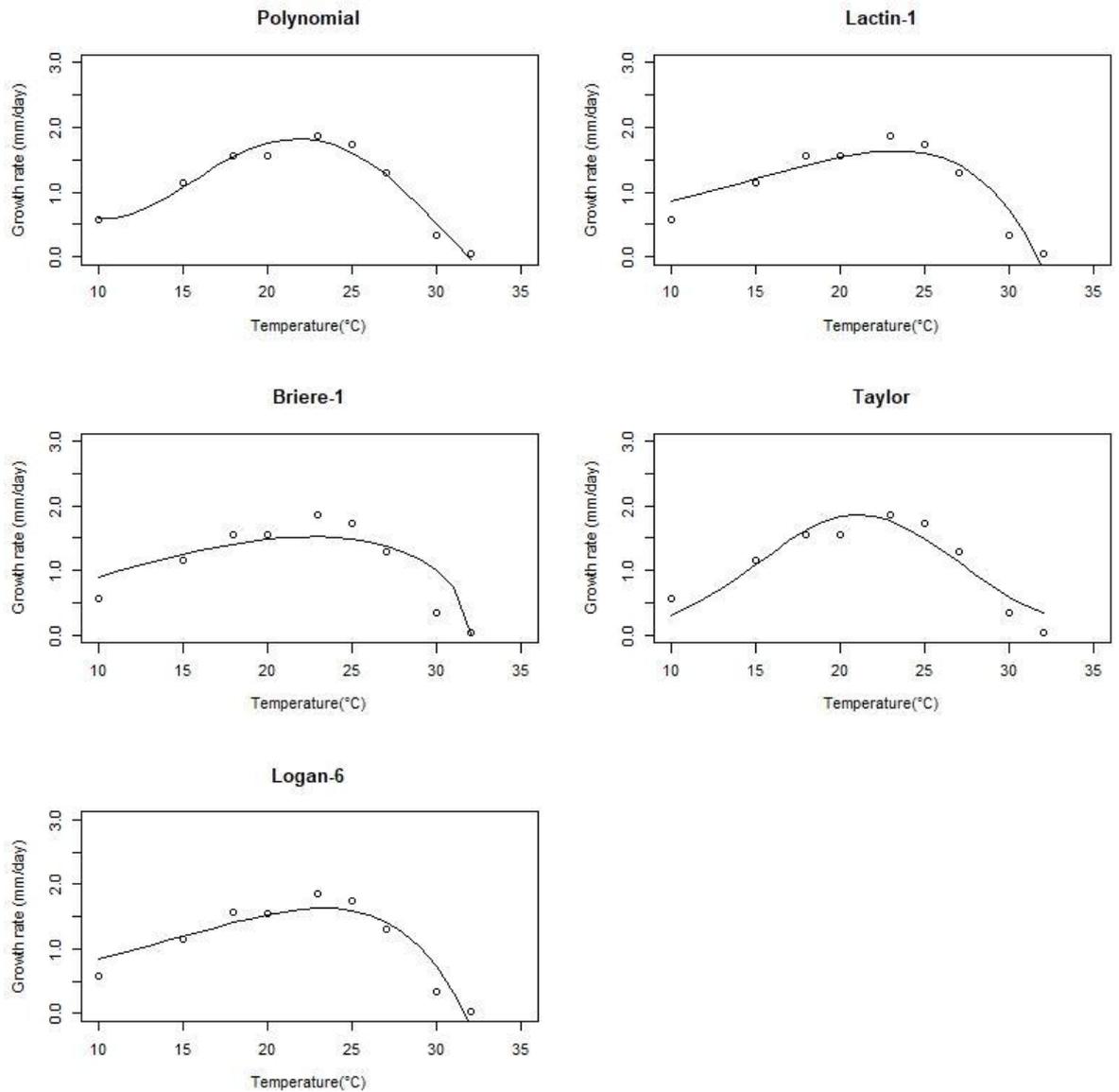


Figure 4.17 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Akanthomyces muscarium* ATCC 6544.

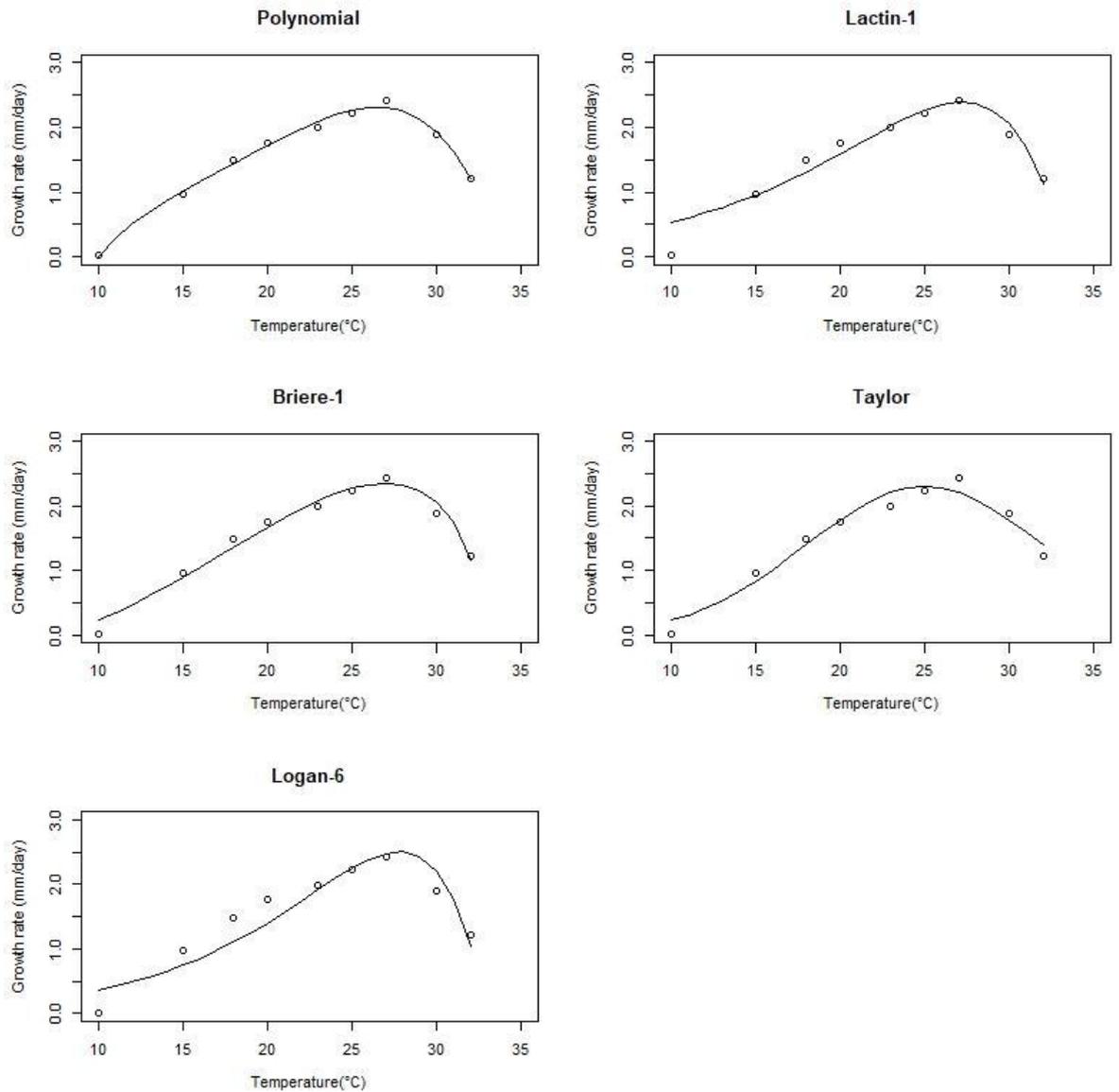


Figure 4.18 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Metarhizium anisopliae* Bioblast.

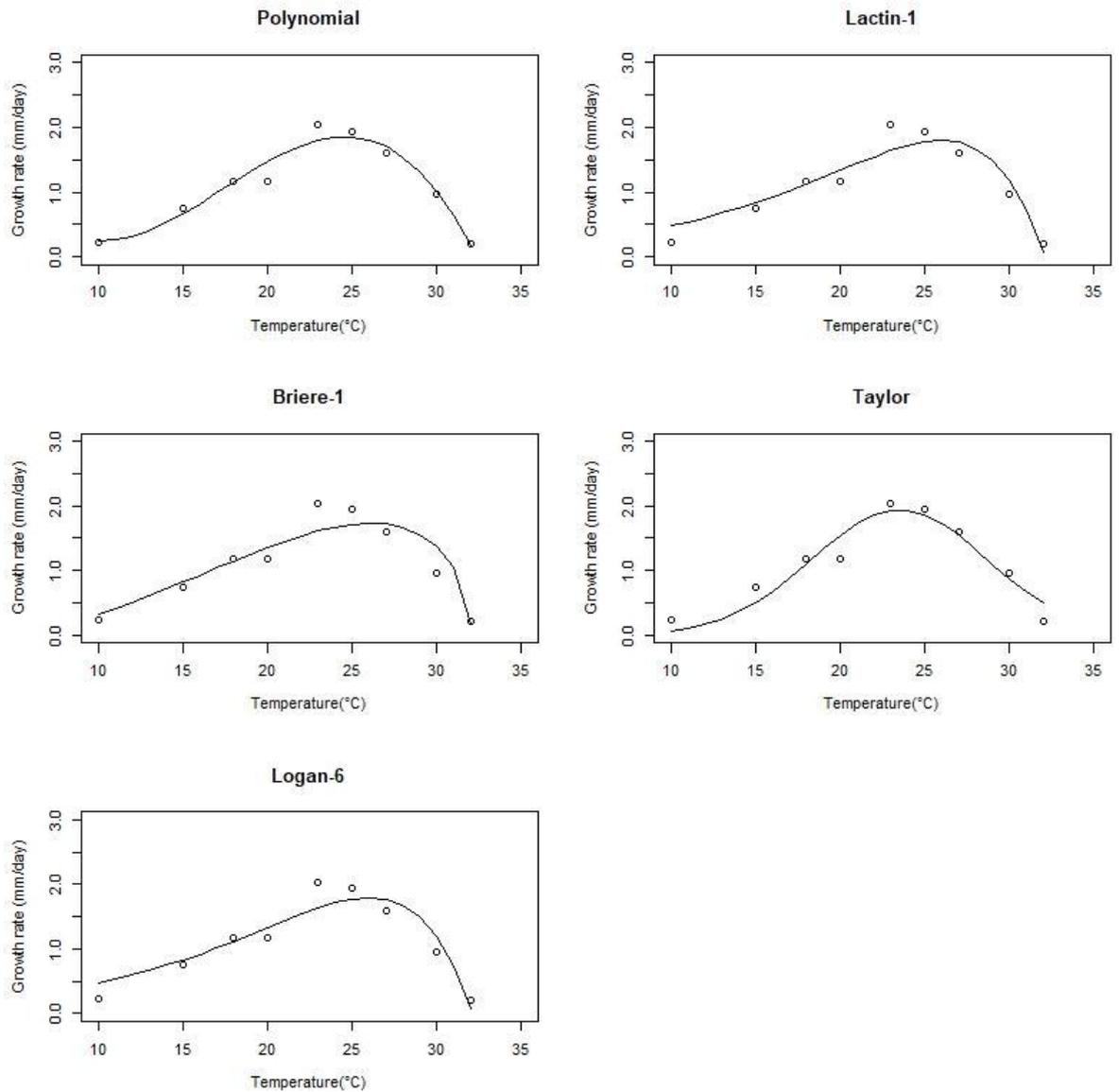


Figure 4.19 Five non-linear models fitted to mean growth rate ($\text{mm}^2 \text{day}^{-1}$) at nine temperatures ($^{\circ}\text{C}$) for fungal isolate *Metarhizium brunneum* Met52

4.4.3 Quantifying the effect of temperature on conidial production of fungal isolates.

There were no significant differences between conidia data for the reference isolates *B. bassiana* PPRI5339 in group 1 and 3 ($\chi^2=0.13$, $df=1$, $p=0.72$) or GHA in group 1, 2 or 4 ($\chi^2=1.18$, $df=2$, $p=0.55$), so all data across groups were compiled for further analysis.

Conidia production counts varied greatly between isolates and ranged from 165 conidia mm^2 - 3.4×10^6 conidia mm^2 (Figure 4.20). Isolates *M. brunneum* (Met 52) and *A. lecanii* (ATCC 4060) produced the greatest number of conidia with counts ranging from 2.6×10^5 – 1.4×10^6 and 1.9×10^5 – 1.3×10^6 conidia mm^2 respectively, depending on incubation temperature.

Whereas, the highest number of conidia was quantified for *B. bassiana* ATCC 6921 and *C. javanica* ATCC 7477 at 5.6×10^4 and 3.9×10^4 conidia mm^2 .

There was no significant interaction between genera and temperature for conidial production ($F=0.58$, $df=3$ $p=0.62$). However, there were significant differences between average conidial production for each genus when averaged across all temperatures, with *Metarhizium* isolates producing significantly more, and *Cordyceps* isolates producing significantly fewer conidia per unit area ($F=11.97$, $df=3$, $p<0.001$; Table 4.4).

Temperature had a significant effect on conidial production for individual isolates, though the response was not the same for all isolates. A linear model with different intercepts and slopes for each isolate fitted the data significantly better than one model for all responses ($F_{35,526}=20.24$, $r^2=0.55$, $p<0.001$). The interaction between temperature and conidial production within the linear regression was not significant for PPRI 5339, ATCC 9451, ATCC 6921, ATCC 6544, ATCC 5278, ATCC 4205, ATCC 4060, GHA, Botanigard and Met 52 (Figure 4.21). Conidial production generally increased with increasing temperature except for isolates ATCC 6920 and ATCC 972, where fewer conidia were produced at higher temperatures (Figure 4.20 & 4.21).

When grouped by Köppen–Geiger climate classification, there was no significant relationship between conidia produced by isolates and temperature ($F=1.71$, $df=5$, $p=0.12$).



Figure 4.20 Box-whisker plots of conidia produced per unit area of eighteen fungal isolates incubated at nine different temperatures (median, 25/75 percentiles, 10/90 percentiles, dots indicate outliers).

Table 4.4 Average natural Log conidial production and standard deviation grouped by Genera. There were significant differences between those without a common letter as determined by ANOVA and Tukey multiple comparisons.

Genus	Average natural Log (conidia mm ⁻²) ± SD	Conidia counts (conidia mm ⁻²)
<i>Akanthomyces</i>	12.28 ± 0.86 ac	297539 ± 196859
<i>Beauveria</i>	12.21 ± 0.68 ab	237413 ± 116715
<i>Cordyceps</i>	11.82 ± 0.53 c	158767 ± 99806
<i>Metarhizium</i>	12.59 ± 0.82 a	402423 ± 282673

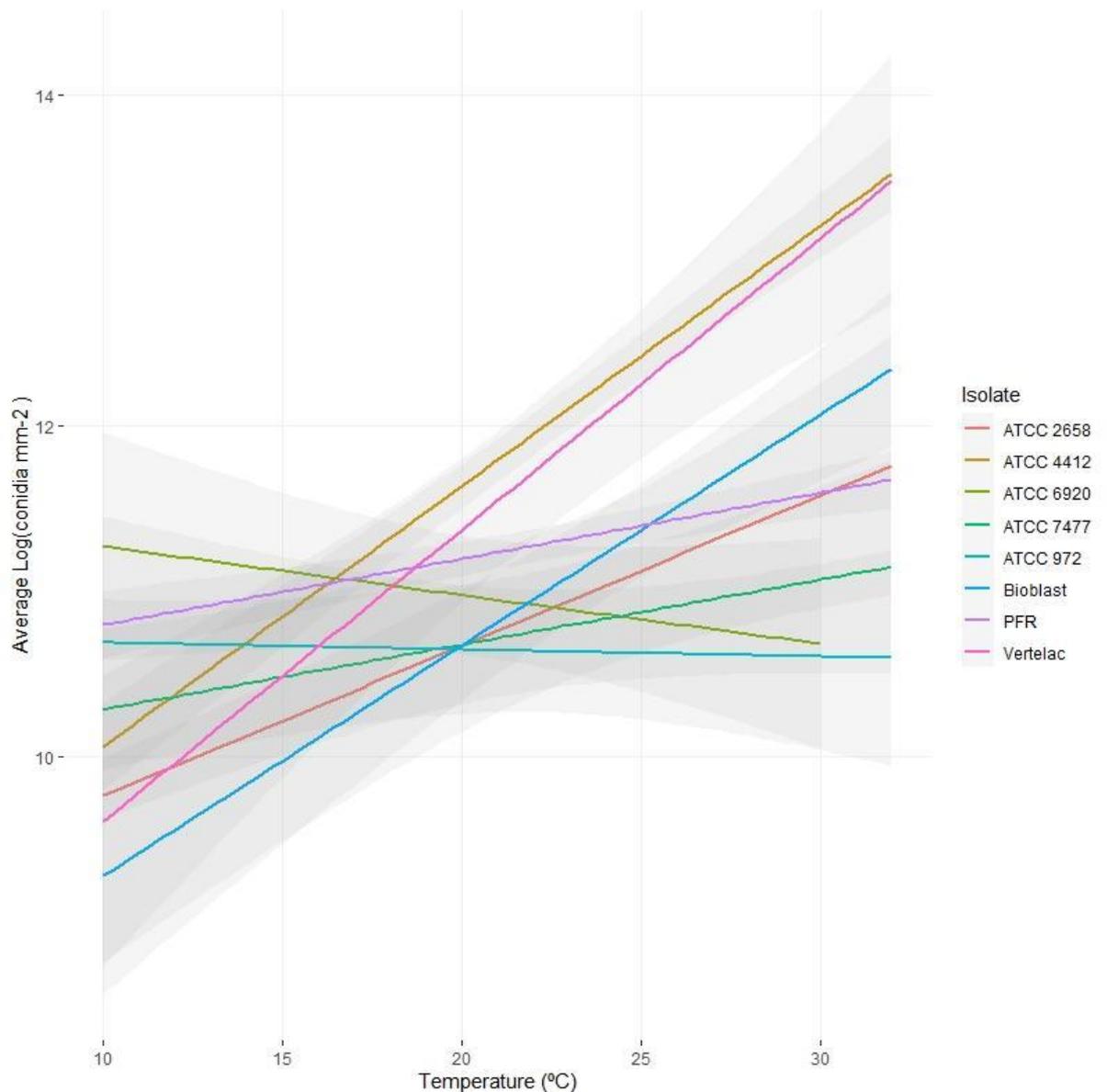


Figure 4.21 Linear regression for conidial production per unit area for isolates demonstrating a significant linear relationship across nine temperatures and 95% confidence intervals. ($F_{35,526}=20.24$, $R^2=0.55$, $p<0.001$)

4.4.4 Quantifying the effect of temperature on *in vitro* germination rate of fungal isolates.

There was no significant difference between the average proportion of germinated conidia at each temperature for *B. bassiana* PPRI 5339 used in group 1 and group 2 ($df=1$, $F=0.166$, $p=0.68$) as indicated in an ANOVA, so data collected in each group was combined for further analysis.

GT₅₀ values were determined for each isolate using a probit analysis (Table 4.5).

Germination rate was significantly affected by temperature though this relationship was not linear and was explained by a quadratic function ($F_{2,45}= 111.3$, $R^2= 0.83$, $p<0.001$).

Germination was slowest at the lowest temperatures (10°C, 15°C and 18°C) and fastest for temperatures above 20°C (20°C, 25°C, 27°C, 30°C and 32°C) for all isolates. There were significant differences between GT₅₀ values for different isolates and model accuracy was improved by fitting isolates independently ($F_{8,39}= 48.62$, $R^2= 0.91$, $p<0.001$, Figure 4.22). Again, similar responses between genera and temperature were observed for growth rate, where *Metarhizium* species (Met 52 and Bioblast) had the slowest GT₅₀ at 10°C and the fastest GT₅₀ at 30°C compared to all other isolates. Whereas, *A. lecanii* (ATCC 6544) had the fastest GT₅₀ at 10°C but the slowest germination rate at 30°C. Data for this isolate at 32°C was missing due to contamination.

Table 4.5 Time for 50% germination and standard error (SE) for seven isolates incubated at seven different temperatures calculated by probit analysis.

Genus	Isolate	GT ₅₀ (hours) ± SE						
		Temperature (°C)						
		10	15	18	20	25	30	32
<i>Beauveria</i>	GHA	49.16±0.98	25.81±0.59	19.64±0.39	14.85±0.16	11.95±0.26	12.66±0.31	16.22±0.24
	ATCC 5278	50.17±0.05	41.49±2.31	28.28±0.60	21.41±0.27	16.81±0.31	15.84±0.19	22.49±0.35
	PPRI 5339	53.29±0.15	34.34±0.28	24.91±0.23	19.52±0.19	16.10±0.28	16.49±0.36	8.98±0.39
<i>Akanthomyces</i>	ATCC 6544	44.51±0.29	26.63±0.13	19.28±0.54	15.46±0.26	13.24±0.21	17.55±0.31	NA*
<i>Cordyceps</i>	ATCC 4412	49.07±0.03	26.56±0.45	16.71±0.69	13.06±0.23	10.94±0.21	11.07±0.29	14.33±0.30
<i>Metarhizium</i>	Met 52	55.92±1.95	27.16±1.48	27.16±1.48	16.11±0.83	10.32±0.56	8.7±0.42	9.0±0.37
	Bioblast	54.76±0.21	33.6±0.47	19.64±0.39	14.27±0.52	10.38±0.33	8.24±0.45	8.98±0.38

* No data available due to contamination.

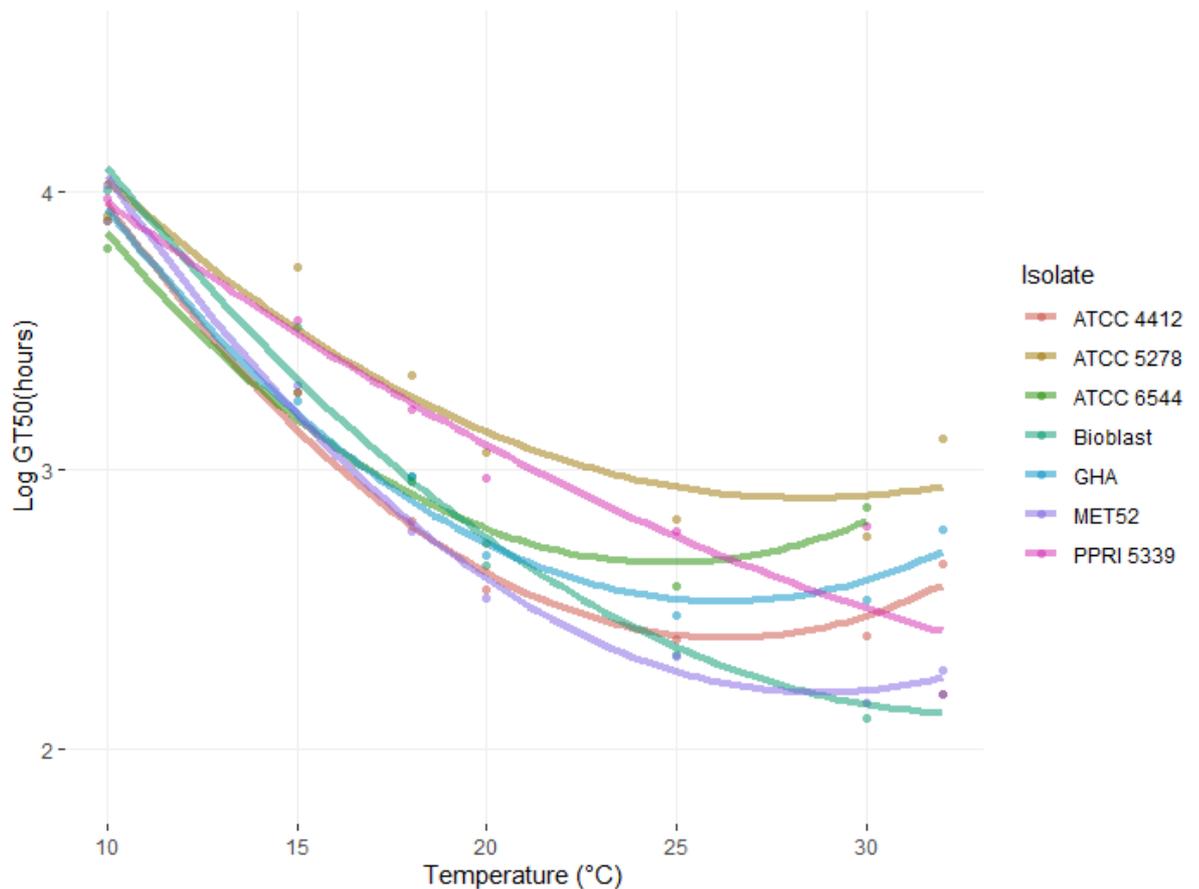


Figure 4.22 The effect of incubation temperature on the in vitro rate of germination of seven entomopathogenic fungi. Time to 50% germination (GT_{50}) was calculated using probit analysis. A quadratic regression with multiple lines described the data well ($F_{8,39} = 48.62$, $R^2 = 0.91$, $p < 0.001$).

4.4.5 Modelling *in vitro* germination data of fungal isolates using non-linear models.

Five non-linear models were fitted to percentage germination data after 16hr incubation at seven temperatures ranging 10-32°C. Percentage germination of conidia of each fungal isolate showed a bell-shaped distribution when plotted against temperature. *Beauveria bassiana* isolates differed in their response to temperature. As a result, germination was best described by different models for each isolate. For isolate *B. bassiana* PPRI 5339, the polynomial and Logan-6 models were determined to be the best fits with r^2 values of 0.99 and 0.93 and AIC values of 33.36 and 54.51 (Table 4.6). These models were also considered the best fit for the growth response of this isolate. Predicted optimal temperature for germination of *B. bassiana* PPRI 5339 varied according to the model fitted but was estimated at 28.56°C for the Logan-6 model.

In comparison, *B. bassiana* isolate GHA was best described by the Briere-1 ($r^2=0.96$, AIC=53.74) and Taylor model ($r^2=0.96$, AIC=54.46), estimating the temperature optimum to be 27.57°C. Whereas, *B. bassiana* isolate ATCC 5278 germination data fitted best to Lactin-1 ($r^2=0.99$, AIC=33.01) and Logan-6 models ($r^2=0.99$, AIC=34.99) which estimated that the temperature optimum for this isolate was relatively higher than other *B. bassiana* isolates at 29.21°C and 28.65°C respectively.

For the *C. farinosa* isolate, a high r^2 value was observed for the polynomial model ($r^2=0.94$, AIC=56.37) followed by the Taylor model ($r^2=0.90$, AIC=63.55). The temperature optimum for germination of this isolate was estimated as 26.61°C.

Germination data for *A. lecanii* ATCC 6544 was not described well by all models except the Taylor model ($r^2=0.98$, AIC=47.52) and polynomial model ($r^2=0.87$, AIC=56.37). Relatively low r^2 values were observed for all other models, ranging from 0.56-0.74.

Metarhizium isolates displayed a flattened bell-shaped curve due to consistent fast germination across all temperatures over 20°C. Therefore, temperature optima were difficult to determine visually. Nevertheless, germination data for *M. brunneum* Met 52 was described well by the polynomial ($r^2=0.9$, AIC=60.06) and Taylor model ($r^2=0.87$, AIC=47.52) which estimated the optima to be 26.99°C. All models fitted well for germination data of *M. anisopliae* Bioblast with R^2 values ranging from 0.87-0.99 and AIC values in the range of 62.13-70.23. The temperature optima for the best fitted model was relatively higher than all other isolates at 30.11°C.

Table 4.6 Fitted parameters, R^2 and AIC values for five non-linear models for percentage germination of seven fungal isolates incubated at seven different temperatures.

Model	Par.	<i>Beauveria</i>			<i>Cordyceps</i>	<i>Akanthomyces</i>	<i>Metarhizium</i>	
		PPRI 5339	GHA	ATCC 5278	ATCC 4412	ATCC 6544	Met 52	Bioblast
Briere-I	<i>a</i>	0.066	0.096	0.054	0.099	0.080	0.093	0.093
	<i>T0</i>	14.72	11.72	14.63	11.10	1.77	10.29	11.95
	<i>Tmax</i>	32.00	32.60	32.27	33.52	32.0	33.96	34.47
	<i>Topt</i>	27.60	27.57	27.80	28.20	25.78	28.42	29.08
	R^2	0.84	0.96	0.77	0.89	0.56	0.86	0.89
	<i>AIC</i>	58.07	53.74	58.05	64.43	70.25	65.89	64.93
Taylor	<i>Rm</i>	64.14	96.65	90.40	112.10	95.86	111.59	110.55
	<i>Topt</i>	26.62	25.88	27.91	26.61	27.76	26.99	27.76
	<i>T0</i>	3.30	5.75	2.04	6.62	6.73	7.28	6.73
	R^2	0.88	0.96	0.90	0.90	0.98	0.87	0.89
	<i>AIC</i>	55.71	54.46	52.41	63.55	47.52	65.73	64.48
Lactin-I	<i>p</i>	0.28	0.21	0.33	0.21	0.21	0.21	0.13
	<i>Tmax</i>	32.03	32.71	32.27	33.24	31.78	33.41	33.02
	<i>Topt</i>	26.62	29.17	29.21	29.73	28.14	29.9	30.11
	<i>A</i>	3.43	3.54	3.063	3.51	3.64	3.51	7.00
	R^2	0.84	0.85	0.99	0.67	0.69	0.61	0.99
	<i>AIC</i>	52.50	63.31	33.011	71.76	67.88	73.13	64.16

Model	Par.	<i>Beauveria</i>			<i>Cordyceps</i>	<i>Akanthomyces</i>	<i>Metarhizium</i>	
		PPRI 5339	GHA	ATCC 5278	ATCC 4412	ATCC 6544	Met 52	Bioblast
Logan-6	Ψ	-0.24	-1.014	-0.0016	-1.57	-2.50	-0.23	-0.97
	p	0.29	0.22	0.39	0.203	0.21	0.23	0.21
	$Topt$	28.56	32.77	28.65	33.88	26.7	27.3	28.9
	$Tmax$	32.03	32.99	32.26	34.078	31.79	34.52	34.54
	ΔT	3.54	5.24	3.61	5.94	5.091	7.22	5.64
	R^2	0.93	0.903	0.99	0.80	0.74	0.77	0.82
	AIC	54.51	51.65	34.99	70.49	68.55	71.56	70.23
Polynomial	a	-378.9	365.3	-558.6	1291	0.74	1338	1193
	b	100.0	-78.89	135.7	-295.0	0.74	-310.4	-268.9
	c	-9.454	5.465	-11.72	23.08	12.48	24.74	20.74
	d	0.375	-0.13	0.43	-0.73	-0.35	-0.801	-0.65
	e	-0.0052	0.0009	-0.0055	0.0082	0.0032	0.0092	0.0072
	R^2	0.99	0.95	0.79	0.94	0.87	0.90	0.87
	AIC	33.36	51.65	53.74	56.37	57.99	60.06	62.13

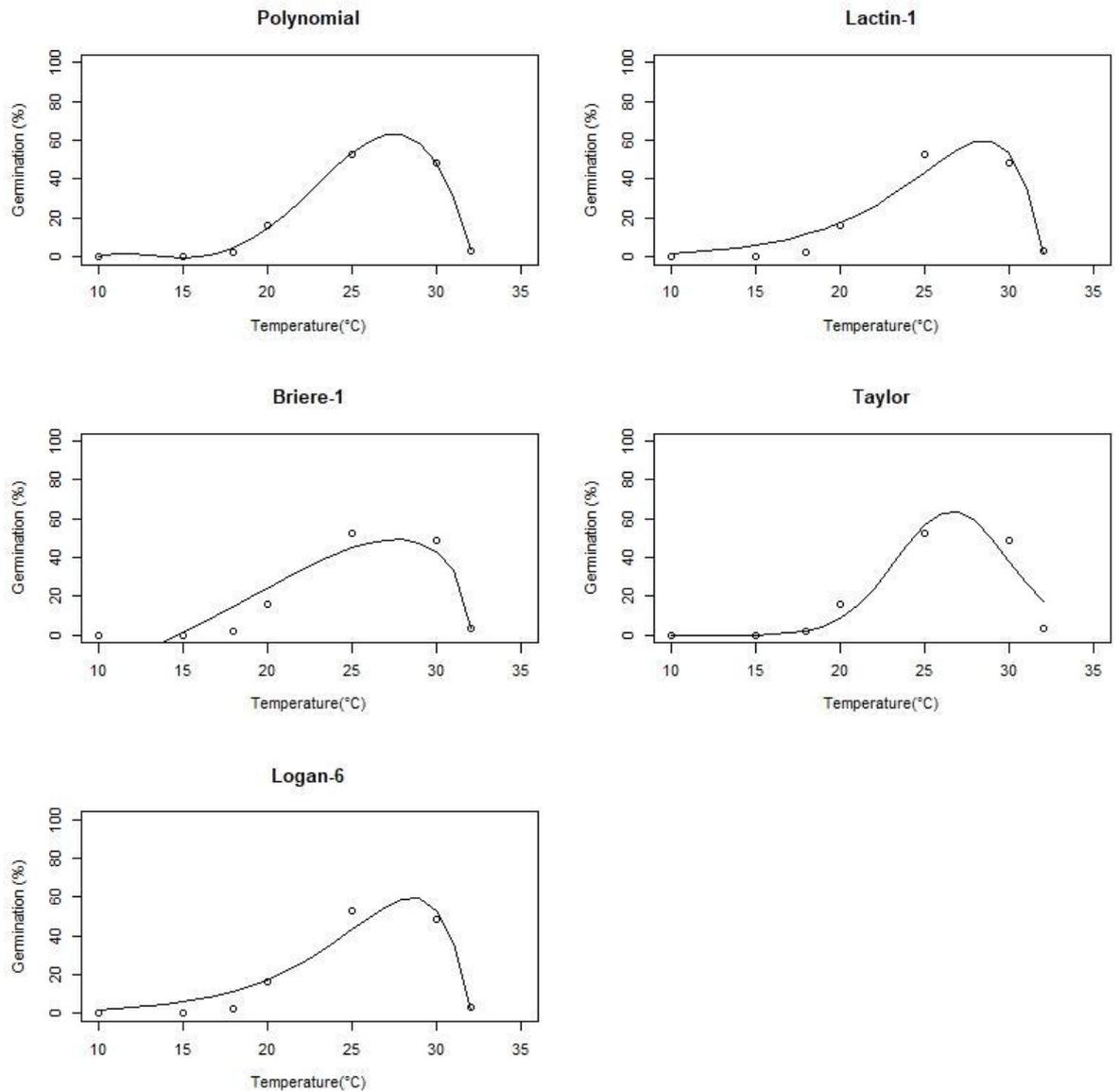


Figure 4.23 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Beauveria bassiana* PPRI 5339.

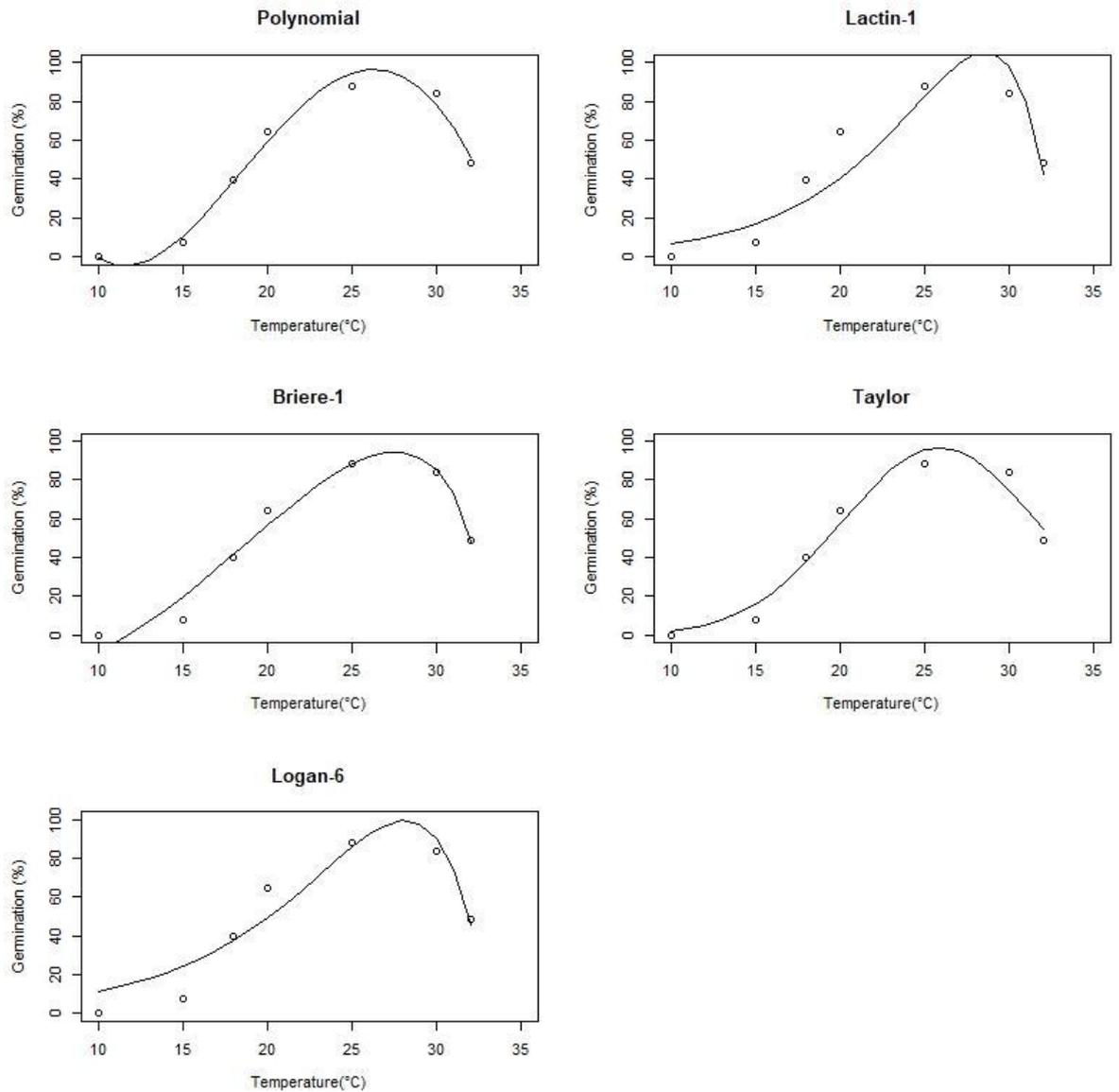


Figure 4.24 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Beauveria bassiana* GHA.

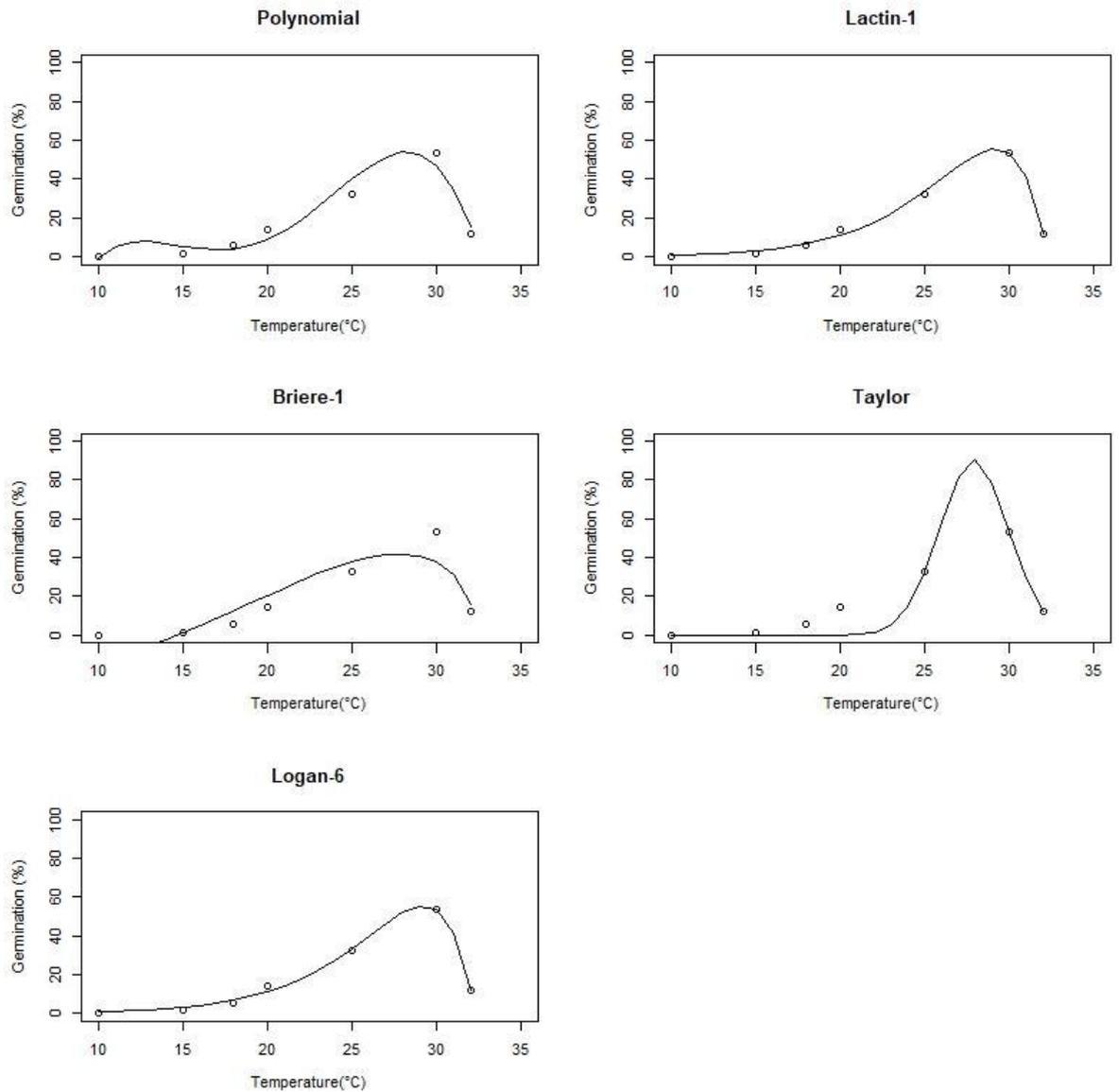


Figure 4.25 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Beauveria bassiana* ATCC 5278.

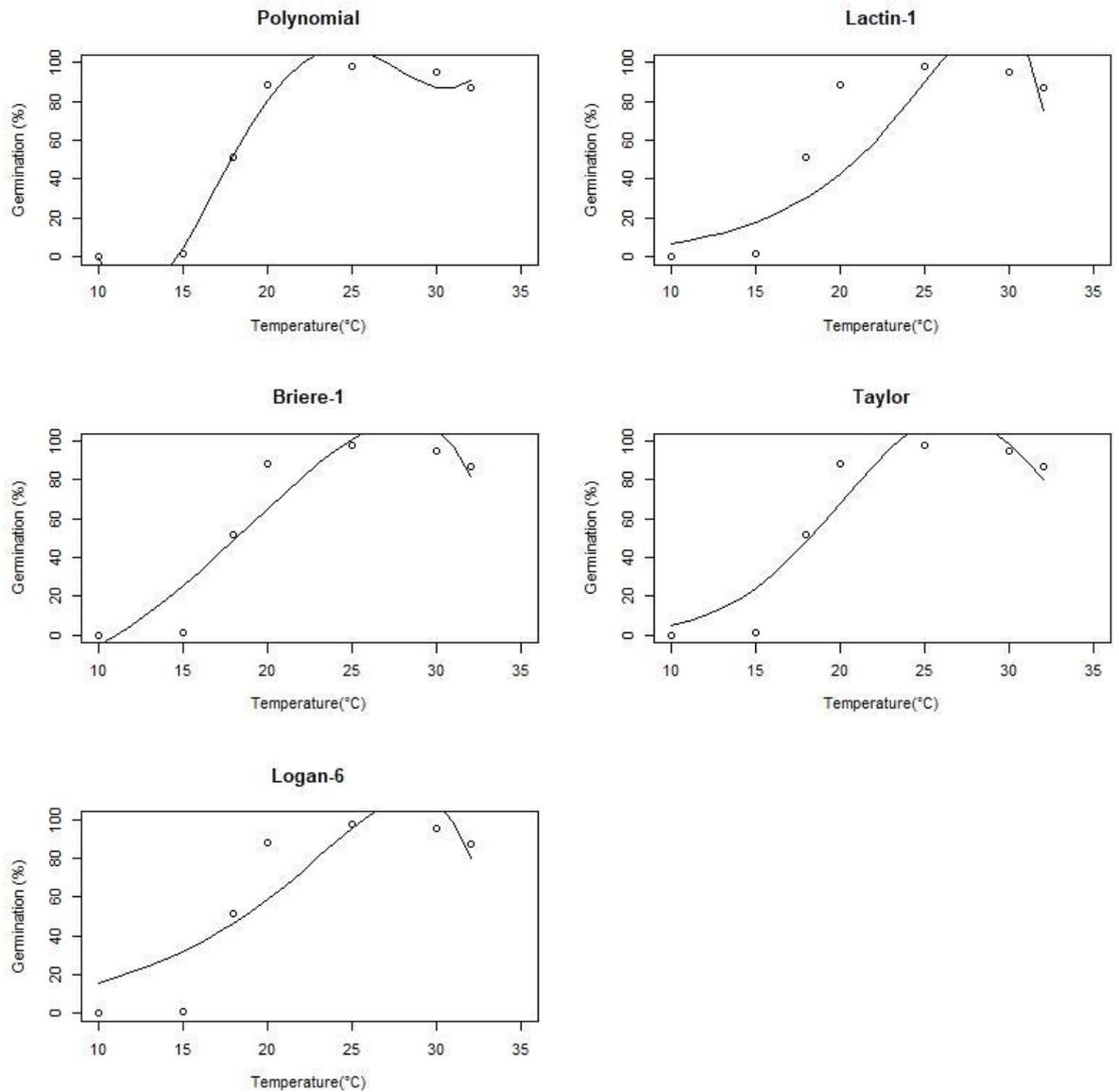


Figure 4.26 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Cordyceps farinosa* ATCC 4412.

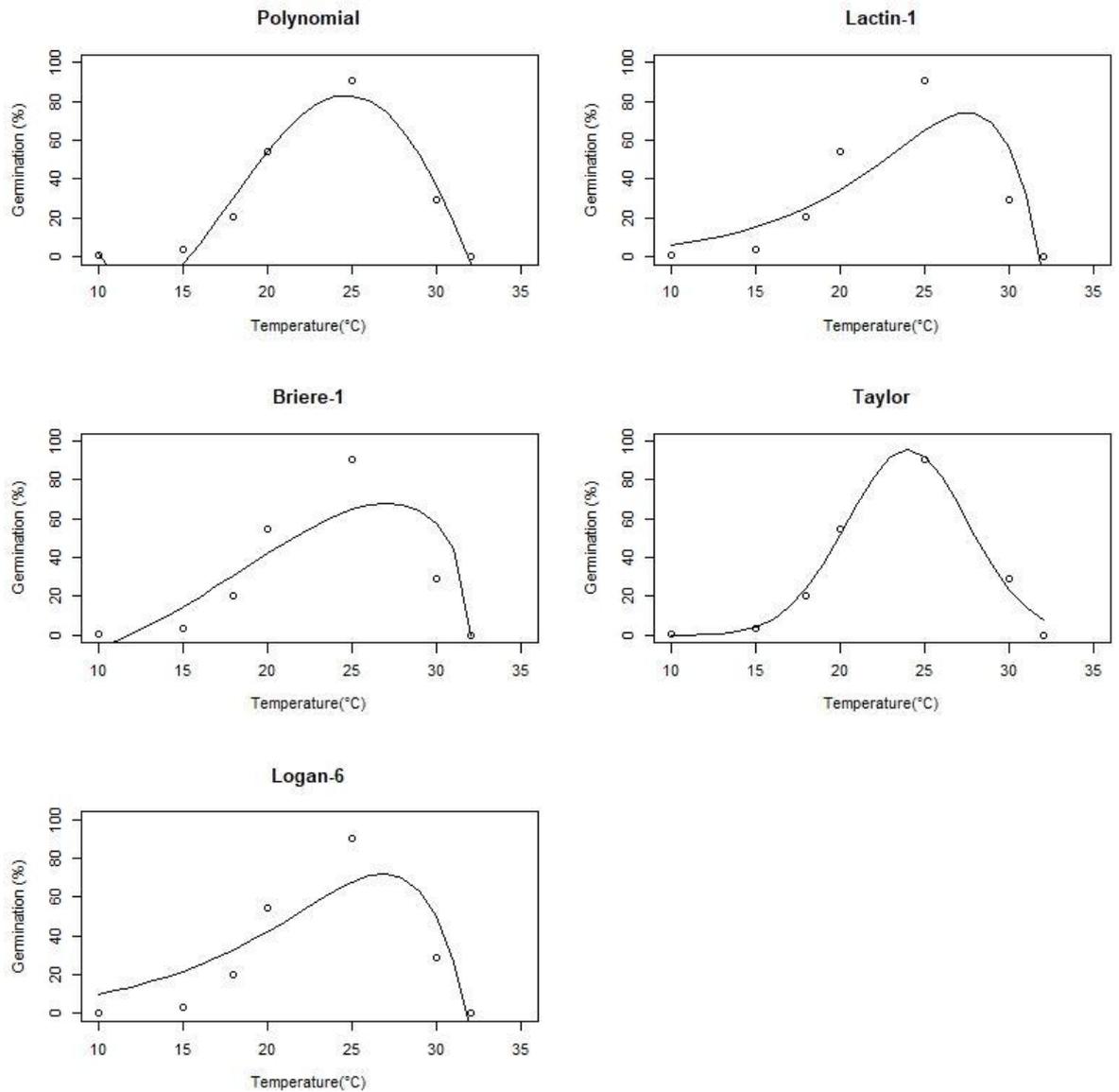


Figure 4.27 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Akanthomyces lecanii* ATCC 6544.

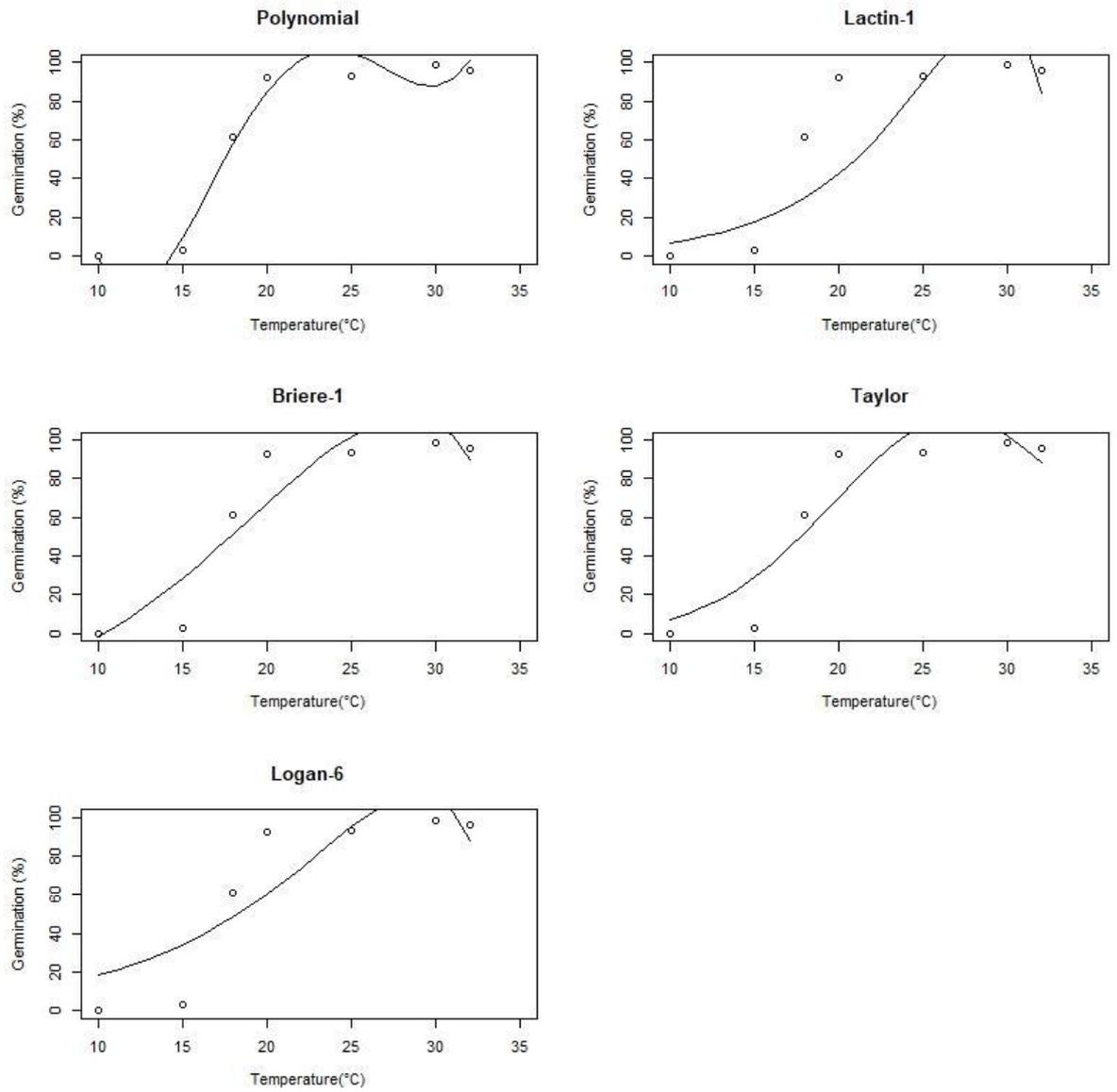


Figure 4.28 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Metarhizium brunneum* Met 52.

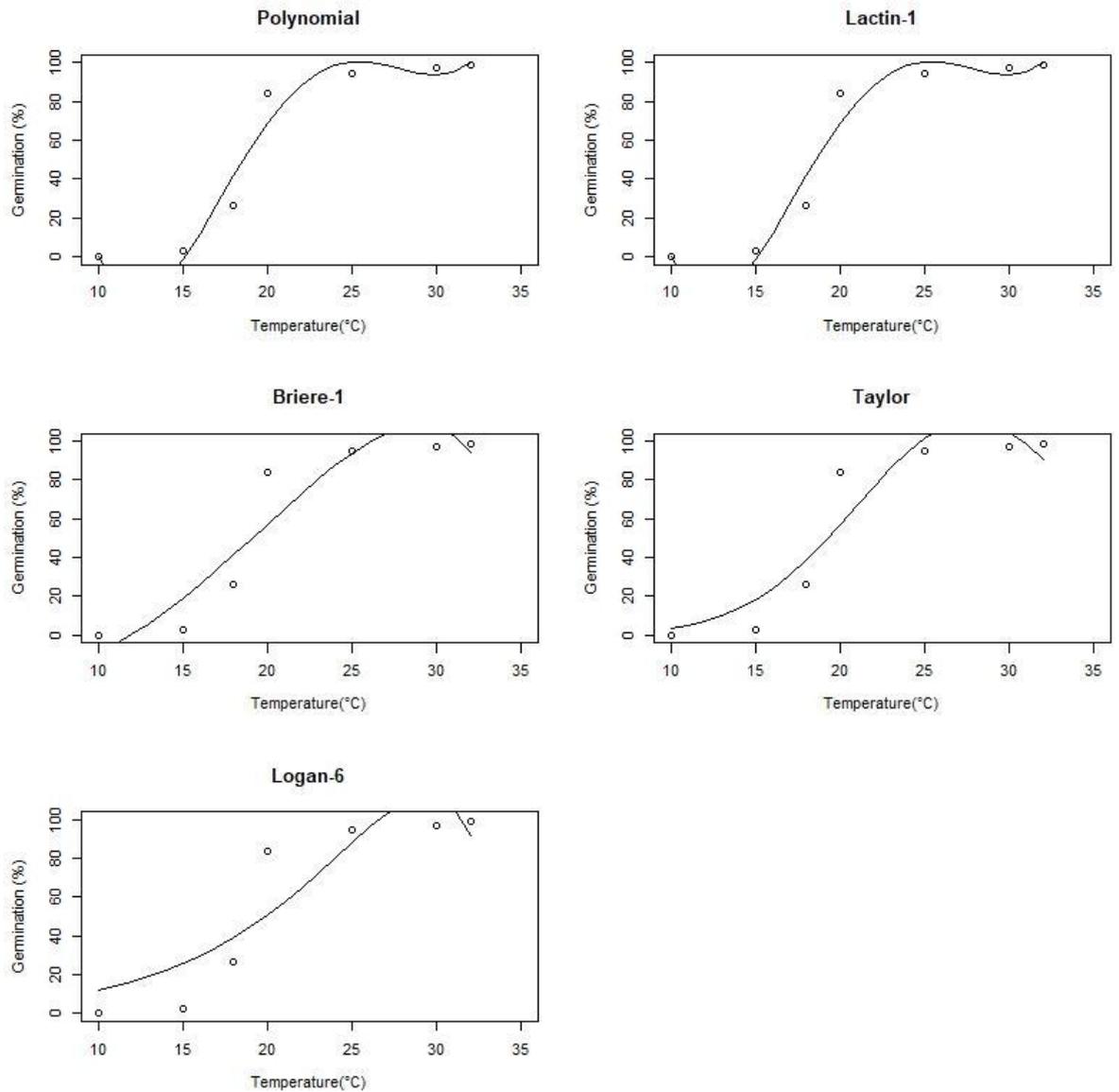


Figure 4.29 Five non-linear models fitted to mean germination after 16hr incubation across nine temperatures for *Metarhizium anisopliae* Bioblast.

4.4.6 Examining the relationship between *in vitro* growth and germination thermal optima.

Comparisons were made between the temperature optima predicted for growth and germination for isolates used in both *in vitro* experiments. Predicted optima were taken from the best fit models, except where the polynomial model provided the best fit. In this case, the next best fit model was chosen in order to extract temperature optima values. A generalised linear regression model was used to determine the relationship between predicted germination and growth optima. Though the output of this model is limited to describing the outputs of the fitted model, and not actual biological parameters.

For each isolate, different models provided the best fit for germination and growth data. *Metarhizium brunneum* (Met 52) was an exception to this trend, with both germination and growth data being described best by the Taylor model. Predicted temperature optima for germination was higher than the predicted optima for growth for all isolates (Table 4.7). However, the difference between germination and growth temperature optima was small for *C. farinosa* ATCC 4412 compared to the other isolates. The linear regression model for growth and germination optima did not provide a significant fit, suggesting that there was no relationship between the two variables ($R^2=0.24$, $p=0.14$, Figure 4.30). However, there was a significant linear relationship between growth and germination optima after the removal of the isolate *C. farinosa* ATCC 4412 ($R^2= 0.86$, $p=0.005$, Figure 4.30).

Table 4.7 Thermal optima for seven entomopathogenic fungi as determined by parameters extracted from the best fitting non-linear model.

Genus	Isolate	Germination Model	Germination Optima (°C)	Growth Model	Growth Optima (°C)
<i>Beauveria</i>	PPRI 5339	Logan	28.56	Lactin	25.59
	GHA	Briere	27.57	Lactin	24.58
	ATCC 5278	Lactin	29.21	Briere	27.03
<i>Cordyceps</i>	ATCC 4412	Taylor	26.61	Briere	26.36
<i>Akanthomyces</i>	ATCC 6544	Taylor	27.76	Lactin	23.85
<i>Metarhizium</i>	Met 52	Taylor	26.99	Taylor	23.51
	Bioblast	Lactin	30.11	Briere	26.96

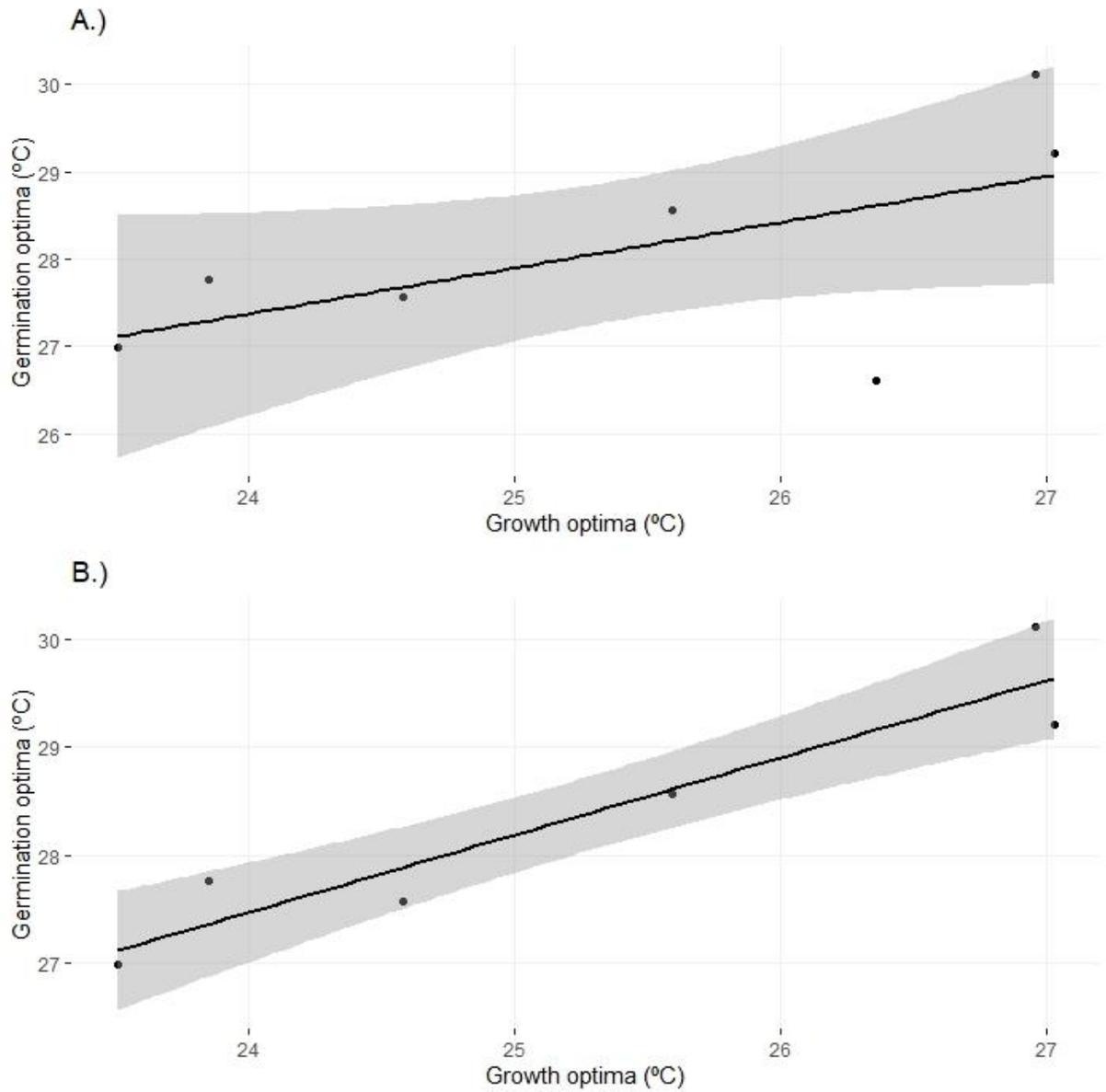


Figure 4.30 Germination and growth optima predicted by one of four non-linear models for A.) seven and B.) six entomopathogenic fungal isolates. Linear regression and 95% confidence intervals are included in each plot. The linear relationship for A.) was not significant, though there was a significant relationship for B.). The linear relationship with six isolates was given by the equation; germination temperature optima ~ growth temperature optimum.

4.4.8 Quantifying the effect of the presence of insecticides on germination of fungal isolates

Exposing conidia to spiromesifen in solutions prior to being applied to agar (method 1) resulted in a significantly different response in germination by different isolates. The data could not be normalised by transformation so a Kruskal- Wallis test was performed followed by pairwise comparisons using a post hoc Dunn test with p values adjusted by the Benjamini & Hochberg method. For *C. farinosa*, treatment had a significant effect on proportion of germinated conidia at the 13H time point ($\chi^2=11.09$, $df=4$, $p=0.025$). There was a significant increase in germination of conidia in the mixture with the LC₅₀ of spiromesifen compared to the control treatment ($p=0.048$), though all other treatments were not significantly different (Figure 4.31). There was no effect of treatment by the 24H time point, as most conidia had germinated in all treatments ($\chi^2=6.13$, $df=4$, $p=0.19$).

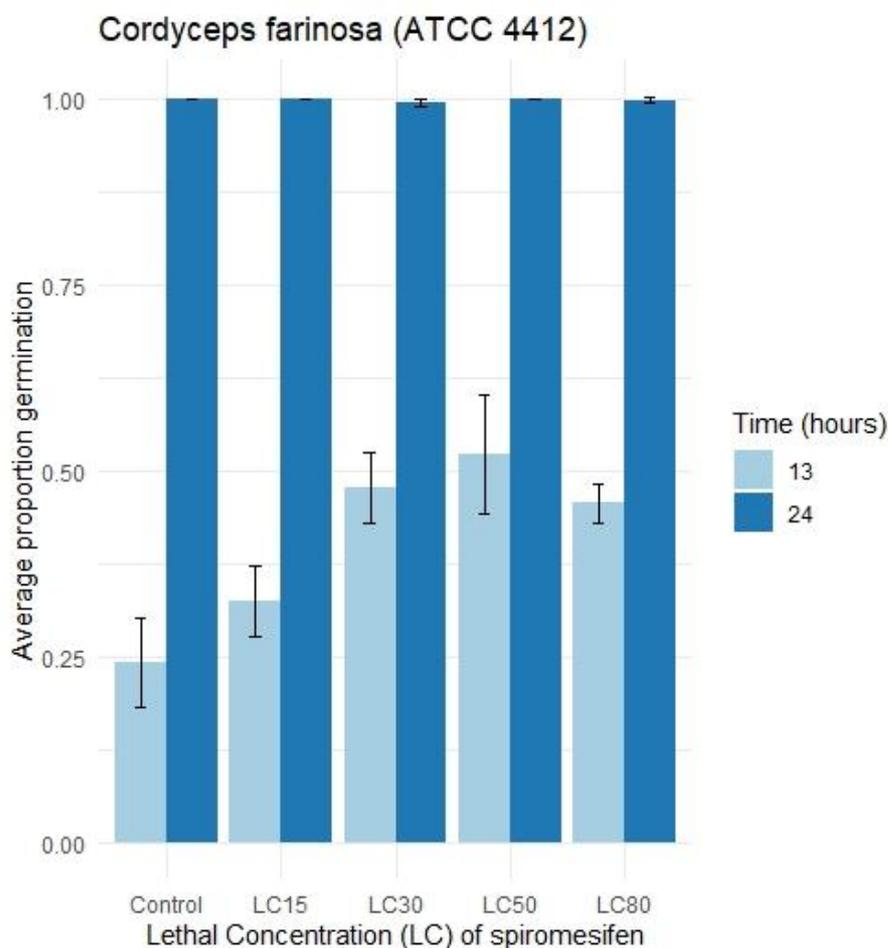


Figure 4.31. Average proportion germination of conidia of *Cordyceps farinosa* (ATCC 4412) after 13 and 24 hours incubation at 25°C when applied as a mixture of fungal conidia and spiromesifen on sabouraud dextrose agar.

Proportion germination data for *B. bassiana* PPRI 5339 showed a normal distribution and equal variances so was analysed by two-way ANOVA and a post hoc Tukey multiple

comparisons test. Proportion germination was significantly affected by duration of incubation ($F=3166.98$, $df=1$, $p<0.001$), treatment ($F=3.77$, $df=4$, $p=0.019$) and there was a significant interaction between time and treatments applied, whereby a greater effect of treatment on germination was observed at the earlier time point ($F=5.09$, $df=4$, $p=0.0054$; Figure 4.32). Proportion germination was significantly higher after treatment of conidia with LC_{80} of spiromesifen compared to the LC_{15} ($p=0.018$) or the control treatment ($p=0.048$).

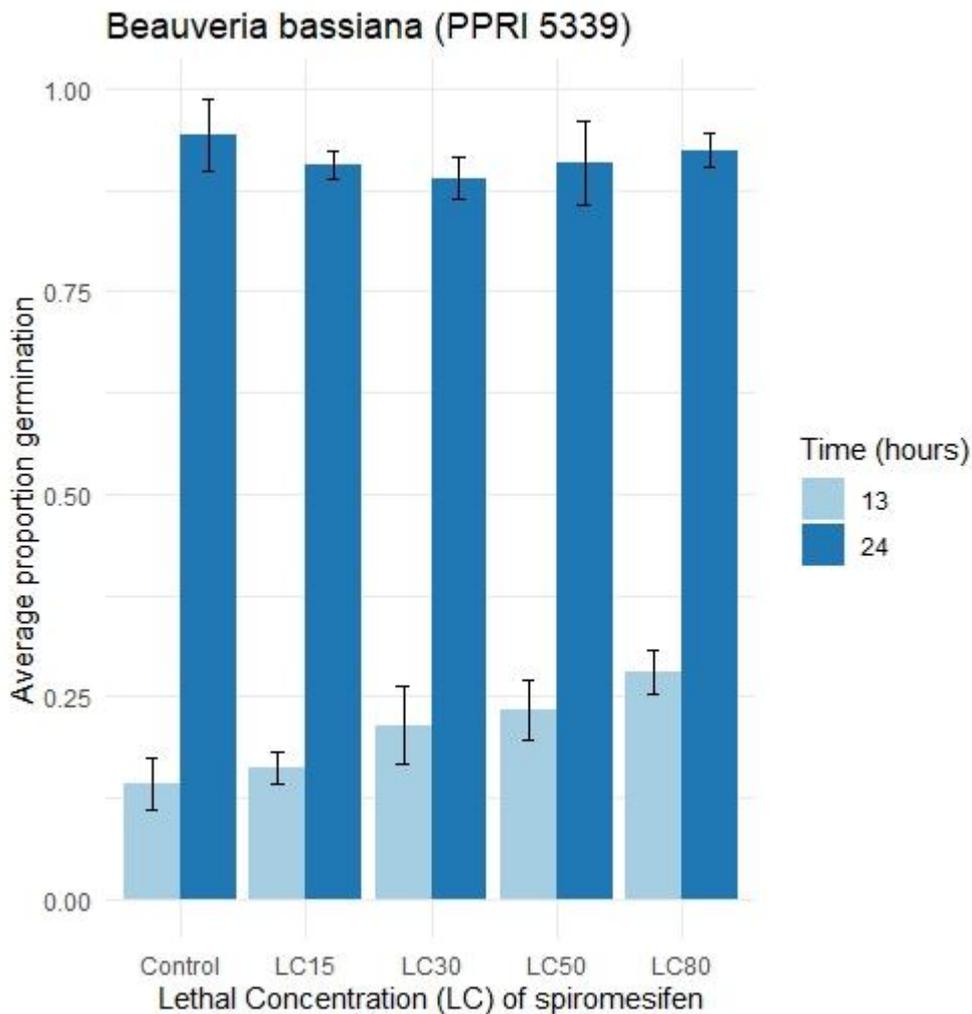


Figure 4.32 Average proportion germination of conidia of *Beauveria bassiana* (PPRI 5339) after 13 and 24 hours incubation at 25°C when applied as a mixture of fungal conidia and spiromesifen on sabouraud dextrose agar.

Metarhizium brunneum (Met 52) conidia germinated faster than all other isolates tested. However, a significantly lower number of conidia germinated in the control treatment at 13H compared to the treatments of spiromesifen ($\chi^2= 9.72$ $df=4$, $p=0.045$; posthoc Dunn $p=0.044$; Figure 4.33).

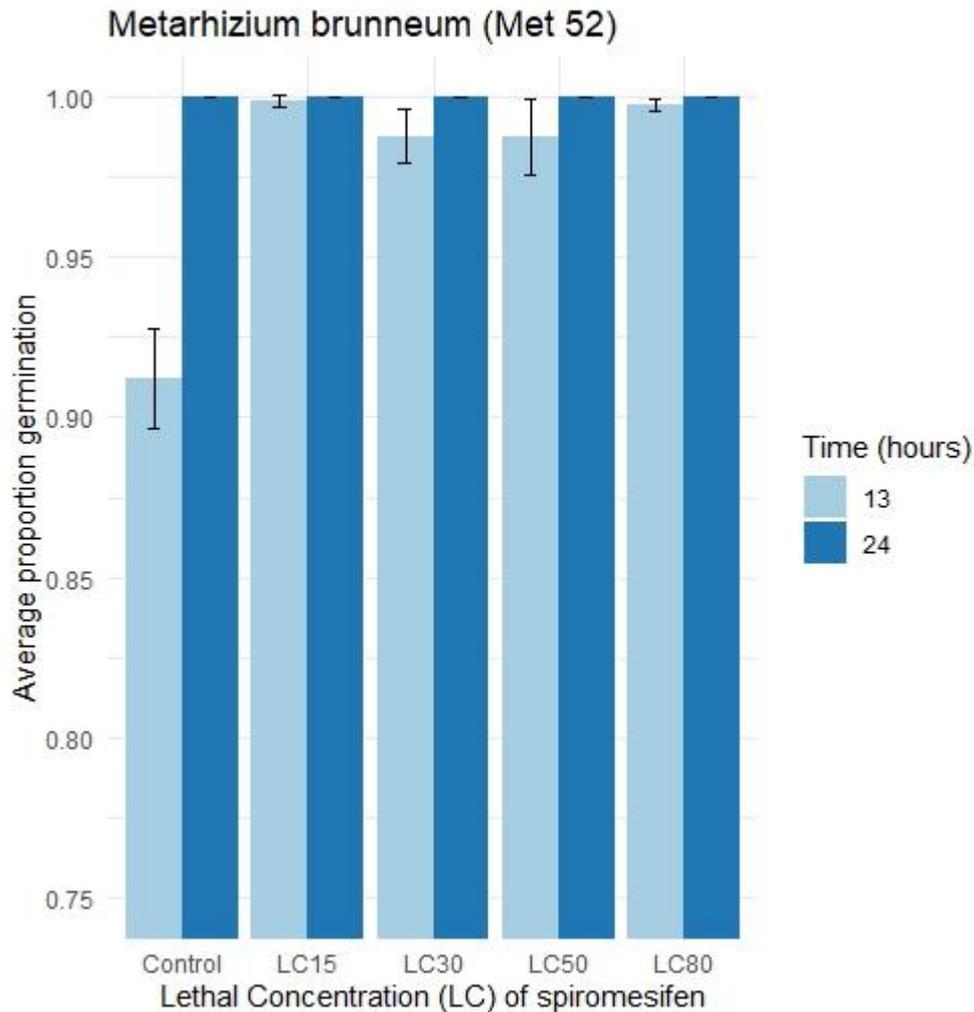


Figure 4.33 Average proportion germination of conidia of *Metarhizium brunneum* (Met 52) after 13 and 24 hours incubation at 25°C when applied as a mixture of fungal conidia and spiromesifen on sabouraud dextrose agar.

There was no significant effect of treatment on the proportion of germinated conidia for isolate *A. lecanii* ATCC 4060 at 13 or 24 hours incubation ($\chi^2= 9.24$, $df=4$, $p=0.055$). All conidia had germinated after the 24 hour incubation period and although there were fewer germinated conidia in the control group compared to other treatments after 13 hours incubation, these differences were not significant (Figure 4.34). Although no significant differences were found for this isolate across each treatment, it was noted that visible hyphal growth on the Petri dish was observed at the 24H time point for treatments with spiromesifen and no hyphal growth was observed for the control group (Figure 4.35).

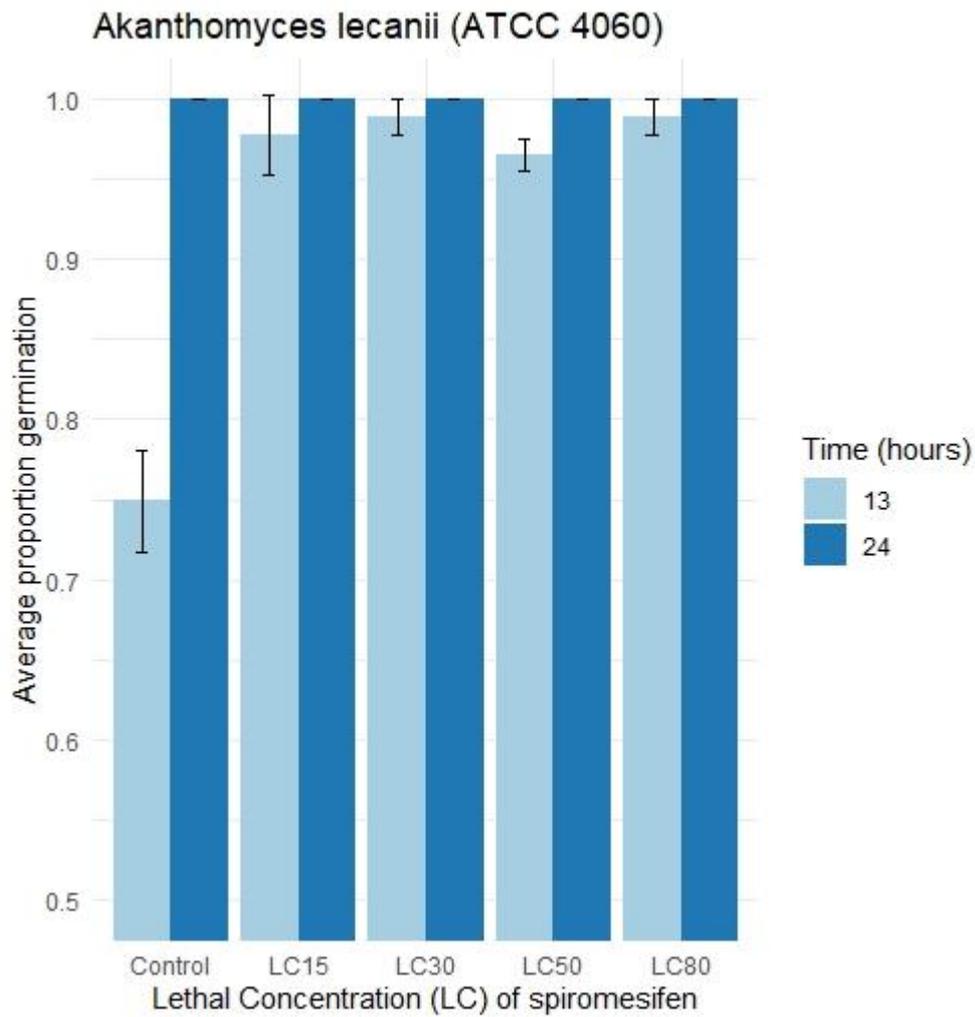


Figure 4.34 Average proportion germination of conidia of *Akanthomyces lecanii* (ATCC 4060) after 13 and 24 hours incubation at 25°C when applied as a mixture of fungal conidia and spiromesifen on sabouraud dextrose agar.

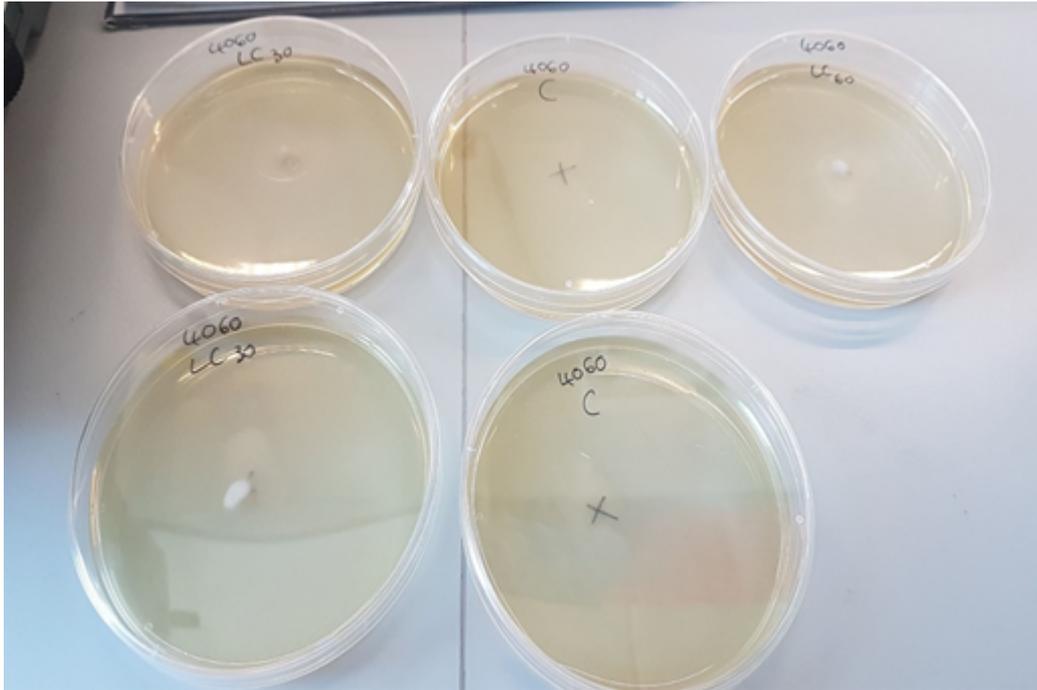


Figure 4.35 Visible hyphal growth observed after 24H incubation for *Akanthomyces lecanii* when co-applied with spiromesifen on sabouraud dextrose agar. Hyphal growth is not observed for the control group.

Proportion germination for conidia exposed to spiked agar with spiromesifen (method 2) was not significantly different for each treatment regardless of the isolate being tested ($F=0.38$, $df=4$, $p=0.82$; Figure 4.36, 4.37 & 4.38). Proportion of germinated conidia was significantly different for 13 and 18 hours incubation for isolate *C. farinosa* ATCC 4412 ($df=1$, $F=552.03$ $p<0.001$) and there was a significant interaction between treatment and time (Two- way ANOVA; $df=4$, $F=8.08$, $p=0.001$), whereby germination was significantly slower in the LC15 treatment group by 18 hours (Figure 4.39).

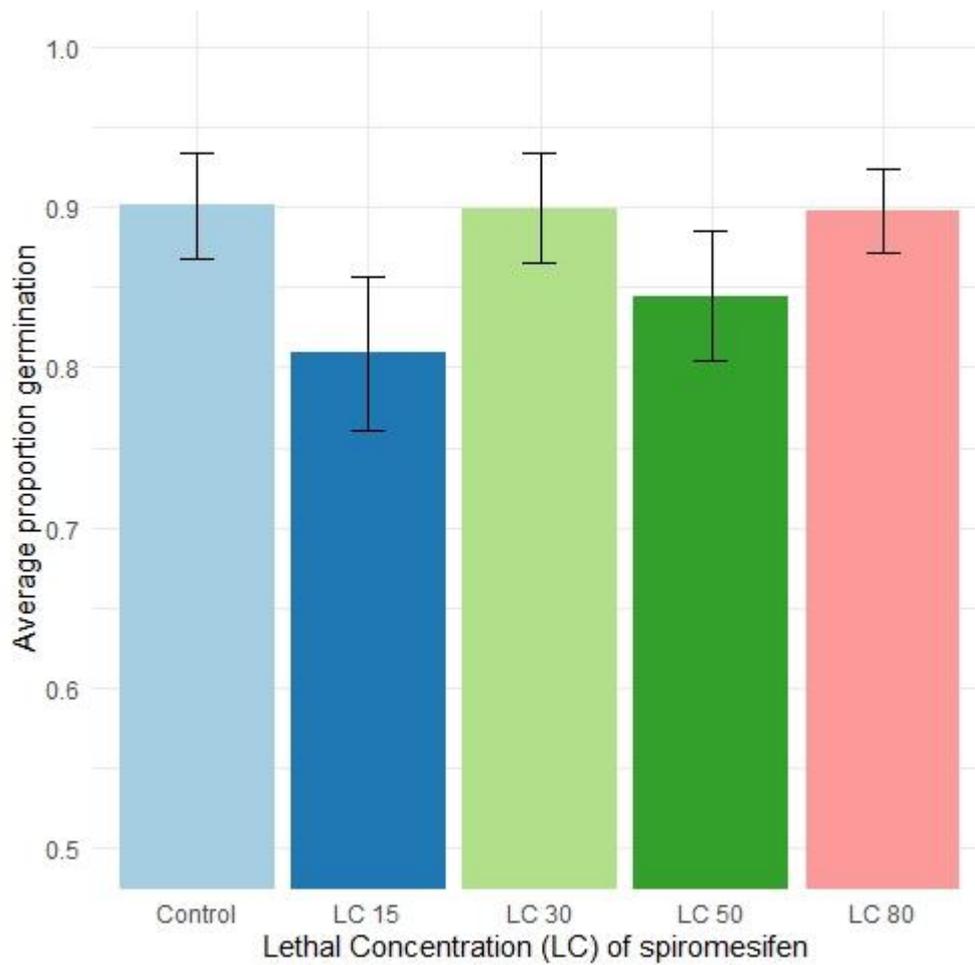


Figure 4.36 Proportion germination of conidia of *Beauveria bassiana* (PPRI 5339) after 18 hours incubation at 25°C and continued exposure to four concentrations of spiromesifen on spiked sabouraud dextrose agar.

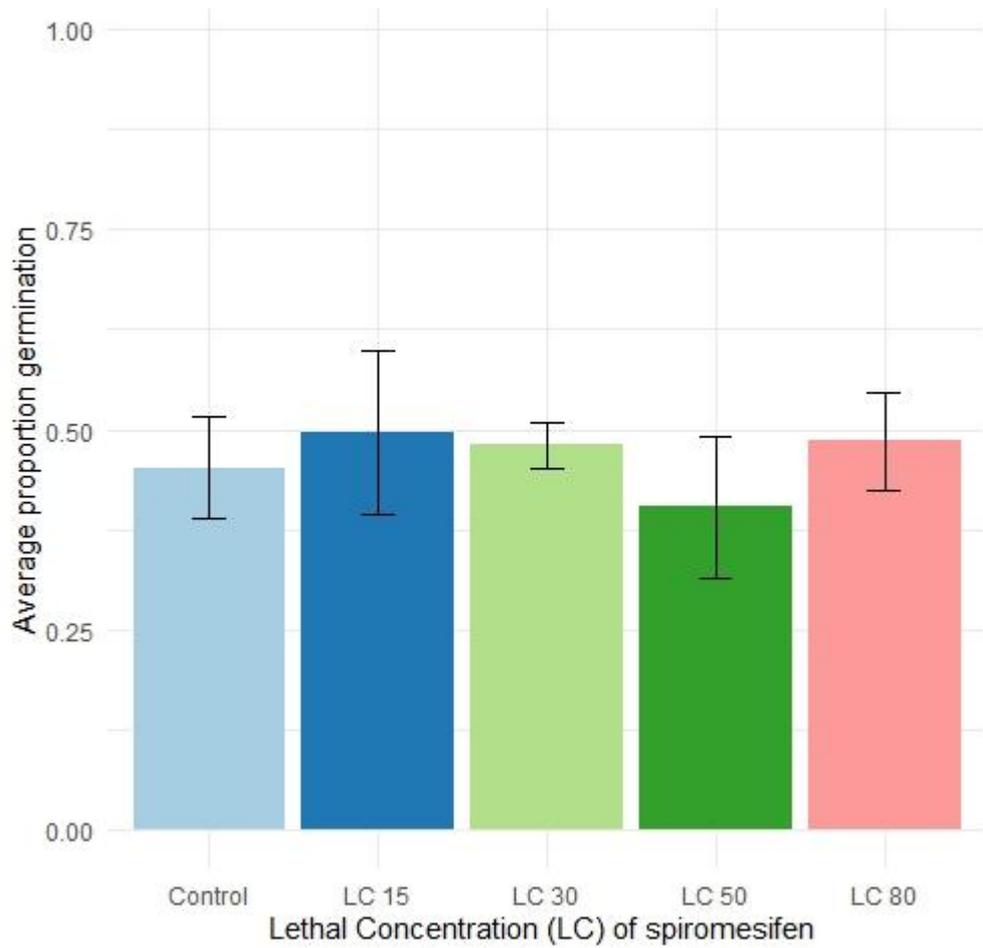


Figure 4.37 Proportion germination of conidia of *Metarhizium brunneum* (Met 52) after 8 hours incubation at 25°C and continued exposure to four concentrations of spiromesifen within spiked sabouraud dextrose agar.

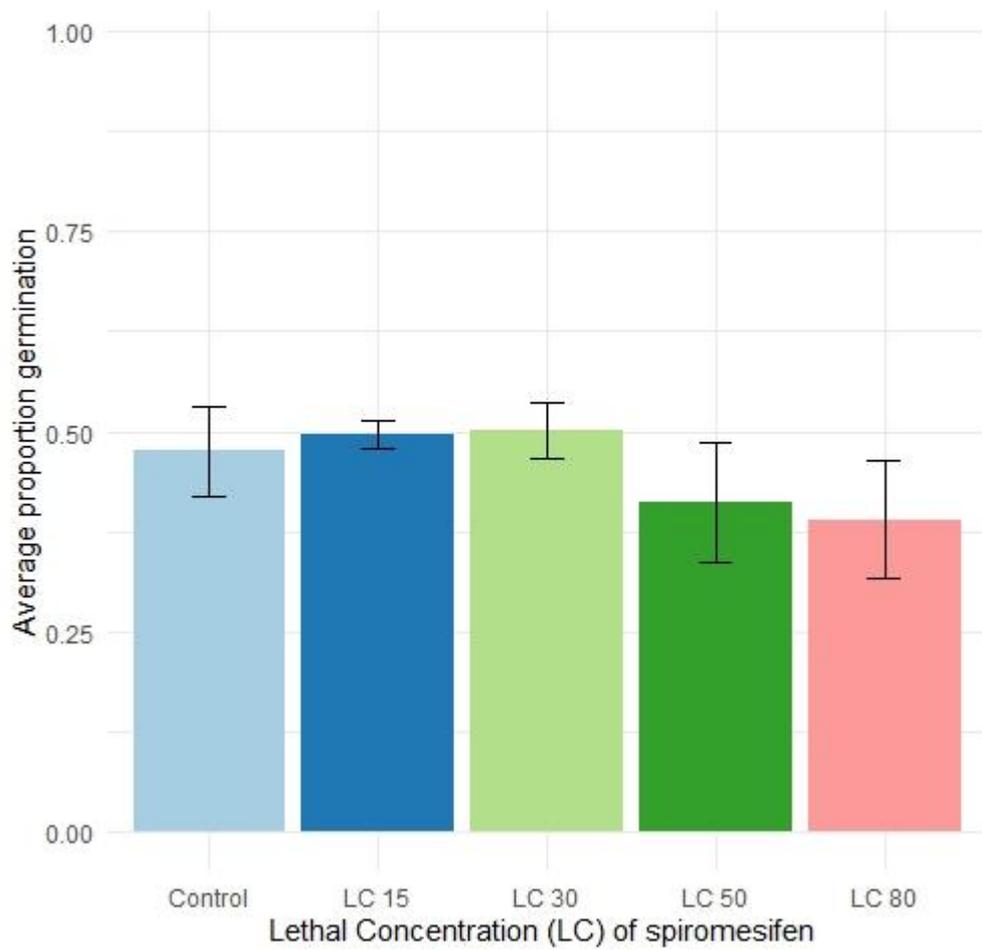


Figure 4.38 Proportion germination of conidia of *Akanthomyces lecanii* (ATCC 4060) after 12 hours incubation at 25°C and continued exposure to four concentrations of spiromesifen within spiked sabouraud dextrose agar.

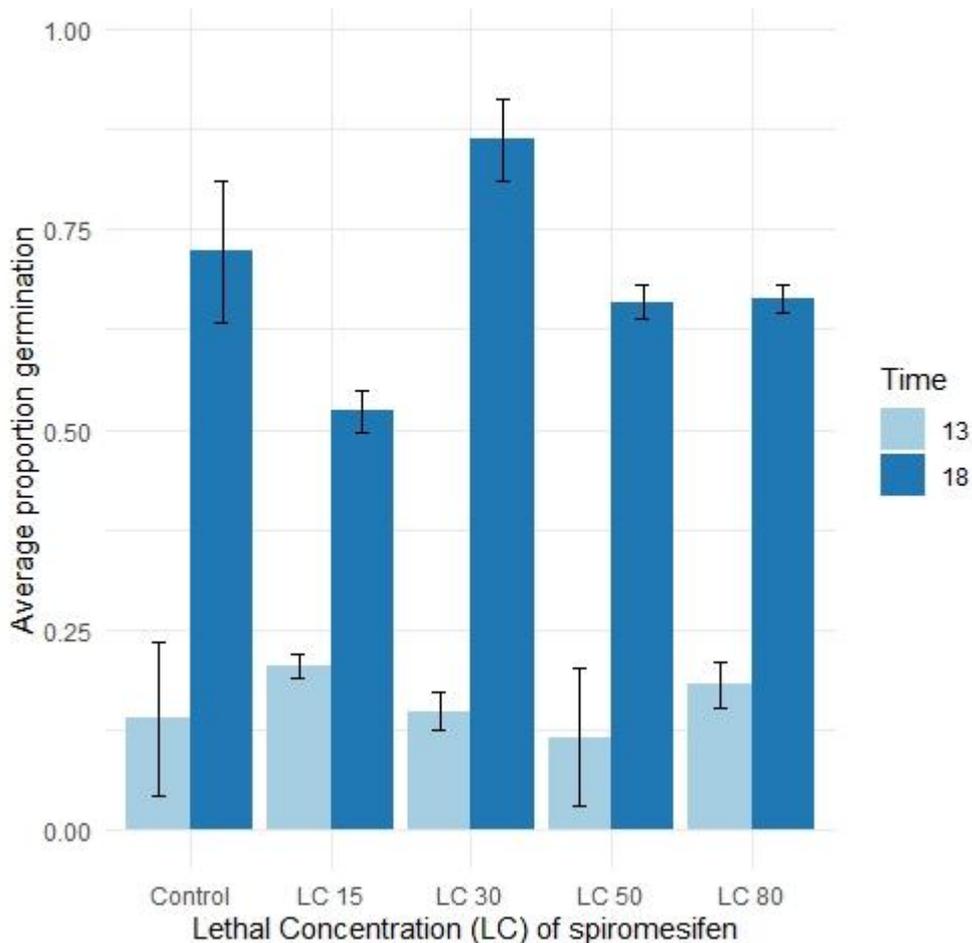


Figure 4.39 Proportion germination of conidia of *Cordyceps farinosa* (ATCC 4412) after 13 and 18 hours incubation at 25°C and continued exposure to four concentrations of spiromesifen within spiked sabouraud dextrose agar.

4.4.9 Quantifying the effect of the presence of insecticides on *in vitro* growth of fungal isolates.

The linear phase of growth was from day three to seventeen. Growth between these dates was used to determine growth rate by plotting a linear regression and extracting the slope of the line. There was no significant difference between growth rate observed for each isolate when exposed to different concentrations of spiromesifen or in the control group ($\chi^2=0.59$, $df=4$, $p=0.96$; Figure 4.40). Growth rate was significantly different for each isolate ($\chi^2=46.46$, $df=3$, $p<0.001$). The fastest vegetative growth rate was observed for isolates *M. brunneum* (Met 52) and *A. lecanii* ATCC 4060 which were significantly faster than *B. bassiana* PPRI 5339 and *C. farinosa* ATCC 4412 ($p<0.001$).

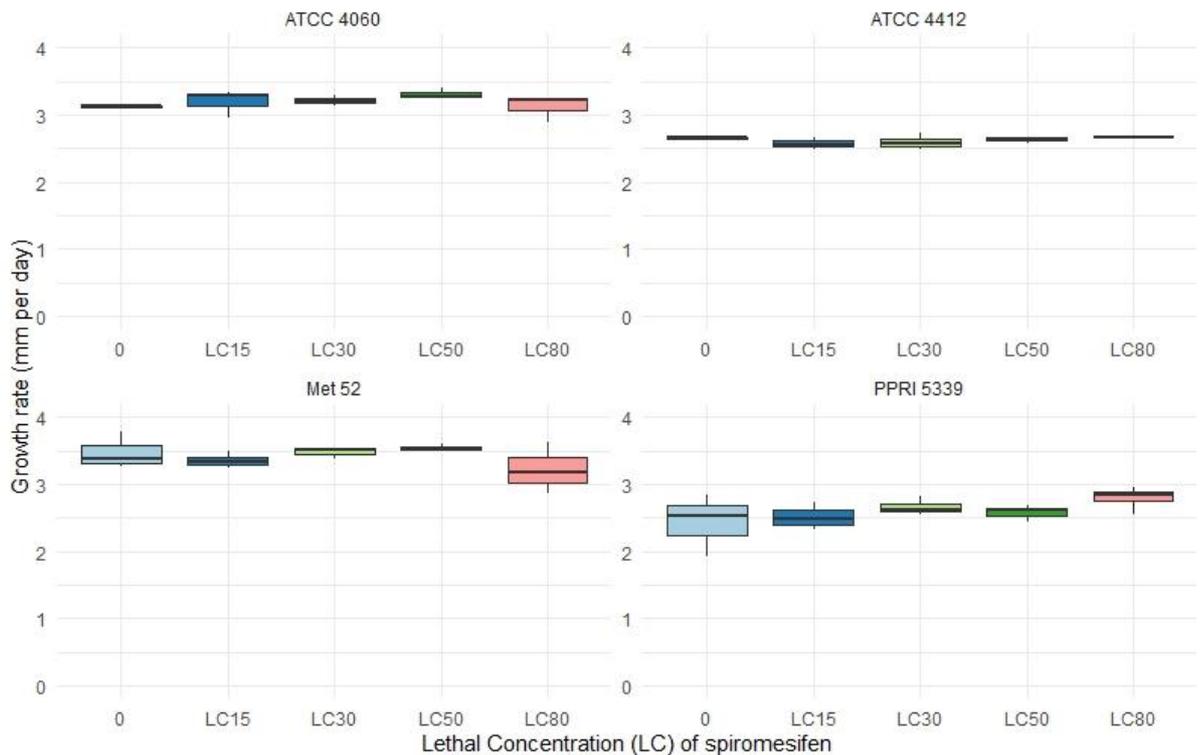


Figure 4.40 Growth rate of four isolates; *Akanthomyces lecanii* (ATCC 4060), *Cordyceps farinosa* (ATCC 4412), *Metarhizium brunneum* (Met 52), *Beauveria bassiana* (PPRI5339) during the linear phase of growth (day 3-17) and exposed to four concentrations of spiromesifen or a negative control.

4.4.10 Quantifying the effect of fungicides and temperature on the *in vitro* growth of an entomopathogenic fungus.

Growth rate of *Beauveria bassiana* PPRI 5339 was significantly reduced by the presence of the fungicides tested ($\chi^2= 65.36$, $df=3$, $p<0.001$). The effect of specific fungicides on *B. bassiana* growth rate was also affected by temperature ($\chi^2=50.19$, $df=2$, $p<0.001$). For example, Luna sensation significantly reduced the growth rate of *B. bassiana* at every concentration however, the extent of the reduction was dependent on the incubation temperature (Figure 4.41). At 10°C, all concentrations of Luna sensation inhibited growth, regardless of concentration applied. At 25°C, increasing concentration of Luna sensation had an increasing inhibitory effect on growth, except for 10% of the recommended application rate which reduced growth by almost 100% compared to the control. At the highest incubation temperature, there was no significant difference between the growth rate of the control and exposure to 1% of the recommended application rate ($p=0.63$), though all concentrations above this caused complete inhibition of growth.

At 10°C, there was no statistically significant difference between growth rates at each concentration of Takumi SC. At 25°C, growth was significantly inhibited by the top two

concentrations of Takumi SC (50 and 100% recommended application rate) compared to the growth rate when exposed to 1% of the recommended application rate ($p=0.026$, $p=0.044$; Figure 4.42). However, growth rate was not significantly different for all treatments compared to the control at this temperature. All treatments of Takumi SC at 32°C resulted in a growth rate that was not significantly different from the control treatment ($\chi^2=10.43$, $df=5$, $p=0.064$). In comparison, there was no effect on growth rate of *B. bassiana* whilst exposed to any concentration of Kumulus at any incubation temperature compared to the control (10°C; $\chi^2=4.9$, $df=5$, $p=0.078$; 25°C $\chi^2=6.94$, $df=5$, $p=0.23$; 32°C, $\chi^2=3.57$, $df=5$, $p=0.61$; Figure 4.43).

A quadratic regression was fit to the temperature and growth rate data with different intercepts and slopes for each fungicide ($F_{11,132}=236.95$, $p<0.001$, $r^2=0.95$). The interaction plot in Figure 4.44 shows the significant inhibition of *B. bassiana* growth by Luna Sensation across all temperatures.

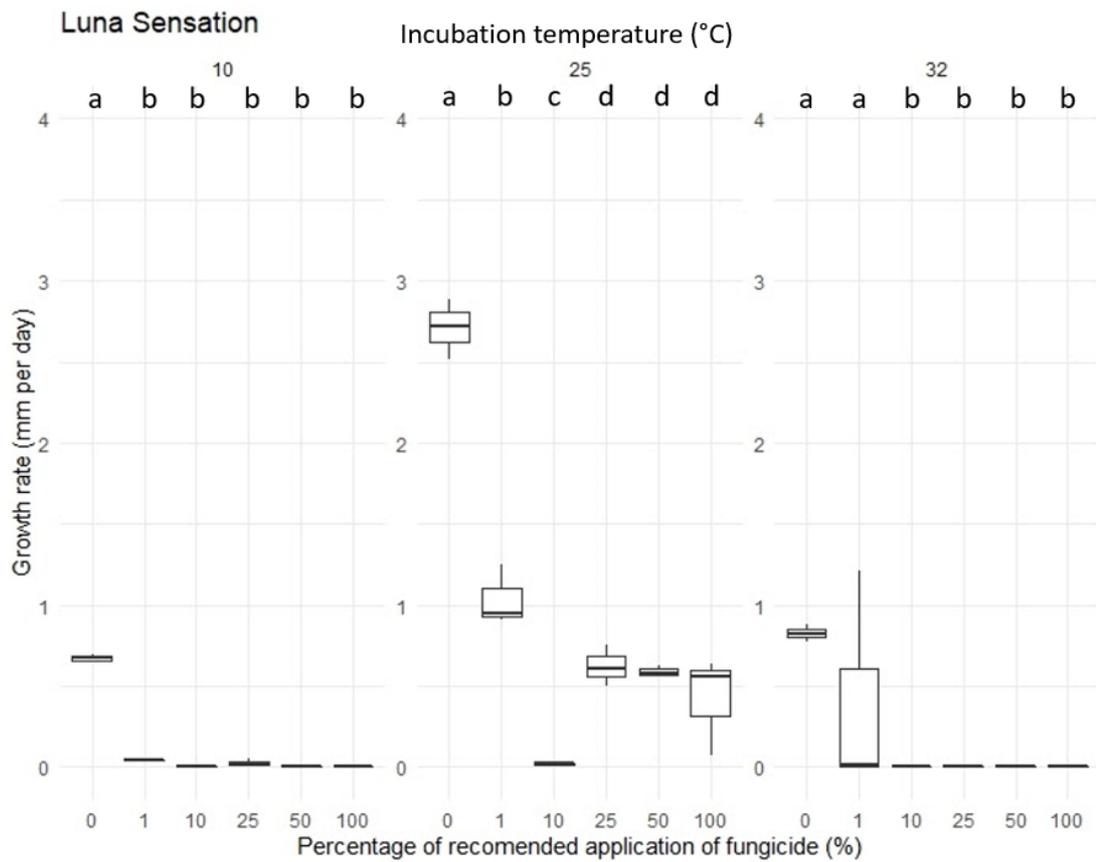


Figure 4.41 Growth rate ($\text{mm}^2 \text{day}^{-1}$) for *Beauveria bassiana* (PPRI 5339) on Sabouraud dextrose agar spiked with a range of concentrations of the fungicide Luna Sensation (active ingredients: fluopyram and trifloxystrobin). Significant differences between growth rates at different exposure concentrations for each incubation temperature are indicated by the presence of a different letter (a-d).

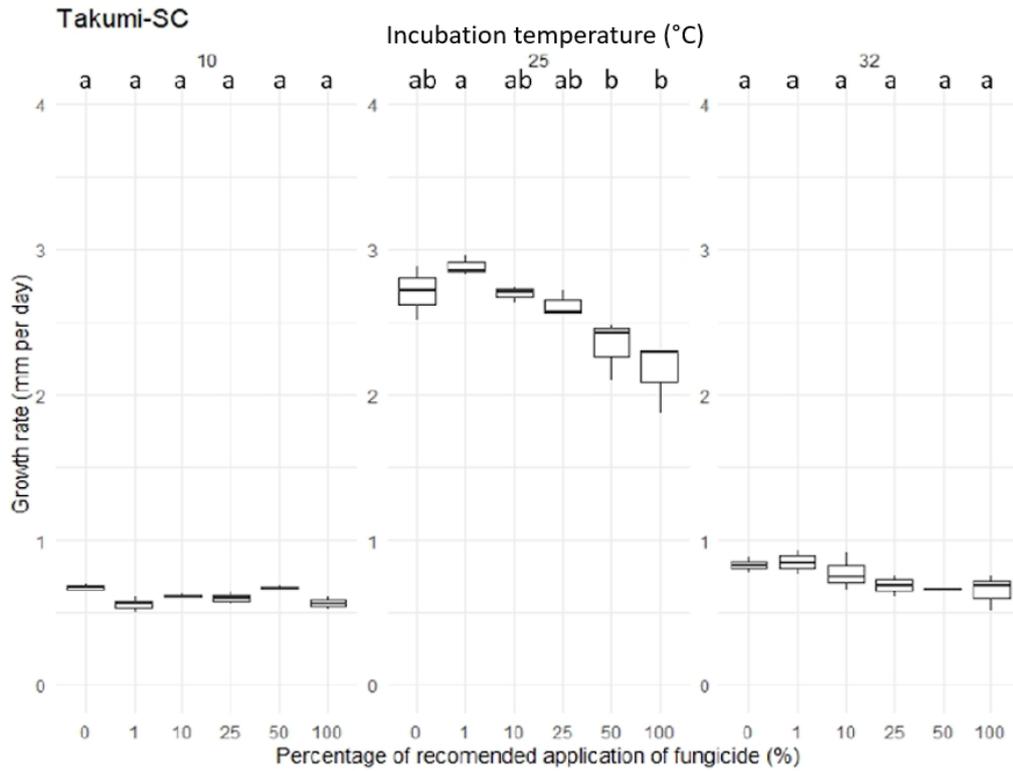


Figure 4.42 Growth rate ($\text{mm}^2 \text{day}^{-1}$) for *Beauveria bassiana* (PPRI 5339) on SDA spiked with a range of concentrations of Takumi SC (active ingredient: cyflufenamid). Significant differences between growth rates at different exposure concentrations for each incubation temperature are indicated by the presence of a different letter (a-b).

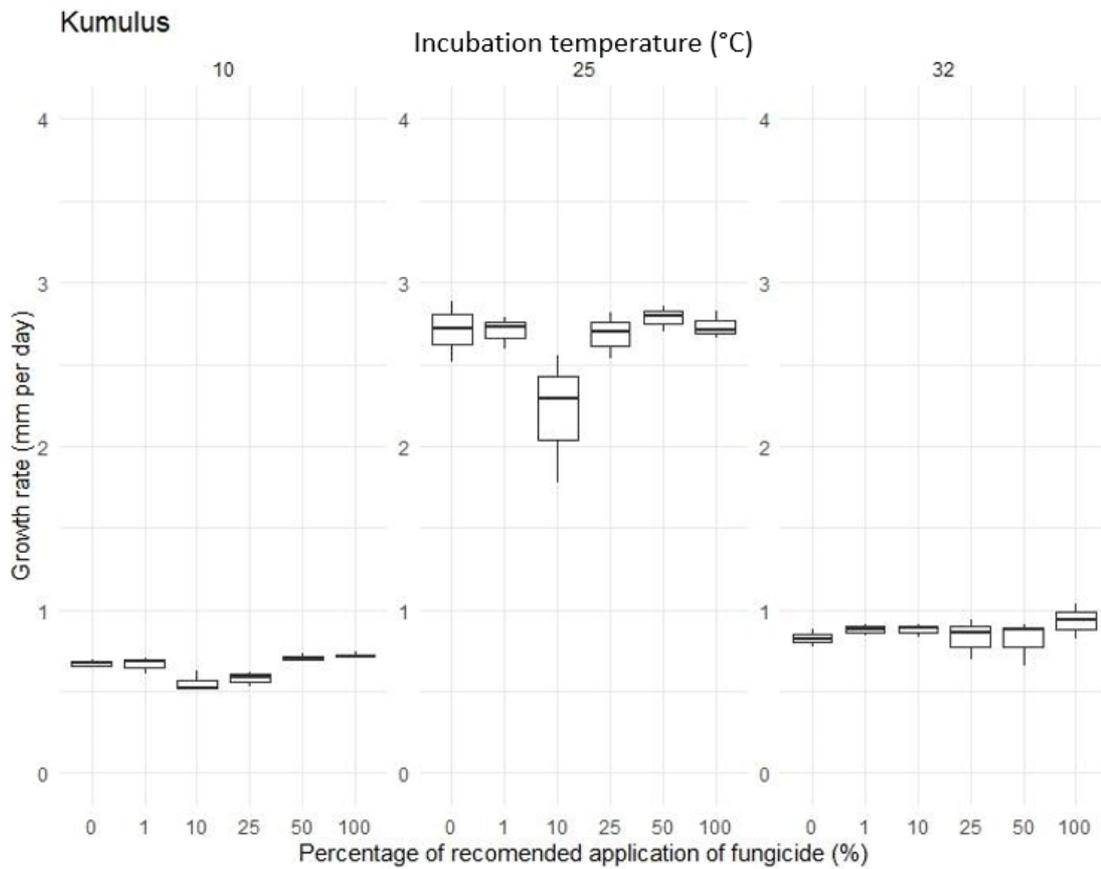


Figure 4.43 Growth rate ($\text{mm}^2 \text{day}^{-1}$) for *Beauveria bassiana* (PPRI 5339) on SDA spiked with a range of concentrations of Kumulus (active ingredient: sulphur). There was no significant effect of different exposure concentrations of Kumulus on the growth rate of *B. bassiana* compared to the control treatment at each incubation temperature.

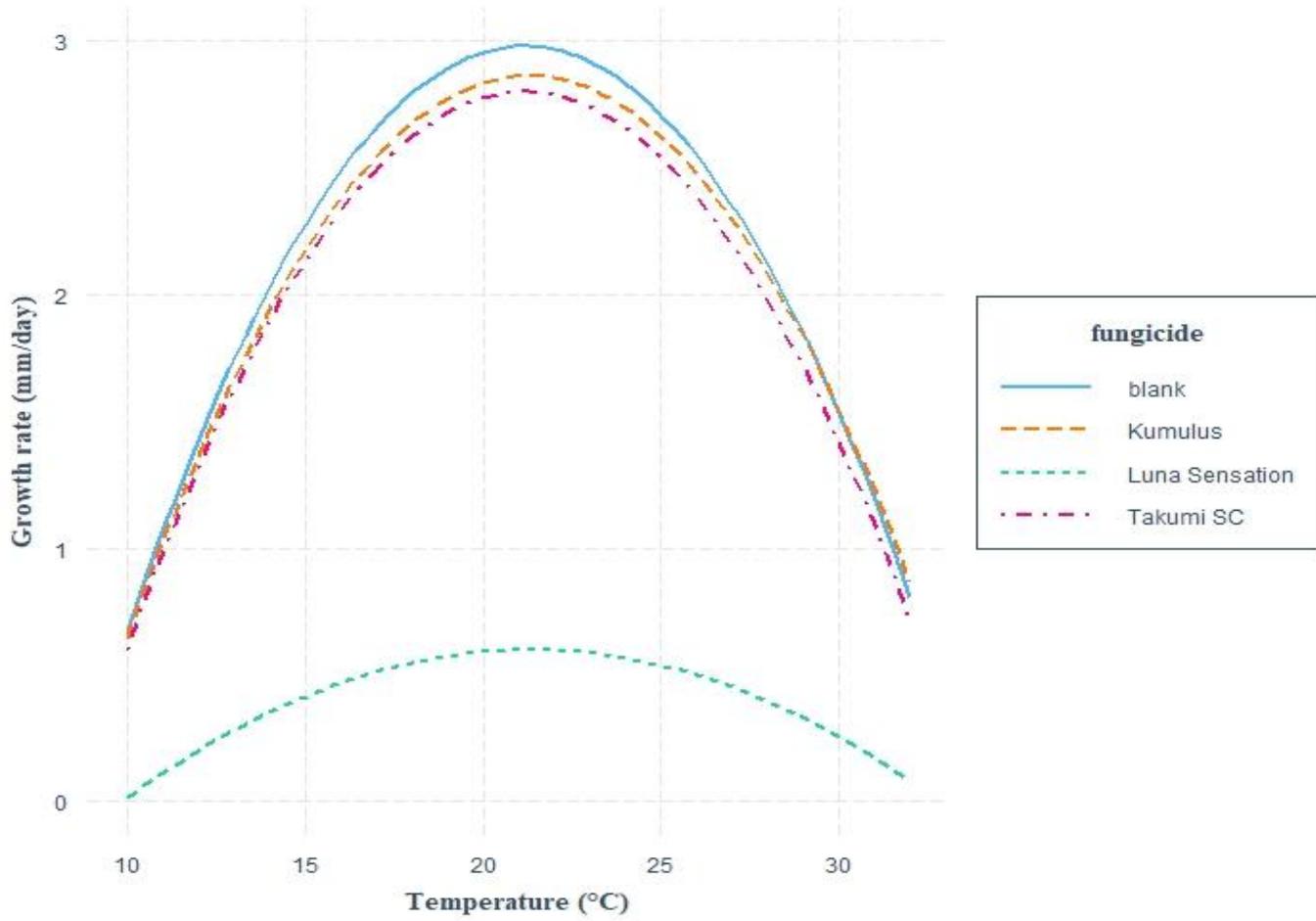


Figure 4.44 Interaction plot displaying the effect of continuous exposure of three fungicides on growth rate of *Beauveria bassiana* (PPRI 5339) when incubated at 10, 25 or 32°C.

4.5 Discussion

After application of EPF conidia onto a crop, the ability of conidia to infect their insect host is directly affected by environmental conditions. Sunlight, temperature, humidity and leaf surface chemistry affect the rate of penetration of the host cuticle, with temperature continuing to influence rate of infection once EPF have entered the host's body (Jaronski, 2009). Selection of EPF for control of greenhouse pests should be based on their suitability to the environmental conditions within the target crop as well as their ability to infect the target insect. Temperatures in a greenhouse can vary from below 10°C to over 30°C, so an isolate that is able to perform over a broad range of temperatures and an optimum close to the daily average temperature would be preferred. In this study, temperature profiles were determined for each isolate through a series of *in vitro* experiments.

There were differences in *in vitro* growth rate both between, and within species across the range of temperatures tested. For all EPF isolates, the response was bell shaped and skewed to the left, with the exception of isolate *A. lecanii* (Vertalec®) which was predicted to have a lower temperature optima than other isolates according to the best fit model, the Taylor model.

The growth response of isolates across all temperatures did not differ between the genera *Beauveria*, *Cordyceps* or *Akanthomyces*. However, *Metarhizium* isolates displayed growth rates that were significantly faster at the higher temperatures and were unable to grow at 10°C. It is characteristic for this genus to be able to tolerate higher temperatures than the other genera investigated in this study, for example, Teja and Rahman (2016), demonstrated that *Metarhizium* isolates were able to grow at 35°C, though at a slower rate than at their optima, but were unable to grow or sporulate at temperatures above 40°C. In comparison, differences between growth rate of *Cordyceps* and *Beauveria* isolates could not be generalised by genera. As these genera are cosmopolitan in their occurrence, temperature profiles differ between isolates rather than species. Comparatively, in this study *Akanthomyces* isolates displayed the fastest growth rates at the lowest temperatures and growth rate was significantly lower at 30 and 32°C compared to other isolates. However, other studies have shown that *Akanthomyces* isolates are capable of growing at 35°C, dependant on the isolate tested (Davidson *et al.*, 2003).

In the current study, there was no relationship between the origin of isolates and their response to temperature, though it should be noted that most isolates of the same genus were similarly grouped by the Koppen-Geiger climate classification, therefore the effect of origin on temperature profiles of EPF within the same genus were unlikely to be found. For

example, temperature optimums for growth of the seven *Beauveria* isolates were predicted to range from 24-27°C by the Briere-1 model. However, all *Beauveria* isolates originated from the same Koppen-Geiger climate (humid continental) so detection of the relationship between origin and temperature optima, if any, was not possible. In a study by Fargues *et al.* (1997), temperature optima were also found to range between 20 and 30°C for *B. bassiana*, but there was no relationship between isolate origin and response to temperature in *in vitro* experiments. Comparatively, in studies involving *Metarhizium* isolates originating from varied climates, it was shown that high temperature tolerance was related to distance from the equator, whereby equatorial EPF are better suited to high temperatures (Rangel *et al.*, 2005). Experiments involving EPF isolates from more varied climates than those used in the current study would be necessary for this type of analysis.

Seven fungal isolates were chosen for germination experiments. These were; *B. bassiana* (PPRI 5339, GHA, ATCC 5278), *C. farinosa* (ATCC 4412), *A. lecanii* (ATCC 6544), *M. anisopliae* (Bioblast) and *M. brunneum* (Met 52). The selection of these isolates was based on their temperature optima and limits, ensuring that there was a mix of isolates that could grow at high or low temperatures, or across a broad range of temperatures. The ability of the isolates to produce large quantities of conidia was also a selection factor. The selection of these isolates was necessary to reduce the size of the experiment, which involved counting 281,383 conidia across 1,200 Petri dishes. However, if more time was available, the study could have benefitted from investigating the germination of all 18 isolates at each incubation temperature in order to determine the relationship between growth and germination for the entire collection of isolates studied.

Germination optima of all isolates tested was higher than the growth optima, except for *C. farinosa* ATCC 4412 which was predicted to have a similar temperature optima for growth and germination based on the best fitting non-linear model. The highest temperature optima was predicted for *M. anisopliae* isolate (Bioblast) at 30.11 °C. Other studies have also noted a high temperature optima for germination of *M. anisopliae* conidia, with some isolates germinating at 35°C (Ouedraogo *et al.*, 1997). Whereas, the lowest predicted optima in this study was 26.61°C for *C. farinosa* ATCC 4412. Germination optima for each isolate could not be grouped by genera, though it is possible that broader patterns amongst isolates would have been identified if the same isolates used in the growth experiment were used in the germination experiment.

Other studies have found that there were no differences in the optimal temperatures for growth and germination for *Metarhizium* (Nussenbaum *et al.*, 2013; Teja and Rahman, 2016), *Beauveria* (Nussenbaum *et al.*, 2013; Ahmad *et al.*, 2016)(Ahmad *et al.*, 2016;

Nussenbaum et al., 2013), *Cordyceps* (previously *Paecilomyces*) (Kiewnick, 2006) and *Akanthomyces* isolates (previously *Lecanicillium*). However, optimum temperatures in these studies were determined by comparisons of bar charts showing rate of growth or germination at the temperatures tested (Ahmad et al., 2016; Teja & Rahman, 2016; Nussenbaum et al., 2013). Another method to determine optimum temperature was to fit a linear regression to rate of growth or germination across the tested temperatures. However, linear regressions can only be fit for suboptimal temperatures increasing towards the optima or temperatures above the optima and again, this method predicts the optimum temperature to be that which resulted in the highest rate of growth or germination limited to the range of temperatures investigated in the experiment (Yeo et al., 2003; Cabanillas and Jones, 2009). The non-linear, asymmetrical, bell shaped curve produced by EPF in response to temperature is similar to those described for insects, bacteria and mites (Logan et al., 1976; Ratkowsky et al., 1982; Wagner et al., 1984). Numerous models have been proposed to describe the shape of these curves, four of which were tested in this study. Application of these types of models provide the most accurate estimation of optimal temperature. For example, the use of a non-linear model by Thomas and Jenkins (1997) predicted that the growth optimum for two *Metarhizium flavoviride* isolates was 27°C and 25.5°C whilst germination optimum was higher, at 30°C, demonstrating a difference between growth and germination optima similar to those observed in the current study. Non-linear models allow the prediction of optimum temperatures which may be between temperatures that were tested in experiments, and often also provide predictions of cardinal temperatures (Davidson et al., 2003; Smits et al., 2003).

Different non-linear models provided the best fit for germination and growth data of the same isolates used in both experiments. The optima for germination was predicted to be ~2°C higher than the growth optima for all isolates except *C. farinosa* ATCC 4412. But this does not explain the discrepancy between best fit models for germination and growth as *C. farinosa* ATCC 4412 was predicted to have a similar growth and germination optima but the shape of the response curves for growth and germination were different and fitted better by different models.

Despite the R^2 and AIC values indicating that the nonlinear models described the germination data well, the bell shaped curves for *M. brunneum* (Met 52), *M. anisopliae* (Bioblast) and *C. farinosa* ATCC 4412 were flattened at all temperatures above 25°C and the models failed to replicate the shape of the curve. Therefore, each fitted model predicted different T_0 and T_{opt} for each isolate, so the type of model used could have a profound influence on EPF isolate selection.

It is likely that the non-linear models are unable to fit to the germination data due to the distribution of the data. The non-linear models best describe bell – shaped data, however, at any given time point a large proportion of the germination data is either at 0 or 100%. The 16H time point was chosen to be modelled as differences between the germination of isolates could be observed across all temperatures tested. At time points later than 16H, 100% germination was observed for most isolates at most temperatures. Whereas, earlier time points displayed too few counts of germinated conidia. Still, at 16H, isolates *B. bassiana* PPRI 5339 and ATCC 5278 had 0% germination across the lowest temperatures tested. Additionally, germination for *M. brunneum* (Met 52), *M. anisopliae* (Bioblast) and *C. farinosa* ATCC 4412 was 100% for most of the higher temperatures tested. For these isolates, the thermal maxima was not reached in the experimental design, so a significant reduction in germination rate at temperatures above the optima was not observed. A potential solution to this issue would be to collect GT_{50} data for each isolate at each temperature. This data distribution would be better suited for the five non-linear models used in this study. However, collection of GT_{50} data requires an experiment with multiple time points for each isolate at each temperature which would not be practical to complete for several isolates.

Another problem encountered was that not all of the non-linear models produced estimates of cardinal temperatures i.e. T_0 , T_{opt} and T_{max} . All models except the polynomial model provided an estimate of T_{opt} , however, T_{max} was only predicted by the Briere-1, Lactin-1 and Logan-6 model. The Briere-1 and Taylor model were the only models capable of predicting T_0 . Thermal range is equally as important as optima when selecting EPF for biological control. Model outputs of T_0 and T_{max} are more difficult to evaluate from raw data than the T_{opt} , making a calculated value useful in isolate selection (Smits *et al.*, 2003). However, the calculation of a T_0 value is not necessary for the selection of an EPF isolate to be used under greenhouse conditions as temperature does not fall to values near the thermal minima of EPF. Though temperatures fall below optimal conditions during the night, these temperatures are unlikely to be an inhibitor for EPF growth or germination as they reach a minimum of approximately 16°C in UK and Spanish greenhouses (Shamshiri *et al.*, 2018). Comparatively, the T_{max} is a valuable model output as internal greenhouse temperatures regularly exceed predicted EPF optima, exceeding 30°C during growing season in the UK and in Europe. Average hourly temperatures recorded in a greenhouse at the BASF crop trial testing facility in Utrera, Spain are shown in Appendix V and highlight the daily oscillations that crops, crop pests and their control agents are exposed to. The difference between the T_{opt} and T_{max} predicted for an isolate can also indicate sensitivity to thermal damage,

whereby EPF isolates with a small difference between T_{opt} and T_{max} are more likely to be negatively impacted by exposure to high temperatures.

Across all isolates, there was not one model that could describe all the data for growth or germination. Therefore, comparisons of thermal optima were made by extracting parameters from the best fit models. In other studies, the Briere-1 model has been capable of fitting thermal response data of different fungal isolates despite different distributions of data (Smits *et al.*, 2003). However, the Briere-1 model did not describe data well for isolates that demonstrated a fast response to increasing temperature towards the optimum and a slow decrease when increasing temperature beyond the optima, such as the growth response of *B. bassiana* PPRI 5339 or the germination of *A. lecanii* ATCC 6544.

It was determined that the linear relationship between temperature optima for growth and germination predicted by the best fitting non-linear models was not significant. However, the statistical test associated with the linear model lacked power due to the lack of data points and upon removal of the isolate *C. farinosa* ATCC 4412, there was a strong linear relationship between the germination optima and growth optima of the remaining six isolates. Further experimentation would be necessary to determine the relationship reliably, however, it is recommended that the growth data, which was modelled significantly better by all non-linear models, be used to estimate cardinal temperature values.

Taking all of these attributes and limitations into consideration, the Lactin-1 model was assessed to be the best of the five non-linear models tested to fit the growth data. The parameters of the Lactin-1 model were found to accurately summarise the growth of each isolate across the temperatures tested and provided accurate T_{opt} and T_{max} values. There were little to no difference between the predicted T_{opt} and T_{max} values produced by the Lactin-1 and Briere-1 model for each isolate. However, the Briere-1 model provided misleading T_0 values as an artefact of the equations describing development rate against temperature. Some output parameters were predicted as zero or less than zero. The Briere-1 model is based on the equation of an S-shaped curve resulting in one half of the model predicting the bell shape of the temperature growth response and the other half crossing the x-axis at a negative. Based on this model, optimums and maximums can be calculated based on the data, however it is a limitation of the model that it does not have any biological relevant meaning and therefore does not limit T_0 to zero or above (Briere *et al.*, 1999). The Lactin-1 model does not cross the x-axis, and therefore cannot provide an estimate of T_0 , though this parameter was not necessary in the current study as previously discussed.

Comparisons of EPF thermal profiles to thermal development rates of *T. vaporariorum* can indicate potential successful EPF isolates for use under greenhouse conditions. Isolates with similar temperature response curves to insect development rate may control the pest more successfully than an EPF isolate which responds differently to temperature than the insect. If the response curves of the EPF and insect differ, there will be temperatures at which the insect is able to develop whilst the EPF is unable to infect the pest, leading to reduced control efficacy (Kryukov *et al.*, 2018). In a study by Gamarra *et al.* (2020), development rates of *T. vaporariorum* nymphs across a range of temperatures were determined and modelled using the Janisch (1932) non-linear model (figure 4.45). *Trialeurodes vaporariorum* nymphs were unable to develop at 10 or 32°C and were predicted to have maximum growth rates at 25°C. According to the Lactin-1 model, EPF isolates tested in this study have optimal temperatures for growth ranging 23.85 - 27.35°C and thermal maximums similar to *T. vaporariorum* (31.65 -33.29°C). This suggests that isolates such as *B. bassiana* ATCC 6921, ATCC 9451, PPRI5339 and *A. lecanii* ATCC 4060 may control *T. vaporariorum* more successfully than isolates predicted to have different temperature optimums, such as *A. lecanii* ATCC 6544 (optimum 23.85°C) and *M. anisopliae* Bioblast® (optimum 27.35°C).

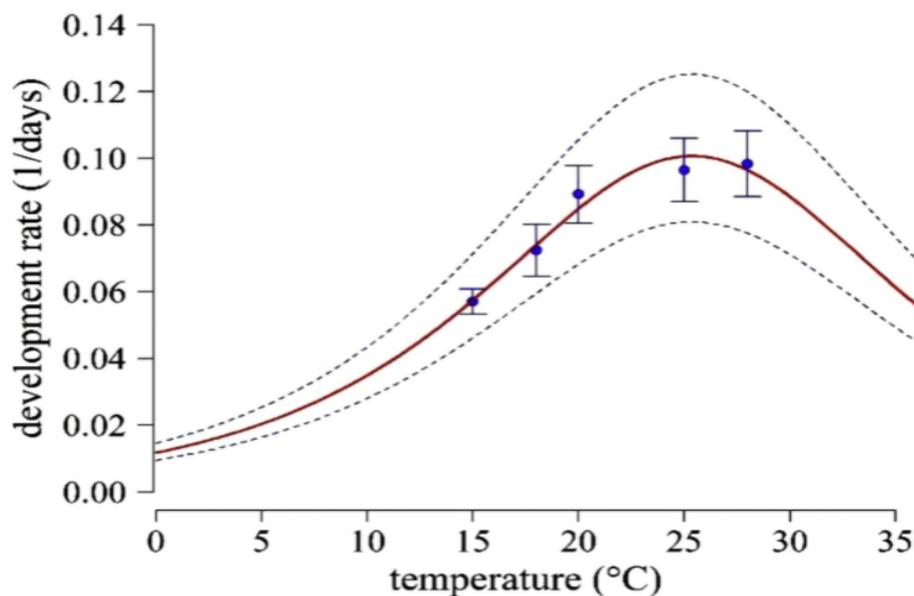


Figure 4.45. The relationship between temperature and median development rates for nymphal stages of *Trialeurodes vaporariorum*. Data on observed median development rates were fitted using the Janisch (1932) model. Broken lines represent 95 % confidence limits for the fitted model. Bars represent 95 % confidence limits of observed data points. This figure was adapted from (Gamarra *et al.*, 2020).

Another important criterion for EPF selection is the ability for large quantities of conidia to be produced on artificial media to allow for mass production. Conidia production varied greatly between isolates, with some producing significantly more conidia across all temperatures such as *M. brunneum* (Met 52) and *C. fumorosea* (PFR), whereas other isolates such as *B. bassiana* (6921) produced significantly less conidia at all temperatures. For mycelial growth, a utilisable carbohydrate and a nitrogen source is required by EPF, with conidial production being activated by depletion of resources (Shah *et al.*, 2005). *Beauveria* and *Metarhizium* isolates begin conidial production in the presence of a carbohydrate when nitrogen has been depleted (Kamp and Bidochka, 2002). However, Kamp and Bidochka (2002) found that starvation of *Akanthomyces* isolates was not required for conidial production. In the current study, significantly more conidia were produced by *Metarhizium* isolates. Similarly, maximal yields of conidia were produced by *Metarhizium* isolates grown on 35:1 CN media (similar to SDA) in a study by Shah *et al.* (2005). Whereas, *Cordyceps* isolates have been shown to produce more conidia on artificial media with a lower CN ratio than SDA on substrates such as rice (Mascarin *et al.*, 2010). *Cordyceps* isolates generally produced significantly less conidia across all temperatures tested, this may have been due to nutritional requirements not being provided by SDA. Nutritional requirements vary for different EPF isolates, with conidial production and quality often being greatest on the insect host (Shah *et al.*, 2005).

For several isolates, significant differences were found between conidia production at different temperatures. Unlike growth and germination data, this relationship was linear. Similarly, a study by Borisade and Magan (2014) found that *in vitro* conidial counts (cm^{-2}) for *Metarhizium*, *Cordyceps* and *Beauveria* EPF increased or decreased with increasing temperature from 25-37°C dependant on the isolate being tested. In their study, humidity had a greater influence on conidial production and EPF were able to sporulate at higher temperatures if water availability was also high. This effect of humidity on conidia production has also been shown in insect hosts. In a study by (Sharififard *et al.*, 2012), temperature variation from 20°C to 30°C had no effect on the sporulation of *B. bassiana* and *M. anisopliae* isolates after infection of *Musca domestica* (Diptera; Muscidae) but reducing relative humidity from 75% to 45% significantly reduced the sporulation of the *B. bassiana* and *M. anisopliae* isolates. The effect of humidity on conidial production was not investigated in this study, as oil based formulations are considered to effectively protect conidia from dry conditions (Jaronski, 2009). Whereas, the impact of sub-optimal temperatures is less easily rectified. Additionally, humidity within the crop is often maintained above 60% to support plant health and maximise production (Shamshiri *et al.*, 2018), whilst simultaneously providing sufficient water availability for EPF germination.

For some isolates, the number of conidia mm⁻² did not have a linear or quadratic relationship with temperature but varied drastically between incubation temperatures, such as *A. lecanii* ATCC 4060, *B. bassiana* GHA and *C. farinosa* ATCC 7477. Conidia production could not be predicted based on temperature for most isolates and there was also random fluctuation between conidia counted and temperature for some isolates. As a result, it is difficult to predict whether temperature optima or temperatures above this result in maximum conidia production. But this study highlighted those isolates which produced more conidia overall across all temperatures, such as *C. farinosa* ATCC 4412, *B. bassiana* PPRI 5339 and *M. brunneum* (Met 52).

The variation between responses to different temperatures for conidia production by the isolates studies could be due to the sampling technique. Plugs of mycelia and conidia were taken from the leading edge of each fungal plate sampled in order to determine conidia production. However, it is possible that some plugs were taken from an area producing more conidia than in another sample. At the temperature extremes, the plug was often the same size as the total area that the isolate had grown. But at 18-30°C, the plug taken was only a small portion of the total sporulating area. An improvement to the method to reduce sampling variation could be to flood the surface of the Petri dish with Tween 80, taking note of the total area of the fungus and determine conidia production per unit area by counting conidia in the Tween 80 sample.

Once EPF have been selected based on the criteria discussed above, it is also important to determine compatibility of the fungus with other commonly applied chemicals in the target crop system. Exposure to fertilisers, herbicides, fungicides and insecticides will occur at varied concentrations depending on whether pesticides are co-applied with the EPF or residues remain on the plant which the EPF is applied onto. It stands to reason that an EPF may be more susceptible to fungicide applications compared to other commonly used products. Pest resurgences are often observed in IPM programmes as a result of fungicide application disrupting EPF populations (Dutcher, 2007).

In vitro compatibility testing of EPF with insecticides can indicate whether co-application would be successful in an IPM strategy. Preliminary *in vitro* mixture experiments showed that germination rate was faster for three out of the four EPF isolates tested when mixed with several concentrations of spiromesifen before being applied to Petri dishes containing SDA. Additionally, there were large differences in hyphal growth after just 24 hours, as shown in the photographic evidence. Therefore, further experiments investigated the growth

and germination of EPF with spiromesifen spiked agar. However, possibly due to the change in method of exposure, when EPF were placed on spiromesifen spiked SDA, there was no significant difference in growth or germination.

A study by Pelizza *et al.* (2018) found that five EPF isolates were equally inhibited in germination experiments when mixtures of the EPF and insecticide were added to SDA and in growth experiments where the agar was spiked with the insecticides. Although this experiment involved the use of different insecticides than in this study, it should be noted that no difference in response by the fungus was observed depending on methods used. In the current study, it may be possible that the fungal isolates in the preliminary compatibility tests benefitted from the short exposure to the active ingredient or one of the components in the formulation of the pesticide. Upon placing the mixture of EPF and insecticide onto the agar for germination experiments, the mixture was absorbed into the agar, effectively reducing the exposure concentration. In the subsequent experiment, where exposure to the chemical was constant due to the change in methods, there was no increased rate of growth or germination.

In vitro compatibility tests between EPF and spiromesifen have shown contrasting results depending on the EPF and the concentration of spiromesifen used. In a study by Sain *et al.* (2019), one *Fusarium*, one *Metarhizium*, three *Cordyceps* (formerly *Isaria*) and five *Beauveria* isolates were exposed to the recommended application rates of one of twelve insecticides, or half the recommended rate. Using similar methods to those used in the current study, agar was spiked with one of the insecticides for *in vitro* growth experiments. Recommended application rates of spiromesifen were compatible with seven of the EPF isolates tested, with no effect on growth observed, similar to the results found in this study. However, Sain *et al.* (2019) also found that growth of one of the *C. javanica* isolates was enhanced and two *B. bassiana* isolates were inhibited. Half concentrations of spiromesifen had a greater effect on EPF tested, with growth of three of the ten EPF isolates being inhibited, unlike in the current study where no effect was observed for any exposure concentration. In another study, no effect was observed in *in vitro* combinations of *C. javanica* (formerly *Isaria javanica*) and exposure concentrations at recommended application rate and three serial dilutions of spiromesifen (Santos *et al.*, 2018b). These results are comparable to those observed for the combination of spiromesifen and the four EPF isolates tested in the current study and indicate potential for improved *T. vaporariorum* control if co-applied in *in vivo* experiments, or onto greenhouse crops. The combined application of spiromesifen and *C. javanica* resulted in an additive or synergistic response in

the *B. tabaci* population, depending on the concentration of spiromesifen applied (Santos *et al.*, 2018b).

In the current study, *in vitro* growth of *B. bassiana* was not affected by any concentration of Kumulus. However, the presence of Takumi SC had an inhibitory effect on growth when the fungus was incubated under optimal conditions, though this negative interaction was not found when incubated at the temperature extremes of 10 and 32°C. Whereas, Luna sensation inhibited growth at all incubation temperatures and growth of *B. bassiana* was severely inhibited at temperature extremes. Although, some studies have shown that negative *in vitro* interactions are not reflected in more realistic experimental designs (Cuthbertson *et al.*, 2005; Roberti *et al.*, 2017). Therefore, further experiments are required to simulate the complexity of an IPM system, whereby EPF and fungicides are often spatially separated. Greenhouse experiments imitating the expected exposure of EPF to these fungicides need to be conducted to effectively determine their compatibility.

4.5.1 Summary

- iv) The relationship between temperature and growth, germination and conidia production of EPF isolates was determined.
 - a. Five non-linear models were used to predict thermal ranges and optima for all EPF isolates.
 - b. Temperature did not have a significant effect on the conidia production of isolates tested.
- v) Growth and germination was not significantly affected by the presence of the insecticide, spiromesifen. Though, different experimental designs in investigations resulted in different interactions between EPF isolates and spiromesifen.
- vi) Compatibility of an EPF isolate with three fungicides was determined. Growth of the EPF isolate *B. bassiana* PPRI 5339 was affected depending on the fungicide present and temperature exacerbated negative interactions between the fungus and the fungicide.

5 Determining the effect of a mixture of entomopathogenic fungi and spiromesifen on *Trialeurodes vaporariorum* under laboratory conditions

5.1 Introduction

The development of effective, safe and sustainable methods of pest management in greenhouse crops is inherently difficult and frequently presents new challenges. The controlled environments established within glasshouses, polytunnels and other protected structures which are designed to optimise crop growth, also provide ideal conditions for arthropod pests, enabling large populations to develop rapidly. In addition, greenhouse crops are vulnerable to a wide diversity of insect and mite pest species, meaning that growers must plan management strategies for a full spectrum of pest types (van Lenteren and Woets, 1988).

The complexity involved in control of greenhouse pests is increased because of pesticide resistance; heritable resistance to broad spectrum, synthetic chemical pesticides was first detected in whiteflies and spider mites in the 1970s and is now commonplace in many target pest species (Shipp *et al.*, 2007). Simply switching to new chemical pesticide modes of action is not sustainable, since many of the most serious pest species have a propensity for rapidly evolving resistance to new active ingredients, due to rapid life cycles and wide host range as a result of their innate capacity to detoxify many plant compounds which are also linked to detoxification mechanisms for pesticides (Pilkington *et al.*, 2010). In addition, with many active ingredients being withdrawn from the pesticide market, over use of active ingredients that remain on the market increases the likelihood of resistance development. Other pressures on pesticide use include: concerns about the safety of residues in fresh produce, long re-entry intervals that prevent staff from working in the nursery after spraying, and pre-harvest spray periods that restrict the time that pesticides remain effective during the final stages of crop growth. For these reasons, greenhouse growers are incentivised to avoid calendar sprays of synthetic chemical pesticides in favour of Integrated Pest Management (IPM), in which different control treatments are combined with decision support tools to maintain pests and diseases below the economic action threshold and minimise chemicals use. The most effective greenhouse insect pest IPM systems are based around preventative applications of arthropod predators and parasitoids (van Lenteren, 2000) done as part on an IPM 'pyramid' approach (Stenberg, 2017). Under this system, IPM compatible pesticides are still applied, but are used as supplementary treatments to biocontrol, acting as a second line of defence should pest numbers increase to levels where natural enemies are unable to control them (Jacobson *et al.*, 2001). The complexities of managing multiple pest species in this way means that increasing attention has to be paid to new methods that help with IPM

planning, organisation and knowledge sharing, as well as ways of helping growers to capitalize on the sustainability benefits of IPM for improved business profitability (Dara, 2019).

Increasingly, IPM practitioners in greenhouse crops are incorporating into their programmes microbial control agents based on entomopathogenic bacteria (predominantly *Bacillus thuringiensis*), viruses, fungi and nematodes (Wraight *et al.*, 2017). These agents can be applied using conventional spray equipment and benefit from the stable, controlled conditions within the greenhouse environment, including favourable temperatures, partial protection from damaging ultraviolet radiation, and protection from run-off caused by rainfall. Humidity is easily manipulated to benefit microbial control agents such as fungi using equipment such as overhead misters (Shipp *et al.*, 2003), though dense crops often provide favourable humidity conditions due to a lack of wind throughout the greenhouse. In addition to their ability to infect and kill target pests, microbial control agents have a number of other attractive features including lack of toxic residue, short pre-harvest and re-entry intervals, and the potential for a certain amount of self-sustaining secondary control through reproduction and spread within the host population (Lacey *et al.*, 2015). They can also work well as a second line of defence to arthropod natural enemies in glasshouse systems (Jacobson *et al.*, 2001). However, compared to fully effective synthetic chemical pesticides, microbial control agents can be slower acting, less efficacious, and more expensive to purchase (Glare *et al.*, 2012). A major impediment to reaching the greatest potential for microbial control agents is a lack of IPM specialists and extension services to educate growers on methods to achieve the most effective pest control (Dara, 2019).

Currently, it is not standard commercial practice to tank mix together a microbial control agent with a conventional chemical pesticide, or to mix different microbial agents together. However, in principle there are some good reasons why such mixtures might be an attractive option. Because conventional pesticides and microbials have different modes of action, there may be synergistic interactions between them that increases the overall level of pest control. A combination treatment may also allow the control of multiple pests or enable pest control over a broader range of environmental conditions (Wang *et al.*, 2002). Importantly, a mixture of a chemical pesticide and a microbial might enable the former to be used at a lower dose while still enabling good levels of control, thereby reducing human and environmental safety risks. However, mixtures of this type must reliably provide high levels of population control to ensure that application of low active ingredients does not result in increased insecticide resistance in the target pest. Some studies have shown that microbial biopesticides can reduce the chances of resistance developing to a chemical pesticide or as a

way of reducing the severity of resistance after it has evolved (Furlong and Groden, 2001; Farenhorst *et al.*, 2009; Delnat *et al.*, 2020)

In order to establish successful mixtures for pest control, interactions between components in the mixture must first be determined. Toxicologists and ecotoxicologists have been investigating the interactions of multiple chemicals in a range of organisms for nearly a century (C.I. BLISS, 1939). Researchers in these fields apply the same basic principles for determining mixture interactions and investigate effects in model organisms which allows for comparable results between studies. In these disciplines, mixture concentration-response analysis can be used to establish the joint effect of two or more chemicals at a range of concentrations (Gestel *et al.*, 2011). Interaction outcomes have clear definitions. Additivity in a mixture of two or more components occurs when the observed combined toxic effect is no different to the expected combined effect calculated from the single compound toxicities using reference models for non-interaction (Jonker *et al.*, 2005). Synergism or antagonism can only be identified by deviation from additivity. Antagonism occurs when there is an interaction between components in a mixture, leading the mixture to cause less mortality than expected (Schäfer and Piggott, 2018). Alternatively, synergism and potentiation results in higher mortality than expected for mixtures (Schäfer and Piggott, 2018). More recently, the study of interacting mixture components have been investigated by terrestrial ecologists, for example the impact on ecosystem services or the potential exploitation of synergistic interactions for pest control (Siviter *et al.*, 2021). However, the language used between and within these different disciplines is not consistent and methods of testing multiple stressors in terrestrial ecology are less refined than those used in ecotoxicological studies (Orr *et al.*, 2020).

The predicted effect of two or more pesticides can be calculated assuming Loewe additivity, if all components of the mixture have the same mode of action (Loewe and Muischnek, 1926). Alternatively, Bliss independence (Bliss, 1939) assumes that each control agent interacting in the mixture kills the target pest by a dissimilar mechanism. It is more reasonable to assume that microbials act entirely independently in a mixture than to assume Loewe additivity, therefore this approach is more commonly used in microbial mixture studies (Cedergreen *et al.*, 2008). This method uses the combination of unaffected fractions to calculate the expected outcome of a mixture.

$$Pm = (pA)(pB)$$

Equation 11

Where the probability of an organism surviving the combined treatment of agent A and agent B (Pm) would be the probability of an organism surviving agent A (pA) multiplied by

the probability of an organism surviving agent B (pB). Therefore, a mixture consisting of two agents which independently each cause 25% mortality when applied alone will result in 56% mortality as a mixture assuming no interaction between the agents, also known as additivity by the calculation of $(1-0.25) * (1-0.25) = 1 - \text{survivorship}$. If the observed mortality is greater than the expected mortality, a synergistic interaction has occurred. Alternatively, antagonism results in lower mortality than expected.

Jonker et al. (2005) developed an eco-toxicological MixTox model which calculates an independent action reference model to determine the expected additive response of a mixture across a range of concentrations and ratios of each mixture component. The MixTox model accounts for interactions in the mixture with added parameters for synergism or antagonism which cause deviation from the reference model. By collecting concentration response data for the individual control agents in a mixture when applied alone, as well as the mixture treatments, the model can calculate the contribution of each chemical in the mixture to the overall toxicity observed and estimate the type of interaction that has occurred. Although MixTox models are regularly used to model outcomes of chemical combinations, this method has never been applied to biological – synthetic combinations before.

Mixtures involving microbial pathogens for control of *T. vaporariorum* and other species of whitefly generally involve entomopathogenic fungi which infect their host via direct penetration of the cuticle. Few studies have investigated the combination of entomopathogenic fungi and chemical pesticides against *T. vaporariorum*. One example is Feng et al., (2004) where three concentrations of imidacloprid were simultaneously applied with *Beauveria bassiana* or *Paecilomyces fumosoroseus* on lettuce in greenhouse experiments. The authors reported that low rate applications of imidacloprid combined with both EPF resulted in increased infection rate and mortality of *T. vaporariorum*. However, the expected mortality in the combined treatments was <90% which does not allow significant increases in mortality caused by synergistic interactions to be identified.

Control of *B. tabaci* by *Akanthomyces muscarius* (previously *Lecanicillium*) was improved by application of the botanical insecticide active ingredient matrine on cotton plants (Ali et al., 2017) as determined by co-toxicity coefficients which identified a synergistic effect following the combination of a range of concentrations of each pest control agent. In another study, *B. tabaci* infested leaves of Chinese hibiscus (*Hibiscus sinensis*) treated with four different concentrations of spirotetramat, acetamiprid, imidacloprid or thiamexotham with conidial suspensions of *Cordyceps* (previously *Isaria*) *fumosorosea* at a ratio of 1:1 resulted in increased *B. tabaci* mortality compared to application of pesticides alone (Zou et al.,

2014). In this study, synergistic effects were identified 2-3 days after simultaneous application of mixture treatments using Mansour's cooperative virulence index (Mansour *et al.*, 1966), though antagonism was observed for all concentrations of every chemical and *C. fumosorosea* mixture by day 7.

Entomopathogens and chemical agents can be applied simultaneously or sequentially throughout the cropping season and either approach may alter the interaction of agents and pest control efficacy. Cuthbertson *et al.* (2005; 2008) investigated the effect of applying recommended application rates of insecticides 24 hours before application of *Akanthomyces* (previously *Lecanicillium*) *muscarium* onto tomato and verbena plants infested with *B. tabaci*. In the first study, buprofezin, nicotine, imidacloprid or teflubenzuron were applied, followed by water or the EPF. Every insecticide caused high pest mortality which was significantly affected by additional application of *A. muscarium*. In the second study, applications of Oberon (Spiromesifen), Majestik (natural plant extract, Certis UK), Agri-50E (alginate/polysaccharide, Fargo Ltd, UK), Spray Oil (petroleum oil, Hortichem Ltd, UK) or Savona (fatty acids, Koppert Biological Systems Ltd, UK) resulted in lower *B. tabaci* mortality compared to chemicals used in the previous study. Application of the EPF 24 hours after each insecticide increased mortality compared to treatments of the insecticides alone. However, the mixture treatments were not significantly more effective than the fungus alone. In both studies, synergistic or antagonistic interactions were not determined.

Such studies indicate a significant potential for combinations of microbials and chemicals to be used in IPM but insight into their interactions is limited by experimental designs which mainly consists of binary application of pesticides at 1 or 2 different dose levels and targeted at one pest. The literature search and analysis of mixture studies described in Appendix VI found that most microbial studies only investigated the effect of two control agents, mixed at one concentration (see figure 5.1). Also, a variety of methods of analysis are used and are not always applied correctly, making comparisons between studies difficult as determination of synergistic or antagonistic effects are varied.

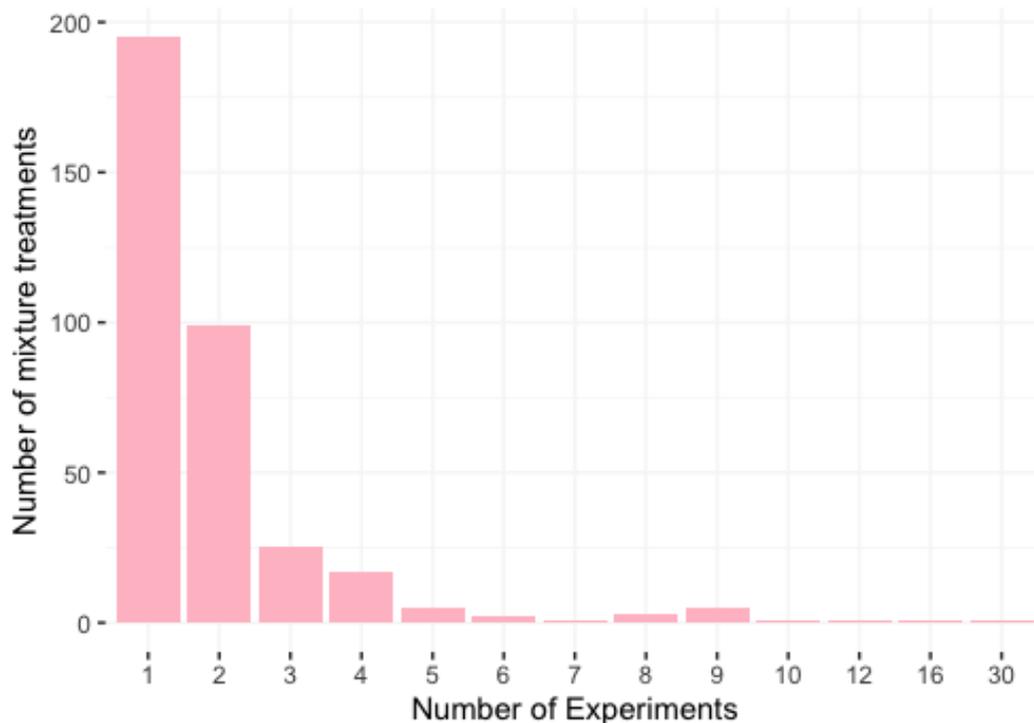


Figure 5.1 Total number of mixture treatments across 351 experiments in 72 papers published between 2000-2020 investigating the combined effect of a microbial pathogen and at least one other control agent. A mixture treatment was defined as the combination of at least two control agents either simultaneously or sequentially applied to the target organism.

Environmental conditions can influence the efficacy of both synthetic and biological pest control agents. Therefore, environmental factors may have an important role in determining the outcome of interactions when applying mixtures in pest control. In a study by Guo *et al.* (2018), exposure of *B. tabaci* to high temperatures (31°C) increased activity of cytochrome P450, responsible for resistance to neonicotinoid pesticides and as a result, increased tolerance of the pest to thiamethoxam. Similarly, Oliver and Brooke (2017) found that short exposure to high temperatures (37°C and 39°C) resulted in increased tolerance of *Anopheles arabiensis* to pyrethroids, including individuals that had no resistance previously.

Temperature oscillation can negatively affect the ability of entomopathogenic fungi to germinate and grow. For example, Ghazanfar *et al.* (2020) found that 100% of *Heliothis virescens* larvae treated with *B. bassiana* succumbed to infection before pupation when maintained at 25°C. In comparison, none of the treated larvae died as a result of *B. bassiana* infection if the moths were exposed to two oscillating temperature regimes ranging 5°C and 10°C away from 25°C. The interactions observed between co-applied control agents may change when conducting experiments in the greenhouse or field compared to the laboratory due to an increase in influential biotic and abiotic factors.

The overall aim of this study was to determine the effect of combined applications of the chemical insecticide spiromesifen and the EPF *Cordyceps farinosa* and *Beauveria bassiana*. Several methods were tested to establish a reliable mixture bioassay. Following this, dose-mortality data were used to determine whether there were interactions occurring between mixtures of *Cordyceps farinosa* and spiromesifen or *Beauveria bassiana* and spiromesifen. Data were analysed using an ecotoxicological approach which had previously only been used to determine interactions in chemical mixtures. Once combinations of specific concentrations of mixture components with potential for improved *T. vaporariorum* control had been identified, the effect of temperature stress on the mixture interactions was investigated.

The component objectives were as follows:

- i) A mixture bioassay method was developed to provide concentration response data following application of individual components of the mixture and simultaneously applied mixture treatments.
- ii) The mixture bioassay was used to quantify the effect of co-application of an insecticide and an EPF against *T. vaporariorum* nymphs.
- iii) Types of interactions, if any, were determined between components of the mixture across a range of concentration combinations using the MixTox analysis.
- iv) Further experiments with previously successful mixture combinations were conducted under temperature oscillation to mimic temperature variation under greenhouse conditions to determine the effect of temperature on the mixture interactions.

5.2 Materials and Methods

5.2.1 Preliminary experimental design for a mixture bioassay against *Trialeurodes vaporariorum*

A preliminary experiment was set up to test the experimental design for a chemical-microbial mixture bioassay against *T. vaporariorum*. Seven-week-old aubergine plants were infested with *T. vaporariorum* nymphs following the methods described in 2.5.1, and left to develop to third instar nymphs. At this stage, plants were grouped into three replicates with at least 100 nymphs in total across the three leaves infested. Target leaves were sprayed with 1mL of *C. farinosa* (isolate ATCC 4412), spiromesifen (Oberon®) or a mixture of the microbial and chemical. Whitefly nymphs were exposed to concentrations of the biological or chemical control agents expected to cause 15, 30, 50 and 80% mortality or a combination of these. These lethal concentrations (LC values) were calculated based on dose response data using the method described in section 2.5.4. Lethal concentrations of *C. farinosa* and spiromesifen are given in Table 5.1. The experimental design is detailed in figure 5.2 which demonstrates the single and mixture treatments. Three treatments have not been included in this experimental design as once control mortality and variation between bioassays is taken into account, there would be no ability to identify synergism at these concentrations which would likely result in 100% mortality following additivity.

Stock solutions of *C. farinosa* and spiromesifen were prepared following the methods described in sections 2.3.3 and 2.4.1 respectively. Then, solutions of twice the target concentration (LC₁₅, LC₃₀, LC₅₀ or LC₈₀) were prepared in 50mL Falcon tubes. For example, if the desired concentration for a treatment of the LC₁₅ of *C. farinosa* was 1×10^4 conidia mL⁻¹, then a stock suspension of 2×10^4 conidia mL⁻¹ was prepared. By preparing double the required concentration, the mixture treatments were diluted to the correct concentration when combined with the second mixture component. Treatments were prepared in 1.5mL tubes (Starlab; Natural flat cap microcentrifuge tubes) by adding 500µL of the double concentrate LC stock of *C. farinosa* and 500µL of one of the stock solutions of spiromesifen. Non-mixture treatments were prepared using the same method, except that *C. farinosa* treatments were combined with 500µL of Tween 80 (0.03%) and spiromesifen was combined with sterile deionised water.

Treatments were applied using the spray tower at 138kPa and dose received per unit area for treatments containing *C. farinosa* were calibrated using methods described in Spence *et al.* (2020). Once solutions applied to the leaf surface had visibly dried (1-2 hours), plants were incubated in plastic containers described in section 2.5.1 and maintained at 24°C under a

16:8h light: dark photoperiod. Instar, emergence and mortality was observed every 48 hours for 2 weeks following spray applications and the entire bioassay was replicated twice in time.

Table 5.1 Lethal concentrations calculated by probit analysis from concentration response bioassays of spiromesifen or *Cordyceps farinosa* against third instar *Trialeurodes vaporariorum*.

Lethal Concentration (%)	Spiromesifen (mg mL ⁻¹)	<i>C. farinosa</i> (conidia mL ⁻¹)
15	0.04	9.5 x 10 ³
30	0.22	6.3 x 10 ⁴
50	1.17	7.9 x 10 ⁵
80	17.73	1.09 x 10 ⁸

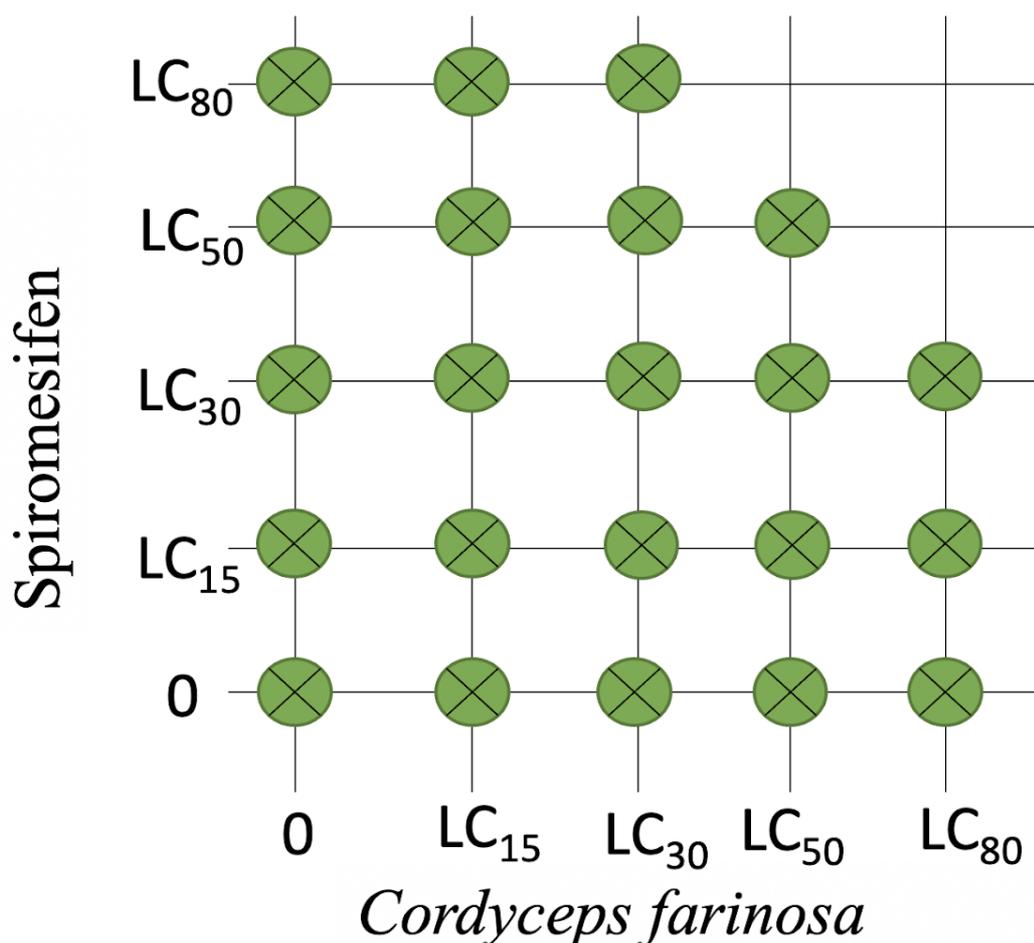


Figure 5.2 Experimental design of the first method used for mixture bioassays involving lethal concentrations (LC) of spiromesifen and *Cordyceps farinosa*. Treatments involved applications of spiromesifen and *C. farinosa* alone and in combination. Lethal concentrations ranged from LC₁₅ - LC₈₀ for each component in the mixture, except for LC₅₀+LC₈₀ and LC₈₀+LC₈₀ mixtures which were not included.

These mixture bioassays produced unexpected results and so further experiments to improve the reliability of the mixture bioassay were undertaken. To determine whether there had been changes in the susceptibility of the *T. vaporariorum* culture to *C. farinosa*, another dose response bioassay was conducted following the same methods described in section 2.5.4. This was done to ensure the validity of the LC values used in the calculation of concentrations used in the mixture bioassays, based on dose response bioassays that had been conducted a year prior. Another dose response bioassay was also conducted for *B. bassiana* PPRI 5339 due to variability in LC values produced in repeats of the dose response experiment. Data from the two previous dose response bioassays and the third additional bioassay were analysed together.

5.2.2 Comparing experimental designs for mixture bioassays against *Trialeurodes vaporariorum*

After determining updated LC values for *T. vaporariorum* and the two EPF isolates, changes were made to the experimental design of the mixture bioassay to improve the reliability of the experiment. Firstly, treatments involving the LC₃₀ were removed due to overlapping conidial deposition and mortality to treatments comprising the LC₁₅ or LC₅₀ of *C. farinosa*.

In addition, two methods of mixing EPF and spiromesifen were tested prior to spraying *T. vaporariorum* on aubergine leaves. For the first method, stock suspensions of *C. farinosa* and solutions of spiromesifen were prepared following the methods described in section 2.3.3 and 2.4.1 respectively. Then, solutions of twice the target concentration of the chemical and microbial (LC₁₅, LC₅₀ or LC₈₀) were prepared in 50mL Falcon tubes. Treatments were prepared in a different 50mL tube by adding 10mL of the double concentrate LC stock of *C. farinosa* and 10mL of one of the stock solutions of spiromesifen. Non-mixture treatments were prepared using the same method, except that *C. farinosa* treatments were combined with Tween 80 (0.03%) and spiromesifen was combined with sterile deionised water. Replicates of each treatment were taken from the same falcon tube to reduce variation in conidia between replicates, but this meant that replicates were pseudo replicates.

The second method involved the same approach in preparing stock solutions of EPF and spiromesifen. However, mixtures were prepared by adding 500µL of the double concentrate LC stock of *C. farinosa* and 500µL of one of the stock solutions of spiromesifen into LoBind® non-stick vials (Eppendorf LoBind® tubes). This was done to reduce conidia sticking to the surface of the tube. Non-mixture treatments were prepared using the same method, except that *C. farinosa* treatments were combined with 500µL of Tween 80 (0.03%) and spiromesifen was combined with sterile deionised water. Each replicate treatment was mixed in a separate LoBind® non-stick vial.

5.2.3 Combined application of *Cordyceps farinosa* and spiromesifen against *Trialeurodes vaporariorum*

Seven-week-old aubergine plants were infested with whitefly nymphs following the methods described in section 2.5.1, and left to develop to third instar nymphs. At this stage, plants were grouped into three replicates with at least 70 nymphs in total across the three leaves infested. Target leaves were then sprayed with 1mL of *C. farinosa* (isolate ATCC 4412),

spiromesifen (Oberon®) or a mixture of these. Whitefly nymphs were exposed to concentrations of the biological or chemical control agents expected to cause 15, 50 and 80% mortality or a mixture treatment involving the combination of *C. farinosa* and spiromesifen. These lethal concentrations (LC values) were calculated based on dose response data using the method described in section 2.5.4. The experimental design is presented in figure 5.3 and the figure demonstrates the single and mixture treatments. Mixtures involving the LC₁₅ for *C. farinosa* or spiromesifen were combined with the LC₁₅, 50 and 80 of the second mixture component. However, mixtures of LC₈₀ with LC₅₀ or LC₈₀ of each mixture component were not conducted.

Stock solutions of *C. farinosa* and spiromesifen were prepared following the methods described in section 2.4.1. Then, solutions of double the target concentration (LC₁₅, LC₅₀ or LC₈₀) were prepared in 50mL Falcon tubes. For example, if the desired concentration for a treatment of the LC₁₅ of *C. farinosa* was 1×10^4 conidia mL⁻¹, then a stock suspension of 2×10^4 conidia mL⁻¹ was prepared. Lethal concentrations of *C. farinosa* and spiromesifen are detailed in Table 5.2. By preparing double the required concentration, the mixture treatments were not diluted beyond the desired LC when combined with the second mixture component. Treatments were prepared in 1mL lobind tubes (Eppendorf LoBind® tubes) by adding 500µL of the double concentrate LC stock of *C. farinosa* and 500µL of one of the stock solutions of spiromesifen. Single component treatments were prepared using the same method, except that *C. farinosa* treatments were combined with 500µL of Tween 80 (0.03%) and spiromesifen was combined with sterile deionised water.

Treatments were applied using the spray tower at 138kPa and dose received per unit area for treatments containing *C. farinosa* were calibrated using methods described in Spence *et al.* (2020). Once solutions applied to the leaf surface had visibly dried (1-2 hours), plants were placed in plastic containers described in section 2.5.1 and incubated at 24°C with a 16:8h light: dark photoperiod. Instar, emergence time and mortality was observed every 48 hours for 2 weeks following spray applications, as described in section 2.5.2. The bioassay was replicated three times.

Table 5.2 Lethal concentrations calculated by probit analysis and used in mixture bioassays for combinations of spiromesifen and *Cordyceps farinosa*.

Lethal Concentration (%)	Spiromesifen (mg mL ⁻¹)	<i>C. farinosa</i> (conidia mL ⁻¹)
15	0.04	3.9 x 10 ³
50	1.17	2.3 x 10 ⁶
80	17.73	3.5 x 10 ⁸

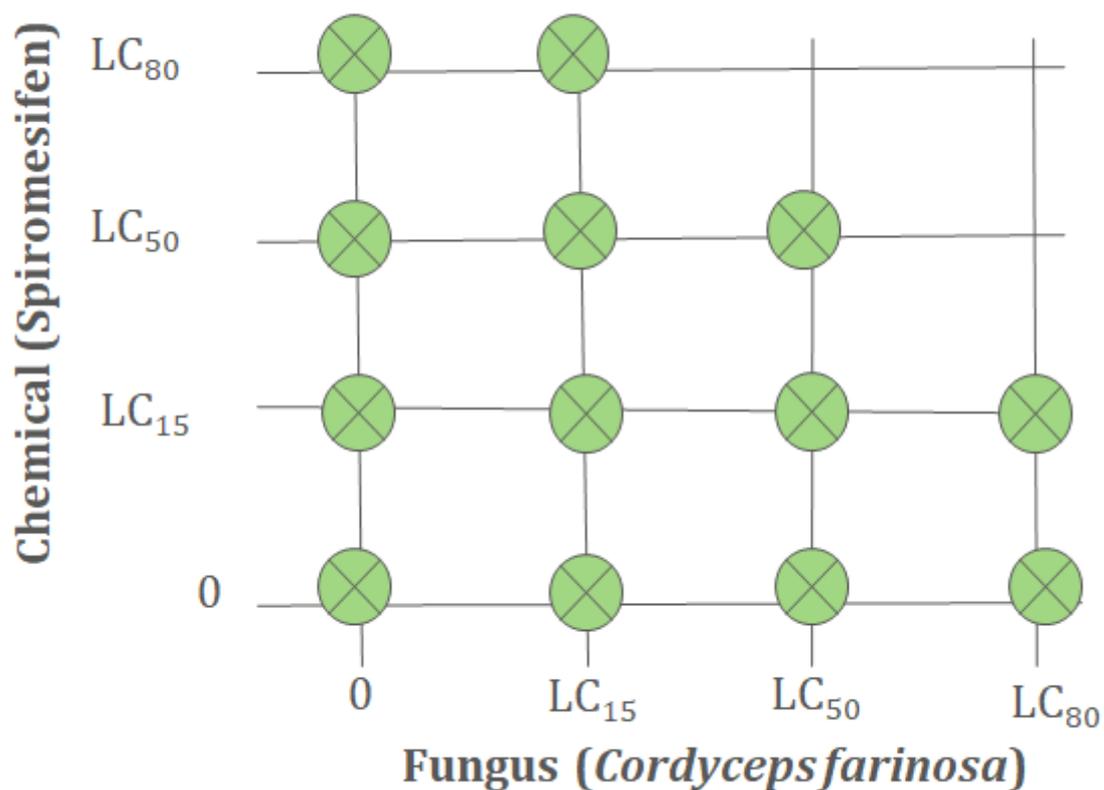


Figure 5.3 Experimental design used for mixture bioassays involving lethal concentrations (LC) of spiromesifen and *Cordyceps farinosa*. Treatments involved applications of spiromesifen and *C. farinosa* alone and in combination. Lethal concentrations ranged from LC₁₅ - LC₈₀.

5.2.4 Combined application of *Beauveria bassiana* and spiromesifen against *Trialeurodes vaporariorum*.

Similarly, a mixture experiment was conducted for the combined effect of spiromesifen and *Beauveria bassiana* PPRI 5339. This experiment followed the same experimental design, except that LC values were adjusted to follow the dose response of *B. bassiana* PPRI 5339 (Table 5.3). This bioassay was also repeated three times but ran for 12 days, rather than the 14 days for other experiments.

Table 5.3 Lethal concentrations calculated by probit analysis and used in mixture bioassays for combinations of spiromesifen and *Beauveria bassiana*.

Lethal Concentration (%)	Spiromesifen (mg mL ⁻¹)	<i>B. bassiana</i> (conidia mL ⁻¹)
15	0.04	1.6 x 10 ²
50	1.17	7.9 x 10 ⁵
80	17.73	7.9 x 10 ⁸

5.2.5 Determining the effect of temperature on interaction outcomes

In order to determine the effect of appropriate temperatures on the interaction between *C. farinosa* and spiromesifen, temperature recordings were taken in an industrial greenhouse using in-house equipment at BASF agricultural experiment station, Utrera Spain. Average hourly temperature was taken during the growing season from the first week of March-May and Sep-Nov 2016. Across each 24 hour period there was a minimum temperature of 17°C and maximum of 30°C resulting in an average daily temperature of 23.5°C.

Plants and whitefly were prepared as described in section 5.2.4.1. Mixtures of Oberon and *C. farinosa* were prepared as previously described in section 5.2.4 and applied following the same procedure. In this experiment, only mixtures of high concentrations of *C. farinosa* and low concentrations of Oberon were applied. Combinations of the LC₅₀ and LC₈₀ of *C. farinosa* with the LC₁₅ of Oberon were tested due to increased proportion mortality of *T. vaporariorum* being observed for these mixtures in previous bioassays.

Once treatments had been applied, plants were placed on top of trays holding capillary matting soaked in water and stored in two incubators for the duration of the experiment. The two incubators were prepared with one incubator with temperature oscillating over a 24 hour period and the second incubator maintained at the average temperature of the oscillations; the average temperature taken across the oscillations over 24 hours was determined to be

23.5°C and this was used as the set temperature for the second incubator. Each incubator was set to a 16:8h light: dark photoperiod. The oscillating temperature was influenced by the average hourly temperature recordings for the first week of every month during the growing seasons at BASF agricultural experiment station, Utrera Spain in 2016 (March - May & September - November). The hourly changes in temperature are shown in figure 5.4. The whole bioassay was replicated twice.

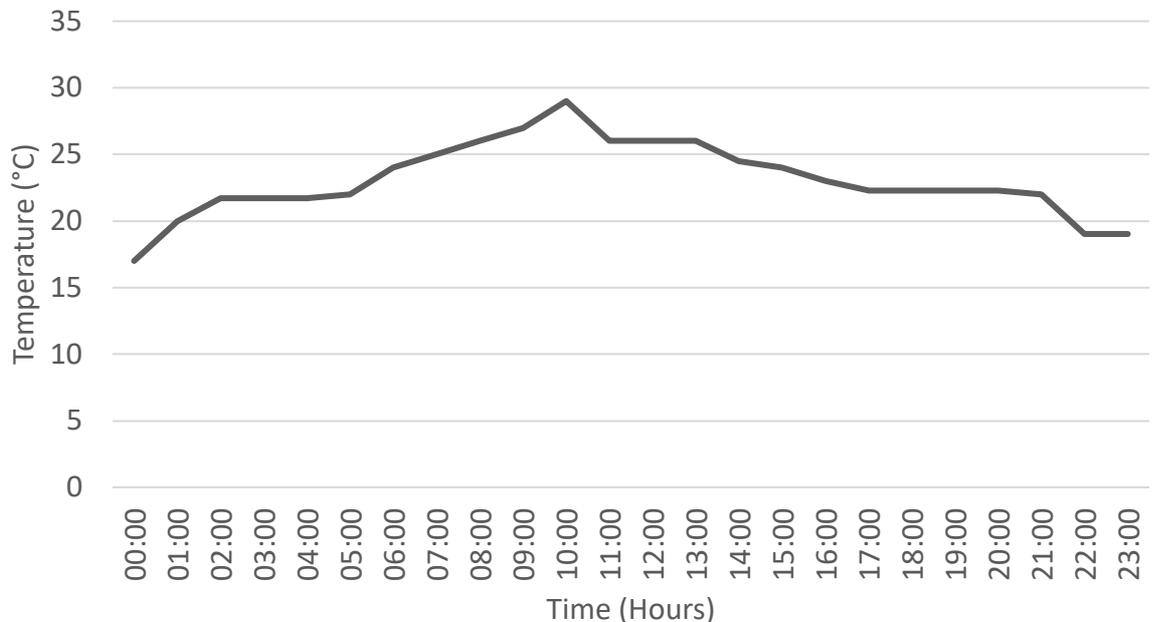


Figure 5.4. Hourly temperature changes of the incubator during oscillating temperature experiments. The oscillating temperature was influenced by the average hourly temperature recordings for the first week of every month during the growing seasons at BASF agricultural experiment station, Utrera Spain in 2016 (March - May & September - November). The average temperature across the 24 hour period was 23.5°C.

5.2.6 Statistical Analysis

Mortality at the end of each bioassay was corrected for control mortality using the Schneider-Orelli approach (detailed in section 2.5.3). Differences in total mortality at the end of the bioassay between treatments and between bioassays were determined by ANOVA, except for the repeat of the concentration response analysis for *C. farinosa* which was analysed by fitting several concentration response curves in the DRC package in 'R' (detailed in section 2.5.4).

The effect of pathogen - spiromesifen mixtures were determined using the MixTox analysis (Jonker *et al.* 2005). The MixTox analysis takes into account the control mortality, so

uncorrected data was used for this analysis. In this analysis, a reference model is produced based on mortality observed following the single application of each mixture component. The reference model describes the expected outcome of applications of the mixture across a range of concentrations assuming independent action of components based on the single outcomes of the single applications. Mixture effects are characterised based on the deviation of observed mixture data compared to the independent action reference model (i.e. the expected outcomes). Deviations from the independent action model can differ across the model axes. Patterns in the deviation from the reference model can be categorised as absolute synergism/antagonism, dose-level dependent deviation or dose ratio dependent deviation. Alternatively, if there are no interactions between components in the mixture, there may be no deviation from the reference model. Absolute synergism/ antagonism occurs when all concentration combinations result in the same deviation from the reference model, either synergism or antagonism. Dose-level dependent deviation occurs when the deviation differs at a low dose compared to the deviation at a high dose. For example, there may be synergism at low concentrations of both components and antagonism at high concentrations of both components. Dose-ratio dependent deviation occurs when deviation from the reference model is dependent on the proportion of components in the mixture. For example, there may be antagonism when the mixture mainly consists of component 1 and synergism when increasing the proportion of component 2 in the mixture (Jonker *et al.*, 2005). Deviations from the reference independent action model are determined by the addition of several parameters to the model and subsequent comparisons between fitted models to see which describes the data most accurately. The value of each parameter can vary and define the functional form of the deviation pattern. Parameter values and their meaning are shown in Table 5.4.

Table 5.4 Interpretation of parameter values substituted into the MixTox independent action reference model to explain mixture interactions causing observed insect mortality to deviate from the reference model following the application of a mixture. Adapted from (Jonker *et al.*, 2005)

Model type	Parameter	Value	Meaning
Synergism/Antagonism	a	>0	Antagonism
		<0	Synergism
Dose ratio dependence	a	>0	Antagonism, except for those mixture ratios where significant negative b_i indicate synergism
		<0	Synergism, except for those mixture ratios where significant positive b_i indicate synergism
	b_i	>0	Antagonism where the toxicity of the mixture is mainly caused by toxicant i
		<0	Synergism where the toxicity of the mixture is mainly caused by toxicant i
Dose level dependence	a	>0	Antagonism low dose level and synergism high dose level.
		<0	Synergism low dose level and antagonism high dose level.
	BDL	>2	Change at lower dose level than the EC_{50}^*
		=2	Change at the EC_{50} level
		$1 < BDL < 2$	Change at higher dose level than the EC_{50}^*
		<1	No change, but the magnitude of synergism/antagonism is effect level (IA) dependent

* EC_{50} = median effect concentration

In cases where the MixTox analysis was not appropriate, interactions between mixture components were determined using χ^2 analysis. Expected mortality was calculated assuming no interaction and Bliss independence of the components. A χ^2 value was calculated following the equation:

$$\chi^2 = (MNC - ME)^2 / ME \quad \text{Equation 12}$$

Where MNC is the observed mortality for the mixture treatment and ME is the expected mortality. The interaction was determined by the difference in observed compared to expected mortality. The calculated χ^2 value was compared to the χ^2 table value for 1 df to determine significance (Finney, 1971).

Estimated rate of kill or time to 50% mortality (LT_{50}) was calculated using probit analysis in the DRC package in R.

5.3 Results

5.3.1 Preliminary experimental design for a mixture bioassay against *Trialeurodes vaporariorum*

Mortality in the control treatment group was $4.3\% \pm 5.2$ and $19.2\% \pm 19.5$ in the first and second repeat of the mixture bioassay respectively. Mortality observed in repeats of the mixture bioassay were significantly different for different treatments for both single and mixture results according to a two-way ANOVA ($F=2.56$, $df=21$, $p<0.01$). However, dose received by the target area (conidia mm^{-2}) following application of the same concentration of EPF was not significantly different between repeats of the mixture bioassay ($F=0.96$, $df=9$, $p=0.49$). Despite this, single applications of *C. farinosa* ranging from LC_{15} - LC_{80} resulted in *T. vaporariorum* mortality that was not significantly different according to concentration applied or dose received in bioassay one ($F=0.91$, $df=3$, $p=0.47$) and two ($F=1.34$, $df=3$, $p=0.33$). Dose received was less than that in the previous dose response bioassay for all application concentrations except the LC_{30} (Chapter 3 and seen in Table 5.5), despite preparation and application of similar concentrations. Differences between dose received during single dose response bioassays and single application treatments from the first two mixture bioassays is detailed in Table 5.5.

There was a concentration response observed for increasing concentration of spiromesifen whereby higher *T. vaporariorum* mortality was observed following the single application of increasing concentrations of spiromesifen in both repeats of the mixture bioassay (Table 5.6).

MixTox analysis was not suitable for this dataset because there was no concentration response for the application of *C. farinosa* alone. The resultant additive effect reference model could not be reliably produced and therefore deviations from the reference model, as a result of mixture interactions, were not determined.

Mixture interactions were investigated using χ^2 analysis and are given in Table 5.6.

In the first bioassay, mixture treatments involving the two highest application concentrations of spiromesifen resulted in additivity, regardless of the concentration of *C. farinosa* applied, whereas, all treatments involving the LC_{30} of spiromesifen and any concentration of *C. farinosa* resulted in antagonism. Mixture treatments involving the lowest application concentration of spiromesifen resulted in additivity or antagonism depending on the concentration of *C. farinosa* in the mixture.

Comparatively, in the repeat of the bioassay, mixture treatments involving the lowest concentration of spiromesifen and all concentrations of *C. farinosa* resulted in antagonism. There was no significant difference between the expected and observed mortality for the two highest application concentrations of spiromesifen and all concentrations of *C. farinosa* except with the mixture of LC₅₀ spiromesifen and LC₁₅ *C. farinosa* that resulted in synergism. A range of interactions occurred between spiromesifen and *C. farinosa* following the application of a mixture of the LC₃₀ spiromesifen and all concentrations of *C. farinosa* whereby antagonism, synergism or additivity was observed depending on the concentration of *C. farinosa* in the mixture. However, for unknown reasons, control mortality was significantly higher for this bioassay (19%) which reduces the accuracy of treatment mortalities. Average control mortality was high across the three replicate control treatments in the second bioassay but data was kept in the analysis as two of the three replicates had mortality below 10%. The third replicate resulted in 41% mortality and was treated as an outlier. Data sets are usually discarded if control mortality is higher than 20% (World Health Organization, 2018).

Data logger recordings showed that a recurring electrical fault caused the controlled temperature room temperature to drop to 17°C every 24 hours for 30 minutes for the duration of both mixture experiments. Despite this unplanned oscillation in temperature, average temperature during the bioassay was 21.5 ± 1.16 °C and relative humidity was $87 \pm 18.6\%$.

Table 5.5 Concentrations applied and doses received by the target area following applications of *Cordyceps farinosa* in dose response bioassays conducted over 2 years.

Dose response bioassay 2019		Single application applied during mixture bioassay 2020	
Concentration applied (conidia mL⁻¹)	Dose received (conidia mm⁻²)	Concentration applied (conidia mL⁻¹)	Dose received (conidia mm⁻²)
1 x10 ⁴	2.26 ± 0.91	9.5 x 10 ³	0.96 ± 0.12
1 x10 ⁵	3.87 ± 0.51	6.3 x 10 ⁴	5.69 ± 0.48
1 x10 ⁶	328 ± 38	7.9 x 10 ⁵	185 ± 15
1 x 10 ⁸	2342 ± 580	1.09 x 10 ⁸	969 ± 69

Table 5.6 Observed and expected mortality of *Trialeurodes vaporariorum* after 14 days in two separate bioassays following the applications of lethal concentrations (LC) of the entomopathogenic fungus, *Cordyceps farinosa* and the chemical insecticide, spiromesifen. Both control agents were applied independently and in combination. Expected mortality was calculated assuming Bliss independence and using observed data from the application of single control agents. Significant differences between expected and observed mortality was determined through χ^2 analysis.

Concentration (LC)		Mixture Bioassay 1				Mixture Bioassay 2			
Spiromesifen	<i>C. farinosa</i>	Observed mortality	Expected mortality	Chi squared value	Response	Observed mortality	Expected mortality	Chi squared value	Response
0	15	54.3	NA	NA	NA	29.8	NA	NA	NA
0	30	66.7							
0	50	49.6							
0	80	53.6							
15	0	41.5							
30	0	50.6							
50	0	75.0							
80	0	82.9							
15	15	38.2	73.3	32.3	Antagonism	23.4	59.8	56.8	Antagonism
15	30	36.7	80.5	52.3	Antagonism	23.4	61.5	62.04	Antagonism
15	50	61.3	70.5	1.4	Additivity	24.9	60.2	50.2	Antagonism
15	80	78.5	72.9	0.4	Additivity	37.5	74.8	37.1	Antagonism
30	15	58.1	77.42	6.4	Antagonism	63.1	56.9	0.61	Additivity
30	30	60.0	83.5	9.2	Antagonism	95.9	58.7	14.4	Synergism
30	50	54.6	75.1	7.7	Antagonism	42.9	57.3	4.9	Antagonism
30	80	56.3	77.1	7.7	Antagonism	85.5	72.9	1.9	Additivity
50	15	87.7	88.6	0.0092	Additivity	92.3	67.1	6.9	Synergism
50	30	84.1	91.7	0.69	Additivity	80.7	68.4	1.9	Additivity
50	50	81.4	87.4	0.44	Additivity	84.1	67.4	3.31	Additivity
80	15	91.3	92.1	0.007	Additivity	93.4	83.0	1.3	Additivity
80	30	88.2	94.3	0.42	Additivity	86.1	79.5	0.51	Additivity

Linear regression models were fitted to the dose response data of 2019 and 2020 for *C. farinosa*. The best fit to the data was the 3-parameter logistic regression (IC=50.59, Lack of fit $p=0.9$, figure 5.5). The model distinguishing between bioassays did not fit the data significantly better than a simplified model ($p=0.97$) indicating that slope and LC_{50} values for both years were not significantly different. Calculation of LC values for further experimentation were used from the model describing data from 2019 and 2020 (Table 5.7).



Figure 5.5 Three parameter logistic regression fit for corrected proportion mortality observed across a range of concentrations of *Cordyceps farinosa* comparing bioassay results from the same experiment conducted on two separate occasions separated in time.

Table 5.7 Lethal concentrations (conidia mL⁻¹) and standard error calculated for concentration response bioassays with *Cordyceps farinosa* against *Trialeurodes vaporariorum* in 2019 and LC values predicted following the addition of single dose response data collected in 2020.

LC value	Concentration (conidia mL ⁻¹) ± SE	
	2019	2019 & 2020
15	9.5 x 10 ³ ± 25	3.9 x 10 ³ ± 13
50	7.4 x 10 ⁵ ± 12	2.3 x 10 ⁶ ± 6
80	1.09 x 10 ⁸ ± 12	3.5 x 10 ^{8.5} ± 68

For dose response data of *B. bassiana* against *T. vaporariorum*, the 3-parameter logistic regression fit the data best (IC=50.59, Lack of fit p=0.9). The model distinguishing between bioassays did not fit the data significantly better than a simplified model (p=0.97) indicating that slope and LC₅₀ values for both years were not significantly different. Calculation of LC values for further experimentation were used from the model describing data from 2019 and 2020 (Table 5.8).

Table 5.8 Lethal concentrations (conidia mL⁻¹) and standard error calculated for concentration response bioassays with *Beauveria bassiana* against *Trialeurodes vaporariorum* in 2019 and LC values predicted following the addition of data collected in 2020.

LC value	Concentration (conidia mL ⁻¹) ± SE
15	1.5 x 10 ² ± 41
50	7.9 x 10 ⁵ ± 24
80	7.9 x 10 ⁸ ± 9

5.3.2 Comparing experimental design for mixture bioassays against *Trialeurodes vaporariorum*

Control mortality during the bioassay to test mixture methods was 9.5% ± 13. Comparisons of corrected mortality between treatments set up using different methods were not significantly different according to method (F=3.76, df=1, p=0.57), though mortality between treatments was significantly different (F=4.61, df=12, p<0.001). Mortality observed increased with increasing application concentrations for treatments of *C. farinosa* alone and spiromesifen alone. Despite no significant difference between methods, method two was chosen for further experiments to avoid the production of pseudo-replicates i.e. dilutions were made in separate LoBind® Eppendorf tubes for each treatment.

5.3.3 Combined application of *Cordyceps farinosa* and spiromesifen against *Trialeurodes vaporariorum*

A two-way ANOVA showed that there were significant differences between corrected mortality observed between treatments ($F=8.53$, $df=12$, $p<0.001$) and across bioassays ($F=3.66$, $df=2$, $p=0.029$). Though there was no significant difference in conidia received per unit area for each treatment during each bioassay ($p=0.83$; Table 5.9). A post hoc pairwise t-test (p values adjusted for multiple comparisons by Bonferroni method (Bonferroni, 1936)) showed that mortality in the third bioassay was significantly different to the second bioassay ($p=0.029$), though the third was not significantly different from the first bioassay ($p=0.12$), nor were there any differences between bioassay one and two ($p=0.81$). Total mortality for bioassays that were not significantly different (1&2, 1&3), were compiled for further analysis.

Control mortality was 2.7%, 14.3% and 3.3% in each replicate mixture bioassay involving *C. farinosa* which was corrected for using Schneider Orelli's formula (section 2.5.3).

MixTox analysis was conducted to determine if interactions occurred between components in mixture treatments. Mortality was modelled against dose received for EPF applications in order to account for variation in suspensions applied between bioassays. Observed data from the first two bioassays could be described using the Independent Action (IA) model ($R^2=0.38$, $p<0.001$), though adding the additional parameters to the model significantly improved the fit. The Dose-Ratio (DR) model provided a significantly better fit for the data ($R^2=0.57$, $p<0.001$). The model indicated there was antagonism across mixture combinations where the toxicity of the mixture was caused mainly by spiromesifen ($a=0.1$). However, there was a switch to synergism at low concentrations of spiromesifen when the toxicity of the mixture was mostly caused by *C. farinosa*. The DR model predicted that this switch occurs when the toxicity of spiromesifen is 1.07×10^{-3} times the concentration of *C. farinosa* and shows that spiromesifen was 1.07 times more toxic than *C. farinosa* (see figure 5.6).

Similarly, when applying the MixTox analysis to bioassays one and three, the DR model also provided the best fit for the data ($p<0.001$) and described a similar proportion of the variation about the mean ($R^2=0.57$). However, the model parameters for bioassays one and three were slightly different than those in the model describing bioassays one and two. There was antagonism for all mixture combinations except where the toxicity of *C. farinosa* was greater than that of spiromesifen. In this model, the switch from antagonism to synergism occurred when the concentration of spiromesifen was 1.2×10^{-3} times the effective concentration of *C. farinosa* (see figure 5.6).

Table 5.9 Average dose received by 22 x 22 mm coverslips during spray applications of lethal concentrations (LC) of *Cordyceps farinosa*, spiromesifen or simultaneous applications of both control agents in three replicate mixture bioassays.

Treatment		Dose received (conidia mm ⁻²) ± SD		
Spiromesifen	<i>C. farinosa</i>	Mixture 1	Mixture 2	Mixture 3
0	LC ₁₅	11 ± 0.74	12 ± 0.7	10 ± 1
0	LC ₅₀	83 ± 14	123 ± 5.9	110 ± 6.9
0	LC ₈₀	731 ± 44	716 ± 126	882 ± 177
LC ₁₅	LC ₁₅	12 ± 1.4	14 ± 0.85	10 ± 2
LC ₁₅	LC ₅₀	106 ± 30	118 ± 10.31	101 ± 18
LC ₁₅	LC ₈₀	1047 ± 98	1085 ± 72	1105 ± 137
LC ₅₀	LC ₁₅	11 ± 1.4	15 ± 0.66	11 ± 2
LC ₅₀	LC ₅₀	NA	126 ± 11	123 ± 5.4
LC ₈₀	LC ₁₅	9 ± 1.35	1267 ± 107	1105 ± 137

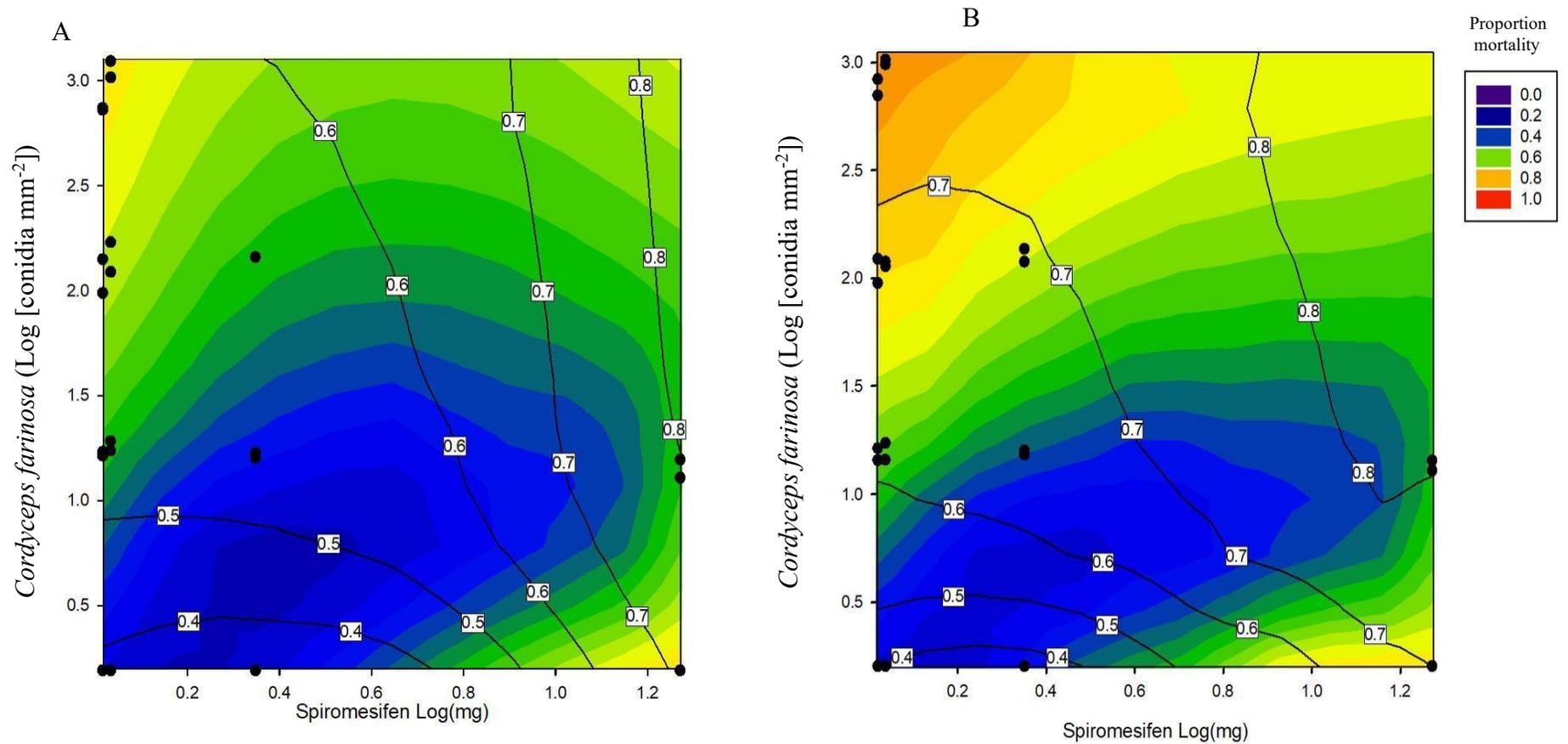


Figure 5.6 Mixture interactions 14 days after the simultaneous application of *Cordyceps farinosa* and spiromesifen (mg a.i.) across a range of concentration combinations against third instar *Trialeurodes vaporariorum*. In A.) proportion mortality was combined across bioassay 1 & 2, whereas B.) shows results from bioassays 1 & 3. Observed mortality is displayed by the coloured contours. Expected (additive) proportion mortality based on the effect of each mixture component applied individually is shown as black isobols. Deviation of the contours from the isobols indicates a mixture interaction. Black points on the plot show the dose combinations tested in the experiment.

Time to kill analysis was performed using probit linear regression. The four-parameter model significantly fitted the data better than other models tested ($p=0.76$). Constraining the LT_{50} value across all treatments did not improve the fit of the model, due to differences between predicted LT_{50} values for different treatments ($p=0.031$). Increasing application concentration in single applications of *C. farinosa* or spiromesifen reduced LT_{50} estimates, as shown in Table 5.10. However, LT_{50} values were not significantly reduced following the application of mixture treatments compared to single treatments of spiromesifen or *C. farinosa*.

Table 5.10 Total corrected mortality observed by the end of the bioassay and LT₅₀ values following the application of *Cordyceps farinosa*, spiromesifen or a combination of these against third instar *Trialeurodes vaporariorum* in laboratory based experiments. Mortality was corrected using the Schneider-Orelli approach. LT₅₀ values were calculated by probit analysis

Lethal concentration of <i>C.farinosa</i> (LC)	Lethal concentration of spiromesifen (LC)	Corrected mortality (proportion treated)			LT ₅₀ (days)		
		Bioassay 1	Bioassay 2	Bioassay 3	Bioassay 1	Bioassay 2	Bioassay 3
LC ₁₅	0	0.07 ± 0.14	0.05 ± 0.04	0.11 ± 0.032	19.11 ± 1.67	48.32 ± 52.56	19.01 ± 2.59
LC ₅₀	0	0.24 ± 0.26	0.31 ± 0.16	0.5 ± 0.036	16.27 ± 1.39	20.61 ± 6.54	12.01 ± 0.53
LC ₈₀	0	0.37 ± 0.18	0.62 ± 0.08	0.87 ± 0.045	15.68 ± 1.69	10.27 ± 1.59	9.38 ± 0.50
0	LC ₁₅	0.07 ± 0.015	0.27 ± 0.21	0.12 ± 0.2	20.29 ± 2.67	16.94 ± 2.51	18.22 ± 3.25
0	LC ₅₀	0.74 ± 0.13	0.65 ± 0.05	0.83 ± 0.012	9.75 ± 0.56	10.80 ± 0.84	8.42 ± 0.56
0	LC ₈₀	0.86 ± 0.06	0.73 ± 0.11	0.84 ± 0.058	8.72 ± 0.46	10.74 ± 1.05	9.23 ± 0.65

Lethal concentration of <i>C.farinosa</i> (LC)	Lethal concentration of spiromesifen (LC)	Corrected mortality (proportion treated)			LT ₅₀ (days)		
		Bioassay 1	Bioassay 2	Bioassay 3	Bioassay 1	Bioassay 2	Bioassay 3
LC ₁₅	LC ₁₅	0.75 ± 0.32	0.35 ± 0.21	0.45 ± 0	9.53 ± 0.72	16.31 ± 1.68	13.70 ± 0.88
LC ₁₅	LC ₅₀	0.15 ± 0.06	0.66 ± 0.21	0.57 ± 0.15	18.95 ± 1.75	10.90 ± 1.59	10.64 ± 1.17
LC ₁₅	LC ₈₀	0.81 ± 0.27	0.74 ± 0.11	0.76 ± 0.19	9.19 ± 0.68	12.59 ± 1.58	7.48 ± 1.20
LC ₅₀	LC ₁₅	0.91 ± 0.08	0.40 ± 0.11	0.93 ± 0.091	18.95 ± 1.75	10.90 ± 1.59	10.64 ± 1.17
LC ₅₀	LC ₅₀	0.13 ± 0.09	0.67 ± 0.04	0.75 ± 0.35	18.93 ± 1.58	10.45 ± 0.50	9.21 ± 0.88
LC ₈₀	LC ₁₅	0.65 ± 0.29	0.33 ± 0.34	0.66 ± 0.20	10.31 ± 0.87	11.36 ± 1.33	9.45 ± 1.19

5.3.4 Combined application of *Beauveria bassiana* and spiromesifen against *Trialeurodes vaporariorum*.

There were significant differences between the total mortality observed at the end of the bioassay for each treatment in the mixture bioassays involving *Beauveria bassiana* and spiromesifen ($F= 16.36$, $df=12$, $p<0.001$). Despite the use of day 12 in the third bioassay as the final mortality count, there were no significant differences between mortality for each treatment across bioassays ($F=2.35$, $df=2$, $p=0.10$) or conidia deposition for application of the same EPF concentration (Table 5.11) so further analysis was conducted with data compiled across all bioassays. Control mortality was 3.5%, 3.2% and 10.6% in each bioassay respectively. Total corrected mortality ranged from 0.2% to 88% depending on the treatment applied.

Data were successfully described by the IA model ($R^2= 0.63$, $p<0.001$) and the addition of parameters to allow for antagonism or synergism did not improve the fit ($p=0.73$). Therefore, all mixture outcomes for concentrations applied of *B. bassiana* and spiromesifen resulted in additivity, whereby the observed mortality was not significantly different to the expected mortality based on the single dose response of each component, assuming that they follow independent action, as shown in Figure 5.7.

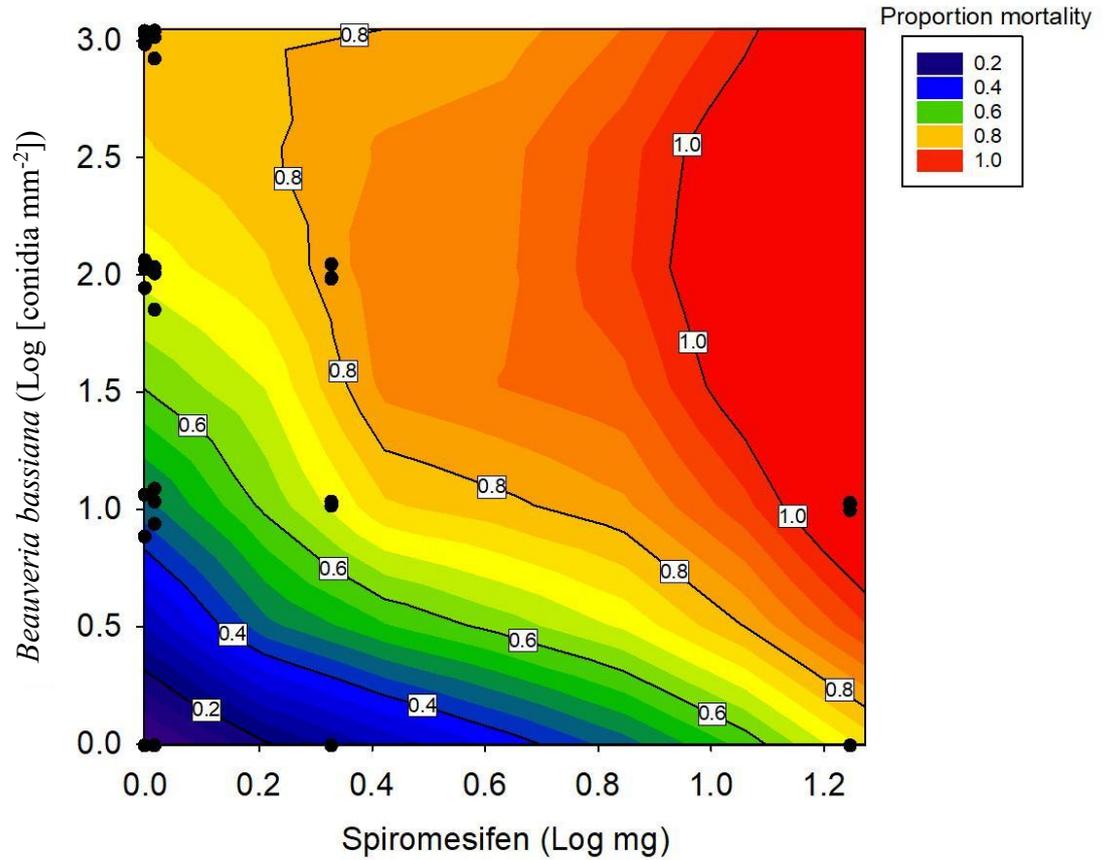


Figure 5.7 Mixture interactions 14 days after the simultaneous application of *Beauveria bassiana* and spiromesifen (mg a.i.) across a range of concentration combinations against third instar *Trialeurodes vaporariorum*. Proportion mortality was combined across three bioassays. Observed mortality is displayed by the coloured contours. Expected (additive) proportion mortality based on the effect of each mixture component applied individually is shown as black isobols. Deviation of the contours from the isobols indicates a mixture interaction. Black points on the plot show the dose combinations tested in the experiment.

Mortality over the duration of the bioassay was significantly different for treatment (df=12, F=11.27, P<0.001) and time point (df=7, F=448.1, p<0.001), though there was no difference between the mortality over time for the three replicate bioassays (df=2, F=2.12, p=0.12). Total observed mortality did not significantly deviate from the calculated expected mortality, therefore particular interest was taken to investigate if LT₅₀ values were significantly different for mixture treatments compared to single applications in each bioassay.

Time to kill analysis was performed using probit linear regression. The four-parameter model gave a significantly better fit to the data, compared to other models tested (p= 0.87). Constraining the LT₅₀ value across all treatments did not improve the fit of the model, due to differences between predicted LT₅₀ values for different treatments (p<0.01). Increasing concentrations of *B. bassiana* and spiromesifen applied singly resulted in reduced LT₅₀ estimates, except in the second bioassay where the two highest concentrations of spiromesifen resulted in similar rates of mortality, as seen in Table 5.12. Though a mixture of LC₅₀ *B. bassiana* and LC₅₀ spiromesifen did not cause mortality significantly higher than expected, the LT₅₀ of this combination was the lowest across all treatments in every repeat of the bioassay, reducing the time to 50% *T. vaporariorum* mortality by up to 5 days compared to applications of LC₅₀ of either mixture component alone.

Towards the end of the study, aubergine leaves chosen to be used in experiments were underdeveloped compared to those used in previous bioassays due to the limited time to complete all replicate bioassays. As a result, several leaves fell off in between whitefly eggs being laid on the target leaf and nymphs being ready for treatment, so some treatments had fewer replicates.

Table 5.11 Average dose received by 22 x 22 mm coverslips during spray applications of lethal concentrations (LC) of *Beauveria bassiana*, spiromesifen or simultaneous applications of both control agents in three replicate mixture bioassays.

Treatment		Dose received (conidia mm ⁻²) ± SD		
Spiromesifen	<i>B. bassiana</i>	Mixture 1	Mixture 2	Mixture 3
0	LC ₁₅	7 ± 0.85	10 ± 1.81	9 ± 1.2
0	LC ₅₀	88 ± 4.9	117 ± 8.9	107 ± 20
0	LC ₈₀	976 ± 69	1114 ± 106	1081 ± 189
LC ₁₅	LC ₁₅	8 ± 0.99	11 ± 0.37	10 ± 0.42
LC ₁₅	LC ₅₀	71 ± 4.6	102 ± 22	109 ± 14
LC ₁₅	LC ₈₀	849 ± 156	1047 ± 199	1117 ± 163
LC ₅₀	LC ₁₅	10 ± 1.0	9.5 ± 1.4	10 ± 1.9
LC ₅₀	LC ₅₀	97 ± 7.5	98 ± 14	112 ± 14
LC ₈₀	LC ₁₅	9 ± 1.1	9.8 ± 2	10 ± 0.84

Table 5.12 Total corrected mortality observed by the end of the bioassay and LT_{50} values following the application of *Beauveria bassiana*, spiromesifen or a combination of these against third instar *Trialeurodes vaporariorum* in laboratory based experiments. Mortality was corrected using the Schneider-Orelli approach. LT_{50} values were calculated by probit analysis.

Concentration of <i>B. bassiana</i>	Concentration of spiromesifen	Corrected mortality (proportion treated) \pm SD			LT_{50} (days) \pm SE		
		Bioassay 1	Bioassay 2	Bioassay 3	Bioassay 1	Bioassay 2	Bioassay 3
LC ₁₅	0	0.13 \pm 0.10	0.016 \pm 0.023	NA	16.07 \pm 0.55	20.65 \pm 2.84	NA
LC ₅₀	0	0.49 \pm 0.47	0.39 \pm 0.23	0.48 \pm 0.18	12.51 \pm 1.49	13.42 \pm 0.82	10.09 \pm 0.96
LC ₈₀	0	0.56 \pm 0.05	0.59 \pm 0.12	0.9 \pm 0.10	10.5 \pm 0.9	9.90 \pm 0.48	6.51 \pm 0.57
0	LC ₁₅	0.19 \pm 0.03	0.12 \pm 0.2	0.14 \pm 0	18.77 \pm 4.16	18.22 \pm 3.21	9.02 \pm 1.16
0	LC ₅₀	0.53 \pm 0.094	0.83 \pm 0.012	0.61 \pm 0.002	12.11 \pm 0.61	8.42 \pm 0.56	10.02 \pm 0.51
0	LC ₈₀	0.78 \pm 0	0.84 \pm 0.058	0.79 \pm 0.03	9.67 \pm 0.52	9.23 \pm 0.65	7.31 \pm 0.61

Concentration of <i>B. bassiana</i>	Concentration of spiromesifen	Corrected mortality (proportion treated) \pm SD			LT ₅₀ (days) \pm SE		
		Bioassay 1	Bioassay 2	Bioassay 3	Bioassay 1	Bioassay 2	Bioassay 3
LC ₁₅	LC ₁₅	0.19 \pm 0	0.002 \pm 0	0.23 \pm 0.39	17.86 \pm 1.8	16.1 \pm 1.63	14.81 \pm 3.85
LC ₁₅	LC ₅₀	0.73 \pm 0.06	0.51 \pm 0.31	0.34 \pm 0	8.56 \pm 0.59	8.97 \pm 0.63	12.6 \pm 1.14
LC ₁₅	LC ₈₀	0.57 \pm 0.12	0.81 \pm 0.19	0.74 \pm 0.15	10.8 \pm 0.51	8.84 \pm 0.68	7.79 \pm 0.59
LC ₅₀	LC ₁₅	0.59 \pm 0.27	0.29 \pm 0.06	0.50 \pm 0.29	12.65 \pm 1.28	14.36 \pm 1.71	9.59 \pm 1.11
LC ₅₀	LC ₅₀	0.80 \pm 0.21	0.85 \pm 0.06	0.88 \pm 0.12	8.22 \pm 0.62	8.64 \pm 0.49	6.28 \pm 0.56
LC ₈₀	LC ₁₅	0.56 \pm 0.26	0.56 \pm 0.14	0.48 \pm 0.16	12.84 \pm 0.64	9.17 \pm 0.8	14.39 \pm 1.12

5.3.5 Determining the impact of temperature on interactions

Control mortality was low in both bioassays; in the oscillating incubator, average control mortality was 1.9% and 1.5% in bioassay one and two respectively. In the constant temperature incubator the control mortality was 0.5% in the first bioassay. There was no control mortality observed in the second repeat of the constant temperature bioassay. Mortality data for treatments were corrected accordingly. The average temperature in the constant temperature incubator was $23.79 \pm 0.21^{\circ}\text{C}$. Average temperature in the oscillating incubator was 23.14°C with a maximum of 31.26°C and minimum of 16.18°C .

Results for both bioassays were deemed to be significantly different according to ANOVA analysis ($F=15.4$, $df=1$ $p<0.001$). There was also a significant interaction between bioassay and treatment ($F=3.71$, $df=4$, $p=0.01$) therefore bioassays were analysed separately.

In the first bioassay, there were significant differences in mortality observed for different treatments ($F=7.2$, $df=4$, $p<0.001$). Single applications of the insecticide or EPF resulted in different mortalities than expected based on estimated LC values calculated from data in previous bioassays, as shown in Table 5.13. Mortality for treatments in constant or oscillating temperature incubators did not differ significantly ($F=2.17$, $df=1$, $p=0.15$) and there was no significant interaction between treatment and incubation conditions ($F=0.84$, $df=4$, $p=0.51$). Application of the mixture of LC_{15} spiromesifen and LC_{50} *C. farinosa* when incubated at a constant temperature of 23.5°C resulted in additivity as *T. vaporariorum* mortality did not significantly differ from the expected mortality. Whereas, synergism was observed for the mixture of LC_{15} spiromesifen and LC_{80} *C. farinosa*. These results are similar to those observed in the mixture experiments involving a larger number of mixture treatments (section 5.3.2.1).

Mixture treatments incubated at a 24h oscillating temperature resulted in different interaction outcomes for both mixtures compared to the constant temperature treatments. When plants were incubated at an oscillating temperature, the mixture of LC_{15} spiromesifen and LC_{50} *C. farinosa* resulted in antagonism, where the observed mortality was significantly less than expected. Comparatively, the LC_{15} of spiromesifen applied with the LC_{80} *C. farinosa* resulted in additivity. Although the mortalities of mixture treatments in the constant and oscillating conditions were very similar, the interaction outcome was different because the mortality for spiromesifen and *C. farinosa* when applied independently was higher than expected. Therefore, the calculated expected mortality was higher than that for the constant

temperature condition. The independently applied control agents caused greater mortality than expected under the oscillating temperature conditions, but this increase in mortality was not seen in the mixture treatments.

Table 5.13 Corrected observed mortality for the first mixture experiment conducted at constant and oscillating temperatures ranging between 17 and 30°C every 24 hour, with an average daily temperature of 23.5°C. Mixtures of *Cordyceps farinosa* and spiromesifen were applied to third instar *Trialeurodes vaporariorum*. Significant interactions were calculated by χ^2 test ($p < 0.05$).

Treatment	Incubation temperature (°C)	Observed	Expected	Interaction
Spiromesifen LC ₁₅	23.5	0.29 ± 0.14	NA	NA
<i>C. farinosa</i> LC ₅₀	23.5	0.40 ± 0.21	NA	NA
<i>C. farinosa</i> LC ₈₀	23.5	0.67 ± 0.15	NA	NA
LC ₁₅ x LC ₅₀	23.5	0.49 ± 0.24	0.57	Additivity
LC ₁₅ x LC ₈₀	23.5	0.94 ± 0.29	0.76	Synergism
Spiromesifen LC ₁₅	oscillating	0.48 ± 0.23	NA	NA
<i>C. farinosa</i> LC ₅₀	oscillating	0.66 ± 0.07	NA	NA
<i>C. farinosa</i> LC ₈₀	oscillating	0.73 ± 0.14	NA	NA
LC ₁₅ x LC ₅₀	oscillating	0.53 ± 0.11	0.82	Antagonism
LC ₁₅ x LC ₈₀	oscillating	0.89 ± 0.08	0.86	Additivity

In the replication of the mixture bioassay incubated at constant and oscillating temperatures, there were significant differences in the mortality observed for each treatment ($F=4.6$, $df=4$, $p=0.009$). Similar to the results of the previous bioassay, the single applications of spiromesifen and *C. farinosa* resulted in mortalities differing from those predicted by LC values calculated from probit analysis of prior dose response bioassays (see Table 5.14). As found in the previous bioassay, mortality for treatments in constant or oscillating temperature incubators did not differ significantly ($F=0.007$, $df=1$, $p=0.93$) and there was no interaction between treatment and incubator conditions ($F=0.72$, $DF=4$, $p=0.59$). Application of the mixture of LC₁₅ spiromesifen and LC₅₀ *C. farinosa* when incubated at a constant temperature of 23.5° C resulted in additivity. The mixture of LC₁₅ spiromesifen and LC₈₀ *C. farinosa* resulted in higher mortality than expected, but the difference was not significant according to the χ^2 test.

In the second bioassay, mixture treatments incubated at a 24h oscillating temperature resulted in the interaction outcome changing for one of the mixture treatments compared to the constant temperature treatments. When plants were incubated at an oscillating temperature, the mixture of LC₁₅ spiromesifen and LC₅₀ *C. farinosa* resulted in synergism. The LC₁₅ of spiromesifen applied with the LC₈₀ *C. farinosa* resulted in additivity.

Table 5.14 Corrected observed mortality for the second mixture experiment conducted at constant and oscillating temperatures ranging between 17 and 30°C every 24 hour, with an average daily temperature of 23.5°C. Mixtures of *Cordyceps farinosa* and spiromesifen were applied to third instar *Trialeurodes vaporariorum*. Significant interactions were calculated by χ^2 test (p<0.05).

Treatment	Incubation temperature (°C)	Observed	Expected	Interaction
Spiromesifen LC ₁₅	23.5	0.19 ± 0.071	NA	NA
<i>C. farinosa</i> LC ₅₀	23.5	0.37 ± 0.075	NA	NA
<i>C. farinosa</i> LC ₈₀	23.5	0.58 ± 0.23	NA	NA
LC ₁₅ x LC ₅₀	23.5	0.49 ± 0.16	0.49	Additivity
LC ₁₅ x LC ₈₀	23.5	0.51 ± 0.07	0.66	Additivity
Spiromesifen LC ₁₅	oscillating	0.14 ± 0.04	NA	NA
<i>C. farinosa</i> LC ₅₀	oscillating	0.37 ± 0.17	NA	NA
<i>C. farinosa</i> LC ₈₀	oscillating	0.47 ± 0.15	NA	NA
LC ₁₅ x LC ₅₀	oscillating	0.63 ± 0.19	0.45	Synergism
LC ₁₅ x LC ₈₀	oscillating	0.44 ± 0.12	0.54	Additivity

5.4 Discussion

Quantification of the effects of a mixture involving a microbial is possible using approaches used to describe chemical interactions. The process of infection of an insect by a pathogen is more variable than the toxicity caused by a chemical because of the complex interactions occurring between the two biological organisms. However, much like the dose response of an insecticide on a target organism, increasing the dose of a microbial pathogen also results in an increased effect and their dose response curves follow the same sigmoidal shape if the host is susceptible to infection. This is because the effect of the pathogen concentration on the mortality of the target pest can also be described as a complex chemical reaction using enzyme kinetics. Though abiotic conditions, conidial viability and host susceptibility influence EPF virulence, the rate of the infection process of the insect by the microbial pathogen is ultimately determined by the slowest step i.e. a single rate limiting enzyme reaction within the EPF (Ortiz-Urquiza and Keyhani, 2013). The rate of many biological processes are calculated under this assumption (Schoolfield *et al.*, 1981). Therefore it is reasonable to use the same concentration effect model used for chemicals as a simplification of the behaviour of a microbial pathogen.

Despite this, more informative mixture analyses, such as the MixTox analysis, have not been utilised in insect pathology. MixTox analysis includes all data points from each mixture and single treatment, allowing the visualisation of patterns across all the concentration combinations tested (Jonker *et al.*, 2005). The relationship between interactions across concentration combinations can also be quantified allowing calculation of important mixture compositions to avoid or aim to improve pest mortality. This understanding is essential for both applied mixtures, differential deposition of control agents throughout the crop and coincidental mixture exposures that occur due to chemical residues. These different exposures influence the efficacy of control and reliability of the mixture. Instead, the most commonly used method of analysis is the χ^2 test which determines interactions through a simple calculation that requires the mortality caused by the mixture and the mortality caused by the two components in the mixture (Finney, 1971).

Alternatively, another approach to interpret interaction outcome is to use co-toxicity coefficients (Sun and Johnson, 1960). This analysis uses toxicity indexes to incorporate the dose response curve of each mixture component into the calculation of expected mixture mortality. Sun and Johnson (1960) state that three dose response curves should be conducted for effective use of this analysis; two dose response curves of the individual mixture

components and one dose response of both components applied together. Three equations are used for this analysis;

$$\text{Toxicity index (TI) of agent} = \left(\frac{\text{LC50 of standard agent}}{\text{LC50 of supplied agent}} \right) \times 100 \quad \text{Equation 13}$$

$$\begin{aligned} \text{Theoretical toxicity index of the mixed formulation} = \\ (\text{toxicity index of agent 1} \times \% \text{ of agent 1 in the mixed formulation}) + \\ (\text{toxicity index of agent 2} \times \% \text{ of agent 2 in the mixed formulation}) \end{aligned} \quad \text{Equation 14}$$

$$\text{co - toxicity coefficient} = \left(\frac{\text{actual toxicity index of the mixed formulation}}{\text{theoretical toxicity index of the mixed formulation}} \right) \times 100 \quad \text{Equation 15}$$

A co-toxicity coefficient equal to 100 indicates that the observed mortality is identical to the expected mortality. If the calculated co-efficient is more than 100, synergism has occurred. In contrast, if the coefficient is less than 100, antagonism has occurred.

Though these methods of analysis are appropriate for experiments which investigate the effect of two control agents combined in one mixture treatment, several mixture studies with elaborate experimental designs and large data sets also use these methods of analysis. For example, the combined effect of *B. bassiana* and azadirachtin on third instar *T. vaporariorum* nymphs was investigated by (Wei, 2020). Five mixture treatments of *B. bassiana* and azadirachtin with different ratio combinations were tested. Antagonism was observed with high relative doses of azadirachtin or *B. bassiana* and synergism was found when the ratio was 1:1 or 1:4 (azadirachtin: *B. bassiana*). In addition, χ^2 analysis used to identify synergistic interactions after twelve treatments of the fungus *Akanthomyces muscarium* (previously known as *Lecanicillium muscarium*) was combined with the alkaloid insecticide, matrine, in three different ratio combinations simultaneously applied to *Bemisia tabaci* and in four treatments where matrine was applied sequentially, 24 hours after *A. muscarium* (Ali et al., 2017). Despite the elaborate experimental design conducted by Ali et al. (2017), the amount of information available from the results is limited by the type of analysis used. The disadvantage of analysing mixture outcomes using χ^2 or co-toxicity coefficients is that interactions can only be determined for the specific combination tested and relationships between different concentration combinations are difficult to decipher. However, increasing complexity of the analysis can allow for greater interpretation and visualisation of results.

Similarly, a study conducted by Kary et al. (2018) investigated the effect of combining the LC₁₀, LC₃₀ or LC₅₀ of the insecticide abamectin with the LC₃₀ or LC₅₀ of *Steinernema carpocapsae* in control of *Phthorimaea operculella* (Lepidoptera, Gelechiidae) in laboratory bioassays. Results from the χ^2 analysis suggested that synergistic interactions were occurring between the nematode and abamectin except at the highest doses of the chemical, where antagonism occurred. If this analysis had been undertaken using a more complex approach, such as the MixTox analysis (Jonker et al., 2005), the relationship between abamectin and *S. carpocapsae* could have been determined as dose level or dose ratio dependent. This analysis would allow the identification of the specific concentrations causing the switch between antagonism and synergism, important additional information which could be applied in pest management to avoid negative interactions.

In contrast, generalised linear models were used by Raymond et al. (2006) to describe the interactions occurring between the *Bacillus thuringiensis* (*Bt*) toxin Cry1Ac and the nucleopolyhedrovirus AcMNPV in *Plutella xylostella* larvae. This experiment co-infected larvae with five virus doses and five toxin doses in a full factorial design. A dose response curve was fitted for the singly applied virus or toxin only treatments. Linear or polynomial curves were then fitted to the mixture treatments and comparisons of slope for each model were determined by analysis of covariance. If the slope deviated from the model fitted for individually applied control agents, there was synergism or antagonism occurring. The addition of higher-order polynomial terms to the mixture models indicated whether there were changes in the type of interaction occurring across the dose response curve.

Antagonism was observed in combinations of low doses of virus and toxin. But, whilst it was noted that the curvature of mortality against virus dose reversed as toxin dose increased, synergism was undetectable because 100% mortality was observed in many of the high toxin treatments. Though this analysis was able to incorporate all the mixture data collected, it was unable to discriminate between complex interaction patterns such as dose ratio or dose-level dependency or estimate parameter values to define important switching points between antagonism, additivity and synergism as provided by the MixTox analysis (Jonker et al. 2005).

The first step in designing a successful mixture experiment is to conduct a dose response of each component to be mixed. In doing so, it is possible to calculate lethal concentrations (LC) which are the concentrations causing an expected response in the pest organism. By conducting preliminary dose response experiments before the mixture is applied, it is possible to select a range of LC values for the mixture experiment so that differences in interaction across a range of doses can be identified. As an example, in order to calculate

LC₂₅ values, Hamama et al. (2015) applied nine concentrations of imidacloprid or acetamiprid and eight concentrations of *Spodoptera littoralis* nucleopolyhedrosis virus or *Bacillus thuringiensis kurstaki* onto castor leaves infested with *Spodoptera littoralis* allowing the accurate determination of LC₂₅ values by probit analysis to be used in the subsequent mixture experiment. In this study, a range of LC values (LC₁₅, LC₃₀, LC₅₀ and LC₈₀) were determined for *C. farinosa* (ATCC 4412), *B. bassiana* (PPRI 5339) and spiromesifen.

Several mixture concentrations of each mixture component were investigated because variation in dose received by individual pests within a crop following the application of one concentration is unavoidable. Spatial, temporal, and environmental variation within the crop causes variation in dose of each component in a mixture reaching target insects. The outcome of a mixture depends on the relative doses of the components at the site of interaction. Hence, the interaction outcome of the components will vary depending on whether small changes in dose received can influence a change in interaction. Additionally, mixture components may behave differently during spray applications and so they may travel different distances during spraying, adhere to the target differently, or persist on the crop for different periods. This will also cause variation in the doses received by the insect relative to the amounts used in a tank mix. Ideally, the type of interaction occurring between mixture components would have little variation depending on concentration or ratio applied to allow for predictable levels of pest control. This demonstrates the complexity involved in mixtures and the need for robust experimental design and analysis in order to determine interaction outcomes and capture the potential benefits.

Once expected lethal concentrations were calculated, it was possible to calculate the expected effect of a mixture application. Mixture concentration combinations were chosen based on expected mortality values that did not exceed >90% assuming independent action, therefore a full factorial experimental design was not conducted. This was to ensure that all interactions, whether resulting in increased (synergism) or decreased (antagonism) mortality could be detected. Several of the previous mixture studies in insect pathology have not used this approach, resulting in redundant treatment groups. For example, a study by Russell et al. (2010) investigated the effect of combining applications of imidacloprid and *Metarhizium brunneum* to control Asian long horned beetles (*Anoplophora glabripennis*). However, because every beetle exposed to *M. brunneum* died before the end of the bioassay, interactions were unable to be determined. In another study, Wu et al. (2017) investigated mortality of fungus gnat (*Bradysia odoriphaga*) using the nematode species *Steinernema feltiae* in combination with thiamethoxam. Three concentrations of *S. feltiae* and four

concentrations of thiamethoxam were used in mixture experiments based on previous exposures indicating a potential synergism between the control agents. In total, 20 treatments were applied in this mixture experiment which included seven treatments of each control agent applied alone, twelve mixture treatments of each possible concentration combination and a control treatment. However, based on their calculations, three of the mixture treatments were calculated to cause an expected mortality higher than 100%. Therefore, basing mixture treatments on LC values of each component allows researchers to predict the expected outcome of a mixture before conducting the experiment and avoid those treatments which do not allow interactions to be determined.

In order to analyse potential mixture interactions across a range of application concentrations using the MixTox analysis, single applications of components of the mixture must also be applied alongside the mixture treatments. This is because the expected mixture mortality or additive effect is calculated from the mortality observed in single application treatments. It is important that both individual and mixture treatments are applied during the same bioassay so that variation in preparation or application of solutions/suspensions is accounted for as well as reducing the variability in population susceptibility which may be observed between bioassays. Because the individually applied treatments were included alongside mixture treatments in the same bioassay, it was possible to identify issues in the initial mixture experimental design. It was evident that differences in mixture treatment mortality could not be determined because there was no significant difference between EPF induced mortality of *T. vaporariorum* following the application of LC₁₅ – LC₈₀ of *C. farinosa*. This was despite application concentrations ranging from 1×10^4 conidia mL⁻¹ to 1×10^8 conidia mL⁻¹. As a result, a replicate dose response bioassay was performed to confirm predicted LC values and to ensure that there had not been changes in the *T. vaporariorum* population susceptibility.

In addition, doses received during mixture bioassays for single applications of *C. farinosa* were less than the previous dose response bioassays. This may have been caused by an additional dilution step required during the set-up of the mixture bioassays and/or it is also possible that conidia adhered to the inside of pipette tips and vials during serial dilutions. The extra dilution steps required during the mixture set up could have resulted in the final suspensions containing fewer conidia than in previous bioassays as conidia were successively removed through adhering to plastic ware, though no evidence was collected to support this. To resolve this, LoBind® non-stick vials were used in preparation of mixtures in further experiments.

Although fewer conidia reached the target area during initial mixture bioassays, the observed mortality was higher than expected for LC₁₅ and LC₃₀ applications of *C. farinosa* and lower than expected for applications of LC₈₀. Treatments of LC₃₀ were removed from further experiments due to similarity in observed mortality and dose received when compared to LC₁₅ treatments, making the treatment redundant. The LC₃₀ of spiromesifen was also removed from further experiments to improve the ability to distinguish between mortality caused by different treatments. The removal of this treatment allowed for longer post-treatment assessments as time was less constrained to assess all treatments in one day.

The initial mixture bioassays were also subject to an electrical fault which caused the temperature to drop significantly every 24 hours. Fluctuating temperatures have been shown to influence insect fitness and development (Ratte, 1984) and deviation from the optimal temperature for *C. farinosa* will affect EPF virulence (Kryukov *et al.*, 2018). In this instance, the temperature did not vary to an extreme, but differences in conditions between bioassays will have had an impact on *T. vaporariorum* development and mortality (Roermund and van Lenteren, 1992).

Antagonism between spiromesifen and *C. farinosa* was identified through χ^2 analysis of initial mixture bioassays. Increasing the concentration of *C. farinosa* relative to spiromesifen resulted in an additive response. The same pattern was found in the second replicate of the bioassay except that synergism was also identified following the application of LC₅₀ spiromesifen and LC₁₅ *C. farinosa* or LC₃₀ of both mixture components. It is difficult to determine whether these interactions are reliable as expected mortality was calculated using the single dose response data which did not follow a dose response curve.

Following the previously mentioned adjustments to the mixture bioassay and ensuring that temperature was maintained throughout the duration of each bioassay resulted in a dose response effect for single applications of *C. farinosa* and spiromesifen in subsequent experiments, which allowed the determination of mixture effects in co-application treatments. Dose received by target leaves was consistent between treatments of single and mixture applications of *B. bassiana* and *C. farinosa* in separately conducted mixture experiments. This provides further evidence of the capability of the calibrated spray tower to accurately deliver a specific dose of conidia mm⁻² (Spence *et al.* 2020).

Despite significant differences in the mortality observed between treatments in separate bioassays, the same mixture effect pattern was produced following MixTox analysis. Spiromesifen was found to interact with *C. farinosa* either synergistically or antagonistically

against *T. vaporariorum* depending on the ratio of components in the mixture. Synergism was found for applications of LC₈₀ and LC₅₀ *C. farinosa* with LC₁₅ spiromesifen whereas increasing the concentration of spiromesifen resulted in additivity or antagonism. The dose ratio model provided a significant fit for all mixture bioassays involving *C. farinosa* and spiromesifen. This dose ratio relationship suggests that spiromesifen has a negative effect on *C. farinosa* when it is the most toxic component in the mixture. Though, it is unknown whether the chemical is directly impairing the EPF during the infection process, or whether the antagonism is mediated through changes in the host toxicokinetics. Previous *in vitro* experiments described in section 4.2.2 showed that the presence of high concentrations of spiromesifen had no significant effect on the growth and germination of *C. farinosa in vitro*. Cuthbertson (2008) reported similar findings whereby *Akanthomyces muscarium* (was *Lecanillium*) conidia showed high proportion germination following direct exposure to recommended application rates of spiromesifen (Oberon®). Although interactions between mixture components in their study were not mathematically determined, approximately 70% mortality was observed in second instar *B. tabaci* following the recommended application rate of either the fungus or the chemical. Sequential application of *A. muscarium* 24 hours after spiromesifen resulted in approximately 85% mortality. Other studies have also shown that *in vitro* compatibility tests may not accurately predict the interactions which occur *in vivo*. For example, gamma-cyhalothrin, lambda-cyhalothrin, rynaxypyr, lufenuron and methoxyfenozide showed variable levels of inhibition on three strains of *B. bassiana*, one *M. anisopliae* strain and one *M. robertsii* strain for conidial germination, vegetative growth and conidial production. However, no antagonistic effects were observed for combinations of each fungus with recommended field doses of each insecticide against third instar *Rachiplusia nu* (Pelizza *et al.*, 2018).

Several studies have indicated the potential for co-application of low concentrations of insecticides with entomopathogenic fungi to increase mortality of pest species. For example, Santos *et al.* (2018b) found that co-application of a *Cordyceps javanica* strain with sub lethal concentrations of spiromesifen resulted in additive or synergistic effects against *B. tabaci* nymphs depending on the concentration applied. Though, in contrast to results from this study, Santos *et al.* (2018) found that increasing the relative concentration of spiromesifen in the mixture resulted in a change from additivity to synergism. Kpindou *et al.* (2008) combined *M. anisopliae* with lambda-cyhalothrin against Sahelian grasshoppers (*Oedaleus senegalensis*) resulting in EPF mortality occurring as early as 2 days after application.

However, conclusions can only be drawn from model outputs generated from observed data. For example, in analysis of *C. farinosa* and spiromesifen combinations, the MixTox analysis

and contour plot indicate that co-application of a concentration smaller than the LC₁₅ *C. farinosa* and the LC₈₀ spiromesifen may result in synergism. But, further experiments testing co-application of these specific concentrations would need to be conducted to confirm the model output.

Overlaying the experimental design with the MixTox model output in a contour plot allows for the visualisation of patterns across the mixture combinations and also identifies gaps in the data set. For example, contour plots showing interactions between *C. farinosa* and spiromesifen indicate that there may be synergism between LC₁₅ of spiromesifen and a range of concentrations of *C. farinosa* between the LC₅₀ and LC₈₀. These results could be confirmed by co-application of 0.04 mg mL⁻¹ spiromesifen with concentrations of *C. farinosa* ranging 2.3x10⁶ – 3.5x10⁸ conidia mL⁻¹ in further experiments.

Identifying the mechanisms behind synergistic interactions is an important step in discovering successful combinations of crop protection products for improved pest control. For example, synergism discovered during the application of sub lethal concentrations of imidacloprid and *B. bassiana* onto second instar *Leptinotarsa decemlineata* larvae was determined to be caused by starvation stress which increased larval susceptibility to the pathogen (Furlong and Groden, 2001). In another study, the mechanisms behind synergistic interactions of *M. anisopliae* and low concentrations (0.01mg/L – 0.025mg/L) of chlorantraniliprole against *Locusta migratoria* were investigated. Activity of glutathione-S-transferase, general esterases and phenol oxidase (important detoxifying enzymes) was reduced following co-application of the insecticide and the EPF. The authors speculate that metabolites produced by *M. anisopliae* prevent the activation of detoxifying enzymes by Ca²⁺ disruption, increasing the host's susceptibility to the insecticide (Jia *et al.*, 2016). Whereas, the synergistic interaction of the chemical matrine and *Akanthomyces muscarium* against *B. tabaci* was determined to be related to both matrine and the EPF secondary metabolite, bassianolide, binding to acetylcholine receptors, causing decreased activity of acetylcholinesterase. In addition, co-application of *A. muscarium* and matrine caused an overall reduction in the activity of carboxylesterases (CarE) and glutathione-S-transferase, host enzymes essential for the detoxification of insecticides and pathogens (Ali *et al.*, 2017).

The mechanisms behind interactions observed in this study were not investigated, however it is known that insect growth regulators (IGR) which prevent or reduce the insects' ability to develop can improve control efficacy of EPF. Spiromesifen is an insect growth regulator which disrupts insect development through inhibition of lipid synthesis (Bi and Toscano, 2007b). Shedding EPF conidia by moulting before conidia have penetrated the cuticle is an

effective way to avoid EPF infection (Kim and Roberts, 2012). If time between successive instars is increased, EPF have a longer period of time in which to penetrate and infect the host (Nian *et al.*, 2015). Further experiments into the effect of low concentrations of spiromesifen on development time of *T. vaporariorum* could indicate whether an increased infection period for the pathogen is the mechanism determining synergism between *C. farinosa* and spiromesifen in this study. However, an increased infection period would also be beneficial for the infection of *T. vaporariorum* by *B. bassiana*. Due to the different interactions observed between spiromesifen and *B. bassiana* or *C. farinosa*, it is likely that the mechanism behind the interactions observed involves EPF specific compounds produced by the EPF or by the host in response to infection. Determining the secondary metabolites produced by *B. bassiana* (PPRI 5339) and *C. farinosa* (ATCC 4412) as well as the changes in enzyme activity in *T. vaporariorum* in response to application of a range of concentrations of EPF and insecticide could indicate how the mixture interactions are mediated.

Temperature oscillations were shown to have a significant effect on the types of mixture interactions that occurred following the application of *C. farinosa* and spiromesifen. Concentration combinations which resulted in additivity at a constant temperature, resulted in an antagonistic effect under the oscillating temperature regime. Additionally, the combination of LC₁₅ spiromesifen and LC₈₀ *C. farinosa* which produced a synergistic interaction in all previous mixture bioassays resulted in additivity under the oscillating temperature regime. However, observed mortality in the repeat of this experiment was not significantly different from expected mortality in mixture treatments maintained at constant temperature, whereas synergism occurred following the application of LC₅₀ *C. farinosa* and LC₁₅ spiromesifen. This is a potential indicator that under greenhouse conditions where the number of factors influencing interaction outcome is increased, synergistic interactions identified in the laboratory may be difficult to exploit. Although, some studies have found consistent interactions when conducting experiments in the laboratory and in the greenhouse. In a study by Al-Mazraáwi (2006), only additive mortality was observed after applying *B. bassiana* and imidacloprid at nine concentration combinations against *Thrips tabaci* on greenhouse grown tomato plants after mostly additivity and some antagonism being observed in laboratory-based experiments.

Applications of mixtures which result in additivity or antagonism may be acceptable under certain conditions. Using two control agents can still have an increased effect compared to application of one agent despite an antagonistic effect. For example, the combination of two control agents which independently cause 50% mortality would be expected to cause 75%

mortality following co-application. If co-application caused 65% mortality, despite this being defined as an antagonistic effect, the increase in mortality may be worth the additional effort of preparing two crop protection products for application assuming the cost of purchasing both was not significantly more. As such, there needs to be a change in usage of these terms in insect pathology; defining interactions by their ecotoxicological definitions rather than classifying interaction outcomes as 'positive' or 'negative' (as shown in appendix VI).

There were no significant interactions influencing total *T. vaporariorum* mortality following co-application of *B. bassiana* and spiromesifen across all concentrations tested. However, co-application of *B. bassiana* and spiromesifen reduced LT₅₀ estimates by up to 5 days depending on the combination applied. Following the application of each EPF alone, comparisons between LT₅₀ values showed that infection rate was generally faster for *B. bassiana* treated *T. vaporariorum* nymphs compared to those treated with *C. farinosa*. A significant increase in speed of kill as a result of the mixture treatment, despite no change in overall mortality is beneficial as it reduces the amount of time that pests are on the crop causing damage. For other pest species, increasing rate of kill is essential to prevent increasing pest populations and migration within and between crops. Mixture treatments involving *C. farinosa* and spiromesifen did not result in changes to LT₅₀ values. Ye *et al.* (2005) also found that simultaneous application of imidacloprid at a concentration of 0.1-0.5 µg mL⁻¹ with *B. bassiana* against *Myzus persicae* resulted in an increased rate of kill.

In order to improve our understanding of mixture interactions, simplistic experiments informed by the theory behind mixture calculations must first be conducted. A priority is to establish the interactions between different elements used within an IPM system, to ensure that one element does not inhibit another. In particular, whether combinations of entomopathogens plus chemical pesticides, interact synergistically, antagonistically or give additive effects for selected pests of importance in agriculture and horticulture. It is important to determine these interactions under conditions emulating those of the target environment, as abiotic conditions affect interactions between the plant, the pest and the control agents applied. Though the desired interaction between mixture components in IPM results in synergism and increased pest control, an emphasis should be made to ensure all mixture outcomes are published, as other outcomes can still be beneficial as a whole depending on the aims of the stakeholder.

Several interdisciplinary reviews into the effects of combinations of drugs (Chou *et al.*, 2006), microbial agents (Xu 2011) or multiple ecological stressors (Orr *et al.*, 2020) have highlighted key issues restricting the impact and applicability of mixture experiments. Despite the different context of these reviews, the same conclusions were drawn; that researchers rarely provided a mathematically rigorous method for identifying synergism or antagonism, and as such faulty or unsubstantiated claims of synergy are published. Researchers also often failed to identify what is meant by synergism, potentiation, antagonism and the additive effect. Similar outcomes were found in a systematic review summarising IPM mixture experiments involving microbial control agents against greenhouse pests conducted by Spence *et al* (Appendix VI). There is a need for researchers to conduct mixture experiments using standardised methods of experimental design, analysis and reporting of results in order to allow formal meta-analysis of results.

A unified approach to mixture applications in IPM will improve our understanding of the underlying mechanisms resulting in desired interactions. Once mechanisms of successful mixtures can be determined, targeted research can be conducted to improve pest control efficacy and as a result, increase uptake of microbial and microbial-chemical mixtures in IPM.

5.4.1 Summary

- Results indicate that mixtures of *C. farinosa* or *B. bassiana* with the chemical spiromesifen are compatible for improved control of *T. vaporariorum*.
- Using the ecotoxicological analysis, MixTox, a dose ratio relationship was identified between *C. farinosa* and spiromesifen following the simultaneous application of a range of concentration combinations.
- Synergism was found following applications of high concentrations of *C. farinosa* and low concentrations of spiromesifen.
- Temperature had a significant effect on the interactions occurring between high concentrations of *C. farinosa* and low concentrations of spiromesifen.
- Mixtures of *B. bassiana* and spiromesifen resulted in an additive response, though the rate of mortality was significantly increased compared to applications of control agents independently.

6 Determining the effect of a mixture of entomopathogenic fungi and spiromesifen on *Trialeurodes vaporariorum* under greenhouse conditions

6.1 Introduction

Greenhouse systems are developed to maximise crop growth and within many systems, there is the capability to regulate temperature, humidity, light and CO₂ levels to provide ideal abiotic conditions for crop production (Montero *et al.*, 2011). The protected environment of the greenhouse is also conducive for use of microbial control agents and allows pest control using inundative applications of Hypocrealean fungi, at levels that are more difficult to achieve under field conditions (Wraight *et al.*, 2017).

The use of entomopathogenic fungi as part of a suite of control options is common practise in greenhouse production. As discussed in chapter 1, EPF are ideal for use within an IPM programme as they are often compatible with other natural enemies (Chandler *et al.*, 2011). For crops with frequent harvests, EPFs are beneficial as there is no or a very short pre-harvest interval, and there are little to no residues left on the crop (Wraight *et al.*, 2017). However, the greatest limitation in the use of EPF for pest control is their variability in efficacy of control. Even within the protected environment of the greenhouse, abiotic conditions can determine the success or failure of a mycoinsecticide, especially during the critical period between application of EPF conidia and penetration of the host insect cuticle. During this time, conidia are susceptible to ultraviolet radiation, humidity and temperature within the microclimate surrounding the leaf. Because of this, formulations for many mycoinsecticides are designed to protect conidia, improve their stability and increase efficacy (Boyetchko *et al.*, 2003).

Direct exposure of conidia to solar radiation, especially the UV-B fraction of the spectrum, can reduce germination, decrease virulence or deactivate conidia in just a few hours (Braga *et al.*, 2001; Fernández-Bravo *et al.*, 2017). However, under greenhouse production, the glass and plastic structures filter damaging UV radiation and can reduce UV-B intensity by 90% (Lasa *et al.*, 2007). Additionally, for pesticide applications that are specifically targeting *T. vaporariorum*, direction of spray is upwards towards the abaxial side of the leaf, so UV damage is unlikely once conidia have reached the leaf surface due to leaf shading.

In comparison, humidity and temperature are likely to be more influential over EPF virulence under greenhouse conditions. For example, under fluctuating temperature and relative humidity conditions ranging from 43-75% humidity, and temperatures between 15-28, 20-25, 25-28, and 25-35°C, Fargues and Luz (2000) determined that relative humidity

was the most important factor determining mortality of first instar *Rodnius prolixus* by *Beauveria bassiana*. After inoculation at 3×10^5 conidia cm^{-2} , at least 97% relative humidity was required for $\geq 12\text{H}$ per day for the first two days to ensure at least 50% mortality of *R. prolixus* under fluctuating temperatures. More recent studies have however indicated that relative humidity has little effect on EPF performance, assuming that the microclimate surrounding the insect and leaf surface fulfils the humidity requirements of the EPF (Devi *et al.*, 2005); studies have shown that humidity can be increased considerably up to 5mm surrounding the leaf surface, due to transpiration (Shipp *et al.*, 2011). Fargues *et al.* (2005) described the microclimate conditions of *T. vaporariorum* on the under-leaf boundary layer as ‘disconnected from that of both inside and outside the greenhouse’. Relative humidity in the Fargues *et al.* (2005) study ranged from 53.5% during the day to 82.9% at night. Despite this variation, *T. vaporariorum* mortality following application of *Akanthomyces muscarium* (formerly *Lecanicillium*) were not significantly different. Additionally, in a study by Athanassiou *et al.* (2017), there was no difference in the mortality of *Ephesia kuehniella* (Mediterranean flour moth) following the application of *Metarhizium anisopliae* when larvae were maintained at 55% or 75% relative humidity. Whereas, increasing incubation temperature of treated larvae from 20°C to 30°C caused *E. kuehniella* mortality to double.

As discussed in detail in chapter 3, temperature can have a significant effect on the ability of an EPF to germinate and grow. The temperature requirements of EPF varies depending on the strain being applied but most EPF have an optimum temperature within the range of 23-28°C and temperatures above 30°C generally significantly reduce rates of germination and growth (Jaronski, 2009). Short exposures to thermal extremes can have a significant effect on the viability of conidia. For example, *in vitro* exposure of *Cordyceps fumosoroseus* conidia to 40°C for one hour, either before 18 hours incubation at 25°C, or halfway through the incubation period, resulted in 30% reduction in conidial viability compared to control conidia regardless of when the temperature stress occurred (Beris, 2021). Additionally, exposure of EPF to heat shock during mycelial growth can result in the production of conidia that are more tolerant of temperature stress and more virulent towards target hosts (Rangel *et al.*, 2015). Temperature also affects the speed of kill achieved by the EPF by directly affecting rate of enzymatic degradation of the cuticle and growth throughout host haemolymph post-penetration (Tang and Hou, 2001).

Temperature also influences susceptibility of insect pests following the application of chemical pesticides. In constant temperature assays, exposure to temperatures between 17-37°C resulted in increasing or decreasing susceptibility of *Diaphorina citri* (Hemiptera: Psyllidae) to several insecticides. Toxicity of the neonicotinoids acetamiprid, imidacloprid

and thiamethoxam increased with increasing temperature, whilst the pyrethroids zeta-cypermethrin, fenpropathrin, and lambda-cyhalothrin were less effective with increasing temperature (Boina *et al.*, 2009). Short periods of exposure to high temperatures can also have a significant effect on the susceptibility of insects to chemical insecticides. Heating *Frankliniella occidentalis* (western flower thrips) to 45°C for two hours before returning to 25°C increased their susceptibility to acetamiprid, spinosad, beta-cypermethrin and methomyl as adults and second instar nymphs. Susceptibility peaked following varied periods of recovery ranging from 2-36 hours which was found to relate to fluctuating activity of important metabolic detoxification enzymes, which are known to be inhibited by high temperatures (Zhang *et al.*, 2016). A similar effect was found in *Bemisia tabaci* whereby exposure of adults to 35°C for two or six hours increased susceptibility of *B. tabaci* to thiamethoxam and correlated with a decrease in expression of six P450 genes, which are associated with detoxification (Guo *et al.*, 2018).

In order to use mixtures for pest control and exploit positive interactions between control agents such as those described in chapter 4, several incremental steps are needed to determine both the relationship of interactions occurring between the control agents and how increasing complexity of the system can affect those interactions. As entomopathogenic fungi, insect pests and the host plant respond and interact differently when exposed to different environmental conditions, it is reasonable to assume that interactions between co-applied chemical and microbial mixtures may change under different conditions, though the effect of abiotic conditions on chemical-microbial mixtures has not yet been investigated. Whilst the effects of some abiotic conditions could be tested under laboratory conditions, the multitude of other influential factors affecting control efficacy in the greenhouse can cause disparaging results when comparing laboratory assays to greenhouse trials. Aside from differences in abiotic conditions, differences in application method, crop architecture, and presence of other natural enemies or competitors are just some of the factors that could affect the ability of a mixture to control a pest population (Gonzalez *et al.*, 2016). For example, combined applications of *B. bassiana* and imidacloprid were determined to result in antagonism or additivity when applied to *Thrips tabaci* in laboratory based leaf-dip bioassays. However, application of the same concentration combinations in nine mixture treatments resulted in only additive mortality in greenhouse applications (Al-Mazraáwi, 2006). In some cases, laboratory experiments can identify successful mixture combinations which show unchanging interactions whether applied on a laboratory or greenhouse scale. For example, combinations of the pyrethroid insecticide, bifenthrin, and the fungus *B. bassiana* were found to be synergistic in laboratory and greenhouse bioassays against the annual bluegrass weevil (*Listronotus maculicollis*) (Wu *et al.*, 2017).

In this study, the overall aim was to determine the effect of combined applications of the chemical insecticide spiromesifen and the EPF *Cordyceps farinosa* when co-applied against *T. vaporariorum* nymphs in greenhouse experiments.

The component objectives were;

- i) Determine the effect of concentration combinations previously identified as having additive and synergistic interactions when co-applied against *T. vaporariorum* nymphs in laboratory based bioassays, when scaled up for application in a greenhouse trial.
- ii) Investigate the effect of combined applications on total *T. vaporariorum* mortality and rate of mortality when applied to aubergine plants under greenhouse conditions.
- iii) Determine types of interactions, if any, between components of the mixture at two mixture concentration combinations.

6.2 Materials and Methods

6.2.1 Greenhouse trial design

Greenhouse trials were conducted in the GroDome at UKCEH Wallingford during May and June 2021. The GroDome is a polycarbonate greenhouse structure containing several chambers where light and temperature conditions can be controlled. Aubergine (*Solanum melongena* var. Paris; Ramiro Arnedo, Spain) seedlings were prepared as described in section 2.2. Once seedlings were 8 weeks old, they were re-planted in 20L plastic bag plant pots (Hadopots Ltd; Polythene plant pots).

Large plant pots were prepared by mixing 10L compost (John Innes Seed and Cutting compost) and perlite (Swiftair Ltd; standard perlite 2-5mm) in a 3:1 ratio. Perlite was used to increase water retention of the compost. Capillary matting (Nutley's Aquamat capillary matting) was cut into strips (approximately 60cm long, 5cm wide). Two strips were inserted into the plant bags amongst the compost and pushed through the drainage holes at the bottom of the bags. At least 30cm of capillary matting was exposed underneath each bag.

A watering system was prepared by filling 55L capacity black plastic containers (Bigdug; Eco Recycled Plastic Black Attached Lid Containers) with 20-30L water. Made-to-fit white acrylic lids were placed on top of the water containers. Each lid had twelve 10cm diameter holes cut into the acrylic. Six bags, each containing one aubergine plant, were placed evenly across the lid with the strips of capillary matting exposed below each plant bag threaded through the holes in the acrylic lid and inserted into the water (see figure 6.1). During the course of the study, roots from each plant emerged through the drainage holes in the plastic pots and extended down the capillary matting towards the water source.

Fertiliser was added to the water containers once a week (Phostrogen®All-purpose plant food; 1g/L). Water levels were monitored every week and supplemented when required. Plants were supported by bamboo canes secured with jute twine (Tildenet Ltd Brown Jute Twine). Aubergine plants were used in greenhouse trials when they were 12 weeks old and approximately 60-70cm tall with at least 9 leaves unfolded on the main shoot classified as BBCH-19 (Meier *et al.*, 2009).

Forty aubergine plants were prepared for each greenhouse trial. A pooter was used to place 20-30 whitefly adults inside a perforated polythene bread bag (30 x 20 cm) which was then placed over one leaf per plant. The leaves chosen were newly emerged and of similar size (approximately 250 cm²). The bread bags were secured using autoclave tape, which was more easily removed without damaging the plant than sellotape. The bread bags were left on

the leaves for 24 hours. After this period, adult whitefly were removed using a pooter and eggs were left to develop on the aubergine leaves *in situ*. Whitefly infested plants were then maintained in the CT room at 24°C on a 16:8H light cycle. Before treatment application, an approximation of the number of *T. vaporariorum* nymphs on each leaf was made and plants were grouped into replicates of six. Each treatment had at least 300 nymphs across the six target leaves, though actual counts were much higher in some treatments.

In the laboratory based experiments, 1 mL applications delivered uniform spray coverage onto the central 1590mm² area under the bench top sprayer. In order to scale the application volume to a larger leaf surface, leaf length and width was measured and an approximate area of the leaf surface was calculated (length x width) for leaves used in both trials. It was determined by visual inspection that a 10mL application provided a similar coverage of the leaf compared to that of the laboratory bioassay. Thirty six plants were used in each greenhouse trial. Applications of the LC₅₀ and LC₈₀ of *C. farinosa* and the LC₁₅ of spiromesifen were applied individually and as mixture treatments. The control group was treated with 0.03% Tween 80.

In order to collect enough conidia for the greenhouse trials, a similar procedure was followed to that described in section 2.3.3, although conducted on a larger scale. Ten cryovials of *C. farinosa* were removed from storage in the -80°C freezer and spread across 20 Petri dishes containing 10mL SDA. Plates were sealed with Parafilm® and incubated at 25°C for 14 days in the dark. Following this, conidia were collected by adding 3mL Tween 80 (0.03%) onto each Petri dish and agitating the surface using a sterile pestle. As before, conidial suspensions were filtered through double folded muslin cloth, but suspensions were collected in multiple 50mL falcon tubes rather than smaller tubes used in previous assays. Suspensions were left on ice in the 4°C fridge overnight. The following day, conidia had concentrated at the bottom of each tube and the lighter coloured supernatant at the top of the suspension was removed. Suspensions were re-agitated on a vortex for 2 minutes before being combined in one 50mL falcon tube before enumeration of conidia. Stock suspensions of double an LC₈₀ and LC₅₀ of *C. farinosa* were prepared by serial dilutions of the original concentrated suspension, following enumeration of conidia using an Improved Brightline Neubauer haemocytometer (x400 magnification).

A stock solution of spiromesifen was prepared using methods described in section 2.4.1. Then, replicate samples of 5mL of double the target concentration of spiromesifen (LC₁₅) were prepared in 18 x 25mL falcon tubes by diluting the stock solution appropriately. These aliquots were then combined with 5mL double the concentration of the LC₅₀ of *C. farinosa*, double the concentration of the LC₈₀ of *C. farinosa* or sterile deionised water. Treatments of

the LC₅₀ and the LC₈₀ of *C. farinosa* alone were prepared using the same approach and combined with 5mL Tween 80 (0.03%). Treatment combinations are presented in Table 6.1. For each greenhouse trial, this resulted in 36 x 25mL tubes containing 10mL of treatment.

Table 6.1 Lethal concentrations calculated by probit analysis in chapter 5 and used in mixture treatments for combinations of spiromesifen and *Cordyceps farinosa* in greenhouse trials.

Mixture treatment	Concentration of Spiromesifen (mg mL ⁻¹)	Concentration of <i>C. farinosa</i> (conidia mL ⁻¹)
LC ₁₅ Spiromesifen x LC ₅₀ <i>C. farinosa</i>	0.04	2.3 x 10 ⁶
LC ₁₅ Spiromesifen x LC ₈₀ <i>C. farinosa</i>	0.04	3.5 x 10 ⁸

Treatments were applied using the spray gun from the portable spray tower (section 3.2.1); this can be detached from the acrylic cylinder which forms the tower and used separately as a handheld spraying device. For each spray application, target leaves were turned abaxial side upward whilst holding the spray gun 20cm away. During the spray application the spray gun was moved constantly across the surface of the leaf to provide an even coverage of liquid. The reservoir on the spray gun was only able to hold a maximum volume of 5mL so two applications of 5mL were necessary to cover the leaf with minimal run-off. All spray applications were conducted in a class II safety cabinet before plants were left to dry on the laboratory bench top and subsequently transferred to the GroDome.

Plants sprayed with the same treatment were placed on top of the same water container, as described previously. Individual treated leaves were covered by bread bags for the duration of the experiment. The containers supporting six replicate plants for each treatment were placed randomly within a GroDome chamber (8.54m x 6.77m) and covered by an aluminium ‘fruit cage’ structure wrapped in voile fabric. Aluminium cages were assembled using aluminium tubing from Harrod Horticultural Ltd [(1.46m x 16mm) x (0.65m x 16mm) x (0.96m x 16mm)] and using three-way ‘slot and lock’ connectors. The cage was covered by attaching voile fabric (Megachest Fabrics & Curtains) to the aluminium poles by cable ties, leaving a detachable section of fabric at the front of each cage to allow access for trial monitoring and assessments. The detachable section of fabric was secured at the top of the cage using cable ties and Velcro placed at the bottom of the cage and each side (see figure 6.2).

6.2.2 Determining the effect of conidial deposition during spray application of *Cordyceps farinosa* alone and in combination with spiromesifen in greenhouse trials

During spray application, three coverslips (22 x 22 mm) were randomly attached to the surface of the leaf ensuring that they did not cover any target *T. vaporariorum* nymphs (see figure 6.3). Following treatment application, cover slips were removed and placed inside 50mL falcon tubes holding 3mL of Tween 80 (0.03%). Calibration of conidia deposition per unit area was conducted following the same procedure described in section 3.2.3.

6.2.3 Monitoring environmental conditions during greenhouse trials

Environmental conditions in the GroDome were monitored using data loggers (Lascar electronics Ltd; EL-SIE-1). Three data loggers were placed within bread bags alongside randomly selected treated leaves, suspended on bamboo canes, to closely monitor conditions surrounding the leaf (see figure 6.4).

Following extreme temperatures during the first greenhouse trial, the second trial was set up in a smaller GroDome chamber which was more capable of quickly reducing the room temperature despite high external temperatures (4.26m x 8.86m).



Figure 6.1 Aubergine plants used in greenhouse trials. 55L capacity black plastic containers (Bigdug; Eco Recycled Plastic Black Attached Lid Containers) were filled with 20-30L water. Made-to-fit white acrylic lids supported six plants per container. Each lid had twelve 10cm diameter holes cut into the acrylic. Strips of capillary matting within each plant pot emerged from below the pots, through the holes in the acrylic lid and into the water in the container.



Figure 6.2 Randomly arranged aubergine plants infested with *Trialeurodes vaporariorum* nymphs and maintained within aluminium cages covered in voile fabric in greenhouse trials. Aluminium cages were assembled using aluminium tubing (Harrod Horticultural Ltd; (1.46m x 16mm) x (0.65m x 16mm) x (0.96m x 16mm) and three-way slot and lock connectors). Voile fabric was attached by cable ties, leaving a detachable section of fabric at the front of each cage to allow access for trial monitoring. The detachable section of fabric was secured at the bottom and side of the cages using Velcro.



Figure 6.3. Randomly placed 22 x 22 mm² coverslips on the abaxial side of an aubergine leaf sprayed with 10mL of a treatment of *Cordyceps farinosa*, spiromesifen or a mixture of these components in greenhouse trials.



Figure 6.4 Data loggers (Lascar electronics Ltd; EL-SIE-1) used in greenhouse trials. Data loggers were placed within bread bags alongside randomly selected treated leaves, suspended on bamboo canes, to closely monitor conditions surrounding the leaf.

6.2.4 Monitoring greenhouse trials

Plants were monitored every 48 hours. Leaves were visually inspected using a hand held lens and whitefly mortality was counted with the aid of a clicker counter on days 4, 7, 10 and 14.

6.2.5 Analysis

Total mortality of *T. vaporariorum* at the end of each trial was corrected for control mortality using the Schneider-Orelli approach (section 2.5.3). Differences in total mortality at the end of the bioassay between treatments and between bioassays were determined by ANOVA. Following a significant result, multiple pairwise comparisons were made with p values adjusted using the Bonferoni method. Significant differences between repeats of the greenhouse trials were determined using a two way ANOVA.

Interactions between mixture components were determined using the χ^2 analysis. Expected mortality was calculated assuming no interaction and Bliss independence of the components. A χ^2 value was calculated using Equation 12. The interaction was determined by the difference in observed compared to expected mortality. The calculated χ^2 value was compared to the χ^2 table value for 1 df to determine significance (Finney, 1971).

Time to 50% mortality (LT_{50}) was calculated by probit analysis using the DRC package in R. Significant differences between estimates LT_{50} values were identified using pairwise t-tests in the DRC package.

Differences between conidial depositions of each replicate within each treatment and between trials were determined by ANOVA. The relationship between conidial deposition and *T. vaporariorum* mortality was determined by fitting linear and polynomial models and comparing them.

6.3 Results

6.3.1 The effect of simultaneous applications of *Cordyceps farinosa* and spiromesifen on *Trialeurodes vaporariorum* mortality in greenhouse trials

In the first greenhouse trial, the lowest number of nymphs counted across six replicate leaves was within the control treatment where 380 nymphs were treated. Leaves with fewer nymphs were intentionally chosen for the control group, keeping leaves with larger numbers of nymphs for the treatment applications. The total number of nymphs across six replicate leaves for treatments of single and mixture treatments ranged from 683 – 1295. Before treatment application in trial one, leaves measured an average of $250 \pm 124 \text{ cm}^2$. Leaves in trial two had an average surface area of $274 \pm 105 \text{ cm}^2$. The same application volume was used in both trials (10mL).

Mortality of nymphs was observed as early as four days after application of treatments in both greenhouse trials. However, significant differences were observed in total mortality between treatments applied in the first and second greenhouse trial ($F=2.65$, $df=5$, $p=0.032$). Therefore, trials were analysed separately.

In the first greenhouse trial, control mortality was 1.8% across the six replicate plants. Applications of an LC_{50} or LC_{80} concentration of *C. farinosa* resulted in $43 \pm 10.7 \%$ and $38 \pm 23\%$ respectively, though these mortalities were not significantly different ($p=0.58$). Mixture applications of LC_{15} spiromesifen and the LC_{50} of *C. farinosa* resulted in fewer *T. vaporariorum* mortalities than expected due to a significant antagonistic interaction (figure 6.5). Whereas, the mixture treatment involving the LC_{15} of spiromesifen and LC_{80} *C. farinosa* resulted in an additive response (Table 6.2, figure 6.6).

Variation between replicates of spiromesifen treatments was less than EPF treatments. Rate of kill was not significantly different between treatments of singly applied control agents and the application of a mixture treatment did not increase or decrease LT_{50} estimates significantly (Table 6.3).

In the second greenhouse trial, the total number of *T. vaporariorum* nymphs per treatment ranged from 779 - 1059. Again, the lowest number of nymphs were selected for the control group though the difference in number of nymphs in the control and treatment groups were not as significant as in the first trial. During the second greenhouse trial, five leaves in total from across all treatments except the control showed signs of premature senescence and detached from the plant. These leaves were not included in the analysis. Control mortality was 8.7% across six replicate plants by the end of the experiment. In this trial, applications

of LC₁₅ spiromesifen and LC₈₀ *C. farinosa* resulted in higher mortality than the previous greenhouse trial, though mortality of *T. vaporariorum* treated with LC₅₀ *C. farinosa* was similar. Applications of LC₅₀ or LC₈₀ of *C. farinosa* caused $41 \pm 15.4\%$ and $65 \pm 23\%$ mortality respectively which was determined to be significantly different ($p=0.02$). Similarly to the previous trial, mixture applications of LC₁₅ spiromesifen and the LC₅₀ of *C. farinosa* resulted in fewer *T. vaporariorum* mortalities than expected due to a significant antagonistic interaction (figure 6.7). Additionally, the mixture treatment involving the LC₁₅ of spiromesifen and LC₈₀ *C. farinosa* also resulted in an additive response, as seen in the previous trial (figure 6.8). The highest mortality for all treatments was observed following the application of LC₁₅ of spiromesifen and LC₈₀ *C. farinosa*.

Rate of infection was fastest in *T. vaporariorum* nymphs treated with a mixture of the LC₁₅ of spiromesifen and LC₈₀ *C. farinosa*, though this increase in rate of mortality was determined not to be significant compared to the application of individual components of the mixture (see Table 6.4).

Table 6.2 Observed and expected mortality of *Trialeurodes vaporariorum* at the end of two 14 day greenhouse trials following the applications of two lethal concentrations (LC) of the entomopathogenic fungus, *Cordyceps farinosa* and one concentration of the chemical insecticide, spiromesifen. Both control agents were applied independently and in mixture treatments. Expected mortality was calculated assuming Bliss independence and using observed data from the application of single control agents. Significant differences between expected and observed mortality was determined through χ^2 analysis.

Concentration (LC)		Trial 1				Trial 2			
Spiromesifen	<i>C. farinosa</i>	Observed (%)	Expected (%)	Chi squared value	Response	Observed (%)	Expected (%)	Chi squared value	response
LC ₁₅	0	11.0	NA			34.2	NA		
0	LC ₅₀	43.2				41.0			
0	LC ₈₀	38.5				65.0			
LC ₁₅	LC ₅₀	21.9	49.3	15.23	antagonistic	43.9	61.2	4.89	antagonistic
LC ₁₅	LC ₈₀	41.0	45.2	0.39	additive	71.0	76.9	0.45	additive

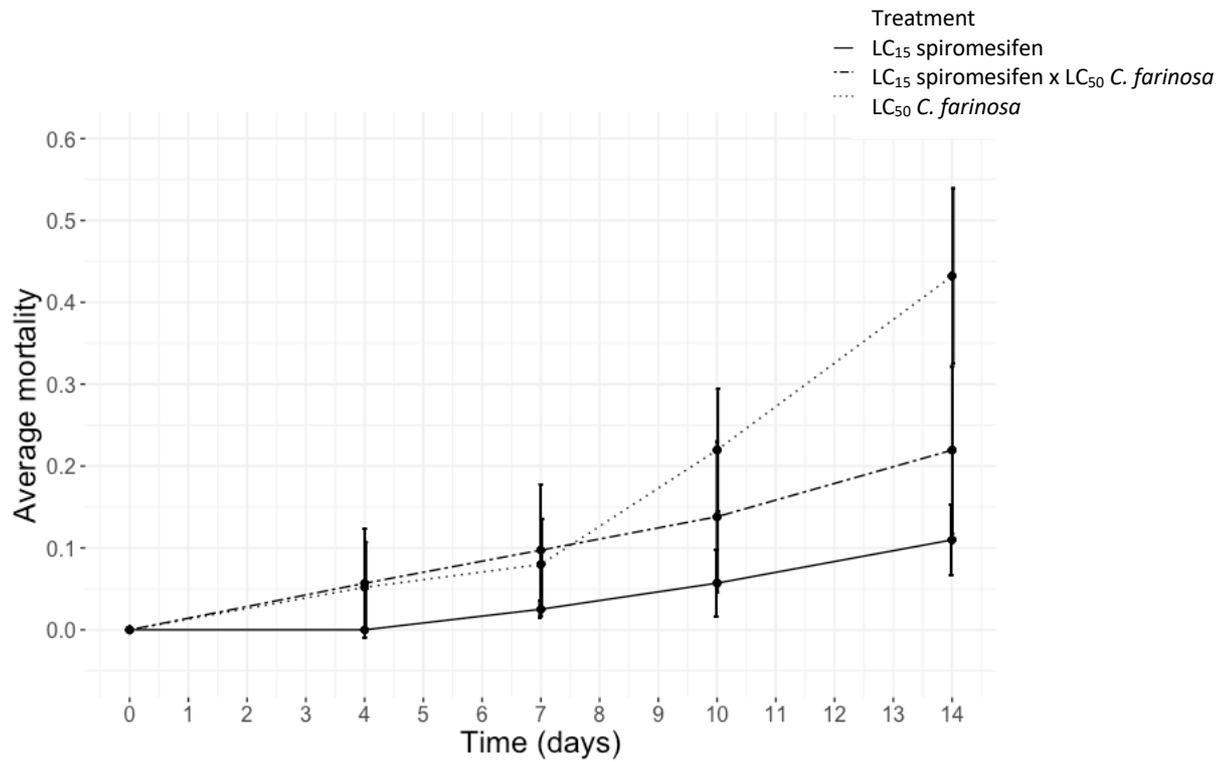


Figure 6.5 Average cumulative mortality of third instar *Trialeurodes vaporariorum* nymphs exposed to the LC₁₅ of spiromesifen, the LC₅₀ of *Cordyceps farinosa* or a combination of both treatments in a simultaneous application during the first greenhouse trial. Standard deviation is shown by error bars. Mixture treatment resulted in significant antagonism by day 14.

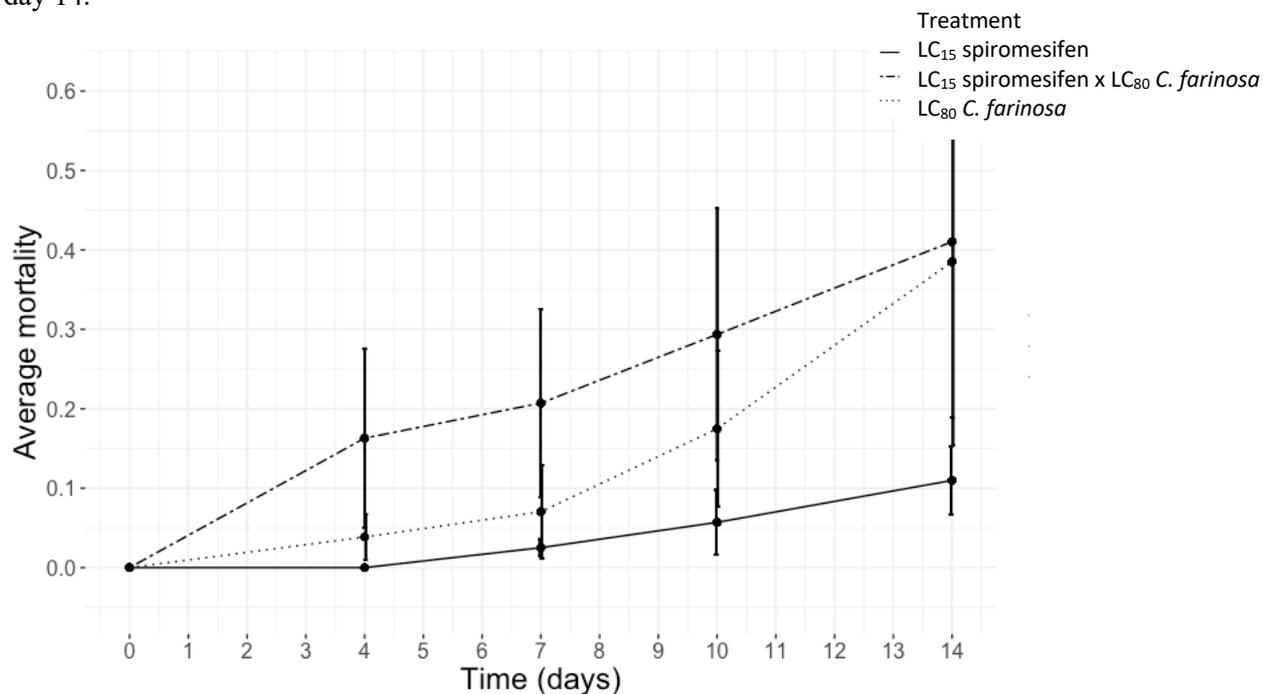


Figure 6.6 Average cumulative mortality of third instar *Trialeurodes vaporariorum* nymphs exposed to the LC₁₅ of spiromesifen, the LC₈₀ of *Cordyceps farinosa* or a combination of both treatments in a simultaneous application during the first greenhouse trial. Standard deviation is shown by error bars. Mixture treatment resulted in additivity by day 14.

Table 6.3 Total corrected mortality observed by the end of the first greenhouse trial and LT_{50} values following the application of *Cordyceps farinosa*, spiromesifen or a combination of these against third instar *Trialeurodes vaporariorum*. Mortality was corrected using the Schneider-Orelli approach. LT_{50} values were calculated by probit analysis. Significant differences between mortalities were determined by pairwise t-tests.

Spiromesifen	<i>C. farinosa</i>	Total average mortality \pm SD	LT_{50} (days) \pm SE
LC_{15}	0	0.11 ± 0.043 a	20.82 ± 1.26
0	LC_{50}	0.43 ± 0.11 b	14.69 ± 0.55
0	LC_{80}	0.39 ± 0.23 b	15.38 ± 0.81
LC_{15}	LC_{50}	0.22 ± 0.10 a	20.10 ± 2.20
LC_{15}	LC_{80}	0.41 ± 0.22 c	15.07 ± 1.46

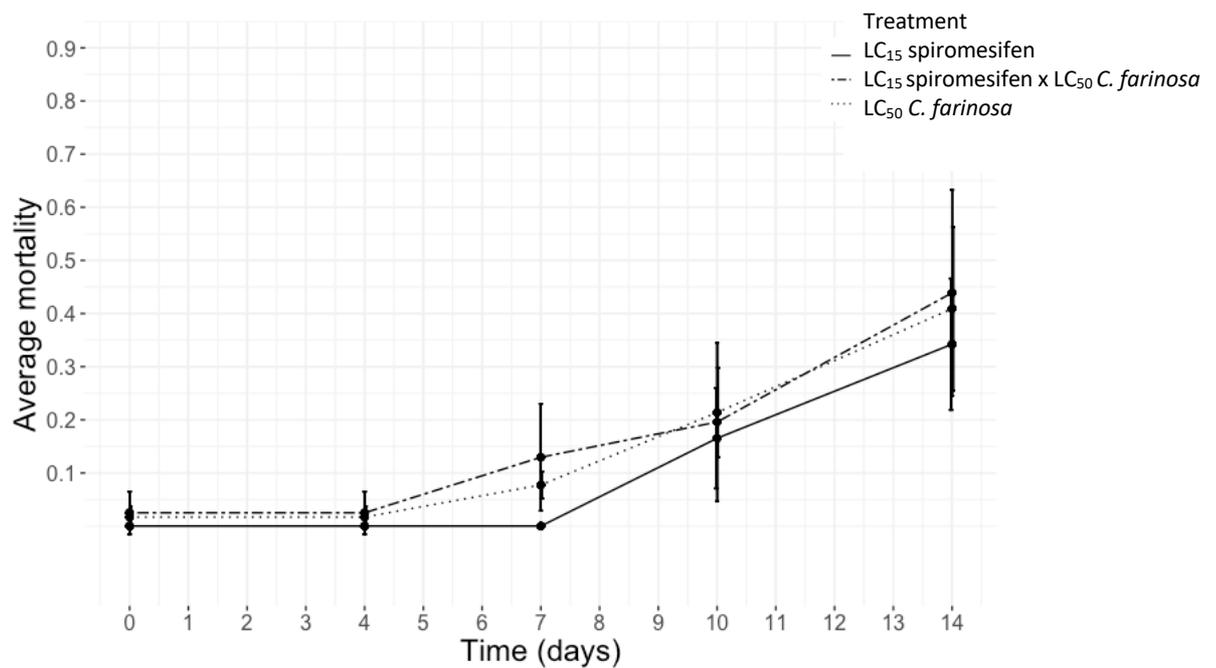


Figure 6.7. Average cumulative mortality of third instar *Trialeurodes vaporariorum* nymphs exposed to the LC_{15} of spiromesifen, the LC_{50} of *Cordyceps farinosa* or a combination of both treatments in a simultaneous application during the second greenhouse trial. Standard deviation is shown by error bars. Mixture treatment resulted in significant antagonism by day 14.

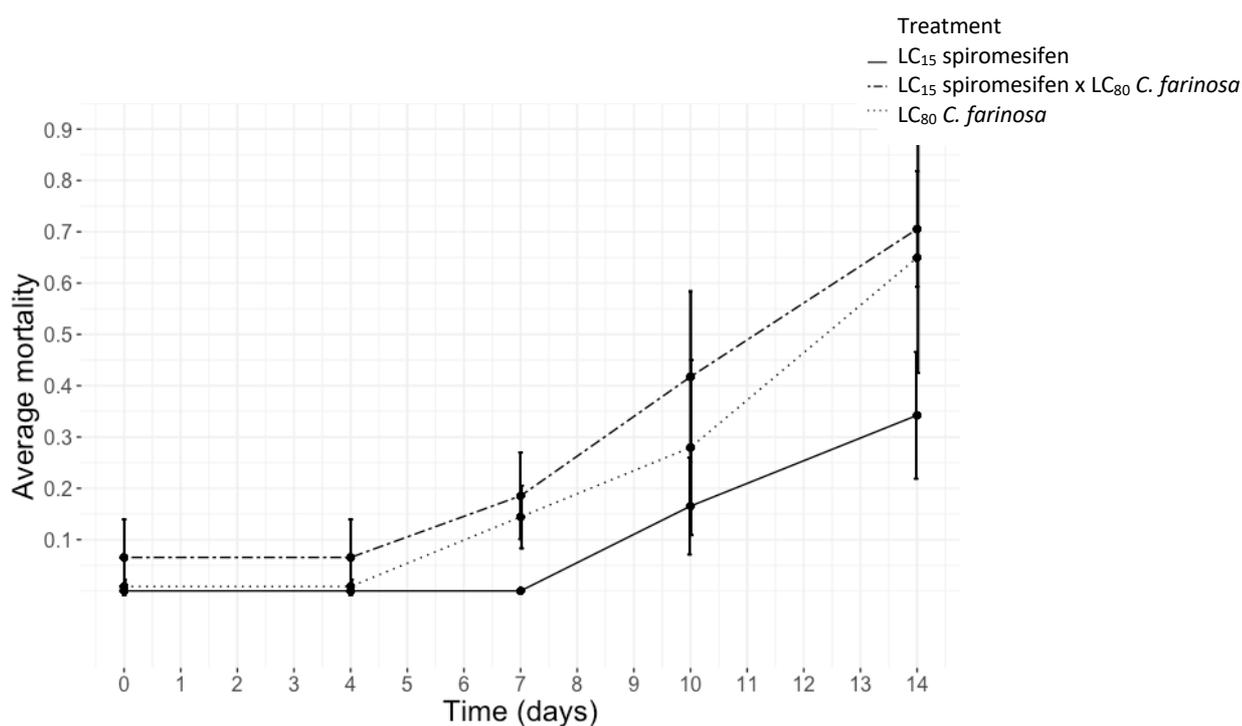


Figure 6.8 Average cumulative mortality of third instar *Trialeurodes vaporariorum* nymphs exposed to the LC₁₅ of spiromesifen, the LC₈₀ of *Cordyceps farinosa* or a combination of both treatments in a simultaneous application during the second greenhouse trial. Standard deviation is shown by error bars. Mixture treatment resulted in additivity by day 14.

Table 6.4 Total corrected mortality observed by the end of the second greenhouse trial and LT₅₀ values following the application of *Cordyceps farinosa*, spiromesifen or a combination of these against third instar *Trialeurodes vaporariorum*. Mortality was corrected using the Schneider-Orelli approach. LT₅₀ values were calculated by probit analysis. Significant differences between mortalities were determined by pairwise t-tests.

Spiromesifen	<i>C. farinosa</i>	Total average mortality ± SD	LT ₅₀ ± SE
LC ₁₅	0	0.34 ± 0.12 a	14.39 ± 0.51
0	LC ₅₀	0.41 ± 0.15 a	14.09 ± 0.48
0	LC ₈₀	0.65 ± 0.23 b	11.90 ± 0.42
LC ₁₅	LC ₅₀	0.44 ± 0.19 a	13.96 ± 0.87
LC ₁₅	LC ₈₀	0.71 ± 0.11 b	10.89 ± 0.49

6.3.2 Determining the effect of conidial deposition during spray application of *Cordyceps farinosa* alone and in combination with spiromesifen in greenhouse trials

Average conidial deposition did not differ for each treatment between repeats of the greenhouse trial ($F=1.93$, $df=3$, $p=0.14$). Additionally, there were no significant differences between conidial counts for applications of the same concentration of *C. farinosa* whether applied individually or in combination with the LC₁₅ spiromesifen (see Table 6.5). However, significantly higher conidial deposition was observed following the treatment of the LC₈₀ of *C. farinosa*, as a mixture or individually applied compared to the LC₅₀ of *C. farinosa* when applied individually or as a mixture. There was large variation between conidial counts for replicates within each treatment for both greenhouse trials.

Despite conidial deposition being significantly higher for treatments of the LC₈₀ of *C. farinosa* compared to the LC₅₀, there was no relationship between conidia received (per mm²) and total mortality in the first greenhouse trial between replicates of the same treatment and between treatments of different *C. farinosa* application concentrations ($F_{1,22}=0.83$, $p=0.37$). There was a significant linear relationship between conidial deposition and total *T. vaporariorum* mortality in the second greenhouse trial ($F_{1,19}=13.01$, $p=0.0019$, $R^2=0.38$), whereby increasing conidial deposition resulted in higher *T. vaporariorum* mortality. Modelling the data with a second, third or fourth order polynomial did not improve the fit compared to the linear model.

Table 6.5. Average conidial deposition during application of *Cordyceps farinosa*, or a mixture of *Cordyceps farinosa* and spiromesifen on aubergine leaves during two greenhouse trials.

Treatment		Conidial deposition (conidia mm ⁻²) ± SD		Equivalent field deposition (conidia ha ⁻¹)	
Spiromesifen	<i>C. farinosa</i>	Trial 1	Trial 2	Trial 1	Trial 2
LC ₁₅	0	NA	NA	NA	NA
0	LC ₅₀	45 ± 12	48 ± 11	4.5 x10 ¹¹	4.8 x10 ¹¹
0	LC ₈₀	588 ± 90	468 ± 119	5.8 x10 ¹²	4.7 x10 ¹²
LC ₁₅	LC ₅₀	57 ± 8.3	50 ± 13	5.7 x10 ¹¹	5.0 x10 ¹¹
LC ₁₅	LC ₈₀	591 ± 59	506 ± 134	5.9 x10 ¹²	5.1 x10 ¹²

6.3.3 Environmental conditions during greenhouse trials

Conditions in the greenhouse during each trial were difficult to control due to high external temperatures. During the first greenhouse trial, internal temperatures reached 48.7°C within the first 48 hours following application of treatments. The average temperature was 24.2°C with oscillations throughout each 24-hour period. Temperatures reached a maximum of 52.9°C and minimum of 19.5°C. Variation in relative humidity was not as pronounced as the temperature variation. The average humidity recorded for the duration of the trial was 81.8% with a minimum recording of 59.8% and maximum of 91.8%.

The second greenhouse trial was conducted in a smaller chamber, to improve control of environmental conditions. In the second greenhouse trial, temperatures did not reach as high as the previous trial, with a maximum temperature recording of 42.3°C. During the night, temperatures were as low as 16.3°C. The average temperature for the duration of the trial was 25.4°C. Humidity was more variable than in the previous trial; average relative humidity was 73.5%, with recordings ranging from 39.5% to 91.4%. Temperature and humidity recordings are given in Appendix VII.

6.5 Discussion

A few days prior to and during greenhouse trials, other insects were found on the experimental aubergine plants, namely thrips and aphids. Insecticidal treatments were not applied due to potential interactions with the experimental treatments. A small number of leaves were damaged due to thrips larvae (species not determined). Damaged leaves were removed before spray applications and no thrips were found in treated leaves nor was any further damage observed. In the first greenhouse trial, a small number of *Myzus persicae* were found on experimental plants on untreated leaves. Individual *M. persicae* were removed by hand and were not found on treated leaves nor was any further damage observed. However, ants (species suspected to be *Lasius niger*) entered the GroDome chamber via drainage holes and several were found inside the bread bags surrounding treated leaves. Despite moving the experiment to a different chamber, ants were also found amongst *T. vaporariorum* nymphs in the second greenhouse trial. Whilst the effect of the presence of these insects was not determined in this study, it is possible that interactions between the plant, other pests and the ants may have affected the efficacy of treatments applied.

Ants and whitefly have a mutualistic relationship whereby ants protect whitefly from parasitoids and predators whilst whitefly supply ants with energy rich honeydew (Way, 1963). Tending ants have also been shown to reduce fungal infection by naturally occurring EPF and increase whitefly adult emergence (Queiroz and Oliveira, 2001). This interaction may have influenced *C. farinosa* efficacy in the current study. Comparatively, ants may have increased the spread of conidia across the leaf surface, passively picking up conidia and infecting *T. vaporariorum* nymphs whilst moving across the leaf consuming honeydew produced by *T. vaporariorum*. As *T. vaporariorum* nymphs are immobile, conidia must be sprayed directly onto the target insect. However, there has been evidence of foraging insects dispersing EPF conidia amongst pest populations (Ekesi *et al.*, 2005). A study by Bird *et al.* (2004) found that ants (*L. niger*) were capable of vectoring *Akanthomyces longisporum* (previously *Lecanicillium*) to rosy apple aphids (*Dysaphis plantaginea*) when artificially inoculated with conidia. Conidia were passively carried on ant tarsi, mandibles and antennae, causing infection of *D. plantaginea* in laboratory, semi field and field trials.

The presence of additional plant pests may potentially also induce a ‘bodyguard interaction’ between the plant and the EPF (Elliot *et al.*, 2000). In this situation, high pest numbers induce the production of plant volatiles which can increase the susceptibility of pests to EPF’s. This tri-trophic interaction was observed in a recent study by Lin *et al.* (2021), where the plant volatiles produced by tomato plants in the presence of feeding of *Bemisia tabaci*

caused increased virulence of *Akanthomyces lecanii* by promoting chitinase activity of the EPF, resulting in 28% higher *B. tabaci* mortality. However, the level of pest infestation required to trigger this effect is not known.

In this study, applications of a high concentration of *C. farinosa* (LC₈₀) and the LC₁₅ of spiromesifen resulted in an additive response in mortality of *T. vaporariorum* in both greenhouse trials. In previous experiments (see chapter 5), this concentration combination resulted in synergism when bioassays were performed under laboratory conditions and maintained at a constant temperature (24°C), except in one of the five mixture bioassays, where an additive response was observed. However, laboratory mixture bioassays investigating the impact of temperature oscillation also resulted in additive mortality of *T. vaporariorum* following the application of *C. farinosa* (LC₈₀) and spiromesifen (LC₁₅). Therefore, the laboratory-based experiment investigating the effect of an oscillating temperature regime, simulating the daily variation observed in a greenhouse, was capable of predicting the interaction outcome of this mixture combination under greenhouse conditions.

Comparatively, the combination of *C. farinosa* (LC₅₀) and spiromesifen (LC₁₅) resulted in an additive response in laboratory-based experiments conducted at 24°C, but interactions were determined to be antagonistic and synergistic in separately conducted laboratory bioassays in which treatments were exposed to oscillating temperatures. Therefore, temperature had an unpredictable effect on interaction outcomes for this concentration combination. Whilst the first oscillating temperature laboratory mixture assay resulted in the same interaction outcomes as those observed in greenhouse trials, contradictory results between laboratory bioassays meant that the interaction outcome for the combination of *C. farinosa* (LC₅₀) and spiromesifen (LC₁₅) under greenhouse conditions could not be accurately predicted.

The MixTox analysis (chapter 5) showed that there was a dose ratio dependent relationship between the two control agents, with synergistic interactions becoming antagonistic in mixtures where the ratio of the effective concentration of *C. farinosa* to spiromesifen was less than 1: 0.012. Experiments conducted in the laboratory indicated that temperature can significantly affect the ‘switching point’ in the ratio of *C. farinosa*: spiromesifen.

The antagonistic interaction observed following the application of LC₅₀ *C. farinosa* and LC₁₅ spiromesifen could be explained if the dose of *C. farinosa* reaching *T. vaporariorum* was reduced relative to spiromesifen, though dose received of spiromesifen was not determined in this study. As discussed in chapter 5, spiromesifen has a negative interaction with *C. farinosa* at higher relative concentrations.

It was noted during experimental setup that some spray applications resulted in even coverage of the leaf surface but treatments applied to smaller leaf areas were beyond runoff point. It is not recommended to apply mycopesticides beyond runoff as EPF conidia are washed off the target area. Though, in the current study, the number of conidia per unit area counted on cover slips was not significantly different between replicates of the same treatment. The three coverslips sprayed during each treatment application were combined in Tween 80 solution, therefore differences in conidial deposition across the leaf surface were not determined. There may have been variation in dose received per *T. vaporariorum* nymphs following hand-held application of treatments, unlike in bioassays using the spray tower application. However, these small variations more closely emulate coverage of greenhouse crops using a knapsack sprayer, where leaf coverage is varied due to overlapping leaves and differences in angle of application (Hill, 1987).

Differences between mortality in the first and second greenhouse trial cannot be attributed to conidial deposition. Despite significant differences in conidial deposition for single applications of LC₈₀ and LC₅₀ in the first trial, total *T. vaporariorum* mortality was not significantly different. Whereas, a linear relationship between conidial deposition and mortality was found for single applications of *C. farinosa* in trial two.

Total mortality was significantly lower in the first greenhouse trial, which may have been caused by exposure to extreme temperatures. In the first trial, temperatures reached >50°C in the first 48 hours following application of treatments. Thermal profiling experiments (described in Chapter 4) and estimates from Lactin-1 non-linear models indicated that the maximum temperature for growth and germination for *C. farinosa* was 32°C and 33.5°C respectively. Above these temperatures, it was predicted that the EPF will not be able to develop, therefore affecting its ability to infect an insect host. However, it is possible that above 50°C, irreversible damage could have been caused, reducing conidial viability by denaturing essential EPF enzymes. Chai *et al.* (2014) found that exposing conidia of *Cordyceps fumosoroseus*, *B. bassiana* and three strains of *Metarhizium anisopliae* to 50°C for 15 minutes before returning to ambient temperature resulted in no *in vitro* conidial germination. However, the three *M. anisopliae* strains were able to withstand 45°C for up to 30 minutes with conidial viability reducing only by 20% compared to optimal germination conditions. In addition, a study by Jaronski, Keyser and Roberts (unpublished data, reported in Jaronski 2010) demonstrated that EPF growth does not always resume upon return to moderate temperature conditions under temperature oscillation. In that study, exposure of *M. anisopliae* or *B. bassiana* to 6 hours at 41°C or 3 hours at 44°C resulted in up to 24 hour delays in resuming growth when returned to 27°C. The length of the delay correlated with

increasing temperature exposure and the length of time for the exposure (Jaronski, 2010). Therefore, the extreme temperature oscillations observed in trial one may have been detrimental to EPF virulence despite average temperature being similar for both trials.

Cordyceps farinosa was also exposed to temperatures above the thermal maxima in trial two, however, the maximum temperature within the first 72 hours was 39°C. Germination results for *C. farinosa* in *in vitro* studies (chapter 4) estimated GT₅₀ values ranging from 11-27 hours at temperatures of 15-32°C. Similarly, (Jaronski, 2010) reported that most EPF conidia invade their host within 24 hours. In this study, EPF induced mortality was observed as early as four days post-application. Therefore, conditions prior to this time point, during the activation and germination period, may have influenced conidial viability in the first trial, resulting in reduced overall mortality.

Once inside the host, EPF are still influenced by temperature but indirectly, through its effect on the insect host. Temperature shock has been shown to increase the hosts' ability to fight infection from EPF. *Galleria mellonella* infected with *B. bassiana* and exposed to 43°C for just 15 minutes resulted in increased lysozyme activity in host haemolymph and prolonged host survival. By day 8, 60% of *G. mellonella* had succumbed to infection whereas 80% of those not exposed to a temperature shock had died (Wojda *et al.*, 2009).

Humidity was variable for the duration of the bioassay with the lowest recording of 59.8% and 39.5% in the first and second greenhouse trial respectively. Whilst humidity is one of the most important factors influencing EPF efficacy, ambient humidity is known to have little impact on the level of pest control achieved. For example, Fargues *et al.* (2003) found similar levels of infectivity of *T. vaporariorum* by *B. bassiana* and *A. lecanii* on greenhouse tomato crops under oscillating humidity conditions. The number of hours spent below 60% and above 90% relative humidity was altered, with average humidity ranging from 62 to 82% in the Fargues *et al.* (2003) study. However, no significant difference in *T. vaporariorum* mortality was found (>85% mortality). Similarly, experiments by Fargues *et al.* (2005) showed that ventilation throughout the greenhouse did not affect *A. muscarium* efficacy against *T. vaporariorum* despite humidity reaching as low as 35.9% on plants found on the outermost row of the greenhouse.

Despite differences in *T. vaporariorum* mortality for each treatment applied in trial one and two, the addition of LC₁₅ spiromesifen to the LC₈₀ of *C. farinosa* (approximately 5x10¹² conidia hectare⁻¹), consistently increased total mortality with no interaction between the control agents. Recommended application rates for entomopathogenic fungi are within the

range of $1 \times 10^{13-14}$ conidia ha^{-1} (Wraight and Carruthers, 1999). However, the results from this study challenge this recommended application rate. Here, it was demonstrated that a reduced application rate of *C. farinosa* can be used simultaneously with 0.04 mg mL^{-1} spiromesifen to achieve $>70\%$ mortality of *T. vaporariorum* nymphs. Using reduced EPF concentrations will reduce application costs. The perception that successful augmentative control using EPF requires large quantities of viable conidia is one of the main factors limiting uptake of EPF for use as microbial control agents. Such coverage is expensive and restricts the use of mycopesticides to high margin crops (Jaronski, 2010). However, successful control can be achieved at concentrations under the recommended application rate of $1 \times 10^{13-14}$ conidia ha^{-1} by improved product formulation, targeted applications with the use of specialised sprayers (Jaronski, 2010) or combinations with other control agents, as shown in this study.

Results from this study also indicate that reduced EPF concentrations should be used with caution when applying alongside low concentrations of spiromesifen, as reducing the number of *C. farinosa* conidia to 5×10^{11} conidia hectare^{-1} can lead to antagonistic interactions. Determination of the mechanism causing this interaction in the mixture could improve its application, allowing growers to avoid negative interactions. Additionally, understanding the mechanisms behind positive interactions will allow for the identification of other successful mixture combinations.

6.5.1 Summary

- i) Results indicate that a low concentration of spiromesifen is compatible with a concentration of *C. farinosa* equivalent to 5×10^{12} conidia ha^{-1} and can be used in an IPM system within the greenhouse to control *T. vaporariorum*.
- ii) Reducing the concentration of *C. farinosa* relative to spiromesifen resulted in an antagonistic interaction between the mixture components and reduced overall control of *T. vaporariorum*.
- iii) Interactions between different concentration combinations of *C. farinosa* and spiromesifen were consistent between greenhouse trials despite differences in abiotic conditions.

7 General Discussion

MixTox modelling is an innovative approach that allows new insights into the effects of combining conventional and microbial biopesticides against insect pests. This improved understanding underpins the development of integrated pest management (IPM) as it enables predictions to be made of the effects of combining chemicals and biopesticides based on a mathematical model. Following selection of entomopathogenic fungal (EPF) isolates through *in vitro* and *in vivo* laboratory based experiments, the characterisation of interactions occurring with mixtures of EPF and chemical insecticides for control of the greenhouse whitefly (*T. vaporariorum*) was demonstrated using the MixTox modelling approach. This is a significant step forward from a simple ‘trial and error’ approach to developing IPM. There is a lack of published research on how different components of IPM interact and instead, most research focuses on single components of an IPM system (Stenberg 2017). This is hampering the identification of compatible groups of control agents, and in identifying different agents that have positive effects on each other.

Laboratory studies investigating effects of biopesticides on target pests are done under controlled conditions and provide useful information underpinning the development of biopesticides for successful deployment in IPM. Field or greenhouse crops present a far more complex environment than the laboratory and as such, the amount of control seen in the crop is likely to be significantly more variable than that observed in the laboratory. For example, Gwynn *et al.* (2015) found a 40% variation in effect between 10 independent trials of a biopesticide compared to only a 5% variation for a chemical pesticide standard. There is inherent variability in a biological system, in which the efficacy of the biopesticide has multiple, complex and interacting determinants, in comparison to that with a conventional chemical insecticide. The amount of variation can be reduced by increasing understanding of the system and adapting accordingly, but this requires complex and in-depth research. For example, EPF used as control agents in hot climates are often applied to crops later in the evening in order to prevent immediate exposure of conidia to high midday temperatures and relative humidity is maintained within greenhouses by periodically misting the crops (personal communication with Rocio Espejo, trials manager, BASF research station, Utrera, Spain). This project has demonstrated that a detailed understanding of EPF and chemical interactions

for control of a glasshouse pest can be obtained by the applying an ecotoxicological approach which categorises complex interactions occurring in laboratory and greenhouse trials.

7.2 Isolate selection

A series of laboratory experiments were conducted to identify EPF candidates with potential to control *T. vaporariorum* by choosing those that met several selection criteria. Ravensberg (2011) listed the most important selection criteria for the development of a pathogen into a commercial biopesticide for inundative control of pests which related to mortality, production and safety:

- (1) Mortality: dose rate, mode of action, speed of kill, host range and sensitivity to abiotic factors.
- (2) Production efficiency.
- (3) Safety: effects on humans and the environment.

In this study, the selection criteria applied to the EPF were related to lethal concentration and dose response, speed of kill, sensitivity to abiotic factors as well as the compatibility of EPF with chemicals used in IPM. Though the mass production of EPF was not investigated specifically in this project, the ease of rearing isolates on artificial media and the number of conidia produced across a range of temperatures was used as a proxy to indicate production and secondary spread of a biopesticide (Borisade and Magan, 2014; Sharififard *et al.*, 2012).

Candidate isolates were obtained from several sources and were reported to have potential as control agents against *T. vaporariorum*. Isolates had either been originally isolated from *T. vaporariorum* or the closely related whitefly species *B. tabaci*. In addition, several mycoinsecticide products were included in the study as standards. It is important to include commercially sold EPF products in research so that results are applicable to the field and can be comparable to other studies where commercial EPF are used as standards. Un-formulated EPF isolates were obtained from mycoinsecticide products to allow direct compatibility with non-product isolates.

7.2.1 Determining thermal optima and maxima of entomopathogenic fungi

Thermal optima and maxima were predicted for all EPF isolates by modelling *in vitro* growth. Predicted optima were within the range of 23-27°C, with maxima predicted at 31-33°C. Predicted temperature optima and maxima were similar to those for 22 *Beauveria*, *Akanthomyces* and *Metarhizium* isolates in a previous study (Davidson *et al.* 2003) with the exception of two *Akanthomyces* isolates predicted to have thermal maxima >38°C (Davidson *et al.* 2003). One of the main factors influencing EPF efficacy is exposure to sub-optimal or variable environmental conditions (Fargues *et al.*, 1997). In greenhouse vegetable production,

temperature can vary from below 10°C to over 30°C (Shamshiri *et al.*, 2018), though the degree of temperature variation depends on factors such as the type of crop and whether cropping is in a glasshouse or polytunnel (Verdoliva 2021). Throughout the literature, a common approach to determine the response of EPF to abiotic conditions, such as temperature, is through *in vitro* growth and germination experiments, which constitute a genotype x environment interaction (G x E) (Davidson *et al.*, 2003; Thomas and Jenkins, 1997; Yeo *et al.*, 2003). This provides useful information, but some caution is required when extrapolating the results to predict the effect of temperature on infection of insects (a genotype x genotype x environment interaction, G x G x E), as the cardinal temperatures and thermal response curve will be influenced by the thermal biology of the host and its pathogen (Davidson *et al.*, 2003; Yeo *et al.*, 2003). In order to extract useful estimates from this data, such as temperature optima and limits, the effect of temperature on growth and germination of EPF are best described using non-linear models (Smits *et al.*, 2003). Generally, the effect of temperature on a physiological response follows a bell shaped curve, often skewed slightly to the left. Several models have been developed to fit this distribution (Briere *et al.*, 1999; Lactin *et al.*, 1995; Logan *et al.*, 1976; Taylor, 1981) and data in this study was successfully described by the Lactin-1 model. In a similar study, Perry (2017) found that growth and germination data of EPF were best described using the Briere-1 model. This model is capable of predicting T0 as well as the estimates provided by the Lactin-1 model. Whilst the Briere-1 model fit the data in this project significantly, T0 estimates were misleading and not biologically relevant. In addition, estimates of temperature minima are unlikely to be necessary for EPF used in the greenhouse.

Distribution of the germination data meant that estimates of cardinal temperatures were less precise than those predicted for growth across all models tested. Germination temperature optima can be several degrees higher than that for growth (Thomas and Jenkins, 1997). This study showed that exposure to 32°C, the highest temperature tested, had a more detrimental effect on growth than germination. As a result, distribution of germination data across the temperature range tested was not bell-shaped and could not be described adequately by the same model as the growth data. In order to improve the ability of the non-linear models to fit to the germination data, a larger temperature range needed to be tested, with temperatures higher than 32°C. Germination of EPF at a range of temperatures has previously been described using the Briere-1 model, though a larger range of temperatures were tested than in the current study (Perry 2017). Further analysis demonstrated that there was a relationship between the rate of growth and germination across the temperatures tested, so cardinal temperatures for growth were ultimately used in the selection process.

7.2.2 Pathogenicity and virulence of EPF to *Trialeurodes vaporariorum*

To determine the pathogenicity and virulence of EPF in this study, an important and fundamental step was the development of a reliable method to deliver controlled, repeatable doses of conidia to a target. An affordable spray tower was constructed based on a design by Mascarin *et al.* (2013a) and calibrated using methods akin to those implemented by Silsoe Spray Applications Unit Ltd (Spence *et al.*, 2020). This is the first time that this portable tower has been used throughout a research project, providing consistent spray deposition in laboratory and greenhouse experiments. These methods are accessible to other researchers, as instructions for 3-D printing of the spray tower have been published by Erdos *et al.* (2020). Using the approach developed in this study, the volume of liquid reaching the target area and accurate quantification of the number of viable conidia mm^{-2} can be determined. Standardisation of methods across experiments aids in the dissemination of results between studies, but differences between conidia received following the application of similar concentrations using the spray tower in different laboratories highlights the importance of calibration of dose of EPF in each bioassay (Erdos *et al.*, 2021; Mascarin *et al.*, 2013a; Spence *et al.*, 2020).

The calibrated spray tower was used for bioassays to determine the pathogenicity and speed of kill of eighteen EPF candidate isolates and the virulence of five of these against *T. vaporariorum* nymphs. An essential requirement of a biopesticide is to cause significant mortality against the target pest. Ideally, there would be a fast speed of kill but this is generally not the case for pathogen-based biopesticides (Ravensberg, 2011). Pathogenicity is the ability of a pathogen to cause disease and virulence is defined as the disease producing power of the pathogen (Shapiro-Ilan *et al.*, 2005). In this study, pathogenicity of EPF to *T. vaporariorum* nymphs was determined at 1×10^7 conidia mL^{-1} . Whilst dose response curves are required to determine the virulence of an EPF against its host, mortality in pathogenicity bioassays was used as a selection criterion to identify EPF to be used in further experiments. If significantly higher conidial numbers than 1×10^7 conidia mL^{-1} were required for infection of *T. vaporariorum*, the EPF would not be suitable as a biopesticide as scaling up for commercial production would not be feasible. Generally, concentrations of EPF within the range of 10^{12} to 10^{14} ha^{-1} are applied for effective control of pests in the greenhouse or field (Jaronski, 2009); the application of 1×10^7 conidia mL^{-1} in pathogenicity bioassays resulted in a dose received by *T. vaporariorum* equivalent to that of a field dose of $\sim 3 \times 10^{12}$ conidia ha^{-1} .

- Based on temperature profiles and pathogenicity bioassays, five EPF isolates were selected for further experimentation, namely; *Akanthomyces lecanii* (ATCC 4060) *C. farinosa* (ATCC 4412), *Beauveria bassiana* (PPRI 5339) *B. bassiana* (ATCC 5278) and Met52 (*M. brunneum*).
- These isolates caused high proportion mortality of *T. vaporariorum* and faster rates of kill relative to other EPF tested.
- These isolates also displayed a range of thermal optima for growth, germination and spore production. For example, *C. farinosa* (ATCC 4412) displayed fast *in vitro* growth and germination across a range of temperatures whereas, Met 52 (*M. brunneum*) was chosen as it was predicted to have a high temperature optimum and may cause contrasting results compared to other isolates in later experiments involving temperature.

7.2.3 Compatibility of entomopathogenic fungi with chemical plant protection products

Unfortunately, it is often the case that EPF with potential for pest control identified in the laboratory, do not perform well in the greenhouse or field. In some cases, this is due to exposure to other incompatible plant protection products (PPPs). In this study, the five selected EPF were considered to be compatible with the insecticide spiromesifen as growth and germination of the fungi were not affected by the presence of the chemical in *in vitro* experiments. Spiromesifen was compatible with some EPF isolates in a study by Sain *et al.* (2019), but inhibited the growth of other EPF. Compatibility varied depending on the species and strain of the fungus (Santos *et al.*, 2018b; Sain *et al.*, 2019). In addition, several experiments were done using three fungicides commonly applied in greenhouse production. These fungicides were found to have significant insecticidal activity against *T. vaporariorum* nymphs; Luna Sensation®, Takumi SC® and Kumulus® caused 34-80% mortality in laboratory based bioassays. These findings are similar to those found by Van De Veire *et al.* (2004), where significant mortality of *T. vaporariorum* nymphs was caused by the fungicides mancozeb and maneb.

Little is known about the combined application of mixtures of fungicides and biopesticides to improve pest control although many studies have investigated the potential negative interactions between EPF and fungicides applied in IPM (Jaros-Su *et al.*, 1999; Avery *et al.*, 2013; Roberti *et al.*, 2017). *In vitro* compatibility tests identified a negative interaction

between *B. bassiana* (PPRI 5339) and the synthetic fungicides Luna Sensation® and Takumi SC®. The severity of the interaction varied with concentration of the fungicide and incubation temperature. However, Kumulus® had no effect on *B. bassiana* growth that may indicate the potential for co-application of control agents for pest and disease management in an IPM programme. Although, *in vitro* compatibility testing does not always indicate the compatibility of components under *in vivo* conditions, as the direct interactions of co-applied components would be short lived and the complex interactions occurring once the pathogen is inside the insect host cannot be accurately simulated *in vitro* (Meyling *et al.*, 2018). As a result, *in vitro* and *in vivo* compatibility are often disconnected when mixtures are applied in the greenhouse or field (Roberti *et al.*, 2017). There could be significant benefits to combined applications of control agents that target plant pests and diseases, including a reduction in labour required for separate applications and a reduction in cost. Therefore, further studies into the potential interaction of these IPM components are needed.

- Spiromesifen was determined to be compatible with five EPF isolates as growth and germination was not significantly affected by the presence of the insecticide in *in vitro* experiments.
- Growth of an EPF standard isolate was affected differentially by the presence of fungicides in *in vitro* experiments.

7.3 Determining mixture interactions

7.3.1 Co-application of EPF and chemical in laboratory and greenhouse

One of the major components of this research was the development of a MixTox modelling approach (Jonker *et al.*, 2005) for biopesticides, previously only used to describe the joint effect of chemical mixtures, and was used successfully here to describe the interactions between a chemical and a microbial pathogen. The model was used to make predictions of the interactions between the chemical and microbial pathogen based on the outcome of interactions observed in laboratory bioassays, before being tested in greenhouse trials. Using this approach, the relationship between *C. farinosa* and spiromesifen was determined to be a dose ratio dependency, where synergism occurred at high relative concentrations of *C. farinosa* and antagonism resulted from a relative increase in spiromesifen. Using the MixTox analysis enabled the switching point to be determined between synergism and antagonism related to the ratio of *C. farinosa* and spiromesifen in the mixture.

The current study showed that oscillating temperature, emulating greenhouse temperature variation, had a significant effect on the interactions of co-applied EPF and chemicals on *T. vaporariorum* mortality. These results are a valuable addition to this area of research as there is currently limited information available regarding the effect of temperature on interactions between co-applied insecticidal agents. (Inglis *et al.*, 1999). Because temperature is such an important determinant of EPF virulence, development of *T. vaporariorum* and susceptibility of *T. vaporariorum* to EPF and chemical insecticides, it was deemed likely that the interactions between EPF and spiromesifen co-applied to *T. vaporariorum* could be influenced by temperature. In a study by Inglis *et al.* (1997), the combined application of *Metarhizium flavoviride* and *B. bassiana* caused an increase in *Melanoplus sanguinipes* mortality relative to application of either EPF alone under constant temperature conditions, indicating compatibility between the control agents. However, under an oscillating temperature regime, simulating the daily oscillation of diurnal grasshopper body temperatures, co-applied EPF interacted antagonistically. Changes in temperature may have resulted in competitive interactions between the EPF. It is also possible that the oscillating temperature resulted in biochemical changes in the host which reduced the capability of both EPF to infect. Most likely, the observed antagonistic interaction was caused by a complex combination of these factors. These results are similar to those found in the current study, whereby addition of temperature variation resulted in antagonism between *C. farinosa* and spiromesifen at a concentration combination that resulted in additivity in constant temperature assays. However, in two repeats of the mixture experiment, oscillating temperature conditions resulted in significantly different interactions between mixture components. The first laboratory based mixture experiment exposed to temperatures emulating greenhouse conditions resulted in the same interactions as those observed later in both greenhouse trials. However, the second laboratory based experiment resulted in disparate results compared to the greenhouse trials. Therefore, it was difficult to predict the effect of temperature on mixture efficacy in greenhouse trials based on the laboratory based experiments. Such a lack of predictability when scaling from laboratory to glasshouse is not uncommon, with other researchers showing the co-application of fungus and chemical resulting for example in negative interactions under laboratory conditions but additive when applied under greenhouse conditions (Al-Mazraáwi, 2006). Whilst this change in interaction could have been caused by the temperature variation of the greenhouse, it is difficult to conclude as several other abiotic and biotic factors will influence interactions observed in greenhouse experiments.

Under greenhouse conditions, there were no interactions between *C. farinosa* and spiromesifen at concentrations of 3.2×10^8 conidia mL⁻¹ and 0.04mg mL⁻¹ respectively. Co-application of these control agents resulted in >70% mortality of *T. vaporariorum*. This

combination provided consistent control in two greenhouse trials whilst using reduced application concentrations compared to recommended application rates. There is an urgent need to reduce the amount of active ingredients required to sustain control due a reduction in number of active ingredients available and a desire to prevent or delay resistance development to the active ingredients that remain on the market, so identifying methods such as those explored in this project, has the potential to provide economical solutions for sustainable control in the future.

In laboratory based mixture bioassays, all combinations of spiromesifen and *B. bassiana* resulted in an additive response in mortality of *T. vaporariorum*. The compatibility between *B. bassiana* and spiromesifen in these studies indicates a potential for combined application in control of *T. vaporariorum*. As there were no significant interactions between the control agents, the efficacy of the co-application of the EPF and insecticide could be accurately predicted. The co-application of *B. bassiana* and spiromesifen resulted in a maximum of 88% mortality. In addition, applications of *B. bassiana* and spiromesifen resulted in an increase in rate of mortality of *T. vaporariorum*, reducing the amount of time that the pest would potentially be causing damage to a crop.

- Mixtures of *B. bassiana* and spiromesifen resulted in an additive response, though the rate of mortality was significantly increased compared to applications of control agents independently.
- A dose ratio relationship was identified between *C. farinosa* and spiromesifen whereby, synergism was found following applications of high concentrations of *C. farinosa* and low concentrations of spiromesifen.
- Temperature had a significant effect on the interactions occurring between high concentrations of *C. farinosa* and low concentrations of spiromesifen.
- A low concentration of spiromesifen co-applied with a concentration of *C. farinosa* equivalent to 5×10^{12} conidia ha⁻¹ resulted in additivity in greenhouse trials against *T. vaporariorum*.
- Antagonism occurred when the concentration of *C. farinosa* was reduced relative to spiromesifen in greenhouse trials.

7.3.2 Current impediments in the use of mixtures for IPM.

Several studies have indicated the potential benefit of combining low concentrations of a chemical insecticide with an EPF to improve the total mortality or rate of kill achieved (Meyling et al., 2018; Russell et al., 2010; Sun et al., 2011). However, few studies in insect pathology have determined how interactions between mixture components change relative to concentrations or ratios applied. Similar to results found in this project, there was synergism between low concentrations of abamectin and the nematode *Steinernema carpocapsae* in control of *Phthorimaea operculella*, and antagonism when the relative chemical concentration was increased (Kary et al. 2018). However, in the study by Kary et al. (2018), only sub sets of the data were analysed at one time, meaning the relationship between mixture treatments could not be explored. Some mixture studies identify interaction outcomes for each mixture treatment and attempt to identify overall patterns by comparing treatments that were analysed separately (Ansari et al., 2008; Kary et al., 2018; Jia et al., 2016). Another method of mixture analysis applied in insect pathology which incorporated the entire data set was unable to identify complex interaction patterns such as dose ratio or dose level dependency or quantify switching points between interaction outcomes (Raymond et al., 2006). Whilst determination of synergism in mixture applications may be beneficial for pest control, the relevance of this information to a grower is minimal if it is unknown under which circumstances antagonism may occur. Abiotic conditions, application methods, differential deposition of mixtures throughout the crop, interactions with other PPP's are just some of the many factors influencing mixture interaction outcomes. Therefore, research should be conducted to determine the extent to which such factors affect pest control when using mixtures and provide suggestions to avoid antagonism of IPM elements.

Though the potential for mixtures in IPM has been known for several years (Purwar and Sachan, 2005) understanding and implementation of mixture theory for the determination of interactions is limited. Most of these studies have tested mixtures without using mixture theory to help explain their findings. In this study, the outcome of six mixture treatments, including combinations of three concentrations of each mixture component, were tested. The experiment was designed to exclude redundant treatments, i.e. those that would give a mortality that was too high to determine if an interaction had occurred. Researchers often investigate the combined effect of control agents which already provide high levels of control independently, meaning that any increase in mortality cannot be determined (Cuthbertson et al., 2010; Raymond et al., 2006; Russell et al., 2010). There have been few studies into the effect of a range of concentrations or different ratios of mixture components in insect pathology, with most studies involving the application of two mixture components at one concentration (for

example; Cuthbertson et al., 2010; De Nardo & Grewal, 2003; Koppenhöfer et al., 2000; Otieno et al., 2016). Not only do these experiments miss the potential variation in interactions between mixture components, depending on concentration or ratio applied, they also ignore the fact that the amount of active ingredient reaching the target insect varies following spray applications to the crop.

In some cases, the mathematical approach to quantify interactions between mixture components is incorrect, for example, in a study by Wu et al.(2017), combinations of the nematode species *Steinernema feltiae* and the chemical thiamethoxam were predicted to cause over 100% mortality of fungus gnat (*Bradysia odoriphaga*) in three mixture treatments. Some studies do not use a mathematical approach to determine the interaction between mixture components (Hamama et al., 2015) or do not statistically determine the difference between the expected and observed mortality (Logan & Birkett, 2007), leading to unsubstantiated claims of synergism. Furthermore, interpretation of data from experiments with complex designs are often limited by the analysis approach which do not incorporate the whole data set (Kary et al., 2018). Similar problems were described by Xu et al. (2011) in a review exploring the use of microbial mixtures to manage plant diseases. Xu et al. (2011) found that most studies did not refer to strict definitions for synergism or antagonism, nor were these outcomes determined through appropriate statistical testing.

In order to improve the uptake of mixtures in IPM, researchers must understand the basic principles behind the mathematics of mixture interactions and how these relate to the desired outcomes for a grower. With this in mind, experiments can be appropriately designed to elucidate the complex ecological interactions between mixture components, the host and the environment whilst also identifying the characteristics required to build compatible and practical IPM systems.

7.4 Future research directions and recommendations

7.4.1 Determining the mechanism of synergism between *C. farinosa* and spiromesifen

It has been suggested that combinations of insect growth regulators (IGRs) and EPF can result in synergism due to the prevention of moulting by the insect, and increase in time for the EPF to penetrate the insect cuticle (Nian et al., 2015). Several studies could be done to determine whether the synergism between *C. farinosa* and spiromesifen was caused by such an effect. Firstly, experiments to determine whether mixture applications affect the rate of development of *T. vaporariorum* compared to applications of the EPF alone. Preventing the development of *Plutella xylostella* larvae by applications of low concentrations of insecticides containing the active ingredients, *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*), thiacloprid or azadirachtin resulted in synergism following the application of *B. bassiana* (Perry, 2017). It is possible that a similar effect is driving the synergism between *C. farinosa* and spiromesifen and that difference in the rate of germination of conidia on the cuticle of each EPF caused the differentiation in the interactions observed between each EPF isolate and spiromesifen.

Although not considered in this study, potential molecular mechanisms underlying the observed interactions of *C. farinosa* and spiromesifen in *T. vaporariorum* could be investigated using a series of targeted molecular and biochemical studies. Nymphs treated with the EPF, spiromesifen or a mixture of these components could be sampled over time and changes in key enzymes common in insect signalling pathways and effectors in response to pathogens and insecticides, including common detoxification enzymes, could be quantified. In particular, quantification of the changes in acetyl-coA-carboxylase as well as important enzymes in the insect response to toxic chemicals and pathogens, such as esterases, acetylcholinesterase, glutathione-S-transferase and multifunction oxidases (Li et al., 2007), in relation to time after application of individual or mixture treatments may elucidate the process resulting in synergistic or antagonistic interactions *C. farinosa* and spiromesifen. The increase in mortality of *B. tabaci* following the application of *Akanthomyces muscarium* and the plant alkaloid matrine was suggested to be caused by a combined effect of the fungus and insecticide targeting acetyl choline receptors, causing a decrease in acetylcholinesterase (Ali et al., 2017). In a separate study, it was found that *Metarhizium anisopliae* and chlorantraniliprole both independently affected Ca^{2+} concentration in *Locusta migratoria* cells (Jia et al., 2016). It was suggested that the synergism between these control agents could be caused by a reduction in the activity of phenoloxidase, an essential enzyme in the insect immune response, which is regulated by Ca^{2+} (Jia et al., 2016). Determination of the mechanisms behind successful mixture outcomes (i.e. synergistic or additive) could aid in the

discovery of other effective IPM strategies or allow for further improvements to be made based on the modes of action.

7.4.2 Identifying the potential for a mixture of *B. bassiana* and spiromesifen

Based on the laboratory results demonstrating additivity across all combinations of *B. bassiana* and spiromesifen, there could be potential for this combination to be used in an IPM programme. Chemical pesticides are considered to provide more reliable control than biopesticides (Gwynn *et al.*, 2015), but using a combination of a chemical with an EPF could reduce the variability of control often seen when the EPF is used on its own, whilst also allowing for reduced pesticide load. This could result in benefits for the environment and insecticide resistance management (Butt and Ansari, 2011). Other studies support these findings; for example there is evidence that applying *B. bassiana* in combination with azadirachtin, acetamiprid, flonicamid, bifenthrin and avermectin can effectively suppress *T. vaporariorum* populations in strawberry crops, whilst reducing reliance on chemical insecticides (Dara 2016). However, further investigations into the effect of abiotic conditions and ultimately, whether additivity between *B. bassiana* and spiromesifen occurs under greenhouse conditions would need to be conducted. In general, the application of synthetic chemicals at low or sublethal concentrations is not recommended as it may lead to increased risk of development of resistance (IRAC, 2006). But, in this case, the application of multiple insecticidal components with different modes of action, especially in combination with other parts of IPM, are likely to reduce development of resistance (Ambethgar, 2009). Further studies into the susceptibility of *T. vaporariorum* to spiromesifen after repeated exposure to EPF and spiromesifen mixtures over multiple generations could determine whether this approach reduces the evolution of resistance in the population.

The aim of IPM is to maintain pest populations below the economic damage threshold by integrating multiple controls in a complementary way. Therefore it does not normally require the total elimination of the target pest population, although on some high value crops this may be necessary. In some systems, sequential applications of control agents may be required. Determination of the effect of sequential applications of spiromesifen and *B. bassiana* could elucidate whether cross-over from separate applications of one control agent may impact on the efficacy of another. Dara (2016) found that replacing one or more chemical spray applications with *B. bassiana* or a mixture of *B. bassiana* with low concentrations of a chemical was an effective way to maintain aphid, thrip and whitefly populations below the economic threshold in strawberry crops whilst reducing the total amount of chemical applied. However, interactions between sequentially applied pesticides and biopesticides can differ

from co-applied (simultaneous) control agents and are influenced by timing of application and the order that each component is applied. For example application of sublethal concentrations of alphacypermethrin followed by *B. bassiana* 24 hours later on *Tenebrio molitor* larvae resulted in additive mortality of the beetle (Meyling *et al.*, 2018). However, application of *B. bassiana* at least 48 hours before application of alphacypermethrin resulted in a synergistic interaction. The impact of sequential versus simultaneous application of control agents is another essential aspect of mixture interactions that needs to be examined when considering an EPF-chemical mixture approach.

7.4.3 Improving the applicability of mixtures research to commercial crop protection

Fixed aged populations are often used to determine the efficacy of EPF and insecticides (Karatosol *et al.*, 2010; Kim *et al.*, 2010; Mascarin *et al.*, 2013b), as in this study, but in reality, the crop would be infested with all developmental stages of *T. vaporariorum*. It is known that insects can avoid infection by EPF by ecdysis, meaning that *T. vaporariorum* stages closer to moulting are less likely to be infected (Ortiz-Urquiza and Keyhani, 2013). In addition, those newly moulted individuals with thin, non-sclerotized cuticles are more vulnerable to infection (Ortiz-Urquiza and Keyhani, 2013). Spiromesifen and EPF have been shown to cause mortality of *T. vaporariorum* eggs, all nymphal stages and adults, though their efficacy differs between each developmental stage (Bi and Toscano, 2007; Pineda *et al.*, 2007). Therefore, further studies into the effect of the mixture on each stage of *T. vaporariorum* will elucidate the effect of the mixture on a naturally occurring infestation. In the greenhouse, it is likely that multiple applications would be applied throughout a crop life cycle; intervals between applications that are based on biologically relevant periods of time related to the development rate of *T. vaporariorum* can improve pest control during the crop cycle. For example, mortality of adult *B. tabaci* in field grown crops of cantaloupe melons, cucumbers and courgette following the application of *Cordyceps fumosoroseus* (formerly *Paecilomyces*) or one of four *B. bassiana* isolates, was minimal. However, up to five applications of each EPF at rates ranging from $1.25 \times 10^{13} - 1 \times 10^{14}$ conidia ha⁻¹ with intervals between 4 and 7 days resulted in a 50% reduction of *B. tabaci* adults and >90% mortality of *B. tabaci* nymphs by the end of the trial in each crop (Wraight *et al.*, 2000).

Conidial deposition in relation to concentration of the chemical insecticide was important in determining the interactions between *C. farinosa* and spiromesifen under greenhouse conditions. An average conidial deposition of 538 conidia mm⁻² combined with 0.04mg mL⁻¹

spiromesifen resulted in additivity, whereas combinations of 50 conidia mm⁻² *C. farinosa* and the same concentration of spiromesifen, resulted in antagonism. Whilst there is a ten-fold difference in these conidial depositions, other studies have found that depending on the spray technique used, conidial deposition can vary to this magnitude despite the application of the same concentration (Wraight and Ramos, 2002). Application of 2.5 x10¹³ conidia ha⁻¹ *B. bassiana* to the abaxial leaf surface of *Solanum tuberosum* using a knapsack sprayer resulted in 484 conidia mm⁻² or 50 conidia mm⁻² depending on the angle of spray nozzles relative to the crop. In the current study, individual leaves were targeted for spray applications, ensuring even coverage of the leaf surface. In authentic crop applications, leaves overlap and protrude at different angles causing differential deposition of spray; in some cases, it is estimated that only 0.1% of the spray reaches the target (Pimentel and Levitan, 1986).

In order to better understand the effect of application on the efficacy of this mixture, the experiments described in this thesis could be taken further. In particular, the applicability of the study could be improved by the use of a knapsack sprayer on whole plants infested with *T. vaporariorum*, to test the hypothesis that differential deposition of mixture components affects the interactions between *C. farinosa* and spiromesifen and the average level of control provided. The ratio of interacting mixture components may differ throughout the canopy, causing antagonism, additivity or synergism depending on the ratio of active ingredients reaching the pest. Furthermore, several repeats of the greenhouse trials would need to be conducted in order to determine the average efficacy of the mixture. As a guideline, at least ten efficacy trials must be conducted before any PPP can be registered on the market in Europe (European Commission, 2009). One of the key issues with expanding successful use of biopesticides is through providing growers the appropriate training and education on application methods. Applied research should test appropriate equipment and conduct research experiments that can inform specific instructions and general training for the improved application of biopesticides.

7.4.4 Future direction

In order to improve our current knowledge on EPF and interactions within IPM, there needs to be a shift in focus for research that produces transferable academic results that are applicable to growers using biopesticides in applied situations. There are many studies selecting isolates for biocontrol, describing virulence of different strains of fungi against many different hosts (for example; Anderson *et al.*, 2011; Santos *et al.*, 2018; Shahriari *et al.*, 2021). Whilst undoubtedly an isolate must have a level of efficacy against a host, rather than improving control methods and searching for increased levels of control, identification of unsuccessful

combinations could potentially be just as useful when assembling an IPM approach. Additionally, reporting of all aspects of the experimental system such as methods to prepare mixtures, application techniques and environmental conditions during trials are essential to identify patterns amongst successful and unsuccessful combinations. This requires researchers to publish IPM combinations that result in antagonism and additivity as well as those that demonstrate synergism so that we can begin to understand factors influencing the breakdown of IPM strategies.

Following such recommendations, eventually, a knowledge bank of interactions between control agents and the effect on target pests could be collated. Digitalised platforms that allow interactive knowledge exchange between researchers, advisors and farmers are already in place across Europe on national and/or regional levels in the form of informal groups on social media (Twitter, Whatsapp and Facebook), indicating a need for more direct connections between science and practice (Bogaardt *et al.*, 2019). Advanced knowledge banks already exist in other research disciplines and review of these would assist in the development of a database. For example, The Drug Combination Database (DrugCombDB) holds data from 450,000 drug combinations (H. Liu *et al.*, 2020) and in pharmacology, databases are used to compile the results of protein-protein interactions (Szklarczyk *et al.*, 2017) or chemical-protein interactions (*STITCH: Chemical Protein Interactions.*, 2021). These knowledge banks could improve identification of ‘gaps’ in the research, as well as expediting information exchange to growers via agronomists and consultants.

7.4.5 Conclusion

In conclusion, this thesis has demonstrated the complexity involved in investigating the interactions between multiple components in pest control. However, it has also demonstrated that there are ecotoxicological models capable of describing these complex interactions which can identify synergism between mixture components, and most importantly, predict and explain the reasons why pest control may not work under certain circumstances. There is still a pressing need for improved understanding of mixture theory in insect pathology before the full potential of these analysis techniques and others like them can be fully realised. Improving comparability between experiments through standardisation of approaches could allow for a database to be collated, as highlighted previously, for interactions in IPM. Researchers should report on both successful as well as unsuccessful combinations of IPM elements and the difference in efficacy when applied against different pest species, on different crops and under different environmental conditions. Ultimately, if all experimental conditions are reported in depth in each mixture experiment, patterns of synergy or antagonism could emerge in relation

to environmental conditions as demonstrated in this thesis, which will allow identification of appropriate integration of EPF biopesticides in IPM and successful, reliable pest management outcomes.

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Appendix I

Pareto dominance table. Rows contain the number of properties for which an isolate was out-performed or equalled by another isolate. A value of 20 indicates an isolate is Pareto-dominated (outperformed or equalled across all desirable properties).

	Vert-elac	Met 52	ATCC 4412	ATCC 9451	ATCC 4205	ATCC 2658	Botani-gard	GHA	ATCC 5278	ATCC 4060	Bio-blast	PFR	PPRI 5339	ATCC 7477	ATCC 6544	ATCC 972	ATCC 6921	ATCC 6920	Total
Vert-elac	n	18	15	10	12	9	11	10	10	12	11	9	14	11	10	8	11	11	192
Met 52	2	n	4	5	2	1	3	3	5	6	7	3	2	2	3	3	4	1	56
ATCC 4412	5	16	n	9	1	1	7	6	8	9	9	4	6	4	8	2	5	2	102
ATCC 9451	10	15	11	n	9	9	9	10	13	16	9	10	12	10	9	8	8	9	177
ATCC 4205	8	18	19	11	n	10	9	8	12	12	15	10	13	12	11	9	11	13	201
ATCC 2658	11	19	19	11	10	n	11	8	10	14	17	11	15	12	12	10	12	13	215
Botani-gard	9	17	13	11	11	9	n	8	14	14	11	9	11	9	9	9	9	9	182
GHA	10	17	14	10	12	12	12	n	12	14	13	11	15	10	10	10	10	12	204
ATCC 5278	10	15	12	7	8	10	6	8	n	13	9	7	11	8	10	6	7	8	155

	Vert-elac	Met 52	ATCC 4412	ATCC 9451	ATCC 4205	ATCC 2658	Botani-gard	GHA	ATCC 5278	ATCC 4060	Bio-blast	PFR	PPRI 5339	ATCC 7477	ATCC 6544	ATCC 972	ATCC 6921	ATCC 6920	Total
ATCC 4060	8	14	11	4	8	6	6	6	7	n	9	7	8	8	8	2	7	7	126
Bio - blast	9	13	11	11	5	3	9	7	11	11	n	5	9	3	7	7	7	6	134
PFR	11	17	16	10	10	9	11	9	13	13	15	n	15	8	9	9	9	12	196
PPRI 5339	6	18	14	8	7	5	9	5	9	12	11	5	n	8	7	3	9	6	142
ATCC 7477	9	18	16	10	8	8	11	10	12	12	17	12	12	n	13	9	9	10	196
ATCC 6544	10	17	12	11	9	8	11	10	10	12	13	11	13	7	n	6	6	8	174
ATCC 972	12	17	18	12	11	10	11	10	14	18	13	11	17	11	14	n	11	11	221
ATCC 6921	9	16	15	12	9	8	11	10	13	13	13	11	11	11	14	9	n	9	194
ATCC 6920	9	19	18	11	7	7	11	8	12	13	14	8	14	10	12	9	11	n	193

Appendix II

In order to determine the effect of storing conidia at -20°C for short periods of time, a preliminary experiment was conducted to test whether Tween 80 or Glycerol provided better protection to conidia when exposed to low temperatures. *Beauveria bassiana* (GHA) was prepared using methods described in section 2.3.6. After 28 days growth of *B.bassiana* (GHA) on SDA incubated in the dark at 15°C or 20°C , samples of conidia were taken using a cork borer with a diameter of 15mm and stored in 1mL of aqueous glycerol (10% per volume) or Tween 80 (0.03% per volume). There were two replicates of *B.bassiana* (GHA) grown at each temperature and stored in each solution. Samples were stored at -20°C for two weeks before being removed, broken down using a sterile pestle and filtered in twice folded muslin cloth. Concentration of each solution was determined through serial dilutions and counting conidia microscopically using a haemocytometer. There was no significant difference between the conidial counts for GHA when stored in glycerol (10% per volume) or Tween 80 (0.03% per volume) at 15°C (Two-sample t test: $t=-1.184$, $df=2$, $p=0.179$) or 20°C ($t=0.613$, $df=2$, $p=0.699$). Therefore, Glycerol was used for further experiments as this is the protocol for storage at -80°C .

Appendix III

A preliminary germination experiment was conducted to investigate the average time taken for 50% conidial germination by each fungal isolate in the presence of Oberon. Sabouraud dextrose agar was autoclaved and left to cool to 40°C before being spiked with 396 µl of Oberon. The spiked agar solution was poured into 90mm diameter Petri dishes in 10mL aliquots and left to cool. Conidial suspensions of *Beauveria bassiana* (PPRI 5339), *Metarhizium brunneum* (Met 52), *Akanthomyces lecanii* (ATCC 4060) and *Cordyceps farinosa* (ATCC 4412) were collected and diluted to 1×10^6 conidia mL⁻¹ using methods described in 2.3.3. For each isolate, 4µl of suspension was placed in the centre of a Petri dish containing the spiked agar or untreated agar, which acted as the control. Each fungal isolate had three replicates per treatment which were stored in the dark at 25°C in a constant temperature incubator until germination was stopped by adding a drop of lactophenol and cotton blue onto the centre of the Petri dish. Germination was stopped at three time points for each fungal isolate. Petri dishes containing *Metarhizium brunneum* (Met 52), *Akanthomyces lecanii* (ATCC 4060) and *Cordyceps farinosa* (ATCC 4412) conidia were incubated for 8, 10 and 12 hours, whereas *Beauveria bassiana* (PPRI 5339) was incubated for 14, 16 and 18 hours after previous experiments showed a slower germination rate at this temperature. Following the application of Lactophenol cotton blue, each dish was stored overnight in the fridge (4°C). The following day, the proportion of germinated conidia were counted on each dish using methods previously described in section 2.3.5.

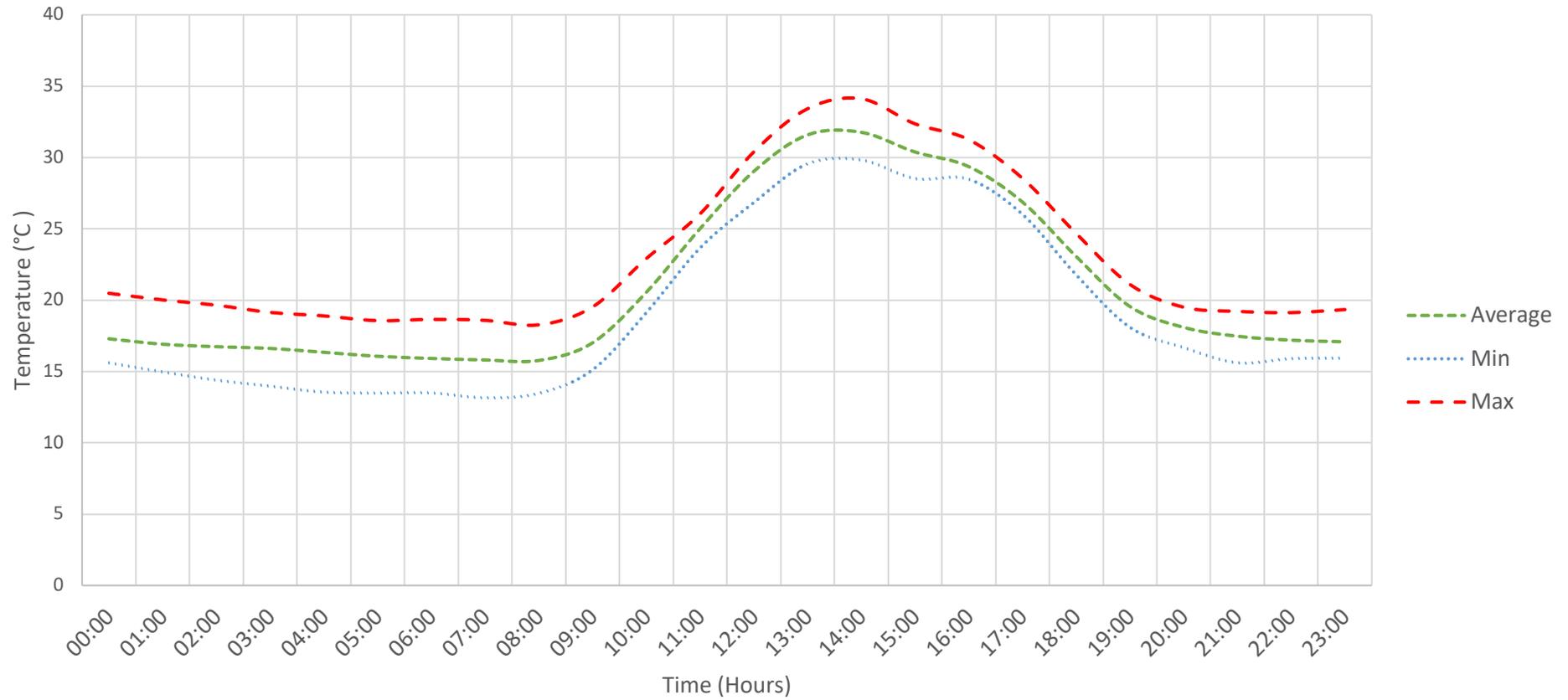
Appendix III

Genus	Isolate	Growth rate (mm day ⁻¹)								
		Temperature (°C)								
		10	15	18	20	23	25	27	30	32
<i>Beauveria</i>	ATCC 6921	0.53 ± 0.024	1.072 ± 0.015	1.14 ± 0.043	1.21 ± 0.058	1.49 ± 0.065	1.34 ± 0.088	0.76 ± 0.075	0.76 ± 0.034	NA
	ATCC 6920	0.39 ± 0.016	0.71 ± 0.038	0.89 ± 0.041	1.12 ± 0.029	1.26 ± 0.038	1.39 ± 0.037	1.42 ± 0.030	0.86 ± 0.040	0.043 ± 0.011
	ATCC 5278	0.11 ± 0.017	0.45 ± 0.023	0.74 ± 0.014	0.96 ± 0.035	0.88 ± 0.027	1.002 ± 0.047	1.11 ± 0.026	1.12 ± 0.031	0.64 ± 0.027
	ATCC 9451	0.23 ± 0.022	0.42 ± 0.033	0.53 ± 0.032	0.59 ± 0.026	0.59 ± 0.02	0.65 ± 0.060	0.81 ± 0.061	0.34 ± 0.036	0.074 ± 0.011
	PPRI5339	0.34 ± 0.010	0.60 ± 0.013	0.91 ± 0.022	1.12 ± 0.024	1.19 ± 0.033	1.21 ± 0.045	1.06 ± 0.064	0.80 ± 0.031	0.35 ± 0.026
	GHA	0.34 ± 0.012	0.61 ± 0.017	0.79 ± 0.030	0.95 ± 0.037	1.11 ± 0.092	1.25 ± 0.066	1.09 ± 0.085	0.84 ± 0.043	0.44 ± 0.026
	Botanigard	0.21 ± 0.017	0.41 ± 0.013	0.55 ± 0.024	0.79 ± 0.022	0.86 ± 0.029	0.94 ± 0.065	1.06 ± 0.044	0.87 ± 0.046	0.37 ± 0.035
<i>Akanthomyces</i>	ATCC 4060	0.47 ± 0.026	0.77 ± 0.023	0.86 ± 0.025	0.98 ± 0.034	1.20 ± 0.043	1.35 ± 0.030	1.06 ± 0.053	0.39 ± 0.024	0.21 ± 0.011
	Vertalec	0.15 ± 0.009	0.51 ± 0.026	0.92 ± 0.027	1.14 ± 0.056	1.12 ± 0.026	1.04 ± 0.028	0.96 ± 0.015	0.88 ± 0.027	0.41 ± 0.014
	ATCC 972	0.39 ± 0.022	0.53 ± 0.022	0.72 ± 0.017	1.08 ± 0.047	1.02 ± 0.104	1.05 ± 0.063	0.96 ± 0.054	0.26 ± 0.0086	0.012 ± 0.0061
	ATCC 6544	0.57 ± 0.023	1.15 ± 0.039	1.56 ± 0.042	1.55 ± 0.11	1.86 ± 0.057	1.74 ± 0.063	1.30 ± 0.034	0.34 ± 0.017	0.037 ± 0.005
<i>Cordyceps</i>	ATCC 7477	0.13 ± 0.011	0.50 ± 0.021	0.93 ± 0.069	1.36 ± 0.057	1.38 ± 0.044	1.55 ± 0.047	1.66 ± 0.060	1.32 ± 0.050	0.38 ± 0.047
	ATCC 2658	0.17 ± 0.013	0.56 ± 0.058	0.89 ± 0.027	1.02 ± 0.067	1.15 ± 0.038	1.19 ± 0.073	1.35 ± 0.14	0.84 ± 0.042	0.39 ± 0.024
	ATCC 4205	0.13 ± 0.014	0.58 ± 0.028	0.86 ± 0.016	1.12 ± 0.045	1.32 ± 0.036	1.35 ± 0.048	1.36 ± 0.06	1.11 ± 0.038	0.44 ± 0.020
	ATCC 4412	0.19 ± 0.015	0.72 ± 0.029	1.016 ± 0.062	1.30 ± 0.048	1.39 ± 0.045	1.55 ± 0.047	1.44 ± 0.068	1.36 ± 0.08	0.46 ± 0.036
	PFR	0.25 ± 0.0081	0.52 ± 0.023	0.42 ± 0.173	1.092 ± 0.065	1.078 ± 0.037	1.028 ± 0.16	1.24 ± 0.044	1.083 ± 0.043	0.54 ± 0.023
<i>Metarhizium</i>	Bioblast	0.016 ± 0.004	0.97 ± 0.019	1.49 ± 0.057	1.76 ± 0.097	1.99 ± 0.056	2.22 ± 0.041	2.42 ± 0.068	1.89 ± 0.023	1.21 ± 0.015
	Met52	0.23 ± 0.011	0.75 ± 0.028	1.16 ± 0.048	1.65 ± 0.048	2.037 ± 0.032	1.93 ± 0.073	1.59 ± 0.044	0.96 ± 0.020	0.21 ± 0.015

Table. Average growth rate and standard error of fungal isolates across nine temperatures as determined by the slope of a linear regression plotted for the linear phase of growth for each isolate.

Appendix V

315



Average, maximum and minimum internal temperatures of one of the greenhouses at BASF crop trials facility in Utrera, Spain during growing season (September 2016). Average hourly temperature was calculated for the first day of each week in September.

Appendix VI

Systematic review of microbial mixtures for greenhouse pest control

A range of mixture studies have been published in the scientific literature, but they have not yet been reviewed as a whole. This might be preventing valuable lessons being learnt from the literature which could be applied to improve IPM practice. With this in mind, a systematic review of the literature on combination treatments of microbial control agents (MCAs), or MCAs and conventional chemical pesticides, against greenhouse pests from the last 20 years was conducted. The review had the following component objectives: (i) to determine which combination treatments have been investigated and against which greenhouse pests and (ii) to ascertain whether experiments have been conducted and analysed in a way that allows the outcome of mixtures to be determined reliably and consistently.

Systematic review methods and analysis

All literature included in this analysis was selected using an evidence review protocol. A literature search in all databases in Web of Science was conducted to identify peer reviewed papers presenting the results of combinations of different microbial control agents or microbial control agents combined with chemical pesticides in greenhouse cropping systems or laboratory based studies aimed at common greenhouse pests. Chemical pesticides included conventional pesticides as well as alternatives classed as bioinsecticides such as Neem. The search terms used are shown in Table A.1. Searches involved all databases in web of science from 2000-2020. Titles were selected if they described the combination of at least two agents and their effect on an insect pest in the lab or greenhouse. Agents included anything that had lethal or sublethal effects, but excluded parasitoids and predators. Potentiators, which have no insecticidal activity alone, but can improve the efficacy if used in combination with another agent were included but formulation chemicals, which improve the efficacy of microbial and chemical pesticides by increasing their shelf life, survival time on foliage, spray characteristics etc were excluded. An example of a potentiator would be an optical brightener which increases the efficacy of baculoviruses by affecting host cell membranes and thereby enhancing virus infection (a potentiating effect) but they also protect virus particles from damage by u.v. radiation (i.e. a formulation effect).

Some of the papers involved more than one experiment, either those based in the laboratory and the greenhouse or those involving combinations of different control agents targeting different pests. For this reason, the literature was summarised by experiment rather than by paper with treatments of different pest species or different combinations of control agents being listed as separate experiments. Several observations were entered into a database for each experiment, including; target pest, host plant, the number of components in the mixture, how many mixture treatments were tested which involved the combination of different concentrations of each agent and we recorded how these doses were determined. We also noted the scale of the experiment (laboratory or greenhouse), whether agents were applied simultaneously or sequentially, the type of analysis used to determine the outcome of the interaction (if any) and terminology used to describe the interaction found. In order to determine whether redundant mixture combinations were included in the experimental design, the number of treatments expected to result in >90% mortality was calculated assuming independent action.

To calculate the number of expected combination treatments that would result in >90% mortality, Bliss independence was assumed. However, in some cases, the data required for this calculation was not available due to experimental design or data being unreported. If the calculation was not possible, NA was entered into the database.

Table. A.1. Search terms used in a systematic literature search.

	Search Terms used
1	Insect AND
2	Chemical OR synthetic OR pesticide* OR insecticide* AND
3	Bio*cont* OR pathog OR entomopath* OR biocont* OR botanical* OR biorational* OR microbial* OR organic* AND
4	G*house OR protected OR enclosed OR hoop*house OR polytunnel* AND
5	Interact* OR synergis* OR antagonis* OR mixed* OR co infect* OR dual OR inocul* OR competit* OR neutr* OR benefi* OR enhance* OR additiv* OR IPM OR integrate* OR compat* OR multiple

Systematic review results

The literature search with terms shown in Table A.1 returned 6,209 results. Of these, 395 were selected based on paper titles alone. A further 109 were chosen based on experimental descriptions found in paper abstracts and 73 papers were included in the final analysis.

The final database used for analysis contained 356 experiments from 73 papers (Table A.3). The increasing interest in the application of mixtures for pest control is reflected in the increase in publications in this area of research. The number of publications fitting the review criteria had nearly doubled during 2010-2020 compared to the 10 year period prior, as seen in Figure A.1. However, there was large variation in the number of publications and none were published in 2009 or 2011 that fit our criteria.

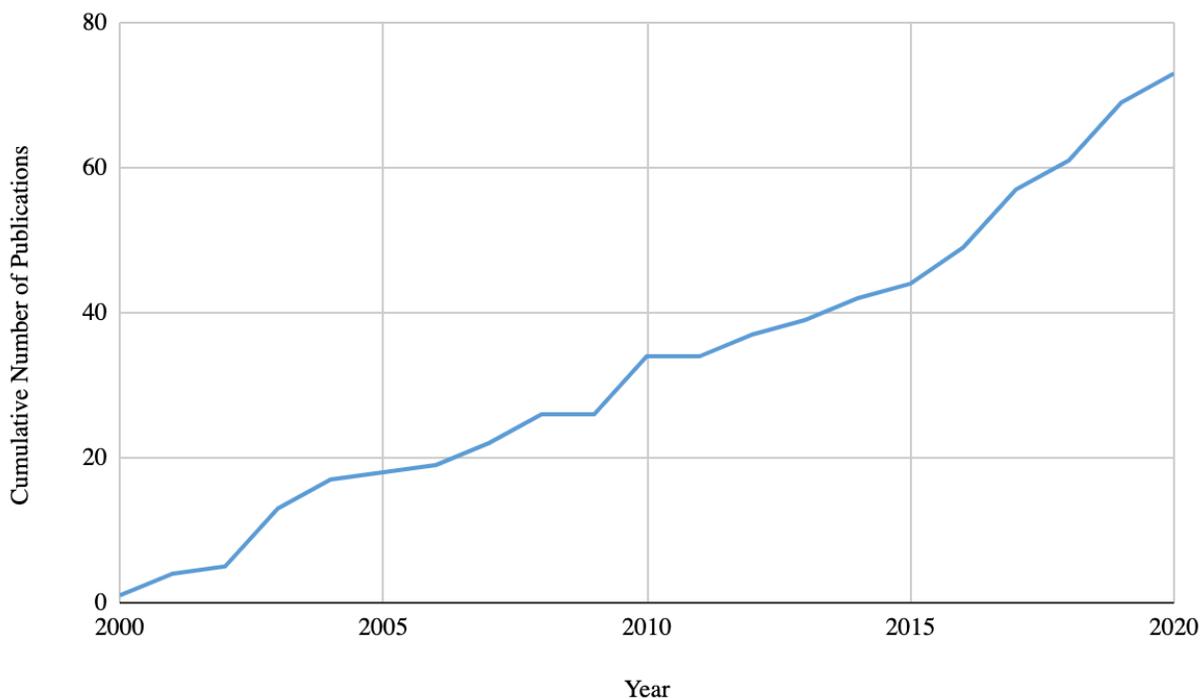


Figure A.1. Cumulative number of mixture publications involving at least one microbial pathogen for the control of greenhouse crops published between 2000-2020.

Of the 356 experiments, 108 were conducted on crops or horticultural plants grown in the greenhouse. The majority of these studies were conducted on perennial ryegrass (32% of experiments) or tomato plants (11%) though there were a wide range of plants used as pest hosts throughout the literature. The remainder of experiments were conducted in the laboratory in microcosm style bioassays and similarly to greenhouse experiments, perennial ryegrass (7%) and tomato (14%) were also popular choices for experiments. However, a large proportion of laboratory studies were conducted by maintaining pest insects on artificial media whilst conducting Petri dish inoculations (38%).

A large variety of target pests were used in experiments, the order of these insects are shown in Figure A.2. Lepidopteran, Coleopteran and Hemipteran pests were the most commonly targeted. In particular, the most popular species within the literature were *Bemisia tabaci* (Hemiptera) (12%), *Tuta absoluta* (Lepidoptera) (10%) and *Galleria mellonella* (Lepidoptera) (7%).

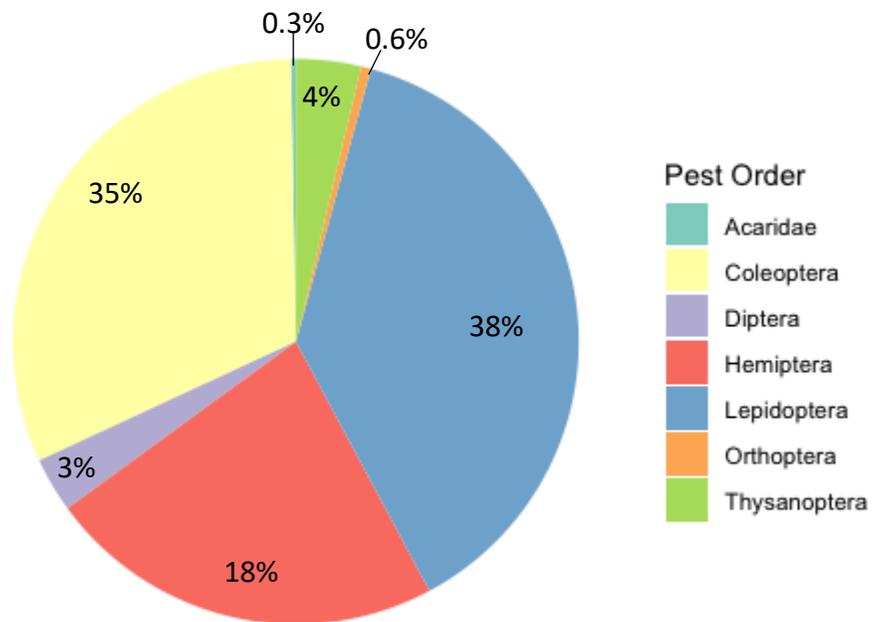


Figure A.2. Proportion of different insect orders across 351 experiments in 71 papers published between 2000-2020 investigating the combined effect of a microbial pathogen and at least one other control agent.

In the majority of experiments, two control agents were combined at one concentration against a target pest (Figure A.3). There were 87 experiments involving the combination of different microbial pathogens. The majority of experiments were investigating the combination of a microbial pathogen with a chemical or biopesticide. Microbial pathogens were combined with a

variety of biological and synthetic insecticides, fungicides, herbicides and potentiators which are shown in Table A.2 and Figure A.4. Entomopathogenic fungi were the most commonly used microbial pathogen to be combined with another non-microbial control agent. In particular, the most investigated combination was the neonicotinoid, imidacloprid with *Beauveria bassiana*.

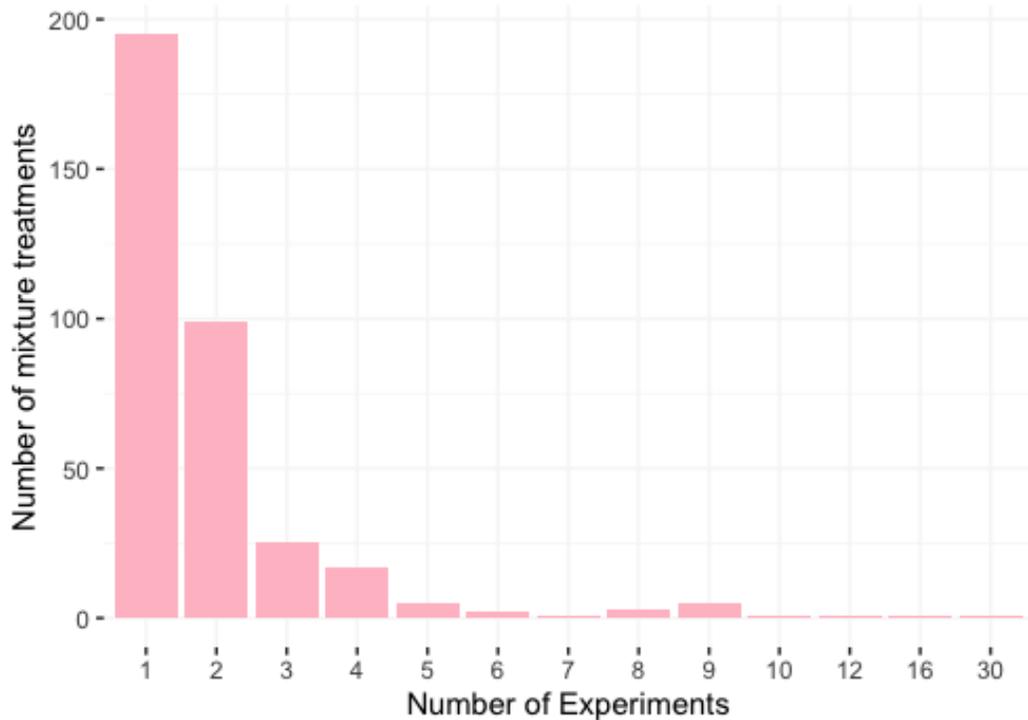


Figure A.3. Total number of mixture treatments across 351 experiments in 72 papers published between 2000-2020 investigating the combined effect of a microbial pathogen and at least one other control agent. A mixture treatment was defined as the combination of at least two control agents either simultaneously or sequentially applied to the target organism.

Summarising the interaction outcomes across all experiments was difficult due to high variation in target pest and control agents applied. However the main issue in summarising interaction outcomes was due to inconsistencies in experimental design, statistical determination of interactions and misuse of terms when reporting results. Some mixture combinations were described as having a ‘positive’ or ‘significant’ interaction or as being ‘compatible’ without being defined as synergistic or additive. For analysis purposes, if data was not available to calculate the interaction, results from these papers were described as positive. Similarly, if there was a negative term used to describe the interaction, without data provided in order to check for antagonism, interaction outcomes were classed as negative.

It was notable that of the 351 experiments included in this review, 41% reported there to be synergism or a ‘positive’ outcome as a result of their mixture experiment. A large proportion of studies reported additivity (38%) (see figure A.5).

Comparatively, only 7% of experiments described the interaction as negative or antagonistic. A further 44 experiments (13%), described the interactions occurring as several outcomes depending on the concentration applied, the order of application or the duration of the interval in between application of each component in the mixture.

However, several of the studies describing the interactions seen as synergistic or antagonistic, did not collect the data necessary or conduct appropriate calculations in order to determine each of these outcomes (39% of mixture combination experiments). In several studies, experiments were carried out using mixture treatments only, so the effect of the individual control agents was not tested, making the calculation of expected outcomes and determination of interaction impossible (3% of studies). Additionally, the concentrations of some control agents when applied alone resulted in >90% mortality of the pest, therefore addition of another control agent often resulted in 100% mortality, and additivity or synergism could not be determined. Authors also often claimed synergism as a result of increased pest mortality in mixture treatments, though this increase was an expected result of using multiple control agents and actually did not deviate from additivity.

Table A.2. Types of combinations studied in experiments investigating the effect of two or more control agents published between 2000-2020.

Mixture investigated	No. experiments
Fungus x fungus	30
Virus x virus	13
Bacteria x fungus	6
Bacteria x virus	4
Bacteria x nematode	1
Fungus x nematode	32
Bacteria x fungi x nematode	1
Fungi x nematode x non-microbial*	2
Bacteria x non-microbial	21
Fungi x non-microbial	94
Virus x non-microbial	30
Nematode x non-microbial	122

*Non-microbial includes biological and synthetic insecticides, fungicides, herbicides and potentiators.

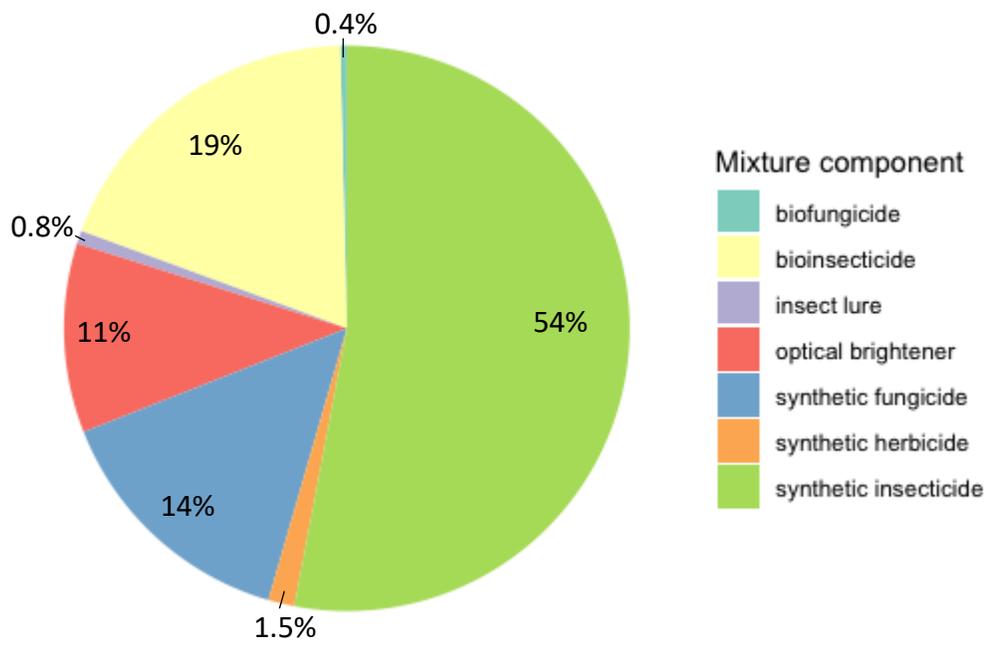


Figure A.4 Proportion of non-microbial control agents used in combination with a microbial pathogen for pest control across 268 experiments published 2000-2020.

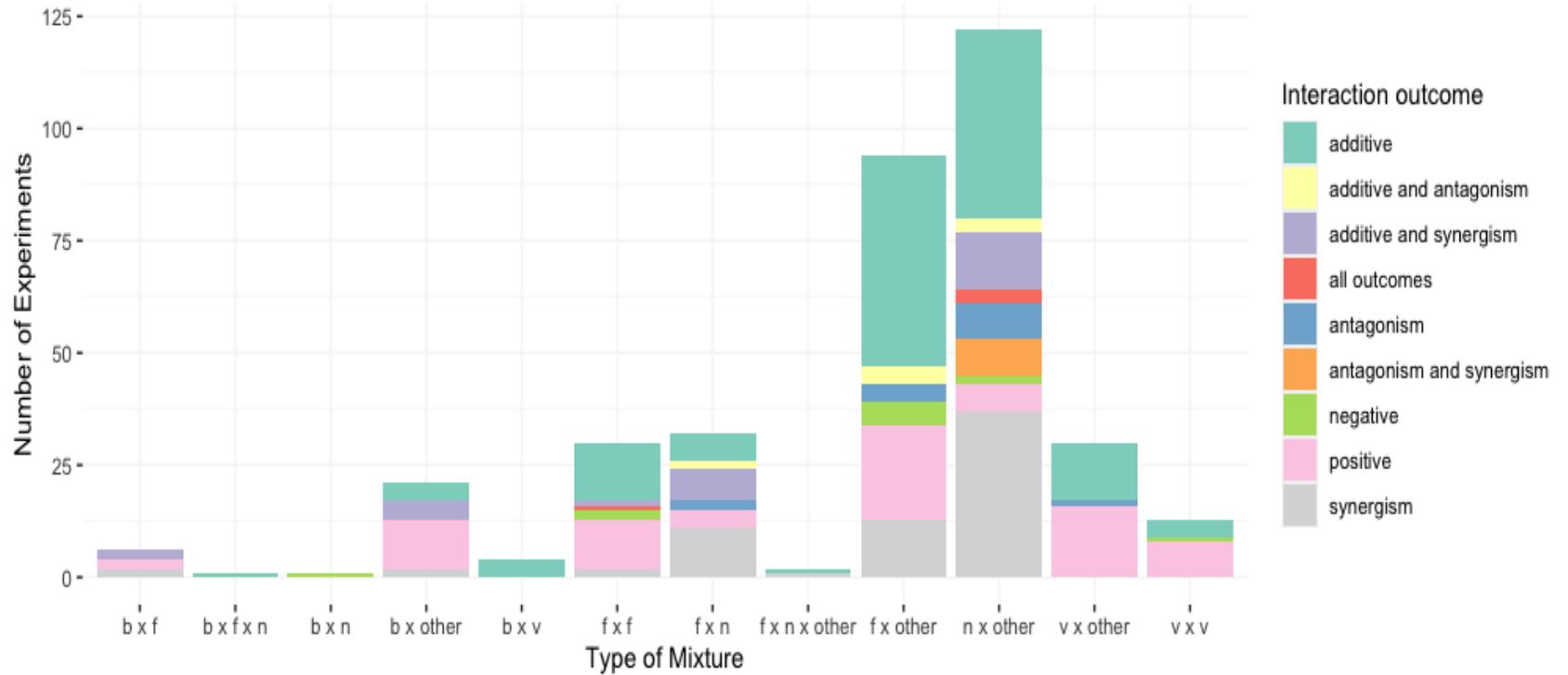


Figure A.5 Outcomes reported for mixtures of a microbial pathogen and at least one other control agent applied to control greenhouse pests in 353 experiments across 72 papers published between 2000 and 2020. Type of mixture refers to combinations of entomopathogenic bacteria (b), fungi (f), nematodes (n), viruses (v) or another non-microbial control agents (other). Non-microbial control agent refers to biological and synthetic insecticides, fungicides, herbicides and potentiators.

Table A.4. Papers investigating the effect of mixtures of a microbial pathogen and at least one other control agent applied to greenhouse pests published between 2000 and 2020 used in the systematic review.

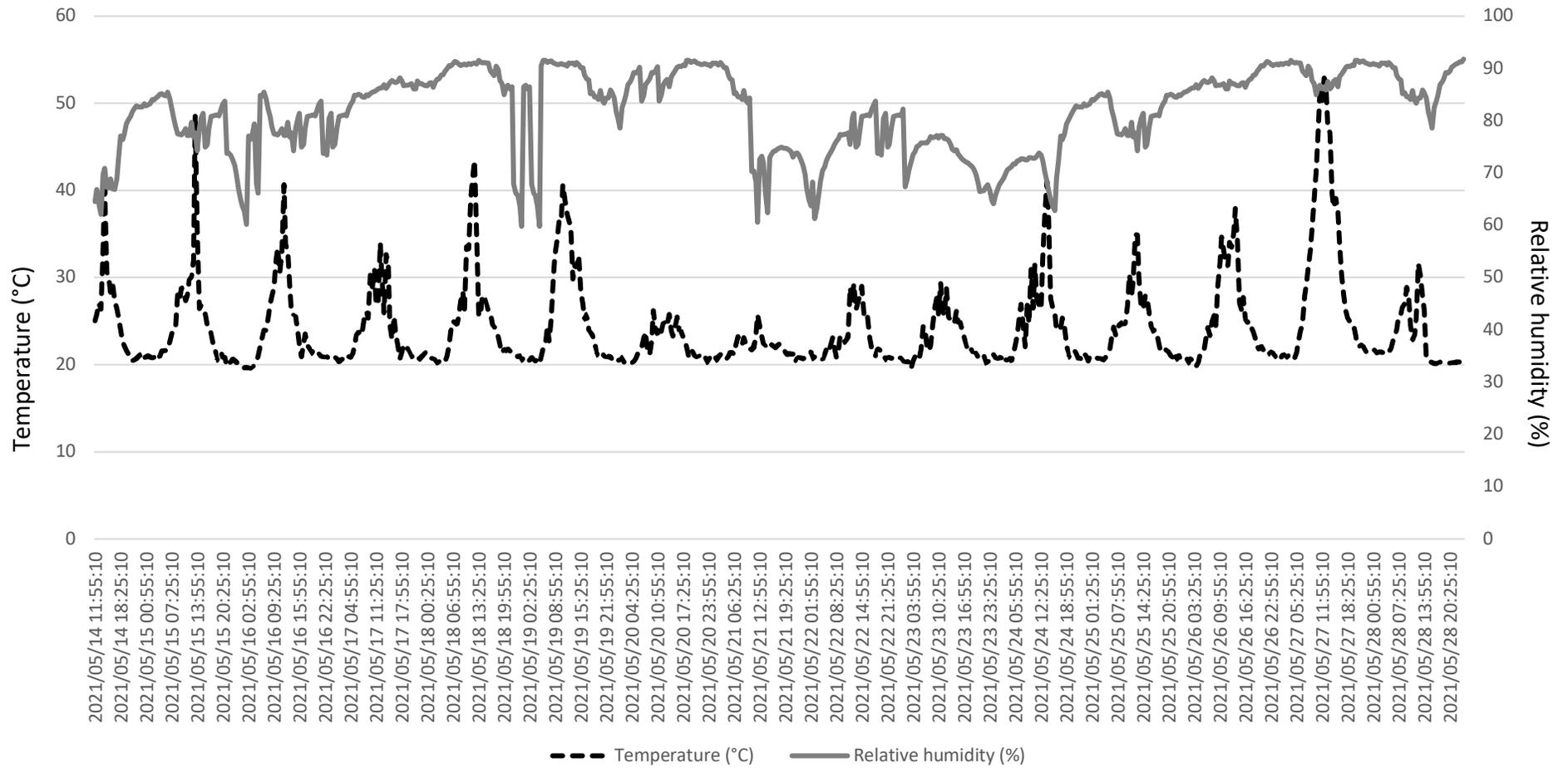
Author	Year	Target Pest	Crop	Number of components in the mixture	Application method	Simultaneous or Sequential Application
Koppenhöfer et al.	2000	<i>Cyclocephala hirta</i>	Perennial ryegrass	2	treated with'	simultaneous
Liu & Meister	2001	<i>Bemisia argentifolii</i>	Cantaloupe	2	drip irrigation and spray application	sequential
Morales et al.	2001	<i>Anticarsia gemmatalis</i>	NA	2	diet fed	simultaneous
Furlong & Groden	2001	<i>Leptinotarsa decemlineata</i>	Potato	2	Spray and topical application	both
Koppenhöfer et al.	2002	<i>Eutetranychus orientalis</i>	Perennial ryegrass	2	treated with'	simultaneous
De Nardo & Grewal	2003	<i>Galeria mellonella</i>	NA	2	soil application	simultaneous
Farrar et al.	2003	<i>Helicoverpa zea</i>	Lima bean	3	diet fed	simultaneous
Koppenhöfer & Fuzy	2003	<i>Maladera castanea</i>	Perennial ryegrass	2	spray applicaiton	simultaneous
Koppenhöfer et al.	2003	<i>Popillia japonica</i>	NA	2	soil application	simultaneous
Lugwig & Oetting	2003	<i>Frankliniella occidentalis</i>	Chrysanthemum	2	spray application	simultaneous
Maniania et al.	2003	<i>Frankliniella occidentalis</i>	Chrysanthemum	2	spray application	simultaneous
Maniania et al.	2003	<i>Frankliniella occidentalis</i>	Chrysanthemum	2	spray applicaiton	simultaneous
Yildirim & Hoy	2003	<i>Delia antiqua</i>	Welsh onion	2	seed treatment & soil application	sequential
Ansari et al.	2004	<i>Hoplia philanthus</i>	NA	2	soil application	simultaneous
Dani et al.	2004	<i>Lacanobia oleracea</i>	NA	2	injection	sequential
Feng et al.	2004	<i>Trialeurodes vaporariorum</i>	Lettuce	2	spray application	simultaneous
Martin	2004	<i>Lymantria dispar dispar</i>	NA	2	diet fed	simultaneous
Jaramillo et al.	2005	<i>Cyrtomenus bergi</i> <i>Froeschner</i>	Maize	2	soil application	simultaneous
Al-Mazraáwi	2006	<i>Thrips tabaci</i>	Tomato	2	dipping	simultaneous
Lasa et al.	2007	<i>Spodoptera exigua</i>	NA	2	diet fed	simultaneous
Logan & Birkett	2007	<i>Helicoverpa armigera</i>	artificial	2	pipette and diet fed	simultaneous
Polavarapu et al.	2007	<i>Anomala orientalis</i>	highbush blueberry	2	watering can	simultaneous

Ansari et al.	2008	<i>Otiorynchus sulcatus</i>	NA	2	soil application	simultaneous
Author	Year	Target Pest	Crop	Number of components in the mixture	Application method	Simultaneous or Sequential Application
Bardin et al.	2008	<i>Bemisia tabaci</i>	Tomato	2	spray application	simultaneous
Kapongo	2008	<i>Trialeurodes vaporariorum</i>	Tomato	2	vectored	simultaneous
Koppenhöfer & Fuzy	2008	<i>Anomala orientalis</i>	Perennial ryegrass	2	"treated with"	sequential
Cuthbertson et al.	2010	<i>Bemisia tabaci</i>	Poinsettia	2	spray application	sequential
Georgievska et al.	2010	<i>Helicoverpa armigera</i>	NA	2	diet fed	simultaneous
Karthiba et al.	2010	<i>Cnaphalocrocis medinalis</i>	Rice	3	spray application	simultaneous
Kim et al.	2010	<i>Trialeurodes vaporariorum</i>	Tomato	2	spray application	simultaneous
Richards et al.	2010	<i>Lacanobia oleracea</i>	NA	2	injection & diet fed	simultaneous
Russel, Uginé, Hajek	2010	<i>Anaplophora glabripennis</i>	Maple	2	pipetted imidacloprid onto mouthparts, dipped into fungi	sequential
Senthilraja	2010	<i>Aproaerema modicella</i>	Ground nut	2	spray application	simultaneous
Senthilraja et al.	2010	<i>Aproaerema modicella</i>	Ground nut	3	seed, soil, and foliar applications	simultaneous
Cuthbertson et al.	2012	<i>Bemisia tabaci</i>	Poinsettia	2	spray application	sequential
Mascarin & Delalibera	2012	<i>Phthorimaea operculella</i>	potato	2	dipping	simultaneous
Petzold-Maxwell et al.	2012	<i>Diabrotica virgifera virgifera</i>	Maize	6	soil application	simultaneous
Avery et al.	2013	<i>Diaphorina citri</i>	Citrus	2	spray application	simultaneous
Rondelli et al.	2013	<i>Plutella xylostella</i>	Cabbage	2	spray application	simultaneous
Prabhukarthikeyan et al.	2014	<i>Helicoverpa armigera</i>	Tomato	2	seed treatment, dipping and spray	simultaneous
Wu et al.	2014	<i>Cyclocephala lurida</i>	NA	2	soil application	simultaneous
Xu et al.	2014	<i>Helicoverpa armigera</i>	NA	2	diet fed	sequential
Cuthbertson & Collins	2015	<i>Bemisia tabaci</i>	Poinsettia	2	spray application	simultaneous
Hamama et al.	2015	<i>Spodoptera littoralis</i>	Castor	2	diet fed	sequential
Arrizubieta et al.	2016	<i>Helicoverpa armigera</i>	Tomato	2	spray application	simultaneous
Buitenhuis et al.	2016	<i>Bemisia tabaci</i>	Poinsettia	2	dipping	simultaneous
Keyser et al.	2016	<i>Tenebrio molitor</i>	Wheat	2	diet fed	simultaneous
Otieno et al.	2016	<i>Frankliniella occidentalis</i>	French bean	2	soil application	simultaneous

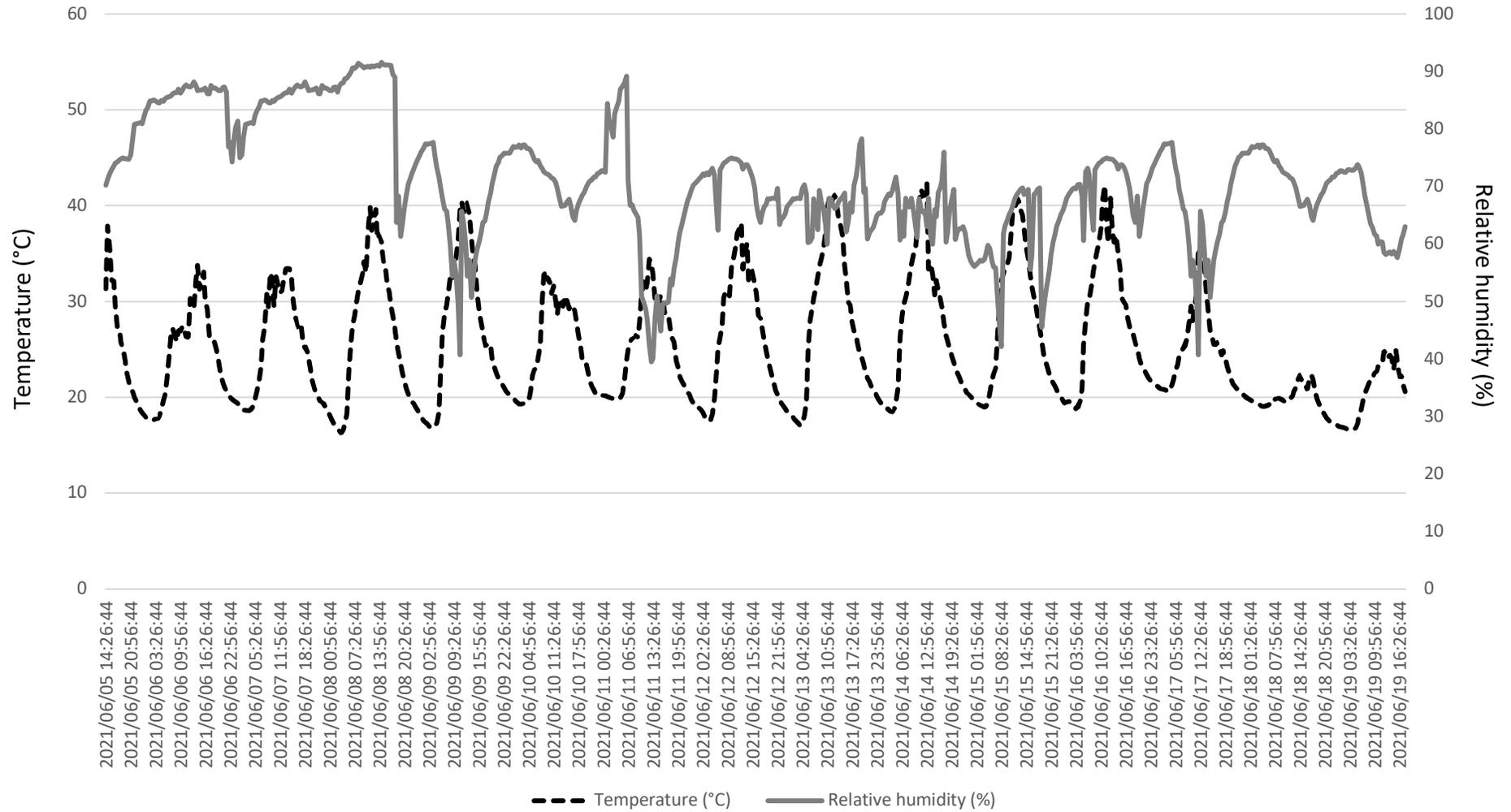
Author	Year	Target Pest	Crop	Number of components in the mixture	Application method	Simultaneous or Sequential Application
Virto, Navarro, Murillo, Williams & Cabarello	2016	<i>Spodoptera exigua</i>	NA	2	diet fed	simultaneous
Antwi, Shrestha, Reddy & Jaronski	2017	<i>Limoniuss californicus & Hypnoidus bicolor</i>	Wheat	2	soil application	simultaneous
Carballo et al.	2017	<i>Spodoptera exigua</i>	NA	2	diet fed	simultaneous
Kumar, Avery, Ahmed, Cave, McKenzie, Osborne	2017	<i>Diaphorina citri</i>	Citrus	2	spray application	simultaneous
Niu, Wang, Liu, Xiao, Wang & Guo	2017	<i>Nilaparvata lugens</i>	Rice	2	spray application	simultaneous
Roberti, Righini, Masetti & Maini	2017	<i>Trialeurodes vaporariorum</i>	Saffron crocus	2	spray application	simultaneous
Wright & Ramos	2017	<i>Leptinotarsa decemlineata</i>	potato	2	spray application	simultaneous
Wu et al.	2017	<i>Bradysia odoriphaga</i>	Chinese chive	2	treated Petri dish	simultaneous
Ali	2017	<i>Bemisia tabaci</i>	Cotton	2	dipping	both
Eivazian et al.	2018	<i>Phthorimaea operculella</i>	potato	2	dipping	simultaneous
Sabino, Negrisoli, Andalo, Filigueiras, Moino & Sales	2018	<i>Tuta absoluta</i>	Tomato	2	spray application	simultaneous
Sabino, Negrisoli, Andalo, Filigueiras, Moino & Sales	2018	<i>Tuta absoluta</i>	Tomato	2	spray application	simultaneous
Somoza-Vargas et al.	2018	<i>Bemisia tabaci</i>	Strawberry	2	spray application	simultaneous
Alencar Velez et al.	2019	<i>Dactylopius opuntiae</i>	Prickly pear	3	spray application	simultaneous
Amizadeh et al.	2019	<i>Tuta absoluta</i>	Tomato	2	spray application	simultaneous
Ayyub et al.	2019	<i>Spodoptera litura Fabricius</i>	Cotton	2	spray application	simultaneous
Canassa et al.	2019	<i>Tetranychus urticae</i>	French Bean	2	seed treatment	simultaneous
Cuartas-Otálora et al.	2019	<i>Spodoptera frugiperda</i>	NA	2	diet fed	simultaneous
Cuartas-Otalora, Gomez-Valderrama, Ramos, Barrera-Cubillos & Villamizar-Rivero	2019	<i>Spodoptera frugiperda</i>	Castor	2	diet fed	simultaneous

Author	Year	Target Pest	Crop	Number of components in the mixture	Application method	Simultaneous or Sequential Application
Dakhel, Latchininsky, Jaronski	2019	<i>Melanoplus sanguinipes</i>	Wheat	2	diet fed	simultaneous
Jallow et al.	2019	<i>Tuta absoluta</i>	Tomato	2	spray application	simultaneous
Gonçalves et al.	2020	<i>Dactylopius opuntiae</i>	Prickly pear	2	spray application	sequential
Konecka et al.	2020	<i>Cydia pomonella</i>	NA	2	diet fed	simultaneous
Sáenz-Aponte et al.	2020	<i>Plutella xylostella</i>	Broccoli	2	spray application	simultaneous
Wari et al.	2020	<i>Bemisia tabaci</i>	Common bean	2	dipping & spray applicaiton	both

Appendix VII



Temperature and humidity recordings taken from data loggers (Lascar electronics Ltd; EL-SIE-1) placed close to the surface of treated leaves, within bread bag containers during the first greenhouse trial.



Temperature and humidity recordings taken from data loggers (Lascar electronics Ltd; EL-SIE-1) placed close to the surface of treated leaves, within bread bag containers during the second greenhouse trial.