Understanding interactions between biopesticides and partial crop resistance for management of aphid pests of *Brassica* crops

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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Life Sciences

University of Warwick, School of Life Sciences

January 2022

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Abbreviations

5PL	Five-parameter logistic concentration-response curve/equation
µl	Microlitre
µm	Micrometre
µmol	Micromole
ABA	Abscisic acid
AIC	Akaike information criteria
ANOVA	Analysis of variance
ARSEF	Agricultural Research Service Collection of Entomopathogenic Fungal Cultures
AVR	Avirulence
B.C.E.	Before Common Era
BLAST	Basic local alignment search tool
C	Celsius
df	Degrees of freedom
DFFS	Diversity fixed foundation set
DNA	Deoxyribonucleic acid
DT	Doubling time
EIL	Economic injury level
EPF	Entomopathogenic fungi
ET	Economic threshold
ET	Ethylene
EU	European union
FAO	Food and Agriculture Organization
g	Gram
GA	Gibberellic acid
GLM	Generalised linear model
GLMM	Generalised linear mixed model
h	Hour
ha	Hectare
hg/ha	Hectogramme per hectare
HPLC	High performance liquid chromatography
hr	Hour
HR	Hazard ratio

IPM	Integrated pest management
ITC	Isothiocyanate
ITS	Internal transcribed spacer
JA	Jasmonic acid
kPA	Kilopascal
LC_{50}	Concentration at which 50% mortality occurs
LC_{70}	Concentration at which 70% mortality occurs
$LC_{70}A$	Concentration at which 70% adult aphid mortality occurs
$LC_{70}N$	Concentration at which 70% nymph aphid mortality occurs
LC_{90}	Concentration at which 90% mortality occurs
L:D	Light:dark
Log	Logarithm
MACE	Modified acetylcholinesterase
MAMP	Microbe associated molecular pattern
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
MRGR	Mean relative growth rate
MST	Median survival time
n	Number
NB-LRR	Nucleotide-binding leucine-rich repeat
NCBI	National Centre of Biotechnology Information
No.	Number
p	Probability
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pH	Power of hydrogen
Pmax	Specific maximum growth response
QTL	Quantitative trait locus
R^2	Coefficient of determination
R genes	Resistance genes
RH	Relative humidity

RLK/RLP	Receptor-like kinase/receptor-like proteins
r_m	Intrinsic rate of increase
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
SA	Salicylic acid
SDA	Sabouraud dextrose agar
SEM	Standard error of the mean
Sig.	Significance
subsp.	Subspecies
ssp.	Species
Tmax	Thermal maxima
Tmin	Thermal minima
Topt	Thermal optima
UK	United Kingdom
UKVGB	UK Vegetable Genebank
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
var.	Variety
VeGIN	Vegetable Genetic Improvement Network

Acknowledgements

I would firstly like to thank my supervisors David Chandler and Graham Teakle. I am very grateful for your help, support, and enthusiasm over the past three years. Thank you also to Gill Prince for your guidance and advice throughout my PhD, and instruction in laboratory methods.

I would like to thank all the staff and students at the Warwick Crop Centre, with whom I spent many enjoyable hours in the lab and common room. Thanks especially to Andy Jukes for teaching and assisting me with HPLC analysis, James Lynn for advice on statistical analysis, and Marian 'Maz' Elliott for expertise on all insect-rearing queries.

Finally, thank you to my family and friends, especially my partner Alec, for all their support and encouragement throughout my PhD.

I acknowledge the funding for this research provided by the MIBTP and BBSRC.

Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. The work in this thesis including the data generated and data analysis is original research carried out by the author unless otherwise stated. It has been composed by me and has not been submitted in any previous application for any degree.

Abstract

The cabbage aphid, *Brevicoryne brassicae* L., is a *Brassica* specialist, and is among the most damaging pests of vegetable *Brassica* crops, causing significant yield losses due to direct feeding damage, virus transmission and crop contamination. It is normally managed using synthetic chemical pesticides. However, with emerging reports of insecticide-resistant *B. brassicae* clones and withdrawal of insecticides from the market because of environmental concerns, there is a need for alternative, sustainable approaches. In this project, a novel Integrated Pest Management (IPM) system was developed and evaluated, combining partially aphid resistant *Brassica* accessions with entomopathogenic fungi (EPF). In recent years it has been suggested that host plant resistance and biological controls such as EPF may interact favourably, resulting in greater than additive control. It was hypothesised that partial host plant resistance may extend the window of opportunity for successful EPF infection resulting in significantly higher levels of control. Through pre-selecting and screening a range of *Brassica* accessions, partially resistant accessions were identified which limited *B. brassicae* population development by approximately one third. Assessment of aphid life history traits showed that partial resistance significantly reduced the rate of juvenile aphid development, aphid weight and adult reproduction. Laboratory bioassays of 10 isolates of EPF showed that all were pathogenic to *B. brassicae* adults but were largely ineffective against nymphs with the exception of one isolate of *Akanthomyces dipterigenus*, which was highly virulent to nymphs. Experiments indicated that this may have resulted from faster germination and growth compared to other isolates. In experiments where *B. brassicae* were reared on partially resistant *Brassica* accessions and treated with the EPF *A. dipterigenus* or *Beauveria bassiana*, it was found that partial host plant resistance caused nymphs to become significantly more susceptible to infection by *B. bassiana* relative to nymph cohorts on more aphid-susceptible plants. Combining partial resistance with *A. dipterigenus* caused a significant increase in aphid population control as a result of a plant x EPF interaction. The results of this study demonstrate the potential of this combined IPM strategy, with the effects of partial resistance upon *B. brassicae* able to activate or improve EPF virulence to aphid nymphs, which appear to be the primary challenge for aphid control using EPF.

1. Introduction

Globally, the most significant causes of yield and quality loss in crops are pests and diseases – accounting for an estimated 20-40% decline in yields (Lobell *et al.*, 2009; Cerda *et al.*, 2017; FAO, 2019). Driven by these significant pest and disease associated crop losses alongside increasing demand from retailers for flawless produce, growers are driven to take increasingly significant action to mitigate pest and disease associated yield losses. Of the potential crop protection approaches available to growers, the routine application of synthetic chemical inputs including pesticides, fungicides, and herbicides has come to dominate as the core component of crop protection in modern conventional agriculture (Skinner *et al.*, 2014).

In recent decades however, a growing scientific and public awareness of the adverse environmental repercussions of synthetic chemical inputs, the emergence and rapid dissemination of heritable resistance to these inputs in crop pest, disease and weed populations, and potential negative impacts of these chemicals upon human health has made the use of synthetic chemical inputs increasingly controversial. In response, legislating bodies in recent years have been taking increasingly significant action to address the issue – most notably by enforcing withdrawals of synthetic chemical inputs from the market (Nicolopoulou-Stamati *et al.*, 2016; Zhang *et al.*, 2018). With access to fewer effective synthetic chemical controls, growers face mounting pressures and difficulty in maintaining current yields.

Despite mounting public and political pressures to decrease the usage of synthetic chemical controls, conventional agriculture nonetheless continues to rely almost solely upon synthetic chemical inputs to control a wide array of crop pests and diseases – particularly in field crops. One such crop/pest combination in which synthetic chemical pesticides remain the mainstay of control are aphid pests of field-grown vegetable and oilseed *Brassica* crops. In total, nine aphid species are currently recognised as potential *Brassica* crop pests, of which two species cause the majority of economic losses: the polyphagous peach potato aphid, *Myzus persicae* Sulzer, 1776 and the monophagous cabbage aphid, *Brevicoryne brassicae* Linnaeus, 1758 (Blackman and Eastop, 2000). Historically synthetic chemical insecticides have proven a hugely successful tool in the control of these aphid pests however, increasing emergence and dissemination of heritable insecticide resistance among

populations of both species alongside increasing withdrawal of previous mainstays of control such as neonicotinoids has left growers few viable options for aphid control (Dedryver *et al.*, 2010). Alternative management tools and strategies are therefore urgently required.

1.1. Integrated pest management

Integrated Pest Management (IPM) has since its inception in the late 1950s risen to prominence among legislating bodies and governments as the foremost alternative pest control strategy - emphasising a holistic, balanced, and diverse systems approach to pest management of which conventional synthetic chemical products play only a supporting role (Brewer and Goodell, 2012). IPM was Pioneered by Stern, Smith, van den Bosch and Hagen in 1959, with its inception being driven by a growing awareness of the potentially harmful side-effects of synthetic chemical pesticides at this time (Stern *et al.*, 1959; Naranjo and Ellsworth, 2009). While IPM gained continued and growing research traction throughout the 1980s, 1990s and 2000s, its recent move to the forefront of agricultural discourse followed a 2009 EU directive obliging all commercial growers within the EU (as of 2014) to utilise IPM and its core principles as their primary means of pest management (Stern *et al.*, 1959; Stenberg, 2017).

1.1.1. Economic injury level and economic thresholds

In contrast to conventional pesticide-centric pest control, IPM strategies are not intended to wholly exclude pests but rather aim to quantitatively supress pest populations below an economic injury level (EIL) – defined as the theoretical lowest pest population density sufficient to cause economic damage (Stern *et al.*, 1959). In practice, IPM typically recommends the use of an economic threshold (ET) for the given crop/pest combination – this being the threshold pest population at which control action must be taken to prevent populations exceeding the EIL and incurring economic damage; continual monitoring of pest population density is thus crucial. The relationship between the EIL and ET is outlined in Figure 1.1. Economic thresholds are invariably difficult to calculate, incorporating numerous complex elements including the EIL, pest and host phenology, pest population growth rate and any time delays associated with deployed control measures. Through determination of the crop/pest EIL and ET and a considered combination of pre-

emptive controls, regular monitoring for pest populations and, where necessary, post-emptive controls - a significant decrease in synthetic chemical pesticide usage is feasible - thus reducing environmental, grower, and public exposure to potentially toxic synthetic chemicals (Higley and Pedigo, 1993; Stenberg, 2017).

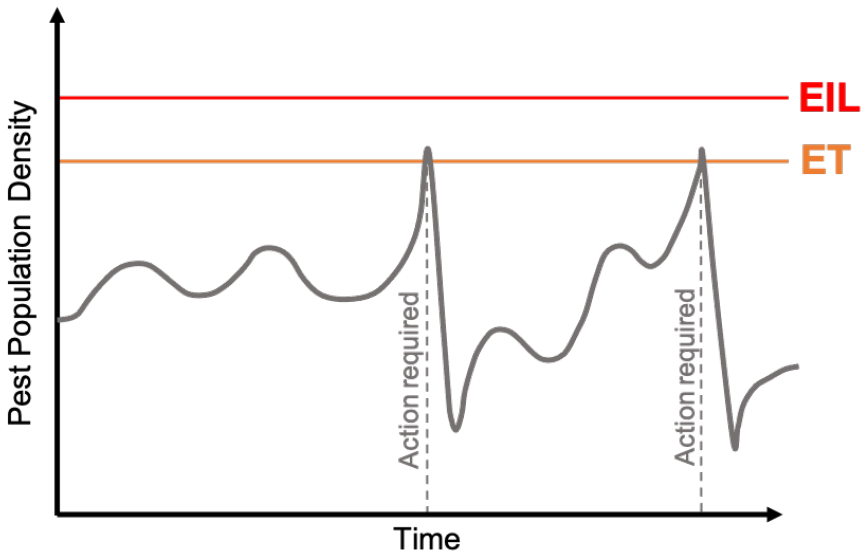


Figure 1.1: Relationship between Economic Injury Level (EIL) and Economic Threshold (ET) in Integrated Pest Management (IPM). The ET incorporates the EIL alongside practical ecological traits of the crop and pest, alongside the speed and efficacy of any control measures to set a threshold pest population density below which no action is necessary and above which action is required to prevent economic damage to the crop.

1.1.2. IPM frameworks

To maintain pest populations and population density below the Economic Threshold, IPM recommends the informed and integrated use of an array of practical tools and management strategies relevant for the given agricultural situation (Dara, 2019). Following the initial proposal of IPM, research into potential IPM tools has grown into a significant and active area of agricultural research, leading to the proposal of a wealth of different methods, approaches and tools for potential inclusion in IPM strategies. They seek to both prevent the initial migration of pests into a crop and limit pest population development once present. IPM tools include the deployment of pest-resistant crop varieties, the promotion of natural enemies to problematic

pests, the use of macro and micro biological control agents/biopesticides, the deployment of physical boundaries, crop pest monitoring and, as a last resort, the use of synthetic chemical pesticides. Owing to the significant number of potential IPM tools, there exists the potential for confusion over the relative importance of each. In an effort to combat this, numerous IPM frameworks have been proposed aiming to convey more simply the options available to growers and clarify the control measures which should be prioritised. Among these frameworks, IPM pyramids have become the most prominent, organising pest control components into three distinct tiers defined by both their effect and optimal order of application (Figure 1.2) (Naranjo and Ellsworth, 2009; Luo *et al.*, 2014).

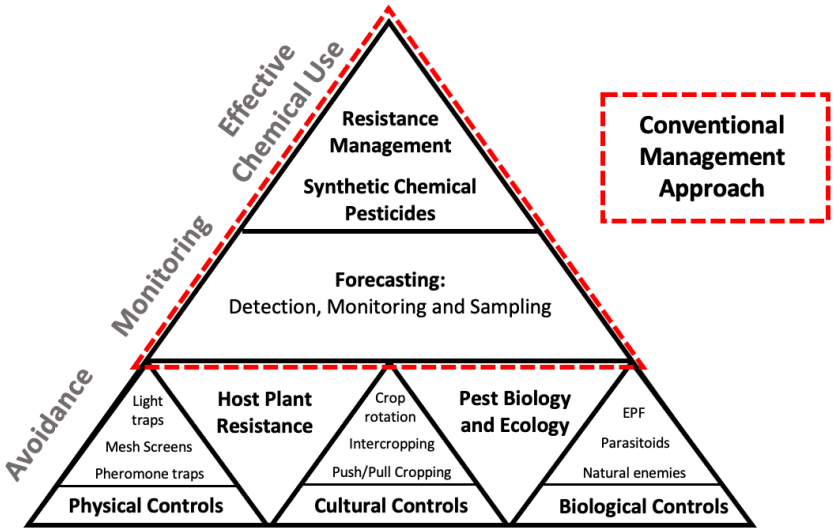


Figure 1.2: IPM Pyramid outlining the principles of Integrated Pest Management alongside the conventional management approach. Through this pyramid model, IPM tools and components are organised into three distinct tiers, demonstrating the focus of each and their respective inter-relationships and importance. The basal tier, Avoidance, includes pre-emptive components which either inform about the biology of, delay or exclude pests from a crop. The second tier, Monitoring, includes measures which allow for the detection and continual observation of pest population abundance and density - informing whether the Economic threshold for that given pest has been exceeded. The final tier, Effective Chemical Use, serves as a final option for pest control, where avoidance measures have been insufficient, and monitoring informs that the economic threshold has been exceeded and thus further control measures are vindicated. Figure based on Naranjo and Ellsworth (2009) and Luo *et al.* (2014).

1.1.3. Successes of IPM: augmentative biological control in protected environments

With the increasing prominence of IPM, particularly from the 1990s onwards, several sophisticated IPM programmes have arisen – most notably in protected growing environments and glasshouse crops where IPM is now standard practice in both the UK and the Netherlands (van Lenteren, 2000). In these environments, avoidance strategies such as augmentative biological control, have been particularly successful in offering reliable pest control sufficient to maintain pest populations below economic thresholds – thereby mitigating the need for synthetic chemical pesticides (van Lenteren and Woets, 1988; van Lenteren 2012; Dara, 2019). While levels of sophistication vary wildly between different protected growing environments, the predictability of environmental conditions that these can provide alongside the semi-closed nature of the system are consistent and likely the most significant factors permitting the significant successes of IPM in these environments - allowing suitable conditions for natural enemies and biopesticides. Glasshouse conditions also ensure biological control agents such as natural predators and parasitoids are contained within the glasshouse alongside limiting access to the crop for external pest migrations (van Lenteren and Woets, 1988). Through predictability and control over glasshouse temperatures (including daily fluctuations), ambient humidity, light-intensity and day-length, growers are also able to accurately predict and monitor pest population development rates (van Lenteren and Woets, 1988). By monitoring and targeted modification of these environmental conditions alongside monitoring and modelling of pest populations, biological and cultural control methods can be precisely targeted and deployed strategically at times where the conditions best suit the known biology of natural enemies or biopesticides, hence maximizing the effectiveness of these products.

1.1.4. IPM in the field

In contrast to the successes of IPM programmes in protected environments, the development of IPM in field crops has proven a significant challenge. Many field growers already successfully employ a range of individual IPM components to limit the migration, establishment, and proliferation of a range of pests into crops including crop rotations, crop management strategies (including removal of plant residues and the application of necessary fertilisers and irrigation), and physical

traps/barriers. Similarly, monitoring of pests and diseases in the crop is also commonplace, with many growers regularly checking pest/disease forecasts and undertaking routine crop walks to assess/monitor pest presence and abundance. While many of the fundamental components are thus already in place for successful field IPM, there remains failings and knowledge gaps across the agricultural system limiting the success of IPM in the field; among the most major being a relative lack of viable alternatives to synthetic chemical pesticides.

In protected environments the closed nature of the system and controllability of the environment conditions permit the successful use of a wealth of alternative pest control approaches – most notably natural enemies, parasitoids and biopesticides. In stark contrast however, biological controls have proven significantly less successful under field conditions.

Within a field context, environmental conditions including light intensity, temperature, humidity, windspeed, wind direction and water availability are innately and profoundly variable – with each fluctuating significantly on an hourly, daily, weekly, and monthly basis.

As primarily live organisms, the success of biopesticides is intimately linked to environmental conditions - with significant to near total declines in efficacy where environmental conditions are unfavourable (Chandler *et al.*, 2008; van Lenteren, 2000). It is therefore paramount to determine the effective operating range of biological control agents for these key variable environmental factors. Further issues for the use of biological controls in the field stem from the open nature of the field, which allows the free migration of pests into and out of the crop alongside the drift of synthetic chemical inputs applied to adjacent fields which may adversely affect biological control agents.

To overcome these challenges to the use of biological control in the field, significant academic and industrial research is required to fully assess and quantify the viability of each control option under variable environmental conditions. Further optimisations in biological control efficacy may also be achieved by determining optimal conditions/timings of application, calculating and disseminating economic

thresholds for specific crop/pest combinations to growers. Through detailed and rigorous research into these underdeveloped areas, it may be possible to improve the relative efficacy of biopesticides and biological controls in the field – providing an alternative to synthetic chemical pesticides.

1.1.5. IPM research

Despite substantial IPM research effort over the past 60 years, synthetic chemical pesticides remain the mainstay of pest control throughout a majority of global field cropping systems (Deguine *et al.*, 2021). It is therefore clear that despite the significant and growing body of research into IPM, there have been failings in translating IPM research progress into deployable IPM strategies and communicating such strategies with growers.

A wealth of complex and interconnected factors have likely contributed to the limited success and inconsistent deployment of IPM in field settings. Described in detail by Deguine *et al.* (2021), many such factors extend beyond the remit of researchers, including a weak grower knowledge base surrounding IPM technologies, potential vested corporate interests in synthetic chemical pesticides and inconsistent global agronomic policies. Nonetheless, significant failings also result directly from the historic and current focuses of IPM research, chiefly the overwhelming attention placed on researching individual IPM tools rather than seeking to develop holistic IPM strategies (Stenberg, 2017).

This research focus has generated a wealth of IPM tools for growers however – in lieu of defined IPM strategies – such an array of tools often serves only to create confusion among growers as to the relative importance of each tool and the optimal course of action at any given time (Stenberg, 2017). While IPM frameworks including the IPM pyramid (Figure 1.2), alleviate this confusion in part, there nonetheless remains uncertainty among growers as to the optimal course of action necessary at different times and pest population densities (Deguine *et al.*, 2021).

Alongside research into isolated IPM tools, significant research efforts have also been focussed on the definition and fundamental principles of IPM. Since its inception, the definition and theoretical basis of IPM has been heavily debated and

amended, with countless authors between 1959-2016 proposing upwards of 67 differing definitions – ballooning further to exceed 100 definitions as of 2021 (Bajwa and Kogan, 2002; Coll and Wajnberg, 2017; Deguine *et al.*, 2021). This number of definitions has led to further confusion among growers and inconsistency in the recommendations of agronomists and researchers dependent upon the particular IPM definition to which they subscribe (Stetkiewicz *et al.*, 2018; Deguine *et al.*, 2021). When combined, the confusing array of IPM tools available to growers and the significant number of often contradictory definitions of IPM in lieu of defined IPM strategies have undoubtedly hindered the success IPM in the field - leading to the inconsistent successes reported by growers and continuing reliance on synthetic chemical pesticides (Deguine *et al.*, 2021).

To address these problems, there is an urgent need for IPM research to refocus upon the development of holistic IPM strategies. IPM strategies are complex and involve the integration and interaction of many different components, thus such research is typically complex and interdisciplinary. While this complexity is necessary to provide the levels of crop protection and durability, it nonetheless poses an obstacle for researchers. Such research would therefore benefit from a logical roadmap, outlining the key steps, knowledge and research focuses necessary to guide the development of IPM strategies. To date, no such logical roadmap has been universally accepted. There do however exist several notable case studies where a logical approach was taken to aid in developing IPM strategies. Through these case studies, it may therefore be possible to devise such a roadmap to determine a logical approach to IPM research – keeping research tightly focused within the context of developing IPM strategies.

1.1.6. IPM strategy development: a case study

Perhaps the most significant case study which sought to take a logical process to guide the development of a field based IPM strategy has been an ongoing project seeking to develop an IPM strategy for the control of Sunn Pest (*Eurygaster integriceps*) on wheat and barley crops in Central and Western Asia (El Bouhssini *et al.* 2009; Skinner *et al.*, 2014). Sunn pest in these regions causes significant yield losses (20-30% in Barley and 50-90% in Wheat) thus control is paramount (Davari and Parker, 2018). Historically, Sunn pest control relied on aerial applications of

chemical pesticides – costing US$42 million per annum and driving the development of insecticide-resistant pest biotypes (Skinner *et al.*, 2014; Davari and Parker, 2018). In 1996 however, recognising the unsustainability of this control approach, an initiative to develop a sustainable IPM control strategy was launched by regional research institutes, agricultural specialists, and universities with a view to creating a sustainable IPM strategy. Recognising the complexity of the challenge to develop such a strategy, the initiative took a logical stepped approach to the problem.

1. **Understanding the pest and crop.** A detailed understanding of both the pest and the crop was chosen as the foundation upon which the IPM strategy would be built. The first step taken was to assess the biology, life cycle and diversity of both the crop and the pest – with a focus also on their relationship, interactions, and the relative susceptibility of the crop to the pest. It was within this context that all other aspects of IPM would be considered.
2. **Current control measures.** Having assessed the problems posed by the pest, researchers moved on to evaluate the currently used range of crop protection products and approaches – particularly their historical and current efficacy, how such control approaches relate to the biology of the pest-plant system and seeking to understand why the pest had become resistant to many such controls.
3. **Natural controls.** Recognising the need for alternative applicable controls to synthetic chemical pesticides as a component of IPM, researchers sought to identify naturally occurring moderators of pest populations – chiefly any predators, parasitoids or entomopathogens which could offer significant natural control and may be viable as biological control agents.
4. **Directing research.** Having identified alternative control approaches which held promise as potential components of an IPM strategy, the final step taken by this initiative was to direct research towards these identified natural controls alongside potential sources of crop resistance potentially highlighted in the first step of the approach.

While this initiative seeking to develop an IPM strategy for Sunn pest on wheat and barley launched over 20 years ago, it continues to drive progress and research in this

field (Davari and Parker, 2018). The success of this initiative is undoubtedly due to the logical stepped approached proposed at its outset, providing a logical grounding for the necessary complex and interdisciplinary research required. This study provides part of a valuable blueprint for the development and investigations required when developing a novel IPM strategy.

1.1.7. A new conceptual framework for IPM

While the value of the stepped approach taken by researchers seeking to develop an IPM strategy for Sunn pest is clear, progress in this project has significantly slowed in recent years (Davari and Parker, 2018). The clear route set out in this project allowed for the identification and evaluation of a range of useful tools which could form part of an IPM strategy, however the failure of this project to set out the subsequent steps involved in integrating these disparate tools has caused progress to stall. The failure to take this subsequent integrative step has consistently plagued IPM research (Stenberg, 2017).

Of the major research gaps present within the field of IPM, perhaps the most significant obstacle remains the development of truly holistic IPM strategies for specific crop/pest combinations for which successful integration of individual IPM tools is paramount. While successes have undoubtedly been seen with many current IPM strategies, typically these strategies combine IPM elements haphazardly with little regard for their interactions – resulting in control significantly less effective than the sum of their individual parts (Stenberg, 2017). To maximise the potential of each individual IPM component it is therefore essential for researchers to undertake truly integrated and interdisciplinary research – exploring in detail the nature, compatibility, and significance of interactions between individual IPM components such that they can be logically integrated to maximise their potential. Despite this urgent need for truly integrated IPM strategies however, a majority of IPM research remains focused on single pest management methods (Stenberg, 2017).

Recognising this fundamental failing of IPM research, in an attempt to refocus the current IPM research paradigm away from research upon singular IPM elements, Stenberg (2017) proposed a new conceptual framework for IPM highlighting seven key interactions between ecological/biological IPM elements towards which

interdisciplinary research must be focused (Figure 1.3) (Stenberg, 2017). Of these highlighted interactions, biological control and heritable plant resistance serve as the most interconnected ecological nodes. Heritable plant resistance is indicated as directly interacting with, and thus altering the performance of, three other IPM elements: biorational volatiles, biological control and plant vaccination. Conversely, biological control is the ecological element most susceptible to the effects of other IPM elements, interacting with four other IPM tools: biorational volatiles, heritable plant resistance, plant vaccination and botanical diversity. While all seven identified interactions are important for IPM research in the future, from a research perspective the two-way interaction between biological control and heritable plant resistance is the most tantalizing. Such a two-way relationship indicates the significant interaction between these two IPM components and highlights the substantial benefits for an IPM strategy if strong levels of each can be identified and integrated to maximise their potential.

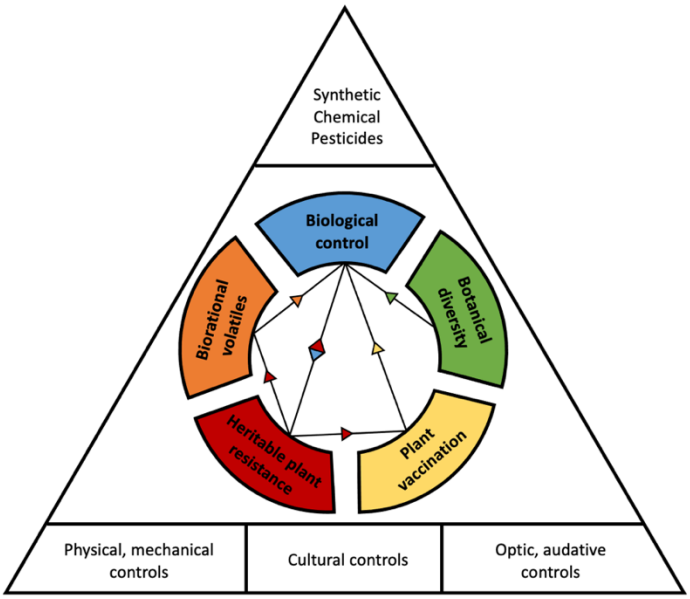


Figure 1.3: Amended IPM Pyramid proposed by Stenberg (2017). The basal layer, similarly to most IPM pyramids, includes the three broad categories of typically pre-emptive abiotic actions available to growers to both mitigate against pest migration into a crop and limit pest population development where present. The second layer includes five categories of 'ecological' IPM tools, emphasising the complex interrelationships and interactions between each. The top layer of the pyramid includes synthetic chemical pesticides – a last resort for growers where abiotic and ecological tools have failed to maintain pest populations below the economic threshold. Through this pyramid, Stenberg highlights seven key interactions between ecological IPM tools for which research is urgently required to characterise the nature of their interactions in order to maximise their integrated potential. Figure based on Stenberg, 2017.

1.2. *Brassica* crops

Within the context of both the Sunn pest case study and the conceptual framework proposed by Stenberg (2017), crop plant varieties and their intrinsic resistance or susceptibility are unquestionably central in determining the outcome of deployed IPM strategies (Stenberg, 2017; Davari and Parker, 2018). To date however, while significant research has been undertaken assessing plant resistance to pests and diseases in isolation - only limited research has sought to explore the importance of such resistance in the context of IPM. A substantial knowledge gap therefore exists relating to how varietal variations in crop plant biology interacts with other aspects of an IPM strategy (Stenberg, 2017). To develop this understanding, a fundamental first step is understanding in isolation the nature of a given crop including: its evolutionary origins, the diversity present within a given crop species, the relationship of a crop species to other important crops, the pests and diseases to which the crop is susceptible, and the nuanced physical and biochemical interactions between the crop and the pest or disease of particular interest.

1.2.1. *Brassica* crops: origins, diversity, and global importance

The genus *Brassica* is classified in the eudicot plant family *Brassicaceae* – one of the most globally important families of cultivated plants. Within the *Brassicaceae* family there exist 372 genera and 4060 currently recognised species, several of which are of significant agricultural and economic importance as vegetable, oil, condiment, or fodder crops (Kim *et al.*, 2018). Of the genera within the *Brassicaceae*, the genus *Brassica* contains the most significant proportion of these economically important crop species. It is unclear precisely when humans began cultivating and domesticating *Brassica* species into recognisable modern crops, though references to cruciferous vegetables in Greek and Roman literature indicate that *Brassica* crops have been cultivated for a minimum of several thousand years by numerous human civilisations dating back to the VI century B.C.E. (Shyam *et al.* 2012; Maggioni *et al.*, 2018).

1.2.2. Significance of *Brassica* crops

Among the 37 recognised species in the genus *Brassica*, most cruciferous vegetables arise from only three species: *Brassica oleracea* L., *Brassica rapa* L. and, to a lesser extent, *Brassica napus* L.. Historically *Brassica oleracea* has been the predominant

source of cruciferous vegetables in Europe and latterly North America, with the majority of *B. oleracea* crop diversification recognised as occurring in Europe likely between the 12th and 18th centuries (Maggioni *et al.*, 2010). Conversely *B. rapa* has historically been the predominant source of cruciferous vegetables in Asia, with much of its diversification also having taken place in this region (Ignatov *et al.*, 2008). Increasing globalisation has eroded somewhat this geographic trend, with cruciferous vegetables of both *B. oleracea* and *B. rapa* now being common in both regions.

Table 1.1: Global production of Cabbage and other vegetable *Brassica* crops by area in 2019. (FAOstat, 2019).

Area	Element	Value	Unit
UK	Area Harvested	8382	ha
	Yield	222450	hg/ha
	Production	186458	tonnes
Americas	Area Harvested	66769	ha
	Yield	314687	hg/ha
	Production	2101133	tonnes
Asia	Area Harvested	1773157	ha
	Yield	308121	hg/ha
	Production	54634684	tonnes
Europe	Area Harvested	325804	ha
	Yield	298336	hg/ha
	Production	9719903	tonnes
Oceania	Area Harvested	3978	ha
	Yield	400214	hg/ha
	Production	159205	tonnes
Global	Area Harvested	2446294	ha
	Yield	286762	hg/ha
	Production	70150406	tonnes

Of these two species, *B. oleracea* is the most agriculturally diverse and economically significant – accounting for a majority of the cruciferous vegetables consumed globally (Mabry *et al.*, 2021). Among its diversity, *B. oleracea* includes six crops of recognised global significance: broccoli, brussels sprouts cabbage, cauliflower, kale, and kohlrabi (Mabry *et al.*, 2021). Owing to these six major crops, *B. oleracea* is recognised among the 10 most economically significant crops globally. Approximately 70.1 million metric tonnes of *B. oleracea* crops were produced globally in 2019, of which 186,458 tonnes were produced in the UK (Table 1.1) (The Food and Agriculture Organization; www.fao.org).

1.2.3. Nutritional significance of *Brassica* crops

Brassica crops have for centuries been, and remain, a staple vegetable in global food systems. In recent years *Brassica* crops have gained increasing prominence owing to recognition of their high vitamin and mineral content (Shaym *et al.*, 2012). *Brassica oleracea* crops have high levels of vitamins C and E alongside carotenoids and folate, high levels of mineral P, S, Cl, Ca, Fe, Sr and K (particularly in kale), high levels of antioxidants and significant concentrations of beneficial phytochemicals including phenolics and glucosinolates (Francisco *et al.*, 2016; Sanlier and Saban, 2018). While there are significant difficulties in drawing direct scientific relationships between specific foods and their potential benefits to human health, increasingly compelling evidence is being reported for the role of regular cruciferous vegetable consumption in potentially reducing the risk of degenerative neurological diseases, cancers, cardiovascular diseases and immunological dysfunction – leading to the proposal and wide-spread marketing of several cruciferous vegetables, most notably Kale, as putative 'superfoods' (Samec *et al.*, 2018).

1.2.4. Intraspecific diversity and classification of *Brassica oleracea*

Relative to its most closely related *Brassica* relatives alongside other vegetable species, the economic and societal importance of *B. oleracea* is unique in stemming largely from the wide diversity which has arisen within the species. Within the intraspecific scope of *B. oleracea* there currently exists 18 distinct and recognised taxa, distinguished primarily at the varietas level (Table 1.2). While suitable for botanical nomenclatorial purposes, there remains continued confusion among both growers and researchers surrounding the formal interspecific botanical classifications

of *B. oleracea* (Mabry *et al.*, 2021). Aiming to simplify this complexity, several competing systems have been proposed for the categorisation of *B. oleracea* diversity into defined horticultural 'Cultivar Groups'. While cultivar groups are intended as horticultural rather than scientific aides, these groupings have nonetheless, and despite their occasionally paraphyletic nature, become the most frequently used method to formally describe *B. oleracea* diversity. In recent years, several competing cultivar grouping systems have been proposed and remain in active use; most notably those devised by Mabberley and Kew (Gladis and Hammer, 2001; Mabberley, 2017). While both proposed systems base their groupings upon the developmental form, relatedness, and edible organ of the mature crop, they differ in the number of groups they propose. The most recent edition of Mabberly's system proposes that *Brassica oleracea* crops be categorised into fifteen cultivar groups while the Kew system proposes a further condensing of *B. oleracea* diversity into eight cultivar groups (Mabberley, 2017). Of these two systems, the Kew cultivar grouping system has seen the widest adoption among both researchers and growers. The Kew cultivar grouping system is centred around the six major *Brassica oleracea* vegetable crops, centring a cultivar group around each: broccoli (Italica Group), brussels sprouts (Gemmifera Group), cabbage (Capitata Group), cauliflower (Botrytis Group), kale (Acephala Group) and kohlrabi (Gongylodes Group). The remaining two cultivar groups (Alboglabra Group and Tronchuda Group) serve as orphan groups into which the remaining, lesser grown, *B. oleracea* crop types are grouped.

Table 1.2: Intraspecific botanical nomenclature of *Brassica oleracea*, corresponding Kew Cultivar Groups and the associated crop types.

Botanical nomenclature, *Brassica oleracea* varietas and forma (var.)	Kew Cultivar Group	Crop types
acephala	Acephala Group	Ornamental Cabbage
alboglabra	Alboglabra Group	Chinese White Kale, Chinese broccoli, Ga laan, Kai Lan
botrytis	Botrytis Group	Cauliflower, Broccoflower, Calabrese, Romanesco broccoli, Broccoli di Torbole
capitata	Capitata Group	Cabbage
capitata f. *sabauda*	Capitata Group	Savoy Cabbage
capitata f. *rubra*	Capitata Group	Red Cabbage
capitata f. *acuta*	Capitata Group	Cone Cabbage
costata	Tronchuda Group	Tronchuda Kale, Portuguese cabbage, Seakale cabbage
gemmifera	Gemmifera Group	Brussels Sprouts
gongylodes	Gongylodes Group	Kohlrabi, Knol-kohl
italica	Italica Group	Purple sprouting, Sprouting broccoli
longata	Acephala Group	Walking Stick Kale/Jersey Cabbage
medullosa	Acephala Group	Marrow Cabbage
oleracea	-	Wild oleracea
palmifolia	Acephala Group	Giant Jersey Kale, Lacinato Kale
ramosa	Acephala Group	Perpetual Kale
sabellica	Acephala Group	Kale, Curly Kale
virdis	Acephala Group	Collards

1.2.5. Interspecific relationships of significant *Brassica* species

Owing to the economic significance of many *Brassica* species, significant research has been undertaken assessing their relative evolutionary relatedness – with advances in understanding providing potentially useful insights to facilitate ongoing crop breeding efforts. The most notable early advancement in understanding of the evolutionary relationship between *B. oleracea* and other economically significant *Brassica* species was the triangle of U theory (Figure 1.4). First proposed by Woo Jang-choon in 1935 and based on his research creating artificial interspecific *Brassica* hybrids, the triangle of U theory postulates that all species within the genus *Brassica* have one of three basic genome types: A, B or C. Three species can be considered diploid progenitor species, *B. rapa* (AA genome, 2n = 20), *B. nigra* (BB genome, 2n = 16) and *B. oleracea* (CC genome, 2n = 18). Through natural or artificial allopolyploidisation, crosses of these progenitor species can give rise to three distinct allopolyploid species – *B. juncea* (AABB genome, 2n = 36), *B. napus* (AACC genome, 2n = 38), and *B. carinata* (BBCC genome, 2n = 34) (Nagaharu, 1935; Kim *et al.*, 2018).

The triangle of U theory has in recent years been scrutinised rigorously through mitochondrial DNA, genomic, and proteomic approaches with each re-confirming the initial theory (Chalhoub *et al.*, 2014; Yang *et al.*, 2016; Kim *et al.*, 2018; Xue *et al.*, 2020). Despite these early advances, a comprehensive understanding of the evolution of the *Brassica* genomes themselves alongside the specific evolutionary relationship of *Brassica* species beyond these six species has not yet been achieved (Kim *et al.*, 2018). Nonetheless, this remains a field of active research, particularly owing to the relative relatedness of *Brassica* crops to the model system *Arabidopsis thaliana*, permitting comparative genomic studies with *B. oleracea* to provide inferences and testable hypotheses about the functions and evolution of genes and chromosomal regions in the *Brassica* genomes (Paterson *et al.*, 2001; Ziolkowski, 2006; Qui *et al.*, 2009; Sharma *et al.*, 2014).

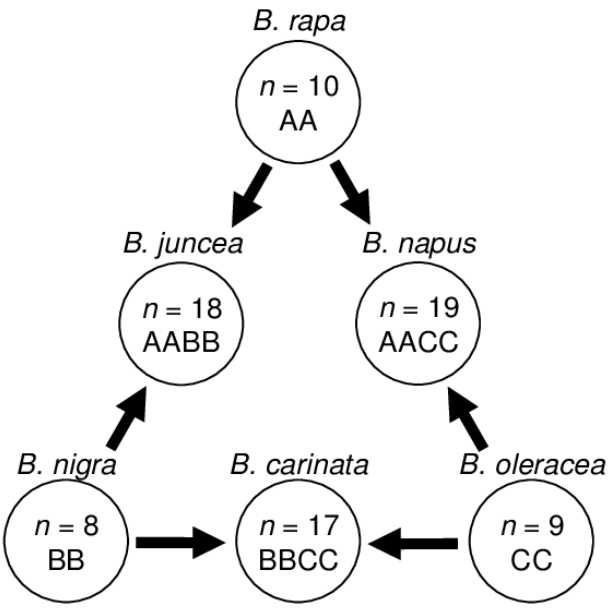


Figure 1.4: Triangle of U, demonstrating the interspecific evolutionary relationship and genomic makeup of six economically significant *Brassica* species (Nagaharu, 1935).

1.2.6. Evolutionary origins of *Brassica oleracea*

Despite extensive research into the interspecific relationships of these six significant *Brassica* species, complimentary research exploring the underlying evolutionary history and initial domestication of *Brassica oleracea* is noticeably lacking – particularly relative to other crop species. Such research holds potentially significant value, with related species if identified serving as both important research tools and potentially valuable sources of novelty and allelic diversity for exploitation in crop breeding programmes (Mabry *et al.*, 2021). This lack of clarity surrounding the evolutionary history of *B. oleracea* stems from a combination of factors including a lack of genetic evidence as to the geographic centre of origin of *B. oleracea*, the significant number of wild *Brassica* species sharing the same cytodeme (C genome species, 2n = 18), the perplexing incompatibility of many of these C genome species with *Brassica oleracea* and the continued confusion surrounding *Brassica* taxonomy (Mabry *et al.*, 2021). Owing to these multiple confounding factors, no consensus has yet been reached as to the evolutionary origins of *B. oleracea* - though countless hypotheses have been proposed over the last century.

The simplest of these proposed theories purports that cultivated *B. oleracea* arose directly from the cultivation of extant wild populations. In its wild and uncultivated

form, *B. oleracea* var. *oleracea* (sometimes referred to as *B. oleracea* var. *sylvestris*) is typically a biennial coastal species, primarily restricted to limestone clifftops with a geographic range spanning the Atlantic and Mediterranean coastlines of Europe. While these populations of wild *B. oleracea* have been proposed as the progenitor of modern *B. oleracea* varieties (Song *et al.*, 1990; Hodgkin, 1995), conflicting evidence indicates that these population may instead represent escapees of historically cultivated *B. oleracea* varieties which through a process of adaptation, evolution or genetic drift over many generations have developed into a distinct *B. oleracea* subspecies (Mitchell and Richards, 1979).

Contrary hypotheses propose that cultivated forms of *B. oleracea* arose from the domestication of a related C-genome *Brassica* species, with potential candidates including:

- *Brassica bourgeaui* (Webb ex H. Christ) Kuntze
- *Brassica cretica* Lam.
- *Brassica hilarionis* Post.
- *Brassica incana* Ten.
- *Brassica insularis* Moris
- *Brassica macrocarpa* Guss.
- *Brassica montana* Pourr.
- *Brassica rupestris* Raf.
- *Brassica villosa* Biv.

While the modern ranges of these species span Europe, largely these species are clustered around the Mediterranean – indicating that this region may be the most likely centre of origin for *B. oleracea* (Maggioni *et al.*, 2015). Proposals surrounding domestication from these related C genome species range from hypotheses suggesting a single domestication event, with all existing *B. oleracea* diversity arising from a single domestication of a single related species (Song *et al.*, 1990; Allender *et al.*, 2007; Mabry *et al.*, 2021), through to the opposite extreme - with multiple domestication events of different C-genome species, each giving rise to separate *B. oleracea* varieties and crop types (Lizgunova, 1959; Song *et al.*, 1990;

Hodgkin 1995; Maggioni *et al.*, 2018). While a majority of these hypotheses stem from the mid to late 20th century, the recent revolution in modern genomic techniques has reignited research in this field. One such recent study by Mabey *et al.* (2021) utilised and integrated phylogenomics, population genomics, and species distribution modelling alongside existing archaeological sources to rigorously assess 224 accessions including 14 cultivar types and 9 wild relatives in an attempt to disentangle the taxonomic origins of *B. oleracea* (Mabry *et al.*, 2021). The results of this study indicate that *B. oleracea* most likely arose from a single domestication event of *Brassica cretica*, supporting the previous work of Schulz *et al.* (1936) and Snogerup (1980) and highlighting *B. cretica* as the most likely closest extant relative of *B. oleracea* (Schulz, 1936; Snogerup, 1980; Mabry *et al.*, 2021).

1.3. Globally significant pests and diseases of *Brassica oleracea* crops

While *B. oleracea* vegetable crops are recognised among the most significant within the global food system, few vegetable crops face such intense and extensive disease and pest pressures. Diseases of *Brassica* crops stem from a range of pathogenic organisms including fungi, oomycetes, bacteria, and viruses while common pests include a wide array of both vertebrates and invertebrates. The diseases and pest pressure experienced by field-grown *Brassica* crops vary depending upon geographic location, climate, soil type, water availability, crop variety and control measures implemented.

1.3.1. Significant diseases of *Brassica* crops

Within the UK, there are currently 18 recognised major diseases of horticultural *Brassica* crops (Table 1.3), ranging in the severity of their impact from moderate blemishing causing loss of yield and/or quality through to potentially complete crop loss - with severity often dependent upon *Brassica* crop type, environmental conditions, and management approach. The most frequently reported and studied diseases including black rot, blackleg, clubroot, downy mildew, Fusarium wilt, stem rot, and Turnip mosaic virus which account for most of the disease related economic losses for growers in the UK (Lv *et al.*, 2020).

Current recommendations for the prevention and control of *Brassica* diseases promote an integrated crop protection approach, including the growing of resistant

plant varieties and regular crop monitoring to evaluate the presence and spread of diseases both in the crop and in the soil. Where detected, diseases may be controlled through an array of physical management approaches (including high temperature treatments and crop rotations), the application of synthetic chemical fungicides and bactericides or the application of suitable biological control agents (such as *Bacillus subtilis*) (Lv *et al.*, 2020). It is also recommended to apply appropriate pesticides where possible to control the populations of known disease vectors, particularly virus vectors (Castle *et al.*, 2009).

Within the scope of disease prevention and control, developing crop varieties which can resist specific problematic diseases has received the most significant research attention and effort. Plant immunity can be broadly categorised into two major types; pathogen/microbe-associated molecular pattern (PAMP/MAMP)-triggered immunity and effector-triggered immunity which is triggered by host resistance (R) genes (Bayer *et al.*, 2018). Effector-triggered immunity has historically been the principle focus of resistance breeding efforts owing to the predominantly gene-for-gene nature of this immunity, meaning identification and breeding of specific R genes into crops should introduce effector-triggered immunity into the resulting progeny (Chisholm *et al.*, 2006). Many plant disease specific R genes have been identified in *Brassica* crops in recent years, aided significantly by the increasingly high quality genomic, pangenomic and proteomic data available for both *B. oleracea,* other economically significant *Brassica* species and the related model species *A. thaliana* (Chalhoub *et al.*, 2014; Yu *et al.*, 2014; Lv *et al.*, 2015; Golicz *et al.*, 2016; Zhang *et al.*, 2016; Alamery *et al.*, 2018; Bayer *et al.*, 2018). Many of these identified R-genes have been successfully bred, typically in a stacked or 'pyramiding' fashion, into new *Brassica* crop varieties producing improved varieties demonstrating quantitatively improved disease resistance (Wang *et al.*, 2011; Chalhoub *et al.*, 2014; Liu *et al.*, 2014; Yang *et al.*, 2016; Lv *et al.*, 2020).

Table 1.3: Common names and causative agents of the 18 most prevalent and damaging recognised diseases of *Brassica* crops in the UK alongside the predominant horticultural *Brassica* crop types affected each (AHDB, 2017).

Disease common name	Causative Agent	Causative Species	Susceptible Crop Types
Black Rot	Bacterial	*Xanthomonas campestris* pv. *campestris*	All cultivated Brassica
Clubroot	Fungal	*Plasmodiophora brassicae*	All cultivated Brassica
Dark Leaf Spot	Fungal	*Alternaria brassicae, Alternaria brassicicola*.	All cultivated Brassica
Downy Mildew	Oomycete	*Hyaloperonospora brassicae*	All cultivated *Brassica*
Light Leaf Spot	Fungal	*Pyrenopeziza brassicae*, anamorph, *Cylindrosporium concentricum*	All cultivated *Brassica*, problematic in Brussels sprout
Phoma	Fungal	*Leptosphaeria maculans* and *Leptosphaeria biglobosa*; asexual stage *Phoma lingam*	All cultivated *Brassica*, problematic in cauliflower, oilseed rape, and swede
Powdery Mildew	Fungal	*Erysiphe cruciferarum*	All cultivated *Brassica*, problematic in Brussels sprout, cabbage, swedes and turnips
Rhizoctonia/Wirestem	Fungal	*Rhizoctonia solani*	All cultivated *Brassica*, problematic in calabrese, swedes, and turnips
Ringspot	Fungal	*Mycosphaerella brassicicola*	All cultivated *Brassica*, problematic in Brussels sprouts
Sclerotinia (watery soft rot, white mould, stem rot, cabbage drop)	Fungal	*Sclerotinia sclerotiorum*	Seed crops most at risk
Spear Rot	Bacterial	*Pseudomonas fluorescens*	Broccoli and Calabrese
White Blister	Oomycete	*Albugo candida*	All cultivated *Brassica*
Turnip yellows virus (TuYV),	Virus	TuYV, vectored by aphids	All cultivated *Brassica*
Cauliflower mosaic virus (CaMV),	Virus	CaMV, vectored by aphids	All cultivated *Brassica*
Turnip mosaic virus (TuMV)	Virus	TuMV, vectored by aphids	All cultivated *Brassica*
Turnip yellow mosaic virus (TYMV)	Virus	TYMV, vectored by aphids	All cultivated *Brassica*
Broccoli necrotic yellows virus (BNYV)	Virus	BNYV, vectored by aphids	All cultivated *Brassica*

1.3.2. Significant pests of *Brassica* crops

The major pests of *Brassica* crops include both vertebrates and invertebrates. The most problematic and abundant vertebrate pest in *Brassica* crops are pigeons, *Columba palumbus* Linneaus 1758, which feed typically on young leaves and shoots causing often extensive cosmetic damage, reducing yield, and sometimes serving as a disease vector where birds have fed on other diseased crops. Other notable vertebrate pests of *Brassica* crops include deer and rabbits. Typically, mammal-associated *Brassica* damage is most common in the Winter months, causing cosmetic damage and reducing yield. Vertebrate pests are most commonly controlled through the use of physical barriers, including fences, netting – complemented with the use of bird scarers.

In stark contrast to the relatively low number of problematic vertebrate pests, there exists significant diversity in the potential invertebrate pests of *Brassica* crops. Within the UK alone there are currently 49 recognised insect pest species of *Brassica* crops, including species from the orders Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera alongside numerous slug species from the order Stylommatophora. Slug associated damage to *Brassica* crops can be severe where crop plant vigour is low and weather conditions are moist. Nonetheless a majority of invertebrate pest damage in *Brassica* crops is caused by insect pests. Within the wider agricultural food production system, insect pest infestations are predicted to account for 20-30% of yield and productivity losses (De Geyter *et al.*, 2007). Insect pests fall broadly into four defined categories: chewing pests, piercing/sucking pests, rasping/sucking pests, and mining-type pests (Santamaria *et al.*, 2018). Herbivory from insect pests in these different categories results in markedly different damage profiles and consequently elicit significantly different defence and physiological responses in infested plants (De Geyter *et al.*, 2007; War *et al.*, 2012).

Many of the 49 recognised insect pest species of *Brassica* crops, are currently infrequent or sporadic. Nevertheless, there exists a sizeable subset of globally significant, hugely damaging, and difficult to control insect pest species within the scope of these 49 species (Table 1.4). Similarly to diseases, the major pests of horticultural *Brassica* crops range in the severity of damage and economic losses they cause; from moderate to major yield loss due to blemishing/marking through to

complete defoliation of the crop - potentially leading to complete crop loss. Damage from insect pests is also often further compounded owing to pest associated damage creating a route of entry for disease-causing pathogens into plant tissues or, more directly, through insect pests acting as primary vectors for plant diseases. The most prominent example of this vectoring potential are aphid pests from the order Hemiptera, which are frequently implicated as the primary vectors of *Brassica* viruses including viruses responsible for 5 of the 18 most significant *Brassica* diseases (Table 1.4) (Dietzgen *et al.*, 2016).

Table 1.4: Most prevalent and damaging insect pest species of UK vegetable *Brassica* crops and their importance owing to the damage type and severity caused (AHDB, 2016; 2017).

Common name	Scientific name	Order	Severity and damage caused
Cabbage Aphid	*Brevicoryne brassicae*	Hemiptera	Infestations reduce yield by distorting plants and contaminating crop. Severe infestations may kill young plants. Vector of viruses.
Cabbage moth	*Mamestra brassicae*	Lepidoptera	Feeding can cause rapid defoliation of large plants and kill small plants.
Cabbage root fly	*Delia radicum*	Diptera	Problematic cosmetic damage reducing yield/marketability, particularly of root *Brassica*. Newly emerged crops most susceptible.
Cabbage stem weevil	*Ceutorhynchus* spp.	Coleoptera	Cosmetic damage caused by tunnelling into leaf stalks/stems, damage promotes fungal infections.
Cabbage whitefly	*Aleyrodes proletella*	Hemiptera	Causes contamination of crop with scales, immature nymphs and excreted honeydew reducing quality.
Cutworm (Turnip moth)	*Agrotis segetum*	Lepidoptera	Can cause severe damage leading to reduced quality and often loss of whole plants.
Diamondback moth	*Plutella xylostella*	Lepidoptera	Typically sporadic but large infestations can cause significant crop loss and contamination.
Flea beetles	*Phyllotreta*, *Psylliodes* spp.	Coleoptera	Particularly damaging to seedlings, sometimes leading to loss or significant leaf pitting.
Garden pebble moth	*Evergestis forficalis*	Lepidoptera	Feeding results in leaf damage, contamination (frass, silk webbing). Localised to specific areas.
Leaf miner	*Scaptomyza flava*	Diptera	Damage caused by adult females leading to characteristic corridor-blotch on lower leaf surface.
Peach-potato aphid	*Myzus persicae*	Hemiptera	Severe infestations cause distortion and may kill young plants. Significant vector of viruses (TuYV).
Pollen beetles	*Meligethes* spp.	Coleoptera	Most problematic in broccoli and cauliflower, where feeding causes browning of florets.
Silver Y moth	*Autographa gamma*	Lepidoptera	Migratory pest into SE UK. Causes leaf damage and crop contamination reducing quality.
Thrips	*Thrips tabaci*	Thysanoptera	Cause cosmetic damage primarily of stored Brussels sprots and cabbage.
White Butterfly (Large)	*Pieris brassicae*	Lepidoptera	Large groups frequently skeletonise leaves causing severe crop damage and reduction in yield.
White Butterfly (Small)	*Pieris rapae*	Lepidoptera	Large groups frequently skeletonise leaves causing severe crop damage and reduction in yield.

1.4. Aphid pests

Aphids (Hemiptera: Aphididae) are among the most significant insect pest species of *Brassica* crops (Bhatia *et al.*, 2011). While ubiquitous globally, a majority of aphid diversity is found in temperate northern regions (Dixon, 1998). Approximately 250 aphid species are considered to be economically important pests of edible and ornamental crops (Bhatia *et al.*, 2011; Dedryver *et al.*, 2010). The development of sustainable systems for managing aphid pests of crops is therefore among the foremost challenges in agricultural science. Aphids are characterised by a short development time, parthenogenetic reproduction, and high fecundity, enabling populations to expand rapidly on host plants where environmental conditions are suitable. As a result, high levels of crop infestation can occur within a few weeks of initial plant colonisation (Dixon, 1992).

Globally, the majority of aphid species are considered to be monophagous, feeding on one or a small group of closely related plant species. Approximately 10% of global aphid species however are recognised as polyphagous – being able to feed on a wide range of plants species and genera - typically varying in their host plant dependent upon local flora and season (Dixon, 1998). Aphid host plant selection is typically driven on a local scale by a combination of visual and chemical cues, with aphid antennae bearing many sensilla competent for the olfactory chemoreception of plant volatile chemical compounds (Dixon, 1998). The small size of migratory alate aphids means however that on a broader scale, aphid host finding is somewhat stochastic in nature driven by wind strength and direction (Irwin *et al.*, 1988; Parry, 2013).

1.4.1. Aphid interactions with host plants

Aphids are classified as piercing/sucking arthropod pests, utilising specialized mouthparts termed stylets to probe plant tissues for phloem vasculature. Upon the stylet piercing into phloem vasculature, aphids can then feed passively on phloem sap due to the positive pressure of the phloem. During early feeding phases, most aphid species typically inject two forms of saliva into the phloem, gelling saliva and watery saliva, with each serving specific functions to promote successful feeding and improve aphid performance. (Will *et al.*, 2007).

Gelling saliva is typically secreted first, forming a contiguous proteinaceous sheath around the aphid stylet, sealing the stylet induced cell puncture site and reducing localised influx of extracellular calcium – thereby inhibiting localised defence responses (Guerrieri and Digilio, 2008; van Bel and Will, 2016). Watery saliva in contrast contains a significantly more complex array of specialised proteins and RNA, with the watery saliva of the peach potato aphid, *M. persicae* containing over 200 distinct polypeptides (Harmel *et al.*, 2008). Watery saliva polypeptides typically include a range of proteins and enzymes which, through their combined action, allow for the circumvention of plant defence responses by inhibiting plant-mediated occlusive phloem-sealing mechanisms, dampening plant immune responses, easing further stylet probing and broadly promoting aphid performance (Will *et al.*, 2007; Guerrieri and Digilio, 2008; van Bel and Will, 2016; Chen *et al.*, 2020). Perhaps the furthest reaching effects of aphid watery saliva is the ability to enhance host plant nutritional quality by redirecting/altering plant amino acid synthesis and transport to increase both the localised and phloem amino acid concentrations, removing nitrogen as a limiting factor for aphid growth and fecundity (Cao *et al.*, 2016). Owing to the complex biochemical makeup of aphid saliva secretions and the far-reaching targets and actions of these secretions, gel and watery saliva thus serve as the most direct chemical interface between aphids and their plant hosts.

Owing to the clandestine nature of aphid phloem feeding, physical damage caused by aphid phloem feeding is typically limited during early phases of aphid infestation, particularly relative to chewing pests (Guerrieri and Digilio, 2008). As the aphid population on the plant expands however, stunting, discolouration and distortion of foliage is increasingly common – resulting primarily from the phytotoxic effects of aphid injected saliva and autotoxicicity of plant defences (Miles, 1999). Many aphid species are also vectors of plant pathogenic viruses which can cause significant crop damage and reductions in yield (Table 1.3) (Dedryver *et al.*, 2010). In general, polyphagous aphid species harbour and transmit a wider spectrum of viruses than plant specialist species, though specialists are also competent virus vectors (Ng and Perry, 2004). Aphid infestations also invariably lead to extensive contamination of the crop with live aphids and ecdysed aphid cuticles, alongside deposition of honeydew on the plant surface promoting the growth of sooty moulds (Dedryver *et al.*, 2010). Through the combination of direct aphid feeding associated damage,

indirect damage through aphid vectored plant pathogenic viruses and concurrent contamination of the crop, aphids infestations can be severely detrimental to the marketability of fresh produce with significant economic consequences if uncontrolled (Tatchell, 1989).

1.4.2. Significance and life cycles of aphid pests of *Brassica* crops

Aphid infestations can be particularly problematic in *Brassica* crops, including both vegetable (*B. oleracea*, *B. rapa*) and oilseed (*B. napus*, *B. juncea*) types. Of the 9 species currently recognised as potential pests of *Brassica* crops, the polyphagous peach potato aphid, *M. persicae* and the monophagous cabbage aphid, *Brevicoryne brassicae* cause the majority of damage (Blackman and Eastop, 2000). These species have evolved different life strategies but frequently coexist on a single *Brassica* crop. Both species share a complex multi-phasic and adaptable life cycle, being biologically capable of alternate oviparous and viviparous phases (Figure 1.5).

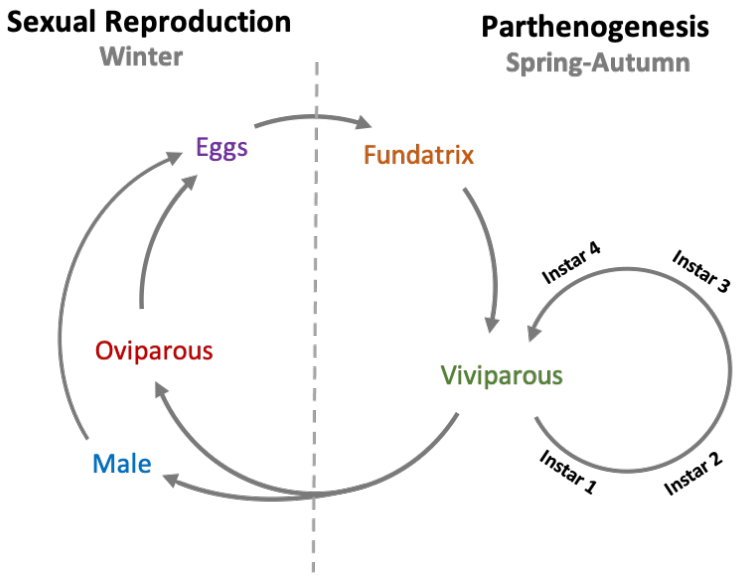


Figure 1.5: Schematic of a typical holocyclic aphid life cycle, defined by seasonally cycling oviparous and viviparous phases. During seasons with warmer temperatures and longer days, typically Spring and Summer, aphids reproduce via thelytokus parthenogenesis. In late Autumn, a combination of shortening day length and decreasing temperatures leads to the production by viviparous females of male aphids and oviparous (sexual) females. Haploid oocytes and sperm are produced via meiosis in oviparous female and males accordingly and, upon mating, diploid eggs are produced. Diploid eggs hatch as viviparous fundatrices the following spring, stimulated by increasing day length and temperatures. Dependent upon aphid species, the fundatrices may be alate or wingless (Ogawa and Miura *et al.*, 2014).

In its native Asian range, *M. persicae* is predominantly holocyclic, exploiting *Prunus* spp., primarily peach (*Prunus persica* L. Batsch 1801), as winter hosts across its global distribution. Despite the relative specialism of its overwintering life-stages, *M. persicae* is polyphagous for summer hosts. As days lengthen and temperatures rise, large waves of *M. persicae* migrate from overwinter hosts in search of suitable summer hosts. On its varied summer hosts, *M. persicae* can cause significant direct and indirect economic damage, particularly on plants in the families Brassicaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Poaceae and Solanaceae (Bass *et al.*, 2014). Alongside its typically holocyclic life cycle, in regions with milder climates *M. persicae* can instead maintain a wholly anholocyclic life cycle, with viviparous females overwintering on herbaceous crops and weeds (Howling *et al.*, 1994). *Myzus persicae* is a highly variable aphid species, with a wide range of biotypes, strains and races being recognised – varying according to biology, colour, insecticide resistance and preferred host plant. *Myzus persicae* is also reported to be the most significant insect vector for over 100 different plant pathogenic viruses - with turnip mosaic virus, cauliflower mosaic virus, and turnip yellow virus the most notable of these in *Brassica* crops (Table 1.3).

In contrast, *B. brassicae* is a specialist, monophagous feeder on members of the Brassicaceae. It occurs frequently on *Brassica* crops across its native range in Europe and is an invasive species in North and South America, Africa and Australasia (Mpumi *et al.*, 2020). Similarly to *M. persicae*, *B. brassicae* is a holocylically competent species and is thus capable of overwintering as eggs. In temperate countries such as the UK however, *B. brassicae* more commonly maintains an anholocyclic life-cycle – with viviparous females overwintering on cultivated *Brassica* crops, agricultural *Brassica* escapees or wild species relatives (Dixon, 1977). Economically, *B. brassicae* is one of the most damaging pests for vegetable *Brassica* growers due to both direct damage and through also serving as a vector of 20-30 plant pathogenic viruses (Valenzuela and Hoffmann, 2014).

Asexually produced nymphs of both species undergo four instars prior to reaching adulthood. Development time from first instar to adulthood typically occurs within 7-10 days in *M. persicae* and 8-15 days in *B. brassicae*, varying in response to environmental conditions and host plant.

1.4.3. Aphid control measures and insecticide resistance

Aphid control on *Brassica* crops is currently based on the routine applications of synthetic chemical pesticides, with high reliance on a small number of chemical classes (chiefly pyrethroids, carbamates and neonicotinoids). Several compounding factors however have raised concern over the long-term viability of this approach. Growers are facing pressures to reduce their use of pesticides due to concerns over residues in food, alongside increasing public recognition of the harmful effects that pesticides may have upon beneficial and non-target organisms. Furthermore, over-reliance on synthetic chemical pesticides for managing aphid pests has resulted in increasingly common control failures as a result of selection for heritable resistance in target populations. To date over 20 aphid species have developed insensitivity or resistance to one or more classes of synthetic chemical pesticides (Silva *et al.*, 2012). *Myzus persicae* has proven particularly difficult to control due to its development of resistance to seven different classes of synthetic chemical pesticides - most notably resistance to carbamates through Modified acetylcholinesterase (MACE), and knockdown (kdr and super-kdr) resistance to pyrethroid insecticides (Bass *et al.*, 2014).

The introduction and high efficacy of neonicotinoids in the late 1990s temporarily overcame the problems associated with widespread insecticide resistance (Jeschke and Nauen, 2008). However, with the introduction of restrictions preventing neonicotinoid usage on field crops in Europe and elsewhere, growers have been left with very few effective control options (Prince and Chandler, 2020). The well-documented and rapid emergence of resistance to insecticides in aphids therefore places increasing pressure on the declining number of novel chemical active products coming to market.

Insecticide resistance in *B. brassicae* has not yet proven a problem in Europe, however there are increasing reports of pesticide insensitivity and resistance in *B. brassicae* populations elsewhere, particularly Pakistan and India (Ahmed and Akhtar, 2013) raising concerns of a similar global trend towards pesticide resistance as typified by *M. persicae*. *Brevicoryne brassicae* also poses specific control problems due to its preference for feeding sites on the growing tips of plants, potentially shielding individuals from spray applications and allow a population to

rebound quickly after pesticide treatment (Blackman and Eastop, 2000). The problems associated with overreliance on pesticides necessitate an urgent shift to a more sustainable management approach.

1.5. Varietal resistance to insect pests

One potential component of an alternative management approach to insect pests of crop plants is the development and deployment of crop varieties demonstrating resistance to insect herbivory. Identified by Stenberg (2017) as a key ecological IPM component, there exists significant benefits to an IPM strategy if robust plant resistance to pests and diseases can be identified and integrated holistically with these other IPM tools (Figure 1.3).

To drive the development of elite varieties harbouring desirable traits such as pest and disease resistance, the cataloguing and effective utilisation of the existing genetic variation in crop accessions, landraces and wild species to allow for this augmentation of existing crop lines is paramount. While breeding for disease resistant crop varieties has been historically and remains commonplace, variety breeding for lines able to resist insect herbivory is far rarer - likely owing to the previously high efficacy and ease of synthetic chemical pesticide-based control (Hervé, 2018).

Plant resistance to insect pests can be conferred through a diverse array of structural or biochemical mechanisms. Structural adaptations in plants such as thicker leaves and increased surface trichome concentration have long been understood to provide defence against pests – providing direct physical barriers to aphid feeding activities. Such traits however are frequently undesirable in crop varieties; thus, the overwhelming focus of resistance screening and breeding efforts to date has been biochemically conferred resistance against herbivory (Stenberg, 2017). A majority of research in the field of varietal resistance has been undertaken with a focus on plant resistance to pathogens however the principles and methods established in plant-pathogen systems are equally applicable and useful in the context of plant resistance to insect pests.

1.5.1. Antibiosis and antixenosis

The resistance of plants to their respective insect pests can broadly be categorised into two distinct phenotypic traits: antixenosis and antibiosis. Antixenosis describes plants which demonstrate a phenotype able to alter herbivore host locating/identifying behaviour, thereby limiting or wholly preventing the initial establishment of a pioneer pest generation. Significant research has focussed upon the biochemical nature of plant host identification, investigating the combinatory cues crop pests (including aphids) use to identify their preferred host plants. Such studies have identified that a combination of visual appearance and olfactory chemosensory cues from complex mixtures of volatile organic compounds are the primary driving forces underlying host plant identification (Hopkins *et al.*, 2017). However, despite extensive research and breeding efforts, only limited progress has been made towards the development of crop plants harbouring markedly increased antixenosis resistance (Diaz-Montano *et al.*, 2006; Koch *et al.*, 2016).

Antibiosis describes plants which are able to mount successful defence; whether immunologically or through lessened nutritional quality, against pests which have already successfully located and populated their desired host. Where plant resistance screens and breeding regimes against insect pests have been undertaken, the overwhelming focus has historically been placed upon antibiosis resistance. This focus may in part be due to the greater ease of screening for antibiosis resistance under laboratory conditions. Laboratory identified antibiosis resistance has through a number of studies been demonstrated as typically transferable into field conditions, with laboratory identified pest reductions likely to be maintained on a field-scale (Ellis *et al.*, 1998). Conversely, antixenosis resistance identified on a small laboratory scale frequently fails when transferred to a field setting, with such monoculture environments likely amplifying visual and olfactory identification cues aiding host-localisation of insect pests. Past research has postulated that only through the successful introduction of plants harbouring both increased levels antibiosis alongside antixenosis can the greatest levels of plant defence be achieved (Ellis *et al.*, 1998).

1.5.2. Complete antibiosis resistance

Within the scope of antibiosis resistance there exists significant quantitative variation. In the most extreme case exists complete antibiosis resistance, this describes resistance of otherwise susceptible host plants which, typically through the action of typically single dominant genes, express a phenotype preventing entirely the survival of pioneer and subsequent pest generations.

The principles of complete resistance are well established through decades of study assessing complete plant resistance to pathogens. Complete resistance is conferred most commonly through highly specific gene-for-gene interactions between plant R-genes and corresponding pathogen avirulence proteins (Brown, 2015). Such R-genes typically encode either nucleotide-binding leucine-rich repeat (NB-LRR) proteins or receptor-like kinase/receptor-like proteins (RLK/RLP) which can recognise and bind pathogen effectors/avirulence proteins (AVR) inducing signalling pathways and ultimately driving the expression of effective plant defences (Brown, 2015). R-genes underlying complete resistance are often referred to as major resistance genes owing to the significant resistance they confer (Pilet-Nayal *et al.*, 2017). While conceptually, complete resistance is highly desirable owing to the near complete exclusion of pests or pathogens it can provide, such resistance has often proven unsustainable in the long-term owing to it promoting rapid evolution in pest or pathogen AVR genes – thereby breaking the gene-for-gene interaction and rendering specific plant R-genes ineffective (Brown, 2015).

Owing to the high efficacy but low durability of complete resistance, multiple strategies have been proposed and tested in an attempt to increase their durability and therefore better capitalise upon the potential of R-genes. The leading strategies proposed to increase the durability of complete resistance and R-genes are (i) the growing of multiple plant lines simultaneously each carrying different R-genes, (ii) the rotation both spatially and temporally of varieties carrying different R-genes and (iii) the breeding of new varieties carrying a combination of different R-genes in a stacked or pyramid fashion (Papaix *et al.*, 2011; Sapoukhina *et al.*, 2013; Brown, 2015; Pilet-Nayal *et al.*, 2017). Of these proposed strategies, breeding schemes seeking to stack or pyramid R-genes have seen the greatest uptake and research interest, resulting in the generation of many resistant varieties of different crop

species which continue to be widely cultivated (Ordon and Kühne, 2014; Ellis *et al.*, 2014; Pilet-Nayal *et al.*, 2017). Despite the increased durability of complete resistance in varieties bred with stacked R-genes, there nonetheless remains a significant risk of resistance in such varieties breaking down over time owing to the shared mode of action/activity of R-gene mediated resistance and the affinity for rapid evolution in pathogen effectors/avirulence proteins (AVR) (Brown, 2015).

Regardless of these caveats, complete antibiosis resistance to insect pests has nonetheless become a desirable trait for plant breeders. Such resistance however has proven a rare trait in a majority of crop species (Dogimont *et al.,* 2010). Despite this rarity, alongside the significant risks of eroding efficacy owing to its gene-for-gene nature, to date dominant gene complete resistance has been the overwhelming focus of studies aiming to identify plant resistance to insect pests. To identify such resistance, studies most commonly employ a top-down methodology, assessing many hundreds or thousands of different accessions in the hopes of identifying a small resistant sub-set which can then probed genotypically in an attempt to identify the underlying R-genes.

The success of identifying aphid resistance in crops through top-down phenotypic screening has been mixed, with sources of resistance proving limited (Dogimont *et al.*, 2010). The most notable successes have been achieved in wheat, *Triticum aestivum*, tomato, *Solanum lycopersicum* and muskmelon, *Cucumis melo*. In wheat, twelve genes have been identified to date which confer biotype-specific resistance against the Russian wheat aphid, *Diuraphis noxia* (Dogimont *et al.*, 2010). In tomato and muskmelon one resistance gene has been successfully identified, cloned and sequenced in each crop: *Mi*-1 in tomato which provides resistance against certain biotypes of the potato aphid, *Macrosiphum euphorbiae* and *vat* in muskmelon which confers resistance to the melon-cotton aphid, *Aphis gossypii* alongside providing resistance to *A. gossypii* vectored viruses (Rossi *et al.*, 1998; Vos *et al.*, 1998; Dogimont *et al.* 2010; Dogimont *et al.*, 2014; Boissot *et al.*, 2016ab).

Following their identification, single dominant gene resistance to aphids has been bred successfully into commercial lines of several crop species, most prominently wheat, tomato, muskmelon, lettuce and raspberry (Dogimont *et al.*, 2010). However,

resistance conferred by single dominant genes carries with it a high risk of selection for resistant-breaking aphid biotypes and subsequent loss of efficacy (Stuthman *et al.*, 2007; McMenemy *et al.*, 2009; Dogimont *et al.*, 2010). This risk is best exemplified by the development, commercialisation and deployment of raspberry, lettuce and wheat varieties bred with the dominant resistance genes *Ag1*, *Nr* and *Dn4*. As predicted, a classical 'boom and bust' pattern of efficacy was observed with high initial resistance to aphid pests being rapidly eroded due to the emergence of novel resistant biotypes of *Amphorophora agathonica*, *Nasonovia ribisnigri* and *Diuraphis noxia* respectively (Xu *et al.*, 2015; Boissot *et al.*, 2016ab; Dossett and Kempler, 2016).

1.5.3. Plant-aphid defence signalling

Continuing advances in understanding of plant defence and immunological signalling against insect pests has opened the possibility for an alternative 'bottom-up' approach to resistance identification and screening. In the context of aphid pests of *Brassica* crops, the *Myzus persicae-Arabidopsis thaliana* model system has been central in untwining the complex immunological interactions, genes and pathways underlying antibiosis resistance (O'Neill and Bancroft, 2001). These studies have demonstrated that aphid feeding induces both a generalised plant response to insect-inflicted tissue damage alongside a more specific gene-for-gene recognition of conserved aphid elicitor and effector molecules: molecules secreted by aphids during early phloem feeding phases which have evolved specifically to interact which plant gene expression, signalling and defences to promote successful phloem feeding and likely downregulate plant defences (Jaouannet *et al.*, 2014; Cui *et al.*, 2019). Both mechanisms have been demonstrated to result in local and systemic defence reactions mediated by phytohormone signalling pathways (Smith and Boyko, 2006; Jaouannet *et al.*, 2014), most notably the jasmonate (JA) and salicylate (SA) pathways, although extensive crosstalk occurs with the abscisic acid (ABA), ethylene (ET) and gibberellic acid (GA) pathways (Åhman *et al.*, 2019).

Across different plant families, the prominence of these pathways in driving defence against aphid pests appears to be variable, and their actions can be either synergistic or antagonistic. In the *M. persicae*/*A. thaliana* model it appears that JA and SA act antagonistically. Overall, aphids appear to show greater susceptibility to plant

defences initiated through the JA pathway compared to SA signalling, particularly where these are constitutively upregulated prior to aphid infestation (Ellis *et al.*, 2002; Mewis *et al.*, 2006; Kersch-Becker and Thaler, 2019).

These observations have led to the suggestion, with subsequent transcriptomic corroboration, that aphids have evolved effectors which act by subversively inducing SA-mediated defence responses in order to down-regulate the more effective anti-aphid defence responses regulated by the JA pathway - exploiting the antagonistic relationship between the two (Thompson and Goggin, 2006; de Vos *et al.*, 2007; Goggin, 2007; Jaouanett *et al.*, 2014). While this proposal is largely supported, several studies have presented evidence seemingly opposing these findings, thus indicating further complexities in plant phytohormone signalling in response to aphid pests (Jaouannet *et al.*, 2014). Additional signalling pathways that act independently of phytohormone pathways, such as protein phytoalexin deficient pathways 3 and 4 (PAD3 and PAD4), have also been implicated in mediating defence against aphids, although their importance remains unclear (Jaouannet *et al.*, 2014). Such bottom-up research in the *Myzus persicae-Arabidopsis thaliana* model system has recently resulted in the identification, cloning and sequencing of a specific R-gene, *Sli1*, in *A. thaliana* which confers defence against *M. persicae* (Kloth *et al.*, 2017). The application of this R-gene however is limited in the context of *Brassica* crops as this gene is not conserved in the *B. oleracea* genome.

1.5.4. Partial resistance

Owing to the durability shortcomings of complete antibiosis resistance, an alternative approach may be to identify and utilise partial, multigene resistance – also referred to as horizontal or quantitative resistance (Smith and Chuang, 2014; Pilet-Nayal *et al.*, 2017). Partial multigene resistance describes a stable and more durable alternative to complete antibiosis resistance which, in the specific case of aphids, acts by quantitatively lowering pest biotic potential rather than excluding them entirely (Gatehouse, 2002; Dogimont *et al.*, 2010). Being less specific in its action than single gene resistance, partial multigene resistance offers potential benefits against a broader spectrum of pest species whilst simultaneously exerting a lower ecological selection pressure for the emergence of resistance-breaking pest biotypes (Rant *et al.*, 2013). Similarly, to complete resistance, much of our

understanding of the principles and molecular mechanisms underlying partial resistance arise from studies in plant-pathogen systems.

While the genes conferring partial resistance to a given variety may be widespread across the genome, such genes are frequently clustered. Genes in these clustered regions typically exert a minor effect upon the specific resistance trait, with such regions referred to as quantitative trait loci (QTL) (Pilet-Nayal *et al.*, 2017). While QTLs may include R-genes and confer significant or near complete resistance, most QTLs exert only a minor-effect, thus conferring partial rather than complete resistance (Rosewarne *et al.*, 2013; Pilet-Nayal *et al.*, 2017). Similarly to methods seeking to improve the durability of R-genes associated with complete resistance, there exists significant potential to further the degree of quantitative resistance minor-effect QTLs offer through stacking them (Niks *et al.*, 2015; Pilet-Nayal *et al.*, 2017). Several studies have been undertaken assessing the viability and efficacy of minor-effect QTL stacking in barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*) and wheat (*Triticum aestivum*) (Stall *et al.*, 2009; St. Clair, 2010). Of these studies, Stall *et al.* (2009) demonstrated most clearly the significant potential of this approach - with stacking of the minor-effect *rx1*, *rx2*, and *rx3* QTLs being found to provide a high degree of resistance in tomato crops to the bacterium *Xanthomonas campestris* (Stall *et al.*, 2009). Despite this success, few further studies assessing minor-effect QTL stacking have been undertaken – potentially because for such stacking to be effective, the QTLs utilised must all have additive effects and be either constitutively expressed or rapidly inducible - traits which are not guaranteed for all QTLs (Bocianowski, 2013).

In stark contrast to R-gene mediated complete resistance, the molecular mechanisms underlying minor-effect QTL conferred resistance are poorly understood. It is likely there exists a multitude of modes of action of minor-effect QTLs upon pests and pathogens, including altering host plant development or morphology, upregulating basal defences, detoxification mechanisms and upregulation or altered transduction of defence signals (Poland *et al.*, 2009). Individual genes in each minor-effect QTL may therefore encode a wide range of proteins involved in both fundamental plant molecular processes and at various stages of defence including pathogen recognition, signal transduction and defence elicitation (Pilet-Nayal *et al.*, 2017). Many genes

involved in minor-effect QTLs also have the potential for far reaching downstream effects, particularly QTL genes encoding transcription factors which can directly moderate plant responses to defence hormones and/or upregulate or direct secondary metabolite production (Kushalappa *et al.*, 2016; Pilet-Nayal *et al.*, 2017).

While the increased durability of partial resistance is thought to arise through numerous mechanisms, it is likely that this increased durability result primarily from(1) the lesser degree of overall resistance conferred by individual minor-effect QTLs and (2) the multiple modes of action active across one or more QTLs. The lesser degree of overall resistance is thought to increase resistance durability by exerting a lower selection pressure upon pest or pathogen population. Conversely, the numerous modes of action are thought to act by placing numerous, potentially contradictory, selection pressures upon pest or pathogens, leading to an increasingly low probability of the multiple simultaneous mutations occurring sufficient to provide a selective advantage. Minor-effect QTL induced resistance mechanisms are likely to also vary significantly at different plant and pest/pathogen life stages, creating a dynamic challenge for pests or pathogens to overcome (Mundt, 2014).

Despite the notable durability of minor-effect QTL induced partial resistance, there nonetheless remains a risk of pests or pathogens overcoming such resistance. This risk has been exemplified directly in real-world examples where QTLs have been identified, successfully cloned and bred into deployed crop varieties. The most notable examples of minor effect QTL-conferred partial resistance breakdown are the erosion of wheat cultivar partial resistance to Septoria leaf blotch (*Mycosphaerella graminicola*), grapevine (*Vitis vinifera*) partial resistance to Grapevine downy mildew (*Plasmopara viticola*) and apple (*Malus domestica*) partial resistance to Scab (*Venturia inaequalis*) (Cowger and Mundt, 2002; Caffier *et al.*, 2016; Delmas *et al.*, 2016; Pilet-Nayal *et al.*, 2017). While the breakdown of these resistances were observed to be significantly slower relative to the boom-and-bust breakdown of R-gene mediated complete resistance, taking upwards of 8 years to degrade, the result nonetheless was the complete breakdown of resistance through the development of resistance breaking pathogen biotypes.

Despite the apparent durability of minor-effect QTLs relative to complete resistance conferred by major-effect R-genes, researchers and breeders have thus come to recognise that deployment of these minor-effect QTLs alone is insufficient to prevent the development of resistance-breaking pest or pathogen biotypes. These findings have led to a recent reappraisal of the optimal means of minor-effect QTL deployment, including suggestions that minor-effect QTL should be integrated into pyramiding strategies alongside R-genes to better manage and protect identified resistance loci and produce varieties with compound complete and partial resistance – whether this approach would be successful however has yet to become apparent (Pilet-Nayal *et al.*, 2017).

While progress in the field of partial resistance to insect pests undoubtedly lags the progress seen for pathogens, there has in recent years been an increasing body of research seeking to identify and understand partial resistance to insect pests in a wide range of crops – chiefly through the identification of minor-effect QTLs in crop plants and wild relatives. While identifying both major and minor effect genes and QTLs for pest resistance is in its relative infancy, continuing advances in genomic and mapping techniques have allowed for increasing progress to be made in recent years. The most notable advances have been made in maize (*Zea mays*), soybean (*Glycine max*) and wild soybean (*Glycine soja*) where several promising minor-effect QTLs have been identified conferring potential partial resistance to Larger grain borer (*Prostephanus truncates*), soybean aphid (*Aphis glycines*), and the Tobacco cutworm (*Spodoptera litura*) respectively (Jun *et al.*, 2013; Oki *et al.*, 2017; Mwololo *et al.*, 2018). There has also been research interest in minor effect QTLs of the tomato relative *Solanum galapagense*, which demonstrates significant partial resistance to a range of problematic pests of tomato crops (Vosman *et al.*, 2018).

While bottom-up studies seeking to identify QTLs conferring resistance to insect pests hold significant promise for the breeding of resistant crop varieties, there also exists a significant established body of research in the literature assessing partial resistance through top-down screens. Prior to the advances in recent years permitting detailed genomic approaches and marked-assisted breeding, large, randomised screens were the most utilised methodology to identify resistance. Typically, such studies sought to identify complete resistance genes, selecting therefore for major-

effect R-genes prior to their first cloning in 1992 (Kourelis and van der Hoorn, 2018). A small number of researchers however, despite an environment of historically high efficacy and low cost of synthetic chemical pesticides (meaning minimal appetite among plant breeders and seed companies for partially resistant material), recognised the significant potential value of partial resistance for future crop breeding programs (Ellis, pers. communication). Among these researchers, and within the specific context of *Brassica* partial resistance to aphid pests, the work of Ellis *et al.* between 1995-2002 represents a valuable resource (Ellis *et al.*, 1995, 1996, 1998). Throughout this period, Ellis *et al.* assessed hundreds of different accessions of *B. oleracea* alongside a range of different related *Brassica* species. While the genes and minor-effect QTLs conferring partial resistance within this identified partially resistant plant accessions remain unknown, this resource nonetheless provides a significant foundation resource for further exploration.

1.5.5. Partial resistance case study: the glucosinolate-myrosinase defence system

QTL identification has become the leading methodology to identify and investigate both partial and complete resistance and the underlying major and minor effect genes and QTLs. Inevitably however, many such identified genes at these loci have unknown or difficult to determine functions, requiring significant further research efforts to determine their means of action and potential for breeding programmes. Within the context of *Brassica* crops however, a major body of research already exists surrounding a specific defence system which is both constitutively active and upregulated in response to pest-associated crop damage and feeding activities.

The glucosinolate-myrosinase defence system, sometimes referred to as the “mustard oil bomb”, is among the most sophisticated and best studied biochemical mechanisms plants employ to provide protection from pests and diseases (Chaplin-Kramer *et al.*, 2011; Chhajed *et al.*, 2020). While infrequently referenced in the literature, glucosinolates do not provide complete resistance to a given pest or disease, offering instead quantitative or partial resistance effective against a wide range of organisms. Glucosinolates are therefore among the best studied and understood examples of partial/quantitative resistance among all crop species.

Characteristic of species in the order Brassicales (which includes all *Brassica* species alongside related species in the *Brassicaceae* family), glucosinolates are the large family of over 120 secondary metabolites which confer the distinctive bitter or spicy flavours of *Brassica* crops and mustards (Ishida *et al.*, 2014; Liu *et al.*, 2021). Glucosinolates are typically localised to S-cells which are present throughout all plant tissues and organs, being particularly abundant within leaf and stem tissues. Relatively inert and stable in their unaltered form, glucosinolates serve as defence compounds activatable by the enzyme myrosinase (Hopkins *et al.*, 2009; Chhajed *et al.*, 2020). Myrosinase is typically localised to myrosin cells, which lie adjacent to glucosinolates housing S-cells (Kliebenstein *et al.*, 2005ab). Only following a breakdown of this compartmentalisation are glucosinolates therefore exposed to myrosinase and converted via an enzyme catalysed reaction into toxic isothiocyanates which are thought to confer the majority of glucosinolate associated partial crop protection (Hopkins *et al.*, 2009; Burow and Halkier, 2017).

The glucosinolate-myrosinase defence system serves as a major constitutive defence mechanism in *Brassica* crops, with significant evidence highlighting its role in protection against biotic stresses including vertebrate/invertebrate herbivory and pathogen infection alongside also conferring resistance to abiotic stresses such as drought, temperature extremes and salt stress (Rask *et al.*, 2000; Kos *et al.*, 2012; Dubuis *et al.*, 2005; Liu *et al.*, 2021). The relationship between glucosinolate content and insect herbivory has for many years been an active area of research, owing to the prevalence of glucosinolates in many economically significant vegetable and oilseed *Brassica* crops. While much research has focussed upon the effects of constitutively expressed glucosinolates on insect pests, transcriptomic and proteomic studies indicate that glucosinolates are also induced as a direct response to insect herbivory – with the range of upregulated glucosinolates typically differing significantly to those constitutively expressed (Hopkins *et al.*, 1998; Traw *et al.*, 2002; Mewis *et al.*, 2006).

There exists significant complexity in determining the effects of glucosinolates on insect herbivory, with outcomes dependent upon inter and intraspecific variation in the biology and activity of both insect pests and plant varieties (Hopkins *et al.*, 2009). Traits of insect pests which can alter the effectiveness of the glucosinolate-

myrosinase defence system include the nature of a pests feeding mechanism (chewing, sucking, or rasping) alongside whether the pest is a specialist or generalist feeder on *Brassica* crops – with specialists more likely to have evolved physiological strategies to resist or overcome the effects of glucosinolate-myrosinase defence system either through enzymatic detoxification, excretion, or sequestration (Mainguet *et al.*, 2000). The likely success of this defence system also relies heavily upon the specific plant variety and its phenotype, with different plant varieties varying significantly in both the range and levels of glucosinolates expressed (Kleibenstein *et al.*, 2005b; Hopkins *et al.*, 2009).

While widely accepted to negatively impact generalist insect pests, glucosinolates and their associated hydrolytic products are not uniform in their activity against all insect pests (Hopkins *et al.*, 2009). Significant evidence exists to suggest glucosinolates can reduce the survival and relative growth rates of many generalist insect pests (Bodnaryk, 1991; Li *et al.*, 2000). While glucosinolates are thought to provide variable resistance to generalist insect pests, their presence and specificity to *Brassica* crops also serve as a common host plant locating, feeding and oviposition chemoreception stimuli for specialist insect *Brassica* pests (Louda and Mole, 1991; Wittstock *et al.*, 2003).

Owing to the phloem sucking feeding habit of aphids and the minimal damage this causes to the cellular compartments housing glucosinolates and myrosinase, the role of glucosinolates in providing *Brassica* resistance to aphid pests has long been debated – with numerous studies demonstrating conflicting results on whether there exists a positive or negative correlation between glucosinolate concentration and aphid performance (Kim *et al.*, 2008). Both *M. persicae* and *B. brassicae* are known to have evolved mechanisms to resist the effects of the glucosinolate-mediated defence, with *B. brassicae* in particular having evolved a specialised mechanism to co-opt the glucosinolate-myrosinase defence system by sequestering *Brassica* glucosinolates (predominantly aliphatic glucosinolates) and producing their own myrosinase enzymes to generate their own "Mustard Bomb" defence against predators and parasitoids (Pratt *et al.*, 2008; Hopkins *et al.*, 2009; Chaplin-Kramer *et al.*, 2011).

While a majority of glucosinolates are compartmentalised in S-cells, research suggests that glucosinolates are also frequently loaded into the phloem at sites of production and transported throughout the plant – particularly indole glucosinolates which frequently dominate where phloem exudate glucosinolate content is analysed (Chen *et al.*, 2001). Experimental assays have demonstrated that *M. persicae* honeydew contains both indole and aliphatic glucosinolates alongside breakdown products of each – the concentrations of indole glucosinolate breakdown products however appear to dominate relative to intact indole glucosinolates indicating myrosinase-independent breakdown of indole glucosinolates within *M. persicae* causing reduced aphid reproduction in artificial diet experiments (Chen *et al.*, 2001; Kim and Jander, 2007). Experiments using the *M. persicae-A. thaliana* model system have demonstrated that aphid feeding also induces the localised synthesis of the indole glucosinolate 4-methoxyindol-3-ylmethylglucosinolate (4MI3G), further supporting a role in plant defence against aphids for this class of glucosinolates (Kim and Jander, 2007; Kim *et al.*, 2008; Chhajed *et al.*, 2020). While this defensive role of phloem-localised indole glucosinolates is well supported in the generalist *M. persicae*, it is unknown whether indole glucosinolates negatively affect aspects of *B. brassicae* biology. Notably, when sequestering glucosinolates *B. brassicae* have a strong preference for accumulating aliphatic glucosinolates rather than indole glucosinolates – potentially indicating that indole glucosinolates may also adversely affect *B. brassicae* thus their accumulation is prevented to limit any potential autotoxicity (Sun *et al.*, 2020).

1.6. Entomopathogenic fungi

While the stacking of major and minor effect QTLs has received the greatest research focus as a method to increase the durability of plant resistance, the integration of plant resistance both complete or partial with complementary IPM tools may also provide a method to increase resistance durability (Stenberg, 2017). This increased durability results from the multiple modes of action such combined deployment exposes pests to, placing an increased number of potentially conflicting evolutionary pressures upon the pest. Among the most promising candidates to combine with varietal plant resistance to confer this increased durability are biological controls owing to their potential for two-way interactions with varietal resistance (Stenberg, 2017).

Within the scope of biological control agents employed as components of IPM, among the most successful are entomopathogenic fungi (EPF). To date, over 700 species of EPF have been described across 90 genera – being defined by a life cycle centered around parasitizing insect hosts (Gul *et al.*, 2014; Skinner *et al.*, 2014; Abdelghany, 2015). Entomopathogenesis as a fungal life-strategy has evolved independently numerous times across an array of fungal taxa however, EPF species developed for biological control primary originate from two group: the Hypocreales (Ascomycota) and the Entomophthoromycota (Chandler, 2017). The host range of different EPF species and isolates varies widely, with some being defined specialists of a single insect species or family while others are able to parasitize species across several insect orders. Within terrestrial ecosystems EPF are widespread, particularly as facultative saprophytes within soils, and are recognised as important natural regulators of insect populations (Ambethgar, 2009; Skinner *et al.*, 2014; Clifton *et al.*, 2015).

1.6.1. EPF mechanism of infection

Entomopathogenic fungi infect insects through conidia which are typically dispersed passively via weather components including wind and rain splash (Meyling and Eilenberg, 2007). Irrespective of dispersal mechanism, upon contacting an insect cuticle, conidia attach strongly via hydrophobic interactions and under favourable environmental conditions, germinate generating a germ tube. Through a combination of mounting mechanical pressure from growth, the production of specialised infection structures including penetration pegs and appressoria, and the production of cuticle-degrading hydrolytic enzymes (proteolytic, chitinolytic and lipolytic), this germ tube is able over the course of several days to penetrate the aphid cuticle – with this duration varying significantly with abiotic conditions and both inter and intra-specifically (Maina *et al.*, 2018). Penetration of the aphid cuticle allows for hyphal invasion of the insect haemolymph and innervation of organs. Invading fungal hyphae secrete exudates which dampen insect immunological responses permitting rapid and extensive hyphal innervation of insect tissues - causing mortality 3-14 days post infection in aphids (Maina *et al.*, 2018). Following death, fungal hyphae grow from the cuticle of the insect cadaver, producing conidia which may disperse passively by wind or in rain droplets: conidia may also be produced on still live

insects allowing for the direct vectoring of EPF from infected to new hosts (Włóka, 2011; Ortiz-Urquiza and Keyhani, 2013; Petrisor and Stoian, 2017).

1.6.2. Biological control using EPF

EPF have been investigated widely for their potential as biological control agents and represent a growing element of biological pest control regimes in many countries (Lacey *et al.*, 2001; Shah and Pell, 2003; Lacey *et al.*, 2015; Maina *et al.*, 2018). EPF can be deployed as biological control agents through several strategies including augmentation (both inundative or inoculative), classical biological control and conservational biological control. To date, EPF been used most commonly as inundative agents (Jaronski, 2010; Chandler, 2017).

To date, over 170 EPF-based commercial crop protection products have been developed and registered – with a significant number commercially available to growers globally (Faria and Wraight, 2007; Skinner *et al.*, 2014; Ravensburg, 2015). EPF possess many advantageous characteristics making them suitable for inclusion in IPM strategies. Typically, EPF are pathogenic only to arthropods, they rarely cause adverse effects to beneficial arthropods, their usage leaves no toxic residues on crops and (where suitable EPF are selected) they can be highly host specific (Skinner *et al.*, 2014). Importantly, EPF also pose minimal risk to human health, particularly relative to synthetic chemical pesticides (Goettel *et al.*, 2001; Zimmermann 2007).

The anamorphic hypocrealean EPF dominate commercially available EPF products owing to their high relative efficacy, ease of formulation and ease of commercial mass-production – traits which, alongside formulation optimization, have been explored experimentally by industrial producers in significant detail (Rohrlich, 2018). A majority of currently available EPF-based products are based upon the anamorphic hypocrealean generalist species *Beauveria bassiana* and *Metarhizium anisopliae* and to a lesser degree *Isaria* and *Lecanicillium* species (Faria and Wraight, 2007; Chandler, 2017; Elkhateeb *et al.*, 2021). Conversely Entomophthoromycota (such as *Pandora neoaphidis*), despite demonstrating devastating natural epizootics within pest populations, remain inviable as commercial biocontrol agents due to profound difficulties in laboratory culturing and

inducing sufficient sporulation of Entomophthoromycota species (Gryganskyi *et al.*, 2012; Fekih *et al.*, 2013).

Commercially available EPF-based products are indicated for use against a wide array of arthropod pest species within the Acari, Coleoptera, Diptera, Hemiptera, Lepidoptera and Orthoptera and are primarily designated for horticultural crop protection - including both glasshouse and field systems (Chandler, 2017). The factors governing the success of EPF as a component of a specific IPM system are complex and multifaceted. Historically, within the context of inundative biological control, the primary aim of EPF usage has been to ensure individual pests acquire sufficient conidia, whether directly from spray or secondarily from contact with a spore-contaminated surface, to kill them under the prevailing environmental conditions at the given location (Jaronski, 2010; Chandler, 2017). From a grower's perspective, the major considerations when applying EPF are therefore the optimal timing, optimum conditions, necessary spray frequency and rate at which the EPF should be applied to achieve a conidia concentration sufficient for control (Jaronski, 2010). Recommendations for these metrics are provided by manufacturers however such recommendations typically focus predominantly on dosage criteria and rarely account for fundamental aspects of EPF biological ecology such as the effects of abiotic and environmental variations upon the interaction between the EPF and target arthropod species.

As live organisms, the control offered by EPF is significantly affected by environmental and abiotic conditions including temperature, UV radiation, relative and ambient humidity, rainfall, phenotype of the plant, local climate, and unrelated agronomic practices/inputs – particularity fungicide usage (Chandler, 2017). To maximise the efficacy of EPF within an IPM system, a detailed understanding of how these environmental variations and other inputs may affect the specific EPF isolate being deployed, the nature of the biological pest control agent (chiefly the effect of any additional elements within the product upon the EPF) and the interaction between the EPF and target insect is paramount. To date however, many such fundamental aspects of EPF biological ecology have received limited research effort, calling into question whether current EPF application recommendations capture the best efficacy from these products (Ugine *et al.*, 2007; Rohrlich, 2018).

1.6.3. EPF in protected environments

Anamorphic hypocrealean EPF-based products have been widely deployed both in protected glasshouse and open field horticultural crop systems globally - with particularly success and significant levels of pest control being seen within edible and ornamental protected glasshouse crops. Notable examples of EPF successes in protected glasshouse conditions include trials assessing the control of Western flower thrips (*Frankliniella occidentalis*) and Two-spotted spider mite (*Tetranychus urticae*) on tomato crops (*Solanum lycopersicum*), where biopesticides based on different *B. bassiana* isolates have demonstrated >75% and 97% control respectively (Jacobson *et al.*, 2001; Chandler *et al.*, 2005).

Similarly, commercial EPF-based products based on *B. bassiana*, *Isaria fumosorosea* and *M. anisopliae* when included in an 8-week cycle alongside a limited number of synthetic chemical pesticides performed indistinguishably from a solely synthetic chemical pesticide-based rotation for the protection of glasshouse *Chrysanthemum* crops from the Western flower thrip (*F. occidentalisis*) (Kivett *et al.*, 2015; Chandler, 2017). The success of EPF in this environment is undoubtedly aided by the predictability and control of environmental conditions permitted by protected glasshouse conditions (van Lenteren and Woets, 1988; Milner, 1997; Chandler *et al.*, 2011; van Lenteren 2012; Dara, 2019). Such environments also protect applied EPF from adverse abiotic conditions, allowing significantly longer persistence of conidia on the plant surface thereby permitting control both directly through sprayed conidia landing on target pests and secondarily, through secondary pickup of EPF as pests move over conidia-contaminated plant surfaces (Gatarayiha *et al.*, 2010; Jaronski, 2010).

1.6.4. Effects of environmental conditions upon EPF in the field

Relative to protected glasshouse environments, the significant variations in environmental conditions on a daily, seasonally, and yearly basis within a field situation poses an immense challenge for the success of any applied EPF biopesticide (Stafford and Allan, 2010). Temperature, humidity, and UV-B exposure are recognised to be the most significant environmental variables posing challenges to field usage of EPF. Conidia for almost all crop protection relevant EPF species remain viable between 0-40°C, with a majority of species also thermally tolerant

between these temperatures when actively growing (Rai *et al.*, 2014; Chandler, 2017). Different EPF isolates of each species however have a unique combination of optimum temperatures for germination, growth, and sporulation - most commonly between 20–30°C (Mishra *et al.*, 2013). Within warmer countries, particularly close to the equator, temperatures in this range are likely to be commonplace, permitting close to optimal EPF operating temperatures for a significant duration of the growing season. At higher and lower and latitudes in the Northern and Southern hemispheres however, temperatures in this range are significantly more uncommon - with lower temperatures therefore likely to significantly affect the biology of any applied EPF biopesticides.

Alongside temperature, humidity is also recognised as posing a significant potential challenge for EPF application in the field. To permit the successful and rapid germination of EPF conidia, relatively high humidity is typically necessary. Historically, humidity has been assessed through measurements of ambient field humidity - with EPF failures being commonly attributed to low ambient humidity (Chandler, 2017). Increasingly however, ambient humidity is recognised as an unreliable metric, with growing recognition instead of the importance of humidity within the specific leaf or stem-surface microhabitat in which insect pests and applied EPF inhabit – an environment in which humidity is typically higher owing to plant transpiration (Yarwood and Hazen, 1944; Roy *et al.*, 2008; Jaronski, 2010). As is the case for temperature, in some regions naturally higher humidity likely benefits the viability of EPF in the field. The exposure of EPF to UV-B radiation also varies significantly by region, with direct exposure to high levels of UV-B being able to render conidia inviable within several hours (Kaiser *et al.*, 2019). While temperature, humidity and UV-B shortcomings can be moderately mitigated through the inclusion of different oils and compounds within an EPF-based biopesticide formulation, where EPF is to be deployed in a specific field context it is paramount to carefully assess a wide range of potential EPF isolates across a range of abiotic conditions to select an isolate suitable for the prevailing conditions within a given climate.

1.6.5. EPF deployment in the field

Despite the significant challenges to EPF success in the field, EPF have nonetheless been trialled and deployed extensively within field environments globally against a

wide range of arthropod pests and through a range of application methods including dipping, foliar sprays and granular soil treatments (Skinner *et al.*, 2014). The most significant field successes for EPF deployment have been seen in regions where high temperatures and/or humidity prevail – particularly regions of Africa, Asia, Australia and South American (Alves *et al.*, 2003; Maina *et al.*, 2018). Perhaps the most notable success of IPM in a field context is the use of *M. anisopliae* against Spittlebug pests (Cercopidae) on Brazilian Sugarcane crops (*Mahanarva* spp.) - with over 750,000ha of sugarcane being protected through application of this EPF (Li *et al.*, 2010; Maina *et al.*, 2018). Similar success has also been seen in Africa and Australia, where use of a biopesticide based upon the EPF species *Metarhizium acridum* has been shown to offer up to 90% control of locust and grasshopper pests across a range of crop species – significantly mitigating the need for broad-spectrum synthetic chemical pesticide usage (Lomer *et al.*, 2001). Beyond horticultural crops, several products based on EPF have also demonstrated a significant ability to control pests of tree crops in these regions, including the control of invasive Eucalyptus snout beetle (*Gonipterus platensis*) in Brazilian *Eucalyptus* plantations through use of biopesticide products based on *Beauveria* spp. and *M. anisopliae* (Jordan *et al.*, 2021).

While these successes of EPF in environmentally favourable regions highlight the significant potential of EPF-based biopesticides, outcomes in less environmentally favourable regions, including Europe and North America, have been more mixed – with the use of EPF-based biopesticides being largely restricted to protected environments in these regions as a result. Nonetheless, there are several examples of successful EPF-based biopesticide usage in the field in these regions. One such example is the use of *Beauveria brongniartii* for the inoculation control of European cockchafer beetles (*Melolontha melolontha*) on orchard and forest trees – providing control for upwards of nine years (Keller *et al.*, 1997; Shah and Pell, 2003).

This success demonstrates the significant potential EPF still have as biological control agents even where environmental conditions are less favourable. Within these regions however, identification and selection of EPF isolates suitable for the prevailing conditions is of particular importance. Furthermore, owing to the unavoidably more adverse environment conditions in many European and North

American regions, small improvements in EPF efficacy achieved through optimisations in spray dosage, time, and volume alongside developing biopesticide formulations permitting rapid EPF germination in the prevailing conditions are significantly important to ensure EPF can offer viable control in these regions

1.6.6. EPF and aphid pests

Of all the crop pests for which EPF have been assessed as potential biological control agents, few have received as much research attention as aphid pests – likely owing to significance of aphid pests across a wide range of crop species and the absence of alternative entomopathogens available for the control of hemipteran pests (Hall and Burges, 1979; Fournier and Brodeur, 2000; Jandricic *et al.*, 2014; Chandler, 2017). Despite this research effort however, no EPF-active biopesticide products specified for aphid control are currently registered or available in the EU for either field or glasshouse aphid control.

In recent years a wide array of aphid pest species have been assessed for their susceptibility to different EPF species and isolates, including the peach-potato aphid (*M. persicae*), soybean aphid (*Aphis glycines*), Cotton aphid (*Aphis gossypii*), cowpea aphid (*Aphis craccivora*), and the wheat aphid (*Sitobion avenae*) (Nielsen and Hajek, 2005; Saranya *et al.*, 2010; Jandricic *et al.*, 2014; Lee *et al.*, 2015; Ali *et al.*, 2018). In these studies, EPF virulence was assessed in controlled environment laboratory assays against fixed age adult aphid populations, with temperatures and humidity optimised for the efficacy of applied EPF. Overwhelmingly in such studies, EPF are found to be significantly virulent against the chosen aphid of study – particularly to adult aphids demonstrating the significant potential of EPF as aphid control agents.

In both fields and glasshouses, throughout the growing season, aphids are primarily produced parthenogenetically from adult aphids - generating complex overlapping populations of juvenile aphids of multiple instar phases alongside variably aged adults. Real-world aphid populations are therefore comprised of an unpredictable and complex mix of developmental stages (Stark and Banken, 1999). Numerous studies have demonstrated that insect pests have varying degrees of susceptibility to EPF dependent upon their age and development stage thus these complex

populations represent a more significant challenge for control than many aphid-EPF studies replicate in assays (Clarkson and Charnley, 1996). Variations in the EPF susceptibility of different insect life stages is thought to be due to variation in cuticle biochemical composition, differences in cuticle physiological characteristics and, perhaps most importantly, the reduced size of juvenile insect life-stages - reducing the effective dosage of EPF inoculum smaller individuals receive (Tang *et al.*, 1999; Kim and Roberts, 2012).

Likely the most significant obstacle for EPF-based biopesticide control of aphids however results from a core component of aphid developmental biology. As juvenile aphids develop, they pass through four distinct instar phases punctuated by cuticle/exuvium shedding events. Developing aphids therefore undergo four phases of moulting during development to adulthood. Several studies suggest that where EPF-based biopesticides are applied to juvenile aphid instars, if the insect host moults prior to the fungus successfully invading the aphid hemocoel, the majority of applied conidia are shed along with the exuvium (James *et al.*, 2003; Kim and Roberts, 2012). Juvenile aphids thus have four distinct opportunities to escape control by EPF - allowing populations to rapidly rebound from these control escapees where the biopesticide is not reapplied (James *et al.*, 2003). The duration of aphid instar phases relative to the germination time of applied EPF in the prevailing environmental conditions is therefore paramount in the context of EPF control of aphid pests.

1.6.7. EPF and aphid pests of *Brassica* crops

Of the two primary aphid pests of *Brassica* crops, *M. persicae* and *B. brassicae*, the overwhelming focus of research investigating the potential of EPF as biological control agents has focused upon *M. persicae* – likely owing to its status as a hugely problematic generalist pest of a wide array of economically significant crops. Perhaps the largest evaluation of EPF against *M. persicae* was undertaken by Lee *et al.* in 2015, where 342 total isolates from the species *B. bassiana*, *Lecanicillium attenuatum, M. anisopliae, Metarhizium flavoviride*, and *Purpureocillium lilacinum* were assessed for their pathogenicity. Of these 342 isolates, all demonstrated pathogenicity against mixed-age adult *M. persicae* at a dosage of 10^8 conidia ml^{-1},

with mortality rates ranging from 33.3-100% over the seven day experimental period (Lee *et al.*, 2015).

In contrast to these findings of significant potential EPF control of adult *M. persicae*, studies exploring virulence against *M. persicae* instar phases have shown significantly lower efficacy. Against first instar *M. persicae*, Jandricic *et al.* (2014) evaluated 48 EPF isolates including *B. bassiana*, *Beauveria pseudobassiana*, *Metarhizium guizhouense*, *Metarhizium robertsii*, *Metarhizium pingshaense*, *M. anisopliae*, *M. brunneum* and *Isaria javanica* at concentrations between 1-2 x 10^8 conidia ml^{-1}. This study demonstrated that aphid nymphs are less susceptible to fungal infection than adults – with EPF induced percentage mortalities ranging from 1.0-61.6% across all 48 isolates - of which higher mortality percentages were outliers relative to the overall trend (Jandricic *et al.*, 2014). Kim *et al.* (2008) had also previously demonstrated low efficacy of four EPF isolates at a concentration of 10^5 conidia ml^{-1} under glasshouse conditions against second instar *M. persicae* nymphs – showing a peak mortality of 32.7% (Kim *et al.*, 2008).

While fewer studies have instigated the efficacy and viability of EPF upon the *Brassica* specialist aphid *B. brassicae* relative to *M. persicae*, there nonetheless exists literature to suggest efficacy of EPF against *B. brassicae* adults (Derakhshan *et al.*, 2007; Asi *et al.*, 2009ab; Farag, 2008; Akbari *et al.*, 2013; Ramanujam *et al.*, 2017; Soleymadzade *et al.*, 2019; Prince and Chandler, 2020; Gebreyohans *et al.*, 2021).

Similarly to experiments assessing *M. persicae*, laboratory assays with *B. brassicae* and EPF have typically focussed upon adult aphids rather than nymphs (Derakhshan *et al.*, 2007; Asi *et al.*, 2009ab; Akbari *et al.*, 2013; Soleymadzade *et al.*, 2019). These studies indicate the potential EPF of a wide range of species and isolates (including *B. bassiana*, *M. anisopliae*, *N. rileyi, Paecilomyces fumosoroseus*) hold for adult *B. brassicae* control, with all referenced studies identifying significant aphid mortalities following treatment with EPF - particularly where higher conidial dosages such as 10^7 conidia ml^{-1} were employed and optimal temperatures and humidity for rapid conidial germination were provided (Derakhshan *et al.*, 2007; Asi

et al., 2009ab; Farag, 2008; Akbari *et al.*, 2013; Ramanujam *et al.*, 2017; Soleymadzade *et al.*, 2019; Prince and Chandler, 2020; Gebreyohans *et al.*, 2021).

1.6.8. Sublethal effects of EPF

Within the field of synthetic chemical pesticide research, a growing area of interest in recent decades has been the potential for sublethal effects of pesticides upon target pests (Lashkari *et al.,* 2007; Waqas *et al.*, 2019; Wen *et al.*, 2021). In the context of aphid pests, sublethal effects are quantifiable impacts upon a range of different aspects of aphid biology, population development or population structure. Direct effects may include reducing overall or aphid instar aphid development rate, relative fitness, and adult aphid reproduction rates. Such effects may thereby drive significant perturbations in aphid population development or structure.

Owing to the complex interaction between EPF and their insect hosts there is growing interest in evaluating whether EPF also elicit such sublethal effects. Owing to the multiple modes of action employed by EPF during the infective process, there exist several mechanisms through which sublethal effects may arise however to date only a limited number of studies have been undertaken exploring whether such sublethal effects arise. Sublethal effects following EPF application have been reported in a range of sucking-insect pest species including psyllids (*Bactericera cockerelli*), whiteflies (*Bemisia tabaci*) and aphids (*Aphid gossypii*) treated with different isolates of *Beauveria bassiana* and *Lecanicillium attenuatum* (Torrado-León *et al.*, 2006; Kim, 2007; Liu *et al.*, 2020). In all three studies, sublethal effects upon reproductive period duration of treated insects were observed alongside potential effects upon the developmental rate, longevity, and fecundity of subsequent generations (Torrado-León *et al.*, 2006; Kim, 2007; Liu *et al.*, 2020).

Contrary to studies assessing EPF overall where most studies have focused upon *M. persicae*, of the small number of undertaken studies assessing sublethal EPF effects, a majority have investigated *B. brassicae*. In these studies, the sublethal effects of many different EPF species and isolates (*Acremonium sclerotigenum*, *Beauveria bassiana*, *Lecanicillium longisporum*, *Lecanicillium muscarium*, *Metarhizium anisopliae*, *Paecilomyces variotii* and *Simplicillium* sp.) have been evaluated

utilising predominantly a life-table approach (Emami *et al.*, 2016; Akbari *et al.*, 2020; Safavi and Sarhozaki, 2020).

Owing to a primary focus upon sublethal effects, significantly lower conidia solution concentrations were assessed in many of these studies; with Emami *et al.* (2016) evaluating a dosage calculated to lead to an average of 30% mortality (LC_{30}) and Akbari *et al.* assessing a LC_{25} dosage. Safavi and Sarhozaki (2020) however evaluated sublethal effects across a more comprehensive range of EPF concentrations, from 1×10^3 - 1×10^7 conidia ml^{-1}. Utilising lower EPF doses, both Emami *et al.* (2016) and Akbari *et al.* (2020) reported significant declines in *B. brassicae* reproduction rates, resulting in significant lessening of *B. brassicae* intrinsic rates of population increase and population doubling time. Safavi and Sarhozaki (2020) reported similarly significant declines in *B. brassicae* intrinsic rates of population increase and population doubling time however mean numbers of offspring per reproducing adult were noted to not significantly differ between any treatment relative to the mock treated control aside from those treated with 1×10^6 conidia ml^{-1} solution – whose reproduction was observed to significantly increase. Alongside evaluating lethal effects, Prince and Chandler (2020) also evaluated *B. brassicae* reproduction post-treatment with high doses of formulated EPF products (as per manufacturer recommendations) (Prince and Chandler, 2020). In common with Safavi and Sarhozaki (2020), Prince and Chandler (2020) detected no significant difference pre-mortality reproduction (Prince and Chandler, 2020).

1.6.9. EPF and IPM

To date, EPF have been overwhelmingly deployed as inundative controls serving effectively as a direct analogue for synthetic chemical pesticides (Figure 1.2) (Jaronski, 2010; Chandler, 2017). While success has undoubtedly been achieved with this approach, increasing evidence is emerging to suggest that greater value can be gained from EPF where they are deployed more strategically within an IPM context (Chandler, 2017). Furthermore, as has been seen with synthetic chemical pesticides, the deployment of EPF in an inundative manner unquestionably places significant selection pressures upon target arthropod species (Dubovskiy *et al.*, 2013). While the multiple modes of action employed by EPF during the infection process confers EPF significant inbuilt durability, a sole means of deployment

nevertheless risks the development of pest biotypes able to resist infection by EPF - thereby endangering a valuable crop protection tool (Dara, 2017; Dubovskiy *et al.*, 2013).

One means of increasing the durability of ecologically based IPM tools and maximising their potential is to strategically combine them in holistic IPM systems. To successfully integrate EPF with different IPM tools requires extensive knowledge of their biology and efficacy in isolation, alongside an understanding and appreciation of factors which may influence their integration including environmental variables and the nature of their interaction (Lacey and Shapiro-Ilan, 2008; Stenbeg, 2017). Historically however, little research considering EPF in an IPM context has been undertaken, with most simply assessing the compatibility of EPF with other IPM tools including fungicides and synthetic chemical pesticides (Ramanujam *et al.*, 2017; Sain *et al.*, 2019; Gebreyohans *et al.*, 2021). There is therefore significant scope for evaluating EPF within a fully realized IPM context, particularly in light of the key interactions between ecological IPM component identified by Stenberg (2017).

1.7. Project aims and objectives

Aphids of *Brassica* crops are of increasing concern to vegetable *Brassica* growers owing to increasing withdrawals of previous mainstay synthetic chemical pesticides from the market, and the development of aphid resistance to commonly used chemical classes. Aphids are therefore becoming increasingly difficult to control on *Brassica* crops, with the risk of complete control failure mounting and growers already having to tolerate increasing levels crop damage and the economic losses this brings.

This research aims to explore the viability of a novel and more durable IPM approach to *Brassica* aphid control, combining varietal partial resistance and entomopathogenic fungi as complementary tools specifically targeted at overcoming the innate difficulties of *B. brassicae* control. Owing to the complex and interdisciplinary research required to develop such an IPM strategy, a logical stepwise approach to this problem was taken - inspired by both the case study of IPM development for the Sunn pest in Asia alongside the work of Stenberg in

highlighting the key interactions between biological IPM components. Through this logical approach, the viability and efficacy of these two IPM components was evaluated individually as a benchmark against which their integration could be assessed – allowing for determination of whether combined deployment was able to offer improvements in aphid control and to reveal the nature of the interaction between partial host plant resistance and EPF when integrated in this manner.

The aims of this project were achieved through the following objectives in each chapter:

- The focus of Chapter 2 was the interaction between *B. brassicae* and their host *Brassica* plants – with a view to identifying and characterising *Brassica* accessions with demonstrable partial resistance to *B. brassicae*. To achieve this, varieties of both *B. oleracea* crop-type and C-genome wild relatives were screened for their relative levels of partial resistance to *B. brassicae*, and the effects of this resistance on the biology of *B. brassicae*.
- The focus of Chapter 3 was the interaction between *B. brassicae* and entomopathogenic fungi, seeking to identify promising EPF candidates for *B. brassicae* control and characterise their interaction with different *B. brassicae* life stages. A range of EPF species were assessed, including both commercial and non-commercial isolates.
- The focus of Chapter 4 was the integration of partial plant resistance and EPF - assessing the effects of their combined deployment upon *B. brassicae*. This chapter sought to evaluate the combined deployment of these IPM components with particular reference to the quantitative effects on the virulence and speed of kill of EPF against both adult and juvenile *B. brassicae* alongside whether such an approach has a significant influence upon *B. brassicae* population development under different EPF spray regimes.

2. Identifying and evaluating *Brassica* plant material for partial antibiosis resistance to the specialist aphid pest *Brevicoryne brassicae*

2.1. Background

2.1.1. Screening for aphid resistant *Brassica* crops

Synthetic chemical insecticides have been the primary method for treating insect pests of *Brassica* crops since the start of the Green Revolution in the 1960s. This reliance on chemical pesticides as 'silver bullets' has meant that alternative, more sustainable methods and IPM systems have not yet entered the mainstream. Owing to the problems associated with injudicious pesticide use and the mounting withdrawal of pesticides as outlined in Chapter 1, the agronomic situation is rapidly changing with aphid pests increasingly difficult to control. While novel and sustainable approaches for aphid control are urgently needed, there remains a shortage of IPM compatible methods for *Brassica* pest management, particularly *Brassica* accessions resistant to insect herbivory. While a growing number of studies have been undertaken seeking to identify insect resistant crop varieties in other crop species (Kloth *et al.*, 2015ab), only a small number of pioneering studies have previously been undertaken seeking to identify aphid-resistance in *Brassica* crops (Ellis *et al.*, 1996, 1998; Ellis *et al.*, unpublished.). It is therefore largely unknown to what extent cultivated *B. oleracea* crops overall, and on a cultivar-specific level, harbour antibiosis resistance to aphid pests.

Historically the most successful aphid-resistance screening systems in other crops have utilised a wide 'top-down' approach, assessing hundreds or thousands of different accessions. This strategy was used by Ellis *et al.* in their screen of 401 *B. oleracea* accessions for *B. brassicae* resistance (Ellis *et al.*, 1998). This approach was necessitated by the relative rarity of aphid resistance, with such screens typically resulting in the identification of only a small subset of promising candidates at the expense of significant resources, time, and labour. In recent years, novel high-throughput aphid-resistance screening methods have been proposed for use in large plant collections and *Brassica* plants specifically (Chen *et al.*, 2012; Kloth *et al.*, 2015ab). However, even such high-throughput methods require extensive time and labour inputs to identify small selections of promising candidate accessions.

Furthermore, such approaches typically rely on the use of isolated pieces of plant tissues rather than intact plants, thus disregarding the importance of the dynamic biochemical interaction between aphids and their host plants.

2.1.2. Effects of partial resistance upon aphid biology and life parameters

Aphids and their host plants have evolved complex and dynamic trophic relationships in which plant genotype, its nutritional quality and the presence/absence of resistance exerts a significant influence upon aphid life history and population growth (Will *et al.*, 2007; Cao *et al.*, 2016; Guerrieri and Digilio, 2008; van Bel and Will, 2016; Gatehouse, 2002; Dogimont *et al.*, 2010). Much of the research on aphid-plant interactions in recent years has built upon earlier studies of aphid life cycles including the construction of detailed life history tables (Carter *et al.*, 1978; Nowierski *et al.*, 1983; Hutchinson and Hogg, 1985; Tsai and Wang, 1999). Life tables typically include a wide range of variables such as the duration of each aphid instar phase, daily reproduction, and aphid weight (Carter *et al.*, 1978; Nowierski *et al.*, 1983; Hutchinson and Hogg, 1985; Tsai and Wang, 1999). Life tables also commonly contain a range of calculated metrics related to population development and fitness and include measures such as intrinsic rate of population increase, population doubling time and mean relative growth rate (Southwood, 1978; Hutchison and Hogg, 1985). While the majority of life tables have been historically developed for use in pest modelling for glasshouse and field crops, researchers have increasingly come to recognise their value when evaluating different crop protection regimes and approaches. To this day, life tables remain a common and useful tool, particularly for aphid pests, for example to evaluate the effects of variable temperature or insecticidal treatments upon aphid biology (Hutchison and Hogg, 1985; Zhanshan and Bechinski, 2009; Gao *et al.*, 2012; Ramalho *et al.*, 2015; Ning *et al.*, 2017).

In recent years, several life table studies have assessed the effects of temperature and *B. oleracea* and *B. napus* cultivars on *B. brassicae* biology (Mirmohammadi *et al.*, 2009; Jahan *et al.*, 2014; Soh *et al.*, 2018). These studies revealed that temperature had a significant effect upon all measured aspects of *B. brassicae* biology including life cycle and reproduction – with temperatures in the range of 20-30°C eliciting the highest net reproduction rates, shortest average generation time and highest finite

rate of population increase (Soh *et al.*, 2018). Similarly different cauliflower cultivars were found to elicit significant differences in *B. brassicae* development rates and reproduction – particularly intrinsic rate of population increase (r_m) and population doubling time (DT) (Jahan *et al.*, 2014). Conversely Mirmohammadi *et al.* noted no significant differences in *B. brassicae* life parameters when reared on four distinct *B. napus* cultivars (Mirmohammadi *et al.*, 2009).

While screening for aphid resistant crop varieties and assessing the life history parameters of aphids on different crop varieties are both areas of growing research interest, rarely are both areas considered together to evaluate antibiosis resistance in detail. Where such an approach has been taken, the assessment of aphid life parameters has notably improved the plant resistance screening process – providing valuable information about the basis of observed aphid resistance (Doryanizadeh *et al.*, 2016; Greenslade *et al.*, 2016). Through such work, the relationship between plant resistance and different aspects of aphid biology can also be better understood. This was the case in the work of Wojciechowicz-Zytko and Emden, who identified that *Vicia faba* L. resistance correlated significantly with mean relative growth rate but not intrinsic rate of population increase (Wojciechowicz-Zytko and Emden, 1995). Such research may ultimately aid in the identification of potential genomic regions underlying resistance or partial resistance (Greenslade *et al.*, 2016).

2.1.3. Glucosinolates and plant defence against aphids

Owing to the phloem sucking feeding habit of aphids and the minimal damage this causes to the cellular compartments housing glucosinolates and myrosinase, the role of glucosinolates in providing *Brassica* resistance to aphid pests has long been debated. A number of studies have demonstrated conflicting results on whether there is a positive or negative correlation between glucosinolate concentration and aphid performance (see Kim *et al.*, 2008). Both *M. persicae* and *B. brassicae* are known to have evolved mechanisms to resist the effects of glucosinolate-mediated plant defence, with *B. brassicae* in particular having evolved a specialised mechanism to co-opt the glucosinolate-myrosinase defence system by sequestering *Brassica* glucosinolates (predominantly aliphatic glucosinolates) and producing their own myrosinase enzymes to generate their own “Mustard Bomb” defence against

predators and parasitoids (Pratt *et al.*, 2008; Hopkins *et al.*, 2009; Chaplin-Kramer *et al.*, 2011).

While a majority of glucosinolates are compartmentalised in S-cells, research suggests that glucosinolates are also frequently loaded into the phloem at sites of production and transported throughout the plant – particularly indole glucosinolates which frequently dominate where phloem exudate glucosinolate content is analysed (Chen *et al.*, 2001). Experimental assays have demonstrated that *M. persicae* honeydew contains both indole and aliphatic glucosinolates alongside breakdown products of each. The concentrations of indole glucosinolate breakdown products however appear to dominate relative to intact indole glucosinolates indicating plant myrosinase-independent breakdown of indole glucosinolates within *M. persicae* causing reduced aphid reproduction in artificial diet experiments (Chen *et al.*, 2001; Kim and Jander, 2007). To date, it remains unknown whether such breakdown is mediated by aphid myrosinases, hydrolysis by other enzymes, or occurs as a result of the detoxifying processes of aphid pests.

Further experiments using the *M. persicae-A. thaliana* model system have demonstrated that aphid feeding also induces the localised synthesis of the indole glucosinolate 4-methoxyindol-3-ylmethylglucosinolate (4MI3G), further supporting a role in plant defence against aphids for this class of glucosinolates (Kim and Jander, 2007; Kim *et al.*, 2008; Chhajed *et al.*, 2020). While this defensive role of phloem-localised indole glucosinolates is well supported in the generalist *M. persicae*, it is unknown whether indole glucosinolates negatively affect aspects of *B. brassicae* biology. Notably, when sequestering glucosinolates, *B. brassicae* have a strong preference for accumulating aliphatic glucosinolates rather than indole glucosinolates – potentially indicating that indole glucosinolates may also adversely affect *B. brassicae*, thus their accumulation is prevented to limit any potential autotoxicity (Sun *et al.*, 2020).

2.1.4. Aims and objectives

The aim of this first component of the PhD was to identify crop-type *B. oleracea* and related C-genome *Brassica* accessions which demonstrate variable degrees of partial resistance to the specialist aphid pest *B. brassicae*. To achieve this, a pragmatic pre-

selection approach was taken, making use of existing resources to pre-select likely partially resistant accessions for formal screening. These resources included promising accessions identified through historic top-down screening alongside advances in immunological and plant defence understanding from the *Myzus-Arabidopsis* model, which guided the pre-selection of further accessions for screening. By reducing the number of accessions to be screened, an emphasis could be placed on high levels of replication to accurately determine the relative levels of partial antibiosis resistance displayed by each accession.

This work was then followed up with detailed experiments assessing the specific impacts of each accession upon three key aspects of *B. brassicae* biology which life table studies highlighted as the most likely to be affected by host plant antibiosis resistance: development rate, weight during development and reproduction. Owing to uncertainty surrounding the role of glucosinolates in *Brassica* defence against *B. brassicae*, the glucosinolate content (sinigrin and glucoraphanin) of *Brassica* accessions was also assessed. Finally, analysis was performed to determine whether there was a correlation between observed levels of *Brassica* accession resistance to *B. brassicae* with different aphid life history parameters and *Brassica* accession glucosinolate content.

2.2. Materials and methods

2.2.1. Aphid clones and colony maintenance

The *Brevicoryne brassicae* biotype used throughout this study was the K3 clone isolated from Brussels sprouts in Lincolnshire, UK (1997). This *B. brassicae* clone is maintained continually at the University of Warwick Crop Centre insect rearing unit and demonstrates no known pesticide resistance. *B. brassicae* cultures were maintained on 5-week-old Brussels sprouts plants, *B. oleracea* var. *Gemmifera* 'Doric F1' raised as described in section 2.2.4 (Elsoms Seeds Ltd, Lincolnshire, UK). Stock cultures of aphids were maintained on 5-week-old plants within mesh cages (60 x 60 x 60cm Bugdorm-6S610, Watkins & Doncaster, Leominster UK) in a controlled environment room (20 ± 2 °C, 60% RH, L:D 16:8 h, fluorescent tube lighting). Every two weeks, new plants were added to the colony and aphids allowed two days to move onto new plants, old plants were then discarded.

2.2.2. Generation of fixed-age *Brevicoryne brassicae* adults

Fixed-age *B. brassicae* cultures were produced by transferring groups of 20 mature apterous virginoparae (female adults without wings which give birth to live young by parthenogenesis) onto ten 5-week-old *B. oleracea* var. *Gemmifera* 'Doric' plants. After 24h all mature virginoparae were removed and the fixed-age progeny maintained for a further nine days generating a population of 10-day old adult *B. brassicae*. Fixed-age *B. brassicae* colonies were maintained within mesh cages (60cm^3 Bugdorm-6S610, Watkins & Doncaster, Leominster UK) in a controlled environment room (20 ± 2 °C, 60% RH, L:D 16:8 h) throughout the course of their production.

2.2.3. *Brassica* accession selection

A total of 18 candidate *Brassica* accessions plus a technical control were selected for evaluation of their partial resistance to *B. brassicae* (Table 2.2; Figure 2.2). All the accessions investigated came from seed lots archived within the UK Vegetable Genebank (UKVGB; formerly known as the Genetic Resources Unit or GRU) at the Warwick Crop Centre, University of Warwick (Walley *et al.*, 2012) (https://warwick.ac.uk/fac/sci/lifesci/wcc/gru/). The technical control used in experiments was the plant accession upon which the aphid clones were maintained in culture, *B. oleracea* var. *Gemmifera* 'Doric F1'. Candidate accessions for resistance screening were chosen in two different ways. Firstly, nine accessions were selected which had been reported previously as exhibiting antibiosis partial resistance to *B. brassicae (*(Ellis *et al.*, 1998; Ellis *et al.*, unpublished).

The second method for candidate selection utilised transcriptomics (mRNAseq) gene expression data from Diversity Fixed Foundation Sets (DFFS) of *Brassica* genotypes developed in the Defra VeGIN project at the University of Warwick Crop Centre, Wellesbourne and maintained at the UK Vegetable genebank (https://warwick.ac.uk/fac/sci/lifesci/research/grc/plant/dffs/) (Pink *et al.*, 2008; Walley *et al.*, 2012). Each DFFS consists of a group of genetically fixed lines which represent a broad sample of diversity across the species and/or crop-type gene pools. A majority of DFFS lines are double haploid and descend from a single seed of a single founder doubled haploid plant, minimising the within-line heterogeneity and within-plant heterozygosity that occurs in many germplasm collections (such as

those maintained in the National Vegetable Genebank collections at Wellesbourne), and which can impede attempts to coordinate trait and genetic studies for crop breeding (Pink *et al.*, 2008; Walley *et al.*, 2012). Two DFFS collections were used in this study: (i) a DFFS of 188 crop-type *B. oleracea* accessions (BolDFFS) for which mRNAseq data was available for 50 accessions (https://warwick.ac.uk/fac/sci/lifesci/research/vegin/brassica/boldffs/); and (ii) a DFFS of 89 accessions representing 14 C-genome *Brassica* wild species (BCgDFFS) for which mRNAseq data was available for 77 accessions (https://warwick.ac.uk/fac/sci/lifesci/research/vegin/brassica/bcgdffs/) (Pink *et al.*, 2008; Walley *et al.*, 2012).

Nineteen genes from the *A. thaliana* JA signaling pathway were selected encompassing signal recognition, signal transduction and transcriptional regulation functions, and orthologues were identified in *B. oleracea* using polypeptide sequence reciprocal best BLAST searches, with orthologues identified based upon a greater than 95% sequence identity match (Table 2.1). Orthologues were also subjected to a reverse BLAST search to ensure the full orthologue polypeptide sequence resulted in a closest identity match to the *A. thaliana* gene. Gene expression data (mRNA reads) for each orthologue was then extracted from each DFFS RNAseq data set and a heatmap generated for each using R-studio demonstrating the relative expression level of each gene orthologue for each accession (Figure 2.1) (R Foundation for Statistical Computing, Vienna, Austria). Nine accessions which demonstrated the highest expression across the widest range of gene homologues (6 from the crop-type *B. oleracea* DFFS and 3 from the wild-*Brassica* species DFFS) and for which seed stocks were sufficient for use in further experiments were selected for phenotype screening.

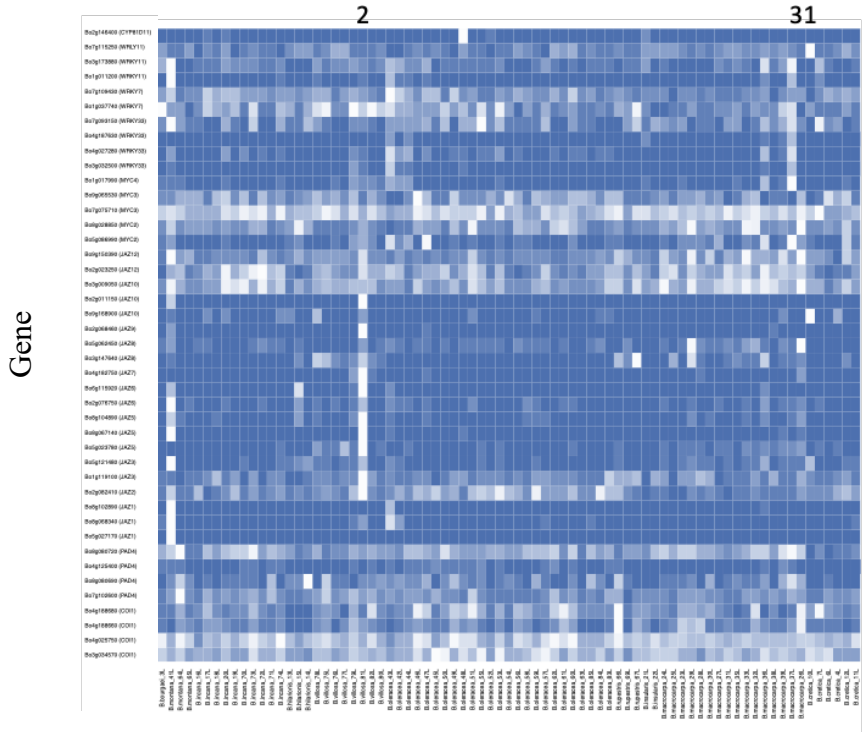
Table 2.1: Nineteen selected *Arabidopsis thaliana* genes acting within the Jasmonic Acid signalling pathway for bioinformatic screening based upon RNAseq data for VeGIN (Vegetable Genetic Improvement Network) Diversity Fixed Foundation Sets (DFFS).

Arabidopsis Gene	No. *B. oleracea* orthologues identified	No. *B. oleracea* orthologues with expression data
COI1	4	4
PAD4	4	4
JAZ1	3	3
JAZ2	3	1
JAZ3	2	2
JAZ5	3	3
JAZ6	2	1
JAZ7	1	0
JAZ8	3	3
JAZ9	2	1
JAZ10	3	3
JAZ12	2	2
MYC2	2	2
MYC3	2	2
MYC4	1	1
WRKY33	4	4
WRKY7	2	2
WRKY11	3	3
CYP81D11	3	2

Table 2.2: Plant accessions selected for antibiosis partial-resistance screening against *Brevicoryne brassicae*. Each accession includes an assigned experimental number, selection criteria, Genus, Species and (where applicable) Subspecies, Cultivar Group, Crop-Type and Cultivar.

No.	Selection Criteria	Genus	Species	Subsp.	Cultivar Group	Crop Type	Cultivar
1	Wild species DFFS	*Brassica*	*macrocarpa*	-	-	-	-
2	Wild species DFFS	*Brassica*	*villosa*	*tinei*	-	-	-
3	Wild species DFFS	*Brassica*	*macrocarpa*	-	-	-	-
4	Crop-type DFFS	*Brassica*	*oleracea*	-	Gongylodes	Kohlrabi	-
5	Crop-type DFFS	*Brassica*	*oleracea*	-	Tronchuda	Tronchuda	-
6	Crop-type DFFS	*Brassica*	*oleracea*	-	Botrytis	Romanesco	-
7	Crop-type DFFS	*Brassica*	*oleracea*	-	Gemmifera	Brussels sprouts	-
8	Crop-type DFFS	*Brassica*	*oleracea*	-	Italica	Broccoli	-
9	Crop-type DFFS	*Brassica*	*oleracea*	-	Alboglabra	Chinese Kale	-
10	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Acephala	Kale	'Butzo'
11	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Acephala	Kale	'Furchehnkohl'
12	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Acephala	Kale	'Arsis F1'
13	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Botrytis	Cauliflower	'Tasman'
14	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Botrytis	Cauliflower	'Mikado March'
15	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	-	Botrytis	Cauliflower	'Marzatico Napoletano'
16	Ellis *et al* wild species	*Brassica*	*cretica*	-	-	-	-
17	Ellis *et al* wild species	*Brassica*	*cretica*	-	-	-	-
18	Ellis *et al* wild species	*Brassica*	*cretica*	-	-	-	-
-	Control	*Brassica*	*oleracea*	-	Gemmifera	Brussels sprouts	'Doric'

(a) Wild *Brassica* DFFS



(b) Crop-type *Brassica oleracea* DFFS

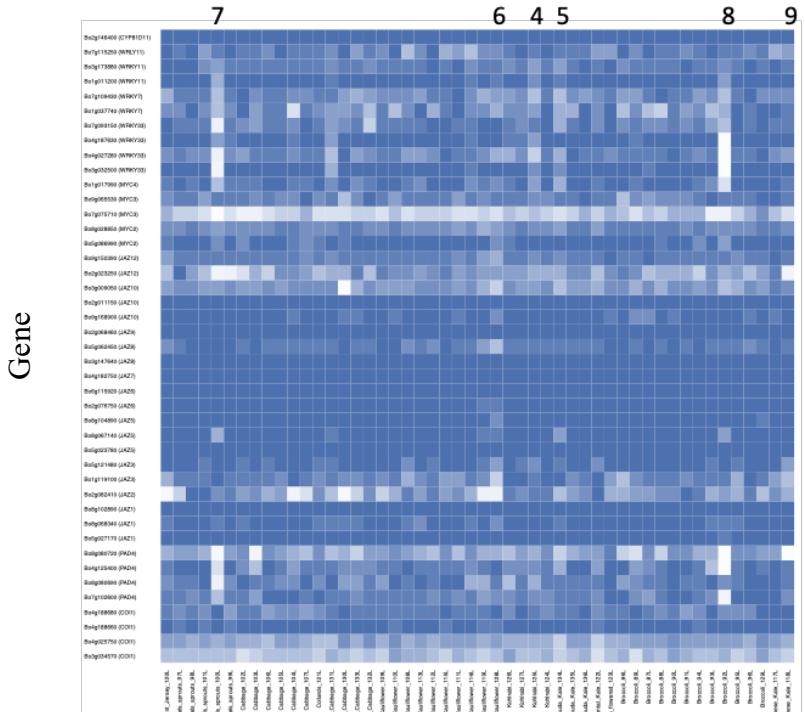


Figure 2.1: Heatmaps of relative gene expression for (a) wild-*Brassica* species DFFS and (b) crop-type *Brassica oleracea* DFFS assessing all identified JA homologues for which transcriptomic data was available. Lighter shades indicate higher gene expression relative to the maximum observed expression level for each gene. Visual inspection of generated heatmaps informed the selection of 9 candidates of interest (three from wild *Brassica* DFFS and six from crop-type *Brassica* DFFS) for subsequent partial-resistance screening against *Brevicoryne brassicae*, with numbers above each heatmap denoting these selected accessions and their assigned numbers.

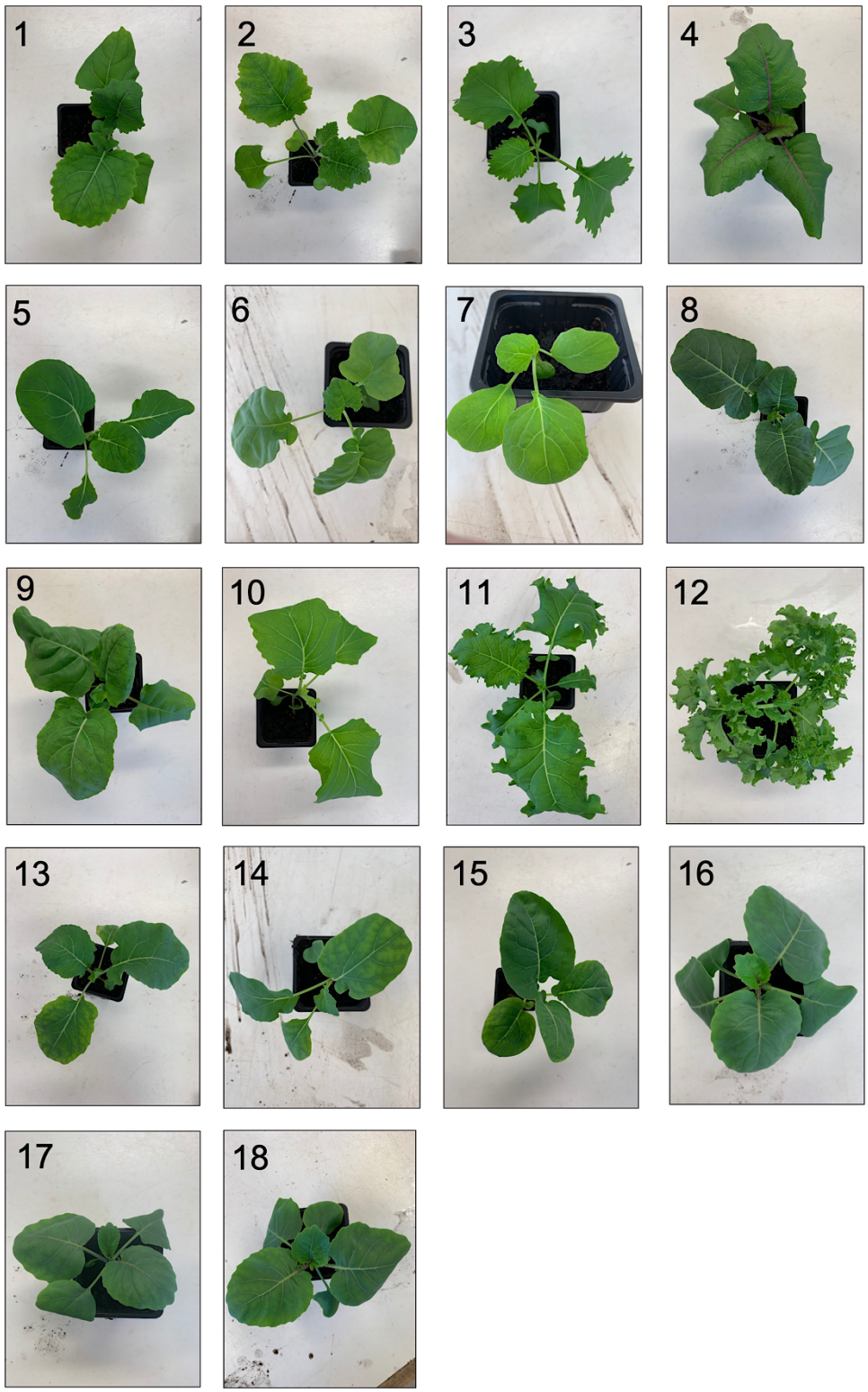


Figure 2.2: Variable phenotypes of the 18 *Brassica* accessions screened for antibiosis partial resistance to *Brevicoryne brassicae*. Labelled numbers correspond with the plant accession experimental numbers described in Table 2.2. Plants shown at 5 weeks old (7 days for germination, 4 weeks growth in 20 ± 2 °C, 60% RH, L:D 16:8 h conditions) immediately prior to resistance screening. Plants were raised in 7cm pots.

2.2.4. Plant raising

Brassica seeds were sown in clear plastic boxes (15cm x 8cm x 6cm) filled with moist vermiculite (BHGS, Evesham, Worcestershire UK), boxes were then sealed with a clear plastic lid and maintained at 20 ± 2 °C and L:D 16:8 h. After seven days, germinated seedlings were transplanted into Levington M2 compost in 75 mm black polyethylene pots and grown on for four weeks (20 ± 2 °C, 60% RH, L:D 16:8 h), watering as required.

2.2.5. Antibiosis partial resistance screening

To determine the relative levels of partial antibiosis resistance to *B. brassicae* present across the 18 selected accessions a rapid phenotyping screen was undertaken. In this screen, aphid population development was quantified over a two-week period from a fixed starting point. The experiment was undertaken using a randomised incomplete block design in which nine (of the eighteen) accessions plus the technical control plant were evaluated per block. A total of six blocks were undertaken with each being a separate occasion. Ten plants of each accession were evaluated per block (with the exception of accessions # 5, 6 and 7 which showed poor germination, meaning that fewer than ten plants were available for some blocks). Thirty plants of each of the 18 accessions were therefore evaluated for partial resistance to *B. brassicae* totalling 540 plants, as the control plant was included in each block 60 plants of this accession were evaluated.

Plants were raised as described in 2.2.4, with 40 seeds of each accession sown per replicate. Because of their slower germination, *Brassica cretica* accessions (16, 17 and 18) were sown a week earlier than all other accessions to permit a longer germination period. After this longer germination period, *B. cretica* accessions were treated identically to all other accessions. As more than ten plants of each accession were typically raised plants, the healthiest and most uniform ten plants of each accession were selected and used for resistance screening.

Three fixed-age, 10-day old apterous virginoparae (generated as described in 2.2.2) were transferred to the fourth true leaf of each of the ten plants per accession and left for 24h to reproduce, after which all adults and all but three *B. brassicae* nymphs were removed. Each plant with three 1-day old nymphs was then sealed in a porous

bread-bag (200 x 250mm perforated polypropylene bags, Cater4you Ltd, High Wycombe, UK) and maintained in a controlled growth room (20 ± 2 °C, 60% RH, L:D 16:8 h) for two weeks, watering as required. After two weeks, plants were removed from bread bags and the number of aphid adults and nymphs per plant was recorded destructively.

2.2.5.1. Antibiosis screening statistical analysis

Total number of recorded aphids per plant at the end of the 14-day experiment period was divided by the starting number of nymphs each plant was inoculated with (3) to determine the average total number of nymphs produced by each starting nymph. Statistical analysis was then performed using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA). Screening data for each aphid species was evaluated for overdispersion to determine whether it conformed to the Poisson distribution and plotted as boxplots with accessions ranked according to the median number of aphids produced per starting nymph. The results were analysed to determine whether the following were significant factors influencing aphid performance:

- Plant Accession
- Plant Accession selection criteria
- Plant Species
- *Brassica oleracea* Cultivar Group

Screening data was rounded to the nearest integer and analysed using four separate one-factor generalised linear models (GLM), each using a negative binomial distribution, log-link function and custom dispersion parameter to account for identified overdispersion. Selection criteria analysis included: wild *Brassica* species DFFS, *B. oleracea* DFFS crop-type, literature selected accessions (Ellis *et al.* 1998 and *Brassica cretica* accessions) and the technical control. Plant species analysis included *Brassica oleracea* (12 accessions), *Brassica cretica* (3 accessions), *Brassica macrocarpa* (2 accessions) and *Brassica villosa tinei* (1 accession). Cultivar group analysis included 7 cultivar groups: Acephala (3 accessions), Alboglabra (1 accession), Botrytis (4 accessions), Gemmifera (1 accession), Gongylodes (1 accession), Italica (1 accession), and Tronchuda (1 accession). Owing to multiple comparisons resulting from these four separate GLMs, a Bonferroni

correction was applied altering significance thresholds to 0.0125. Experimental block and replicate were also included as factors in each analysis to evaluate between block and replicate variation.

2.2.6. *Brevicoryne brassicae* development and reproduction studies

Following determination of relative levels of partial resistance for each plant accession, a screen was undertaken to characterise the impacts of different plant accessions and their different levels of partial resistance upon the biology of *B. brassicae*. Owing to the complexity of these experiments, these biological characteristics were assessed on a subset of *Brassica* accessions (2, 4, 9, 11, 12, 15, 16, 17 and Control), selected to encompass the range of partial resistance seen across the initial screen and to include accessions for which seed stocks were sufficient for further experiments. Four *B. brassicae* biological characteristics were assessed in these studies: the duration of the four individual aphid instar phases, the total time taken for a first instar *B. brassicae* nymph to reach adulthood, reproduction of aphids reaching adulthood and aphid weight throughout their development.

To assess the first three of these aspects of *B. brassicae* biology, a clip cage study was undertaken. Three *Brassica* plants of each accession were raised to 5-weeks old as described in section 2.2.4. A fixed-aged adult *B. brassicae* colony was also established as described in section 2.2.2. Three fixed-age, 10-day old apterous virginoparae were transferred to the fourth true leaf of each of the three plants per accession and left for 24h to reproduce, after which all adults and all but one *B. brassicae* nymphs were removed. The single nymph was then placed within a clip-cage in-situ on the leaf (Figure 2.3). Plants were maintained at 20 ± 2 °C, 60% RH, L:D 16:8 h for 20 days and watered as required. Every 24 hours the clip cage was opened and aphid cuticle shedding events, indicating advancement to the next instar-phase, recorded. Upon aphids reaching adulthood, the number of nymphs produced each day was recorded for the same duration as it took for nymphs to reach adulthood. After recording of the number of nymphs produced each day, all nymphs were removed leaving only the single adult *B. brassicae*, thus daily larviposition could be easily assessed.

All nine plant accessions were screened in each replicate, with three plants assessed per accession per replicate. A total of nine plants were therefore assessed per accession over the course of this experiment.

Figure 2.3: Clip-cage experimental setup used to assess *Brevicoryne brassicae* developmental rate and reproduction.

2.2.6.1. *Brevicoryne brassicae* development and reproduction studies - statistical analysis

Both individual instar durations and overall development time from first instar to adulthood development results were assessed and analysed. To produce graphs, the duration of each *B. brassicae* instar phase on the three plants in each replicate was averaged. Grand means were then calculated and plotted in stacked bar charts including SEM error bars.

To analyse overall development data, the data was transformed via a square root transformation and analysed via ANOVA. Individual instar durations however could not be transformed to conform to a normal distribution and were thus analysed via GLMs each with a gamma distribution and log-link function. Replicate was included in all analyses to evaluate between replicate difference. All analyses were performed using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA).

Daily and gross larviposition (over an 8-day period) were evaluated to determine whether they conformed to the Poisson distribution, plotted as boxplots with accessions ranked according to the median daily/gross larviposition and subsequently analysed through GLM using a negative binomial distribution, log link function and custom dispersion parameter using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA).

Intrinsic rate of population increase (r_m) was calculated according to methodology and formula developed by Wyatt and White (1977), where M_d is the number of nymphs produced by the adult aphid in the first d days (d equalling the number of days taken for the aphid to reach adulthood from first instar phase). The constant (c = 0.738) was devised by Wyatt and White and approximates the proportion of the total reproduction produced in the first days of reproduction. (Wyatt and White, 1977).

$$r_m = \frac{(\ln M_d \times c)}{d}$$

Aphid population doubling time (DT) was calculated using the following equation (Deloach, 1974; Wyatt and White, 1977; Vincent *et al.*, 2004).

$$DT = \frac{\ln(2)}{r_m}$$

Using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA), r_m and DT were plotted as Boxplots with accessions ranked according to the median r_m and DT. Intrinsic rate of increase results were found to conform to a normal distribution thus were analysed through ANOVA. Doubling time results were transformed via Log_{10} transformation and analysed through ANOVA. In each analysis, replicate was included as a factor to assess its significance and evaluate its effect upon analyses.

2.2.7. *Brevicoryne brassicae* development weight

To determine whether identified partially resistant plant accessions impact upon overall aphid size and fitness during development, a screen investigating aphid weight over time (and through multiple generations) was undertaken. Three plants of each accession (2, 4, 9, 11, 12, 15, 16, 17 and Control) were grown to 5-weeks old (6-true leaf stage) as described in section 2.2.4. A fixed-aged 10-day-old adult *B. brassicae* culture was established as described in section 2.2.2 and twenty fixed-age adults were transferred to each plant and left for 24 hours. After 24 hours all adult *B. brassicae* were removed establishing a fixed-age nymph culture on each plant. Five of these aphids per plant were then weighed destructively on days 1, 4, 7 and 10 (Adventurer model AR3130, Oahus, Nanikom, Switzerland). After weighing on day 10, all nymphs were removed, and adults were left 24 hours to reproduce generating a new cohort of 1-day-old second-generation aphid nymphs. Adults were then removed, and the weighing process was started on this second generation with weight again being assessed on days 1, 4, 7 and 10. This process was repeated for the third generation of aphids utilising the same methodology as generation 2. Throughout this experiment, constant conditions of 20 ± 2 °C, 60% RH, L:D 16:8 h were maintained.

2.2.7.1. *Brevicoryne brassicae* development weight statistical analysis

Aphid weight was plotted at each time point over the three assessed generations. The maximum aphid weight reached in each generation (day 10 weight) was then plotted as a boxplot. Day 10 weight was then transformed via a square root transformation and analysed initially using a GLM to determine whether accession, generation and replicate were significant factors. Neither generation nor replicate were found to be significant thus an ANOVA was performed evaluating plant accession as the sole factor using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA).

Mean relative growth rate (MRGR) for *B. brassicae* in each of the three assessed generations was calculated using weights (W_1 and W_2) at days 1 (t_1) and 7(t_2) – with

$$\mathrm{MRGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

this being a period between which all *B. brassicae*, regardless of host plant, will remain nymphs and will thus be actively developing/growing (Radford, 1967). MRGR for each generation of *B. brassicae* on each accession was plotted as a boxplot. MRGR results were found to conform to a normal distribution and were thus analysed initially via two GLMs with a normal distribution and identity link function with three factors: plant accession, generation, and replicate. A second GLM was then performed using identical parameters but reducing the model to two factors: plant accession and replicate. A third analysis was then performed via GLMM, utilising a normal distribution and identity link function with plant accession as a sole factor and replicate as a random effect.

2.2.8. Leaf glucosinolate content testing

Ten plants of each accession for leaf glucosinolate analysis were raised to 5-weeks old as described in section 2.2.4. On three occasions, leaves were harvested by detaching leaves and petioles from the plant stem and placing in a paper bag. Leaves were then dried for 48hrs at 50°C (Hotbox Oven with fan size 2 - CHF097 XX2.5, Gallenkamp, Cambridge, UK) and ground to a fine powder using a pestle and mortar.

Quantification of sinigrin and glucoraphanin in each accession was performed using a simplified hot-water extraction and HPLC method adapted from Tsao *et al.* (2002) and Warmington (2014). Glucosinolate extractions were performed in a round bottomed flask. To each flask, ground leaf material (1g) was added along with some anti-bumping granules (VWR International Ltd, UK). To this sample 100ml of boiling RO water was added and the flask transferred to a heating mantle, a reflux condenser was then connected to the flask and the mixture was kept at boiling point for 30 minutes. The mixture was then allowed to cool before being strained through filter paper into sterilised 25ml plastic bottles. HPLC analysis was undertaken using a HP Agilent 110 series system and a UV diode array detector. In this analysis a reverse-phased Zorbax SB- Aq 4.6 x 250 mm 5µm column (Agilent Technologies, USA) was used, with separations occurring at ~25°C at a running pressure of a~45 bar. An eluent of pH 6.75 0.025M ammonium acetate was used with acetonitrile and samples introduced at a pump rate of 1 ml/min and an injection volume of 20µl. After six minutes, the ammonium acetate gradient was increased from 99% to 50%,

followed by a return to a 99% gradient at 21 minutes. Each sample therefore ran for a total of 26 minutes.

Prior to the running of samples and between every three samples, standards of pure sinigrin, glucoraphanin and allyl ITC (Phytolab GmbH & Co, Germany) (Sigma Aldrich, UK) were run to determine peak locations and sizes. Sinigrin and glucoraphanin retention times were found to vary from 4-8 minutes, with peak locations also varying marginally between HPLC runs. Glucosinolate concentrations were determined by calculating area beneath corresponding peaks and using known standard concentrations and the molecular weight of sinigrin and glucoraphanin to convert units to μmol g^{-1}.

2.2.8.1. Leaf glucosinolate content testing - statistical analysis

Neither leaf sinigrin nor glucoraphanin concentration results were found to conform to a normal distribution and no suitable transformation could be identified to normalise data thus analyses were performed via GLMs and GLMMs, each with a gamma distribution and log link function. Owing to multiple comparisons, a Bonferroni correction was applied thus a significance threshold of P=0.025 was used. To evaluate whether sinigrin and glucosinolate content results clustered by species, a scatter plot with mean content of the two glucosinolates on each axis was plotted. Replicate was included in a factor in analyses to evaluate between replicate variation.

2.2.9. *Brevicoryne brassicae* life tables and stepwise linear regression analysis

Data for eight accessions from initial screening, development monitoring, reproduction monitoring, growth monitoring and glucosinolate assessment were summarised in a life table – including means for each measure/calculated variable, calculated 95% confidence interval upper and lower bounds and median where applicable. Of all the parameters measured, each was noted as a potential contributor to *Brassica* partial resistance to *B. brassicae* thus to evaluate which of these parameters was most closely associated with partial resistance, linear regression/multiple linear regression analysis was undertaken. Each variable was plotted against initial screening results in a scatter plot and a linear trendline fitted to visually inspect linear relationship. Linear regression was then undertaken utilising a

stepwise approach to independent variable addition to the model. Scatter plots were initially evaluated and the independent variable demonstrating the strongest linear relationship with the dependant variable was selected as a sole initial independent. The significance of resultant model relative to a null model was then assessed, R^2 of the model evaluated and significance of the variable assessed, identifying therefore whether the independent variable significantly effects the dependant variable and what percentage of variation within the dataset the independent variable explains. Using the residuals from this analysis, all remaining independent variables were plotted against these residuals and visually inspected for linear relationships - with the most linearly related selected for inclusion in linear regression modelling. Through this stepwise process, a linear regression model was constructed which explained the maximum degree of variation within the dataset with the minimum number of significant independent variables– thereby identifying the variables most related/associated with observed *Brassica* partial resistance screening results.

2.2.10. Evaluating the relationship between *Brevicoryne brassicae* development, reproduction, and weight on screened subset of *Brassica* accessions – correlation analysis

To evaluate potential relationships between observed *B. brassicae* development, weight during development and reproduction, three pairwise scatter plots were plotted - linear trendlines were then fitted to each and R^2 of these trendlines assessed. Correlation was then assessed for these three variables through pairwise two-tailed Pearson correlation coefficient analysis using IBM SPSS Statistics for Macintosh, Version 27.0 (IBM Corp, Armonk, NY, USA).

2.3. Results

2.3.1. Antibiosis partial resistance screening

A total of 12 crop-type *Brassica oleracea* accessions, 6 non-*B. oleracea* C-genome *Brassica* accessions and a technical control (*B. oleracea* var. *Gemmifera* 'Doric F1') were evaluated for their relative partial antibiosis resistance against *B. brassicae* infestation (Table 2.2). In this screen the number of aphids produced by each starting nymph ranged from 0-132 with an overall median of 15. Accession medians ranged from 8-25 representing a 3-fold difference between the observed most partially

resistant and most susceptible plant accessions. The number of aphids produced by each starting nymph in the technical control ranged from 1-49 with an overall median of 13 (Figure 2.4).

An initial GLM analysis, assessing whether plant accession was a significant factor was found to offer a significantly improved fit relative to null model (Wald X^2 (18) = 72.629, p=1.61x10^{-8}). Experimental block and replicate were found to be non-significant and were thus excluded from analyses (replicate: Wald X^2 (2) = 72.629, p=1.61x10^{-8}, block: Wald X^2 (5) = 72.629, p=1.61x10^{-8}). This analysis revealed that accession was a significant factor in determining aphids produced per each starting nymph at the end of the experimental period (Wald X^2 (18) = 79.370, p = 1.10x10^{-9}). The most significant differences in accession performance were observed at the extremes, with the most resistant accession (2) performing significantly different to fifteen of the other assessed accession and the technical control. Conversely the most susceptible accession (12) performed significantly differently to fourteen of the other assessed accession and the technical control. Separate GLM analysis however with plant selection criteria as sole factor was found not to offer a significantly improved model fit relative to null model (Wald X^2 (4) = 8.354, p = 0.079) (Figure 2.5).

To assess whether aphid performance varied between *Brassica* species, results were collated according to species and GLM analysis performed. This analysis was found to offer a significantly improved model fit relative to a null model (Wald X^2 (3) = 31.132, p = 7.97x10^{-7}). *Brassica* species was therefore identified as a significant factor for aphid performance within the scope of these eighteen evaluated accessions (Wald X^2 (3) = 35.766, p = 8.39x10^{-8}) - with post hoc analyses revealing that *Brassica villosa* subsp. *tinei* and *Brassica cretica* both significantly reducing the number of aphids produced by each starting nymph relative to *B. oleracea* while *Brassica macrocarpa* performed indistinguishably to *B. oleracea*. Owing to *B. villosa* subsp. *tinei*, *B. macrocarpa* and *B. cretica* being represented by only 1, 2 and 3 accessions respectively however the significance of these results may only be relevant to the selected plants and not a general property of these species as a whole.

Finally, to assess whether interspecific *B. oleracea* variation significantly affected *B. brassicae* performance, accession results for *B. oleracea* accessions were collated into their respective cultivar groups. Taking account of the Bonferroni correction, this model did not offer an improvement relative to a null model (Wald X^2 (6) = 14.724, p = 0.023) therefore within this analysis and the scope of the twelve evaluated accessions representing seven cultivar groups, *B. oleracea* cultivar group was found not to be a significant factor.

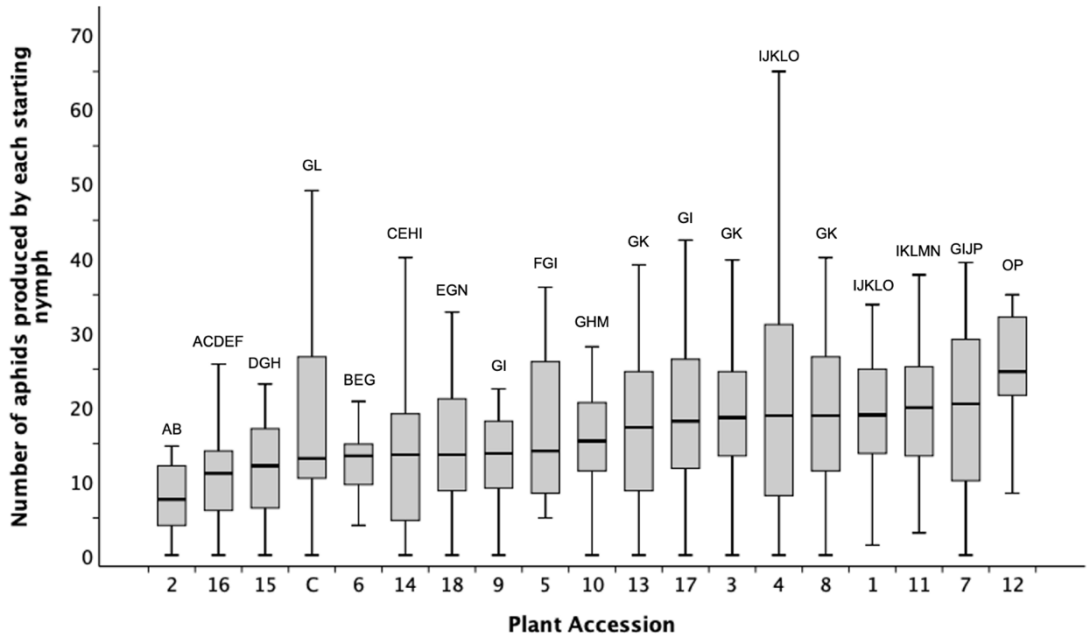


Figure 2.4: Partial-resistance antibiosis screening results for *Brevicoryne brassicae* against the 18 selected *Brassica* accessions and technical control (C, *Brassica oleracea* var. *Gemmifera* 'Doric F1') ranked according to the median number of aphids produced by each starting nymph. Plant accession was found to be a significant factor influencing observed aphid numbers ($P=1.10 \times 10^{-9}$). Between accessions, letters denote GLM post-hoc test results, with accessions not sharing any letters significantly different in their observed partial resistance (=<0.05).

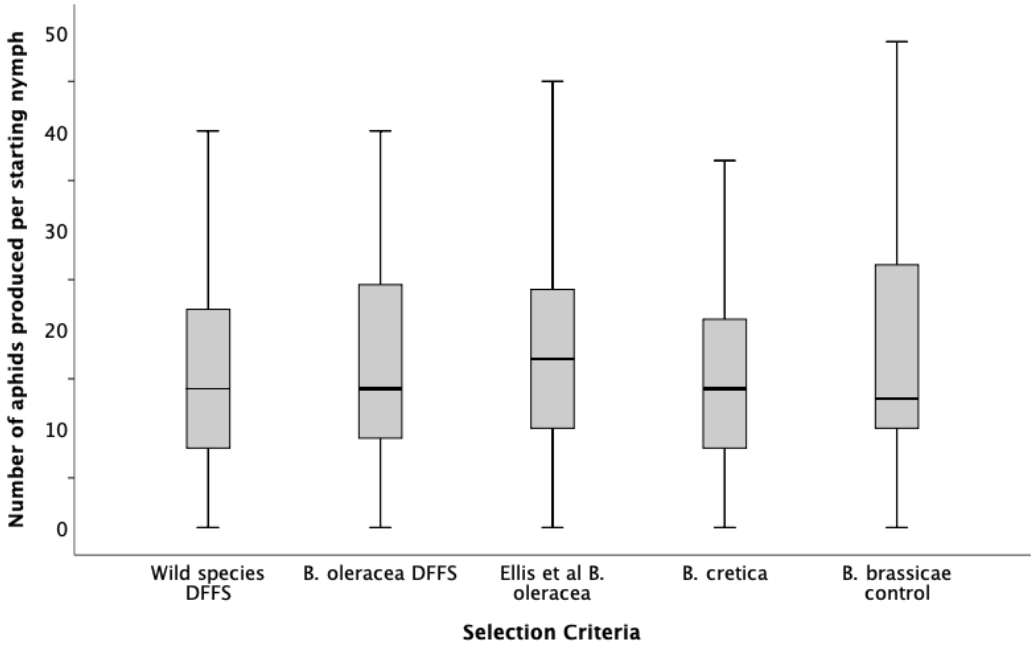


Figure 2.5: Partial-resistance antibiosis screening results for *Brevicoryne brassicae* collated into pre-selection criteria. Including selection criteria as a sole factor, GLM analysis revealed no significant differences between selection criteria.

2.3.2. *Brevicoryne brassicae* development studies

2.3.2.1. *Brevicoryne brassicae* development time

A subset of 8(/18) accessions were assessed for their effect upon overall and individual instar aphid development times (Figures 2.6 and 2.7). Overall *B. brassicae* development time to adulthood across all accessions ranged from 4.5-14.5 days with a median of 9.5 days. The overall duration of instars ranged from 0.5-3.5 days for instar 1, 1.0-4.0 days for instar 2, 1.0-4.0 days for instar 3 and 0.5-4.0 days for instar 4 with medians of 1.5, 3.0, 2.0 and 2.0 days respectively. Experimental replicate was found not to be a significant factor ($F(2)=0.560$, $p=0.808$) thus was excluded from all development time analyses both overall and for specific instars. There was a significant difference in overall development time, with different plant accessions having a significantly different effect ($F(8)=2.095$, $p=0.043$) (Figure 2.6). Tukey post-hoc analysis revealed this significance stemmed from the difference between plant accession 16 and all other accessions aside from accession 2 – with *B. brassicae* on accession 16 requiring an average of 11.25 days relative to averages ranging from 8.90-9.75 days for all other accessions – thus extending total development time by a minimum of 1.5 days. Analysis of each instar duration revealed that only instar 4 duration appeared significantly different between accessions- with a GLM including instar 4 as a sole factor offering a significant improvement over null model (Wald X^2 (8) = 22.993, p = 0.003), therefore confirming plant accession as a significant factor influencing instar 4 duration (Wald X^2 (8) = 26.736, p = 0.003). Conversely, where plant accession was included as a sole GLM factor assessing the impact of resistance in these accessions upon instars 1, 2 and 3 duration, all three resulting models were found to offer no significant improvement over a null model (Instar 1: Wald X^2 (8) = 6.493, p = 0.592) Instar 2: Wald X^2 (8) = 14.845, p = 0.062) (Instar 3: Wald X^2 (8) = 4.304, p = 0.829), therefore indicating that plant accession was insignificant in determining the duration of these three life stages across the *Brassica* accessions evaluated (Figure 2.7). Post hoc GLM analysis revealed that accessions 2, 16, 17 and the control significantly extended the duration of instar 4 relative to all other accessions. Instar 4 duration between all accessions was found to range from 1.2-2.5 days, with accession 16

offering the most significant slowing of this instar and resulting in a duration double that of accession 12 (Figure 2.7).

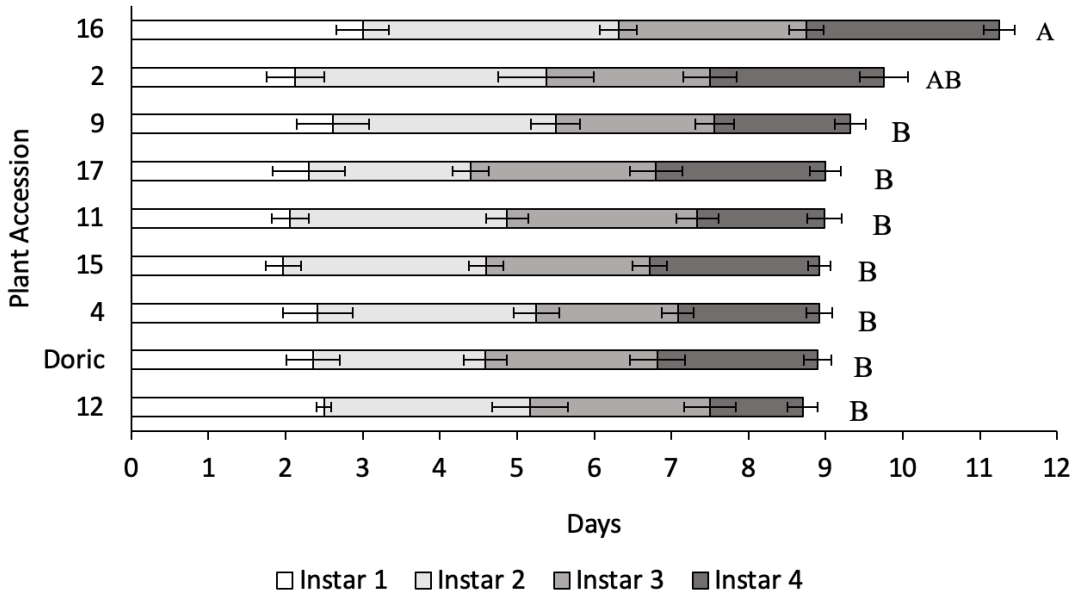


Figure 2.6: *Brevicoryne brassicae* Instar development times on a sub-set of 8 *Brassica oleracea*/C-genome *Brassica* species and the technical control. Overall bar length corresponds to overall development time from birth to adulthood. Individual stacked bars correspond to duration of individual aphid instars. ANOVA analysis revealed plant accession was a significant factor influencing overall aphid development time (p=0.043). Aphids reared on accession 16 demonstrated the longest overall development time, post-hoc analysis revealing that overall development time on accession 16 was significantly extended relative all other *Brassica* accessions aside from 17. Accessions sharing no letters were found to significantly differ in their effect upon overall *B. brassicae* development time to adulthood as indicated by post-hoc ANOVA tests (p=<0.05).

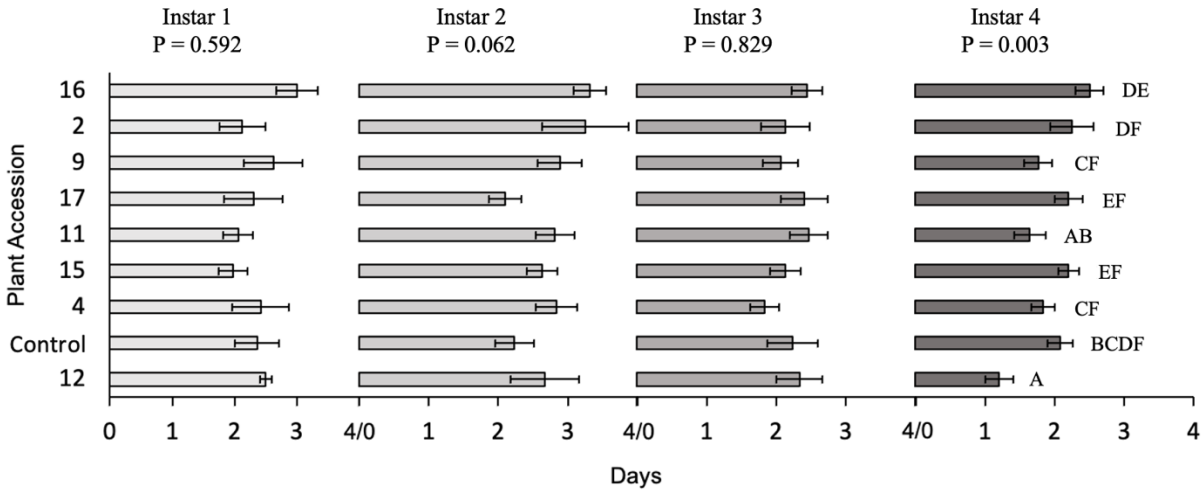


Figure 2.7: *Brevicoryne brassicae* Instar development times on a sub-set of 8 *Brassica oleracea*/C-genome *Brassica* species and the technical control. Overall bar length corresponds to development time/duration of each aphid instar. GLM analysis revealed that plant accession was a significant factor influencing instar development time for only instar 4 (p=0.003). Accessions sharing no letters were found to significantly differ in their effect upon *B. brassicae* instar duration as indicated by post-hoc ANOVA tests (p=<0.05).

2.3.2.2. *Brevicoryne brassicae* development weight

For all evaluated accessions weight increased consistently from day 1-3-7-10 however significant variation in weight was noted between aphids on most accessions – particularly on days 7 and 10 (Table 2.3; Figure 2.8). For maximum weight reach (day 10), the two *B. cretica* accessions 16 and 17 appeared to have the strongest effect - limiting average day 10 weight across all three generations to 16.4 and 17.8 $x10^{-5}$g respectively relative to weights for all other accessions which ranged from 19.9-25.1 $x10^{-5}$g – with accession 12 resulting in this highest average aphid weight. For MRGR (between days 1 and 7) fewer notable differences were identified than for maximum weight (Table 2.4). Accession 17 has the most limiting effect, with an average MRGR of 0.23 closely followed by accessions 11, 9 and 15 - which also resulted in an averaged MRGRs of 0.23. For all other accessions, MRGR ranged from 0.26-0.31, with accession 4 resulting in the highest calculated MRGR (Figure 2.10). Accession 17 therefore had the most significant effect across both maximum weight reached and MRGR.

For the maximum *B. brassicae* weight results (day 10), two GLMs were performed. In an initial model, GLM offered a significantly improved model fit relative to a null model (Wald X^2 (12) = 46.869, p = $5x10^{-6}$). Within this model plant accession was found to be a significant factor (Wald X^2 (8) = 44.736, p = $4.13x10^{-7}$). Generation and replicate however were found to be insignificant factors (generation: Wald X^2 (2) = 5.527, p = 0.063) (replicate: Wald X^2 (2) = 0.924, p = 0.630). This initial GLM model had a model fit score (AIC) of 461.537. Owing to both generation and replicate proving insignificant, GLM analysis was repeated excluding these two factors. This analysis was found to offer a significant improvement over null model (Wald X^2 (8) = 40.474, p = $3x10^{-6}$) and offered a better model fit (AIC), with a score of 459.932. Plant accession was found to be a significant factor determining the maximum (day 10) weight reached by *B. brassicae* (Wald X^2 (8) = 44.135, p = $5.36x10^{-7}$). Tukey post-hoc tests revealed significant differences in day 10 weight between the accessions at the extremes – accession 16 and 17 were found to significantly limit maximum aphid weight relative to accessions 11 and 12 with accession 16 being significantly different to all accessions aside from 17, 9 and 15 – with these results therefore confirming initial conclusions drawn through visual inspection of results.

To analyse MRGR results, two GLMs and a GLMM were performed. Initial GLM analysis (including plant accessions, generation, and replicate as factors) offered a significant improvement over a null model (Wald X^2 (12) = 42.307, p = $3x10^{-5}$). This analysis revealed that generation was not a significant factor in determining MRGR (Wald X^2 (2) = 2.842, P=0.241) however both accession and replicate were found to be significant (accession: Wald X^2 (8) = 33.215, P=5.6 x 10^{-5}) (replicate: Wald X^2 (8) = 33.215, p=0.010). This GLM offered a model fit (AIC) score of -445.12. Owing to generation proving insignificant, a second GLM was undertaken excluding generation as a factor. This second GLM was found to also offer a significant improvement over a null model (Wald X^2 (10) = 39.482, P=2.1 x 10^{-5}), with an improved model fit score (AIC) of -446.29. In this second analysis however, replicate remained a significant factor alongside plant accession (replicate: Wald X^2 (2) = 8.727, p=0.013) (accession: Wald X^2 (8) = 33.393, p=$5.2x10^{-5}$). Through the inclusion of an interaction term in this model (accession x replicate), it was confirmed that there existed no interaction between replicate and accession (Wald X^2 (16) = 22.764, P=0.120).

To account for the added variation introduced by replicate, a GLMM was performed including plant accession as a factor and replicate as a random effect. This model was found to offer a significant improvement over a null model (F (8) = 4.004, p=$1.82x10^{-4}$). Within this model plant accession was confirmed as a significant factor influencing *B. brassicae* relative growth rate (P=$1.82x10^{-4}$). This model however offered a marginal decline in model fit (AIC=-405.25) thus previous model was noted as the best fitting and preferred model. Across both maximum aphid weight and MRGR accessions 17, 16, 9 and 15 offered the most consistent and significant declines in both metrics – with these accessions being in the top 5 accessions most significantly reducing both metrics.

Table 2.3: Minimum, maximum, and median *Brevicoryne brassicae* weights observed across all assessed accessions.

Aphid Generation	Value	Weight ($x10^{-5}$ g)			
		Day 1	Day 4	Day 7	Day 10
Generation 1	Min	1	1	4	11
	Max	8	14	28	47
	Median	3	7	15	21
Generation 2	Min	1	1	4	5
	Max	6	12	29	38
	Median	3	6	15	19
Generation 3	Min	1	3	7	12
	Max	7	16	32	39
	Median	3	7	15	20

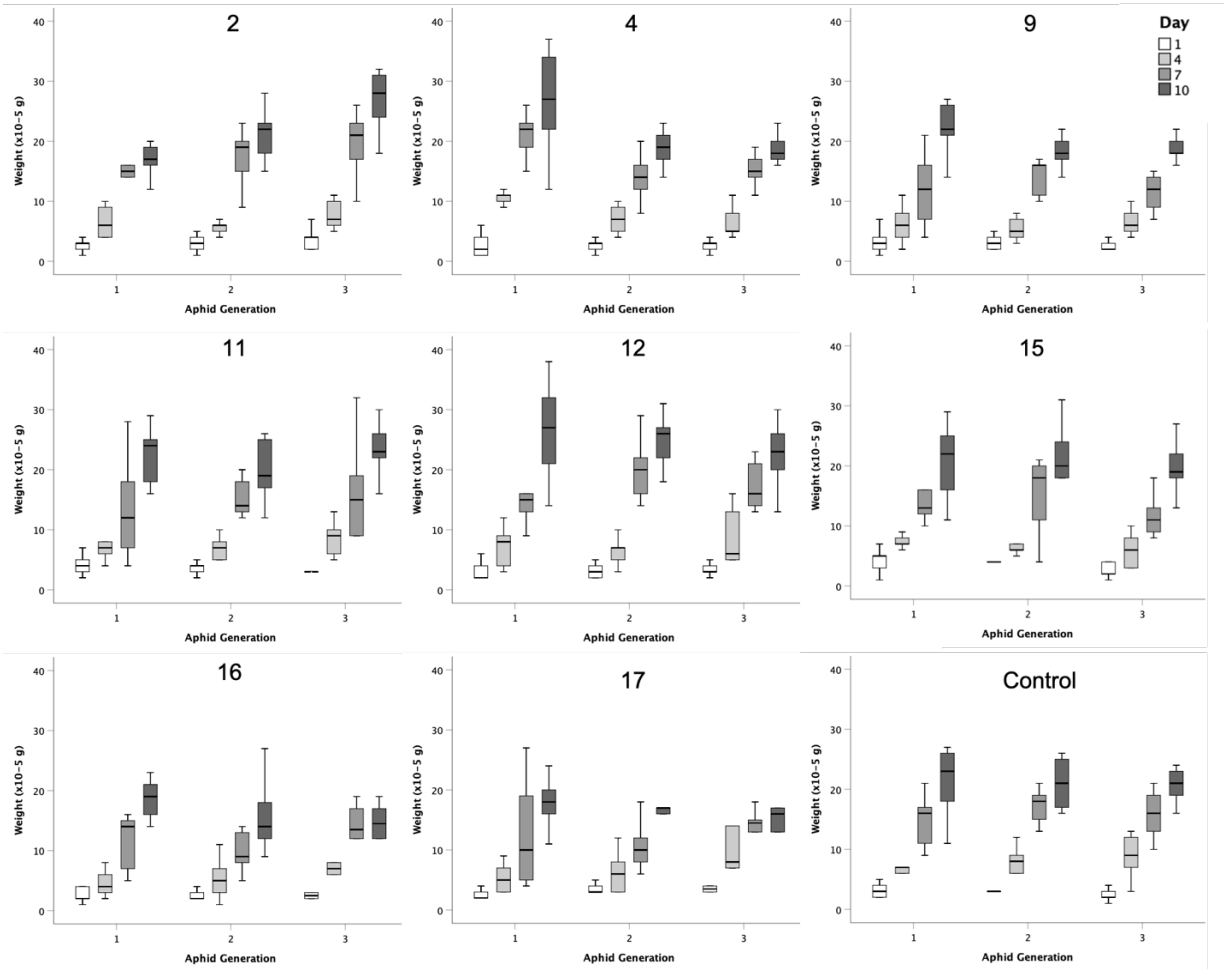


Figure 2.8: *Brevicoryne brassicae* development weight results for the selected subset of 8 *brassica* accessions and technical control. Weight was assessed on days 1, 4, 7 and 10 across three aphid generations on the same plants. Aphids were weighed destructively in batches of 5 aphids.

Table 2.4: Minimum, maximum, and median *Brevicoryne brassicae* MRGRs observed across all assessed accessions.

Aphid Generation	Value	MRGR
Generation 1	Min	0.19
	Max	0.34
	Median	0.25
Generation 2	Min	0.19
	Max	0.31
	Median	0.24
Generation 3	Min	0.23
	Max	0.31
	Median	0.27

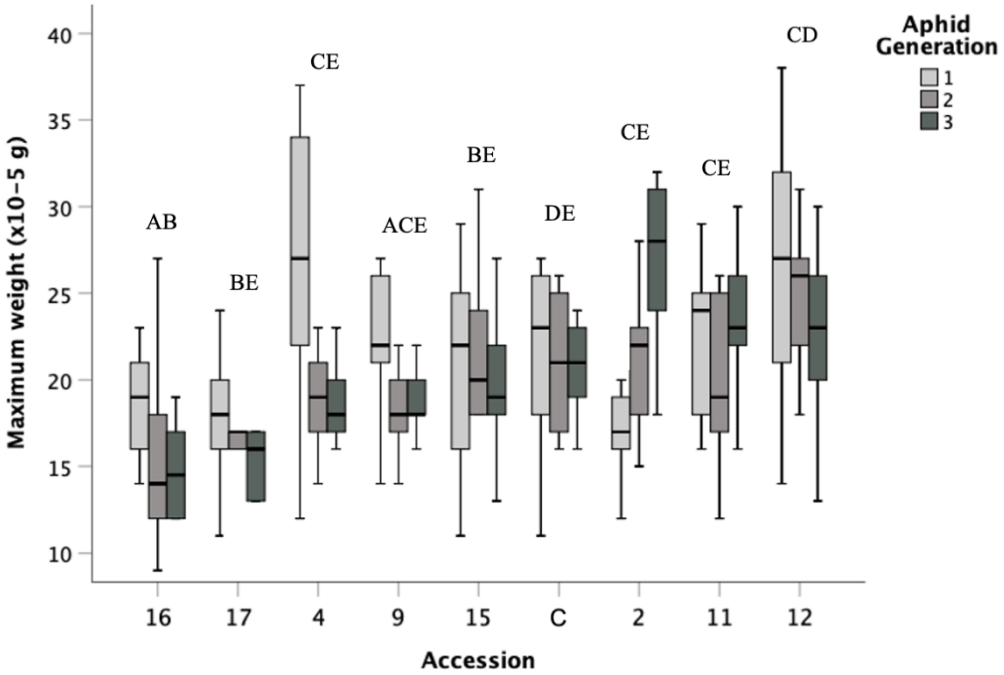


Figure 2.9: Maximum *Brevicoryne brassicae* weight reached (day 10) for each *Brassica* accession across each of the three assessed aphid generations. ANOVA analysis revealed a significant difference in maximum weight reached between accessions ($p=5.36x10^{-7}$) however no overall differences were observed in maximum aphid weight reached between aphid generations. Accessions sharing no letters on this figure were identified through post-hoc analysis as performing statistically differently in their effect on maximum weight ($p=<0.05$).

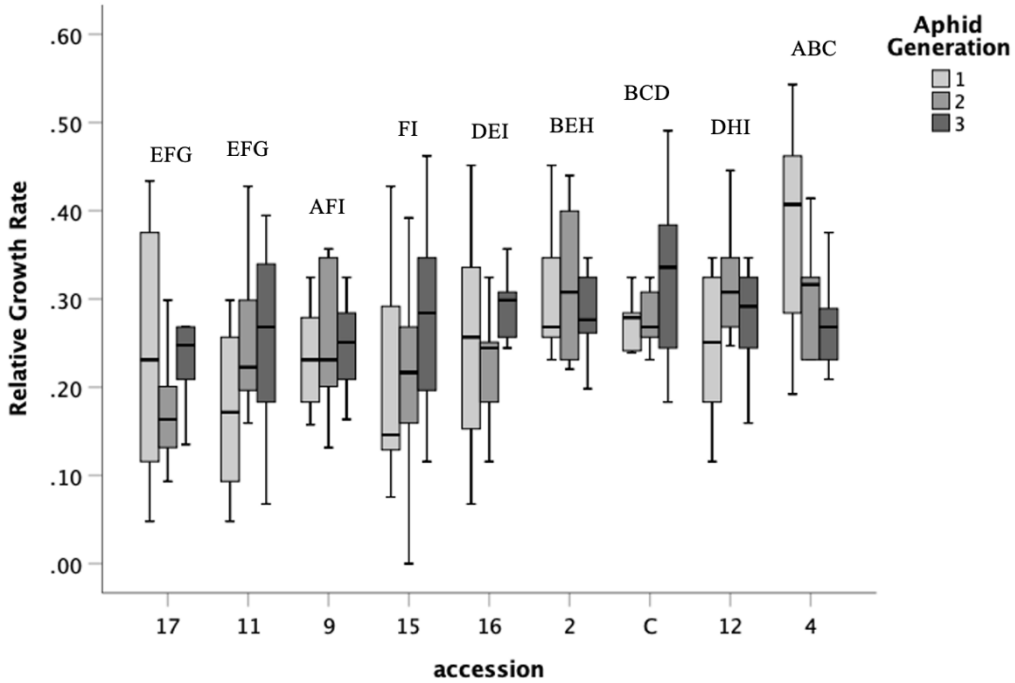


Figure 2.10: Relative growth rate of *Brevicoryne brassicae* reared on selected *Brassica* accessions across three separate generations. GLMM analysis revealed a significant different in relative growth rate of aphids between accessions ($p=1.82 \times 10^{-4}$) but not overall between aphid generations. Accessions sharing no letters on this figure were identified through post-hoc analysis as performing statistically differently in their effect on MRGR (p=<0.05).

2.3.2.3. *Brevicoryne brassicae* reproduction

GLM analysis for daily larviposition with plant accession as a sole factor was found to be a significantly improved model relative to null (Wald X^2 (8) = 25.086, P=0.002), with plant accession therefore proving a significant factor influencing both daily larviposition (Wald X^2 (8) = 24.313, p=0.002). Replicate was not a significant factor in this model (Wald X^2 (2) = 0.320, p=0.852), nor did it interact with accession (Wald X^2 (16) = 0.192, p=0.661) and was thus excluded from analysis. GLM analysis for gross larviposition with plant accession as a sole factor was also found to be a significantly improved model relative to null (Wald X^2 (8) = 18.821, P=0.016), with plant accession therefore proving a significant factor influencing gross larviposition (Wald X^2 (8) = 20.885, p=0.007). Replicate was not a significant factor in this model (Wald X^2 (2) = 2.717, p=0.257), nor did it interact with accession (Wald X^2 (16) = 17.862, p=0.332) and was thus excluded from analysis.

Daily larviposition across all accessions ranged from 0-14 nymphs with a mean of 2.20 and SEM of 0.055 (Figure 2.11). Gross larviposition across all accessions

ranged from 8-37 nymphs with a mean of 17.62 and SEM of 0.632 (Figure 2.12). For daily and gross larviposition a near identical trend was seen within the data with respect to which accessions had to strongest and weakest effect, with significant differences in reproduction primarily being observed between the accessions having the strongest and weakest effect upon reproduction. *B. cretica* accessions 16 and 17 had the most significant negative effect overall, reducing daily larviposition to a median for both accessions of 2 nymphs and mean of 1.5 and 1.8 respectively while for gross larviposition these accessions reduced larviposition to a median of 12.5 nymphs each and a mean of 12.8 and 14.9 respectively. The most poorly performing accession overall was the *B. villosa* subsp. *tinei* accession 2. This accession led to a median daily larviposition of 3 nymphs with a mean of 2.5 while for gross larviposition accession 2 resulted in a median of 22 nymphs and mean of 23.2 nymphs. For daily larviposition, no significant difference in larviposition was identified even between the accessions with the strongest and least effect on reproduction (C and 11 respectively). Similarly for gross larviposition no significant difference between all screened *B. oleracea* accessions were noted even between the best and worst performing accessions (C and 4 respectively).

ANOVA analysis revealed that plant accession had a significant influence upon intrinsic rate of increase and population doubling time of *B. brassicae* (Rm: $F(8)=4.253$, $p=4.70\times10^{-4}$) (DT: $F(8)=5.253$, $p=6.10\times10^{-5}$). Replicate also proved a significant factor in both models (Rm: $F(2)=14.244$, $p=<0.001$) (DT: $F(2)=13.548$, $p=0.001$) however in both cases no significant interaction was seen between plant accession and replicate (Rm: $F(16)=18.099$, $p=0.318$) (DT: $F(16)=16.080$, $p=0.447$). Tukey post hoc analyses revealed that the most significant differences in both intrinsic rate of population increase and population doubling time were again seen between accessions at the extremes. Intrinsic rate of population increase across all accessions ranged from 0.15-0.39 with a mean of 0.26 and SEM of 0.006 (Figure 2.13). Population doubling time across all accessions ranged from 1.79-4.58 days with a mean of 2.78 and SEM of 0.066 (Figure 2.14). *Brassica cretica* accessions 16 and 17 were found to have the most significant limiting effect upon intrinsic rate of population, resulting in a mean r_m of 0.19 and 0.23 respectively with these being significantly lower than *B. oleracea* accessions 9, 11 and 4 (0.27, 0.27 and 0.29 respectively) as well as the *B. villosa* subsp. *tinei* accession 2 (0.29). Between all

other accessions no significant differences in r_m were observed. A similar trend was seen for population doubling time results, with *B. cretica* accessions 16 and 17 most significantly increasing population doubling time to a mean of 3.6 days and 3.2 days respectively. These doubling times were both found to be significantly longer that population doubling times for *B. oleracea* accession 4 (2.39 days) and *B. villosa* subsp. *tinei* accession 2 (2.39 days).

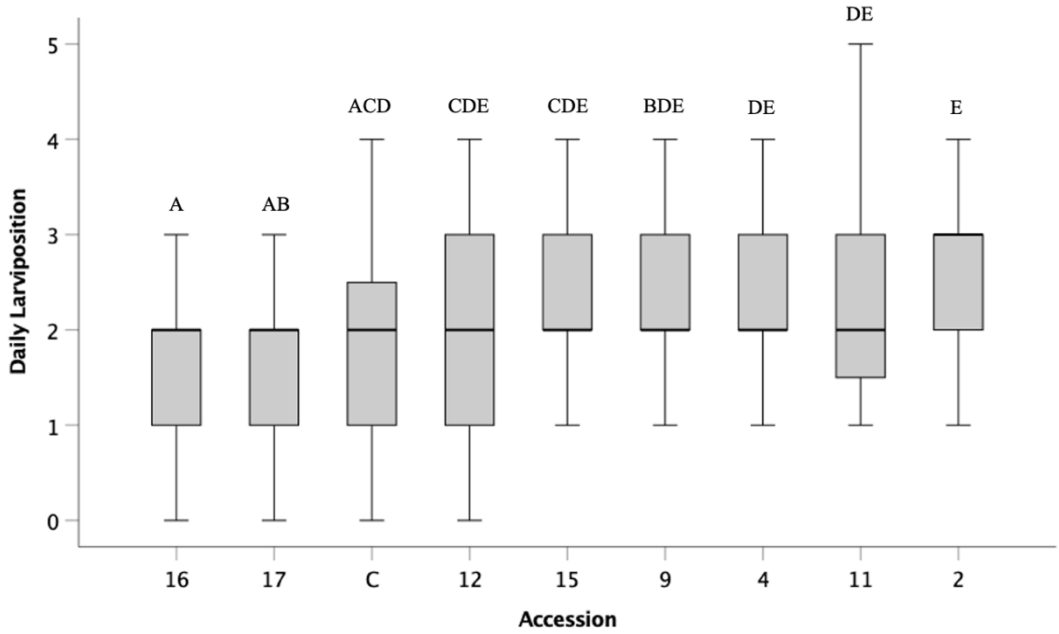


Figure 2.11: Daily larviposition of *Brevicoryne brassicae* when reared on selected plant accessions. GLM analysis revealed a significant difference in aphid daily larviposition between accessions (P=0.002). Accessions sharing no letters in this figure indicates a significant difference in the effect of this accessions upon daily *B. brassicae* larviposition indicated by GLM post-hoc analysis (p=<0.05).

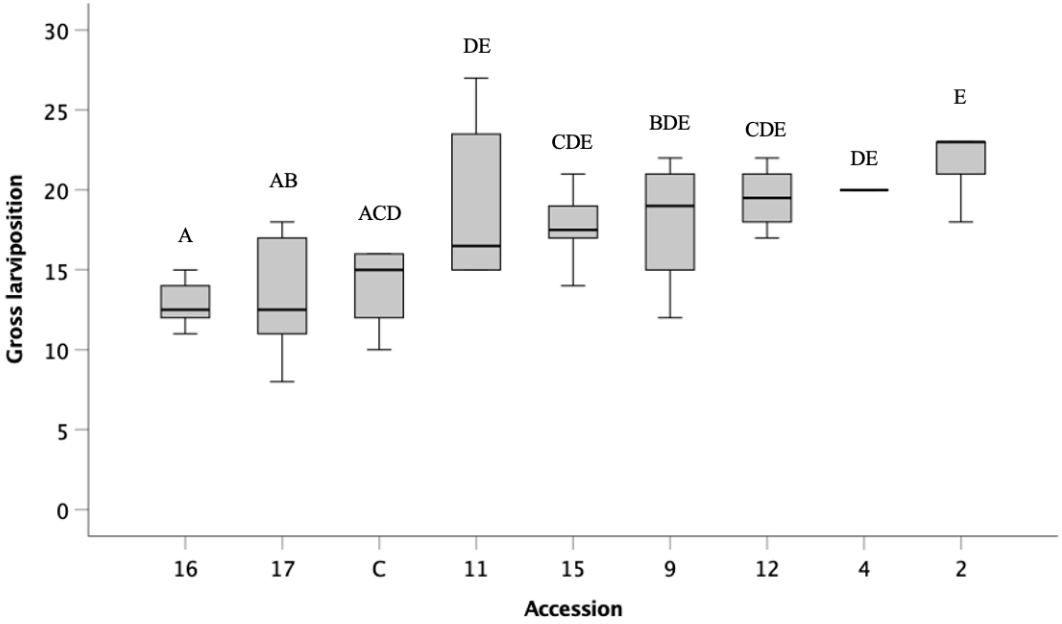


Figure 2.12: Gross larviposition of *Brevicoryne brassicae* when reared on selected plant accessions. GLM analysis revealed a significant difference in gross larviposition between accessions (p=0.007). Accessions sharing no letters in this figure indicates a significant difference in the effect of this accessions upon gross *B. brassicae* larviposition indicated by GLM post-hoc analysis (p=<0.05).

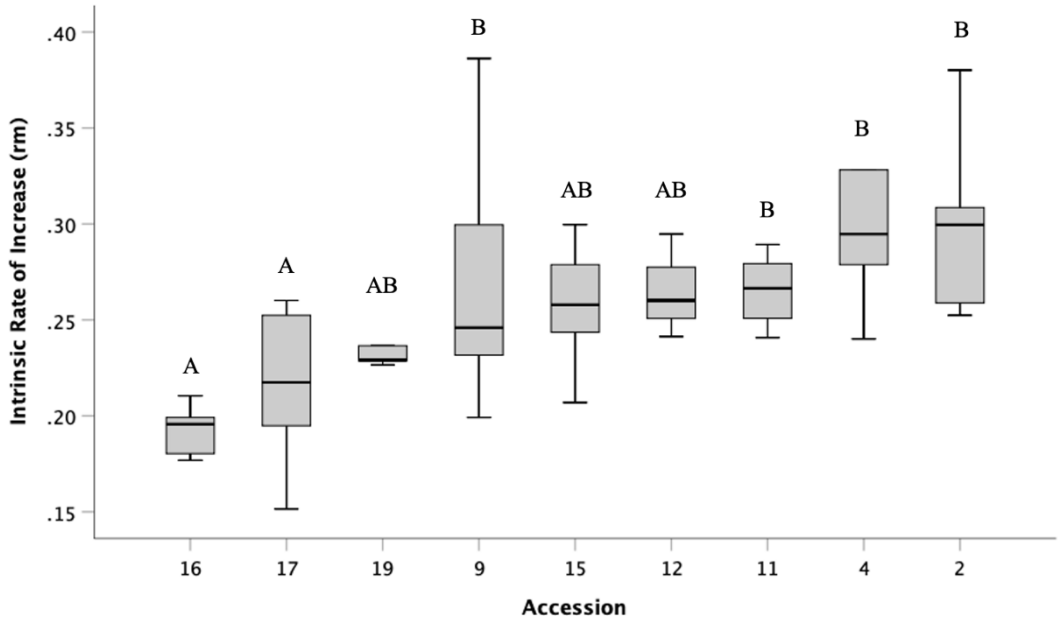


Figure 2.13: Intrinsic rate of increase of *Brevicoryne brassicae* reared on selected plant accessions across three separate generations. ANOVA revealed a significant different in intrinsic rate of increase between accessions ($p=4.70x10^{-4}$). Accessions sharing no letters in this figure indicates a significant difference in the effect of this accessions upon *B. brassicae* intrinsic rate of population increase indicated by post-hoc ANOVA tests (p=<0.05).

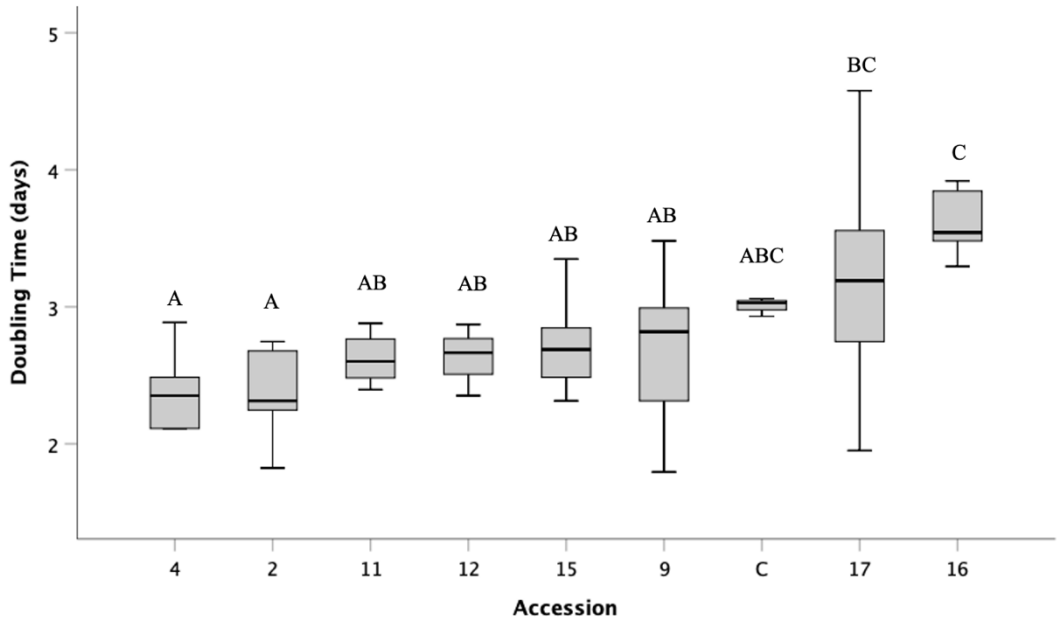


Figure 2.14: Population doubling time of *Brevicoryne brassicae* reared on selected plant accessions across three separate generations. ANOVA (of Log_{10} of doubling time results) revealed a significant difference in population doubling time between accessions ($p=6.10x10^{-5}$). Accessions sharing no letters in this figure indicates a significant difference in the effect of this accessions upon *B. brassicae* population doubling time increase indicated by post-hoc ANOVA tests (p=<0.05).

2.3.3. Glucosinolate content analysis

Sinigrin leaf concentrations across all accessions ranged from 0.39-36.62 µmol g^{-1} with a median of 4.90 µmol g^{-1} (Figure 2.15). GLM analysis of sinigrin concentrations across all accessions offered a significantly improved model relative to null (Wald X^2 (10) = 78.851, P=8.43 x 10^{-13}) with a model fit score (AIC) of 99.572. This GLM revealed that plant accession was a significant factor influencing sinigrin content (Wald X^2 (8) = 651.274, p=<0.001). Replicate was not found to be a significant factor influencing observed sinigrin content (Wald X^2 (2) = 0.548, p=0.760) nor was any interaction between *Brassica* accession and replicate identified (Wald X^2 (16) = 1.027, p=1.000).

Analysis was thus repeated using a GLMM including replicate as a random effect. This GLMM offered a significant improvement over both a null model (F(8)=43.118, p=2.18 x 10^{-9}) and over the previous GLM model, with an improved model fit score (AIC) of 21.858. This GLMM analysis confirmed plant accession was a significant factor influencing observed sinigrin content (F(8)=43.118, p=2.18 x 10^{-9}) (Figure 2.15). *Brassica cretica* accessions 16 and 17 alongside *B. villosa* subsp. *tinei* accession 2 were found to have the highest leaf concentrations of sinigrin, with mean concentrations of 16.0, 11.8 and 20.1 µmol g^{-1} respectively. These were significantly higher than all assessed *B. oleracea* accessions which had mean sinigrin concentrations ranging from 0.4-6.2 µmol g^{-1} with accession 4 having this lowest concentration and accession 11 the highest. Between *B. oleracea* accessions only, a significant difference in sinigrin content was seen for accession 4 relative to all other *B. oleracea* accessions. Sinigrin concentrations results strongly indicated a species level effect. GLM analysis evaluating this offered a significant improvement over a null model (Wald X^2 (2) = 22.332, p=1.4x10^{-5}), revealing that *Brassica* species was a significant factor influencing observed sinigrin content within the scope of the evaluated plant accessions (Wald X^2 (2) = 31.274, p=1.62x10^{-7}) (Figure 2.16). Post hoc testing revealed that *B. cretica* and *B. villosa* subsp. *tinei* had significantly higher leaf sinigrin concentrations, with means of 13.9 and 20.1 µmol g^{-1} relative to a mean concentration of 3.8 µmol g^{-1} across evaluated *B. oleracea* accessions (Figure 2.16).

Glucoraphanin leaf concentrations across all accessions ranged from 0.13-74.18 μmol g^{-1} with a median of 1.90 μmol g^{-1} (Figure 2.17). GLM analysis of glucoraphanin concentrations across all accessions offered a significantly improved model relative to a null model (Wald X^2 (8) = 1135.373, p=<0.001), with this model revealing that plant accession was identified as a significant factor influencing glucoraphanin content (Wald X^2 (8) = 2645.133, p=<0.001). Replicate conversely was not found to be a significant factor in this model (Wald X^2 (2) = 0.018, p=0.991) and furthermore, no interaction was identified between *Brassica* accession and experimental replicate (Wald X^2 (16) = 0.870, p=0.998). *Brassica cretica* accessions 16 and 17 were found to have the highest sinigrin concentrations, with means of 71.2 and 52.4 μmol g^{-1} respectively, significantly higher than all evaluated *B. oleracea* accessions and the single evaluated *B. villosa* subsp. *tinei* accessions – with glucoraphanin concentrations among these accessions ranging from 0.2-2.3 μmol g^{-1}. Significant differences were however still noted among these accessions, with *B. villosa* subsp. *tinei* accession 2 and *B. oleracea* accession 4 being found to have significantly higher glucoraphanin concentrations (2.3 and 2.2 μmol g^{-1}) relative to the *B. oleracea* accessions 15, 12, 9 and the control which had mean concentrations of 0.2, 1.0 and 1.4 μmol g^{-1} respectively. Glucoraphanin concentration results strongly indicated a species level effect. GLM analysis evaluating this offered a significant improvement over null model (Wald X^2 (2) = 63.388, p=1.44×10^{-15}), revealing that *Brassica* species was a significant factor influencing glucoraphanin content (Wald X^2 (2) = 183.349, p=<0.001) (Figure 2.18). Post hoc analysis identified that *B. cretica* demonstrated a significantly higher concentration of glucoraphanin of 61.8 μmol g^{-1} relative to the significantly lower concentrations of 2.3 μmol g^{-1} for *B. villosa* subsp. *tinei* and 1.4 μmol g^{-1} for *B. oleracea* (Figure 2.18).

Having identified a possible species-level effect upon both sinigrin and glucoraphanin concentration, this was further evaluated by plotting a scatter plot of sinigrin concentration against glucoraphanin concentration for each accession (Figure 2.19). This plot demonstrated clear clustering of accessions according to species with respect to their relative sinigrin and glucoraphanin contents further indicating that *Brassica* species was among the most significant factor influencing glucosinolate content overall.

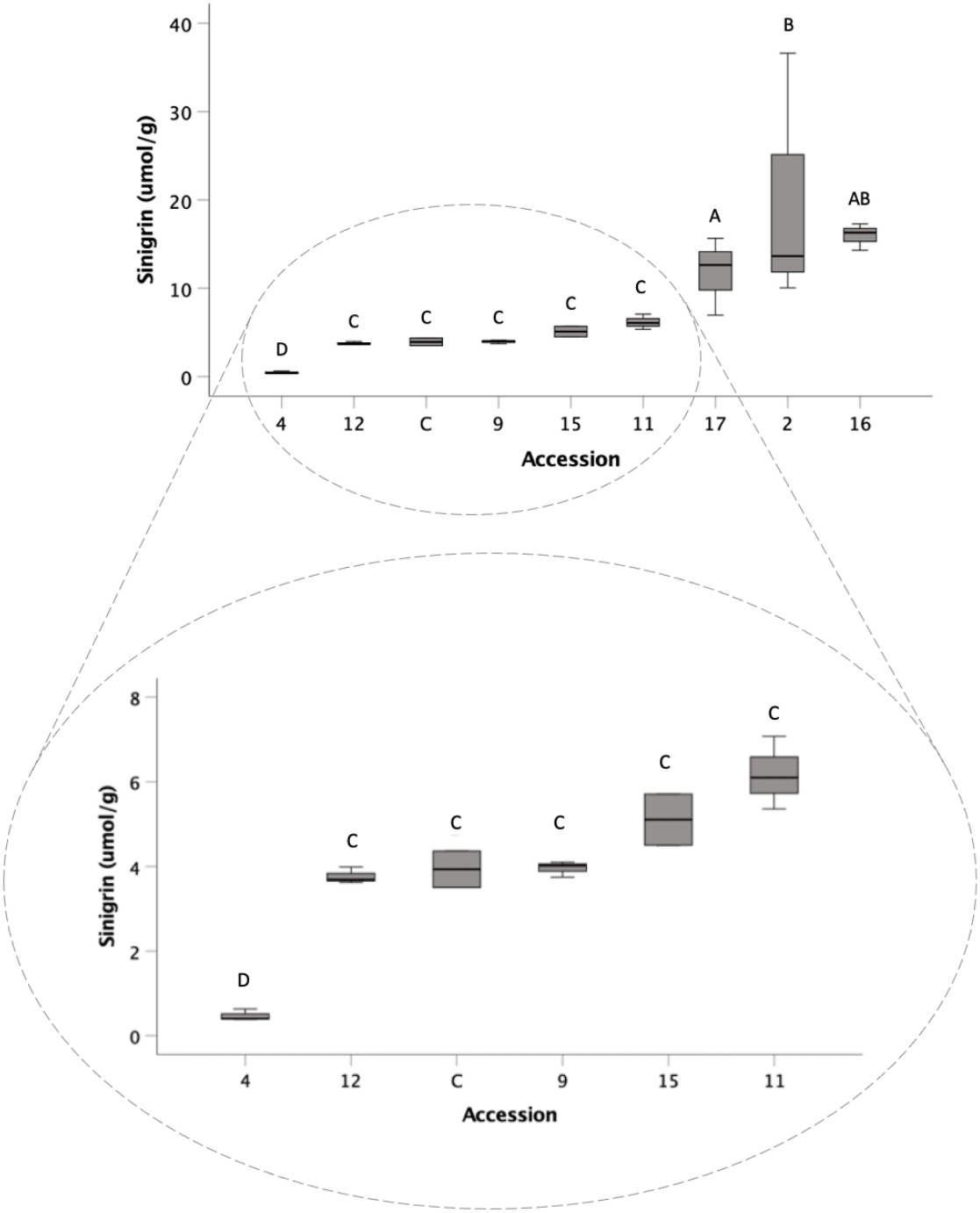


Figure 2.15: Sinigrin concentration in dried leaves of a subset of *Brassica* accession screened for partial resistance to *Brevicoryne brassicae*. GLMM analysis revealed a significant difference in sinigrin content between accessions ($p = 2.18 \times 10^{-9}$).

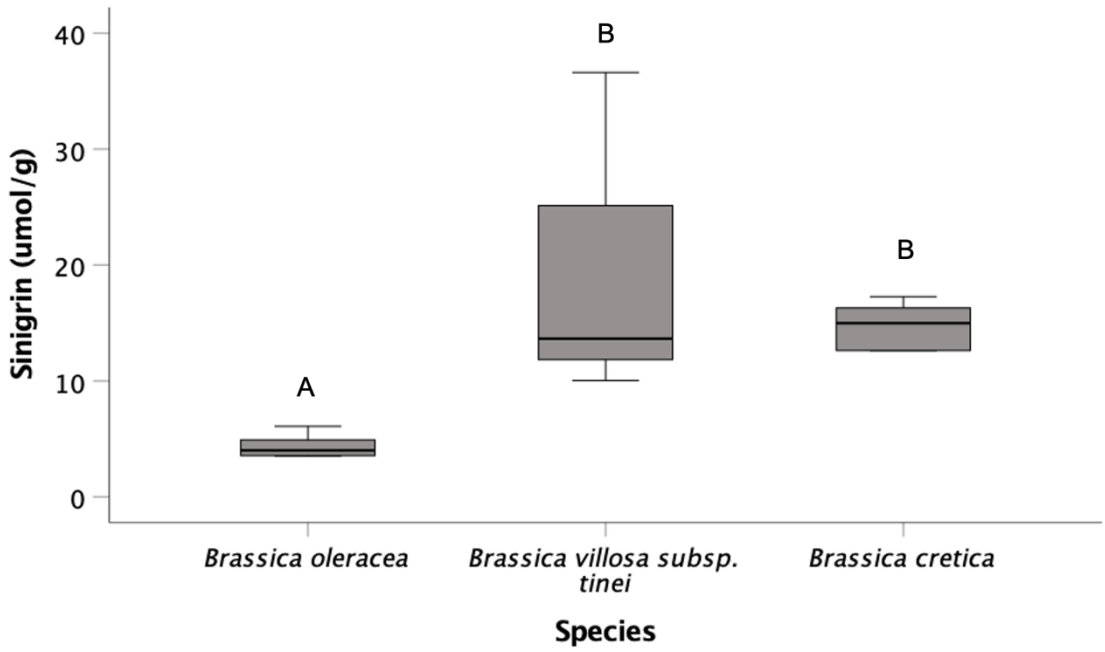


Figure 2.16: Sinigrin concentration in dried leaves of a subset of *Brassica* accession collated into species. GLM analysis revealed a significant difference in sinigrin content between *Brassica* species ($p = 1.62x\ 10^{-7}$).

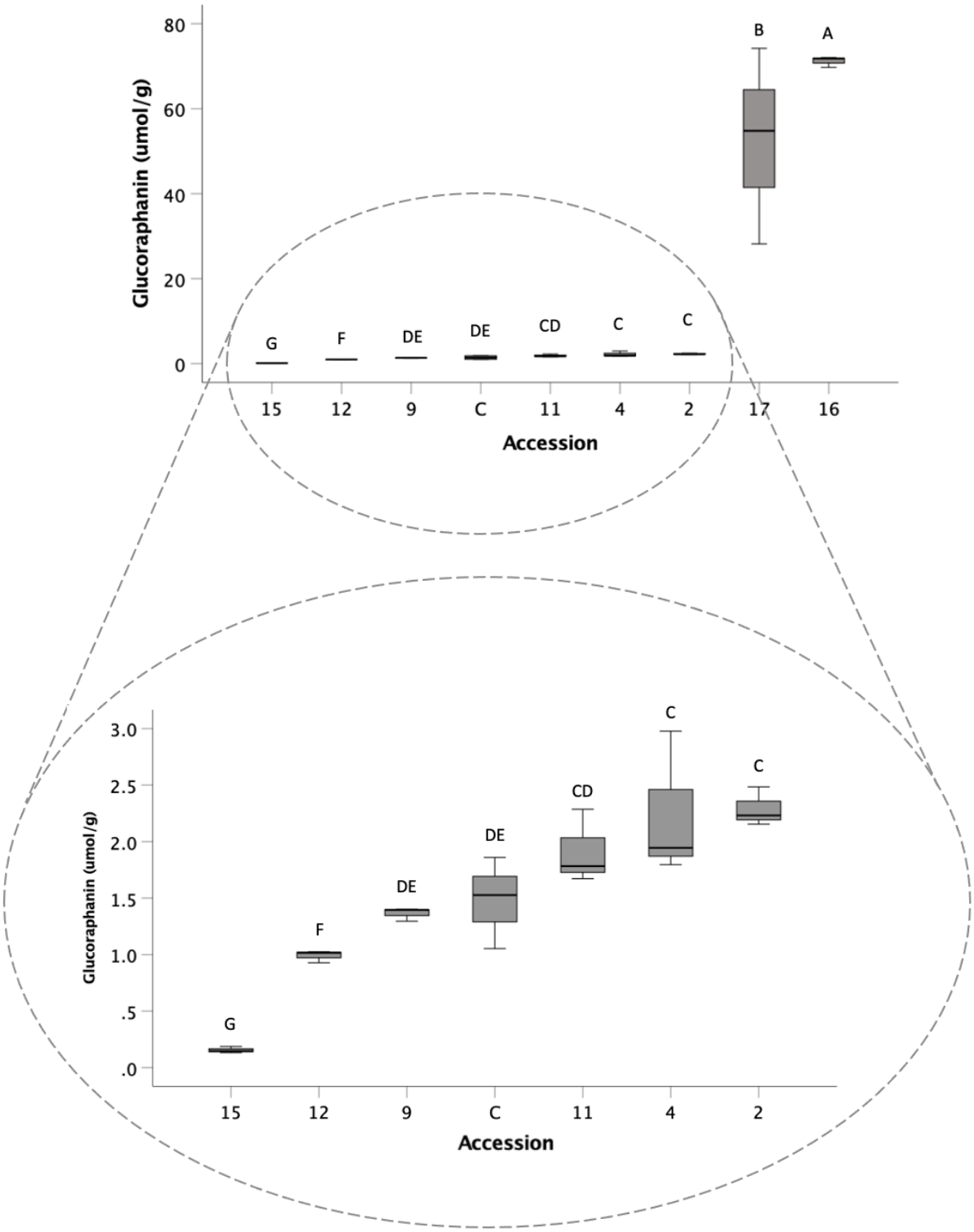


Figure 2.17: Glucoraphanin concentration in dried leaves of a subset of *Brassica* accession screened for partial resistance to *Brevicoryne brassicae*. GLM analysis revealed a significant difference in glucoraphanin content between accessions (p = <0.001).

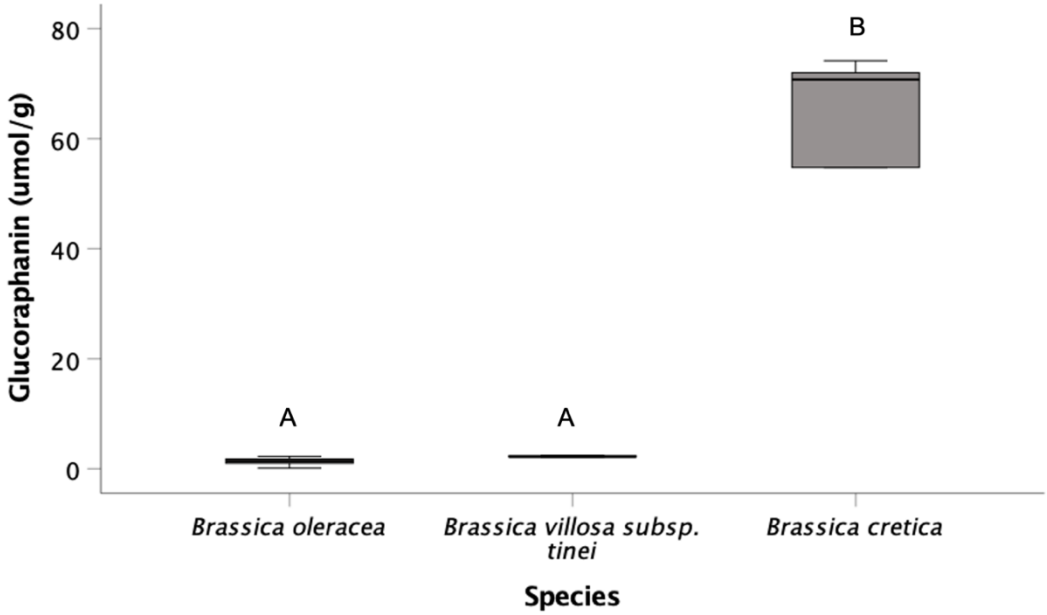


Figure 2.18: Glucoraphanin concentration in dried leaves of a subset of *Brassica* accession collated into species. GLM analysis revealed a significant difference in glucoraphanin content between *Brassica* species (P = <0.001).

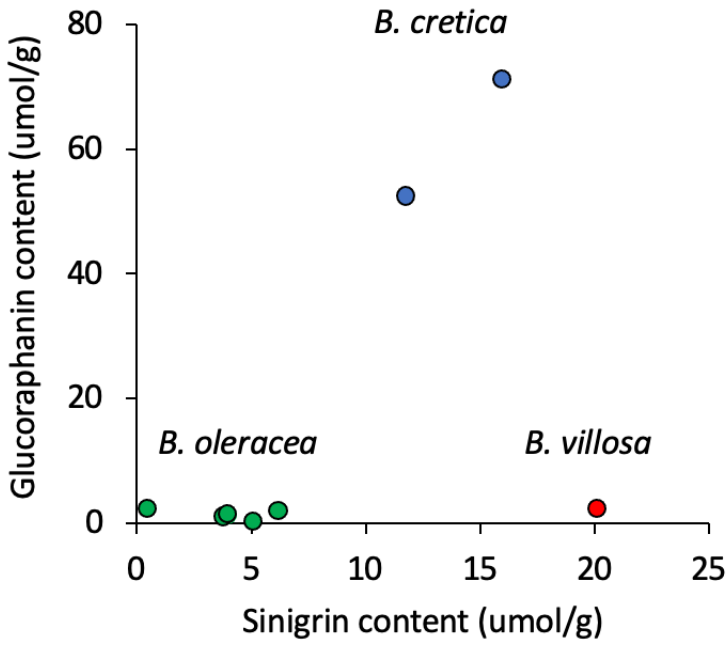


Figure 2.19: Sinigrin and Glucoraphanin concentration in dried leaves of a subset of *Brassica* accessions representing three different species. Within this plot, each datapoint represents the overall mean of the measured sinigrin and glucoraphanin content for 1 of the 9 *Brassica* accessions. Where content of both glucosinolates is considered, clear clustering of data according to species is observed.

2.3.4. Evaluating the relationship between observed life parameters and initial partial resistance screening – multiple linear regression analysis

Having evaluated a wide range of impacts of *Brassica* plants upon *B. brassicae* alongside several physiological attributed of *Brassica* plants, the data were summarised in a life table (Table 2.5). To evaluate which of these parameters most closely related to initial screening results linear regression analysis was undertaken. Initial linear regression analysis with partial resistance screening results as a dependent variable and time to adulthood as a sole independent variable (selected through Figure 2.20 owing to strongest linear relationship) identified a significant fit of regression model to these data relative to a null model, with the model therefore predicting the dependent variable significantly well ($F(1)=8.177$, $p=0.024$, $R^2=0.539$). Time to adulthood alone was therefore a significant factor within the linear regression model ($p=0.024$), accounting for 53.9% of variation within the screening data. Residuals calculated following this initial linear regression analysis were recorded and all remaining observed aphid/*Brassica* parameters were plotted against these residuals in scatter plots, with linear trendlines again fitted to each and R^2 calculated (Figure 2.21). Based upon these plots, instar 3 duration (Figure 2.21C) was noted as a potential second independent variable. Within the biological context of this study however, relative to both overall development time and the development time of instars 1,2 and 4 - instar 3 duration appears a significant outlier – in lieu of any sound biological reasoning instar 3 was rejected as a potential second independent variable for inclusion in the linear regression model. The second factor most linearly related to plotted residuals was therefore selected –intrinsic rate of increase (R_m) (Figure 2.21G) Following inclusion of this second covariate into the linear regression model resulted in a model significantly able to predict the dependent variable and improved the model to account for an estimate 76.7% of dependent variable variation ($F(2)=9.867$, $p=0.013$, $R^2=0.767$) however while 'total time to adulthood' remained a significant coefficient ($p=0.004$), 'intrinsic rate of increase' did not prove significant ($p=0.052$) thus it was removed from the multiple linear regression model. Based upon this finding, it was therefore concluded that 'total time to adulthood' was the sole factor with the greatest relationship with initial screening results and which explained the most significant proportion of the initial screening results. This therefore indicates that the extension of development time from identified partially resistant accessions was the factor which most significantly

influenced the final observed population of *B. brassicae*, with this likely resulting from a reduction in the number of days post development for adult reproduction to occur.

Table 2.5: Life table summary of *Brevicoryne brassicae* on the selected subset of 8/18 *Brassica* accessions and technical control. Each observed metric reported as mean values alongside a calculated upper and lower 95% confidence interval bounds. Medians also presented for aphid development data and reproduction count metrics.

Metric		Statistic	*Brassica* accession								
			2	4	9	11	12	15	16	17	C
Initial screening results (aphids produced per starting nymph)		**mean**	**9.31**	**12.53**	**11.28**	**21.83**	**25.35**	**12.72**	**11.81**	**18.75**	**16.18**
		lower bound	6.23	9.26	8.72	16.09	20.27	9.62	9.43	14.98	13.19
		upper bound	12.39	15.81	13.85	27.58	30.43	15.82	14.2	22.51	19.16
Aphid development (days)	Instar 1	**mean**	**2.13**	**2.42**	**2.62**	**2.06**	**2.5**	**1.97**	**3**	**2.3**	**2.36**
		lower bound	1.24	1.42	1.62	1.55	2.28	1.49	2.26	1.19	1.6
		upper bound	3.01	3.41	3.61	2.56	2.72	2.46	3.74	3.41	3.11
		median	1.5	1.5	1.5	1.5	2.5	1.5	2.75	1.5	2.25
	Instar 2	**mean**	**3.25**	**2.83**	**2.88**	**2.81**	**2.67**	**2.63**	**3.31**	**2.1**	**2.23**
		lower bound	1.78	2.18	2.21	2.22	1.4	2.15	2.81	1.57	1.62
		upper bound	4.72	3.49	3.56	3.4	3.94	3.1	3.82	2.63	2.84
		median	2.5	3	3	2.5	2.5	2	3	2	2
	Instar 3	**mean**	**2.13**	**1.83**	**2.06**	**2.47**	**2.33**	**2.13**	**2.44**	**2.4**	**2.23**
		lower bound	1.3	1.38	1.53	1.88	1.48	1.65	1.96	1.63	1.44
		upper bound	2.95	2.29	2.59	3.05	3.19	2.6	2.91	3.17	3.02
		median	2	2	2	3	2.5	2	2	2	2
	Instar 4	**mean**	**2.25**	**1.83**	**1.76**	**1.64**	**1.2**	**2.2**	**2.5**	**2.2**	**2.08**
		lower bound	1.51	1.47	1.34	1.16	0.64	1.89	2.06	1.75	1.69
		upper bound	2.99	2.2	2.19	2.13	1.76	2.51	2.94	2.65	2.46
		median	2	2	2	1.5	1	2	2	2	2
	Time to adulthood	**mean**	**9.75**	**8.92**	**9.32**	**8.24**	**7**	**8.38**	**11.25**	**9**	**8.43**
		lower bound	6.73	7.58	8.18	6.95	4.48	7.07	10.36	7.64	6.97
		upper bound	12.77	10.26	10.46	9.52	9.52	9.69	12.14	10.36	9.88
		median	8.5	8.5	9.5	8.5	8	8.5	11	9	9
Reproduction	Daily reproduction	**mean**	**2.68**	**2.5**	**2.18**	**2.38**	**2.44**	**2.26**	**1.6**	**1.86**	**1.9**
		lower bound	2.43	2.11	1.97	2.06	1.58	2.02	1.41	1.55	1.57
		upper bound	2.94	2.89	2.39	2.69	3.3	2.51	1.8	2.18	2.23
		median	3	2	2	2	2	2	2	2	2
	Gross reproduction	**mean**	**21.44**	**20**	**17.44**	**19**	**19.5**	**18.1**	**12.83**	**14.9**	**15.2**
		lower bound	18.42	17.37	14.57	14.88	16.19	15.78	11.29	8.88	9.03
		upper bound	24.47	22.63	20.32	23.12	22.81	20.42	14.38	20.92	21.37
		median	23	20	19	16.5	19.5	17.5	12.5	12.5	15
	Intrinsic rate of increase	**mean**	**0.29**	**0.29**	**0.27**	**0.27**	**0.26**	**0.26**	**0.19**	**0.23**	**0.25**
		lower bound	0.26	0.25	0.23	0.25	0.23	0.24	0.18	0.19	0.19
		upper bound	0.33	0.34	0.32	0.28	0.3	0.28	0.21	0.27	0.31
	Population doubling time	**mean**	**2.39**	**2.39**	**2.66**	**2.62**	**2.64**	**2.7**	**3.6**	**3.22**	**2.82**
		lower bound	2.16	1.99	2.26	2.48	2.3	2.49	3.35	2.71	2.27
		upper bound	2.62	2.79	3.06	2.76	2.98	2.91	3.85	3.73	3.36
Weight during development ($x10^{-5}$ g)	Maximum aphid weight (day 10)	**mean**	**22.04**	**21.78**	**20.74**	**22.41**	**25.07**	**19.93**	**16.38**	**17.79**	**21.11**
		lower bound	19.87	19.17	18.25	20.19	22.45	17.39	14.51	15.5	19.46
		upper bound	24.21	24.39	23.23	24.62	27.7	22.47	18.24	20.08	22.76
	Mean relative growth rate	**mean**	**0.3**	**0.32**	**0.24**	**0.23**	**0.28**	**0.23**	**0.25**	**0.22**	**0.29**
		lower bound	0.26	0.28	0.21	0.19	0.25	0.19	0.21	0.17	0.26
		upper bound	0.33	0.37	0.27	0.27	0.31	0.28	0.29	0.26	0.32
Glucosinolate content (umol/g-1)	Sinigrin content	**mean**	**20.11**	**0.47**	**3.96**	**6.18**	**3.77**	**5.11**	**15.96**	**11.76**	**3.94**
		lower bound	0	0.13	3.5	4.04	3.29	0	12.2	0.79	0
		upper bound	55.91	0.82	4.42	8.31	4.25	12.78	19.72	22.73	9.41
	Glucoraphanin content	**mean**	**2.29**	**2.24**	**1.36**	**1.91**	**0.99**	**0.16**	**71.17**	**52.38**	**1.48**
		lower bound	1.86	0.64	1.22	1.1	0.86	0.08	68.03	0	0.47
		upper bound	2.72	3.84	1.51	2.73	1.12	0.23	74.3	109.76	2.49

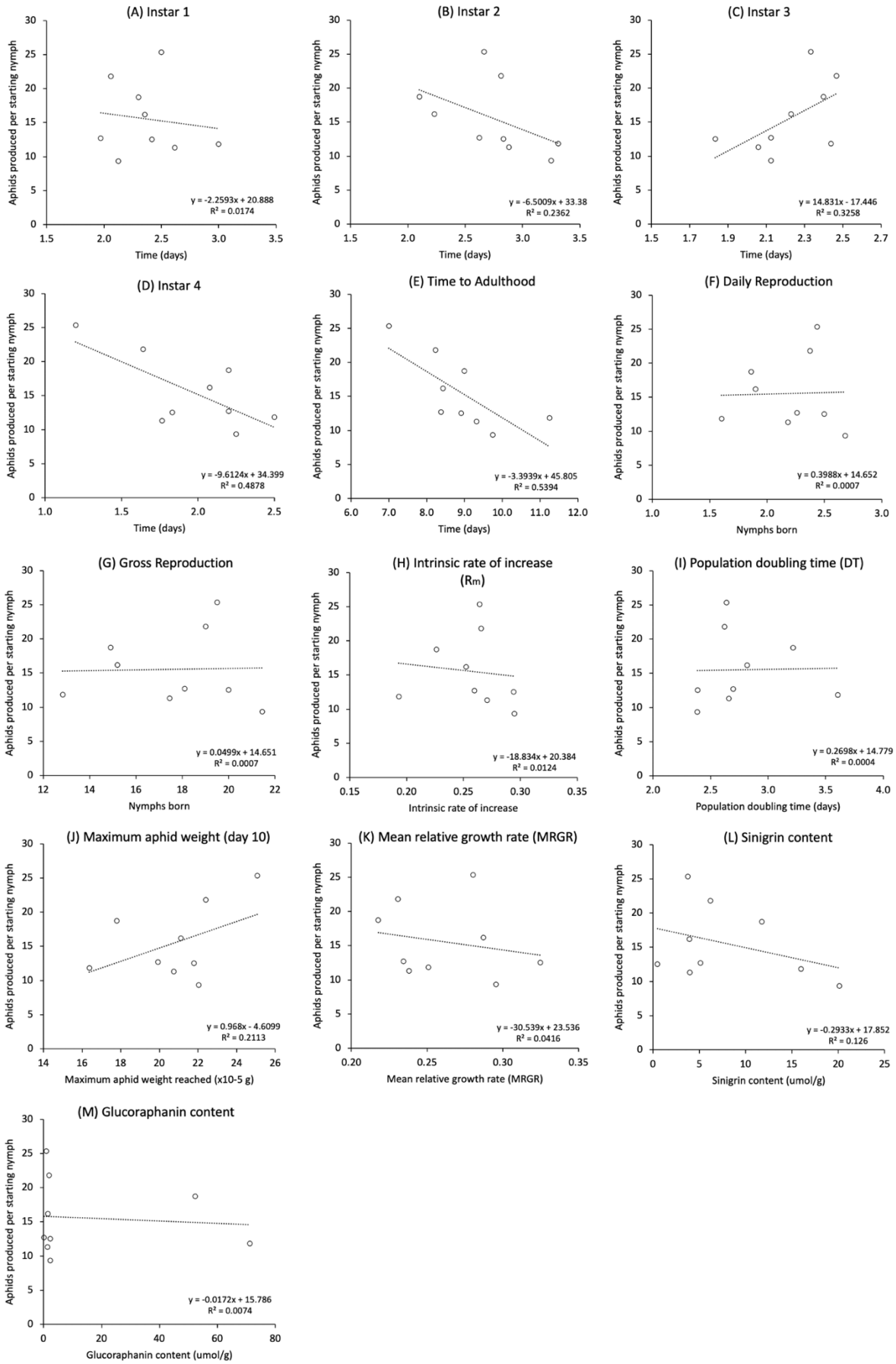


Figure 2.20: Scatter plots evaluating the linear relationship of life table parameters outlined in Table 2.5 with initial partial resistance screening results (aphids produced per starting nymph). Through these plots and calculated R^2 of linear trendlines, (E) time to adulthood was selected as the initial independent variable for inclusions in linear regression analysis.

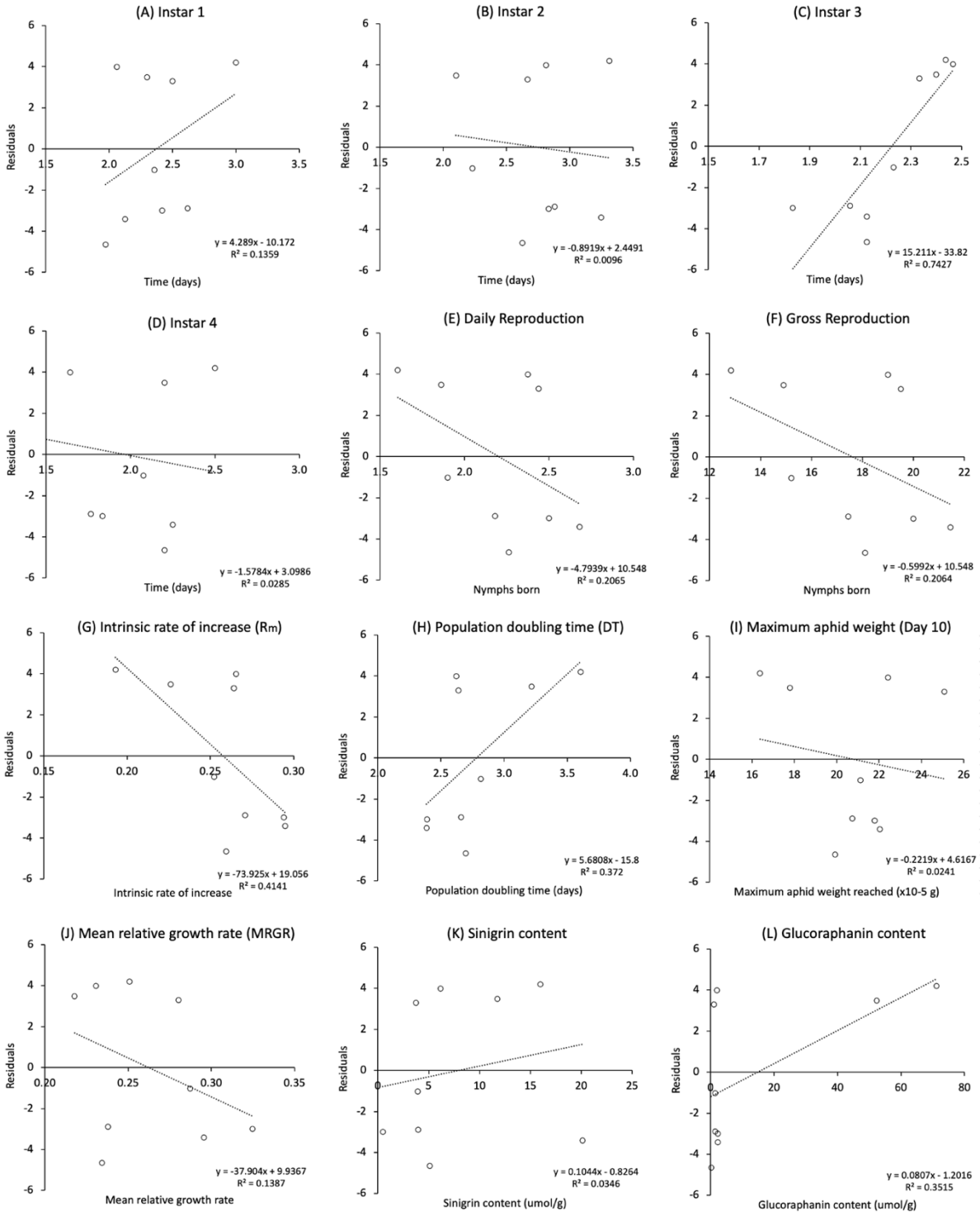


Figure 2.21: Scatter plots evaluating the linear relationship of life table parameters outlined in Table 2.5 with residuals from linear regression analysis including 'time to adulthood' as an independent variable. Through these plots and calculated R^2 of linear trendlines, (C) instar 3 was selected as the second independent variable for inclusions in linear regression analysis.

2.3.5. Evaluating the relationship between *Brevicoryne brassicae* development, reproduction, and weight on screened subset of *Brassica* accessions

To evaluate whether there exists any relationship between observed development, weight, and reproduction data a measured parameter for each was selected. These parameters were 'total time to adulthood' for development, 'gross reproduction' for reproduction and 'maximum aphid weight' for aphid weight results – with these being selected owing to previously confirmed statistically significant differences between accessions for these metrics and these metrics being observed measurements and not calculated variables. Three pairwise scatter plots were produced for these three variables and linear trendlines fitted to evaluate the linear relationship between variables (Figure 2.23). The linear trendline between 'development time to adulthood' x 'gross reproduction' was found to have an R^2 of 0.2076 indicating a relatively poor linear relationship between these factors (Figure 2.23A). Conversely for 'development time to adulthood' x 'maximum aphid weight' and 'maximum aphid weight' x 'gross reproduction', R^2 values were found to be 0.5930 and 0.6308 respectively – indicating stronger linear relationships between these factors (Figure 2.23B and C). Across all three scatter plots, each point represents the two overall mean values for both factors for a single *Brassica* accession, with colours denoting *Brassica* species. Across all three plots, potential species clustering can be observed (Figure 2.23). Notably, accession 12 and 16 across all plots also appear as significant outliers at opposite extremes of the linear trendlines (Figure 2.23)

To formally evaluate whether there existed any significant relationships between these factors, two-tailed bivariate Pearson correlation coefficient analysis was performed. This analysis revealed a significant correlation between 'development time to adulthood' x 'maximum aphid weight' and 'maximum aphid weight' x 'gross reproduction (N=9, p=0.015 and N=9, p=0.010 respectively). No significant correlation was noted between 'development time to adulthood' x 'gross reproduction' (N=9, p=0.217). The first of these significant correlations suggest factors or resistance which negatively affect aphid development rate also significantly reduce aphid weight. The second significant correlation suggests that aphids which weigh less (and are thus likely smaller) are generally less fecund, with significantly lower larviposition over an 8-day period.

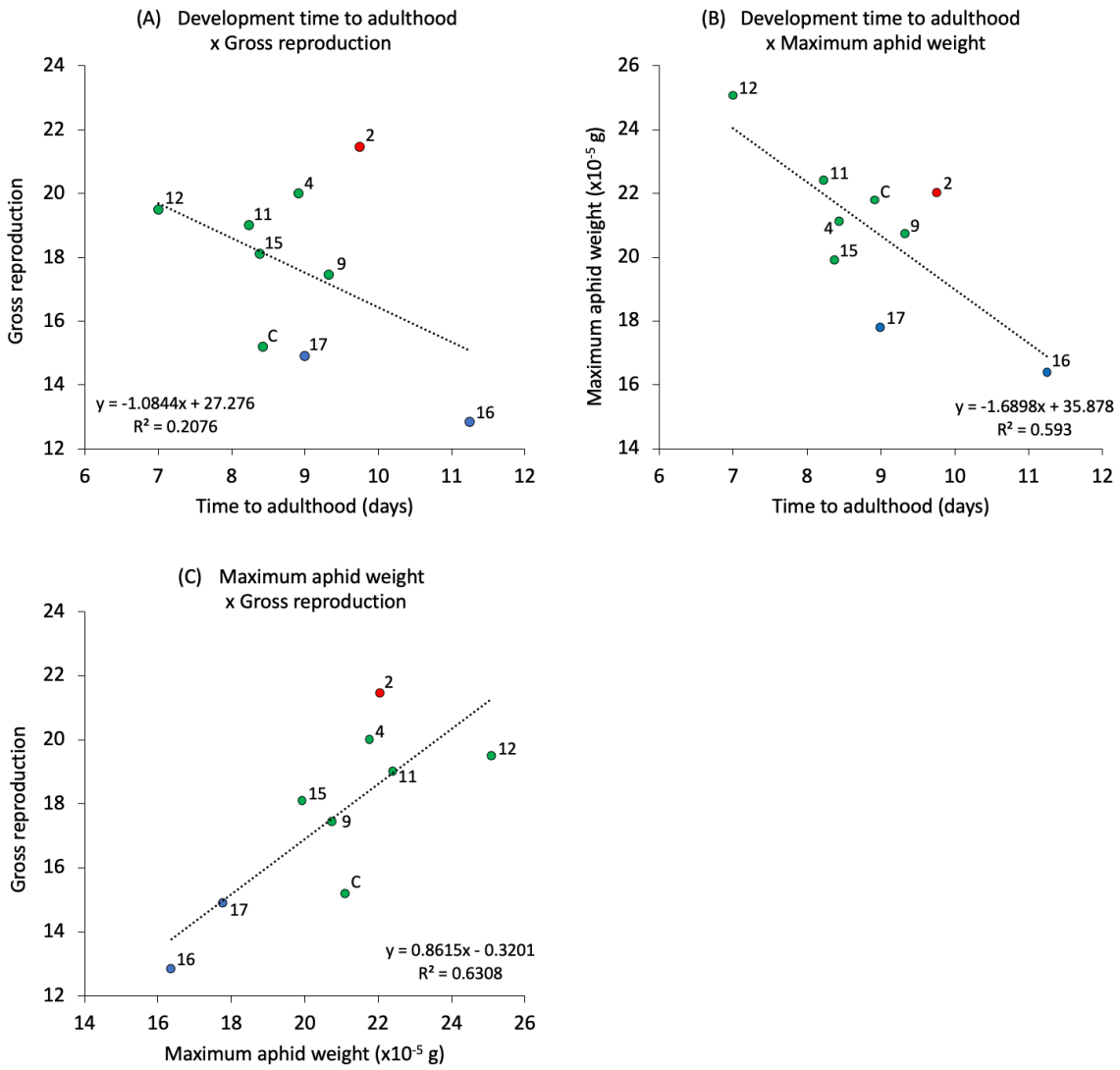


Figure 2.22: Scatter plots evaluating the pairwise linear relationships of overall means of *Brevicoryne brassicae* 'development time to adulthood', 'maximum weight' and 'gross reproduction' for the subset of 8/18 *Brassica* accessions. Each point within these graphs represents the overall means for both variables within a single *Brassica* accession – with all *Brassica oleracea* accessions denoted in green, *Brassica cretica* accessions in blue and the sole *Brassica villosa* subsp. accession denoted in red. Pearson correlation coefficient analysis revealed a significant correlation for factors in plots B and C (p=0.015, 0.010 respectively) however factors in plot A were found not to significantly correlate (p=0.217).

2.4. Discussion

2.4.1. Partial resistance screening

Significant differences were observed in development of *B. brassicae* populations on the 18 Brassica accessions screened in this study, with a 3-fold difference in final population size occurring between the most and least susceptible accessions. The use of partial resistance to *B. brassicae* could help reduce inputs of synthetic chemical pesticides if used as part of an IPM system – as has been observed in other agronomic settings where plant pest resistance has been deployed (Pimentel *et al.,* 1993; Sharma and Ortiz, 2002; Stout, 2014). However, owing to a history of basing aphid control around routine applications of synthetic chemical pesticides, combined with the supermarkets' high-quality standards that results in the rejection of produce with any living or dead aphid 'contaminants' on it, the value of partial resistance has been overlooked in the past (Emmett, 1992; Parker *et al.*, 2002; Ellis, pers. communication). Within recent years, however, attitudes have started to shift, with mounting problems associated with pesticide product withdrawals and the emergence of resistance in target pest populations driving greater interest from growers and plant breeders in complete and partial host plant resistance to insect pests (Dedryver *et al.*, 2010; Hillocks, 2012; Morais and Pinheiro, 2012; Karjari *et al.*, 2017). Partial host plant resistance will not, on its own, provide sufficient aphid control to satisfy growers and meet supermarket quality demands; it does however have significant value in terms of durability and its integration with complementary IPM tools, particularly biological controls (Stenberg, 2017).

In the present study, no single pre-selection criteria outperformed the others. Nonetheless, the combined use of different existing resources to pre-select *Brassica* accessions prior to resistance screening proved effective, allowing for the identification of significant and varied levels of partial resistance for further assessment. This approach therefore warrants consideration as a strategy to limit expensive and time-consuming phenotypic screens to a reduced set of candidate plants in a diversity collection.

2.4.2. Relationships between *Brassica* species and *Brassica oleracea* cultivar groups with identified partial resistance

Within the literature, among the most significant factors reported to influence *Brassica* resistance to insect pests (including aphids) is the species of *Brassica* evaluated (Singh *et al.*, 1994; Ellis *et al.,* 2000; Cole, 2003). Despite only four *Brassica* species being assessed in this study, *Brassica* species was identified as a factor significantly impacting *B. brassicae* population development. Of the species assessed, *Brassica villosa* subsp. *tinei* (one accession, accession 2) and *Brassica cretica* (three accessions, accessions 16, 17 and 18) were found to significantly reduce *B. brassicae* population development relative to *B. oleracea* (accessions 4-15) and *B. macrocarpa* (accessions 1 and 3). These results concur with numerous studies demonstrating the partial resistance of *B. cretica* and *B. villosa* against both *B. brassicae* alongside other pests such as *Delia radicum* (Ellis *et al.*, 1999, 2000; Shuhang *et al.*, 2016). While species was therefore found to be significant within the accessions evaluated within this present study, owing to the low number of accessions representing each species, particularly *B. cretica* and *B. villosa* subsp. *tinei*, these results lack the statistical power for valid extrapolation to consider interspecific variation overall.

To date the mechanisms underlying resistance in different *Brassica* species including those assessed within this study, are unknown. One potential reason for this underutilisation is the relative difficulty and unpredictability in crossing of *B. oleracea* with related species. Owing to linkage drag, the progeny of such crosses typically harbour agronomically undesirable traits – thereby necessitating further breeding efforts to restore agronomic suitability (Walley *et al.*, 2012; Quezada-Martinez *et al.*, 2021). Further compounding the difficulties in isolating resistance from wild species is uncertainty surrounding the underlying mechanisms of resistance, with resistance potentially conferred partly or wholly by physical/structural phenotypes rather than biochemical mechanisms (Ellis *et al.*, 1999). Within this study *B. cretica*, *B. macrocarpa* and *B. villosa* subsp. *tinei* were all noted to have distinctive phenotypes compared with the *B. oleracea* accessions, with leaves of *B. cretica* being thick and waxy, *B. macrocarpa* had moderate leaf surface trichomes and *B. villosa* subsp. *tinei* had a high concentration of leaf surface trichomes (Gladman, A.K. observation). The degree to which these phenotypes may

have conferred partial resistance or susceptibility to *B. brassicae* is unknown, however *B. brassicae* feeding and infestation have previously been found to be largely unaffected by the presence of trichomes (Teakle, unpublished). Owing to the unknown mechanisms resulting in resistance in such species and the potentially significant difficulties associated with crossing C-genome *Brassica* relatives to *B. oleracea*, traditional screening approaches continue to favour *B. oleracea* crop-type and landrace accessions over wild species (Walley *et al.*, 2012).

Alongside *Brassica* species, a further factor considered for its relationship to observed partial resistance in this study was *B. oleracea* cultivar group. Despite the significant intraspecific variation within *B. oleracea*, to date few studies have evaluated for either aphid resistance specifically or pest resistance more generally between different crop types. Of those who have, among the largest screen was undertaken by Ellis *et al.* (1998) who screened 401 *B. oleracea* accessions against *B. brassicae*, identifying that Kale cultivars (Acephala group) were the most promising sources of partial resistance (Ellis *et al.*, 1998). Within this present study however, despite including several of the most significantly resistant Kale accessions identified by Ellis *et al.* (1998), no significant difference in *B. brassicae* performance was observed between *B. oleracea* cultivar groups. While these findings thus in part contradict the conclusions of Ellis *et al.* (1998), the limited number of accessions of several cultivar groups included in this study make definitive conclusion on cultivar groups as sources of resistance difficult to draw – with further research required to comprehensively evaluate the relationship between *B. oleracea* cultivar group and partial host plant resistance to *B. brassicae*. Nonetheless, of the accessions included in this study from the Ellis *et al.* (1998) screen, the majority (83.3%) were re-confirmed as demonstrating significant partial resistance to *B. brassicae* (Ellis *et al.*, 1998).

2.4.3. Pre-selection of accessions with JA pathway gene upregulation

While re-assessing accessions selected for screening from literature sources was a key component of this work, of further significant interest was the performance of those accessions selected on the basis of pre-infestation JA pathway gene orthologue expression, with these accessions being selected owing to research evidence indicating that generalised pre-infestation JA pathway upregulation is related to

aphid resistance (Ellis *et al.*, 2002; Mewis *et al.*, 2006; Kersch-Becker and Thaler, 2019). In this study, while accessions selected through this method did not outperform other selection approaches, they were able to match the performance of all other selection criteria against *B. brassicae*, including matching the performance of accessions already established as partially resistant from the Ellis *et al.* screen (1998). This result therefore appears to support the use of a bottom-up pre-selection approach as a viable method for the pre-selection of partially resistant *Brassica* accessions. Though significant literature resources exist to support pre-infestation JA signalling pathway upregulation as conferring *A. thaliana* resistance against *M. persicae* (and *B. brassicae* to a lesser degree), little work had been undertaken in translating these findings to crop species thus the likely success of this approach was unknown (Kuśnierczyk *et al.*, 2011). This study marks an early step in translating the evolving immunological understanding of plant-aphid defence interactions from model systems into *Brassica* crop screening, warranting future work to expand upon this bottom-up pre-selection approach. The availability of only a pre-infestation transcriptomic dataset for both the *B. oleracea* and wild *Brassica* species DFFSs limited the scope of bottom-up pre-selection in this study however, following these promising results, further work is warranted producing and utilising post-infestation transcriptomic resources to expand the scope of bottom-up selection to post-infestation immunological signalling pathways. As understanding of plant-aphid interactions continues to develop both in model systems and crop plants, further targets of interest for use as pre-selection markers are likely to become increasingly apparent – with aphid saliva effector targets a potentially significant source or interesting future markers as their targets begin to be identified (Hogenhout and Bos, 2011; Jaouannet *et al.,* 2014; Kaloshian and Walling, 2015; Züst and Agrawal, 2016; Rodriguez *et al.*, 2017). In combination with continuing advances in transcriptomics methodology, this greater understanding will allow for increased scope of bottom-up pre-selection therefore driving improvements in pre-selection approaches as employed in this study.

2.4.4. Potential mechanisms underlying partial antibiosis host plant resistance

Having identified significant partial resistance to *B. brassicae* within some of the evaluated *Brassica* accessions, a question arises as to the mechanisms underlying their resistance. While such mechanistic detail was not the focus of this study and

thus not formally evaluated, within the literature there exists several noteworthy case studies of aphid resistance and likely underlying mechanisms in different crop species – with significant advances in understanding made in recent years (Dreyer and Campbell, 1987; Corcuera, 1993; Züst and Agrawal, 2016; Nalam *et al.*, 2019). These studies highlight that resistance can be conferred though either generalised or specific mechanisms – with complete resistance most associated with specific mechanisms and partial resistance primarily attributed predominantly to generalised mechanisms (Dogimont *et al.*, 2010; Züst and Agrawal, 2016). Generalised defence is typically induced by biomolecular recognition of aphid associated damage, leading to the upregulation of phytohormonal signalling pathways (most prominently JA) ultimately leading to the production of toxic plant secondary metabolites (including cardiac glycosides, alkaloids benzoxyazinoids and glucosinolates) and upregulation of proteins involved in phloem-sealing mechanisms (Will *et al.,* 2013; Züst and Agrawal, 2016; Nalam *et al.*, 2019). Specific resistance meanwhile typically requires the presence of host plant R-genes producing receptors capable of direct recognition of pathogen-associated molecular patters (PAMPs) either on the surface of aphid feeding apparatus or of proteins (including effectors) secreted in aphid saliva (Züst and Agrawal, 2016). Owing to the general trend of partial resistance stemming from more generalised resistance mechanisms, it can be assumed that partial resistance identified in some *Brassica* accessions may stem in part from differences in secondary metabolite composition and/or phloem sealing mechanisms (such as callose deposition). Thus, if future research with these accessions is undertaken there would be significant value in assessing whether these mechanisms do indeed underlie partial resistance. One experiment which may be useful to test this would be the evaluation of *B. brassicae* feeding behaviour between accessions using an electrical penetration graph (EPG) approach, with such a method indicating whether phloem feeding on partially resistant plants is more frequently interrupted or disturbed indicating therefore whether phloem localised secondary metabolites and/or phloem sealing mechanisms may be inhibiting aphid feeding activities (Will and van Bel, 2006; Chen *et al.*, 2018; Peng and Walker, 2020).

2.4.5. Aphid development rate, weight, and reproduction studies

In contrast to complete resistance which acts by entirely excluding aphid pests, partial antibiosis resistance works by quantitatively reducing the biotic potential of the target pest (Gatehouse, 2002; Dogimont *et al.*, 2010). From the perspective of later combining partial host plant resistance with biological controls, understanding the specific effects of identified partially resistant *Brassica* accessions upon aphid biotic potential was thus paramount – with the rate of aphid development, aphid weight during development and reproduction therefore evaluated in this study. While each of these metrics were considered in isolation, it should be noted that there is evidence within the literature that they are innately linked. This is supported by the results from the present study of a significant correlation between maximum aphid weight reached and both gross reproduction and time to reach adulthood.

Development time is a well-studied aspect of aphid biology which is known to be significantly influenced by a wide range of biotic and abiotic factors including temperature, aphid population density, plant age, plant fitness and plant nutritional quality (Atkins, 1972; Simon *et al.,* 1991; MacKay *et al.,* 1993; Özder and Sağlam, 2013; Pers and Hansen, 2019). Owing to the four instars aphids must progress through prior to reaching adulthood, aphid development time was considered on an overall scale (from birth to adulthood) and on an instar specific level – where the length of each of the four individual instar phases was considered. Plant accession was found to be a significant factor influencing the overall development time of *B. brassicae*, as has been suggested in other studies where partial antibiosis resistance has been evaluated against different aphid species (Harrington, 1941; Lanteigne *et al.*, 2014). Within this study however, this significance stemmed wholly from a single accession, accession 16 (*B. cretica*), which significantly increased *B. brassicae* development time relative to all accessions except accession 2 (*B. villosa* subsp. *tinei*). While the mechanism through which accession 16 (*B. cretica*) slowed overall development time is unknown, it is noteworthy that a similar extension in overall development time was not observed in the other assessed *B. cretica* accession (17) therefore indicating this is not a species-specific effect. This finding warrants further evaluation owing to the potentially significant knock-on effects upon aphid population development such a difference in development time may cause. On an instar-specific scale, the finding that only instar 4 was significantly affected by plant

partial resistance was unexpected, with no indications in the literature that partial resistance may significantly extend the duration of any specific instars. This result may stem from issues with the design of the experiment, with observations at 24hr intervals potentially too infrequent to detect small differences in instar duration. However, there is evidence in the literature to suggest that significant differences in gene expression between different aphid instars are associated with changes in developmental rate as the aphid comes closer to adulthood (de Vos *et al.*, 2010; Ayyanath *et al.*, 2015). Within this context, this observation of substantially slowed instar 4 duration could therefore represent a phenomenon warranting further research evaluation.

Aphid weight is commonly used to calculate metrics such as mean relative growth rate (MRGR) - with both MRGR and maximum weight being commonly considered as proxy measures of pest fitness within their given environment or habitat (Dykhuizen and Dean, 1990). In this present study a significant difference was noted in both aphid weight and MRGR across the different *Brassica* accessions – as has been suggested as a potential effect of partial host plant resistance upon aphids in other studies (Lanteigne *et al.*, 2014). However, no difference was observed in weight between the three different *B. brassicae* generations studied. Aphid generation was evaluated owing to evidence in the literature that over subsequent generations aphids may either increase in fitness (owing to their ability to prime host plants to optimise their feeding) or decrease in fitness (owing to a compounding effect of plant resistance upon each subsequent aphid generation) (Züst and Agrawal, 2016). The absence of a generational effect in this study was unexpected and most likely attributable to insufficient generations observed for such effects to become apparent. While aphid weight/MRGR have long been used as metrics for evaluating aphid fitness, the link between aphid weight/MRGR and aphid population development is less clear than for time to development and reproduction rate. Significant evidence exists however highlighting how smaller/lighter adult aphids (as can result from resistant plant varieties) may be both less fecund, as was indicated in this study, and potentially more susceptible to parasitoids and disease (Brown and Llewellyn, 1985; Reed *et al.*, 1991; Cai *et al.*, 2009). Both *B. cretica* accessions, 16 and 17, demonstrated the most significant ability to limit aphid weight – with accession 17 also most significantly reducing calculated MRGR. These results may

therefore indicate a mechanism in *B. cretica* which acts to significantly reduce aphid weight during development.

The final aspect of *B. brassicae* biology evaluated was reproduction. Of all three assessed aphid life traits, reproduction rate is conventionally considered the most important – with even small changes in reproduction rate leading to large effects on population size in the field (Lykouressis, 1984; Dixon, 1985; Ma and Bechinski, 2008). Of all three aspects of *B. brassicae* considered here, aphid reproduction is considered to be the most sensitive to antibiosis resistance (Harrington, 1941; Reinink and Dieleman, 1989; Munthali, 2009; Lanteigne *et al.*, 2014; Greenslade *et al.*, 2016). Plant accession in this study was shown to have a significant effect upon both daily and gross larviposition - with *B. cretica* accessions (16 and 17) again being identified as having the most significant effect.

Despite *B. cretica* accessions being consistently demonstrated as both some of the most resistant to *B. brassicae* overall and having among the most significant negative effects upon *B. brassicae* development and reproduction, relatively few other studies have evaluated *B. cretica* aphid resistance (Ellis *et al.*, 1999). Based on the data collected in this present study, further research evaluating the resistance of *B. cretica* against *B. brassicae* and other pest species is warranted. While the effects of assessed *B. oleracea* accessions upon development, weight and reproduction were more pronounced than those for *B. cretica*, significant differences were nonetheless noted between *B. oleracea* accessions though no association between cultivar group or crop type was again noted.

2.4.6. Glucosinolate analysis

As one of the best studied examples of a plant resistance mechanism, the effects of glucosinolates are important to consider – particularly for a *Brassica* specialist pest such as *B. brassicae* for which glucosinolates also serve as an important host-preference factor (Chen *et al.*, 2001; Kim and Jander, 2007; Kim *et al.*, 2008; Mezgebe *et al.*, 2018).

As a large family of secondary metabolites with concentrations varying spatially and temporally within individual plants, usually only the most prevalent glucosinolates in

the target tissue are assessed. The aliphatic glucosinolates sinigrin and glucoraphanin are among the most frequently reported in studies of *B. oleracea* crops (alongside *B. cretica*, *B. macrocarpa* and *B. villosa* subsp. *tinei*), the aliphatic glucosinolates sinigrin and glucoraphanin are among the most frequently reported (Heaney *et al.*, 1987; Breme *et al.*, 2008; Bhandari *et al.*, 2015). In this analysis, both sinigrin and glucoraphanin content were found to vary significantly between *B. oleracea* accessions. The most significant differences however were noted between different species, with *B. cretica* demonstrating significantly higher levels of glucoraphanin relative to *B. oleracea* and both *B. cretica* and *B. villosa* subsp. *tinei* demonstrating significantly elevated levels of sinigrin.

The best evidence to support a role of glucosinolates in plant resistance to aphids concerns indole compounds, whereas sinigrin and glucoraphanin are aliphatic glucosinolates, and hence it is unknown whether / how these two glucosinolates may alter plant resistance to *B. brassicae* or specific aspects of *B. brassicae* biology and what effect significant differences in concentrations between accessions may have (Chen *et al.*, 2001; Kim and Jander, 2007). Regardless of the effect of sinigrin and glucoraphanin upon *B. brassicae*, these results indicate clearly that glucosinolate content is overwhelmingly tied to *Brassica* species rather than being present in higher levels in these resistant *Brassica* accessions. To further investigate the potential significance of glucosinolates in *Brassica* resistance to aphid pests, a likely more comprehensive approach would be to assess glucosinolate content specifically in *Brassica* phloem – with these being the predominant glucosinolates aphid pests are likely to be exposed to (Brudenell *et al.*, 1999; Chen *et al.*, 2001; Kos *et al.*, 2012). Owing to a growing body of evidence, within the context of aphid resistance, further evaluation of glucosinolates would also benefit from the inclusion of indole glucosinolates (Kim and Jander, 2007; Kim *et al.*, 2008; Chhajed *et al.*, 2020; Sun *et al.*, 2020). It should be noted however that high levels of glucosinolates may in itself pose a problem for *Brassica* breeding efforts, likely conferring unpleasant flavour to the resulting crop.

2.4.7. Which measured variables most closely relate to initial screening results?

Having evaluated both the sinigrin and glucoraphanin content of these *Brassica* accessions alongside their specific effects upon aphid development rate, weight, and

reproduction a question arose as to which of these evaluated metrics may most closely relate to initial screening results – with the closest association potentially indicating the metric most related to or affected by identified partial resistance. Through linear regression analysis, total time to adulthood was found to be most closely related to initial screening results, accounting for 53.9% of the observed screening results. Within individual analysis of overall development time however, only accession 16 (*B. cretica*) was noted as significantly extending time to development relative to all other accessions – this finding may therefore suggest that more significant differences were potentially present between accessions in time to adulthood and were simply missed during assessment – potentially due to assessments being too infrequent (24hr intervals). In future studies time to development assessments could be improved by assessing aphid development more frequently and at shorter intervals (e.g., every 12hrs).

A number of studies have been published that sought to identify partial host plant resistance to different pest species, of these however only a relatively small subset evaluated this resistance for its specific effects upon aphid development (van Steenis and El-Khawass, 1995; Sandanayaka *et al.*, 2005; Lanteigne *et al.*, 2014). Within these studies, aphid development time to adulthood and reproduction were the most reported significantly perturbed aspects of aphid biology by partial host plant resistance. Unfortunately, within these studies further analysis was not undertaken to determine whether development rate or reproduction were more related to overall partial resistance (van Steenis and El-Khawass, 1995; Sandanayaka *et al.*, 2005; Lanteigne *et al.*, 2014). The findings of this study therefore raise a question as to whether time to development could be the most commonly and significantly perturbed aspect of aphid biology by host plant resistance in difference crop species – with it having been uniformly reported where partial host plant resistance has been evaluated in detail previously (van Steenis and El-Khawass, 1995; Sandanayaka *et al.*, 2005; Lanteigne *et al.*, 2014). The observed significant relationship between time to development and overall screening results in this assay may also however stem from the methodology used within the initial screen. Within this screen, from a fixed starting point of three 1-day old *B. brassicae* nymphs, total population was recorded after a 14-day period – being taken as a proxy for overall partial host plant resistance. This population however represents a combined metric resulting from the

time taken for these aphids to develop to adulthood, their fitness upon reaching adulthood and their resultant reproduction upon reaching maturity – with the relative resistance level of each *Brassica* accession potentially influencing each of those components. While each of these traits likely contributed in part to the observed final population, regardless of *Brassica* accession it is likely that a majority of this 14-day period was spent by the aphid developing through each instar towards adulthood (requiring ~8.75-11.25 days dependent upon accession as indicated within the more detailed evaluation of development time). Within this context it is thus clear that any partially resistant Brassica accessions were likely to be primarily the result of their effect upon aphid development time.

This notion however raises an interesting and important point for screening plants for partial pest resistance. Where the pest of interest has a complex life cycle, composed of multiple discrete phases, with reproduction confined to a limited number of these phases– the design of an initial screening assay, including its duration, can have a significant impact upon the type of resistance or partial resistance identified. Within this present work, through utilising a 14-day assay period, resistance screening identified primarily accessions which most significantly slowed overall aphid development to reproductive adulthood. Were this experimental period extended however to 28-days it is likely that such an assay would identify plant accessions with more effect upon aphid fitness and/or reproduction. From a plant breeding perspective, this finding is significant as it indicates that resistance screening of crops including Brassicas can be targeted to identify specific sub-components of partial resistance – breaking down the broad and somewhat intangible concept of partial resistance into more defined and manageable quantitative traits such as 'effect upon *B. brassicae* nymph development time' and 'effect upon *B. brassicae* reproduction'. By breaking down such complex traits into more manageable constituent parts, the likelihood of successful mapping of these traits significantly improves – with significantly fewer minor effect QTL likely contributing to these more defined traits than 'partial resistance' overall. The potential to target such screens at specific aspects of partial resistance therefore significantly improves the potential for successfully identifying higher numbers of plant accessions sharing one of these more manageable traits, thereby making possible the process of mapping QTLs underlying this trait and ultimately making breeding for this trait significantly

more achievable. Within the overall context of this present body of research – where partially resistant *Brassica* accessions are intended to be integrated with biological control agents – such a breaking down of partial resistance into these individual more defined traits also offers significant advantages for the formulation and testing of specific hypotheses of how these two IPM tools may interact.

From a plant breeding context however, it must be noted that while a breaking down of resistance into sub-traits is advantageous, this may not fully reflect the underlying genetics and biology of partial resistance – with evidence both within the literature and in this present study that plant effect upon aphid development time, weight during development and reproduction may be interrelated - with the genes underlying these traits in either complete or partial resistance therefore being potentially conferred by the same or genetically linked QTLs (Dogimont *et al.*, 2010).

2.4.8. Conclusions and plants selected for progression to combined experiments

Based upon the results presented in this chapter, four plant accessions were selected for evaluation in combined assessments with entomopathogenic fungal biological control agents. Two *B. oleracea* (11, *B. oleracea* var. Acephala 'Butzo' and 15, *B. oleracea* var. Botrytis 'Marzatico Napoletano') and two *B. cretica* accessions (16 and 17) were selected which demonstrated intraspecific variation in resistance within the two accessions of each species (with one therefore being defined as 'partially resistant' and the other 'susceptible'). These two species were selected owing to indications in assessments of aphid life parameters that there may also exist a species-level difference in partial resistance between these species (with *B. cretica* accessions consistently outperforming a majority of *B. oleracea* accessions). Through selecting accessions demonstrating both significant intra and interspecific variation, the effects of partial resistance within and between species could thus be evaluated in later combined assays.

3. Evaluation of entomopathogenic fungal isolates as biopesticides of the *Brassica* specialist aphid pest *Brevicoryne brassicae*

3.1. Background

3.1.1. Use of entomopathogenic fungi as biopesticides of crop pests

Entomopathogenic fungi (EPF) can be valuable components of IPM and have been widely researched as biocontrol agents of insect pests (Lacey *et al.*, 2001; Gul *et al.*, 2014; Lacey *et al.*, 2015; Maina *et al.*, 2018; Bamisile *et al.*, 2021). To date, over 170 proprietary EPF biopesticides have been developed, the majority based upon hypocrealean species, which are favoured by biopesticide manufacturers owing to their ease of mass production and ability to infect a wide range of pest species (e.g. *B. bassiana* which has been reported to kill over 700 different insect species) (Li, 1988; Glare and Milner, 1991; Goettel *et al.*, 2000; Rohrlich, 2018; Bamisile *et al.*, 2021).

Most commonly, EPF are used according to an inundation strategy, in which pest control relies on the action of a single group of fungal conidia sprayed against the pest cohort, rather than by the subsequent growth, reproduction and persistence of the fungus within the host population to give self-sustaining control (Jaronski, 2010; Chandler, 2017). When sprayed on the crop, conidia are acquired by target pests through two mechanisms: (1) primary acquisition from EPF conidia landing directly upon pests during spraying and (2) secondary acquisition of conidia by pests post-spray while moving across plant surfaces (Roditakis *et al.*, 2000). The amount of pest mortality is determined, therefore, by the lethal dose (i.e., the number of conidia required to kill the insect, which will be the same for both primary and secondary spore acquisition) and the persistence of conidia on leaf surfaces (which will determine the amount of mortality from secondary spore acquisition). Because speed of kill and total mortality are both dose-dependent, the key to successful inundative control is for the IPM practitioner to win the 'numbers game' and deliver a lethal dose of conidia to the target pest (Jaronski, 2010). Thus EPF biopesticide manufacturers place significant emphasis on developing mass production systems and formulations that allow lethal doses to be applied in a cost effective way (Jaronski, 2014; Shubha *et al.*, 2015).

However, there are some important downsides to inundative application of EPF. It can treat the biopesticide as a direct replacement for a conventional chemical pesticide, therefore leading to unrealistic expectations of chemical-like performance (such as rapid kill) and an assumption that applying a large enough dose of conidia to the target can overcome any limitation to efficacy, such as unsuitable environmental conditions (Jaronski, 2010). Furthermore, the focus on inundation and fast mortality ignores the fact that control of pest damage comes not only from insect death, but also from potential sublethal effects on host feeding, growth, development, and reproduction that may extend to subsequent generations (Torrado-León *et al.*, 2006; Kim, 2007; Liu *et al.*, 2020). This aspect of EPF biology is not yet widely exploited in biocontrol programmes, suggesting that greater attention needs to be paid to the ecology of EPF-host interactions in order to maximise their utility in IPM (Roy *et al.*, 2010).

3.1.2. Use of EPF for control of aphid pests

EPF have been used most successfully in protected environments, where environmental conditions are favourable to infection. Good levels of control however can also occur in field crops if conditions are suitable (van Lenteren and Woets, 1988; Milner, 1997; Jacobson *et al.*, 2001; Chandler *et al.*, 2005; Chandler *et al.*, 2011; van Lenteren 2012; Dara, 2019). EPF biopesticides are used in a variety of different circumstances against a range of pest orders including Coleoptera, Lepidoptera, Thysanoptera and Hemiptera (Cherry *et al.*, 2005; Rezende *et al.*, 2009; Skinner *et al.*, 2012; Asi *et al.,* 2013ab; Cruz-Avalos *et al.*, 2019; Sani *et al.*, 2020). With growers having access to a declining number of synthetic chemical pesticides for the control of aphid pests on brassicas and other crops, there is an urgent need for new controls to be used as part of IPM. However, despite many reports within the literature of the potential for EPF as aphid biocontrol agents, to date EPF have demonstrated little commercial success against aphids with only a small number of products having ever been made available for aphid control (Homoptera) (Lee *et al.*, 2015; Nielsen and Hajek, 2005; Jandricic *et al.*, 2014; Saranya *et al.*, 2010; Ali *et al.*, 2018). Among the most prominent of these products are Botanigard® (*B. bassiana*), which advertises efficacy against a broad spectrum of insect pests including whiteflies, thrips, and aphids, and the purportedly more aphid-specific product

Vertalec® (*A. dipterigenus*) (Copping, 2004; Kim, Goettel and Gillespie, 2008; Prince and Chandler, 2020). While Botanigard® continues to be available commercially, Vertalec® which was only indicated for use on protected crops is no longer produced commercially.

3.1.3. Limiters of EPF efficacy against aphid pests of *Brassica* crops

Two main factors are likely to limit EPF efficacy for aphid control on field *Brassica* crops: (1) suboptimal environmental conditions and (2) the short intermoult period of nymphs. Owing to these difficulties, the control of aphid pests using EPF represents a complex problem – with different routes to potential control failure for both *B. brassicae* adults and nymphs (Figure 3.1). These factors therefore warrant research attention.

(1) The conditions within UK and European field crops pose significant challenges for EPF biopesticides (Stafford and Allan, 2010). Temperature and humidity are recognised as the most significant environmental variables affecting EPF conidia germination and growth, while UV-B exposure can reduce conidia persistence on plant surfaces (Ignoffo, 1992; Fernandes *et al.*, 2016; Mora *et al.*, 2017; Tumuhaise *et al.*, 2018; Bernado *et al.*, 2020). Conidia of most EPF species remain viable between 0-40°C but require temperatures between 20–30°C for maximum germination and growth, and different EPF isolates have significantly different optimum temperatures within this range (Mishra *et al.*, 2013; Rai *et al.*, 2014; Chandler, 2017). These optimum temperatures are higher than UK yearly average temperatures and UK average summer temperatures – which in 2020 were noted to be 9.6°C and 14.8°C respectively (Kendon *et al.*, 2021). High leaf/stem surface microhabitat humidity is also required to permit successful conidia germination permitting infection of target pests (Jaronski, 2010). Owing to both the cool climate of the UK and Europe alongside the significant variation during the growing season, within a UK/European field setting applied EPF are subject to suboptimal conditions for a significant duration of the growing season.

(2) The control of aphid nymphs with EPF is a significant challenge because of their short intermoult period, which allows them to 'escape' infection by moulting before conidia have penetrated the cuticle (Kim and Roberts, 2012). During the process of juvenile aphid development, each aphid passes through four instars. If the nymph moults prior to the fungus successfully invading the aphid hemocoel, applied conidia may therefore be shed along with the exuvium (James *et al.*, 2003; Kim and Roberts, 2012). Thus, where EPF treatment is not repeated at regular intervals, aphid populations can rapidly rebound (James *et al.*, 2003).

It is important that EPF isolate selection programmes take these limiting factors into account. Only through a selection approach underpinned by information about EPF thermal biology and the interactions of EPF isolates and aphid nymphs, can EPF isolates with improved chances of successful aphid control be identified. With the context of *B. brassicae* control, there is a need to identify EPF isolates which are (a) able to germinate and grow rapidly at agronomically relevant temperatures and (b) virulent to *B. brassicae*, preferentially with virulence to a majority of pest life stages.

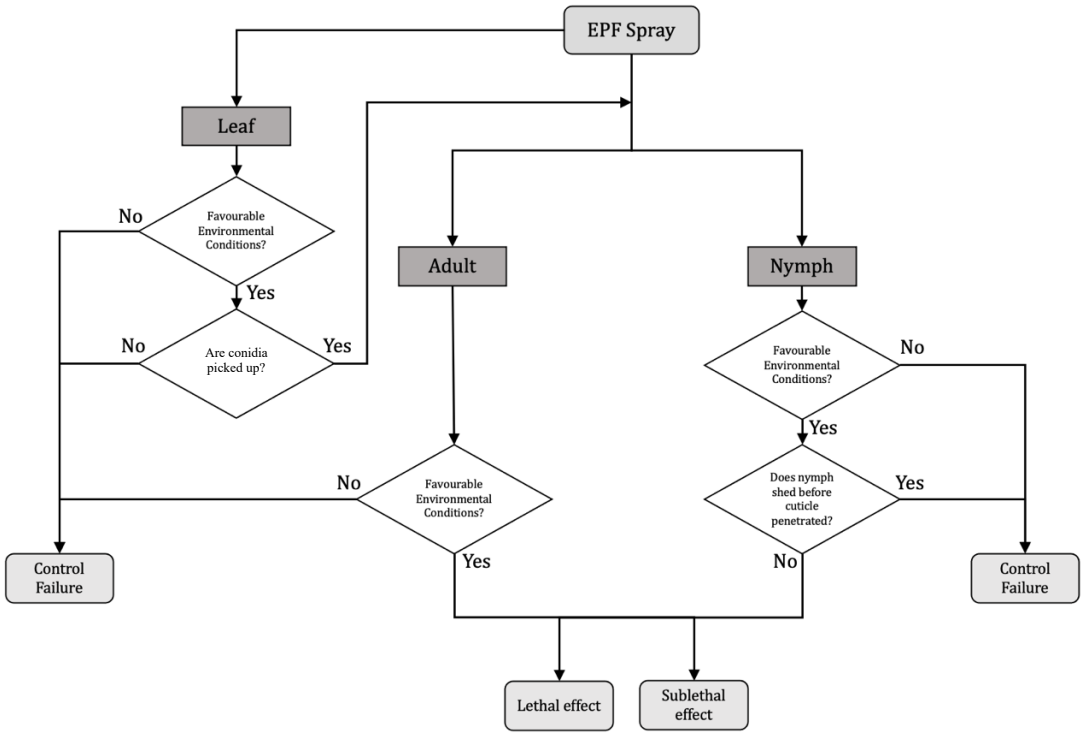


Figure 3.1: Flowchart outlining the potential routes to EPF control failure or success and mediation of outcome by the presence or absence of suitable environmental conditions, aphid nymph ecdysis prior to EPF germination/cuticle penetration and stochastic nature of secondary EPF spore acquisition.

3.1.4. EPF candidate isolate selection: thermal biology

Owing to the intrinsic link between EPF efficacy and temperature, EPF isolate selection programmes universally should include an assessment of the thermal biology of candidate isolates in order to identify those that are capable of functioning well under the prevailing temperature conditions in the crop environment (Omuse *et al.,* 2021). Thermal evaluation is most commonly assessed through in vitro assays, recording the growth and germination of fungal isolates on a growth medium at different temperatures from which the cardinal temperatures can be estimated (Omuse *et al.*, 2021). Like other ectothermic organisms, the response of EPF to temperature takes the form of a skewed normal distribution (Figure 3.2), with growth and germination low at lower temperatures and increasing until reaching its peak at an optimal temperature, followed by a rapid drop with any further temperature increase. Non-linear regression models have been proposed as the most appropriate method to estimate both the thermal tolerance breadth and cardinal temperatures for EPF performance (Krenek *et al.,* 2012; Omuse *et al.*, 2021). Through such an approach, the maximum, minimum and optimum temperatures for germination/growth can be identified and the likely efficacy of EPF at specific temperatures can be inferred. While nonlinear thermal biology models are commonplace within many disciplines including entomology and microbiology, they are not yet standard practice in EPF isolate evaluations (Omuse *et al.*, 2021). Comparatively few nonlinear thermal models have therefore been evaluated for EPF growth and germination (Fargues *et al.*, 1997; Davidson *et al.*, 2003; Smiths *et al.*, 2003; Omuse *et al.*, 2021).

Studies by Smits *et al.* (2003) and Omuse *et al.* (2021) sought to address this problem by evaluating a range of nonlinear models for their goodness of fit and relevance. Within these studies, germination data were noted to be best modelled by the following models: cardinal temperature model with inflection (CTMI), modified Ratkowsky 3 model and the generalised β function model. The best fitting models for growth rate were meanwhile found to be CTMI (cardinal temperature model with inflection), modified Ratkowsky 3 (square root growth rate model), Lactin 1 and generalised β function (flexible sigmoid function of determinite growth) (Ratkowsky *et al.*, 1983; Zwietering *et al.*, 1991; Lactin *et al.*, 1995; Rosso *et al.*, 1995; Bassanezi *et al.*, 1998; Smits *et al.*, 2003; Omuse *et al.*, 2021).

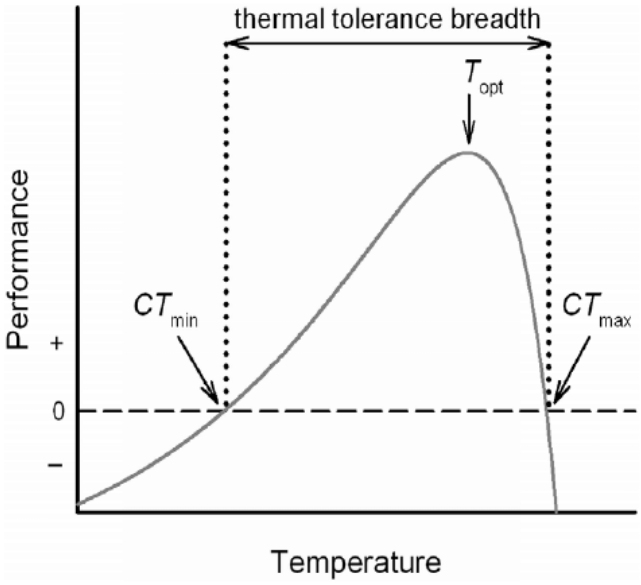


Figure 3.2: Typical right skewed normal curve response of performance at different temperatures of ectothermic organisms. (Figure taken from Krenek *et al.*, 2012).

3.1.5. Selection of EPF candidate isolates as biopesticides of *Brevicoryne brassicae*

The selection of a fungal isolate that can control the target pest is critically important for EPF biopesticide manufacturers. This involves screening candidate isolates against the target in laboratory assays, preferably using methods that are capable of reflecting the conditions that occur within the field. Candidate isolates can be obtained from culture collections (e.g., the USDA ARSEF collection has >13000 isolates of EPF representing >700 taxa that are available for use and lists the host of origin of individual isolates deposited in it) or by sampling isolates from the field (Sharma *et al.*, 2020).

Selection of the candidate pool of isolates for screening from a culture collection is not a trivial task. As a taxonomic order, the anamorphic hypocrealean EPF infect a diverse range of hosts. Within this, individual EPF genera, species and isolates demonstrate widely different levels of host specificity. Thus, while some taxa are true generalists, others show specificity to particular insect orders, families, genera or species (Fargues and Remaudiere, 1977; Rai *et al.*, 2014; Chandler, 2017; Rohrlich, 2018). This is typified by the genus *Metarhizium*, within which individual species occur ranging from the highly host-specific, through species with

intermediate host ranges, to true generalists (Hu *et al.*, 2014; Chandler, 2017). Other genera have narrower host ranges. For example, *Akanthomyces* spp. are mostly associated with hemipteran pests including whiteflies and aphids (Liu *et al.*, 2006; Chandler, 2017). At the within-species level, the highest virulence of an isolate is typically observed against the host of origin, which can be used to define the isolate as a pathotype (Goettel *et al.* 1990, Glare and Milner 1991). A good starting strategy, therefore, is to select candidate isolates from the target pest or its close relatives - however there is no guarantee that this approach will identify the most effective isolate. For aphids the ability to infect and control nymphs is crucial for biocontrol success (Kim and Roberts, 2012). However, most screening studies conducted to date focus solely upon aphid adults (Asi *et al.*, 2009ab; Derakhshan *et al.*, 2007; Akbari *et al.*, 2013; Soleymadzade *et al.*, 2019). Information about aphid-pathogenic isolates in public domain culture collections rarely provide details on whether the isolate is able to infect nymphs or not (Gladman, pers. observation).

To date, most studies of aphid pests of *Brassica* crop have investigated *Myzus persicae* (Filho *et al.,* 2011; Jandricic *et al.*, 2014; Lee *et al.*, 2015; Mohammed and Hatcher, 2017; Javed *et al.*, 2019; Manoussopoulos *et al.*, 2019; Nazir *et al.*, 2019). Fewer studies have been undertaken with the *Brassica* specialist *B. brassicae* (Asi *et al.*, 2009ab; Derakhshan *et al.*, 2007; Ramanujam *et al.*, 2017; Farag, 2008; Akbari *et al.*, 2013; Soleymadzade *et al.*, 2019; Prince and Chandler, 2020; Gebreyohans *et al.*, 2021). The majority of these studies have used adult aphids under fixed (i.e., constant) laboratory conditions (Asi *et al.*, 2009ab; Derakhshan *et al.*, 2007; Akbari *et al.*, 2013; Soleymadzade *et al.*, 2019). They have shown that *B. brassicae* is susceptible to a wide range of EPF species and isolates (including *B. bassiana*, *M. anisopliae*, *N. rileyi, Isaria fumosorosea*) particularly at concentrations of 1 x 10^7 conidia ml^{-1} or above and where optimal temperatures and humidity for rapid conidial germination were provided (Asi *et al.*, 2009ab; Derakhshan *et al.*, 2007; Akbari *et al.*, 2013; Soleymadzade *et al.*, 2019; Prince and Chandler, 2020; Gebreyohans *et al.*, 2021). Little is known about the susceptibility of *B. brassicae* nymphs. Three studies reported high sensitivity of nymphs to both commercial formulations and pure cultures of *M. anisopoliae* and *B. bassiana* (six isolates evaluated in total representing two species) (Farag, 2008; Pacheco *et al.*, 2017; Gebreyohans *et al.*, 2021). However, two studies evaluated mixed age nymph

populations rather than fixed age populations, making interpretation difficult. There thus remains a significant knowledge gap in the literature surrounding the performance of EPF against *B. brassicae* nymphs under laboratory conditions (Farag *et al.*, 2008; Pacheco *et al.*, 2017; Gebreyohans *et al.*, 2021).

3.1.6. Entomopathogenic fungi efficacy against *Brevicoryne brassicae* under glasshouse and field conditions

Soleymanzade *et al.* (2019*)* noted similar overall adult *B. brassicae* susceptibility to EPF in glasshouse conditions relative to a laboratory bioassay (laboratory and glasshouse conditions: 22 ± 2 °C, 70 ± 5 % RH, L:D 12:12h) but discovered a significantly reduced speed of kill (Soleymanzade *et al.*, 2019). Conversely, against adult *B. brassicae*, Gebreyohans *et al.* noted overall reduced EPF efficacy under glasshouse conditions, with higher calculated LC_{50} and LC_{90} *Akanthomyces lecanii* concentrations (Gebreyohans *et al.*, 2021).

Control of *B. brassicae* under field conditions is likely to be more challenging, particularly if abiotic conditions are not conducive to infection (temperature, humidity, UV radiation and rainfall). Conditions will vary significantly between geographic regions, meaning that some regions may be more suitable than others. In both 2014 and 2015, Ramanujam *et al.* (2017) evaluated the performance of three EPF isolates against *B. brassicae* adults in field cabbage crops at ICAR-NBAIR, Yalahanka Farm, Bengaluru, Karnataka, India during the typically warm and humid kharif season (June-September) – with treatment regimens consisting of four rounds of EPF foliar spray application at 10-day intervals. In this study, all three EPF isolates were able to significantly reduce *B. brassicae* populations on cabbage plants relative to untreated controls – with pooled final percentage reductions in aphid populations over the two years ranging from 83.9-87.1% resulting in significantly higher yields with applications of all three EPF relative to controls (Ramanujam *et al.*, 2017).

The effectiveness of four registered EPF-based products against *B. brassicae* has also been assessed by Prince and Chandler (2020) in two fields trials of Brussels sprouts at Warwick Crop Centre, Wellesbourne, Warwickshire, UK. Relative to India, the conditions for these field trials were less favourable for EPF growth and

germination – with a mean temperature throughout the trial of 16.8 °C and range of 14-24 °C. The most successful assessed product (Botanigard®, based on *B. bassiana*) resulted in a significant reduction of 73% total and nymph population size in the first trial following two EPF applications 7-days apart (Prince and Chandler, 2020). In the second trial, significant control was observed after just one spray, reducing *B. brassicae* populations by 54% - rising to 69% after a second application. Both of these studies recorded total population size rather than the population size of different life stages, thus it is unknown to what degree aphid nymphs were controlled relative to adults within this study (Prince and Chandler, 2020).

3.1.7. Aims and objectives

The aim of this chapter was to identify and characterise entomopathogenic fungi able to germinate/grow well at UK agronomically relevant temperatures and which were pathogenic to adult and first-instar *B. brassicae*. Ten EPF isolates were selected, and their growth and germination evaluated across a range of temperatures. The lethal and sublethal effects of these ten EPF isolates were then characterised, in a laboratory bioassay, against adult and first instar *B. brassicae*.

A subset of two EPF isolates were then selected for further assessment in a concentration-response assay and assessment of secondary pickup induced mortality. Finally, speed of germination and growth of this subset were quantified.

3.2. Materials and methods

3.2.1. EPF selection

Ten candidate EPF isolates were selected for inclusion in this study (Table 3.1). Isolates were selected to include: a range of current or historic commercially available isolates, isolates originally isolated from *B. brassicae* or other aphid species and isolates previously evaluated and confirmed for their virulence against aphids. EPF material was obtained from the University of Warwick Crop Centre EPF culture collection. In this collection filtered EPF suspensions of each isolate are combined with glycerol, adhered to porous cryotolerant plastic beads (Microbank Tm, Pro-lab Diagnostics Ltd, Liverpool, UK), and maintained at -80°C (Chandler, 1994).

3.2.2. EPF culturing and culture maintenance

EPF were cultured and maintained on Sabouraud dextrose agar (SDA). SDA was prepared as required by weighing out 40g of powdered Glucose (VWR International Ltd, Lutterworth, UK) 20g of Technical Agar number 3 (Oxoid Ltd, Basingstoke, UK) and 10g of mycological Peptone (Oxoid Ltd, Basingstoke, UK) into a 1L Duran bottle. To this powder, one litre of RO water was added, the bottle was then agitated, and a sterilized magnetic stirrer added. SDA solution was then autoclaved using a benchtop autoclave (121ºC for 21 minutes) after which the Duran was placed on a magnetic stirrer and allowed to cool for 30 minutes. SDA solution was then poured in a laminar flow hood into 9cm Petri dishes (Sarstedt AG and Co, Numbrecht, Germany) and 30ml universal tubes (Sarstedt AG and Co, Numbrecht, Germany) held at an angle to produce an SDA slope.

At 6-month intervals, two beads of each EPF isolate were removed from -80°C storage and spread on SDA slopes using a sterile plastic loop (VWR International Ltd, Lutterworth, UK). These were cultured for 14 days in darkness at 20°C (Sanyo Gallenkamp incubator) then stored at 5°C as a stock culture. Fungal cultures for experiments were produced by subculturing EPF from EPF slopes to a sterile 9cm SDA plate using a sterile loop spreader and cultured for 14 days in darkness at 20°C.

Table 3.1: Entomopathogenic fungi isolates selected for evaluation and screening against *Brevicoryne brassicae*. EPF isolates were obtained from the University of Warwick Crop Centre EPF culture collection. Isolates within the University of Warwick Crop Centre EPF culture collection are identified morphologically.

Isolate number (University of Warwick Culture Collection)	Species	Host (isolated from)	Country of isolation	On-label product name
1.72	*Akanthomyces dipterigenus (*Syns.*Lecanicillium* longisporum, *Verticillium longisporum, Verticillium lecanii*)	*Macrosiphoniella sanborni* (Chrysanthemum Aphid)	UK	Vertalec® (Koppert)*
19.79	*Akanthomyces muscarius* (syn. *Lecanicillium muscarium*)	*Trialeurodes vaporariorum* (Glasshouse Whitefly)	UK	Mycotal® (Koppert)
339.92	*Akanthomyces lecanii* (syn. *Lecanicillium lecanii*)	*Brevicoryne brassicae* (Cabbage Aphid)	UK	NA
347.92	*Beauveria bassiana*	*Delia radicum* (Cabbage Root Fly)	UK	NA
365.92	*Akanthomyces muscarius* (syn. *Lecanicillium muscarium*)	*Delia radicum* (Cabbage Root Fly)	UK	NA
416.96	*Metarhizium brunneum* (syn. *Metarhizium anisopliae*)	Soil	Finland	NA
432.99	*Beauveria bassiana*	*Anthonomus grandis* (Boll Weevil)	USA (Texas)	Naturalis®-L (Fargro®)
433.99	*Beauveria bassiana*	*Bemisia sp. (Whitefly)*	Unknown	BotaniGard®ES (Bioworks®)
1787.17	*Akanthomyces* sp. (syn. *Lecanicillium* sp.)	*Myzus persicae* (Green Peach Aphid)	UK	NA
1808.18	*Beauveria bassiana*	*Myzus persicae* (Green Peach Aphid)	UK	NA

3.2.3. Preparation of EPF conidial suspensions

Conidial suspensions were prepared from 14-day old EPF cultures. 10ml of 0.05% Triton X-100 (BDH Lab supplies, Lutterworth, UK) was added to the culture plate of isolates from which suspensions were required. Using a sterile 'L' shaped spreader (Greiner Bio-one, Gloucester, UK), conidia were then agitated into suspension. The EPF suspension was filtered through a 19cm diameter sterile milk filter (Goat Nutrition Ltd, Kent, UK) to remove mycelial fragments and diluted 10- fold in 0.05% Triton X-100. Conidia concentration was then determined using an Improved Neubauer haemocytometer (Scientific Laboratory Supplies, Nottingham, UK) and suspensions adjusted to the desired concentration with 0.05% Triton X-100.

3.2.4. Confirmation of EPF species identity using nucleotide sequences

All ten EPF isolates were subcultured from slope cultures onto 9cm SDA plates and incubated in the dark at 20°C for 14 days (as described in section 3.2.2). Fungal mycelia were carefully scraped from the surface of each EPF mycelial mass using a sterile scalpel (Swann-Morton, Sheffield, UK) and transferred to a 2ml Eppendorf tube (Eppendorf, Hamburg, Germany). Extraction buffer (Qiagen, Netherlands) was added to the fungal material which was then ground using a sterile micro pestle (Eppendorf, Hamburg, Germany). EPF DNA was extracted using a Qiagen DNeasy plant mini kit (Qiagen, Netherlands) following the manufacturer's guidelines. Extracted DNA was then evaluated using a NanoDrop ® ND-100 Spectrophotometer (ThermoFisher Scientific, USA) to ensure sufficient quality and quantity for PCR amplification. PCR reaction mixtures (25µl) comprised of: 12.5µl RedTaq Ready Mix (Sigma-Aldrich, USA), 1µl of template DNA, 1µl each of forwards and reverse primers (Table 3.2) and 9.5µl of double purified PCR water (Just water, Microzone Ltd, Sturbridge, UK). Separate reaction mixtures were prepared for each EPF isolate-housekeeping gene combination before amplification on a GeneAmp ® PCR System 9700 (Applied Biosystems, USA).

The PCR conditions used for reaction mixtures containing ITS primers were: initial denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 45s, 55°C for 30s and 72°C for 1 min. The conditions for large ribosomal subunit primers were: initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 50°C for 45s and 72°C for 1 min. All PCR reactions were followed by a final incubation for 7

min at 72°C. The resulting PCR products were evaluated via gel electrophoresis on 1.2% agarose gels (Sigma-Aldrich, USA) in TAE buffer (Tris-Acetate EDTA, Fisher Scientific, USA) containing 2µl/50ml of GelRed ® (Biotium, USA). The gels were run in TAE buffer in a horizontal electrophoresis cell (BIO-RAD, USA) at 90V for 1 hour and bands visualised using a UV transilluminator (G:BOX, Synegene, U.K). Successfully amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen, Netherlands) and sequenced by GATC Biotech using forward and reverse primers independently (5 µM) for each gene and EPF isolate.

Using forward and reverse sequences, a consensus sequence was built using BioEdit 7.2 (Tom Hall). Sequence database searches were then performed using determined consensus sequences and the BLAST tool at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997) to identify closest percentage identity matches.

Table 3.2: Primers used for confirmation of EPF species.

Gene	Primer Name	Primer Sequence (5'-3')	Reference
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	White *et al.*, 1990
	ITS4	TCC GCT TAT TGA TAT GC	
Large ribosomal subunit	LROR	ACC CGC TGA ACT TAA GC	Vilgalys and Hester, 1990
	LR5	TCC TGA GGG AAA CTT CG	

3.2.5. EPF physiology assessments

For each of the ten selected EPF isolates, colony extension rates and germination at six different temperatures was evaluated on SDA.

3.2.5.1. Colony extension of EPF isolates at 6 temperatures

Conidial suspensions of 1 x 10^7 conidia ml^{-1} were prepared as detailed in section 3.2.3. To four sterile SDA 9cm petri dishes, 100µl of prepared EPF suspension was added and spread using a sterile L-shaped spreader and the plate incubated in

darkness for 48hrs at 20°C. Using a sterilized metal cork-borer, 14mm plugs of EPF were taken, inverted, and placed centrally on a 9cm SDA Petri dish upon which X and Y axes were pre-drawn. These were then cultured in darkness at either 5, 10, 15, 20, 25 or 32.5°C. Colony extension was assessed every 2 days for a 14-day period using a ruler to measure the size of mycelial mass from the centre of the pre drawn X and Y axis. Two replicates of each EPF/temperature combination were assessed per experimental replicate, with three total experimental replicates giving a total of six plates of each EPF/temperature combination. Following cessation of experiment, results were converted into growth rate (mm day^{-1}).

3.2.5.2. EPF germination at 6 temperatures

20µl 1 x 10^7 ml^{-1} conidial spore suspension (section 3.2.3) was pipetted onto three marked circles on 5cm SDA plates (Sterlin, Newport, UK) (prepared as per section 3.2.2) marked with a circle on the bottom of the plate and incubated for 24 h in the dark at either 5, 10, 15, 20, 25 or 32.5°C. Germination was stopped using lactophenol cotton blue stain (Prolab diagnostics, Liverpool, UK) and the number of germinated and ungerminated conidia of each isolate were counted from a group of ≥100 conidia selected at random under a microscope (Zeiss Axioskop Routine Microscope). Conidia were considered germinated if emerging mycelial germ tube was equal to or greater than the length of the conidia from which it was emerging. Two technical replicates of each EPF/temperature combination were assessed per experimental replicate, with three experimental replicates giving a total of six plates per treatment. The results were expressed as percentage germination.

3.2.5.3. EPF physiology assessment – analysis

Maximum, optimum and minimum temperatures for EPF growth and germination were estimated using a nonlinear modified Radkowsky-3 model for each EPF isolate (Ratkowsky *et al.*, 1983; Zwietering *et al.*, 1991; Omuse *et al.*, 2021).

$$G = [b\,(T - T_{min})]^2\,[1 - e^{\,c(T-T_{max})}]$$

Within this model, the input 'G' can represent either percentage conidial germination (%) or mycelial growth rate (mm day^{-1}). 'T' represents a second input variable for

the temperature at which 'G' was observed. The parameters 'b' and 'c' are 'Ratkowsky parameters', with units $°C^{-1}\ h^{-0.5}$ and $°C^{-1}$, respectively. 'Tmin' and 'Tmax' conversely represent the temperature at which the theoretical minimum and maximum value for germination or growth occur (Omuse *et al.*, 2021).

Fitting the model was performed using the Microsoft Excel Solver add-in program (Microsoft Corporation, Redmond, Washington, USA, 2021). Models were fitted using a GRC nonlinear engine which identified the minimum possible sum of square residuals through modification of the four Ratkowsky-3 model parameters (b, c, Tmin and Tmax). Temperature optima for growth and germination of each isolate were determined through usage of a reparamiterised Ratkowsky-3 model equation developed by Adams *et al.* (2017).

$$b = \frac{P_{max}}{(T_{opt} - T_{min})^2\,(1 - e^{\,c(T - T_{max})})}$$

Within this model, the original modified Ratkowsky model is reparamiterised through mathematical substitution resulting in a removal of the redundant Ratkowsky parameters 'b' and 'c' and integration of additional parameters, 'Topt' and 'Pmax', into the new equation. 'Topt' represents the temperature at which the growth or germination are at their maximum level. 'Pmax' conversely represents the specific maximal growth/germination response at 'Topt'. Input parameters used for 'Tmin' and 'Tmax' for each model were 5 and 35 respectively.

3.2.6. Laboratory bioassay to quantify susceptibility of *Brevicoryne brassicae* to EPF isolates

Ten EPF isolates were assessed against both fixed-age 10-day old adult *B. brassicae* and 1-day old fixed-age *B. brassicae* first instar nymphs. The bioassay methodology used for adult and first instar *B. brassicae* differed marginally owing to the fragility of first instar aphids resulting in significant control mortality if nymphs are handled.

The first step of bioassay setup for both adult and nymphs was raising plants for maintenance of *B. brassicae.* Throughout the course of this first bioassay, 5-week-old technical control plants (*B. oleracea* Var. *Gemmifera* 'Doric F1') were grown as

described in section 2.2.4. Fourteen days prior to bioassay set-up EPF isolates were spread from stock slope cultures onto 9cm SDA Petri dishes (section 3.2.2). On the day preceding bioassay setup, 1 x 10^7 conidia ml^{-1} suspensions for each EPF isolate were prepared (section 3.2.3) and stored overnight at 4°C.

Ten days prior to bioassay setup, fixed-age 10-day old adult *B. brassicae* cultures were established (section 2.2.2). On the day of setup, forty fixed-age adult *B. brassicae* were removed from the fixed-age adult culture and transferred onto damp filter paper in eleven Petri dishes – one for each EPF isolate and a mock/control treatment (0.05% Triton-X100). From the same fixed-age adult *B. brassicae* culture, on the day preceding bioassay setup, 1-day-old *B. brassicae* cultures were established by transferring a cohort of 10 fixed-age adults to leaves of fresh 5-week-old technical control plants. These adults were then contained in a clip-cage and allowed 24hrs to reproduce, producing a fixed-age first-instar cohort. On the day of the bioassay, all adult *B. brassicae* were removed leaving a cohort of 1-day old fixed-age first instar nymphs on the plants.

EPF conidia were applied using a Potter tower, with 2ml of the 1 x 10^7 conidia ml^{-1} suspension being sprayed at 50 kPa per Petri dish/leaf (Potter, 1952). Fixed-age adult cohorts of *B. brassicae* were sprayed separately in Petri dishes, allowed to dry and recover for 1 hour and then transferred to fresh control plants. Fixed-age first-instar *B. brassicae* were sprayed in-situ on plant leaves. Following spraying, 15 adult *B. brassicae* were transferred to leaves of two fresh control plants per treatment. These leaves were enclosed in a Bioassay box alongside damp filter paper to ensure sufficient humidity for conidial germination (Figure 3.3). The leaves of plants upon which sprayed 1-day-old first-instar nymphs were held were treated identically, also being enclosed in a bioassay box containing damp filter paper (Figure 3.3). Following bioassay setup, all plants were transferred to and maintained in a controlled environment room (20°C, 50% RH, L:D 16:8 h). After 24 hours, the solid/unventilated bioassay box lid was swapped to a ventilated lid to allow for a reduction in humidity (Figure 3.3). The bioassay was then read daily recording aphid mortality.

For later experiments assessing EPF in combination with partially resistant plant accessions only two EPF isolates were used. These were selected from the virulence bioassay using the hazard ratio, survival curve and aphid median survival time following the application of ten selected EPF isolates at a fixed concentration of 1 x 10^7 conidia ml^{-1} on control plants (*B. oleracea* var. Gemmifera 'Doric F1') against both adult and first instar *B. brassicae*.

Bioassay box humidity conditions were monitored throughout the experimental period using a humidity monitor (Ibuttons Measurement Systems Ltd, Berkshire, UK), with an observed average relative humidity of 95.65% (0.133 SEM) when the bioassay chamber had a solid plastic lid, and 54.06% humidity (0.232 SEM) when lid was swapped to a ventilated lid. These conditions were selected based on a pilot study as outlined in Figure 3.4.

All deceased aphids were transferred to damp-filter paper in Petri dishes, sealed with Nescofilm (Fisher Scientific, Braunschweig, Germany) and incubated in the dark at 20°C for 5-days. Microscopy confirmed that the applied EPF species was the causative agent of death. All EPF treated aphids showed signs of mycosis This bioassay was repeated 3 times (6 total technical replicates per EPF isolate) encompassing the spraying and subsequent mortality reading of ~90 aphids per isolate.

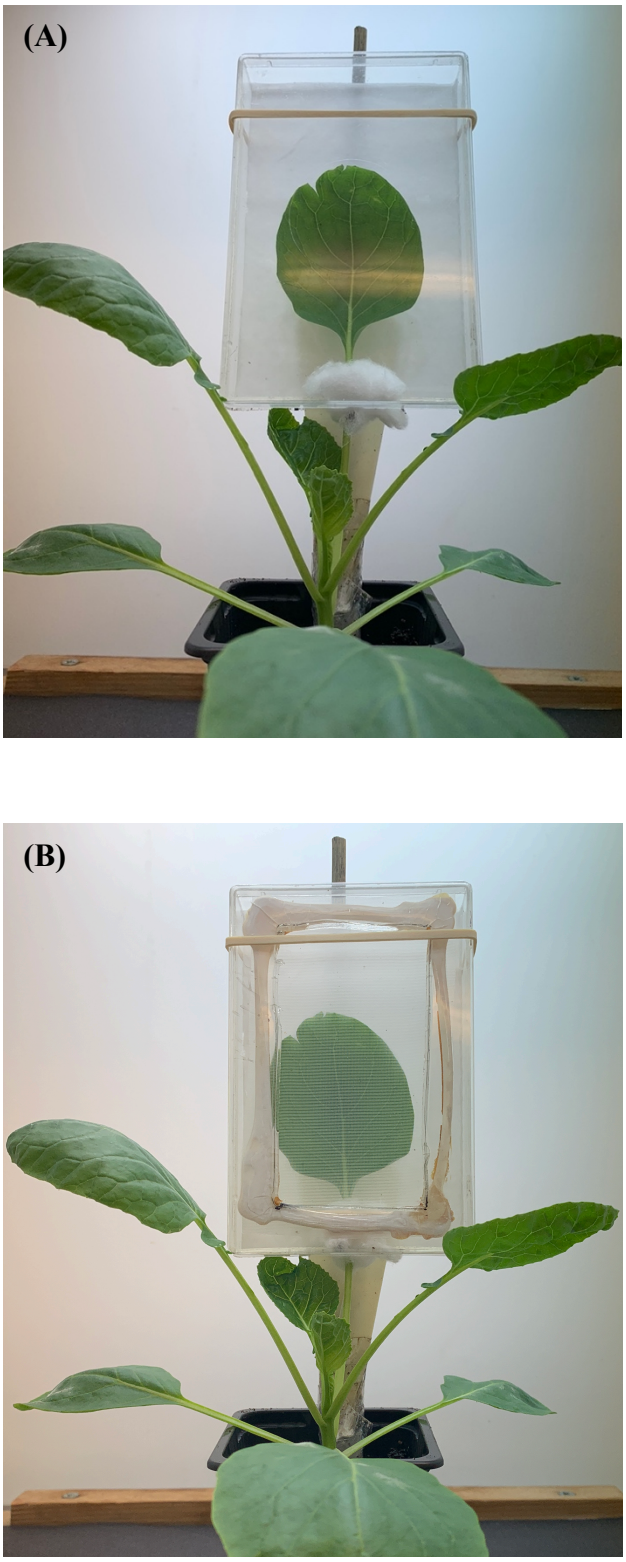


Figure 3.3: Bioassay set-up for EPF bioassay experiments. Following EPF treatment of aphids and transfer to leaves, *Brassica* leaves were enclosed in a clear plastic bioassay box which also contained damp filter paper to increase ambient humidity. For the first 24hrs bioassay box had a solid plastic lid (A) ensuring high humidity to promote conidial germination. After 24 hours, the solid/unventilated bioassay box lid was swapped to a ventilated/mesh fronted lid (B) to allow for a reduction in humidity. The bioassay was then read daily recording aphid mortality.

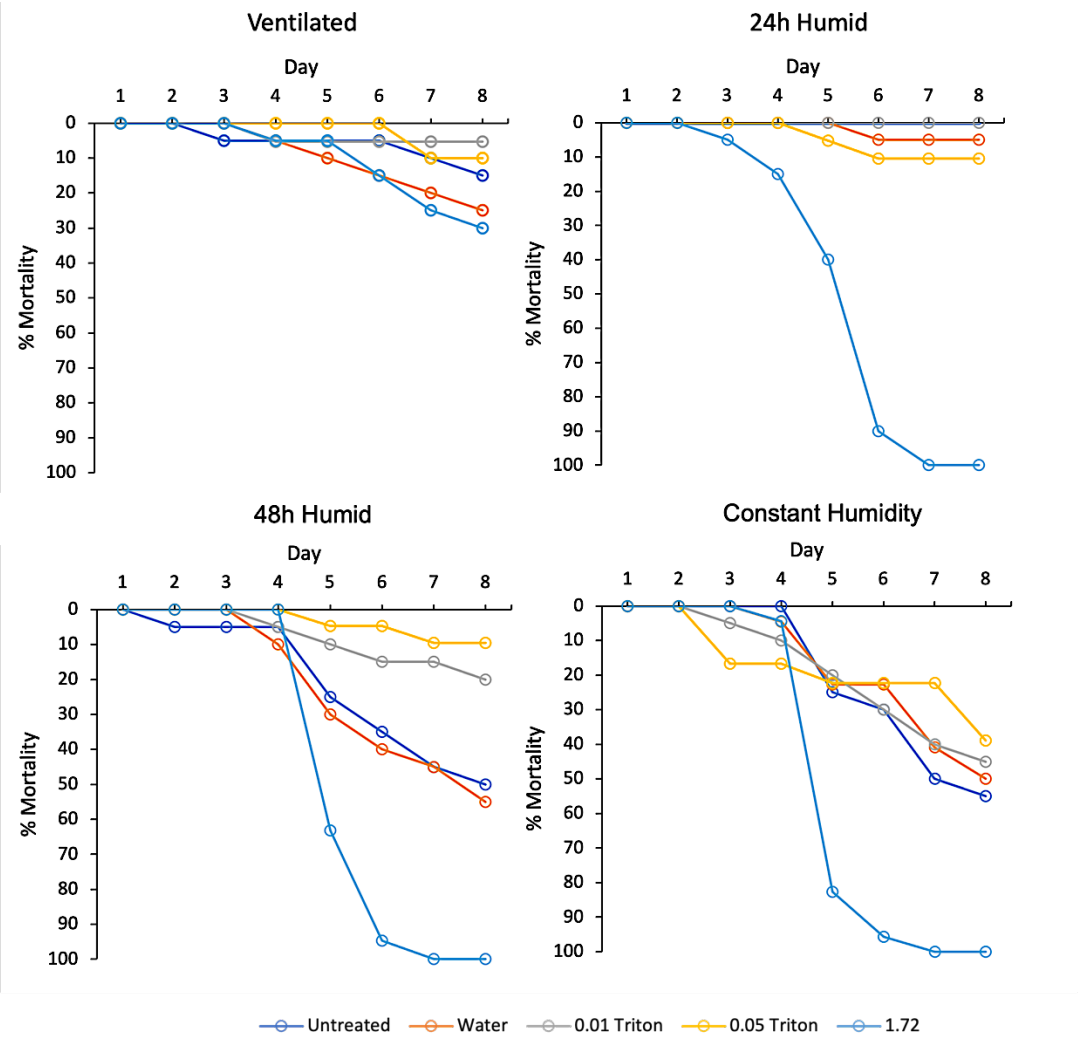


Figure 3.4: Pilot assay assessing the optimal duration of Bioassay chamber high humidity and solution into which EPF conidia were suspended. Four humidity conditions were tested: constant ventilated conditions (bioassay chamber with a ventilated lid at all times throughout the experiment), 24 hours of high humidity (solid plastic bioassay chamber lid which was then swapped after 24 hours to a ventilated lid), 48 hours of high humidity (solid plastic bioassay chamber lid which was then swapped after 48 hours to a ventilated lid) and constant high humidity (bioassay chamber with a solid plastic lid throughout the experiment). Four suspension solutions were evaluated, pure water, 0.01% Triton-X100, 0.05% Triton-X100 and EPF isolate 1.72 (*Akanthomyces dipterigenus*) at a concentration of 1 x 10^7 conidia ml^{-1}

3.2.6.1. EPF bioassay – statistical analysis

Bioassay results were analysed using two methods: survival analysis and GLMM analysis. Survival analysis allows for evaluation of treatment effects across the entire experimental period but is unable to account for factors such as replicate. Conversely through GLM/GLMM analysis, such effects can be accounted for as random effects however the analysis can only be undertaken at specific timepoints. Through

undertaking both statistical approaches, these data could therefore be analysed in complementary ways.

Survival analysis (Kaplan-Meier, and a Cox-proportional hazards model) was undertaken to assess EPF isolate effects across the entire experimental period (days 1-9). Through Kaplan-Meier analysis, survival curves were plotted for each EPF isolate against both aphid life stages, and estimates made of both median survival times and EPF isolate hazard ratios relative to a mock treated control (Goel *et al.*, 2010). The Cox-proportional hazards model was used to evaluate treatment and experimental replicate as factors. Hazard ratios for each EPF isolate relative to a mock treated control were determined, and pairwise log-rank tests were performed between all EPF isolates (Matthew *et al.*, 1999; Goel *et al.*, 2010). (IBM SPSS Statistics for Macintosh, Version 27.0.)

GLMM analysis was undertaken to assess EPF-induced cumulative mortality on experiment days 6 and 9. Through this analysis, the significance of treatment (EPF) overall could be evaluated alongside post-hoc tests which can identify which EPF isolates are resulting in significantly different cumulative mortality relative to one another on these specific days. Within each GLMM analysis a binomial distribution and logit link function were used. Treatment was included as a sole main effect factor while replicate was included as a random effect within each analysis. Owing to evaluation of mortality on both days 6 and 9 for each aphid life stage, a Bonferroni correction was applied altering significance threshold to 0.025. GLMM results were plotted as a series of four bar graphs demonstrating mean cumulative mortality and SEM error bars for variation across experimental replicates. Results from GLMM and GLMM post hoc tests were then added to this graph to demonstrate significant differences between EPF isolates (IBM SPSS Statistics for Macintosh, Version 27.0.).

3.2.6.1.1. Assessing effects of EPF treatment on adult *Brevicoryne brassicae* reproduction

Alongside mortality, adult *B. brassicae* reproduction was recorded each day throughout the entire bioassay period (as described in section 3.2.6). Each day, total

number of nymphs produced were counted and recorded, with all present nymphs then being manually removed. Reproduction was recorded daily for 9-days.

3.2.6.1.2. Assessing effects of EPF treatment on adult *Brevicoryne brassicae* reproduction – statistical analysis

Average daily reproduction per adult was calculated by dividing recorded reproduction on day X by the number of alive adult *B. brassicae* on the previous day (X-1). Reproduction results were analysed using a GLM with a negative binomial distribution, log link function and custom dispersion parameter, including EPF isolate (10 treatments and a mock control) and Day (9 days) (IBM SPSS Statistics for Macintosh, Version 27.0.). Replicate was also included as a factor and interaction between replicate and other factors evaluated.

3.2.7. EPF concentration-response assay

EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were selected for later experiments with partially resistant plant accessions. To determine median lethal concentrations (LC_{50}, LC_{70} and LC_{90}) of both EPF isolates for (a) 10-day-old fixed-age adult *B. brassicae* and (b) 1-day-old fixed-age first-instar *B. brassicae*, a concentration-response bioassay was undertaken. This bioassay experiment utilised identical methodology as the bioassay outlined in 3.2.6, differing only in the EPF concentrations used. Six sequential concentrations of isolates 1.72 and 433.99 were assessed in this bioassay: 4, 4.5, 5.0, 5.5, 6.0 and 7.0 Log_{10} conidia ml^{-1} (1×10^4, 3.2×10^4, 1×10^5, 3.2×10^5, 1×10^6, 1×10^7 conidia ml^{-1}) alongside a control treatment of 0.05% Triton-X100. Each bioassay was repeated 3 times (6 technical replicates per EPF isolate) encompassing the spraying and subsequent mortality reading of ~90 adult aphids per concentration of each isolate. Nymph numbers varied depending upon the number of first-instar aphids produced.

3.2.7.1. EPF concentration-response assay – statistical analysis

Results for the lethal effects of isolates 1.72 and 433.99 in the concentration response assay were analysed by three methods.

Firstly, results were subject to Kaplan-Meier survival analysis (SPSS), Log rank tests, and Cox-proportional hazards models (SPSS). In Kaplan-Meier analysis, EPF

and concentration were considered as a combined treatment thus 12 treatments were assessed relative to control to determine median survival time (where concluding mortality exceeded 50%) and hazard ratios (Matthew *et al.*, 1999; Goel *et al.*, 2010). Pairwise analyses between treatments were then performed using Log rank tests pooled over strata to assess between treatment significant differences in hazard ratio/survival curve (Matthew *et al.*, 1999; Goel *et al.*, 2010). Finally, to assess the significance of factors, two Cox proportional hazards models were created, the first included two factors: Replicate, and combined treatment. The second Cox model included three factors replicate, EPF isolate and concentration – the latter two being constituents of the previously assessed combined treatment. All two-factor interactions including replicate in each model were included to evaluate the influence of replicate variation upon results (IBM SPSS Statistics for Macintosh, Version 27.0.).

Results were also analysed through GLM/GLMM to evaluate mortality on the final day of assessment (day 9) between treatments (EPF isolate + concentration). All undertaken GLM/GLMM models utilised a binomial distribution and logit link function. All analyses also included replicate as a factor alongside the interactions of replicate with other factors (IBM SPSS Statistics for Macintosh, Version 27.0.).

Adults were initially analysed through GLM including both treatment (EPF + concentration) and replicate as factors. Subsequent analysis was undertaken through GLMM analysis including treatment as a sole factor and replicate as a random effect, with post hoc analyses also undertaken to evaluate pairwise significance between all treatments. Nymphs were analysed through GLM with treatment as a sole factor (owing to replicate being insignificant). Post hoc analyses were also undertaken to evaluate pairwise significance between treatments.

Average mortality across the three replicates for each treatment (2 EPF x 6 concentration combinations + 1 control) were plotted for both adult and nymph *B. brassicae*. Post hoc GLM/GLMM test results were included as significance marking letters in these plots. To all four of EPF/concentration scatter plots, a five-parameter logistic concentration-response curve (5PL) was fitted.

$$y = \frac{a + (b - a)}{\left[1 + \left(\frac{c}{x}\right)^{d}\right]^{e}}$$

Within this model variable 'y' was the modelled output while variable 'x' consisted of the six Log_{10} concentrations at which y was assessed. Five parameters were included in the model, 'a' (the theoretical response at zero concentration), 'b' (the theoretical response at infinite concentration), 'c' (mid-range concentration/inflection point ec_{50}), 'd' (slope at inflection point) and 'e' (asymmetry parameter). Model was fitted using the Microsoft Excel Solver tool, minimising the square residuals of observed and modelled y values by modifying the parameters 'a', 'b', 'c', 'd' and 'e' (Gottschalk and Dunn, 2005).

The final analysis undertaken on concentration-response data was probit regression (SPSS) which explored the total number of recorded events at experimental cessation (day 9) relative to the total number of individuals assessed. Probit regression was undertaken to allow for calculation of lethal concentration estimates for each EPF isolate/aphid age combination (LC_{50}, LC_{70} and LC_{90}) (Finney, 1952). Probit regression for *B. brassicae* adults and nymphs for isolates 1.72 and 433.99 was undertaken separately (IBM SPSS Statistics for Macintosh, Version 27.0.)

3.2.7.2. EPF concentration -response assay - assessing effects of EPF treatment upon *Brevicoryne brassicae* reproduction

Alongside mortality, adult *B. brassicae* reproduction was also recorded each day throughout the concentration-response bioassay period. Each day, total number of nymphs produced were counted and recorded, with all present nymphs then being manually removed. Reproduction was therefore recorded daily for 9-days. Per-adult average daily reproduction was calculated by dividing recorded reproduction on day X by the number of alive adult *B. brassicae* on the previous day (X-1).

3.2.7.3. EPF Concentration response assay - assessing effects of EPF treatment upon *Brevicoryne brassicae* reproduction – statistical analysis

Reproduction results were analysed through GLM/GLMMs each with a negative binomial distribution, log link function and custom dispersion parameter (SPSS). One GLM and three GLMMs were undertaken to identify the best model fit to observed reproduction results. Within the first GLM model three factors were included: Treatment (EPF isolate + concentration), day, and replicate. The first subsequent GLMM included Treatment (EPF isolate + concentration) and day as factors while replicate was included as a random effect. The second GLMM included EPF isolate, concentration and day as factors alongside replicate as a random effect while the third GLMM included EPF isolate and concentration as factors while day and replicate were included as random effects. To evaluate these four models, Akaike information criterion (AIC) scores were considered, with the second GLMM model presenting the lowest overall AIC score thus being confirmed as the preferred model. All GLM/GLMM models were undertaken using SPSS (IBM SPSS Statistics for Macintosh, Version 27.0.)

3.2.8. Assessing viable conidia sprayed per unit area by Potter tower

To determine the number of viable conidia sprayed per unit area (i.e. the dose) during the Potter tower spray procedure, three spore suspensions each of the two EPF isolates (1.72, *A. dipterigenus* and 433.99, *B. bassiana*) were prepared (section 3.2.3) at concentrations of 1 x 10^7 conidia ml^{-1} (7 Log_{10} conidia ml^{-1}) and the calculated LC_{70} for adults and nymphs for each isolate. Three glass coverslips (22 x 22 mm, Thermoscientific, Braunschweig, Germany) were placed on damp filter paper in six 9 cm Petri dishes – mimicking the conditions for aphid spraying during the bioassay procedure. Using a Potter tower, 2ml of conidial suspensions was sprayed on each Petri dish at 50 kPa (Potter, 1952). Using sterilised tweezers, each coverslip was transferred to a 20ml universal tube containing 1ml of 0.05% Triton-X100 solution and the tube vortexed for 2 minutes. Resulting conidial suspensions were serially diluted and 100µl of each dilution pipetted onto a 9cm SDA plate and spread using a sterile 'L' shaped spreader. Plates were incubated for 3 days in darkness at 20°C (Sanyo Gallenkamp incubator) and the colonies counted. The experiment was replicated a total of three times. Using colony counts, known dilution, and known area of coverslip, viable conidia mm^{-2} was calculated, and

differences assessed via ANOVA with Tukey post-hoc tests (IBM SPSS Statistics for Macintosh, Version 27.0.).

3.2.9. Secondary pickup assay of first instar *Brevicoryne brassicae* and EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*)

Owing to *B. brassicae* first-instar nymphs being sprayed in-situ on leaves, *B. brassicae* nymphs receive a primary EPF dose directly from the spray but may also acquire EPF conidia secondarily from conidia present on leaves during movement across the leaf surface. A bioassay was therefore undertaken to assess the proportion of observed nymph mortality occurring due to secondary conidia pickup.

This bioassay experiment utilised similar methodology as the nymph bioassay outlined in 3.2.6, however technical control plant leaves were sprayed prior to aphid colony establishment. Sprayed leaves were allowed one hour to dry after which 1-day-old *B. brassicae* cultures were established by transferring a cohort of ten 10-day-old fixed-age *B. brassicae* adults onto them. These adults were then contained in a bioassay box with a solid plastic lid and allowed 24hrs to reproduce, producing a fixed-age first-instar cohort. After 24 hours the solid plastic lid was swapped to a ventilated plastic lid and the instar cohort was monitored daily for mortality. Bioassay box humidity conditions were 95.7% (0.133 SEM) humidity with solid lid and 54.1% humidity (0.232 SEM) when lid was swapped to a ventilated lid. All deceased aphids were transferred to damp-filter paper (Whatman, Maidstone, UK) in 9cm Petri dishes, sealed with Nescofilm and incubated in the dark at 20°C for 5-days. Microscopy confirmed that mycosis by the applied EPF species was the causative agent of death. This bioassay was repeated 3 times (6 total technical replicates per EPF isolate)

3.2.9.1. Secondary pickup assay of first instar *Brevicoryne brassicae* and EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) – statistical analysis

Secondary pickup bioassay results were analysed through survival analysis and GLMM analysis. Survival analyses undertaken were Kaplan-Meier, Log rank tests and Cox-proportional hazards models (SPSS). Through Kaplan-Meier analysis,

survival curves were plotted for the two EPF isolates for which secondary pickup effects were being assessed against *B. brassicae* nymphs. Kaplan-Meier analysis also allowed for the calculation of hazard ratios relative to a control. Note that median survival times could not be calculated in this analysis owing to <50% mortality in all three treatments. Log-rank tests were performed pairwise over all strata allowing for calculation of significant differences in hazard ratios and survival curves between all treatments. Finally, a Cox-proportional hazards model was undertaken to evaluate significant factors within the experiment, with both treatment and experimental replicate being evaluated as factors (IBM SPSS Statistics for Macintosh, Version 27.0.). GLMM analysis was undertaken to assess secondary pickup EPF-induced mortality specifically on day 9. Through this analysis, the significance of treatment (EPF) overall could be evaluated alongside post-hoc tests which were used to identify which differences in cumulative mortality between treatments. GLM analysis used a binomial distribution and logit link function with treatment included as a sole main effect factor (IBM SPSS Statistics for Macintosh, Version 27.0.).

3.2.10. Evaluation of germination and growth of conidia germ tubes over a 24hr time-course of EPF isolates 1.72 (A*kanthomyces dipterigenus*) and *433.99 (Beauveria bassiana*)

Germination assay was prepared as in section 3.2.5.2. 20µl 1 x 10^7 conidia ml^{-1} (7 Log10 conidia ml^{-1}) suspensions (section 3.2.3) were pipetted onto 3 marked areas of 4.5 cm SDA plates (section 3.2.2) and incubated in darkness at 20°C. At 2-hour intervals over a 24-hour period, germination of conidia on three plates was stopped using lactophenol cotton blue stain. The number of germinated and ungerminated conidia on each plate was counted from a group of ≥100 conidia, selected at random under a microscope (Ziess Axioskop Routine Microscope). Conidia were considered germinated if emerging mycelial germ tube was equal to or greater than the length of the spore. Three replicates of each EPF/time combination were assessed. Where >10 conidia had germinated, 10 conidia were randomly selected per SDA plate and their germ tubes measured using ImageJ software (Schneider *et al.*, 2012).

3.2.10.1. Evaluation of germination over a 24hr time-course of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) – statistical analysis

Results for 24-hour germination experiment were plotted as a scatter plot and a three-parameter sigmoidal logistic growth curve was fitted independently for both isolates.

$$y = \frac{L}{1 + e^{-k(x - x_0)}}$$

Within this model variable 'y' was the modelled output while variable 'x' were the timepoints at which y was assessed. Three parameters were included in the model, 'L' (the curves maximum value), 'k' (the logistic growth rate/steepness of the curve) and 'X_0' (the value of the sigmoid midpoint). Model was fitted using the Microsoft Excel Solver tool (GRC nonlinear engine) minimising the square residuals of observed and modelled y values by modifying the parameters 'L', 'k' and 'X_0'. To evaluate germination results, logistic regression was undertaken. Germination data was input into this model in a binomial format (1=germinated, 0=not germinated). This model utilised a binomial distribution and a logit link function. EPF isolate was included as a factor while time (0-24hrs at 2hr intervals) was included as a covariate. An interaction term of EPF isolate x time was also included in the model to determine whether there existed a significant difference in plotted logistic curves indicating a significant different in EPF isolates over time. Through this logistic regression, the median 5%, 50% and 95% germination times of each EPF isolate were also calculated along with 95% confidence intervals for each (IBM SPSS Statistics for Macintosh, Version 27.0.).

3.2.10.2. Evaluation of germ tube growth over a 24hr time-course of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) – statistical analysis

Results for 24-hour germination experiment were plotted as a scatter plot for both isolates. An exponential trendline was found to offer the best model for isolate 1.72 (*A. dipterigenus*) while a linear trendline was plotted for isolate 433.99 (*B. bassiana*) results, with this offering the best model fit. To normalise these data, length results

for both isolates were transformed through a Log_{10} transformation. Results were then analysed through multiple linear regression analysis, including EPF isolate as a factor, time as a covariate and an interaction term (EPF isolate x time) to allow for evaluation of whether lines for each isolate over time were parallel (IBM SPSS Statistics for Macintosh, Version 27.0.).

3.3. Results

3.3.1. Confirmation of EPF species identity

For all ten EPF isolates examined, sequencing for both housekeeping genes confirmed morphological identification with percentage identities ranging from 99.77-100% (Table 3.3).

Table 3.3: EPF sequence identity check results. For each isolate, PCR amplification of ITS and Large ribosomal subunit was performed, and products sequenced. A consensus sequence was constructed, and a nucleotide BLAST search performed, and the closest percentage identity match reported. All closest percentage identities correspond with previous morphological identification of isolates.

Isolate code	Gene	% identity match	Scientific Name	NCBI Accession no.	Reference
1.72	ITS	99.80%	*Akanthomyces dipterigenus*	MZ930384.1	Nagaraj, S. B., Panneerselvam, M., Krishnaswamy, S. and Shanmugam, A. Unpublished
	Large Ribosomal Subunit	99.88%	*Akanthomyces dipterigenus*	NG_058105.1	Park, M. J. and Shin, H. D., Morphological and phylogenetic studies on the genus *Lecanicillium*, Unpublished
19.79	ITS	99.81%	*Akanthomyces muscarius*	MH858369.1	Vu *et al.* (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology,* 93: 135-154
	Large Ribosomal Subunit	99.89%	*Akanthomyces muscarius*	MH867223.1	Vu *et al.* (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology,* 93: 135-154
339.92	ITS	100.00%	*Akanthomyces lecanii*	KJ093501.1	Gonthier, P., Guglielmo, F., Sillo, F., Giordano, L. and Garbelotto, M. A molecular diagnostic assay for the detection and identification of wood decay fungi of conifers. Unpublished
	Large Ribosomal Subunit	99.89%	*Akanthomyces lecanii*	EF026005.1	Jung, H. S. and Kim, K. Selection of Lecanicillium strains for aphid control. Unpublished
347.92	ITS	99.81%	*Beauveria bassiana*	MW534280.1	Imrek, B. and Erler, F. Morphological and molecular diagnosis of entomopathogenic fungus isolated from different regions of Antalya, determination of their efficacy against *Tetranychus urticae*. Unpublished
	Large Ribosomal Subunit	99.88%	*Beauveria bassiana*	MT159433.1	Lehenberger, M. and Biedermann, P. H. Unpublished
365.92	ITS	99.81%	*Akanthomyces muscarius*	MT153619.1	Bien, S. and Damm, U. (2020). Prunus trees in Germany - a hideout of unknown fungi? *Mycological Progress*, 19: 667-690
	Large Ribosomal Subunit	99.77%	*Akanthomyces muscarius*	MH868282.1	Vu *et al.* (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology,* 93: 135-154
416.96	ITS	100.00%	*Metarhizium brunneum*	EU918737.1	Herzig, T., Schweizer, C., Kuhn, R., Grunder, J., GC, Y. D., Rawat, P. R. and Keller, S. Unpublished
	Large Ribosomal Subunit	100.00%	*Metarhizium brunneum*	MH143817.1	Chen, Z.-H. The Species Diversity of Metarhizium in the Gaoligong Mountains of China. Unpublished
432.99	ITS	100.00%	*Beauveria bassiana*	MT528702.1	Li, C. Unpublished
	Large Ribosomal Subunit	100.00%	*Beauveria bassiana*	MT159433.1	Lehenberger, M. and Biedermann. Unpublished
433.99	ITS	99.81%	*Beauveria bassiana*	MW534282.1	Imrek, B. and Erler, F. Morphological and molecular diagnosis of entomopathogenic fungus isolated from different regions of Antalya, determination of their efficacy against *Tetranychus urticae*. Unpublished
	Large Ribosomal Subunit	100.00%	*Beauveria bassiana*	AB576868.1	Mukawa *et al.* (2011). Influence of humidity on the infection of western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), by *Beauveria bassiana*. *Applied Entomology and Zoology*, 46: 255-264
1787.17	ITS	99.81%	*Akanthomyces muscarius*	MH858369.1	Vu *et al.* (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology,* 93: 135-154
	Large Ribosomal Subunit	99.77%	*Akanthomyces muscarius*	MH867223.1	Vu *et al.* (2019). Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology,* 93: 135-154
1808.18	ITS	100.00%	*Beauveria bassiana*	KY471649.1	Amatuzzi, R. F., Poitevin, C. G., Poltronieri, A. S., Zawadneak, M. A. and Pimentel, I. C.. Entomopathogenic fungi as a biological control agent against *Duponchelia fovealis* Zeller. (Lepidoptera: Crambidae). Unpublished
	Large Ribosomal Subunit	99.88%	*Beauveria bassiana*	MW173996.1	Wang, Y., Fan, Q., Wang, D. and Yu, H. Beauveria bassiana complex and Beauveria scarabaeidicola complex. Unpublished

3.3.2. EPF colony extension/growth rate assessment

Optimum temperatures (T_{opt}) were calculated using a reparameterised Ratkowsky-3 equation developed by Adams *et al.* (2017). For all EPF isolates, fitted models were found to fit very well, with R^2 values ranging from 0.920-0.999 with the best model fit for isolate 1.72 (*A. dipterigenus*) and the worst model fit for isolate 1808.18 (*B. bassiana*) (Figure 3.5; Table 3.4). T_{opt} for growth across all isolates ranged from 24.13-25.20°C with a mean of 24.45°C and standard deviation of 0.540°C (Table 3.4). Isolate 433.99 (*B. bassiana*) was found to have the highest optimum temperature of 25.20°C while the lowest optimum temperature of 24.13°C was found for isolate 1.72 (*A. dipterigenus*). Maximum operating temperature (T_{max}), beyond which EPF are unable to grow, were found to range from 33.12-39.55°C with a mean of 36.10°C and standard deviation of 2.63°C, with these highest and lowest maximum operating growth temperatures being seen for isolates 433.99 (*B. bassiana*) and 1.72 (*A. dipterigenus*). All ten EPF isolates demonstrated growth at the lowest evaluate temperature, 5°C.

Table 3.4: Radkowsky-3 model covariates, fit parameters and calculated optimum growth rate temperature (Topt) and Pmax, the specific maximum growth response at calculate Topt. for each of the ten evaluated EPF isolates (Lactin *et al.*, 1995). Optimal growth rate temperatures were calculated using rearranged Radkowsky-3 equation (Omuse *et al.*, 2021).

EPF isolate	Model covariates				Calculated covariates		Sum of square residuals	R^2
	B	c (x10^4)	**Tmin (°C)**	**Tmax (°C)**	**Topt (°C)**	Pmax		
1.72	1.11	6.49	**5.01**	**33.12**	**24.13**	2.36	0.01	0.999
19.79	0.98	4.77	**1.07**	**33.70**	**23.58**	2.38	0.05	0.988
339.92	0.90	5.33	**1.39**	**36.23**	**24.08**	3.00	0.12	0.976
347.92	0.91	3.55	**1.95**	**37.52**	**24.81**	2.14	0.06	0.978
365.92	1.23	2.96	**1.98**	**33.62**	**23.79**	1.70	0.28	0.928
416.96	1.79	2.08	**5.78**	**35.98**	**24.79**	1.50	0.03	0.995
432.99	1.74	2.12	**3.15**	**38.88**	**25.01**	2.44	0.09	0.994
433.99	1.83	1.07	**3.81**	**39.55**	**25.20**	1.28	0.06	0.987
1787.17	2.78	0.50	**1.98**	**33.10**	**24.59**	0.71	0.23	0.954
1808.18	3.54	0.24	**1.02**	**39.34**	**24.52**	0.74	1.13	0.920

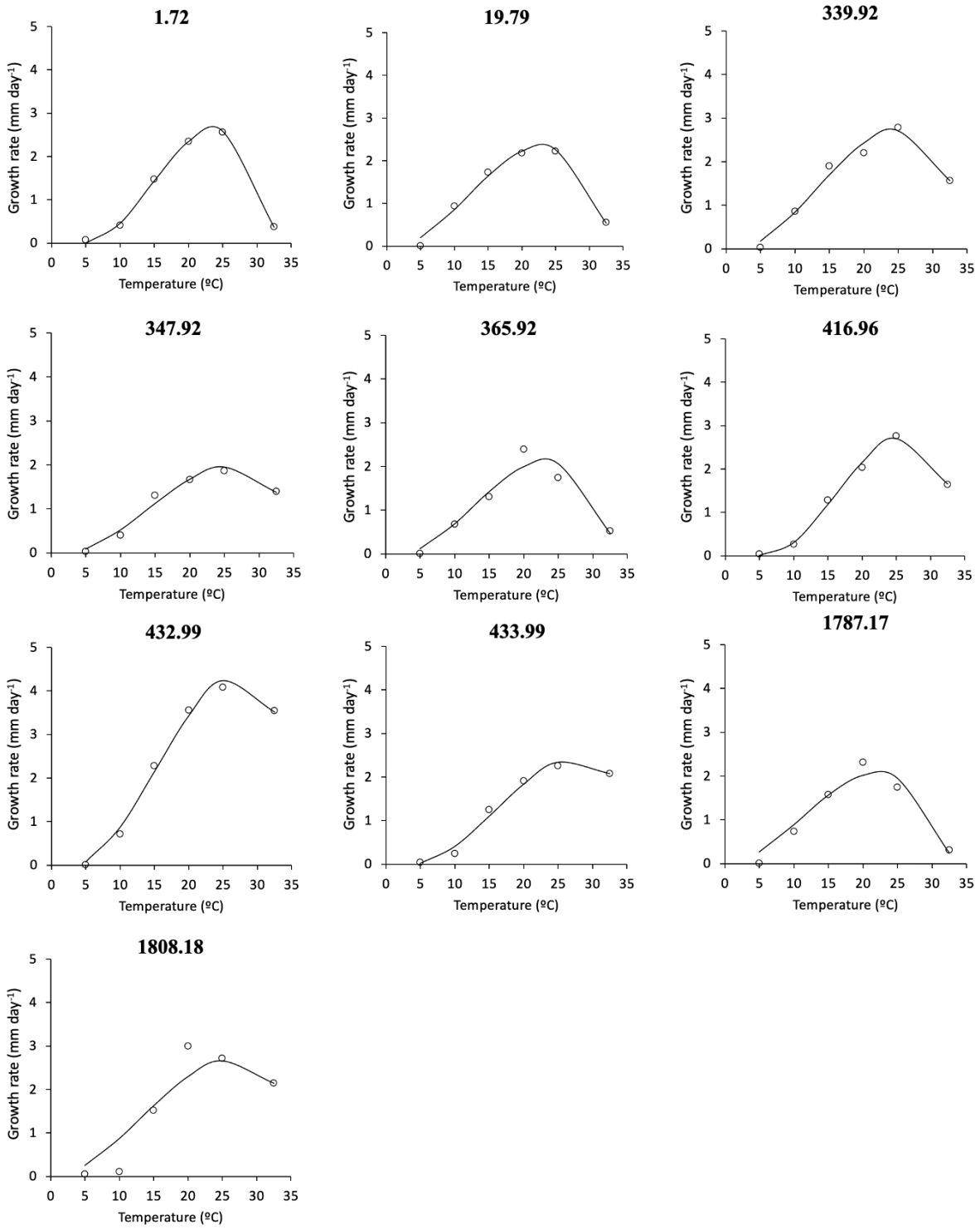


Figure 3.5: Ratkowsky-3 model curves for the effect of temperature upon EPF growth rate (mm day^{-1}) on SDA (Lactin *et al.*, 1995). The nonlinear Ratkowsky-3 model was selected to model EPF growth rate owing to previous usage and confirmed efficacy in modelling temperature-dependant effects upon EPF physiology (Omuse *et al.*, 2021). Growth rate for each isolate (Table3.1) was assessed at six different temperatures (5, 10, 15, 20, 25 and 32.5°C) over a 14-day period and Ratkowsky-3 models fitted to these results.

3.3.3. EPF germination rate assessment

Optimum temperatures (T_{opt}) were calculated using a reparameterised Ratkowsky-3 equation developed by Adams *et al.* (2017). For all EPF isolates, fitted models were found to fit well, with R^2 values ranging from 0.881-0.993 with the best model fit for isolate 433.99 (*B. bassiana*) and the worst model fit for isolate 1.72 (*A. dipterigenus*) (Table 3.5; Figure 3.6). T_{opt} for germination across all isolates was found to range from 24.43-25.04°C with a mean of 24.80°C and standard deviation of 0.221°C (Table 3.5). Isolate 433.99 (*B. bassiana*) was found to have the highest optimum temperature of 25.20°C while the lowest optimum temperature of 24.13°C was found for isolate 1.72 (*A. dipterigenus*). Maximum operating temperature (T_{max}), beyond which EPF are unable to germinate, were found to range from 32.52-34.10°C with a mean of 33.52°C and standard deviation of 0.46°C, with these highest and lowest maximum operating germination temperatures being seen for isolates 416.96 (*M. brunneum*) and 339.92 (*A. lecanii*).

Table 3.5: Ratkowsky-3 model covariates, fit parameters and calculated optimal germination temperature for each of the ten evaluated EPF isolates (Lactin *et al.*, 1995). Optimal germination temperatures were calculated for each EPF isolate by subtracting Δ from Tmax (Roy *et al.*, 2002).

EPF isolate	Model covariates				Calculated covariates		Sum of square residuals	R^2
	B	c ($x10^4$)	**Tmin (°C)**	**Tmax (°C)**	**Topt (°C)**	Pmax		
1.72	6.54	6.03	**3.56**	**33.46**	**25.04**	15.28	1660.93	0.881
19.79	6.54	4.93	**2.00**	**34.00**	**24.89**	18.77	1271.57	0.892
339.92	37.88	0.19	**2.77**	**32.52**	**24.51**	14.90	1589.94	0.886
347.92	47.17	0.09	**1.05**	**33.37**	**24.98**	18.91	1901.73	0.925
365.92	47.17	0.09	**1.80**	**34.00**	**24.77**	19.15	1894.94	0.893
416.96	57.96	0.12	**8.37**	**34.10**	**24.97**	9.90	1152.15	0.953
432.99	65.42	0.08	**6.24**	**33.35**	**24.74**	11.59	604.67	0.972
433.99	64.35	0.08	**6.50**	**33.35**	**24.63**	11.27	257.82	0.993
1787.17	6.54	5.98	**3.47**	**33.46**	**25.01**	15.43	1549.36	0.902
1808.18	37.88	0.16	**2.77**	**33.60**	**24.43**	16.92	1612.22	0.884

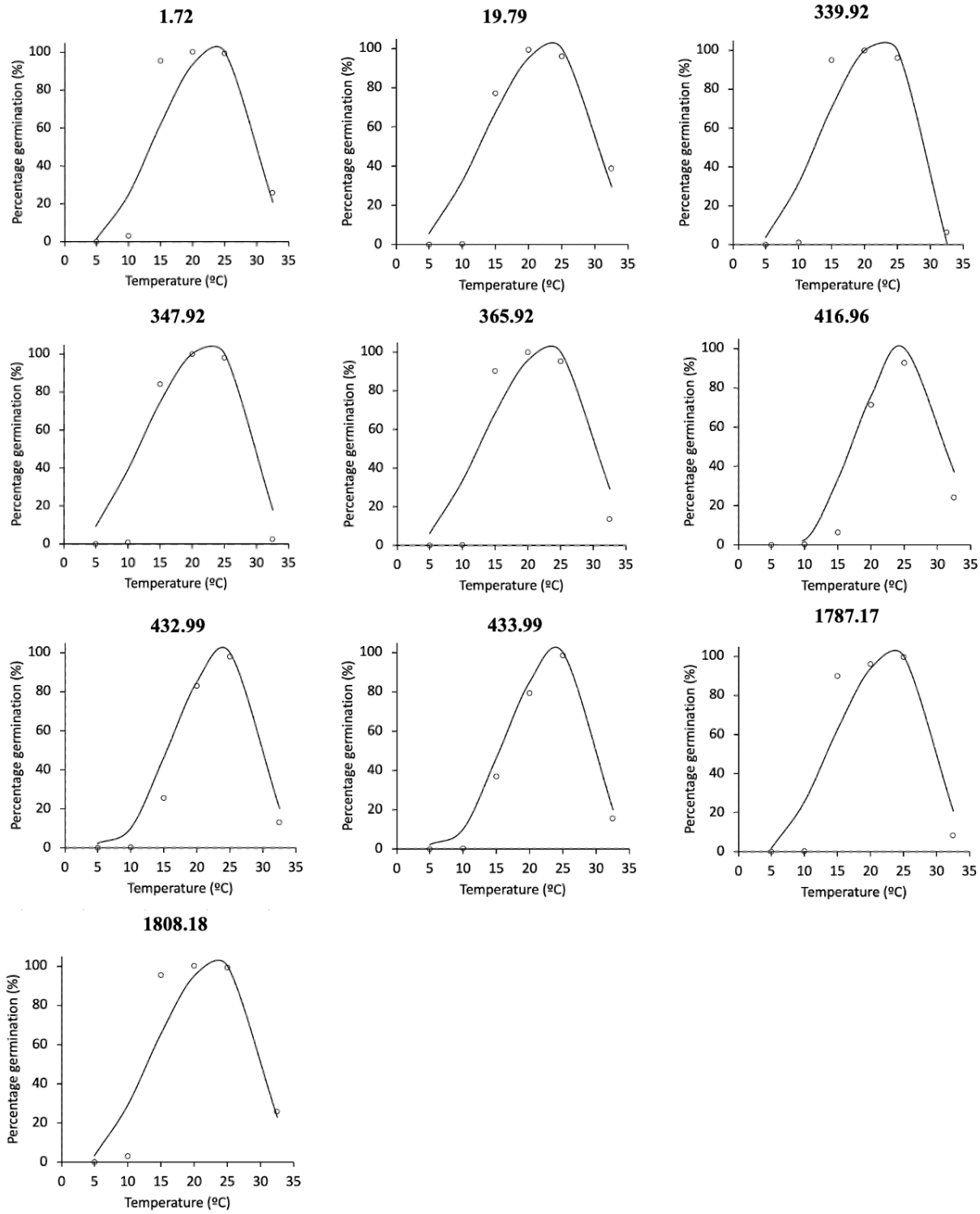


Figure 3.6: Ratkowsky-3 model curves for the effect of temperature upon percentage EPF germination (Lactin *et al.*, 1995). The nonlinear Ratkowsky-3 model was selected to model EPF growth and germination owing to previous usage and confirmed efficacy in modelling temperature-dependant effects upon EPF physiology (Omuse *et al.*, 2021). Germination for each isolate (Table 3.1) was assessed for 24 hours at six different temperatures (5, 10, 15, 20, 25 and 32.5°C) and Ratkowsky-3 models fitted to these results.

3.3.3.1. Evaluation of EPF isolates virulence and speed of kill on adult and first instar *Brevicoryne brassicae* – survival analysis

To evaluate whether EPF treatment overall outperformed mock treatment for adult *B. brassicae* Cox modelling was undertaken. This model was found to offer a significant improvement over a null model (X^2 (12) = 254.781, p = 1.32×10^{-47}). Within this model, both treatment and replicate were found to be significant factors influencing aphid survival (treatment: Wald X^2 (10) = 157.412, p = 1.11×10^{-28}) (replicate: X^2 (2) = 88.845, p = 5.10×10^{-20}), thus confirming that EPF treatment overall resulted in significantly decreased adult survival relative to control treatment. No interaction however was identified between treatment and replicate in this model (Wald X^2 (18) = 0.920, p = 0.761). The hazard ratio of all ten EPF isolates against adult *B. brassicae* ranged from 2.88-11.23, with Log-rank testing confirming that all ten significantly differed in their observed hazard ratio and survival curve relative to control (0.05% Triton-X), further indicating that all ten isolates resulted in significantly lower survival relative to control treatment. Kaplan-Meier survival plots evaluating *B. brassicae* survival following treatment with ten different EPF isolates demonstrated that all treatments resulted in <50% adult survival by assay day 9 (Figure 3.7a). Isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) resulted in the lowest survival (8.3% and 3.9% respectively) (Figure 3.7). Log-rank testing revealed no significant difference in the observed hazard ratio/resultant survival curve between these two isolates thus confirming their shared status as the most virulent EPF screened against adult *B. brassicae*, with both also resulting in a median aphid survival time of 4 days. Within this bioassay, control survival at experimental conclusion was 80% thus isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) resulted in a 71.7% and 76.1% decrease in survival respectively relative to control treatment. Of the remaining eight EPF isolates, 365.92 (*A. muscarius*) resulted in lowest mortality and thus the highest adult survival (48.8%), with the remaining isolates resulting in adult survival ranging from 46.6-22.7%. Despite lower hazard ratios and less extreme effects on cumulative survival in survival curves relative to isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), these remaining eight EPF isolates were also found to significantly increased observed hazard ratio and generate significantly different survival curves relative to the mock treated control. Median survival time of adults following the treatment with these eight EPF isolates ranged

between all ten isolates from 4-7 days (Table 3.6). Across all ten isolates EPF induced adult mortality occurred predominantly between bioassay days 3-6 (Figure 3.7). Isolate 1.72 (*A. dipterigenus*) resulted in the most rapid decrease in survival, with 69.5% adult survival on day 3 relative to 95.4-81.0% survival on day 3 for all other EPF isolates. Based upon these tests, isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were found to demonstrate the most significant overall ability to control *B. brassicae* adults (Figure 3.7a; Table 3.6).

For the *B. brassicae* nymph assay results, a Cox model was also constructed including treatment (EPF isolate) and replicate as factors. This model was found to offer a significant improvement over a null model (X^2 (12) = 674.082, p = 1.55×10^{-136}), confirming both treatment and replicate as significant factors influencing aphid survival (treatment: Wald X^2 (10) = 375.858, p = 1.28×10^{-74}) (replicate: X^2 (2) = 76.323, p = 2.67×10^{-17}) thus confirming that EPF treatment overall resulted in significantly decreased nymph survival relative to control treatment. No interaction was identified between treatment and replicate in this model (Wald X^2 (18) = 0.780, p = 0.052). The hazard ratio of EPF isolate 1.72 (*A. dipterigenus*) was found to be 59.01, with Log rank tests revealing this to be significantly higher than for all other EPF isolates, for which hazard ratios ranged from 0.87-6.05 (p=<0.001 in all cases) (Table 3.7). Isolate 1.72 (*A. dipterigenus*) outperformed all other EPF isolates, as indicated by this significantly higher hazard ratio and a significantly different survival curve (Figure 3.7b). Of the remaining nine EPF isolates, only six EPF isolates resulted in a hazard ratio/survival curve significantly different to the 0.05% Triton-X control – with isolates 19.79 (*A. muscarius*), 365.92 (*A. muscarius*), 1787.17 (*Akanthomyces* sp.) and 1808.18 (*B. bassiana*) being indistinguishable in their effect upon *B. brassicae* nymphs relative to the control (p=0.053, 0.705, 0.080 and 0.991 respectively) (Table 3.7). After only 2-days 1.72 (*A. dipterigenus*) was found to have reduced nymph survival to 44.6%, lower further to 14.7% on day 3 and 3.4% by day 4 before leading to 0% survival (100% mortality) by assay day 7 (Figure 3.7b). In stark contrast, on assay day 7 survival for all other assessed EPF isolates ranged from 95.8-74.3%, with isolates 1808.18 (*B. bassiana*) resulting in this highest level of survival and isolate 339.92 (*A. lecanii*) resulting in this lowest level of survival. Owing to only isolate 1.72 (*A. dipterigenus*) resulting in <50%

survival only for this isolate could median survival time be calculated for *B. brassicae* nymphs, with this being found to be 3 days.

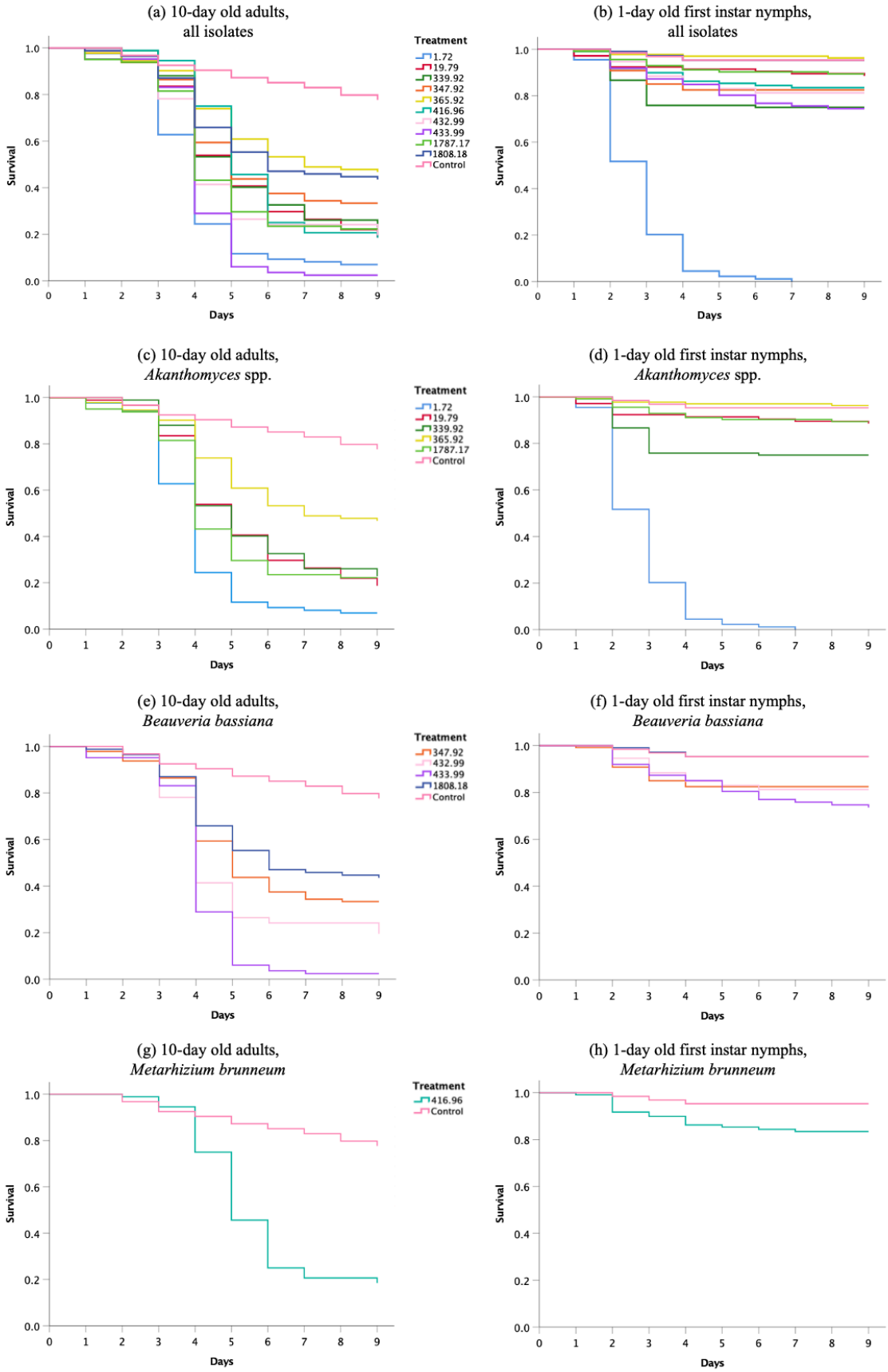


Figure 3.7: Survival of (a) *Brevicoryne brassicae* 10-day old, fixed age adults and (b) *B. brassicae* 1-day old first instar nymphs on control plants (*Brassica oleracea* var. *Gemmifera* 'Doric F1') following treatment with Log_{10} 7 conidia ml^{-1} of ten different EPF isolates and a mock control (0.05% Triton-X100). Survival also presented for *Akanthomyces* spp. isolates only (c)(d), *Beauveria bassiana* isolates only (e)(f) and *Metarhizium brunneum* isolates only (g)(h).

Table 3.6: Median survival time of adult *Brevicoryne brassicae* against ten EPF isolates and hazard ratio of each EPF isolate. Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. HR values followed by different lower-case letters within the column are significantly different (log rank c2 ≥ 3.841, P < 0.05).

a MST = median survival time, given in days
b HR = hazard ratio, compared to the 0.05% Triton-X treated control
* MST upper and lower confidence interval bounds could not be calculated
** Control treatment assigned a hazard ratio score of 1, against which all other treatments are evaluated.

		Factors	**MST[a] (95% Cl)**	**HR[b] (95% Cl)**	**Z (HR)**	**P (HR)**	**df**	**n**
	Treatment	Replicate			88.845	<0.001	2	3
		Treatment			157.412	<0.001	10	11
Mock	0.05% Triton-X100		*	1** a				94
1.72	*Akanthomyces dipterigenus*		4 (3.76 - 4.24)	11.23 (6.90-18.26) b	94.900	<0.001	1	86
19.79	*Akanthomyces muscarius*		5 (4.53- 5.47)	5.85 (3.59-9.51) eg	50.631	<0.001	1	91
339.92	*Akanthomyces lecanii*		5 (4.58 - 5.42)	5.04 (3.10-8.22) eg	42.212	<0.001	1	92
347.92	*Beauveria bassiana*		5 (4.37 - 5.64)	4.48 (2.73 - 7.34) dg	35.287	<0.001	1	96
365.92	*Akanthomyces muscarius*		7 *	2.88 (1.73 - 4.80) c	16.409	<0.001	1	92
416.96	*Metarhizium brunneum*		5 (4.59 - 5.41)	4.73 (2.91 - 7.70) g	39.371	<0.001	1	92
432.99	*Beauveria bassiana*		4 (3.72 - 4.28)	6.78 (4.15 - 11.01) ef	58.575	<0.001	1	87
433.99	*Beauveria bassiana*		4 (3.82 - 4.18)	9.57 (5.88 - 15.57) b	82.753	<0.001	1	83
1787.17	*Akanthomyces muscarius*		4 (3.72 - 4.28)	5.82 (3.55 - 9.56) fg	48.544	<0.001	1	81
1808.18	*Beauveria bassiana*		6 (2.99 - 9.01)	3.31 (1.98 - 5.53) cd	20.906	<0.001	1	85

Table 3.7: Median survival time of one-day old first instar *Brevicoryne brassicae* against ten EPF isolates and hazard ratio of each EPF isolate. Hazard ratio (HR) indicates the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. HR values followed by different lower-case letters within the column are significantly different (log rank c2 ≥ 3.841, P < 0.05).

a MST = median survival time, given in days
b HR = hazard ratio, compared to the 0.05% Triton-X treated control
* MST could not be calculated due to <50% mortality at experimental cessation (Day 9)
** Control treatment assigned a hazard ratio score of 1, against which all other treatments were evaluated.

		Factors	**MST[a] (95% Cl)**	**HR[b] (95% Cl)**	**Z (HR)**	**P (HR)**	**df**	**n**
	Treatment	Replicate			77.207	<0.001	2	3
		Treatment			375.855	<0.001	10	11
Mock	0.05% Triton-X100			1** bcd				128
1.72	*Akanthomyces dipterigenus*		3 (2.88-3.22)	59.01 (25.45 - 136.79) a	90.350	<0.001	1	89
19.79	*Akanthomyces muscarius*		*	3.14 (1.18 - 8.38) cg	5.215	0.053	1	105
339.92	*Akanthomyces lecanii*		*	6.05 (2.52 - 14.55) ef	16.202	<0.001	1	120
347.92	*Beauveria bassiana*		*	4.00 (1.61 - 9.90) eg	8.957	0.001	1	120
365.92	*Akanthomyces muscarius*		*	0.87 (0.27 - 2.85) b	0.052	0.705	1	134
416.96	*Metarhizium brunneum*		*	3.75 (1.49 - 9.45) eg	7.858	0.003	1	109
432.99	*Beauveria bassiana*		*	4.88 (1.97 - 12.11) eg	11.698	0.001	1	112
433.99	*Beauveria bassiana*		*	5.55 (2.25 - 13.70) ef	13.841	<0.001	1	87
1787.17	*Akanthomyces muscarius*		*	2.37 (0.89 - 6.30) bcd	2.962	0.080	1	113
1808.18	*Beauveria bassiana*		*	0.89 (0.27 - 2.90) bcd	0.040	0.991	1	107

3.3.4. Evaluation of EPF isolates virulence and speed of kill on adult and first instar *Brevicoryne brassicae* – GLMM analysis

All four GLMMs evaluating adult and nymph *B. brassicae* survival on days 6 and 9 of the bioassay offered a significant improvement over a null model, with each confirming treatment as a significant factor for the mortality of adults on day 6 (F(10,968)=14.860, P=<0.001) and day 9 (F(10,968)=15.074, P=<0.001) alongside nymphs on day 6 (F(10,1213)=9.295), P=<0.001) and day 6 (F(10,1213)=4.979, P=<0.001). Experimental replicate proved a significant factor in all four models (Adult *B. brassicae*, day 6 [F(2,968)=73.267, P=<0.001], and day 9 ([F(2,968)=104.873, P=<0.001]: *B. brassicae* nymphs, day 6 [F(2,968)=78.384, P=<0.001], day 9 ([F(2,968)=112.923, P=<0.001]). In all cases however, no interaction was noted between treatment (EPF isolate) and replicate (Adult *B. brassicae*, day 6 [F(20,968)=11.068, P=0.944], and day 9 ([F(20,968)=22.018, P=0.340]: *B. brassicae* nymphs, day 6 [F(20,968)=12.889, P=997], day 9 ([F(20,968)=21.231, P=0.413])

For adult *B. brassicae*, on day 6 cumulative proportional mortality across all treatments ranged from 45.5 – 96.1% with a mean of 69.7% and control mortality of 14.6%. Isolates 433.99 (*B. bassiana*), 1.72 (*A. dipterigenus*) and 1787.17 (*Akanthomyces* sp.) resulted in the highest cumulative mortality, resulting in 96.1%, 89.0% and 76.1% adult mortality respectively while isolate 365.92 (*A. muscarius*) resulted in the lowest mortality of 45.5%. Post hoc testing revealed no significant difference in the performance of these top performing three EPF isolates (p=>0.05) however the best performing isolate 433.99 (*B. bassiana*) resulted in significantly greater cumulative mortality relative to EPF isolates 432.99, 416.96, 19.79, 339.92, 347.92, 1808.18 and 365.92 (p=<0.05 in all cases) for which cumulative mortality ranged from 52.6-73.5% (Figure 3.8A). For adult *B. brassicae*, on day 9 cumulative proportional mortality across all treatments ranged from 52.6 - 97.4% with a mean of 75.0% and control mortality of 22.1%. On day 9 all ten evaluated EPF isolates resulted in significantly greater mortality relative to mock treated control (p=<0.05). Isolates 433.99 (*B. bassiana*) and 1.72 (*A. dipterigenus*) resulted in the highest overall mortality of 97.4% and 91.8% respectively. No significant difference however was noted between isolates 1.72 and 433.99 relative to isolates 19.79, 432.99, 416.96, 1787.17 and 339.92 (p=>0.05) for which cumulative mortality

ranged from 76.5 – 80.0%. Isolates 433.99 (*B. bassiana*) and 1.72 (*A. dipterigenus*) resulted in significantly higher adult mortality on day 9 relative to only isolates 347.92 (*B. bassiana*), 1808.18 (*B. bassiana*) and 365.92 (*A. muscarius*), for which cumulative day 9 mortality ranged from 52.6 – 63.1% (p=<0.05).

For *B. brassicae* nymphs, on day 6 cumulative proportional mortality across all treatments was found to range from 4.2 – 99.1% with a mean of 24.1% and control mortality of 6.0%. Isolate 1.72 (*A. dipterigenus*) resulted in the highest cumulative nymph mortality of 99.1% with post hoc testing confirming this to be significantly higher than for all other evaluated EPF isolates (p=<0.05) (Figure 3.8C). Of the remaining nine isolates cumulative nymph mortality was found to average 15.7% with isolate 339.92 (*A. lecanii*) resulting in this highest mortality (25.7%) while isolate 1808.18 (*B. bassiana*) resulted in the lowest mortality (4.2%). Of these nine EPF isolate, only 339.92 (*A. lecanii*), 432.99 (*B. bassiana*) and 433.99 (*B. bassiana*) were found to result in significantly higher mortality relative to the control treatment, with mortality following treatment with these accessions ranging from 22.7 – 25.7% (Figure 3.8C). For *B. brassicae* nymphs the trend in cumulative mortality results on day 9 matched very closely results on day 6, with cumulative proportional mortality across all treatments ranging from 4.2 – 100.0% with a mean of 24.6% and control mortality of 6.0%. Isolate 1.72 (*A. dipterigenus*) resulted in the highest cumulative nymph mortality of 100.0% with post hoc testing confirming this to be significantly higher than for all other evaluated EPF isolates (p=<0.05) (Figure 3.8D). Of the remaining nine isolates, cumulative nymph mortality of only isolates 339.92 (*A. lecanii*), 432.99 (*B. bassiana*) and 433.99 (*B. bassiana*) were found to result in significantly higher mortality relative to the control treatment (p=<0.05), with mortality following treatment with these accessions ranging from 22.7 – 25.7%, identical to observed mortality at day 6 indicating no further mortality in these accessions between days 6 and 9 (Figure 3.8C). On both days 6 and 9 of the bioassay period, isolates 1808.18, 365.92, 1787.17, 347.92, 19.79 and 416.96 did not result in mortality significantly different to the control treatment (p=>0.05) (Figure 3.8).

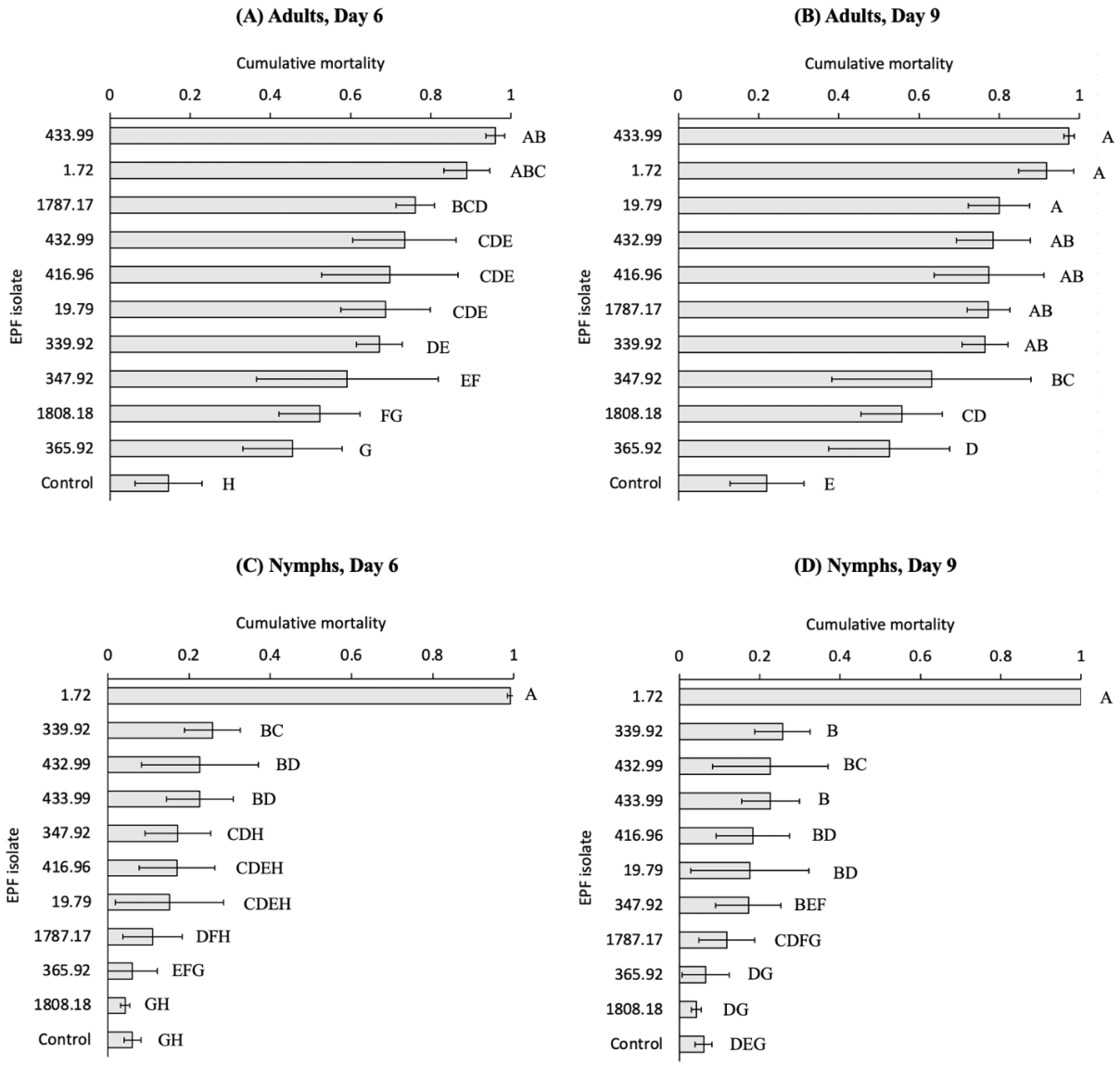


Figure 3.8: Average cumulative mortality of *Brevicoryne brassicae* adults (A, B) and nymphs (C, D) on days 6 and 9 post-Potter tower spray with 2ml of 10^7 conidia ml^{-1} solutions of ten different EPF isolates. Results were analysed via four separate GLMMs, each including treatment as a factor and replicate as a random effect. Owing to two GLMMs per aphid life stage, a Bonferroni correction was applied. All four models were found to be significant ($P<0.001$ in all cases), confirming EPF isolate as a significant factor affecting the mortality of adult and nymph *B. brassicae* on both days 6 and 9.

3.3.4.1. Evaluation of sub-lethal effect of EPF isolates upon adult *Brevicoryne brassicae* reproduction

Reproduction results were initially analysed using a GLM. This model was found to offer a significant improvement over a null model (Wald X^2 (12) = 121.512, p = 1.11×10^{-16}), with a model fit score (AIC) of 7606.812. In this model both treatment and day were found not to be significant factors (treatment: Wald X^2 (10) = 5.347, p = 0.867) (day: Wald X^2 (8) = 10.870, p = 0.290) however replicate was found to be a significant factor (Wald X^2 (2) = 116.116, p = <0.001). No interaction between treatment and replicate was present within this model (Wald X^2 (18) = 16.417, p = 0.563) however, an interaction was noted between day and replicate (Wald X^2 (12) = 47.231, p = 0.413)

To account for the variability introduced by replicate, analysis was therefore repeated utilising a GLMM including replicate as a random effect. This model offered a significant improvement over both a null model (F(18) = 2.157, p = 0.004) and previous GLM, with an improved fit score (AIC) of 934.875. In this model, while treatment was again found not to have a significant main effect upon *B. brassicae* reproduction (F(10)=1.304, p=0.224) day was found to be a significant factor (F(8)=3.314, p=0.001). One further GLMM was thus performed including both day and replicate as random effects and treatment as a sole factor however this model proved insignificant relative to a null model (F(10)=1.245, p=0.259 and resulted in a decline in model fit (AIC = 950.589) thus the second GLMM was identified as the optimal model for interpretation. EPF treatment was therefore noted as insignificant with respect to daily reproduction, with average daily reproduction across all treatments ranging from 0.8-7.6 nymphs per day with an overall mean of 3.3 nymphs per day (Figure 3.9). The day upon which reproduction was assessed however was found to significantly influence observed reproduction, with post hoc analysis revealing that the average reproduction of 2.4 nymphs on day 1 was significantly lower (p=<0.05) than reproduction on days 4, 5, 6,7 and 8 between which reproduction was found to range from 3.5 – 3.8 nymphs per day, indicating an average of 1.1 – 1.4 extra nymphs being born on these later days relative to day 1 (Figure 3.10).

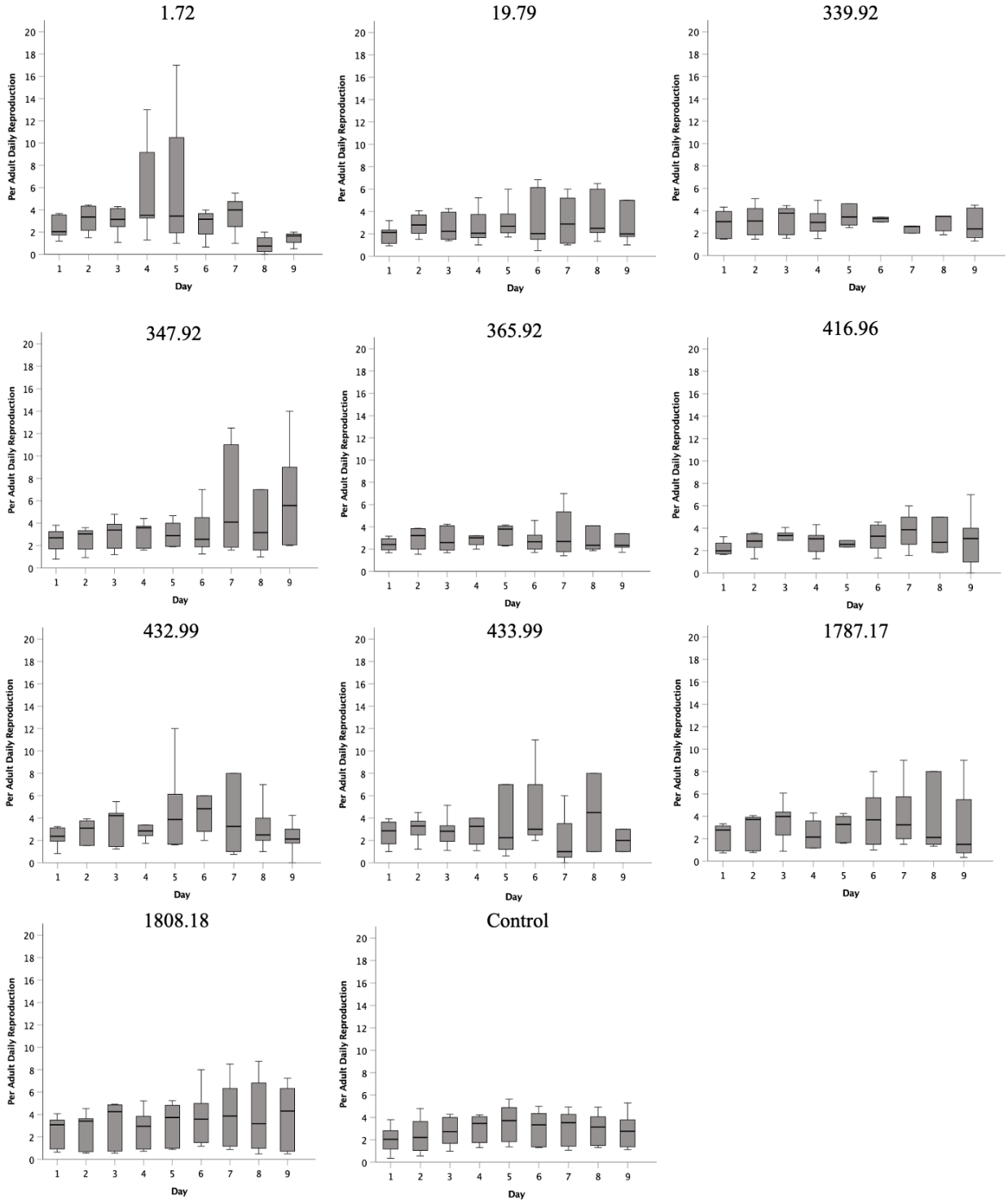


Figure 3.9: Per *Brevicoryne brassicae* adult daily reproduction observed post spray with 2ml of 10^7 conidia ml^{-1} solutions of each EPF isolate independently. These results were analysed through a GLM M which revealed a significant difference in overall mean daily reproduction, however the best model fit confirmed that day was a significant factor (p=0.001) while treatment was not significant (p=0.224).

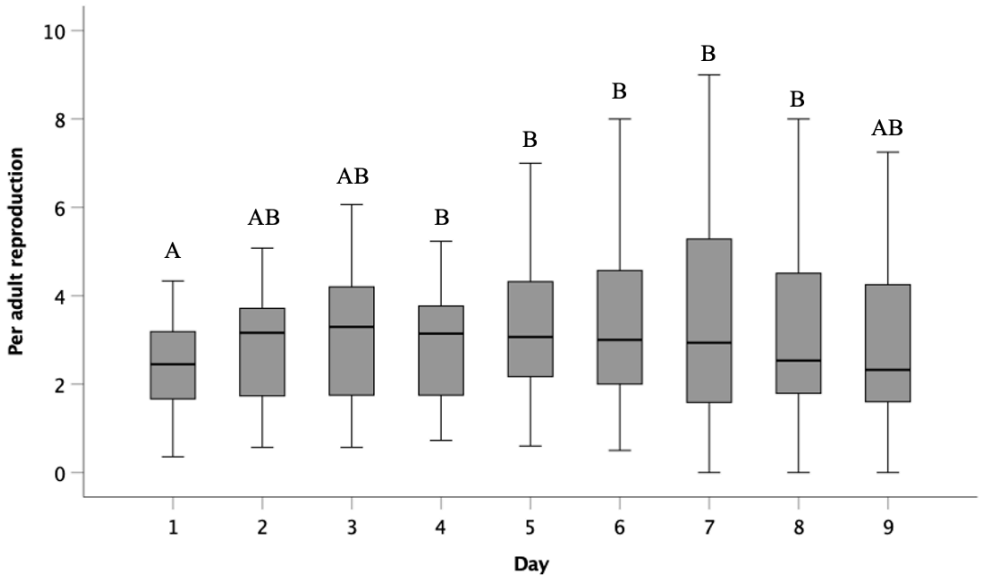


Figure 3.10: Per *Brevicoryne brassicae* adult reproduction observed each day across all EPF treatments. GLMM analysis revealed day as a significant factor in daily reproduction results (p=0.001).

3.3.5. Selection of EPF isolates for further evaluation

Based upon the results of the initial bioassay, assessing the virulence and speed of kill of ten EPF isolates, 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were selected for progression and further evaluation (Table 3.8). These two isolates were selected owing to their contrasting efficacies against *B. brassicae* adults and nymphs. Both isolates demonstrated a statistically indistinguishable significant (p=>0.05) ability to kill *B. brassicae* adults at a concentration of 1 x 10^7 conidia ml^{-1} with respect to hazard ratio and median survival times calculated through survival analysis alongside cumulative day 6 and 9 mortality calculated through GLMM analysis. Conversely however, survival analysis and GLMM analysis confirmed that isolate 1.72 (*A. dipterigenus*) offered significantly greater control over *B. brassicae* nymphs relative to isolate 433.99 (*B. bassiana*).

Table 3.8: Entomopathogenic fungi isolates selected for further evaluation and progression to combinatory experiments with partially resistant *Brassica* accessions.

Isolate number (University of Warwick Culture Collection)	Species	Host (isolated from)	On-label product name
1.72	*Akanthomyces dipterigenus* (Syn.*Lecanicillium longisporum*; *Verticillium lecanii*)	*Macrosiphoniella sanborni* (Chrysanthemum aphid)	Vertalec® (Koppert)
433.99	*Beauveria bassiana*	*Bemisia sp.* (Whitefly)	BotaniGard®ES (Bioworks®)

3.3.6. Concentration-response Assay – lethal effects survival analysis

Concentration-response assay results for adult and first instar *B. brassicae* were first analysed through Kaplan-Meier survival analysis, log rank tests and Cox proportional hazards models (Figures 3.11 and 3.12; Tables 3.9 and 3.10). Two Cox models were undertaken per aphid life to determine whether combined treatment (EPF + concentration) and EPF and concentration separately resulted in significantly different aphid survival relative to control treatment.

The first Cox model for *B. brassicae* adults evaluating combined treatment (EPF isolate and concentration) was found to both offer a significant improvement over a null model (X^2 (14) = 361.702, p = 1.44×10^{-68}), revealing that both treatment (EPF isolate + concentration) and replicate were significant factors influencing aphid mortality (treatment: Wald X^2 (12) = 238.102, p = 4.12×10^{-44}) (replicate: X^2 (2) = 13.646, p = 0.001). No interaction however was noted between treatment and replicate (Wald X^2 (24) = 3.054, p = 0.081). The second Cox model (where treatment was broken down in EPF and concentration) was found to offer a significant improvement over a null model (X^2 (9) = 325.968, p = 8.00×10^{-65}) with EPF isolate, concentration and replication all found to be significantly influencing adult survival (EPF isolate: Wald X^2 (2) = 46.996, p = 6.24×10^{-11}) (concentration: Wald X^2 (5) = 189.922, p = 4.06×10^{-39}) (replicate: Wald X^2 (2) = 15.955, p = 3.43×10^{-4}). No significant interactions were noted in this model, with both EPF x replicate (Wald X^2 (4) = 1.044, p = 0.307) and concentration x replicate (Wald X^2 (10) = 0.752, p = 0.386) proving non-significant.

For adult *B. brassicae*, following treatment with EPF isolate 1.72 (*A. dipterigenus*) hazard ratios were found to range from 3.04 – 60.38 with the lowest evaluated concentration of 4 Log_{10} conidia ml^{-1} resulting in this lowest hazard ratio and the highest evaluated concentration 7 Log_{10} conidia ml^{-1} resulting in the highest hazard ratio (Table 3.9). Kaplan-Meier survival plot confirmed that increasing concentrations of 1.72 (*A. dipterigenus*) related directly to decreasing survival, with survival following treatment with the lowest concentration resulting in 14.5% mortality by day 9 while treatment with the highest concentration resulted in 100.0% mortality by day 6 (Figure 3.11a). Log-rank testing revealed that 1.72 (*A.*

dipterigenus) treatment with concentrations of 4.5, 5.0, 5.5, 6.0 and 7.0 Log_{10} conidia ml^{-1} resulted in significantly increased hazard ratios and survival curves relative to mock treated control (p=<0.05 in all cases) however a treatment with a concentration of 4 Log_{10} conidia ml^{-1} performed indistinguishably relative to mock treated control (p=>0.05). No significant difference was noted in survival following treatment with concentration of 4.0, 4.5 and 5.0 Log_{10} conidia ml^{-1} (p=>0.05) however each subsequent higher concentration (5.5, 6.0 and 7.0 Log_{10} conidia ml^{-1}) resulted in survival significantly lower to all other 1.72 (*A. dipterigenus*) concentrations (p=<0.05) (Table 3.9).

For adult *B. brassicae*, following treatment with EPF isolate 433.99 (*B. bassiana*) hazard ratios were found to range from 4.74 – 44.39 with the second lowest evaluated concentration of 4.5 Log_{10} conidia ml^{-1} resulting in this lowest hazard ratio and the highest evaluated concentration 7 Log_{10} conidia ml^{-1} resulting in the highest hazard ratio (Table 3.9). Kaplan-Meier survival plot revealed that while the lowest survival observed of 11.1% resulted from the highest evaluated concentration of 7 Log_{10} conidia ml^{-1} of isolate 433.99 (*B. bassiana*) survival following treatments with lower concentrations did not increase with each decrease in concentration, with treatment with a concentration of 5 Log_{10} conidia ml^{-1} resulting in the second lowest survival of 40.0% (Figure 3.12a). Log-rank testing revealed that this lack of direct relationship between increasing concentration and decreasing survival may have resulted from a larger margin of error between 433.99 (*B. bassiana*) concentrations, with no significant difference in adult survival between concentration 5, 5.5 and 6 Log10 conidia ml^{-1} between which hazard ratios ranged from 11.02-17.85 and adult *B. brassicae* percentage survival ranged from 66.7 - 40.0%. Similarly, no significant difference in adult survival following treatments with concentration of 4.0 and 4.5 Log_{10} conidia ml^{-1} which had hazard ratios of 5.58 and 4.74 respectively and resulted in 78.0% and 81.0% survival respectively (Table 3.10; Figure 3.11). All evaluated concentrations of isolate 433.99 (*B. bassiana*) did however result in significantly increased adult mortality relative to control treatment (p=<0.05), while a concentration of 7.0 Log_{10} conidia ml^{-1} resulted in significantly decreased survival relative to all other 433.99 concentration (p=<0.05) (Table 3.10). Between treatments 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), no significant difference was noted in adult survival following treatment with concentrations of 4.0, 4.5, 5.5,

and 7.0 Log_{10} conidia ml^{-1} ($p=<0.05$ in all cases). Significant differences in mortality however were noted following treatments at concentrations of 5.0 and 6.0 Log_{10} conidia ml^{-1}, with isolate 433.99 (*B. bassiana*) causing significantly lower survival (40.0% relative to 73.5%) at this lower concentration while at the higher concentration isolate 1.72 (*A. dipterigenus*) caused significantly lower survival (21.9% relative 60.0%) (Table 3.9; Figures 3.11a and 3.12a).

The first Cox model for *B. brassicae* nymphs evaluating combined treatment (EPF isolate and concentration) was found to both offer a significant improvement over a null model (X^2 (14) = 471.508, p =$1.00x10^{-91}$), revealing that treatment was found a significant factor influencing nymph survival (Wald X^2 (12) = 252.654, p = $3.82x10^{-47}$) while replicate was not when the Bonferroni correction was considered (X^2 (2) = 7.032, p = 0.030). No interaction however was noted between treatment and replicate (Wald X^2 (24) = 1.070, p = 0.301). The second Cox model (where treatment was broken down in EPF and concentration) was found to offer a significant improvement over a null model (X^2 (9) =, p =$4.52x10^{-87}$), with both EPF isolate and concentration being found to significantly influence nymph survival(EPF isolate: Wald X^2 (2) = 93.488, p = $5.01x10^{-21}$) (concentration: Wald X^2 (5) = 180.730, p = $3.74x10^{-37}$) while experimental replicate again prove an insignificant factor (Wald X^2 (2) = 6.875, p = 0.032). No significant interactions were noted in this model, with both EPF x replicate (Wald X^2 (4) = 0.008, p = 0.930) and concentration x replicate (Wald X^2 (10) = 0.309, p = 0.578) proving non-significant.

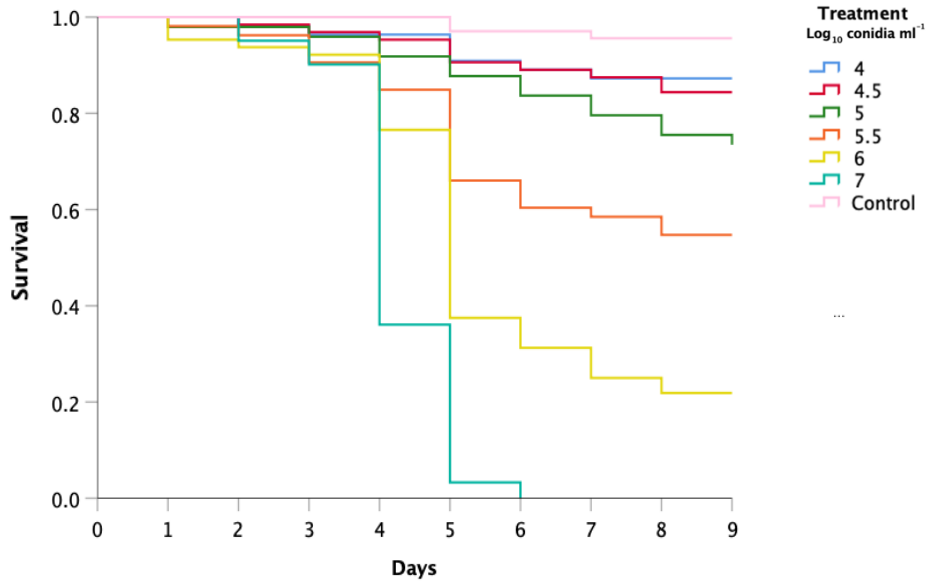
For *B. brassicae* nymphs, following treatment with EPF isolate 1.72 (*A. dipterigenus*) hazard ratios were found to range from 2.28 – 82.31 with the second lowest evaluated concentration of 4.5 Log_{10} conidia ml^{-1} resulting in this lowest hazard ratio and the highest evaluated concentration 7.0 Log_{10} conidia ml^{-1} resulting in the highest hazard ratio (Table 3.10). Kaplan-Meier survival plot confirmed that, aside from concentration 4.0 and 4.5 Log_{10} conidia ml^{-1} being reversed, increasing concentrations of 1.72 (*A. dipterigenus*) related directly to decreasing survival, with survival following treatment with the second lowest concentration resulting in 9.8% mortality by day 9 while treatment with the highest concentration resulted in 100.0%

mortality by day 4 (Figure 3.12a). Log-rank testing revealed that 1.72 (*A. dipterigenus*) treatment with concentrations of 5.0, 5.5, 6.0 and 7.0 Log_{10} conidia ml^{-1} resulted in significantly increased hazard ratios and survival curves relative to mock treated control (p=<0.05 in all cases) however 1.72 (*A. dipterigenus*) treatment with a concentration of 4 and 4.5 Log_{10} conidia ml^{-1} performed indistinguishably relative to mock treated control (p=>0.05). Treatment with a 1.72 (*A. dipterigenus*) concentration of 7 Log10 conidia ml^{-1} resulted in a significantly higher hazard ratio and thus significantly lower survival relative to all other evaluated 1.72 concentrations (Table 3.10). Between concentrations of 5.5 and 6.0 Log_{10} conidia ml^{-1} no significant difference in hazard ratio and survival was observed, with hazard ratios of 8.56 and 15.97 respectively resulting in nymph survival of 63.3% and 51.9% respectively on assay day 9. No significant differences in nymph survival were also noted between treatment with 1.72 (*A. dipterigenus*) at concentrations of 5.0 and 5.5 Log_{10} conidia ml^{-1}, which were found to have hazard ratios of 8.07 and 8.56 respectively resulting in nymph survival of 71.7% and 63.3% respectively (Table 3.10).

For *B. brassicae* nymphs, following treatment with EPF isolate 433.99 (*B. bassiana*) hazard ratios were found to range from 0.83 – 13.58 with the lowest evaluated concentration of 4.0 Log_{10} conidia ml^{-1} resulting in this lowest hazard ratio and the highest evaluated concentration 7 Log_{10} conidia ml^{-1} resulting in the highest hazard ratio (Table 3.10). Kaplan-Meier survival plot revealed that overall, with increasing 433.99 (*B. bassiana*) concentration, nymph survival was incrementally decreasing. The lowest survival observed of 55.2% resulted from the highest evaluated concentration of 7 Log_{10} conidia ml^{-1} while the lowest concentration evaluated (4.0 Log_{10} conidia ml^{-1}) resulted in the highest nymph survival of 96.6% (Figure 3.12b). Log-rank testing revealed that treatment with 433.99 (*B. bassiana*) at concentrations of 4.0, 4.5, 5.0 and 5.5 Log_{10} conidial ml^{-1} resulted in hazard ratios (which ranged from 0.83 – 3.38) and nymph survival (which ranged from 96.4 – 85.7%) indistinguishable to control treatment (p=>0.05). (Table 3.10). Treatment with 433.99 concentrations of 6.0 and 7.0 Log10 conidia ml^{-1} however both resulted in significantly higher hazard ratios (4.36 and 13.58 respectively) and decreased nymph survival (79.5% and 55.3% respectively), with Log-rank testing also confirming that a concentration of 7.0 Log10 conidia ml^{-1} resulted in a significantly higher hazard

ratio and significantly lower nymph survival relative to treatment with a concentration of 6.0 Log_{10} conidia ml^{-1} ($p=<0.05$) (Table 3.10). Between treatments 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), no significant difference was noted in nymph survival following treatment with concentrations of 4.0, 4.5 Log_{10} conidia ml^{-1} ($p=<0.05$ in all cases). Significant differences in mortality however were noted following treatments at concentrations of 5.0, 5.5, 6.0 and 7.0 Log_{10} conidia ml^{-1}, with isolate 1.72 (*A. dipterigenus*) causing significantly decreased survival at each of these respective concentrations (5.0 Log_{10} conidia ml^{-1}: 71.7% vs 85.7%, 5.5 Log_{10} conidia ml^{-1}: 63.3% vs 89.5%, 6.0 Log_{10} conidia ml^{-1}: 51.9% vs 79.5%, 7.0 Log_{10} conidia ml^{-1}: 0.0% vs 55.3%). Isolate 433.99 (*B. bassiana*) at a concentration of 7.0 Log_{10} conidia ml^{-1} however resulted in an indistinguishable hazard ratio and nymph survival relative to isolate 1.72 (*A. dipterigenus*) at a concentration of 6.0 Log_{10} conidia ml^{-1} (Table 3.10). These results indicate that isolate 1.72 (*A. dipterigenus*) results in significantly decreased nymph survival relative to isolate 433.99 (*B. bassiana*) when applied at higher equal concentrations however where isolate 433.99 (*B. bassiana*) is applied at a notably higher concentration relative to 1.72 (*A. dipterigenus*) equal levels of nymph mortality was achieved.

(a) 10-day old adults



(b) 1-day old nymphs

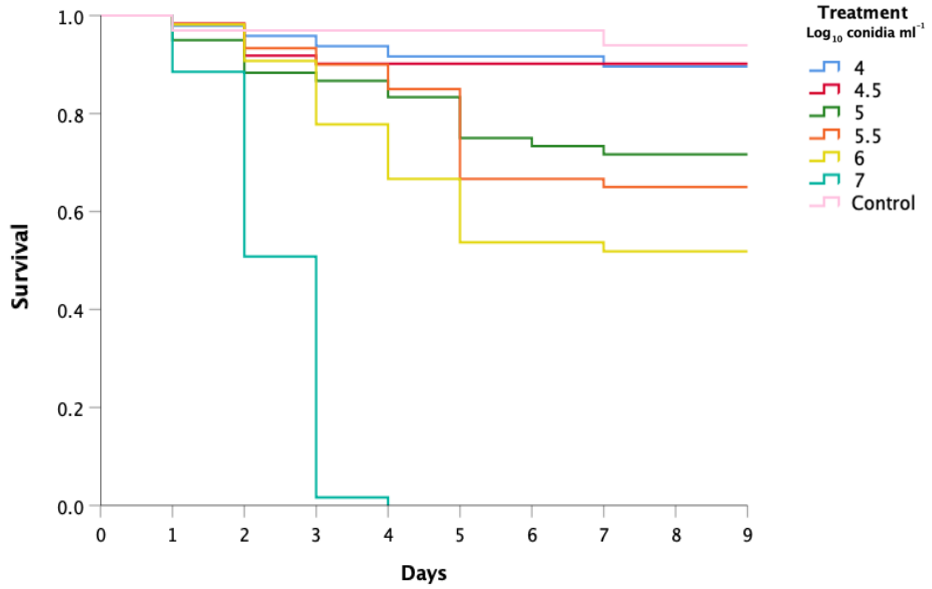
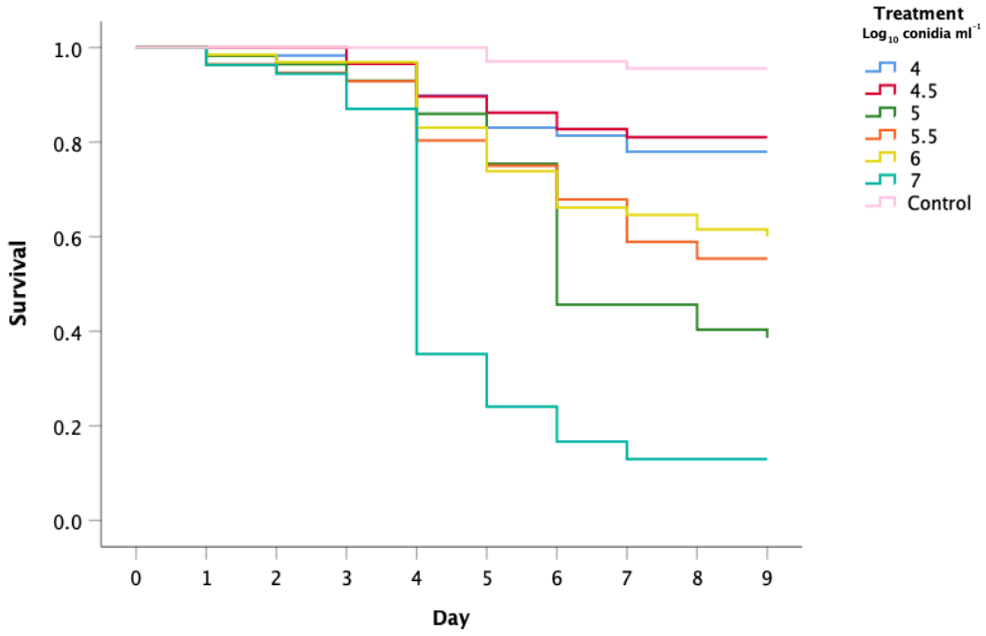


Figure 3.11: Cumulative survival of *Brevicoryne brassicae* (a) 10-day old, fixed age adults and (b) first instar nymphs on control plants (*Brassica oleracea* var. *Gemmifera* 'Doric F1') following treatment with six different concentrations from 4 –7 Log10 conidia ml^{-1} of EPF isolate 1.72 (*Akanthomyces dipterigenus*) and a control (0.05% Triton-X100).

(a) 10-day old adults



(b) 1-day old nymphs

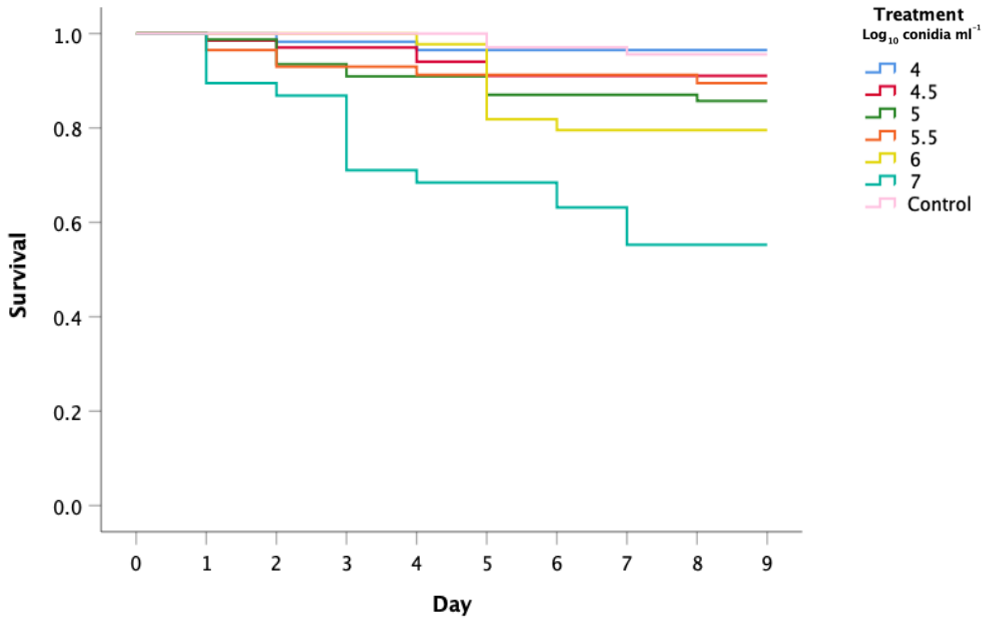


Figure 3.12: Cumulative survival of *Brevicoryne brassicae* (a) 10-day old, fixed age adults and (b) first instar nymphs on control plants (*Brassica oleracea* var. *Gemmifera* 'Doric F1') following treatment with six different concentrations from 4 –7 Log10 conidia ml^{-1} of EPF isolate 433.99 (*Beauveria bassiana*) and a control (0.05% Triton-X100).

Table 3.9: Median survival times of adult *Brevicoryne brassicae* when evaluated independently against six different concentrations of two EPF isolates and corresponding isolate/concentration hazard ratio. Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. HR values followed by different lower-case letters within the column are significantly different (log rank c2 ≥ 3.841, p < 0.05).

a MST = median survival time, given in days
b HR = hazard ratio, compared to the 0.05% Triton-X treated control
* MST, upper, and lower confidence interval bounds could not be calculated
** Control treatment assigned a hazard ratio score of 1, against which all other treatments are evaluated.

Treatment		Factors	MST[a] (95% Cl)	HR[b] (95% Cl)	Z (HR)	P (HR)	df	n
Cox model 1	Rep				13.646	0.001	2	3
	Treatment (EPF, Concentration)				238.102	<0.001	12	13
Cox model 2	Rep				15.955	<0.001	2	3
	EPF + mock				46.996	<0.001	2	3
	Concentration				189.922	<0.001	5	6

Treatment		Concentration (Log10 conidia ml^{-1})						
Control	0.05% Triton-X	-	*	1**a			1	68
1.72	*Akanthomyces dipterigenus*	4	*	3.04 (0.79-11.75) ae	2.593	0.107	1	55
1.72	*Akanthomyces dipterigenus*	4.5	*	3.74 (1.03-13.59) e	4.017	0.045	1	64
1.72	*Akanthomyces dipterigenus*	5	*	6.59 (1.88-23.12) de	8.663	0.003	1	49
1.72	*Akanthomyces dipterigenus*	5.5	*	13.10 (3.94-43.49) c	17.638	<0.001	1	53
1.72	*Akanthomyces dipterigenus*	6	5 (4.969-5.304)	28.14 (8.77-90.34) f	31.451	<0.001	1	64
1.72	*Akanthomyces dipterigenus*	7	4 (3.777-4.223)	60.38 (18.82-193.48) b	47.558	<0.001	1	61
433.99	*Beauveria bassiana*	4	*	5.58 (1.59-19.58) e	7.204	0.007	1	59
433.99	*Beauveria bassiana*	4.5	*	4.74 (1.32-16.98) e	5.704	0.017	1	58
433.99	*Beauveria bassiana*	5	6 (4.894-7.106)	17.85 (5.49-58.06) c	22.935	<0.001	1	57
433.99	*Beauveria bassiana*	5.5	*	12.59 (3.80-41.71) c	17.184	<0.001	1	56
433.99	*Beauveria bassiana*	6	*	11.02 (3.33-36.40) cd	15.482	<0.001	1	65
433.99	*Beauveria bassiana*	7	4 (3.754-4.246)	44.39 (13.78-142.97) b	40.393	<0.001	1	54

Table 3.10: Median survival times of first instar *Brevicoryne brassicae* when evaluated independently against six different concentrations of two EPF isolates and corresponding isolate/concentration hazard ratio. Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. HR values followed by different lower-case letters within the column are significantly different (log rank c2 ≥ 3.841, P < 0.05).

a MST = median survival time, given in days
b HR = hazard ratio, compared to the 0.05% Triton-X treated control
* MST, upper, and lower confidence interval bounds could not be calculated
** Control treatment assigned a hazard ratio score of 1, against which all other treatments are evaluated.

Treatment			Factors	MST[a] (95% Cl)	HR[b] (95% Cl)	Z (HR)	P (HR)	df	n
		Cox model 1	Rep			7.032	0.030	2	3
			Treatment (EPF, Concentration)			252.654	<0.001	12	13
		Cox model 2	Rep			6.875	0.032	2	3
			EPF + mock			93.488	<0.001	2	3
			Concentration			180.730	<0.001	5	6
Treatment		Concentration (Log10 conidia ml^{-1})							
Control	0.05% Triton-X	-		*	1** fh				68
1.72	*Akanthomyces dipterigenus*	4		*	2.54 (0.61-10.62) hi	1.624	0.203	1	48
1.72	*Akanthomyces dipterigenus*	4.5		*	2.28 (0.57-9.13) hi	1.359	0.244	1	61
1.72	*Akanthomyces dipterigenus*	5		*	8.07 (2.36-27.65) de	11.054	0.001	1	60
1.72	*Akanthomyces dipterigenus*	5.5		*	8.56 (2.55-28.74) bde	12.083	0.001	1	60
1.72	*Akanthomyces dipterigenus*	6		*	15.97 (4.80-53.12) bc	20.431	<0.001	1	54
1.72	*Akanthomyces dipterigenus*	7		3 (2.927-3.073)	82.31 (25.31-267.69) a	53.724	<0.001	1	61
433.99	*Beauveria bassiana*	4		*	0.83 (0.14-4.94) fh	0.044	0.834	1	57
433.99	*Beauveria bassiana*	4.5		*	2.06 (0.52-8.25) hi	1.048	0.306	1	67
433.99	*Beauveria bassiana*	5		*	3.38 (0.94-12.10) gh	3.487	0.062	1	77
433.99	*Beauveria bassiana*	5.5		*	2.71 (0.68-10.86) hi	1.982	0.159	1	57
433.99	*Beauveria bassiana*	6		*	4.36 (1.18-16.13) egi	4.864	0.027	1	44
433.99	*Beauveria bassiana*	7		*	13.58 (3.97-46.42) cd	17.298	<0.001	1	38

3.3.6.1. Concentration-response Assay – lethal effects GLMM analysis

To further assess concentration-response assay results on adult *B. brassicae* a GLM was undertaken including treatment (EPF/concentration) and replicate as factors. This model offered a significant improvement over a null model (Wald X^2 (14) = 334.669, p = <0.001), with a model fit score (AIC) of 207.216. In this model both treatment and replicate were found to be significant factors influencing aphid mortality (treatment: Wald X^2 (12) = 143.846, p = <0.001) (replicate: Wald X^2 (2) = 13.231, p = 0.001). No significant interaction was present between treatment and replicate (Wald X^2 (24) = 31.793, p = 0.132).

To account for between replicate variation data was reanalysed utilising a GLMM including treatment as a sole factor and replicate as a random effect. This model was also found to be significant relative to null model (F(12,750) = 11.995, p = <0.001) however this model offered an inferior model fit (AIC score of 4274.868) thus the initial GLM model was used for data interpretation. Across all six evaluated concentrations of 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), only a concentration of 4 Log10 conidia ml^{-1} failed to result in mortality significantly higher than control treatment, resulting in 14.5% mortality (Figure 3.13). A concentration of 7 Log_{10} conidia ml^{-1} of isolate 1.72 (*A. dipterigenus*) resulted in the highest observed mortality of 100.0% across all three experimental replicates. The second highest observed adult *B. brassicae* mortality was 88.9% resulted from treatment with isolate 433.99 (*B. bassiana*) at a dosage of 7 Log_{10} conidial ml^{-1} however post-hoc analysis revealed that this treatment performed identically (p=>0.05) to treatment with isolate 1.72 (*A. dipterigenus*) at a concentration of 6 Log_{10} conidial ml^{-1} for which an average aphid mortality of 78.1% was observed (Figure 3.13). Treatment with isolate 1.72 (*A. dipterigenus*) at a concentration of 5.5 Log_{10} conidia ml^{-1} was found to result in 45.3% adult mortality, with post hoc test confirming that this was significantly indistinguishable (p=>0.05) to 1.72 (*A. dipterigenus*) treatment at the lower concentrations of 4.0, 4.5 and 5.0 Log_{10} conidia ml^{-1} (which resulted in mortality of 14.5%, 15.6% and 26.5% respectively). This treatment was also found to be significantly indistinguishable (p=>0.05) relative to treatment with isolate 433.99 (*B. bassiana*) at concentrations of 5.0, 5.5 and 6.0 Log_{10} conidia ml^{-1} (which resulted in mortality of 33.3%, 44.6% and 40.0% respectively). No significant

differences in resultant adult mortality were noted in post hoc analysis between isolate 1.72 (*A. dipterigenus*) at concentration of 4.0, 4.5 and 5.0 Log_{10} conidia ml^{-1} and 433.99 (*B. bassiana*) at concentrations of 4.0 and 4.5 Log_{10} conidia ml^{-1} (Figure 3.13). Fitted logistic curves were found to fit these concentration response data for adult *B. brassicae* well, with an R^2 score of 0.9927 and 0.9198 for isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) respectively.

To further assess concentration-response assay results on *B. brassicae* nymphs a GLM was undertaken including treatment (EPF/concentration) and replicate as factors. This model offered a significant improvement over a null model (Wald X^2 (14) = 278.040, p = <0.001), with a model fit score (AIC) of 161.181. Within this model treatment was found to be a significant factor (Wald X^2 (12) = 77.208, p = $1.40x10^{-11}$) while replicate was found not to be significant (Wald X^2 (2) = 3.959, p = 0.138). No significant interaction was present between treatment and replicate (Wald X^2 (24) = 29.092, p = 0.217). Replicate was therefore removed as a factor and GLM analysis repeated. This second GLM was also found to significantly improve upon a null model (Wald X^2 (12) = 274.133, p =<0.001), with treatment therefore being a significant factor (Wald X^2 (12) = 75.180, p =$3.40x10^{-11}$). This second GLM was also found to offer a better model fit, with an AIC score of 70.833 therefore this model was used for data interpretation. Across all six concentrations of EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), six EPF/concentration treatments were found to perform indistinguishably relative to control treatment (p=>0.05) in their effect upon nymph mortality, with these treatments being isolate 1.72 (*A. dipterigenus*) concentrations of 4.0 and 4.5 Log_{10} conidia ml^{-1} (which resulted in nymph mortality of 10.4% and 9.8% respectively) and isolate 433.99 (*B. bassiana*) concentrations of 4.0, 4.5, 5.0 and 5.5 Log_{10} conidia ml^{-1} (which resulted in nymph mortality of 3.6%, 9.0%, 14.3% and 10.5% respectively) (Figure 3.14). The highest average nymph mortality was seen following treatment with isolate 1.72 (*A. dipterigenus*) at a concentration of 7.0 Log_{10} conidia ml^{-1}, with this level of mortality significantly higher than for all other observed treatments (p=<0.05) (Figure 3.14). the second highest level of nymph mortality was observed following treatment with isolate 1.72 (*A. dipterigenus*) at a concentration of 6.0 Log_{10} conidia ml^{-1}, which resulted in 48.1% nymph mortality (Figure 3.14). Post hoc analysis however

revealed that this treatment was significantly indistinguishable relative to treatment with lower 1.72 (*A. dipterigenus*) concentrations of 5.0 and 5.5 Log_{10} conidia ml^{-1} (which resulted in 28.3% and 36.7% nymph mortality) and treatment with isolate 433.99 (*B. bassiana*) at concentrations of 5.0, 6.0 and 7.0 Log_{10} conidia ml^{-1} (which resulted in 14.3%, 20.5% and 44.7% average mortality respectively). Fitted logistic curves were found to fit these concentration response data for nymphs well, with an R^2 score of 0.9885 and 0.9352 for isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) respectively.

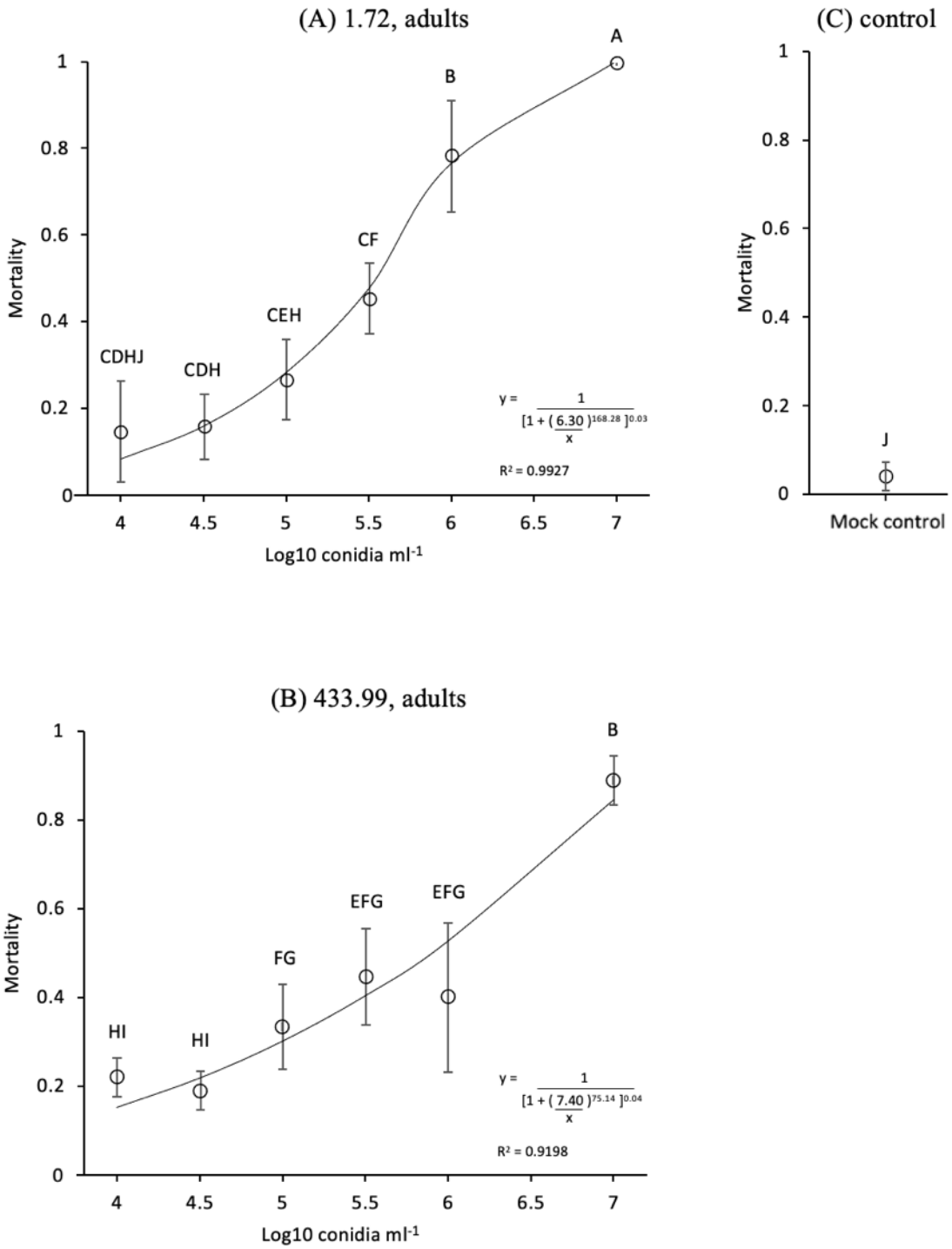


Figure 3.13: Concentration response assay mortality results for adult *Brevicoryne brassicae* when treated with six serial Log10 concentrations (4, 4.5, 5, 5.5, 6 and 7) of EPF isolates (A) 1.72 (*Akanthomyces dipterigenus*), (B) 433.99 (*Beauveria bassiana*) and (C) a mock treated control. An asymmetrical (5-factor) logistic concentration-response curve was fitted to the six evaluated concentrations for both isolates 1.72 and 433.99. Between treatment (EPF isolate + concentration) differences were evaluated through a GLMM which revealed treatment was a significant factor (p=<0.001). Post hoc analyses of pairwise treatment differences represented as letters across (A), (B) and (C), allowing for comparison of pairwise differences within and between treatments.

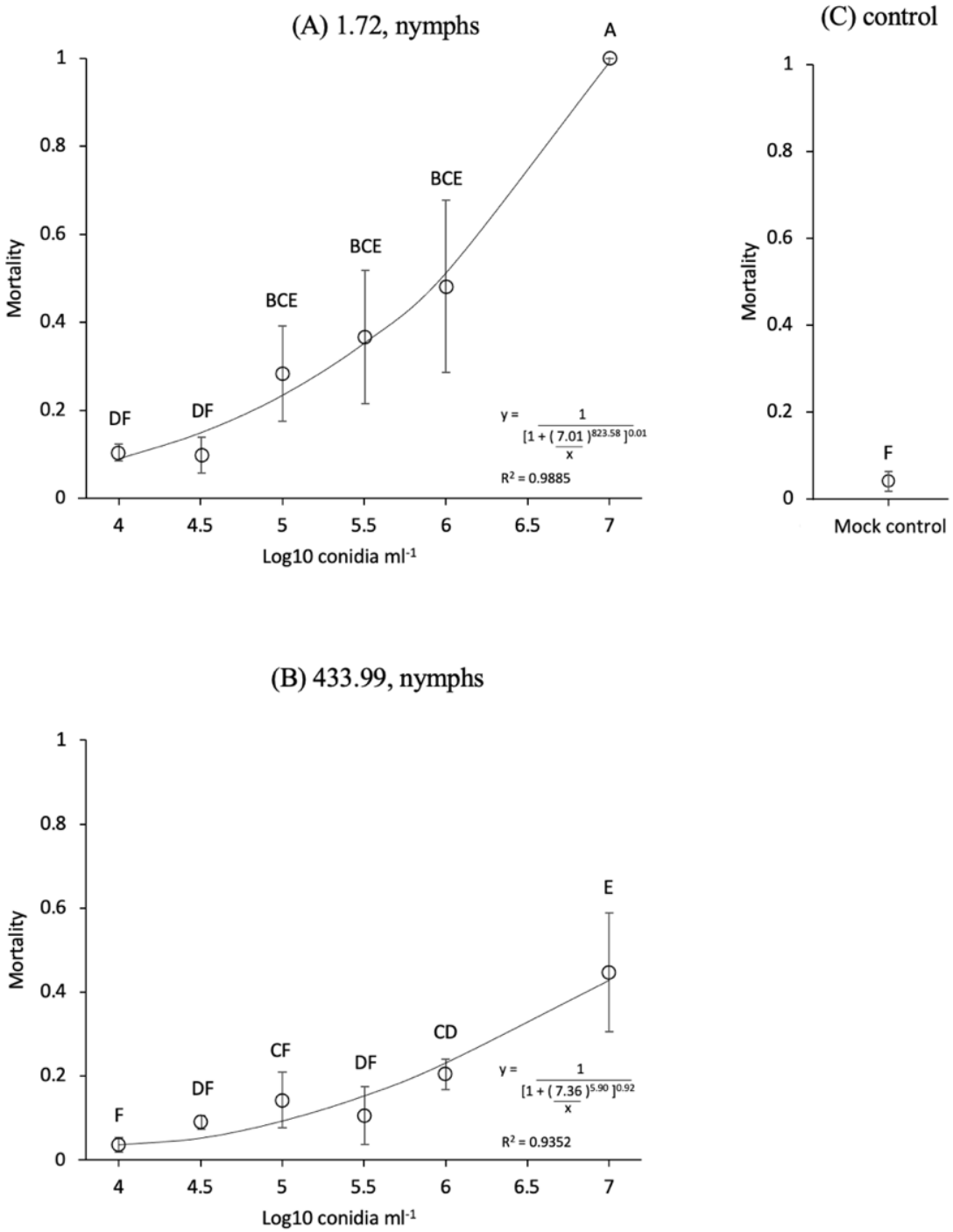


Figure 3.14: Concentration response assay mortality results for *Brevicoryne brassicae* nymphs when treated with six serial Log10 concentrations (4, 4.5, 5, 5.5, 6 and 7) of EPF isolates (A) 1.72 (*Akanthomyces dipterigenus*), (B) 433.99 *(Beauveria bassiana*) and (C) a mock treated control. An asymmetrical (5-factor) logistic concentration-response curve was fitted to the six evaluated concentrations for both isolates 1.72 and 433.99. Between treatment (EPF isolate + concentration) differences were evaluated through a GLM which revealed treatment was a significant factor ($p=3.40x10^{-11}$). Post hoc analyses of pairwise treatment differences represented as letters across (A), (B) and (C), allowing for comparison of pairwise differences within and between treatments.

3.3.6.2. Concentration-response Assay – lethal concentrations

Median lethal concentrations leading to 50%, 70% and 90% mortality (LC_{50}, LC_{70} and LC_{90}) of both EPF isolates for both adult and nymph *B. brassicae* were calculated through probit regression (Table 3.11). Within all four regression analyses, concentration was found to be a significant factor (1.72/adults: $Z(5)=10.698$, $p=1.05x10^{-26}$) (433.99/adults: $Z(5)= 7.608$, $p=2.79x10^{-14}$) (1.72/nymphs: $Z(5)=10.244$, $p=1.25x10^{-24}$) (433.99/nymphs: $Z=5.105$, $p=3.32x10^{-7}$). As concentration was found to be significant in all regression analyses, estimates for LC_{50}, LC_{70} and LC_{90} were therefore calculated alongside upper and lower 95% confidence interval limits for both EPF isolates against both aphid life stages (Table 3.11). In all cases, probit estimated lethal concentrations of isolate 433.99 were noted to be higher than isolate 1.72 (Table 3.11). Calculated LC_{50}, LC_{70} and LC_{90} estimates for adult *B. brassicae* treated with EPF isolate 433.99 (*B. brassicae*) were found to be 2.45x, 6.22x and 23.75x higher relative to those seen for adult *B. brassicae* treated with isolate 1.72 (*A. dipterigenus*). In each case however, 95% CI lower and upper bounds were observed to overlap indicating equal virulence between these two EPF isolates against *B. brassicae* adults. The differences in calculated LC_{50}, LC_{70} and LC_{90} for first-instar *B. brassicae* nymphs treated with 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) was starker, with isolate 433.99 (*B. bassiana*) requiring concentrations 67.37x, 250.32x and 1658.88x higher than isolate 1.72 (*A. dipterigenus*) respectively to reach 50%, 70% and 90% median lethality respectively. Unlike the confidence intervals seen for adult *B. brassicae*, the 95% CI lower and upper bounds for isolates 1.72 and 433.99 against first instar *B. brassicae* did not overlap for LC_{50}, LC_{70} indicating a substantial difference in nymph virulence between the two isolates.

Table 3.11: Lethal concentration (LC50, LC70 and LC90) estimates and 95% confidence limits of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) for *Brevicoryne brassicae* 10-day old adults and 1-day old first instar nymphs. Lethal concentrations were calculated using probit regression (SPSS) to analyse concentration-response assay where both aphid life stages were treated with six concentrations of each EPF isolate. Lethal concentrations presented in conidia ml^{-1}.

EPF Isolate	Aphid life-stage	LC50			LC70			LC90		
		Estimate	Lower bound	Upper bound	**Estimate**	Lower bound	Upper bound	**Estimate**	Lower bound	Upper bound
1.72 *A.dipterigenus*	Adults	**$2.34x10^5$**	$9.96x10^4$	$6.34x10^5$	**$7.03x10^5$**	$3.01x10^5$	$3.28x10^6$	**$3.44x10^6$**	$1.09x10^6$	$4.82x10^7$
	Nymphs	**$4.75x10^5$**	$1.64x10^5$	$2.40x10^6$	**$1.55x10^6$**	$5.14x10^5$	$2.36x10^7$	**$8.56x10^6$**	$1.87x10^6$	$9.13x10^8$
433.99 *B.bassiana*	Adults	**$5.74x10^5$**	$1.30x10^5$	$1.20x10^7$	**$4.37x10^6$**	$8.20x10^5$	$3.43x10^9$	**$8.17x10^7$**	$5.92x10^6$	$2.42x10^{13}$
	Nymphs	**$3.2x10^7$**	$7.31x10^6$	$7.10x10^8$	**$3.88x10^8$**	$4.70x10^7$	$3.84x10^{10}$	**$1.42x10^{10}$**	$6.60x10^8$	$1.27x10^{13}$

3.3.6.3. Concentration-response Assay – sublethal effects

Concentration-response reproduction results (Figure 3.15) were analysed through a GLM/GLMM approach. One GLM and three GLMMs were undertaken with each being significant improvements over null models (Table 3.12). Within the initial GLM analysis, replicate proved a non-significant factor in the model (Wald X^2 (2) = 0.231, p = 0.891). No significant interactions were noted between replicate and (1) treatment (Wald X^2 (22) = 13.160, p = 0.929), (2) day (Wald X^2 (14) = 6.617, p = 0.0.948), (3) EPF isolate (Wald X^2 (2) = 3.270, p = 0.195), and (4) concentration (Wald X^2 (10) = 7.601, p = 0.668).

Model fit (AIC) scores identified that a GLMM model including EPF isolate, concentration and day as factors offered the best model fit (AIC score of 250.5) thus this model was used for interpretation (F(14,278)=5.217, p=9.29 x 10^{-9}) (Table 3.12). From this model, concentration (F(5)=3.426, p=0.005) and day (F(8)=6.351, p=1.43 x 10^{-7}) were found to be significant factors influencing observed reproduction while EPF isolate was found not to be a significant factor (F(1)=3.297, p=0.059). Post hoc analysis revealed that daily reproduction was significantly higher following a treatment with an EPF concentration of 7.0 Log10 conidia ml^{-1} (1 x 10^7 conidia ml^{-1}) (regardless of the EPF isolate) relative to all other evaluated concentrations (p=<0.05), with an average of 3.3 nymphs born per day following this treatment and a range of 0 – 9 nymphs (Figure 3.16C). No significant difference in reproduction was noted between the remaining five treatment concentrations, for which average daily reproduction ranged from 2.5 - 2.7 nymphs. Post hoc analysis to evaluate between day differences in reproduction revealed that the average reproduction of 2.0 on day 1 was significantly lower all other days aside from day 9 (average of 2.2) (p=<0.05). Between days 2-8 average reproduction ranged from 2.6 - 3.2 nymphs with this lowest level of reproduction being seen on both days 3 and 8 and the highest on day 6 – with the only significant difference in reproduction within days 3-8 being seen between days 3/8 and 6 (p=<0.05) (Figure 3.16D).

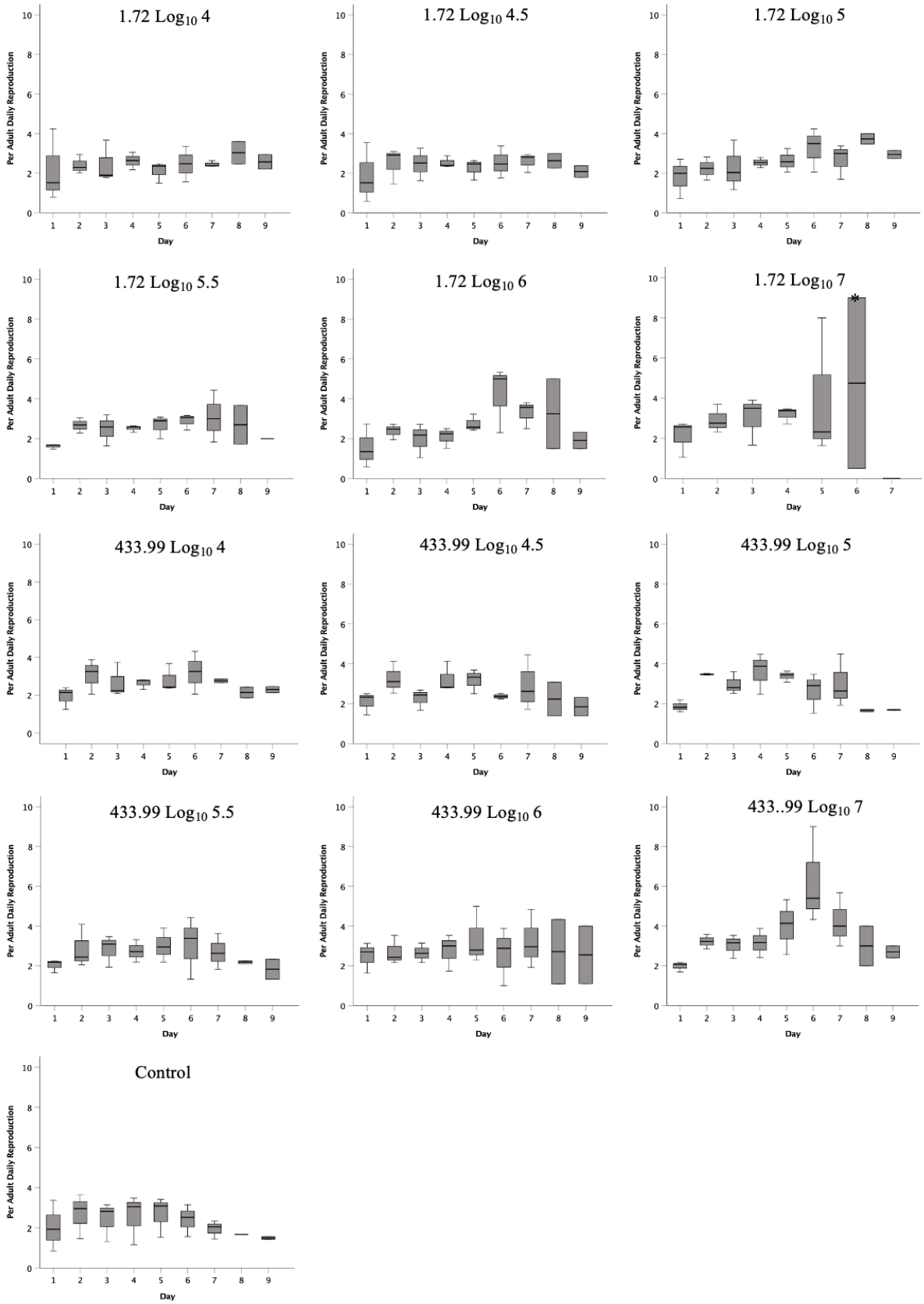


Figure 3.15: Per *Brevicoryne brassicae* adult daily reproduction observed post spray with 2ml of conidia solutions of 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*). These results were analysed through a GLMM which revealed a significant difference in overall mean daily reproduction ($P = 9.29 \times 10^{-9}$).

* Owing to 100% mortality at day 7, per adult daily reproduction for EPF isolate 1.72 were unavailable for days 8 and 9.

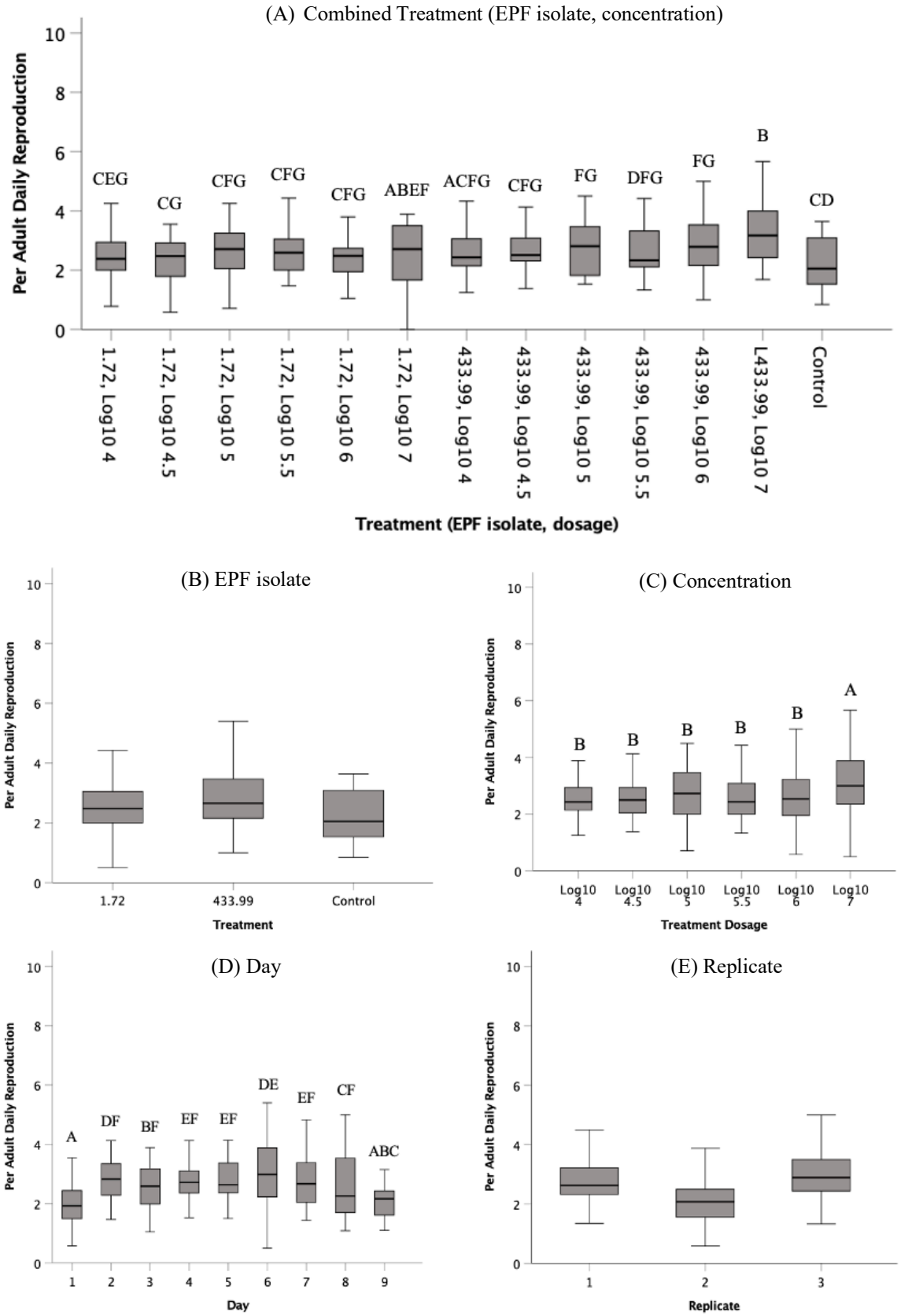


Figure 3.16: Effects and significance of the five assessed factors upon per adult daily reproduction observed post spray within a concentration-response assay of adult *Brevicoryne brassicae* against six different concentrations of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*). These factors were analysed through a GLMM which included (B) EPF isolate, (C) concentration and (D) day as factors while (E) replicate was included as a random effect. Within this model, concentration and day were found to be significant factors influencing *B. brassicae* reproduction (p=0.005, <0.001 respectively).

Table 3.12: Summary of four models used to evaluate sublethal effects of treatments upon adult *Brevicoryne brassicae* daily reproduction during concentration-response assay. The third evaluated model (GLMM with EPF isolate, concentration and day as factors alongside replicate as a random effect) was found to offer the best model fit and thus this analysis was preferred in interpretation of findings.

Model	Distribution	Link	Omnibus test (Sig.)	Model fit (AIC)	Factors (Sig.)				Random effects	
GLM	Negative binomial	Log	**1.23×10^{-13}**	3798.7	Treatment (EPF isolate and concentration)		Day	Replicate	-	
					0.001		**1.15×10^{-9}**	**1.15×10^{-10}**		
GLMM	Negative binomial	Log	**5.67×10^{-8}**	288.2	Treatment (EPF isolate and concentration)		Day		Replicate	
					0.005		**1.25×10^{-7}**			
GLMM	Negative binomial	Log	**9.29×10^{-9}**	250.5	EPF isolate	Concentration	Day		Replicate	
					0.059	**0.005**	**1.43×10^{-7}**			
GLMM	Negative binomial	Log	**0.004**	257.5	EPF isolate	Concentration			Replicate	Day
					0.055	**0.011**				

3.3.7. Assessing viable conidia per unit area sprayed by Potter's tower

ANOVA analysis revealed a significant difference in Log_{10} viable conidia mm^{-2} resulting from spraying with different conidial suspensions of different concentrations ($F(5)=3393.045$, $p=6.14 \times 10^{-60}$). Tukey post hoc analysis revealed no significant difference in the viable conidia mm^{-2} resulting from spraying 2ml of the 1×10^{7} conidia ml^{-1} for isolates 1.72 and 433.99. All other treatments were significantly different in viable conidia per unit area ($p=<0.001$). ANOVA analysis was also undertaken to assess between replicate variation, with no significant variation being identified demonstrating high between replicate conidial dose ($F(2)=0.002$, $p=0.998$). Descriptive statistics were also calculated for each isolate and concentration to evaluate the averages and spread of data (Table 3.13).

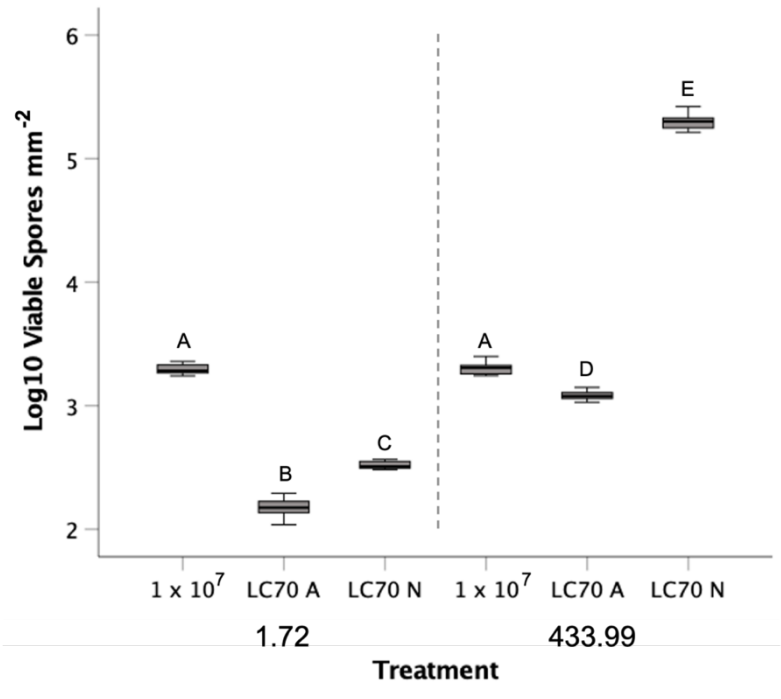


Figure 3.17: Viable conidia mm^{-2} received by *Brevicoryne brassicae* during bioassay procedure, wherein 2ml of conidia suspensions is sprayed by Potters tower. LC70 concentrations for adults (10-day old fixed aged adults) and nymphs (1-day old, first instar nymphs) evaluated in this experiment are shown in Table 3.5. ANOVA analysis was undertaken to determine whether applied real-term doses varied. ANOVA confirmed that between different treatments and concentrations, applied viable conidia mm^{-2} significantly varied (P=6.14 x 10^{-60}) aside from the two evaluated 1 x 10^{7} conidia ml^{-1} (7 Log10 conidia ml^{-1}) suspensions for 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*), where the number of viable conidia delivered per unit area did not significantly differ.

Table 3.13: Descriptive statistics outlining the averages and variation seen within Log10 viable conidia mm^{-2} following Potters tower spraying of 2ml each of six EPF suspensions.

EPF Isolate	Conc	Mean	Standard Deviation	SEM	Min	Max	Range	Interquartile Range
1.72	1 x 10^{7}	3.2961	0.04266	0.01422	3.24	3.36	0.12	0.08
1.72	LC70 A	2.1802	0.07755	0.02585	2.04	2.29	0.25	0.12
1.72	LC70 N	2.5108	0.04635	0.01545	2.41	2.57	0.15	0.06
433.99	1 x 10^{7}	3.3062	0.053	0.01767	3.24	3.4	0.16	0.09
433.99	LC70 A	3.0838	0.04074	0.01358	3.03	3.15	0.12	0.07
433.99	LC70 N	5.3019	0.06649	0.02216	5.21	5.42	0.21	0.11

3.3.8. Secondary Pickup Assay

To evaluate whether secondary pickup of EPF induced significant mortality, a Cox proportional hazards model analysis was undertaken. This model was found to offer a significant improvement over a null model (X^2 (4) = 74.007, p = 3.23×10^{-15}). Pre-infestation EPF leaf treatment with EPF was found to be a significant factor influencing aphid survival within this model (Wald X^2 (2) = 60.256, p = 8.23×10^{14}). Replicate meanwhile was found to be insignificant within this model (Wald X^2 (2) = 4.527, p = 0.104). This finding was also reflected in a Kaplan-Meier survival plot where EPF isolate 1.72 (*A. dipterigenus*) after 9 days resulted in 20.0% mortality while isolate 433.99 (*B. bassiana*) resulted in 40.2% mortality (control mortality, 10%) (Figure 3.18). Isolate 1.72 was found to have a hazard ratio of 2.123 while isolate 433.99 had a hazard ratio of 4.877, with Log rank testing revealing that the hazard ratios and survival curves of both isolates were significantly different to both the control and to one another (p=<0.05 in all cases) (Table 3.14; Figure 3.18).

Secondary pickup assay was also evaluated through GLM analysis of day 9 mortality results including treatment (secondary EPF isolate pickup) as a sole factor. This model was found to offer a significant improvement relative to null model (Wald X^2 (2) = 74.089, p = 1.11×10^{-16}) and confirmed that EPF isolate was a significant factor influencing secondary pickup associated mortality (Wald X^2 (2) = 63.843, p = 1.37×10^{-14}). Subsequent post-hoc analysis revealed a significant difference between all three pairwise combinations (1.72 *A. dipterigenus*, 433.99 *B. bassiana* and control treatment (p=<0.05 in all cases) as was also revealed through Log-rank tests. Relative to the nymph assay described in section 3.3.4 pre-treatment with isolate 1.72 (*A. dipterigenus*) in this assay led to only 20.0% mortality relative to 100.0% mortality where EPF was applied directly to nymphs as well as the leaf surface. In contrast, within this assay leaf pre-treatment with isolate 433.99 (*B. bassiana*) led to 40.2% mortality compared to 22.7% in the nymph assay described in section 3.3.4. This difference may in part be accounted for by variation between replicates in the observed mean mortality (Figure 3.8D).

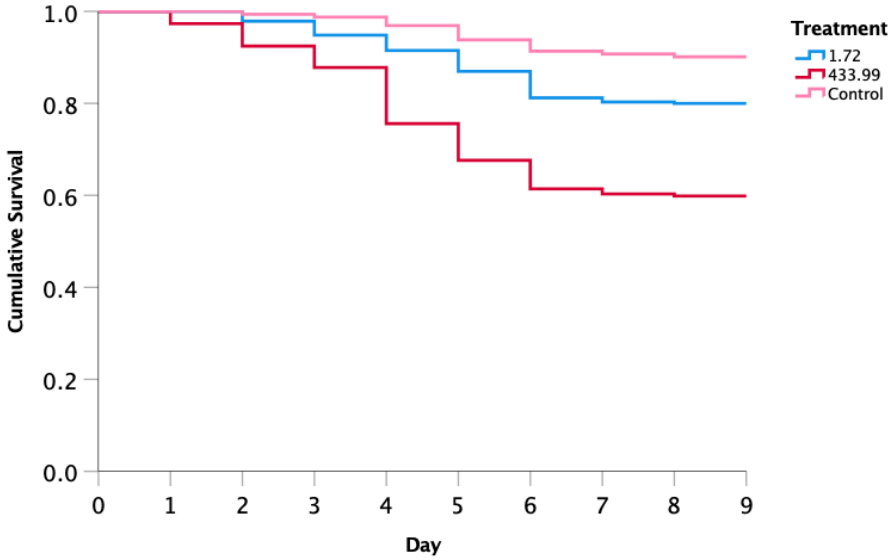


Figure 3.18: Cumulative survival of *Brevicoryne brassicae* 1-day old nymphs raised on leaves pre-sprayed with EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*). Owing to the relative fragility of first-instar *B. brassicae* nymphs, aphids of this life-stage assessed in bioassays were sprayed directly on the leaves to mitigate inviable levels of handling damage. This treatment regime however potentially results in mortality due not only to primary EPF pickup (directly from the spray) but also from later secondary pickup of EPF conidia as aphid nymphs move on the leaf. This assay therefore sought to investigate whether there is any significant mortality owing to secondary pickup of EPF conidia by *B. brassicae* nymphs**.**

Table 3.14: Mean survival time of one-day old first instar *Brevicoryne brassicae* reared on leaves pre-sprayed with the determined LC70 (against *B. brassicae* nymphs) of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) and hazard ratio of secondary pickup for both isolates 1.72 and 433.99. Hazard ratio (HR) indicates the relative average daily risk of death compared to the 0.05% Triton-X treated control. Median survival time which gives the proportional cumulative survival of 50% of the populations could not be calculated owing to <50% mortality at experimental cessation (Day 9). HR values followed by different lower-case letters within the column are significantly different (log rank c2 ≥ 3.841 [73.58], P < 0.05 [<0.001]).

a MST = median survival time, given in days
b HR = hazard ratio, compared to the 0.05% Triton-X treated control
* MST could not be calculated due to <50% mortality at experimental cessation (Day 9)
** Control treatment assigned a hazard ratio score of 1, against which all other treatments were evaluated.

Treatment		Factors	MST[a] (95% Cl)	HR[b] (95% Cl)	Z (HR)	P (HR)	df	n
		Rep			60.256	0.104	2	3
		Treatment			4.527	<0.001	2	3
Control	0.05% Triton-X100		*	1** a				162
1.72	*Akanthomyces dipterigenus*		*	2.123 (1.230 – 3.666) b	6.969	0.008	1	330
433.99	*Beauveria bassiana*		*	4.877 (2.924- 8.132) c	36.006	<0.001	1	451

3.3.9. Assessing germination and germ tube growth of isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) over a 24hr period

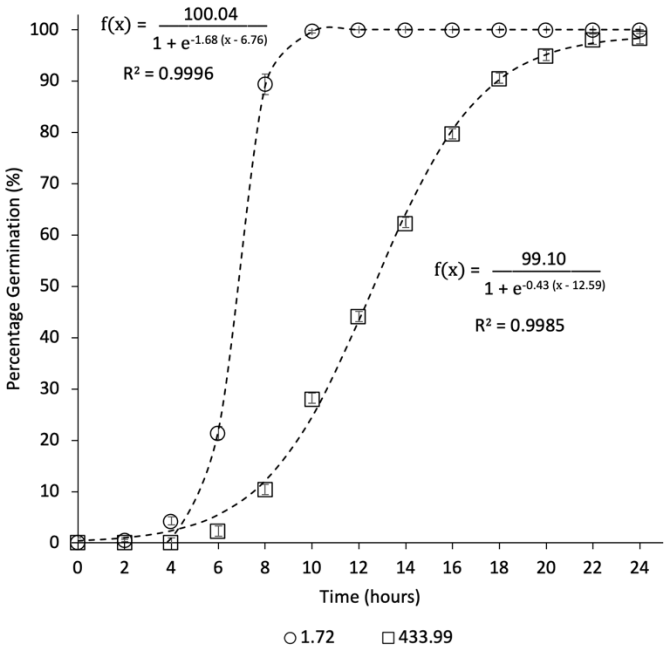
Germination and germ tube growth rate between EPF isolates 1.72 and 433.9, were plotted initially as two scatter plots for each dependant variable (Figure 3.19). To germination data for EPF isolates 1.72 and 433.99, two 3-parameter logistic growth curves were fitted. These curves were found to describe the data well, with calculated R^2 values of 0.9996 and 0.9985 respectively. To germ tube growth plots trendlines were also fitted, with an exponential trendline fitting isolate 1.72 data well ($R^2 = 0.9947$) while a linear trendline was found to best describe isolate 433.99 germ tube growth results ($R^2 = 0.9797$).

For percentage germination, for both isolates minimal germination was noted between 0-4hrs (Figure 3.19A). At 6hrs however a notable difference in the germination between the two isolates had emerged, with isolate 1.72 (*A. dipterigenus*) having exceed 20% germination which germination for isolate 433.99 (*B. bassiana*) was still only ~2-4%. The difference in germination between isolates was the greatest at 8hrs, with isolate 1.72 (*A. dipterigenus*) having reached 90% germination while germination for isolate 433.99 (*B. bassiana*) was ~10%. By 10hrs isolate 1.72 (*A. dipterigenus*) had reached 100% germination while isolate 433.99 (*B. bassiana*) had reached ~28% germination. Between hours 10-16 the rate of germination for isolate 433.99 (*B. bassiana*) was linear, reaching ~80% germination after 16hrs. This rate began to slow from 16hrs onwards, with 433.99 (*B. bassiana*) having reached ~90%, ~93% and ~96% germination after 18, 20 and 22hrs respectively before reaching a concluding germination level of ~98% after 24hrs. This difference in germination between isolates can be seen in the microscope photographs taken at each timepoint for isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*)

For germ tube length, no difference in germ tube growth was observed between hours 0-8 between isolate, with average germinated conidia germ tube length at this time point for both 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) being approximately 10µm (Figure 3.19B). from 10hrs onwards however the average germ tube length of isolate 1.72 (*A. dipterigenus*) became increasingly high relative to 433.99 (*B. bassiana*). After 10hrs, the average germ tube length of 1.72 (*A.*

dipterigenus) was ~18 µm while for 433.99 (*B. bassiana*) length was ~15µm. At 12hrs the difference in germ tube length was relatively similar to 10hrs, with lengths of ~20µm and ~~17µm for isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) respectively. From 14hrs onwards however this difference became increasingly high, with isolate 1.72 (*A. dipterigenus*) having an average germ tube length of ~45µm, ~60µm, ~78µm and ~98µm at 14, 16, 18 and 20hrs post setup relative to ~18 µm, ~20µm, ~22µm and 24µm for isolate 433.99 (*B. bassiana*). By 22hrs and 24hrs post experiment setup, the germ tube length of isolate 1.72 (*A. dipterigenus*) had reached ~150 and ~175µm respectively, ~125µm and ~148µm longer than the germ tube of germinated 433.99 (*B. bassiana*) conidia.

(A) Percentage Germination



(B) Germ Tube Length

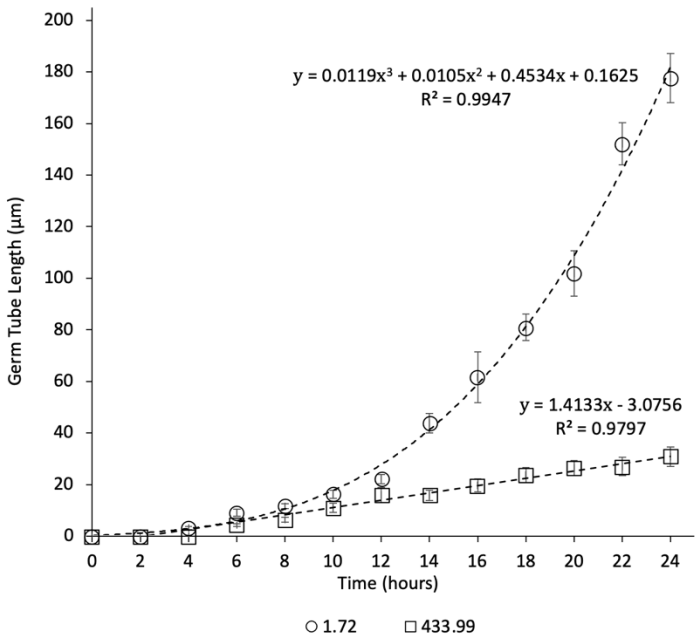


Figure 3.19: Germination percentage (A) and germ-tube length (B) results following 24-hour monitoring at 2-hour intervals. Percentage germination results for both isolates conformed well to logistic trendlines. Germ tube length results for isolate 1.72 (*Akanthomyces dipterigenus*) was modelled best by an exponential trendline while for isolate 433.99 (*Beauveria bassiana*) a linear trendline best matched experimental results.

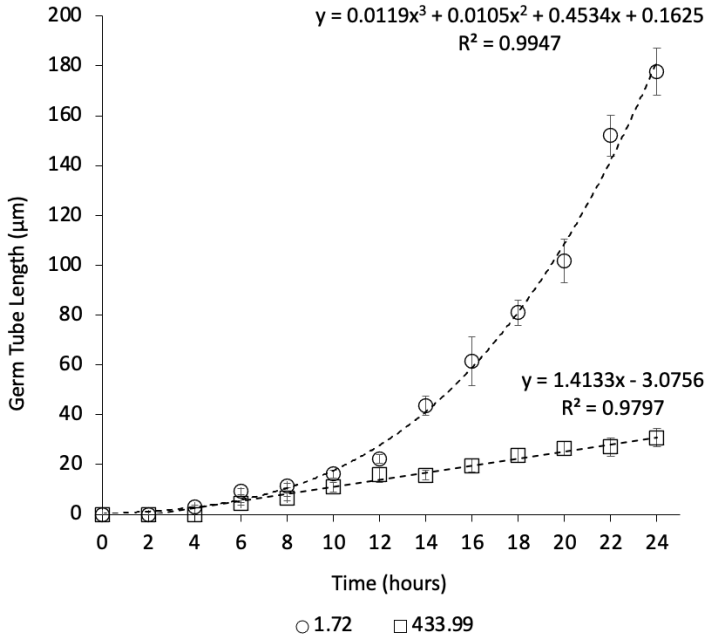
Percentage germination results between isolates were assessed using logistic regression. This model included EPF isolate as a factor, time as a covariate and an interaction term (EPF isolate x time) to determine whether there existed a difference in fitted logistic lines. This model was found to be a significant improvement over a null model (X^2 (3)=24017.755, p<0.001). Within this analysis, the interaction term EPF isolate x time was found to be a significant factor (Wald X^2(1)=501.430, p<0.001 therefore indicating a significant difference in logistic EPF curves over time. Both time and EPF isolate were found to be significant factors (EPF: W(1) = 2710.041, p=<0.001) (time: W(1) = 5263.637, p=<0.001) therefore also indicating a difference in intercepts between curves. Through logistic regression, median times were calculated for 5%, 50% and 95% germination of both isolates (Table 3.15). Calculated median germination times differed between EPF isolates; with isolate 1.72 (*A. dipterigenus*) germinating faster than isolate 433.99 (*B. bassiana*). At the lowest germination percentage assessed (5%), median time for 5% germination for EPF isolate 1.72 (*A. dipterigenus*) was 4.047 hours relative to 7.772 hours for isolate 433.99 (*B. bassiana*) with no evidence of overlaps in calculated 95% confidence intervals. This difference in time for germination was further apparent for 50% germination, with isolate 1.72 (*A. dipterigenus*) requiring a median time of 6.447 hours to reach this percentage while isolate 433.99 (*B. bassiana*) required 12.382 hours therefore requiring almost double the amount of time relative to isolate 1.72 *(A. dipterigenus*). A similar trend was also noted for 95% mortality, with isolate 1.72 requiring only 10.272 hours to reach this relative to 19.728 hours for isolate 433.99 (*B. bassiana*).

Table 3.15: Calculated median germination times for 5%, 50% and 95% total germination for conidia of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria* bassiana) at 20°C. Germination times calculated through logistic regression analysis.

EPF Isolate	**Time to 5% germination (hours)**			**Time to 50% germination (hours)**			**Time to 95% germination (hours)**		
	Estimate	Lower bound	Upper bound	**Estimate**	Lower bound	Upper bound	**Estimate**	Lower bound	Upper bound
1.72 *A. dipterigenus*	**4.047**	3.625	4.427	**6.447**	6.02	6.884	**10.272**	9.505	11.26
433.99 *B. bassiana*	**7.772**	7.073	8.373	**12.382**	11.839	12.919	**19.728**	18.537	21.308

Germ tube growth data were analysed by first transforming through a Log_{10} transformation and plotting as a scatter plot with linear trendlines fitted to germ tube length for each isolate, with both being found to offer good model fits (R^2=0.9602 and 0.8799 respectively) (Figure 3.20). Transformed data were then assessed through multiple linear regression analysis, including EPF isolate as a factor, time as a covariate and an interaction term (EPF isolate x time). This analysis revealed that a linear regression model including these three variables was a significant improvement over a null model, accounting for 90.2% of the overall variation with the data ($F(3)=787.687$, $p=6.57x10^{-129}$, $R^2=0.902$). Within the model, as the interaction term 'EPF isolate x time' was found to be significant ($p=3.09x10^{-14}$), the lines for EPF isolates 1.72 and 433.99 were confirmed as non-parallel therefore indicating a significant difference in the growth rates of the germ tubes of these two isolates, with isolate 1.72 (*A. dipterigenus*) having therefore being found to have a significantly faster rate of germ tube growth relative to isolate 433.99 (*B. bassiana*) ($p=<0.05$).

(A) Length (µm)



(B) Log10 Length (µm)

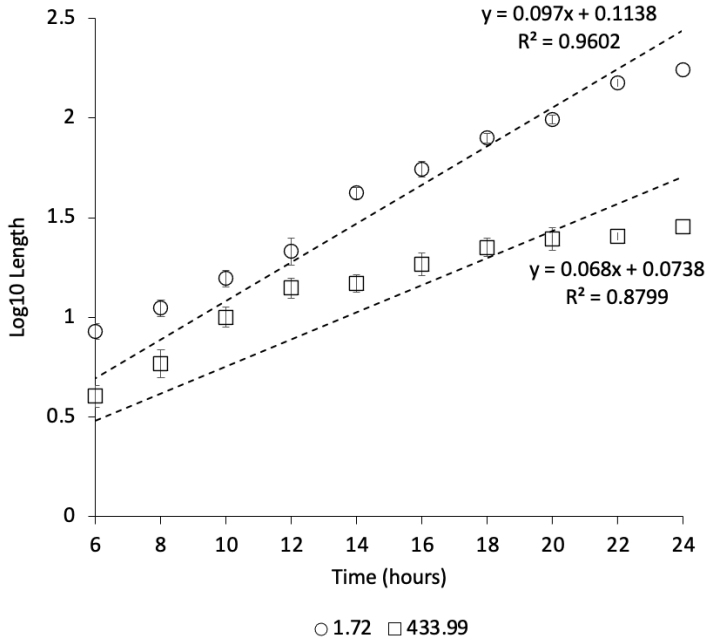


Figure 3.20: (A) Germ tube lengths (B) and Log10 germ tube lengths observed at 2-hour intervals over a 24-hour monitoring period for EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*). Multiple linear regression analysis confirmed a significant difference between the growth rate of isolates 1.72 and 433.99 over time ($p=3.09x10^{-14}$).

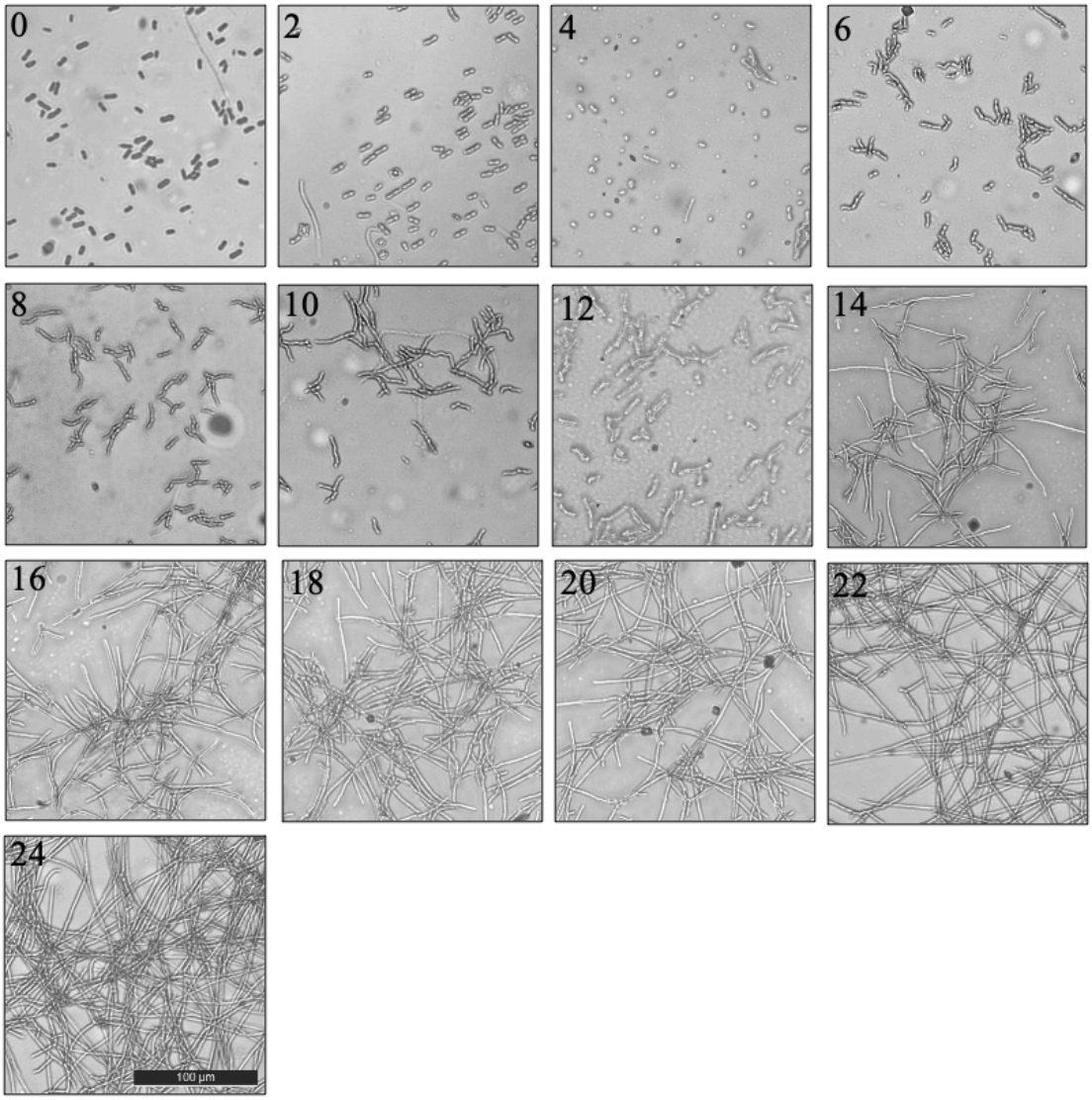


Figure 3.21: 20x magnification images of isolate 1.72 (*Akanthomyces dipterigenus*) conidia when growing on SDA at 20°C over a 24-hour period. At experimental setup, a separate plate was inoculated with 3 x 20ul spots of 1 x 10^7 conidia ml^{-1} for each timepoint. Plates were then incubated at 20°C and a plate taken every 2-hours onto which lactophenol cotton blue stain was added to halt further germination/growth and stain EPF conidia and developing germ tubes.

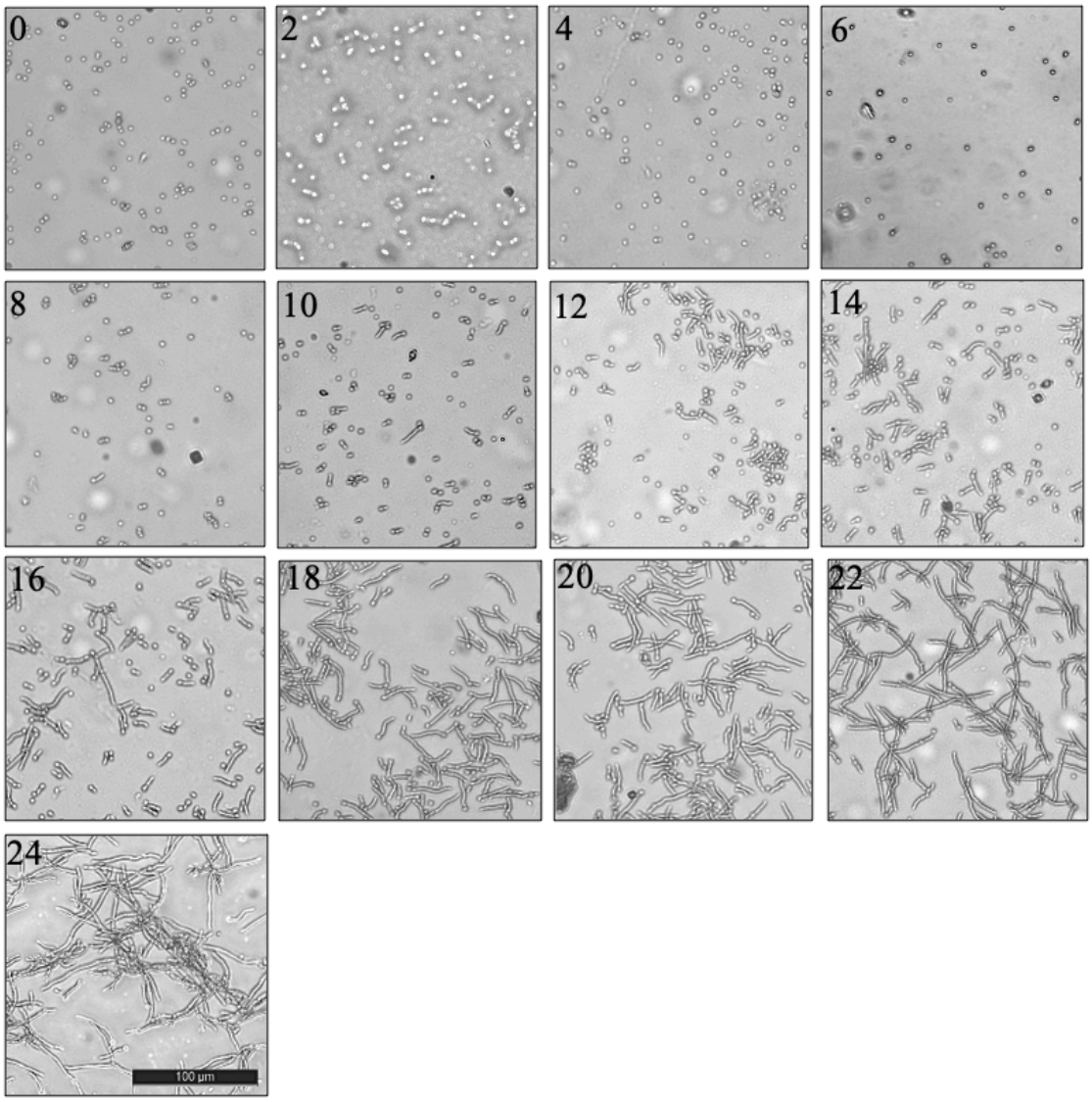


Figure 3.22: 20x magnification images of isolate 433.99 (*Beauveria bassiana*) conidia when growing on SDA at 20°C over a 24-hour period. At experimental setup, a separate plate was inoculated with 3 x 20ul spots of 1x 10^7 conidia ml^{-1} for each timepoint. Plates were then incubated at 20°C and a plate taken every 2-hours onto which lactophenol cotton blue stain was added to halt further germination/growth and stain EPF conidia and developing germ tubes.

3.4. Discussion

3.4.1. Candidate EPF isolate selection

To maximise the efficacy of EPF within a field IPM system, it is important that EPF are selected which are (1) able to germinate and grow effectively at field realistic temperatures and (2) virulent to a majority of the life stages of the target pest species. Within this study, ten EPF isolates were selected for evaluation of both their range of effective operating temperatures and virulence to adult and nymph *B. brassicae*. A pragmatic approach was taken to selecting candidates for screening, and included isolates used in commercial biopesticides (*A. muscarius* 19.79, *B. bassiana* 432.99 & 433.99), isolates isolated from aphids (*A. dipterigenus* 1.72 [which was also used as a commercial biopesticide, Vertalec®], *A. lecanii* 339.92, *Akanthomyces* sp. 1787.17, *B. bassiana* 1808.18) or isolates isolated from other pests of brassica crops (*A. muscarius* 365.92 & *B. bassiana* 347.92) (Table 3.1). To broaden the represented EPF species one further EPF isolate was also assessed (*M. brunneum*, 416.96), with this isolate having been isolate in Finland and thus likely to be adapter to cooler climates. All candidate isolates were obtained from the Warwick Crop Centre collection of EPF cultures (which has c. 2000 isolates) – with isolates being preferentially sourced from this collection because most had been isolated within the UK and thus likely to be better adapted to UK climatic conditions. Within this collection isolates are primarily classified morphologically (Thiery and Frachon, 1997). Before proceeding further, the identity of the selected isolates was first verified by sequencing two housekeeping genes per isolate and homology searches against the reference sequence database.

3.4.2. EPF isolate thermal evaluation

For both datasets modified Ratkowsky 3 models offered a good data fit, however R^2 values identified a better model fit for growth rate data relative to germination percentage.

For growth rate, optimum temperatures were found to range from 23.6 - 25.2°C, while for germination optimum temperatures were found to range from 24.4 - 25.0°C. These predictions for growth rate temperature optima broadly agree with

previous literature estimates for species including *B. bassiana*, *A. lecanii* and *A. muscarius*, with optimal growth temperatures typically reported to fall between 25-28°C (Fargues *et al.*, 1997; Davidson *et al.*, 2003; Vu *et al.*, 2007; Hussain *et al.*, 2018; Islam *et al.*, 2021). However, germination optimal temperatures identified within this study were lower than estimates of optimal EPF germination temperatures reported in the literature, which typically fall between 25-30°C (Scholte *et al.,* 2004; Omuse *et al.*, 2021). Such overall EPF germination and growth temperature estimates however can be misleading, with a growing body of evidence highlighting differences in thermal tolerance between EPF isolates isolated from different countries and regions – with particularly strong evidence of isolates adapted to cooler climatic conditions (Bidochka *et al.,* 1998; Hetsch *et al.*, 2002; Scholte *et al.*, 2004; Kryukov *et al.*, 2012; Klingen *et al.*, 2015). Owing to the majority of evaluated isolates within this study having been isolated within the UK, the findings of this study may therefore demonstrate such geographic thermal tolerance effects – with these isolates potentially demonstrating adaptation to cooler UK conditions. This discrepancy between identified temperature optima in this study and literature estimates may also result from differing statistical methodology. Within this study a nonlinear model was used to evaluate temperature dependant effects, as is commonplace for thermal evaluations in other fields including entomology (Damos and Savopoulou-Soultani, 2012). While nonlinear models undoubtedly provide a more accurate prediction of EPF isolate thermal tolerance and optimal temperature, this approach remains underutilised for EPF research (Omuse *et al.*, 2021). A majority of EPF studies continue to either not model temperature dependant effects (reporting instead the optimal evaluated temperature as an overall optima) or utilise regression methods for thermal evaluation (typically logistic or probit) – in spite of right-skewed temperature data being unlikely to meet the assumptions of such models (Mietkiewski *et al.* 1994; Vu *et al.,* 2007; Garrido-Jurado *et al.,* 2011; Couceiro *et al.*, 2021; Omuse *et al.*, 2021).

Despite lower optimal temperatures identified for these isolates than indicated in the literature, these optima are nonetheless still higher than UK annual average field temperatures (9.4°C in 2019 and 9.6°C in 2020) and summer average temperatures (June-September: 15.1°C in 2019 and 14.8°C in 2020) (Kendon *et al.*, 2020, 2021).

These averages do not reflect the difference between day and night-time temperatures, with higher temperatures present during the day (Kendon *et al.,* 2019, 2020). It should be noted however that for EPF biopesticides, it is not the ambient climatic conditions which influence the efficacy of EPF but the specific microclimatic conditions (particularly temperature and humidity) on the leaf/plant surface which is not necessarily linearly related to ambient conditions (Jaronski, 2010; Pincebourde and Woods, 2012; Caillon *et al.*, 2014). Leaf microclimates can vary significantly between different plant species owing to both their physical phenotypes and biochemical processes (Caillon *et al.*, 2014). To date no detailed study had been undertaken to evaluate the microclimatic conditions on the leaf surface of different field *Brassica* crops in the UK and its potential effect upon insect pests and pest-pathogen interactions, thus it remains unclear precisely how close or distant to optimal temperatures EPF isolates such as those evaluated here are likely to experience in UK field conditions.

3.4.3. EPF isolate virulence evaluation against *Brevicoryne brassicae* adults and nymphs

Through the thermal evaluation of EPF a temperature of 20°C was selected to evaluate EPF against *B. brassicae* – with this temperature being both relevant to UK daytime temperatures in the summer season (at which time *B. brassicae* pose the greatest risk to *Brassica* crops) while also being a temperature at which selected EPF isolates were demonstrated to germinate and grow well (Kendon *et al.,* 2020; 2021).

Previous studies have shown that adult aphids of many species are susceptible to a wide range of EPF (Derakhshan *et al.*, 2007; Asi *et al.*, 2009ab; Akbari *et al.*, 2013; Lee *et al.*, 2015; Nielsen and Hajek, 2005; Jandricic *et al.*, 2014; Saranya *et al.*, 2010; Ramanujam *et al.*, 2017; Ali *et al.*, 2018; Soleymadzade *et al.*, 2019). It was therefore anticipated that a majority of evaluated EPF isolates in this study would demonstrate pathogenicity to *B. brassicae*. In contrast, there is much less published information on the effect and efficacy of EPF against aphid nymphs - with the limited available evidence suggesting that nymphs have a low susceptibility to EPF infection because of the short intermoult period during nymph development which enables nymphs to escape infection by shedding conidia on the exuvium before they

have penetrated the haemocoel (Kim and Roberts, 2012; Jandricic, 2014). Despite this, there are reports that EPF can control mixed-age *B. brassicae* colonies under both controlled environment and field conditions – suggesting that they can infect and kill nymphs (Farag, 2008; Pacheco *et al.*, 2017; Prince and Chandler, 202: Gebreyohans *et al.*, 2021).

Within this study, all ten evaluated EPF isolates demonstrated significant virulence to *B. brassicae* adults (relative to control). Performance between EPF isolates however was not uniform, with 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) significantly outperforming most other isolates. Despite potential evidence in the literature suggesting some EPF genera such as *Akanthomyces* may be more specialist to hemipteran pests, no clear association between EPF species and virulence was observed (Fargues and Remaudiere, 1977; Liu *et al.*, 2006; Rai *et al.*, 2014; Chandler, 2017; Rohrlich, 2018). Within the context of population development, while overall virulence is important for control, speed of kill is also crucial – with more rapid kill resulting in a shorter adult reproductive period and thus significantly reduced progeny, the effect of which is likely to be exponentially amplified in subsequent aphid generations (Hesketh *et al.*, 2009). While indistinguishable in their virulence, isolate 1.72 (*A. dipterigenus*) demonstrated notably more rapid lethality in this bioassay relative to isolate 433.99 (*B. bassiana*).

Against *B. brassicae* nymphs, evaluated EPF isolates performed poorly – with only six of the ten evaluated EPF isolates resulting in significantly higher control relative to a control and only isolate 1.72 (*A. dipterigenus*) resulting in >30% cumulative mortality. This study thus provides further evidence supporting the hypothesis that EPF are less effective against aphid nymphs (Kim and Roberts, 2012; Jandricic *et al.*, 2014). Most commonly this is attributed to the short intermoult period of aphid nymphs - with the relationship between nymph intermoult period and the rate of germination, growth, and cuticle penetration of EPF therefore crucial in determining EPF efficacy (James *et al.,* 2003; Kim and Roberts, 2012). Emerging evidence highlights that aphid nymphs (alongside other pest species) may also employ further mechanisms to resist EPF infection including the production of (epi) cuticular antimicrobial molecules (including lipids, proteins, microRNAs and secondary metabolites) alongside exhibiting innate or learned behaviours to either prevent EPF

initial conidia acquisition or reduce acquired conidial load (Anderson *et al.*, 1995; Kim and Roberts, 2012; Ortiz-Urquiza and Keyhani, 2013; Kaczmarek *et al.*, 2020; Wang *et al.*, 2021). While mechanisms underlying aphid nymph insusceptibility to EPF are significantly understudied, the underlying fitness costs and pressures driving the evolution of these mechanisms are clear – with any lethal EPF infection of nymphs having a significantly greater population-level fitness cost than to adults owing to nymphs not yet having reached a reproductive stage. This is further underlined by reports in the literature and evidence in this study that suggest EPF infected adult aphid reproduction is unaffected until death (Prince and Chandler, 2020; Safavi and Sarhozaki, 2020). While aphid nymphs are undoubtedly a more difficult target for EPF, if EPF can kill nymphs the effect on *B. brassicae* population on a per-individual basis for nymphs is likely to be significantly higher than per adult (James *et al.,* 2003; Kim and Roberts, 2012).

3.4.4. Selection of a subset of EPF isolates and nymph susceptibility to EPF isolate 1.72 (*Akanthomyces dipterigenus*)

Isolate 1.72 (*A. dipterigenus*) demonstrated significant virulence to *B. brassicae* nymphs resulting (at a concentration of 1 x 10^7 conidia ml^{-1}) in consistent total nymph control (100% mortality and mycosis). Despite having been the EPF isolate included in the now commercially unavailable formulated biopesticide product Vertalec®, sold as an aphid-specific biopesticide, relatively few reports of the efficacy of this EPF isolate specifically against nymphs exist in the literature (Askary *et al.,* 1998; Safavi *et al.*, 2002; Faria and Wraight, 2007). Based upon the significant virulence of isolate 1.72 (*A. dipterigenus*) to nymphs identified in this study and the results of both the adult and nymph EPF screens overall, EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were selected for further and more detailed evaluation Owing to both isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) having been the basis of formulated products (Vertalec® and Botanigard® respectively), significant information is available regarding the host ranges of these two EPF isolates – with 1.72 (*A. dipterigenus*) recognised as primarily an aphid specialist while 433.99 (*B.* bassiana) is a broad generalist virulent to a wide range of insect orders (Brodeur, 2012; Prince and Chandler, 2020).

For isolate 1.72 (*A. dipterigenus*) the LC_{50} was approximately equal for adults and nymphs. In stark contrast, for isolate 433.99, the calculated LC_{50} for nymphs was two Log orders of magnitude higher than for adults. Interestingly the median survival times of nymphs (3 days) following treatment with 1.72 (*A. dipterigenus*) were markedly reduced relative to adults (4 days) – indicating that where EPF are able to overcome nymph defence mechanisms, mortality can occur quicker.

Owing to the suggestion that it is the intermoult period of nymphs which may primarily underlie the resistance of aphid nymphs to EPF, one reason for the high virulence of isolate 1.72 (*A.*) to *B. brassicae* nymphs was hypothesised to be more rapid germination and growth of this isolate –potentially allowing it to successfully infect prior to nymph exuvium shedding, thereby circumventing this means of defence (Kim and Roberts, 2012). Within this study, such rapid germination and growth of EPF isolate 1.72 was confirmed – taking approximately half the number of hours to reach 5, 50 and 95% germination relative to isolate 433.99 and growing at a significantly faster rate once germinated. As EPF were not exposed to the complex (epi) cuticular proteins and lipids which aphids employ to resist EPF adhesion, germination, and growth this study is not without caveats – with a relationship between growth and germination on artificial media and the aphid cuticle yet to be fully established (Ortiz-Urquiza and Keyhani, 2013). Nonetheless owing to a stark significant difference between both the virulence to nymphs of EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) and an equally significant difference in their growth and germination – there is a significant scope for further experiments exploring this observation:

- Firstly, having only observed such rapid germination/growth on artificial media, there is the need to investigate this phenomenon on the cuticle of both adult and nymph *B. brassicae* to identify whether such a difference is also present in the presence of cuticular and epicuticular proteins and lipids. Through such an experiment, further evidence would also be generated as to the relationship between EPF studies on artificial media and directly on insect cuticles.
- Secondly, owing to the link between EPF performance and temperature, there would be significant value in evaluating the rate of germination and growth

of EPF isolate 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) (alongside other isolates for comparison) at different temperatures.

- Thirdly, with isolate 1.72 (*A. dipterigenus*) previously having been described as an aphid specialist when marketed as the product Vertalec®, the results of this study raise the question whether rapid germination and growth could be a specific adaptation of aphid specialist EPF isolates. While the determinants of host range are undoubtedly more complex than speed of germination/growth alone, if such an association between aphid specialisms and germination/growth rate was confirmed, evaluating speed of germination in vivo could serve as a pre-selection approach for potentially aphid-specialist EPF isolates prior to the screening of EPF isolates against *B. brassicae* adults and nymphs.

3.4.5. EPF secondary pickup

Within both the initial screen and concentration-response bioassays in this study, isolate 1.72 (*A. dipterigenus*) universally outperformed isolate 433.99 (*B. bassiana*) in its virulence to *B. brassicae* nymphs. However, when EPF isolates were applied to directly to Brassica leaves, isolate 1.72 exhibited lower virulence than previously observed and isolate 433.99 (*B. bassiana*) resulted in double the mortality compared to 1.72 (40% and 20% respectively), indicating that EPF isolate 433.99 (*B. bassiana*) virulence to nymphs can increase by secondary pickup of conidia while isolate 1.72 (*A. dipterigenus*) is virulent primary through direct spray treatment.

Within this experiment, as in deployed agronomic settings, secondary pickup induced mortality is likely proportionate to (1) the persistence of viable conidia on the leaf surface owing to its resistance to damaging environmental conditions (e.g. UV and desiccation due to low humidity), (2) the ability of sufficient conidia (to constitute a lethal dose) to successfully adhere to the cuticle of any passing aphid pest and (3) the amount of movement of by the particularly pest species, with *B. brassicae* being noted as relatively sedentary (McLaren and Pottinger, 1969; Boucias *et al.*, 1988; Kouassi *et al.*, 2002; Holder and Keyhani, 2005; Bernardo *et al.,* 2020). While conidial viability on *Brassica* leaf surface was not assessed in this study, owing to relatively humid bioassay conditions and low UV due to artificial lighting - with low humidity and high UV the primary factors reported to reduced conidial

viability in the field Thompson *et al.*, 2006 - it can be assumed that the persistence of viable conidia on the leaf surface during the experimental period was significantly high. This finding of marked differences in secondary pickup mortality between isolates was unexpected, indicating a difference in ability of conidia of the two EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) to adhere successfully to passing nymphs at sufficiently high doses to cause mortality - potentially resulting from either a species level difference or a difference owing to host specificity. Some differences have been noted in the literature in the adhesion processes employed by these two EPF species which may partially explain this finding (Qu *et al.*, 2017).

EPF conidial adhesion to insect cuticles is well characterised, being broadly governed by nonspecific hydrophobic and electrostatic interactions between EPF conidia and the host insect epicuticle (Boucias *et al.*, 1988; Holder and Keyhani, 2005), *A. dipterigenus* conidia however have been noted to employ a further adhesion mechanism - being covered in an amorphous mucilage which facilitates the adhesion of its conidia to host insect cuticles (Qu *et al.*, 2017). Owing to bioassay methodology used in these evaluations, such mucilage was likely washed away thus removing one of the mechanisms employed by *A. dipterigenus* for adhesion. While this could account wholly for experimental observations of minimal 1.72 (*A. dipterigenus*) secondary pickup related mortality, these findings may also reflect differences in adhesion according to host specificity. As a generalist EPF, isolate 433.99 (*B. bassiana*) is able to infect a wide range of hosts, this however comes with the caveat that the population density of some hosts is likely to be low thus conidia may not be directly transferred to a new host and may instead be deposited on a leaf surface – thus having strong leaf surface to passing insect cuticle adhesive capabilities could be an advantageous generalist EPF adaptation. Conversely for an aphid-specialist EPF isolate such as 1.72 (*A. dipterigenus*), new potential aphid hosts are likely to be in abundance within the immediate area of an actively infected host – it is therefore evolutionary advantageous for such an EPF to have adhesion mechanisms best suited for host-host transmission – with the amorphous mucilage of *A. dipterigenus* having been hypothesised as such an adaptation (Qu *et al.*, 2017). Such differences in EPF adhesion according to host specificity have yet to be investigated in the literature and represents an avenue for potential research.

3.4.6. Aphid-specialist EPF – identification and agronomic value

The significant virulence of isolate 1.72 (*A. dipterigenus*) to both aphid adults and nymphs raises the question of whether further such highly virulent aphid-specialist anamorphic hypocrealean EPF isolates conducive to mass production exist. While evaluation of EPF against aphids has been historically and remains a significant area of research, there exist very few reports of similarly aphid specific EPF with demonstrably high virulence to both adults and nymphs (Vu *et al.*, 2007; Herlinda *et al.*, 2010; Jandricic *et al.*, 2014).

While aphid specialism as demonstrated by isolate 1.72 (*A. dipterigenus*) may therefore be significantly rare within the anamorphic hypocrealean EPF, its value for the control of both adult and nymph aphids is clear - likely having a profoundly more significant population level effect than the control of adults alone. While significant further research is required to both corroborate the efficacy of isolate 1.72 (*A. dipterigenus*) against *B. brassicae* nymphs and investigate them in different situations and against different aphid pests, these data present a clear argument in favour of the re-commercialisation of EPF isolate 1.72 (*A. dipterigenus*) as an aphid-specific biopesticide. Such commercialisation however should be taken with care. Owing to the longstanding evolutionary 'arms race' between EPF and their insect hosts, interactions between the two are profoundly complex – with physical, biochemical, and immunological battles being fought both on the insect cuticle surface and within the haemolymph (Butt *et al.*, 2016; Wang and Wang, 2017; Pedrini, 2018). From a conventional pest control perspective, EPF can therefore be considered to act through multiple modes of action to result in aphid mortality (Altinok *et al.*, 2019). Aphids however have consistently demonstrated a remarkable propensity for developing resistance to both plant resistance and synthetic chemical pesticides (Stuthman *et al.*, 2007; McMenemy *et al.*, 2009; Dedryver *et al.*, 2010; Dogimont *et al.*, 2010; Bass *et al.*, 2014; Xu *et al.*, 2015; Boissot *et al.*, 2016ab; Dossett and Kempler, 2016). While EPF undoubtedly represent among the most significant evolutionary challenges to aphids, widescale adoption and deployment of EPF inundatively (as it the current predominant application approach) would undoubtedly place a profoundly high selection pressure upon aphid populations and thus, however unlikely, risk the development of more EPF-resistant aphid clones. While such a phenomenon has received minimal research attention, some evidence

does highlight that aphid resistance to EPF may not be wholly hypothetical (Serebrov *et al.*, 2005; Kraaijeveld and Godfray, 2008; Dubovskiy *et al.*, 2014). Even with this study evidence of the strength of EPF as a selection pressure is apparent, with a significant increase in the reproduction of surviving adult *B. brassicae* when treated with the highest EPF concentration (of both isolate 1.72 and 433.99) in a concentration-response assay (7 Log10 conidial ml^{-1} / 1 x 10^7 conidia ml^{-1}) relative to those treated at lower concentrations – indicating only the fittest and most fecund adults survived treatment forming a smaller yet more fecund subpopulation.

Self-evidently relying on EPF, even one as virulent to aphids as 1.72 (*A. dipterigenus*), is potentially problematic. Two questions are therefore raised: (1) how can valuable EPF isolates such as 1.72 (*A. dipterigenus*) be deployed agronomically but protected from the risk of pests developing EPF resistance and (2) are there any methods through which the performance of otherwise less optimal EPF (e.g. isolate 433.99, *B. bassiana*) can be improved to provide increased virulence to aphid nymphs alongside adults and thus better overall aphid control. The solution to both questions may lie within the deployment of EPF as components of an IPM system, alongside other complementary IPM tools such as physical controls, pest monitoring, resistant plant varieties and the judicious usage of synthetic chemical pesticides (Barzman *et al.,* 2015; Stenberg, 2017). A majority of IPM tool acts through distinct mechanisms and impose upon aphids one or several selection pressures dependent upon through their mode(s) of action. To prevent resistance development in aphid pests, whether to EPF, plant resistance or synthetical chemical pesticides, longstanding research evidence highlights that a fundamental approach is to ensure aphids (or pests more widely) are placed under numerous potentially conflicting selection pressures - thereby significantly increasing the evolutionary challenge to overcome relative to each IPM tool in isolation (Ambethgar, 2009; Barzman *et al.*, 2015; Stenberg, 2017). Alongside potentially limiting resistance development however, Stenberg (2017) noted that many IPM tools may also favourably interact, potentially improving their efficacy (Stenberg, 2017). Any such improvements in EPF-mediated speed of kill of improved virulence of generalist EPF isolates such as 433.99 (*B. bassiana*) are thus likely to have a significant effect upon EPF efficacy and aphid population management in the field – potentially circumventing in part

lost EPF efficacy in the field stemming from inconsistency of spray application and suboptimal environmental conditions.

3.4.7. Summary and conclusions

Within this chapter ten EPF isolates were selected and screened for their thermal tolerance and virulence to *B. brassicae* adults and nymphs. No significant differences in thermal tolerance/optima between isolates were identified however while all proved virulent to adults, only one isolate demonstrated notable control over aphid nymphs. EPF treatments overall demonstrated no sublethal effects upon adult aphid reproduction. Isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were selected (based on differing efficacy against *B. brassicae* nymphs) for further evaluation. Concentration-response assays revealed that isolate 1.72 (*A. dipterigenus*) performance is relatively indistinguishable against adults and nymphs while isolate 433.99 (*B. bassiana*) requires significantly higher concentrations (and thus a higher dose) to achieve equivalent adult and nymph mortality. Conversely, isolate 433.99 (*B. bassiana*) demonstrated a greater ability to kill nymphs from secondary pickup than 1.72 (*A. dipterigenus*).

This provides both a solid logical basis upon which to further evaluate isolate 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) performance against adult and nymph *B. brassicae* in combination with partially aphid resistant and susceptible *Brassica* accessions and a valuable resource to aid in the interpretation of such combined assays.

4. Combining partial host plant resistance and entomopathogenic fungi to evaluate the efficacy of combined deployment

4.1. Background

The previous chapters in this thesis described research that (i) identified Brassica accessions with partial resistance to *B. brassicae*, and (ii) selected EPF isolates with contrasting virulence to *B. brassicae* adults and nymphs. This chapter presents the results of laboratory studies to quantify the effects of combining host plant resistance and EPF against *B. brassicae*.

Interactions between natural enemies and plants have been known to determine insect fitness traits (including survival) for many years (Cory and Hoover, 2006). However, it is only recently that these interactions have been formally addressed in a theoretical framework for IPM (Stenberg, 2017). Interactions between host plants and biological controls are thought to be especially important in IPM because of their potential for mutual reinforcement or antagonism (Stenberg, 2017). In principle, the combined effects of the host plant and a biocontrol agent on the insect pest can be: (i) additive (i.e., realised through the individual, independent effects of host plant and biological control agent on the target insect); (ii) interactive, and expressed within the insect pest; (iii) interactive and expressed without the insect pest. Hypocrealean EPF are particularly interesting in this regard because they have evolved from endophytes (Vega *et al.*, 2009) and some EPF species retain the ability to grow within plants and induce antibiosis resistance with negative effects on insect feeding, reproduction and survival (Vidal and Jaber, 2015; Bamisile *et al.,* 2018, Dash *et al.,* 2018; Gange *et al.,* 2019). There is some evidence to suggest that spray applications of EPF to foliage can result in endophytic colonisation, however little is known about its effects on insect herbivores (Guesmi-Jouini *et al.,* 2014; Allegrucci *et al.,* 2017; Silva *et al.,* 2020). It has even been suggested that EPF can act as 'plant bodyguards', in similar ways that plants are able to recruit arthropod natural enemies for defence against insect herbivores (Elliot *et al.*, 2000; Gange *et al.*, 2019), and it is hoped that new knowledge of EPF-plant interactions will lead to novel ways of exploiting them in IPM (Mantzoukas and Eliopoulos, 2020).

4.1.1. Plant resistance effects upon EPF

In contrast to the growing body of research into the interaction and effects of EPF endophytism upon plant resistance, the reverse interaction investigating how plant resistance may impact EPF efficacy and virulence has received significantly less attention. In 1994 Knudsen *et al.* evaluated *B. bassiana* efficacy against Russian wheat aphid (*Diuraphis noxia*) on both host preference (*Triticum aestivium* L.) and non-host preference (*Avena sativa* L.) plants – with these plants serving as a proxy for high levels of partial host plant resistance (Knudsen *et al.*, 1994). Within this study, a significant difference in aphid population size and development was noted between host and non-host preference plants following EPF treatment, with a significant difference in aphid spatial patterns/distribution also noted (Knudsen *et al.*, 2014). Building upon this work, Hatting *et al.* (2010) identified at 65% increase in the efficacy of *B. bassiana* for the control of the *D. noxia* on partially aphid-resistant wheat cultivars under field conditions relative to mock treated control – however the lack of a susceptible control in this study against which percentage population control could be directly compared likely diminished the significance of this finding (Hatting *et al.*, 2010). Complementary research by Tkaczyuk *et al.* (2007) confirmed a difference in the virulence of *Pandora neoaphidis* (Entomophthorales) between different host plant cultivars and species (*Pisum sativum* and *Vicia fabia*) against *Acyrthosiphon pisum* -providing further evidence in favour of a plant resistance interaction with EPF efficacy (Tkaczuk *et al.*, 2007).

While the number of studies investigating specifically the ability of host plant resistance to result in improvements of EPF efficacy is limited, there do exist studies demonstrating the significant effects that resistant host plants can have upon aphid pest biology. Partial host plant resistance in some cases has been shown to act through quantifiable increases in aphid development time – thereby increasing intermoult periods (Amjad and Peters, 1992; Simon *et al.*, 2021). In principle this should increase the susceptibility of nymphs to EPF infection by allowing more time for conidia to germinate on and penetrate the host cuticle. Given that several studies have highlighted that aphid nymphs are significantly more challenging to control with EPF than adults, such interactions that may increase the virulence of EPF to nymphs warrant significant investigation (Kim and Roberts, 2012; Jandricic *et al.,* 2014).

4.1.2. Interaction analysis

Interaction analysis is a statistical approach commonly used within multifactorial regression analyses to investigate whether individual independent variables significantly influence the effects of one another, resulting in an effect upon the dependant variable greater or lesser than the additive sum of their individual effects (Berrington de González and Cox, 2007; Beck and Bliwise, 2014). Through follow-up analyses including simple contrast tests and the plotting of interaction plots, the significant interactions between individual components of each independent variable can be identified (Cox, 1984). Through this approach, the presence of absence of a specific interaction between partial host plant resistance and EPF-induced virulence could therefore be investigated.

4.1.3. Aims and objectives

The aim of this chapter was to quantify the effects of *Brassica* host plant genotype (partial resistance versus susceptibility) combined with EPF on *B. brassicae*. The central hypothesis was that partial host plant resistance facilitates increased virulence of EPF, and hence the combined effects of host plant resistance and EPF (and their interaction) would give significant improvements in crop pest control than using host plant resistance or EPF separately. This hypothesis was evaluated in two ways:

1. The effect of integration upon *B. brassicae* adult and nymph survival was assessed in a bioassay experiment, seeking to determine the main effects and interactions of each factor.

2. The effect of integrated treatment under several different spray regimes was assessed to identify effects upon the population development of *B. brassicae*.

4.2. Materials and methods

4.2.1. *Brassica* plant accessions and EPF isolates

Four *Brassica* accessions (Table 4.1) and two entomopathogenic fungal isolates (Table 4.2) were selected for combined evaluation. *Brassica* accessions included two each of crop-type *B. oleracea* and *B. cretica*, with one accession of each being previously categorised as partially resistant to *B. brassicae* and the other accession more susceptible. The two EPF isolates (*A. dipterigenus* 1.72 and *B. bassiana* 433.99) were both pathogenic to *B. brassicae* adults but had contrasting effects on first instar *B. brassicae* nymphs: 1.72 (*A. dipterigenus*) had high virulence to nymphs while 433.99 (*B. bassiana*) had low virulence.

Table 4.1: *Brassica* accessions selected for combinatory experiments with biological control. Two contrasting accessions were selected of both *Brassica oleracea* and *Brassica cretica*, one demonstrating partial resistance and one more susceptible to *Brevicoryne brassicae* (as indicated by initial screening results).

Assigned Number	Selection Criteria	Genus	Species	Cultivar Group	Crop Type	Cultivar	Relative resistance
11	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	Acephala	Kale	'Furchehnkohl'	Susceptible
15	Ellis *et al.* 401 screen	*Brassica*	*oleracea*	Botrytis	Cauliflower	'Marzatico Napoletano'	Partially Resistant
16	wild species	*Brassica*	*cretica*	-	-	-	Partially Resistant
17	wild species	*Brassica*	*cretica*	-	-	-	Susceptible
-	Control	*Brassica*	*oleracea*	Gemmifera	Brussels sprouts	'Doric F1'	Control

Table 4.2: Entomopathogenic fungi isolates selected for further evaluation and progression to combinatory experiments with partially resistant *Brassica* accessions. EPF were selected on the basis of their contrasting virulence; both isolates demonstrated significant virulence against adult *Brevicoryne brassicae* however only isolate 1.72 (*Akanthomyces dipterigenus*) demonstrated significant virulence against first instar *B. brassicae* nymphs.

*No longer available commercially

Isolate number (University of Warwick Culture Collection)	Species	Host (isolated from)	On-label product name
1.72	*Akanthomyces dipterigenus*	*Macrosiphoniella sanborni* (Chrysanthemum aphid)	Vertalec® (Koppert)*
433.99	*Beauveria bassiana*	*Bemisia sp.* (Whitefly)	BotaniGard ®ES (Bioworks ®)
Control treatment		0.05% Triton X100	

4.2.2. Evaluating the effects of combining partial varietal resistance with EPF against *Brevicoryne brassicae*

To evaluate whether the combined deployment of *Brassica* plants demonstrating partial antibiosis resistance to *B. brassicae* and entomopathogenic fungi can lead to a significant increase in EPF efficacy against adult and/or first instar *B. brassicae* a bioassay was undertaken. Within this bioassay, all 12 combinations of *Brassica* accessions 11, 15, 16 and 17 and EPF isolate 1.72 (*A. dipterigenus*), 433.99 (*B. bassiana*) and mock treatment (Table 4.4) were evaluated. This experiment included 3 factors: EPF isolate (1.72, 433.99 or control), *Brassica* species (*B. oleracea* or *B. cretica*) and whether the accession was resistant or susceptible (resistant or susceptible) – having therefore a 2x2x3 design.

Similarly to previous bioassays, different methods were used to inoculate adult and first instar *B. brassicae* owing to the fragility of instar aphids and likelihood of significant control mortality if nymphs are handled – with adults sprayed in 9cm Petri dishes and first-instar nymphs sprayed in-situ on *Brassica* leaves.

Five-week-old plants of the four accessions and technical control (Table 4.1) were raised as described in 2.2.4. Fourteen days prior to bioassay set-up, both EPF isolates (Table 4.2) were cultured from stock EPF slopes (as described in 3.2.2). On the day preceding bioassay set-up, conidia suspensions were prepared (as described in 3.2.3), diluted to calculated $LC_{70}A$ (LC70 for adult *B. brassicae*) and $LC_{70}N$ (LC_{70} for nymph-stage *B. brassicae*) concentrations, as determined in 3.3.6.1, for each EPF isolate (Table 4.3) and stored overnight at 4°C.

Ten days prior to bioassay setup, fixed-age 10-day old adult *B. brassicae* cultures were established (as described in 2.2.2) on control plants (*B. oleracea* var Gemmifera 'Doric F1'). On the day of bioassay set-up, forty fixed-age adult *B. brassicae* were removed from the fixed-age adult culture and transferred onto damp filter paper in a Petri dish for each of the 12 EPF isolate/plant accession combination and a mock/control treatment (Table 4.4). From the same fixed-age adult *B. brassicae* culture, on the day preceding bioassay set-up, 1-day-old *B. brassicae* first instar nymph cultures were also established by transferring a cohort of 10 fixed-age *B. brassicae* adults to leaves of fresh 5-week-old *Brassica* plants of each of the five

Brassica accessions to be assessed. These adults were then contained in a clip-cage and allowed 24hrs to reproduce and produce a fixed-age first-instar cohort. On the day of the bioassay, all adult *B. brassicae* were removed from these plants leaving a cohort of 1-day old fixed-age first instar nymphs in-situ on the leaves of the *Brassica* accessions to be evaluated.

The LC_{70} concentrations (2ml) were applied as described in 3.2.6.1. Following spraying, 15 adult *B. brassicae* were transferred to leaves of two fresh/uninfested Brassica plants per treatment (Table 4.4). These leaves were then enclosed in a Bioassay box alongside damp filter paper to ensure sufficient humidity for conidial germination. The leaves of plants upon which sprayed 1-day-old first-instar nymphs were held were treated identically, also being enclosed in a bioassay box containing damp filter paper. Following bioassay setup, all plants were transferred to and maintained in a controlled environment room (20 ± 2 °C, 50% RH, L:D 16:8 h). After 24 hours, the solid/unventilated bioassay box lid was swapped to a ventilated lid to allow for a reduction in humidity. Bioassay box humidity conditions when the bioassay chamber had a solid plastic lid had an observed average relative humidity of 95.65% (0.133 SEM), dropping to 54.06% humidity (0.232 SEM) when the lid was swapped to a ventilated lid. This bioassay was read daily for nine days, recording aphid mortality each day.

All deceased aphids were transferred to damp filter paper in Petri dishes, sealed with Nescofilm and incubated in the dark at 20ºC for 5 days. Microscopy confirmed that the applied EPF species was the causative agent of death. The bioassay procedure was repeated on three separate occasions.

Table 4.3: Lethal concentration (LC50, LC70 and LC90) estimates of EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) for *Brevicoryne brassicae* 10-day old adults and 1-day old first instar nymphs. Estimated LC70 concentrations were used in combinatory assays.

EPF Isolate	Aphid life-stage	LC_{50} Conidia ml^{-1}	LC_{70} **Conidia ml^{-1}**	LC_{90} Conidia ml^{-1}
1.72 *A. dipterigenus*	**Adults**	$2.34x10^5$	**$7.03x10^5$**	$3.44x10^6$
	Nymphs	$4.75x10^5$	**$1.55x10^6$**	$8.56x10^6$
433.99 *B. bassiana*	**Adults**	$5.74x10^5$	**$4.37x10^6$**	$8.17x10^7$
	Nymphs	$3.2x10^7$	**$3.88x10^8$**	$1.42x10^{10}$

Table 4.4: Twelve combined EPF and plant accession treatments. Each combined treatment was evaluated through bioassays against fixed-age 10-day-old *Brevicoryne brassicae* adults and fixed-age 1-day-old first instar *B. brassicae* nymphs. (R) and (S) refer to the identification of accessions as either significantly more partially resistant to *B. brassicae* or significantly more susceptible within the assays of chapter 2.

Combined Treatments			
EPF isolate		**Plant accession**	
mock	0.05% Triton-X	Control	*B. oleracea* var. Gemmifera 'Doric F1'
1.72	*Akanthomyces dipterigenus*	11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'
1.72	*Akanthomyces dipterigenus*	15 (R)	B. oleracea var. Botrytis 'Marzatico Napoletano'
1.72	*Akanthomyces dipterigenus*	17 (S)	*B. cretica*
1.72	*Akanthomyces dipterigenus*	16 (R)	*B. cretica*
433.99	*Beauveria bassiana*	11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'
433.99	*Beauveria bassiana*	15 (R)	B. oleracea var. Botrytis 'Marzatico Napoletano'
433.99	*Beauveria bassiana*	17 (S)	*B. cretica*
433.99	*Beauveria bassiana*	16 (R)	*B. cretica*
mock	0.05% Triton-X	11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'
mock	0.05% Triton-X	15 (R)	B. oleracea var. Botrytis 'Marzatico Napoletano'
mock	0.05% Triton-X	17 (S)	*B. cretica*
mock	0.05% Triton-X	16 (R)	*B. cretica*

4.2.2.1. Combined partial varietal resistance/EPF bioassay – survival analysis

Results were analysed through two methods. Firstly, they were analysed through a survival analysis approach using Kaplan-Meier survival analysis, Log rank tests and three Cox-proportional hazards models (SPSS) – with this analysis allowing for assessment of the general trends within the dataset (Matthew *et al.*, 1999). Through Kaplan-Meier survival analysis, four survival curve plots were produced demonstrating cumulative *B. brassicae* survival post EPF spray when raised on resistant or more susceptible accessions of *B. oleracea* and *B. cretica*. The median survival time and hazard ratios of all twelve combined treatments were calculated relative to a mock EPF spray on a control plant (*B. oleracea* var. Gemmifera 'Doric F1'). Mock control treated results were excluded from further analyses, being included within Kaplan-Meier as a simple comparison reference. Log rank tests were performed to determine between treatment (EPF isolate and plant accession) pairwise differences in hazard ratio and survival curves. To determine which factors were significant main effects in determining resultant *B. brassicae* survival, three Cox-proportional hazards models were undertaken (Table 4.5). The first model included two factors: replicate and treatment – with treatment being a combination of EPF isolate and plant accession. The second Cox model divided treatment into two separate factors and thus included three factors: replicate, EPF isolate and plant accession. The third Cox model further broke down the plant accessions factor into two further factors (*Brassica* species and whether the accession was resistant or susceptible) thus this third model included four factors: replicate, EPF isolate, *Brassica* species and resistant/susceptible. The fourth Cox model included the same four factors as the third model and also included four interaction terms within the model:

- 'EPF isolate' x '*Brassica* species'
- 'EPF isolate' x 'Resistant or Susceptible'
- 'Resistant or Susceptible' x '*Brassica* species'
- 'EPF isolate' x 'Resistant or Susceptible' x '*Brassica* species'

Interaction terms were included to evaluate whether there exist any interactions between these three factors, lower order interactions considered only within the

context of the highest order interaction identified. Interaction terms between experimental factors and replicate were also included in the first three cox models to determine whether between replicate variation influenced observed results.

Table 4.5: Factors utilised and relationship between factors in the four undertaken Cox-proportional hazards models. In the first model, treatment was assessed alongside replicate however in subsequent models, treatment was divided into its component sub-factors to better assess whether each contributed a main effect. Alongside factors, the number of variables within each factor is noted alongside the four interaction terms included within Cox proportional hazards model 4.

Effects and interactions	Factors							
	Cox model 1		Cox model 2		Cox model 3		Cox model 4	
Main effects	Replicate	1-3	Replicate	1-3	Replicate	1-3	Replicate	1-3
	Treatment	1-12	EPF isolate	1-3	EPF isolate	1-3	EPF isolate	1-3
			Plant accession	1-4	*Brassica* species	1-2	*Brassica* species	1-2
					Resistant or Susceptible	1-2	Resistant or Susceptible	1-2
Interaction terms							EPF isolate x *Brassica* species	
							EPF isolate x Resistant or Susceptible	
							Brassica species x Resistant or Susceptible	
							EPF isolate x *Brassica* species x Resistant or Susceptible	

4.2.2.2. Combined partial varietal resistance/EPF bioassay – GLMM analysis

Results were firstly evaluated through a GLM with a binomial distribution and logit link function. This model was undertaken chiefly to evaluate between replicate variation. Within this model, EPF isolate, *Brassica* species, Resistant or Susceptible and replicate were included as main factors. Three interaction terms were also evaluated within this model: (1) replicate x EPF isolate, (2) replicate x *Brassica* species, and (3) replicate x Resistant or Susceptible.

Results were subsequently analysed using a GLMM with a binomial distribution and logit link function. This model was used to analyse aphid survival at 6- and 9-days post EPF treatment. Four GLMMs were performed. Each GLMM included three factors: EPF isolate, *Brassica* species and Resistant/Susceptible – thereby having a 2x2x3 design. Replicate was identified as a significant factor thus was also included in each GLMM as a random effect. Alongside these three factors, three 2-factor interaction terms and one 3-factor interaction term were also evaluated within each GLMM:

- 'EPF isolate' x '*Brassica* species'
- 'EPF isolate' x 'Resistant or Susceptible'
- 'Resistant or Susceptible' x '*Brassica* species'
- 'EPF isolate' x 'Resistant or Susceptible' x '*Brassica* species'

These interaction terms were included to evaluate whether there exist any interactions between the three included factors. Where interactions were identified, interaction was plotted graphically in an interaction plot to visually evaluate the nature of interaction and pairwise simple contrasts tests performed to identify the specific causes and results of identified interactions, with a focus upon between contrasts between plant accessions with intraspecific differences in resistance and between *Brassica* species owing to potential interspecific resistance effects. For each GLMM analysis, only the highest order identified interaction term was evaluated in this way, with lower order interactions considered within the context of the highest order interaction.

4.2.3. Evaluating the effects of combined partial varietal resistance/EPF deployment upon *Brevicoryne brassicae* population development

To evaluate whether the combined use of *Brassica* plants with partial antibiosis resistance to *B. brassicae* and entomopathogenic fungi can significantly limit *B. brassicae* population development, a 20-day monitored population development assay was undertaken.

This experiment included four factors. Of these factors, three were shared with the bioassay described in section 4.2.2: EPF isolate (*A. dipterigenus* 1.72 or *B. bassiana* 433.99), *Brassica* species (*B. oleracea* or *B. cretica*) and whether the accession was partially resistant or susceptible (resistant or susceptible). Within this assay however, an additional fourth factor of spray regime was also investigated to evaluate how *B. brassicae* populations on partially resistant and susceptible plants of both *Brassica* species respond to one EPF spray vs two EPF sprays (alongside a twice mock sprayed control) – with the second sprays four days after the first (assay day 5). Three different spray regimes were evaluated within this experiment: mock, mock, EPF, mock and EPF, EPF. Each regime included two separate sprays with the first on experimental day 1 and the second on experimental day 5. This experiment therefore had a 2x2x2x3 design – with twenty different treatments evaluated overall (Table 4.6). Throughout the 20-day experimental period, *B. brassicae* adult and nymph populations were counted non-destructively at 24hr intervals.

The first step of experimental setup for both adult and nymphs was raising plants for assessment, upon which *B. brassicae* would be held throughout the course experimental assessment. Five-week-old plants of the four *Brassica* plants of interest were raised as described in section 2.2.4. Fourteen days prior to bioassay set-up, both EPF isolates (Table 4.2) were spread from stock EPF slopes onto 9cm SDA Petri dishes (as described in 3.2.2). On the day preceding bioassay set-up, conidia suspensions for each EPF isolate were prepared (as described in section 3.2.3), diluted to $LC_{70}A$ concentrations (Table 4.3) and stored overnight at 4°C.

Ten days prior to bioassay setup, fixed-age 10-day old adult *B. brassicae* cultures were established (as described in section 2.2.2) on control plants (*B. oleracea* var

Gemmifera 'Doric F1'). On the day of bioassay set-up, forty fixed-age adult *B. brassicae* per treatment (Table 4.6) were removed from the fixed-age adult culture and transferred onto damp filter paper in twenty Petri dish – one for each of the combined treatment/spray regimes to be assessed (Table 4.6).

Fixed-age adult *B. brassicae* were sprayed in Petri dishes and allowed 1hr to recover. Spraying was done using a Potter's tower (at 50 kPa), with 2ml of conidia suspension at the estimated LC_{70} for both isolates for adult B. brassicae (Table 4.3) (Potter, 1952). Ten adult aphids were then transferred to a leaf, with two plants per treatment. These leaves were enclosed in Bioassay boxes with unvented lids lined with damp filter paper and incubated for 24 h, after which they were swapped with ventilated lids. Bioassay box humidity conditions were 96% (0.13 SEM) humidity with solid lid and 54% humidity (0.23 SEM) with a ventilated lid.

The bioassay was read daily, non-destructively recording numbers of both adults and nymphs. On experiment day 4, using a second batch of pre-prepared EPF cultures, $LC_{70}N$ conidia suspensions for each EPF isolate were prepared as described in section 3.2.3 (Table 4.4) and stored overnight at 4ºC. On day 5 following counting of the number of adults and nymph-stage *B. brassicae* present, all *B. brassicae* were sprayed in-situ on the experimental plant leaves EPF conidia spraying was performed using a Potter's tower, as described previously with 2ml of the preprepared $LC_{70}N$ conidia suspensions for each EPF isolate being sprayed on both the front and the back of the leaves as aphids were present on both sides.

These leaves were then enclosed in Bioassay boxes with a solid/unventilated lid alongside damp filter paper to ensure sufficient humidity for conidial germination. After 24 hours, the *B. brassicae* population was counted and the bioassay box lid changed to a to a ventilated lid to allow for a reduction in humidity. The number of adult and nymph *B. brassicae* was then counted at 24h intervals for a further 14 days. A total of 20 days of population counts was therefore undertaken. Throughout the course of the experiment, deceased aphids were removed daily and mortality due to EPF treatment confirmed by microscopy. The experiment was repeated on three occasions.

Table 4.6: Combined treatments performed during evaluation of effects of EPF and partially resistant *Brassica* accessions upon B*revicoryne brassicae* population development. During the first spray, all present *B. brassicae* were fixed age 10-day-old adults were sprayed with calculated $LC_{70}A$ concentrations calculated for the corresponding EPF isolate (Table 4.3). Conversely, at day 5, *B. brassicae* populations were of mixed ages (adults and nymphs). At this timepoint, *B. brassicae* population was sprayed in-situ with calculated $LC_{70}N$ concentrations calculated for the corresponding EPF isolate (Table 4.3)

Plant accession		Spray regime			
		Spray 1 (day 1) - $LC_{70}A$		Spray 2 (day 5) $LC_{70}N$	
11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'	mock	0.05% Triton-X100	mock	0.05% Triton-X100
11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'	1.72	*Akanthomyces dipterigenus*	mock	0.05% Triton-X100
11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'	1.72	*Akanthomyces dipterigenus*	1.72	*Akanthomyces dipterigenus*
11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'	433.99	*Beauveria bassiana*	mock	0.05% Triton-X100
11 (S)	*B. oleracea* var. Acephala 'Furchenkohl'	433.99	*Beauveria bassiana*	433.99	*Beauveria bassiana*
15 (R)	*B. oleracea* var. Acephala 'Furchenkohl'	mock	0.05% Triton-X100	mock	0.05% Triton-X100
15 (R)	*B. oleracea* var. Acephala 'Furchenkohl'	1.72	*Akanthomyces dipterigenus*	mock	0.05% Triton-X100
15 (R)	*B. oleracea* var. Acephala 'Furchenkohl'	1.72	*Akanthomyces dipterigenus*	1.72	*Akanthomyces dipterigenus*
15 (R)	*B. oleracea* var. Acephala 'Furchenkohl'	433.99	*Beauveria bassiana*	mock	0.05% Triton-X100
15 (R)	*B. oleracea* var. Acephala 'Furchenkohl'	433.99	*Beauveria bassiana*	433.99	*Beauveria bassiana*
17 (S)	*B. cretica*	mock	0.05% Triton-X100	mock	0.05% Triton-X100
17 (S)	*B. cretica*	1.72	*Akanthomyces dipterigenus*	mock	0.05% Triton-X100
17 (S)	*B. cretica*	1.72	*Akanthomyces dipterigenus*	1.72	*Akanthomyces dipterigenus*
17 (S)	*B. cretica*	433.99	*Beauveria bassiana*	mock	0.05% Triton-X100
17 (S)	*B. cretica*	433.99	*Beauveria bassiana*	433.99	*Beauveria bassiana*
16 (R)	*B. cretica*	mock	0.05% Triton-X100	mock	0.05% Triton-X100
16 (R)	*B. cretica*	1.72	*Akanthomyces dipterigenus*	mock	0.05% Triton-X100
16 (R)	*B. cretica*	1.72	*Akanthomyces dipterigenus*	1.72	*Akanthomyces dipterigenus*
16 (R)	*B. cretica*	433.99	*Beauveria bassiana*	mock	0.05% Triton-X100
16 (R)	*B. cretica*	433.99	*Beauveria bassiana*	433.99	*Beauveria bassiana*

4.2.3.1. Combined partial varietal resistance/EPF population development assay – GLMM analysis

Assay results were firstly plotted as a series of stacked bar charts to allow for evaluation of overall trend within the dataset. Results for this assay were evaluated initially through a GLM approach, primarily to determine whether any between-replicate variation was present. GLM analysis was undertaken using a negative binomial distribution and log link function for total aphid counts (adults and nymphs) on days 5, 10 and 20. These GLMs included five factors: EPF isolate (species name 1.72 or 433.99), *Brassica* species (*B. oleracea* or *B. cretica*), Resistant/Susceptible (resistant or susceptible), EPF spray regime (mock, mock, EPF, mock or EPF, EPF) and experimental replicate. All two-factor interaction terms between the four experimental factors and replicate were also evaluated to evaluate variation in each factor between replicates.

As replicate proved a significant factor in some GLM analyses, for consistency between analyses and to account for this variation, all population count results were subsequently analysed using a GLMM approach with a negative binomial distribution and log link function for days 5, 10 and 20, including replicate as a random effect factor. A Bonferroni correction was applied altering significance threshold to 0.0167. Each GLMM included four factors: EPF isolate, *Brassica* species, Resistant/Susceptible and EPF spray regime – thereby having a 2x2x2x3 design.

Alongside these four factors, six 2-factor interaction terms, four 3-factor interaction terms and one 4-factor interaction term were also evaluated in each GLMM:

- ‘EPF isolate’ x ‘*Brassica* species’
- ‘EPF isolate’ x ‘Resistant or Susceptible’
- ‘EPF isolate’ x ‘spray regime’
- ‘*Brassica* species’ x ‘Resistant or Susceptible’
- ‘*Brassica* species’ x ‘spray regime’
- ‘Resistant or Susceptible’ x ‘spray regime’

- ‘EPF isolate’ x ‘*Brassica* species’ x ‘Resistant or Susceptible’
- ‘EPF isolate’ x ‘*Brassica* species’ x ‘spray regime’
- ‘EPF isolate’ x ‘Resistant or Susceptible’ x ‘spray regime’
- ‘*Brassica* species’ x ‘Resistant or Susceptible’ x ‘spray regime’
- ‘EPF isolate’ x ‘*Brassica* species’ x ‘Resistant or Susceptible’ x ‘spray regime’

Interaction plots were drawn for those cases where interactions were identified, and pairwise simple contrasts tests performed. For each GLMM analysis, only the highest order identified interaction term was evaluated, with lower order interactions considered within the context of the highest order interaction. Where significant higher-orders interactions were identified, further GLMMs were undertaken evaluating aphid and nymph populations separately as follow-up secondary analyses. As supplementary analyses, Bonferroni corrections were not applied.

4.3. Results

4.3.1. Evaluating the effects of combined partial varietal resistance/EPF deployment upon EPF efficacy against *Brevicoryne brassicae* – survival analysis

For adult *B. brassicae*, all eight EPF treatments resulted in >50% mortality The median survival time for each treatment (MT50s) ranged from 4-5 days (Table 4.7). On *B. oleracea* accessions, following mock treatment no difference in survival was noted between accessions 11 and 15, with both resulting in ~80% survival (Figure 4.1A). For *B. cretica* accessions however a marginal difference was evident in adult survival, with marginally higher survival noted on accession 16 relative to 17 on experimental day 9 (with ~85% survival on accession 16 relative to ~75% on accession 17) (Figure 4.1C). On *B. oleracea* accessions, following EPF treatment with either isolate 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*) adult survival was noted to markedly fall ranging from 10-30% across all treatments - with a treatment of 1.72 (*A. dipterigenus*) on accession 15 (*B. oleracea* partially resistant) leading to the lowest overall survival observed on experiment day 9 (10%). Conversely, of these EPF treated adults on *B. oleracea*, treatment of 433.99 on accession 11 (*B. oleracea* susceptible) led to the highest EPF treated survival (~30%). The difference

between partially resistant/susceptible accessions was noted to be more evident following treatment with 433.99 (*B. bassiana*) relative to 1.72 (*A. dipterigenus*). On *B. cretica* a similar trend was observed following treatment with either 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*), with survival ranging from 12-30% (Figure 4.1C). As was observed on *B. oleracea* accessions, a more notable difference in survival was identified between the partially resistant (16) and susceptible (17) *B. cretica* accessions following treatment with 433.99 (*B. bassiana*) relative to 1.72 (*A. dipterigenus*), with 433.99 also leading to lower survival overall (15% on accession 16 relative to 20% on accession 17). A pairwise Log-rank test (pooled across strata revealed that all treatments (aside from mock treated *B. brassicae* on plant accession 11 and 15) significantly increased hazard ratio relative to mock treated control plants. All mock treatments on plant accessions 11, 15, 16 and 17 were indistinguishable in hazard ratios (p=>0.05) while all non-mock treatments (1.72, *A. dipterigenus* and 433.99, *B. bassiana* treatments on all plant accessions) significantly (p=<0.05) increased hazard ratio relative to all mock treatment on all plant accessions. When considering the two pre-selected resistant and susceptible accessions of the two *Brassica* species, only one significant difference in hazard ratio and survival was detected with this being a significantly increased (p=<0.05) hazard ratio in accession 15 (10.27) relative to accession 11 (6.41) when treated with EPF isolate 433.99 (Table 4.7).

For *B. brassicae* nymphs, seven of the eight EPF treatments resulted in >50% mortality. The Mt50s ranged from 4-5 days for *B. oleracea* accessions and 3 days across all four treatments including *B. cretica* plant accessions (Table 4.9). On *B. oleracea* accessions 11 and 15, in the absence of EPF treatment no significant difference in survival was noted in aphid nymphs with both resulting in ~85% survival. In contrast however, untreated nymph survival on *B. cretica* accession 17 (susceptible) appeared to result in lower survival from day 5 onwards relative to accession 16 (partially resistant), with ~88% survival on accession 16 at day 9 relative to ~78% survival on accession 17 (Figure 4.1D). Following EPF treatment with either isolate 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*) on *B. cretica* accessions, survival was noted to be very consistent across EPF isolate and *B. cretica* accessions, with survival ranging from 25-35%. In contrast, for *B. brassicae* nymph survival on *B. oleracea* accessions the most significant difference within this

bioassay was isolate 433.99 (*B. bassiana*) related survival being significantly lower on *B. oleracea* accession 11 (susceptible) and much higher on *B. oleracea* accession 15 (partially resistant) indicating a potentially significant interaction between EPF isolate 433.99 (*B. bassiana*) and partial resistance in *B. oleracea*. A similar difference however was not noted following isolate 1.72 treatment (*A. dipterigenus*), which resulted in a consistent level of nymph survival of ~35-40% regardless of *B. oleracea* accession. A pairwise Log-rank test (pooled across strata) revealed that, similarly to findings for adult *B. brassicae*, all treatments significantly increased hazard ratio relative to mock treated control plants. All mock treatments on plant accessions 11, 15, 16 and 17 were again all found to be indistinguishable in hazard ratios while all non-mock treatments (1.72, *A. dipterigenus* and 433.99, *B. bassiana* treatments on all plant accessions) significantly increased hazard ratio relative to all mock treatments on all plant accessions. When considering the pre-selected resistant and susceptible accessions of the two *Brassica* species, only one significant difference in hazard ratio and survival was detected with this being a significantly increased hazard ratio in accession 15 (15.06) relative to accession 11 (8.24) when treated with EPF isolate 433.99 (Table 4.9).

The first Cox model for both life stages evaluated combined treatment (EPF isolate and plant accession) alongside replicate. For both adult and nymph *B. brassicae*, both Cox models offered a significant improvement over a null model (adult: X^2 (13) = 260.256, p = 4.73×10^{-48}) (nymph: X^2 (13) = 235.091, p =7.91×10^{-43}). Within these Cox models for EPF against adult *B. brassicae*, both treatment (EPF isolate + plant accession) and replicate were found to be significant factors influencing aphid mortality (treatment: Wald X^2 (11) = 201.762, p = 3.21×10^{-37}) (replicate: X^2 (2) = 15.612, p = 4.10×10^{-4}). For *B. brassicae* nymphs, treatment and replicate were also both found to be significant factors (treatment: Wald X^2(11) =180.591, p = 7.74×10^{-33}) (replicate: X^2 (2) = 12.626, p = 0.002). For adults and nymphs, owing to the Bonferroni-adjusted significance threshold (0.125), no significant interaction was noted between treatment and replicate in the first model (adults: Wald X^2 (11) = 16.486, p = 0.124, nymphs: Wald X^2 (11) = 21.228, p = 0.031).

A second set of Cox models were undertaken wherein replicate remained included as a factor however treatment was broken down into two separate factors: EPF isolate and plant accession. Similarly to the previous Cox models, both of these analyses offered a significant improvement over a null model (adult: X^2 (7) = 239.682, p = $4.34x10^{-48}$) (nymphs: X^2 (7) =, p =$5.92x10^{-42}$). Within this second Cox model for adult *B. brassicae*, EPF isolate, and replicate were found to be significant factors (EPF isolate: Wald X^2 (2) = 180.047, p = $8.00x10^{-40}$) (Replicate: Wald X^2 (2) = 15.953, p = $3.43x10^{-4}$) however plant accession was not found to be a significant factor (plant accession: Wald X^2 (3) = 7.229, p = 0.065). No significant interactions were noted between replicate and EPF isolate (Wald X^2 (2) = 6.751, p = 0.034) and replicate and *Brassica* accession (Wald X^2 (3) = 3.689, p = 0.297). Within this second Cox model for *B. brassicae* nymphs however, EPF isolate and plant accession were found to be significant factors (EPF isolate: Wald X^2 (2) = 144.181, p = $4.91x10^{32}$) (Plant accession: Wald X^2 (3) = 28.573, p = $3.00x10^{-6}$) while owing to the Bonferroni correction threshold, replicate was found to no longer be a significant factor (Replicate: Wald X^2 (2) = 8.026, p = 0.018). No significant interactions were noted between replicate and EPF isolate (Wald X^2 (2) = 8.627, p = 0.013) and replicate and *Brassica* accession (Wald X^2 (3) = 2.210, p = 0.530).

Within the third Cox model the factor 'Plant accession' was further subdivided into '*Brassica* species' and 'Resistant or Susceptible' – this model also included replicate and EPF isolate as factors. As was seen in previous Cox models, both Cox models for adult and nymph *B. brassicae* offered a significant improvement over a null model (adult: X^2 (6) = 234.140, p = 1.00^{-47}) (nymphs: X^2 (6) = 206.278, p =$8.74x10^{-42}$). Within this third Cox model for adult *B. brassicae*, EPF isolate and replicate were again found to be significant factors (EPF isolate: Wald X^2 (2) = 178.263, p = $1.95x10^{-39}$) (Replicate: Wald X^2 (2) = 15.178, p = 0.001), however both *Brassica* species and whether the accession was resistant or susceptible were not found to be significant factors (*Brassica* species: Wald X^2 (1) = 0.146, p = 0.702) (resistant or susceptible: Wald X^2 (1) = 1.064, p = 0.302). No significant interactions were noted between replicate and *Brassica* species (Wald X^2 (1) = 1.008, p = 0.013) and replicate and resistant/susceptible (Wald X^2 (1) = 0.059, p = 0.808). A significant interaction was however noted between replicate and EPF isolate. (Wald X^2 (2) = 8.768, p =

0.012). Within this third Cox model for *B. brassicae* nymphs, EPF isolate, *Brassica* species and replicate were found to be significant factors (EPF isolate: Wald X^2 (2) = 142.466, p = 1.16×10^{-31}) (*Brassica* species: Wald X^2 (1) = 22.205, p = 2.00×10^{-6}) (Replicate: Wald X^2 (2) = 8.522, p = 0.014), while whether the accession was resistant or susceptible proved insignificant with this model (resistant or susceptible: Wald X^2 (1) = 0.873, p = 0.350). No significant interactions were noted between replicate and *Brassica* species (Wald X^2 (1) = 0.168, p = 0.682) and replicate and resistant/susceptible (Wald X^2 (1) = 0.935, p = 0.682). A significant interaction was however noted between replicate and EPF isolate. (Wald X^2 (2) = 10.274, p = 0.006)

A final Cox proportional hazards model was undertaken including the same four factors as the third Cox model however this model also included all three pairwise 2-factor interaction terms and the sole three factor interaction for the three primary independent variables (no interaction terms with replicate were included). For both adult and nymphs this fourth model offered a significant improvement over a null model (adult: X^2 (13) = 235.091, p = 7.91×10^{-43}) (nymphs: X^2 (13) = 206.256, p =4.72×10^{-43}).

Within this fourth Cox model for adult *B. brassicae*, EPF isolate and replicate were again found to be significant main effect factors (EPF isolate: Wald X^2 (2) = 45.519, p = 1.31×10^{-10}) (Replicate: Wald X^2 (2) = 15.612, p = 4.07×10^{-4}), however both *Brassica* species and whether the accession was resistant or susceptible were found not to be significant main effect factors (*Brassica* species: Wald X^2 (1) = 0.188, p = 0.665) (resistant or susceptible: Wald X^2 (1) = 0.711, p = 0.399). No significant interactions were noted in this model between the three cross evaluated factors (Table 4.8). Conversely, within this fourth Cox model for *B. brassicae* nymphs, EPF isolate, and replicate were found to be significant main effect factors (EPF isolate: Wald X^2 (2) = 41.718, p = 8.74×10^{-10}) (Replicate: Wald X^2 (2) = 12.626, p = 0.002). Contrary to Cox model 3, *Brassica* species was not found to be a significant main effect (*Brassica* species: Wald X^2 (1) = 2.246, p = 0.134), while resistant or susceptible again proved an insignificant main effect factor (resistant or susceptible: Wald X^2 (1) = 0.488, p = 0.485). Within the interaction terms, a potentially significant interaction was noted for the three-way interaction between EPF isolate x

Brassica species x resistant or susceptible (Wald X^2 (1) = 6.144, p = 0.046), with a lower order significant two-way interaction noted between EPF isolate x *Brassica* species (Wald X^2 (1) = 16.285, p = 2.91 x 10^4). Thus, *Brassica* species was found to significantly interact with EPF isolate – with this two-way interaction potentially being further moderated by whether accessions were noted as susceptible or partially resistant (Table 4.10).

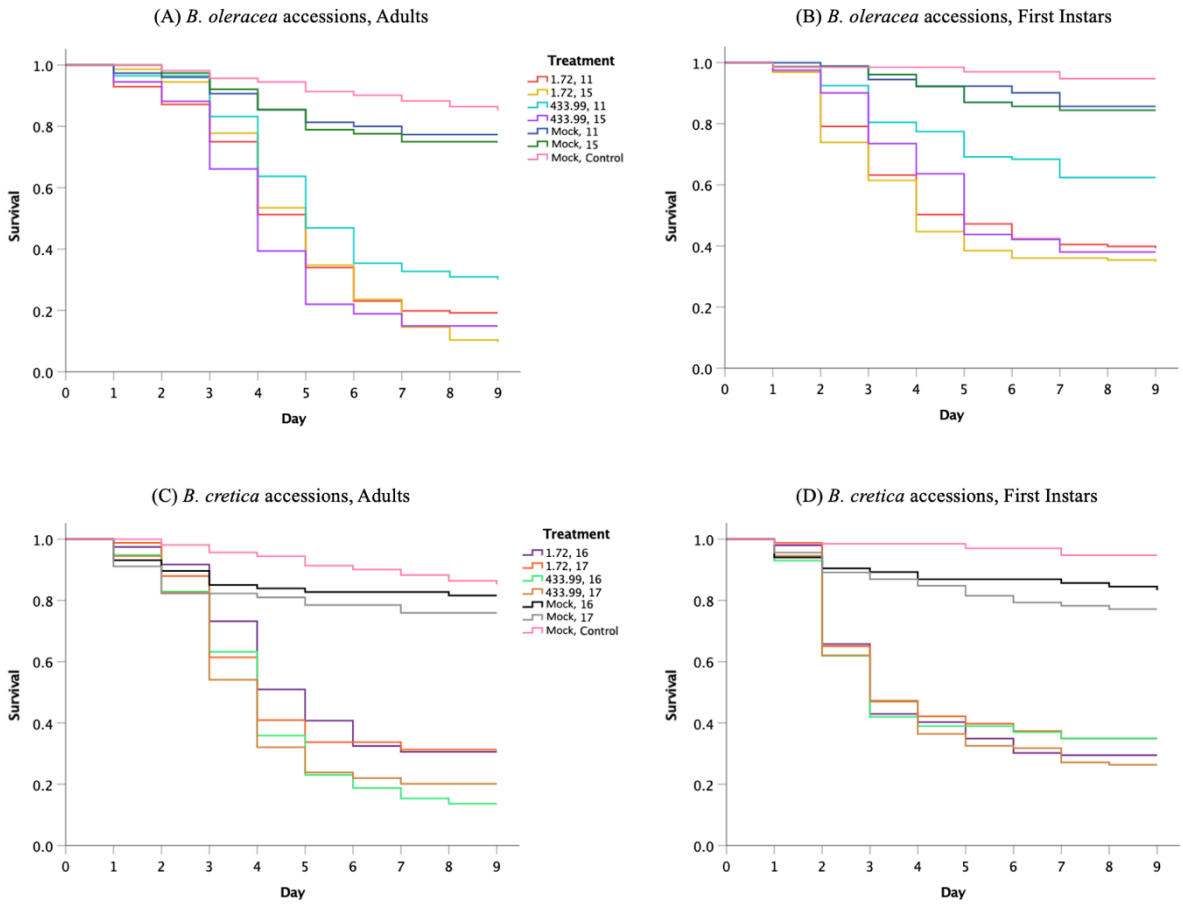


Figure 4.1: Cumulative survival curves for *Brevicoryne brassicae* adults and nymphs when raised on four different *Brassica* accessions from two species and treated independently with two different EPF isolates; 1.72 (*Akanthomyces dipterigenus*) or 433.99 (*Beauveria bassiana*). Each figure includes a mock treatment on control *Brassica* plant (*Brassica oleracea* var. Gonglyodes 'Doric F1') against which all other treatments can be assessed.

Table 4.7: Kaplan-Meier calculated median survival times of adult *Brevicoryne brassicae* when evaluated independently against twelve combinations of two EPF isolates and four *Brassica* plant accessions (table 4.4) and hazard ratios of combined treatment. Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. The median survival time (MST) gives the proportional cumulative survival of 50% of the populations. HR values followed by different lower-case letters within the column are significantly different (log rank $\chi 2 \geq 3.841$, $P < 0.05$). The pairwise differences in hazard ratios/survival curves between combined treatments was evaluated through Log-rank tests, with significant differences indicated where no letters are shared.

a MST = median survival time, given in days, b HR = hazard ratio, compared to the 0.05% Triton-X100 treated control,
* MST, upper, and lower confidence interval bounds could not be calculated,
** Control treatment assigned a hazard ratio score of 1, against which all other treatments are evaluated

Combined Treatment									
	EPF isolate		**Plant accession**	**MST[a] (95% Cl)**	**HR[b] (95% Cl)**	**Z (HR)**	**P (HR)**	**df**	**n**
Mock	0.05% Triton-X100	Control	*B. oleracea* var. Gemmifera 'Doric F1'	*	1** a				162
1.72	*Akanthomyces dipterigenus*	11	*B. oleracea* var. Acephala 'Furchenkohl'	5 (4.64-5.36)	8.647 (5.58-13.39) fh	93.378	<0.001	1	156
1.72	*Akanthomyces dipterigenus*	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	5 (4.64-5.36)	9.29 (6.00-14.39) eh	100.075	<0.001	1	144
433.99	*Beauveria bassiana*	11	*B. oleracea* var. Acephala 'Furchenkohl'	5 (4.35-5.65)	6.41 (4.06-10.12) bc	63.354	<0.001	1	113
433.99	*Beauveria bassiana*	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	4 (3.68-4.32)	10.27 (6.59-16.01) eh	105.818	<0.001	1	127
Mock	0.05% Triton-X100	11	*B. oleracea* var. Acephala 'Furchenkohl'	*	1.65 (0.88-3.06) adg	2.47	0.116	1	76
Mock	0.05% Triton-X100	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	*	1.81 (0.99-3.31) adg	3.763	0.052	1	76
1.72	*Akanthomyces dipterigenus*	16	*B. cretica*	5 (4.53-5.47)	7.08 (4.55-11.02) cf	75.099	<0.001	1	157
1.72	*Akanthomyces dipterigenus*	17	*B. cretica*	4 (3.48-4.52)	7.80 (4.83-12.54) bfh	70.861	<0.001	1	83
433.99	*Beauveria bassiana*	16	*B. cretica*	4 (3.68-4.32)	10.86 (6.95-16.97) e	109.554	<0.001	1	117
433.99	*Beauveria bassiana*	17	*B. cretica*	4 (3.65-4.35)	10.52 (6.69-16.55) eh	103.561	<0.001	1	109
Mock	0.05% Triton-X100	16	*B. cretica*	*	1.37 (0.73-2.58) dg	0.943	0.332	1	87
Mock	0.05% Triton-X100	17	*B. cretica*	*	1.88 (1.03-3.42) dg	4.189	0.041	1	79

Table 4.8: Four Cox proportional hazard models were undertaken to evaluate the significance of different factors upon observed adult *Brevicoryne brassicae* survival data with the first three subsequent models splitting factors from the previous model into constituent sub-factors (Treatment > EPF isolate + Plant accession > Plant species + resistant or susceptible). Within the fourth model, three 2-factor interaction terms and one 3-factor interaction term was included to determine whether any significant interactions were present between the three primary independent variables (EPF isolate, *Brassica* species and Resistant or Susceptible). Technical control plants were excluded from these analyses to allow for direct comparison between resistant and susceptible accessions of the two *Brassica* species.

Model	Effects and interactions	Factors	Z (HR)	P (HR)	df	n
Cox model 1	Main effects	Rep	15.612	<0.001	2	3
		Treatment	201.762	<0.001	11	12
Cox model 2	Main effects	Rep	15.953	<0.001	2	3
		EPF isolate	180.047	<0.001	2	3
		Plant accession	7.229	0.065	3	4
Cox model 3	Main effects	Rep	15.178	0.001	2	3
		EPF isolate	178.263	<0.001	2	3
		Brassica species	0.146	0.702	1	2
		Resistant or Susceptible	1.064	0.302	1	2
Cox model 4	Main effects	Rep	15.612	<0.001	2	3
		EPF isolate	45.519	<0.001	2	3
		Brassica species	0.188	0.665	1	2
		Resistant or Susceptible	0.711	0.399	1	2
	2-factor interaction terms	EPF isolate x *Brassica* species	5.918	0.052	2	3
		EPF isolate x Resistant or Susceptible	1.122	0.571	2	3
		Brassica species x Resistant or Susceptible	0.579	0.447	1	2
	3-factor interaction term	EPF isolate x *Brassica* species x Resistant or Susceptible	0.164	0.921	2	3

Table 4.9: Kaplan-Meier calculated median survival times of nymph *Brevicoryne brassicae* when evaluated independently against twelve combinations of two EPF isolates and four *Brassica* plant accessions (table 4.4) and hazard ratios of combined treatment. Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X100 treated control. The median survival time (MST) gives the proportional cumulative survival of 50% of the populations. HR values followed by different lower-case letters within the column are significantly different (log rank $\chi 2 \geq 3.841$, $P < 0.05$). The pairwise differences in hazard ratios/survival curves between combined treatments was evaluated through Log-rank tests, with significant differences indicated where no letters are shared.

a MST = median survival time, given in days, b HR = hazard ratio, compared to the 0.05% Triton-X100 treated control,
* MST, upper, and lower confidence interval bounds could not be calculated,
** Control treatment assigned a hazard ratio score of 1, against which all other treatments are evaluated

Combined Treatment									
EPF isolate		Plant accession		MST[a] (95% Cl)	HR[b] (95% Cl)	Z (HR)	P (HR)	df	n
Mock	0.05% Triton-X100	Control	*B. oleracea* var. Gemmifera 'Doric F1'	*	1**a				136
1.72	*Akanthomyces dipterigenus*	11	*B. oleracea* var. Acephala 'Furchenkohl'	5 (3.90-6.1)	16.00 (7.43-34.45) jl	50.221	<0.001	1	163
1.72	*Akanthomyces dipterigenus*	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	4 (3.54-4.46)	18.08 (8.41-38.88) ikl	54.938	<0.001	1	161
433.99	*Beauveria bassiana*	11	*B. oleracea* var. Acephala 'Furchenkohl'	*	8.24 (3.74-18.17) b	27.305	<0.001	1	133
433.99	*Beauveria bassiana*	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	5 (4.55-5.45)	15.06 (6.94-32.69) ij	47.064	<0.001	1	121
Mock	0.05% Triton-X100	11	*B. oleracea* var. Acephala 'Furchenkohl'	*	2.80 (1.12-7.01) ch	4.814	0.028	1	91
Mock	0.05% Triton-X100	15	*B. oleracea* var. Botrytis 'Marzatico Napoletano'	*	3.08 (1.21-7.82) ch	5.594	0.018	1	77
1.72	*Akanthomyces dipterigenus*	16	*B. cretica*	3 (2.65-3.35)	21.52 (10.01-46.29) gk	61.715	<0.001	1	149
1.72	*Akanthomyces dipterigenus*	17	*B. cretica*	3 (2.06-3.94)	19.22 (8.74-42.25) eikl	54.075	<0.001	1	83
433.99	*Beauveria bassiana*	16	*B. cretica*	3 (2.52-3.48)	20.30 (9.31-44.30) fkl	57.212	<0.001	1	100
433.99	*Beauveria bassiana*	17	*B. cretica*	3 (2.3303.67)	23.07 (10.70-49.74) efg	64.129	<0.001	1	129
Mock	0.05% Triton-X100	16	*B. cretica*	*	3.47 (1.40-8.59) c	7.220	0.007	1	84
Mock	0.05% Triton-X100	17	*B. cretica*	*	4.85 (2.06-11.40) c	13.078	<0.001	1	92

Table 4.10: Four Cox proportional hazard models were undertaken to evaluate the significance of different factors upon observed nymph *Brevicoryne brassicae* survival data with the first three subsequent models splitting factors from the previous model into constituent sub-factors (Treatment > EPF isolate + Plant accession > Plant species + resistant or susceptible). Within the fourth model, three 2-factor interaction terms and one 3-factor interaction term was included to determine whether any significant interactions were present between the three primary independent variables (EPF isolate, *Brassica* species and Resistant or Susceptible). Technical control plants were excluded from these analyses to allow for direct comparison between resistant and susceptible accessions of the two *Brassica* species.

Model	Effects and interactions	Factors	Z (HR)	P (HR)	df	n
Cox model 1	Main effects	Rep	12.626	0.002	2	3
		Treatment	180.591	<0.001	11	12***
Cox model 2	Main effects	Rep	8.026	0.018	2	3
		EPF isolate	144.181	<0.001	2	3
		Plant accession	28.573	<0.001	3	4***
Cox model 3	Main effects	Rep	8.522	0.014	2	3
		EPF isolate	142.466	<0.001	2	3
		Brassica species	22.205	<0.001	1	2
		Resistant or Susceptible	0.873	0.350	1	2
Cox model 4	Main effects	Rep	12.626	0.002	2	3
		EPF isolate	41.718	<0.001	2	3
		Brassica species	2.246	0.134	1	2
		Resistant or Susceptible	0.488	0.485	1	2
	2-factor interaction terms	EPF isolate x *Brassica* species	16.285	<0.001	2	3
		EPF isolate x Resistant or Susceptible	1.849	0.397	2	3
		Brassica species x Resistant or Susceptible	0.683	0.308	1	2
	3-factor interaction term	EPF isolate x *Brassica* species x Resistant or Susceptible	6.144	0.011	2	3

4.3.2. Evaluating the effects and interactions of combined partial varietal resistance/EPF deployment upon EPF efficacy against *Brevicoryne brassicae* – GLMM analysis

Through GLM analysis, replicate was identified as a significant factor influencing *B. brassicae* adults and nymph mortality on both days 6 and 9 of bioassay procedure (day 6 adults [Wald X^2 (2) = 14.843, p = 5.98x10^{-4}], day 9 adults [Wald X^2 (2) = 9.889, p = 0.007], day 6 nymphs [Wald X^2 (2) = 15.031, p = 5.45x10^{-4}], and day 9 nymphs [Wald X^2 (2) = 2.265, p = 0.002]. Several interactions between replicate and other experimental factors were also noted in this analysis. Within the day 6 adults results a significant interaction was noted between replicate and EPF isolate (Wald X^2 (4) = 16.100, p = 0.003). In the day 9 adult results a significant interaction was also noted between replicate and EPF isolate (Wald X^2 (4) = 23.776, p = 8.9x10^{-5}). Within the day 6 nymphs results, significant interactions were noted between replicate and EPF isolate (Wald X^2 (4) = 14.540, p = 0.006) and between replicate and *Brassica* species (Wald X^2 (2) = 6.258, p = 0.044). In the day 9 nymph results, three significant interactions were identified between (1) replicate and EPF isolate (Wald X^2 (4) = 14.228, p = 0.007), (2) between replicate and *Brassica* species (Wald X^2 (2) = 8.429, p = 0.015), and (3) between replicate and resistant/susceptible (Wald X^2 (2) = 6.958, p = 0.031).

To account for this between replicate variation, results were subsequently analysed using a GLMM approach, including replicate as a random effect factor. All four GLMMs were found to be significant improvements over a null model (P<0.001), with main effect and interaction term Sig. (p), F statistics and degrees of freedom for each of the four undertaken GLMMs shown in Table 4.11.

For adult *B. brassicae* at both 6 and 9 days, EPF isolate was found to be a significant main effect factor (P<0.001 in both cases) while neither *Brassica* species nor 'Resistant vs Susceptible' proved significant as main effects. There was a significant 2-factor interaction on both days 6 and 9 between EPF isolate and *Brassica* species (P=0.021, P=<0.001 respectively). To evaluate this interaction, these factors were plotted in interaction plots and contrast tests performed (Figures 4.2 and 4.3, Tables 4.12 and 4.13). These tests showed that isolate 1.72 (*A. dipterigenus*) caused more *B.*

brassicae mortality on *B. oleracea* (day 6: ~78%, day 9: ~83%) relative to *B. cretica* (day 6: ~62%, day 9: ~64%) on both days 6 and 9 (p=0.01, p=1.58 x 10^{-5} respectively) (Figures 4.2 and 4.3). Through simple contrasts testing, this observation was confirmed to be statistically significant on both days 6 and 9 (p=0.01, p=1.58x10^{-5} respectively). No further significant differences were observed between *Brassica* species - with isolate 433.99 and mock control treatments performance statistically indistinguishable between *Brassica* species on both days 6 and 9 (Tables 4.12 and 4.13).

For *B. brassicae* nymphs on both 6 and 9 days, EPF isolate, and *Brassica* species were found to be significant main effect factors (P<0.001 in all cases) while "resistant or susceptible" proved insignificant as main effect (Table 4.11). A significant 3-factor interaction was identified however between EPF isolate, *Brassica* species and whether the accession was partially resistant or susceptible on both days 6 and 9 (p=0.003, p=0.024 respectively) (Table 4.11). To evaluate this interaction, these factors were plotted in interaction plots and contrast tests performed, firstly in the context of intraspecific variation in resistance (*B. oleracea* 11 vs 15, *B. cretica* 16 vs 17) and secondly in the context of interspecific variation in resistance (*B. oleracea* vs *B. cretica*) (Figures 4.4 and 4.5, Tables 4.14, 4.15, 4.16 and 4.17). The same significant differences and trends were observed on days 6 and 9, with differences marginally more pronounced and survival marginally lower on day 9 relative to day 6 (with virulent treatments resulting in ~20% less survival on day 9 relative to day 6). These tests showed that between the partially resistant (15) and susceptible (11) accessions of *B. oleracea* there was a significant difference in EPF isolate 433.99 (*B. bassiana*) performance (p=<0.001), with ~65% survival on the susceptible accession (11) dropping to ~30% survival on the partially resistant accession (15) on day 9 (Figure 4.5A). No significant difference was noted in *B. brassicae* nymph survival between the partially resistant (16) and susceptible (17) *B. cretica* accessions following treatment with EPF isolate 433.99 (*B. bassiana*) with both of these treatments resulting in ~10-20% nymph survival (Figure 4.5A; Table 4.16). No significant differences in nymph survival were noted following nymph treatment with isolate 1.72 (*A. dipterigenus*) both intra- and interspecifically, with all 1.72 treatments resulting in nymph survival of ~10-22% (Figure 4.5; Table 4.16 and 4.17). Interspecifically, the only identified significant difference in nymph mortality

was observed following treatment with isolate 433.99 (*B. bassiana*) between the susceptible *B. oleracea* accession (11) and susceptible *B. cretica* accession (17), with day 9 nymph survival on accession 11 being ~65%, significantly higher than the ~15% survival seen on accession 16 ($p=<0.001$) (Figure 4.5B; Table 4.17). No significant difference was observed in nymph mortality following 433.99 (*B. bassiana*) treatment on the partially resistant B. *oleracea* accession (15) relative to the partially resistant *B. cretica* accession (16), with both resulting in ~20-30% survival (Figure 4.5B; Table 4.17).

Table 4.11: Statistical output from four GLMMs assessing the main effects and interactions of EPF isolate, *Brassica* species and whether accessions were resistant or susceptible upon adult and first instar *Brevicoryne brassicae* mortality on day 6 and 9 of a 9-day bioassay. In all cases, GLMM omnibus test was significant (P=<0.001), with EPF being found to be a significant main effect and factor influencing *B. brassicae* mortality in all four models (P<0.001), however no significant main effects were detected for either *Brassica* species or resistant/susceptible (aside from against adults on day 9 where a marginal significant effect was noted between resistant and susceptible accessions). In all cases however, there was a significant interaction detected between EPF isolate and *Brassica* species. A significant interaction was also detected against nymphs on days 6 and 9 between EPF isolate and resistant/susceptible accessions where these accessions were evaluated within the context of *Brassica* species.

GLMM analysis		Statistic	Adults		Nymphs	
			6 days	9 days	6 days	9 days
Model vs null		**Sig. (P)**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
		F	23.006	26.638	19.558	19.417
		df	11, 1311	11, 1311	11, 1371	11, 1371
Factors (Main effect)	EPF isolate	**Sig. (P)**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
		F	119.566	137.646	85.315	87.308
		df	2, 1311	2, 1311	2, 1371	2, 1371
	Brassica species	**Sig. (P)**	**0.500**	**0.047**	**0.001**	**0.001**
		F	0.455	3.960	12.151	11.223
		df	1, 1311	1, 1311	1, 1371	1, 1371
	Resistant/ Susceptible	**Sig. (P)**	**0.319**	**0.035**	**0.196**	**0.508**
		F	0.994	4.453	1.674	0.439
		df	1, 1311	1, 1311	1, 1371	1, 1371
2-factor interaction terms	EPF isolate x *Brassica* species	**Sig. (P)**	**0.021**	**<0.001**	**0.013**	**0.021**
		F	3.888	8.759	4.329	3.868
		df	2, 1311	2, 1311	2, 1371	2, 1371
	EPF isolate x Resistant/Susceptible	**Sig. (P)**	**0.158**	**0.114**	**0.388**	**0.468**
		F	1.848	2.176	0.947	0.760
		df	2, 1311	2, 1311	2, 1371	2, 1371
	Resistant/Susceptible x *Brassica* species	**Sig. (P)**	**0.257**	**0.054**	**0.003**	**0.022**
		F	1.288	3.715	4.593	5.247
		df	1, 1311	1, 1311	1, 1371	1, 1371
3-factor interaction term	EPF isolate x Resistant /Susceptible x *Brassica* species	**Sig. (P)**	**0.717**	**0.645**	**0.003**	**0.024**
		F	0.322	0.439	4.593	3.699
		df	2, 1311	2, 1311	2, 1371	2, 1371

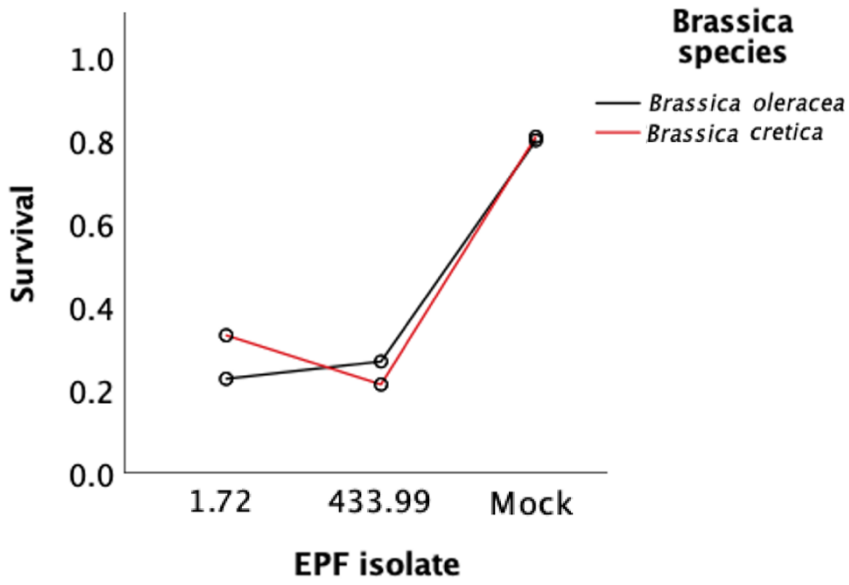


Figure 4.2: Interaction plot between EPF isolate and *Brassica* species on day-6 against adult *Brevicoryne brassicae*. GLMM analysis revealed this to be a significant interaction (P=0.021). Within this interaction, *Brassica* species upon which *B. brassicae* are feeding appears to significantly affect the performance of EPF isolate 1.72 (*Akanthomyces dipterigenus*). Pairwise contrasts to evaluate simple effects of *Brassica* species within the scope of each EPF isolate confirmed this observation, with a significant difference being found in EPF isolate 1.72 performance between the two assessed *Brassica* species (P=0.005). All other simple effects were found to be non-significant.

Table 4.12: Interaction analysis simple contrasts tests results for adult *Brevicoryne brassicae* on assessment day 6. These results confirmed a significant difference in the performance of EPF isolate 1.72 (*Akanthomyces dipterigenus*), 433.99 (*Beauveria bassiana*) relative to mock treatment for against aphids on both *Brassica* species. While a difference in performance of EPF isolates 1.72 and 433.99 could be detected against aphids reared on *Brassica cretica*, at this timepoint there was no significant difference noted in the performance of these two EPF isolates on *Brassica oleracea*. These analyses do however also confirm a significant difference in EPF isolate 1.72 performance dependent upon whether sprayed aphids were on *B. oleracea* or *B. cretica* plants.

Independent variable 1	Independent variable 2: Pairwise Contrasts	t	df	Adj. Sig.
1.72	*B. oleracea* x *B. cretica*	-2.565	1311	**0.01**
433.99	*B. oleracea* x *B. cretica*	1.354	1311	0.176
Mock	*B. oleracea* x *B. cretica*	-0.198	1311	0.843
	1.72 x 433.99	-1.09	1311	0.276
B. oleracea	1.72 x mock	-14.121	1311	**<0.001**
	433.99 x mock	-12.072	1311	**<0.001**
	1.72 x 433.99	2.731	1311	**0.006**
B cretica	1.72 x mock	-10.48	1311	**<0.001**
	433.99 x mock	-14.412	1311	**<0.001**

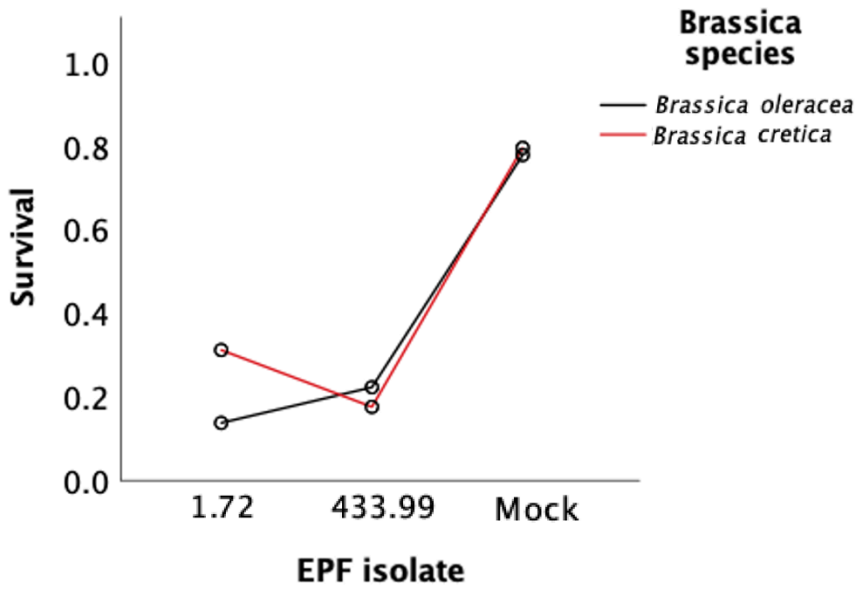


Figure 4.3: Interaction plot between EPF isolate and *Brassica* species on day-9 against adult *Brevicoryne brassicae*. GLMM analysis identified this as a significant interaction (P=<0.001). Within this interaction, *Brassica* species upon which *B. brassicae* are feeding appears to significantly affect the performance of EPF isolate 1.72 (*Akanthomyces dipterigenus*). Pairwise contrasts to evaluate simple effects of *Brassica* species within the scope of each EPF isolate confirmed this observation, with a significant difference being found in EPF isolate 1.72 performance between the two assessed *Brassica* species ($P=6.41x10^{-6}$). All other simple effects were found to be non-significant.

Table 4.13: Interaction analysis simple main effect testing results for adult *Brevicoryne brassicae* on assessment day 9. These results confirmed a significant difference in the performance of EPF isolate 1.72 (*Akanthomyces dipterigenus*), 433.99 (*Beauveria bassiana*) and mock treatment for both *Brassica* species. These analyses also confirmed a significant difference in EPF isolate 1.72 performance dependent upon whether sprayed aphids were on *Brassica oleracea* or *Brassica cretica* plants.

Independent variable 1	Independent variable 2: Pairwise Contrasts	t	df	Adj. Sig.
1.72	*B. oleracea* x *B. cretica*	-4.334	1311	**$1.58x10^{-5}$**
433.99	*B. oleracea* x *B. cretica*	1.227	1311	0.22
Mock	*B. oleracea* x *B. cretica*	-0.367	1311	0.714
	1.72 x 433.99	-2.406	1311	**0.016**
B. oleracea	1.72 x mock	-15.674	1311	**<0.001**
	433.99 x mock	-12.643	1311	**<0.001**
	1.72 x 433.99	3.23	1311	**0.001**
B cretica	1.72 x mock	-10.648	1311	**<0.001**
	433.99 x mock	-15.025	1311	**<0.001**

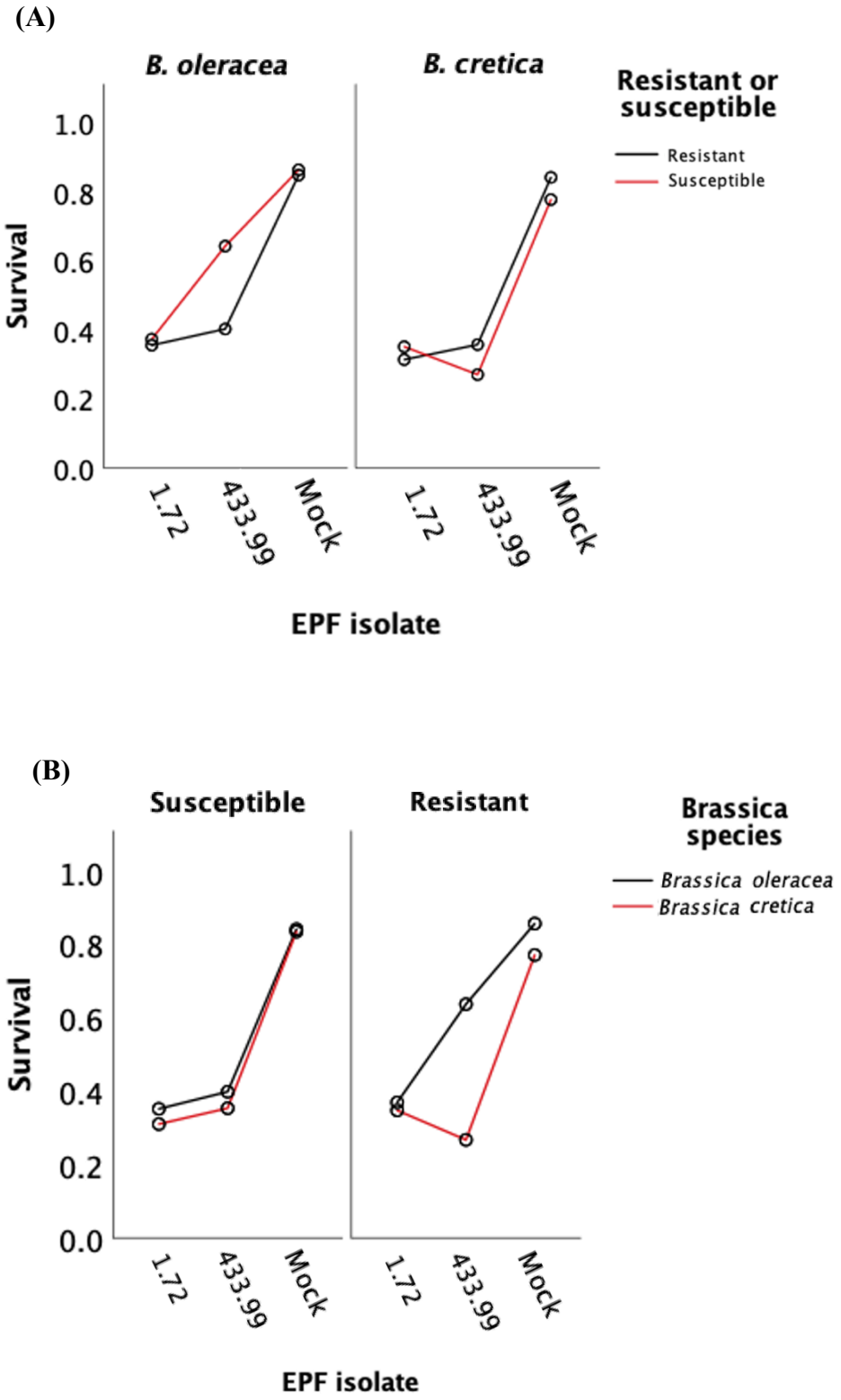


Figure 4.4: Interaction plots between EPF isolate, *Brassica* species and relative resistance level on day-6 against *Brevicoryne brassicae* nymphs contrasted on resistant and susceptible accessions with a focus upon (A) intraspecific differences and (B) interspecific differences. GLMM analysis revealed this to be a significant 3-way interaction (P=0.003). Within this interaction, whether plants are resistant or susceptible appears to be moderating the two-way interaction between EPF isolate and *Brassica* species – particularly for EPF isolate 433.99 (*Beauveria bassiana*) on *Brassica oleracea* plants. Pairwise contrasts confirmed this, with a significant difference in isolate 433.99 performance on a resistant *B. oleracea* plant relative to a more susceptible *B. oleracea* plant (P=<0.001).

Table 4.14: Interaction analysis simple contrasts test results for *Brevicoryne brassicae* nymphs on assessment day 6. These tests confirmed a significant difference in the performance of EPF isolate 433.99 (*Beauveria bassiana*), between resistant and susceptible accessions of *Brassica oleracea* (p=<0.001).

Brassica species	EPF isolate/ treatment	Partially resistant vs Susceptible: Pairwise Contrasts	t	df	Adj. Sig.
Brassica oleracea	1.72	Resistant vs Susceptible	-0.564	1371	0.573
	433.99	Resistant vs Susceptible	-4.206	1371	**<0.001**
	mock	Resistant vs Susceptible	-0.903	1371	0.367
Brassica cretica	1.72	Resistant vs Susceptible	-0.783	1371	0.434
	433.99	Resistant vs Susceptible	0.797	1371	0.426
	mock	Resistant vs Susceptible	1.377	1371	0.169

Table 4.15: Interaction analysis simple contrasts test results for *Brevicoryne brassicae* nymphs on assessment day 6. These tests confirmed a significant difference in the performance of EPF isolate 433.99 (*Beauveria bassiana*), between resistant accessions of *Brassica oleracea* and *Brassica cretica* (p=<0.001).

EPF isolate	Partially Resistant or Susceptible	*Brassica* species: Pairwise Contrasts	t	df	Adj. Sig.
1.72	Resistant	*B. oleracea* vs *B. cretica*	0.765	1371	0.444
	Susceptible	*B. oleracea* vs *B. cretica*	0.333	1371	0.739
433.99	Resistant	*B. oleracea* vs *B. cretica*	0.675	1371	0.5
	Susceptible	*B. oleracea* vs *B. cretica*	6.438	1371	**<0.001**
Mock	Resistant	*B. oleracea* vs *B. cretica*	0.115	1371	0.908
	Susceptible	*B. oleracea* vs *B. cretica*	1.484	1371	0.138

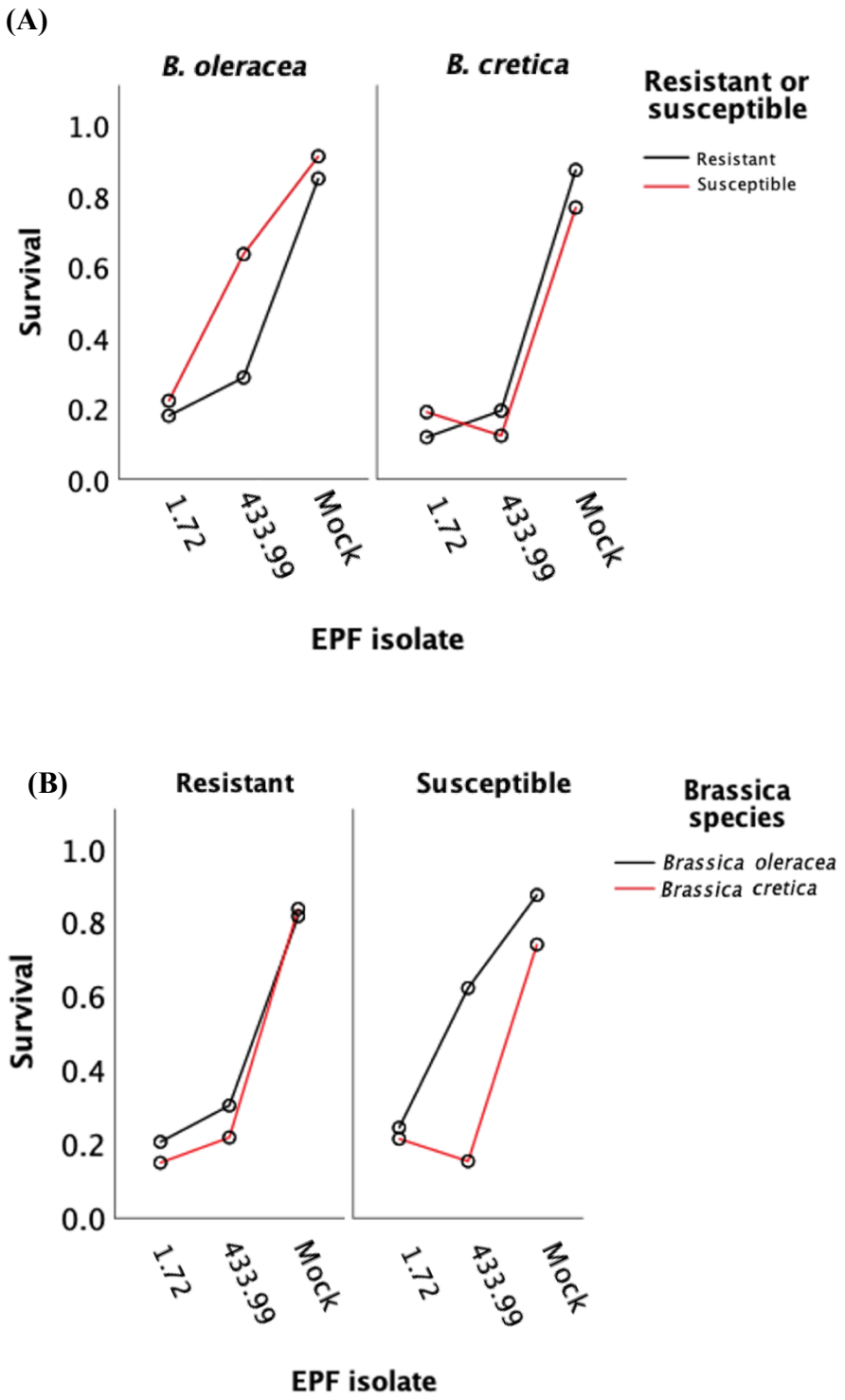


Figure 4.5: Interaction plot between EPF isolate and *Brassica* species on day-9 against *Brevicoryne brassicae* nymphs contrasted on resistant and susceptible accessions with a focus upon (A) intraspecific differences and (B) interspecific. GLMM analysis revealed this to be a significant 3-way interaction (P=0.024). Within this interaction, whether plants are resistant or susceptible appears to be moderating the two-way interaction between EPF isolate and *Brassica* species – particularly for EPF isolate 433.99 (*Beauveria bassiana*) on *Brassica oleracea* plants. Pairwise contrasts confirmed this, with a significant difference in isolate 433.99 performance on a resistant *B. oleracea* plant relative to a more susceptible *B. oleracea* plant (P=<0.001).

Table 4.16: Interaction analysis simple contrasts test results for *Brevicoryne brassicae* nymphs on assessment day 9. These tests confirmed a significant difference in the performance of EPF isolate 433.99, *B. bassiana*, between resistant and susceptible accessions of *Brassica oleracea* (p=<0.001).

Brassica species	EPF isolate/ treatment	Partially resistant vs Susceptible: Pairwise Contrasts	t	df	Adj. Sig.
Brassica oleracea	1.72	Resistant vs Susceptible	-0.324	1371	0.746
	433.99	Resistant vs Susceptible	-3.897	1371	**<0.001**
	mock	Resistant vs Susceptible	-0.275	1371	0.783
Brassica cretica	1.72	Resistant vs Susceptible	-0.571	1371	0.568
	433.99	Resistant vs Susceptible	1.376	1371	0.169
	mock	Resistant vs Susceptible	1.068	1371	0.286

Table 4.17: Interaction analysis simple contrasts test results for *Brevicoryne brassicae* nymphs on assessment day 9. These tests confirmed a significant difference in the performance of EPF isolate 433.99, *B. bassiana*, between resistant accessions of *Brassica oleracea* and *Brassica cretica* (p=<0.001).

EPF isolate	Partially Resistant or Susceptible	*Brassica* species: Pairwise Contrasts	t	df	Adj. Sig.
1.72	Resistant	*B. oleracea* vs *B. cretica*	0.827	1371	0.409
	Susceptible	*B. oleracea* vs *B. cretica*	0.366	1371	0.715
433.99	Resistant	*B. oleracea* vs *B. cretica*	1.027	1371	0.305
	Susceptible	*B. oleracea* vs *B. cretica*	6.557	1371	**<0.001**
Mock	Resistant	*B. oleracea* vs *B. cretica*	0.296	1371	0.767
	Susceptible	*B. oleracea* vs *B. cretica*	1.99	1371	0.047

4.3.3. Evaluating the effects and interactions of combined partial varietal resistance/EPF deployment upon *Brevicoryne brassicae* population development

Within this population development monitoring assay, distinctive trends in population development were observed between different treatment regimes, between the two EPF isolates and between the two *Brassica* species (Figures 4.6 and 4.7). Where two mock sprays were applied (mock, mock), both the partially resistant and susceptible *B. oleracea* accessions resulted in a population development rate which followed broadly an exponential trend both overall and for *B. brassicae* adults and nymphs separately, with concluding (day 20) population reaching ~250 aphids on the partially resistant accession (15) and ~300 aphids on the more susceptible accession (11) (Figure 4.6). The same general trend was observed for the susceptible *B. cretica* accession; however, a more pronounced difference was observed between the partially resistant accession and the susceptible accession, with a concluding (day 20) population of ~220 on accession 16 relative to ~310 on accession 17 (Figure 4.7). For the partially resistant *B. cretica* accession, the population development appears to not follow a uniform exponential trend, appearing to begin to slow from the maximum linear rate of population increase on the later days of assay monitoring (Figure 4.7).

Where a single treatment of EPF isolate 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*) was applied on assay day 1, universally across all *Brassica* accessions (both *B. oleracea* and *B. cretica*), between days 4-9 adult population decreased (typically from 10 to ~2-5 adults). Overall *B. brassicae* population however became arrested, remaining ~40-50 for this period regardless of *Brassica* accession or EPF isolate (Figures 4.6 and 4.7), with this population arrestment indicating that the rate of population decrease owing to EPF induced mortality was matched by the rate of population increase from remaining adult reproduction (Figures 4.6 and 4.7). Following this period of population arrestment, which lasted from days 4-9 on *B. oleracea* accessions and 4-12 on *B. cretica* accessions, population began to increase reaching on day 20 (following 1.72 or 433.99 treatment); ~160/180 aphids on the susceptible *B. oleracea* accession (11), ~160/180 aphids on the partially resistant *B. oleracea* accession (15), ~140/260 aphids on the susceptible *B. cretica* accession (17) (with population following 433.99 on accession 17 appearing a significantly high outlier) and ~150/140 aphids on the partially resistant *B. cretica* accession (16)

(Figures 4.6 and 4.7). Overall, these concluding populations were notably low relative to concluding day 20 population of mock, mock treated *B. brassicae* cohorts (for which population was ~250-300 aphids) – with this difference most likely a result of the population arrestment as a result of day 1 EPF treatment.

Under a two-spray regime, with EPF treatment on both days 1 and 5 of assay procedure, more notable differences in population development arose between different combination of the two EPF isolates (1.72, *A. dipterigenus* and 433.99, *B. bassiana*) and *Brassica* species (*B. oleracea* and *B. cretica*). On *B. oleracea* accessions 15 (partially resistant) and 11 (susceptible), regardless of EPF isolate, between days 1-5 the same pattern of diminishing adult population was observed as was noted following a single EPF spray on day 1, dropping from 10 to ~1-3 (Figure 4.6). More notable differences in population development arose however following the second EPF spray on day 5. On the twice sprayed cohorts, following day 5 EPF treatment of the mixed *B. brassicae* population with either isolate 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*) a lag period of 2-3 days until EPF effect was noted prior to any lethal EPF effects (Figure 4.6). Following this lag, 1.72 (*A. dipterigenus*) treated cohort population dropped from ~45 aphids on day 7 to ~10 aphids on days 10-12 before beginning to recover and eventually reaching ~40-50 aphids by day 20, with no discernible different between the partially resistant (15) and susceptible (11) *B. oleracea* accessions. Following the lag period, 433.99 (*B. bassiana*) treated cohort population dropped from ~30 aphids on day 7 to ~15 following 433.99 (*B. brassicae*) treatment before beginning to recover and reaching ~65-70 aphids by day 20 with no difference discernible in concluding population between the partially resistant (15) and susceptible (11) *B. oleracea* accessions (Figure 4.6). While no substantive differences were noted between the partial resistant and susceptible accessions in this assay, isolate 1.72 (*A. dipterigenus*) appeared to have a more pronounced effect upon *B. brassicae* population after the second spray on assay day 5 (Figure 4.6).

On *B cretica* accessions 16 (partially resistant) and 17 (susceptible), between days 1-5 the same pattern of diminishing adult population was observed as was noted following a single EPF spray on day 1, dropping from 10 to ~1-3 (Figure 4.7). As was seen for *B. oleracea* accessions, more pronounced differences in population

development arose following the second EPF spray on day 5 (Figure 4.7). On these twice sprayed cohorts, following day 5 EPF treatment of the mixed *B. brassicae* population with either isolate 1.72 (*A. dipterigenus*) or 433.99 (*B. bassiana*) a lag period of 2 days until EPF effect was noted prior to any lethal EPF effects (Figure 4.7). Following this lag, 1.72 (*A. dipterigenus*) treated cohort population on the partially resistant accession (16) dropped from ~40 on day 6 to ~5 on day 10 before then slowly decreasing until total *B. brassicae* eradication on day 17 (Figure 4.7). A similar trend was observed on the more susceptible accession (17), with population following treatment with isolate 1.72 (*A. dipterigenus*) dropping from ~45 on day 6 to ~3 on day 15 before then slowly beginning to recover as nymphs born early in the assay period reached adulthood and began reproducing (Figure 4.7). Thus, while adult eradication occurred following 1.72 (*A. dipterigenus*) treatment on accession 17, a small population of nymphs survived the second EPF treatment allowing population to start to rebound from day 17 onwards, reaching ~7-8 aphids by day 20 (Figure 4.7). It should be noted however that this population was still notably small than the concluding populations following two 1.72 (*A. dipterigenus*) treatments on *B. oleracea* accession 11 and 15.

Relative to *B. brassicae* population development on *B. cretica* accessions following a second (day 5) treatment with 1.72 (*A. dipterigenus*), population development following a second treatment with 433.99 (*B. bassiana*) was markedly different (Figure 4.7). On the partially resistant accession (16), following the second 433.99 (*B. bassiana*) treatment, B. *brassicae* population dropped from ~30 aphids on day 7 to ~15 aphids on day 10 – a notably small drop relative to twice 1.72 (*A. dipterigenus*) cohorts. Owing to a small population of adults having survived both 433.99 sprays and nymphs born at the start of the assay period beginning to reach adulthood by day 10, from this day onwards the population began to rebound, reaching ~65 aphids by day 20 (Figure 4.7). On the more susceptible *B. cretica* accession (17), following the second treatment with 1.72 (*A. dipterigenus*) on day 5, *B. brassicae* population dropped from a high of ~55 on day 5 to a low of ~30 on day 13 before then rapidly recovering and reaching a population of ~105 on assay day 20 – markedly higher than the concluding population on *B. cretica* accession 16 (Figure 3.7). This difference may have resulted from a difference in the rate of nymph development between *B. cretica* accession 16 and 17, with adult *B. brassicae*

population on the susceptible accession (17) appearing to increase from day 8 onwards indicating that nymph produced early in the bioassay period were beginning to reach adulthood whereas on the more partially resistant accession (16) the *B. brassicae* adult population did not appear to begin increasing until assay day 10 onwards (Figure 4.7).

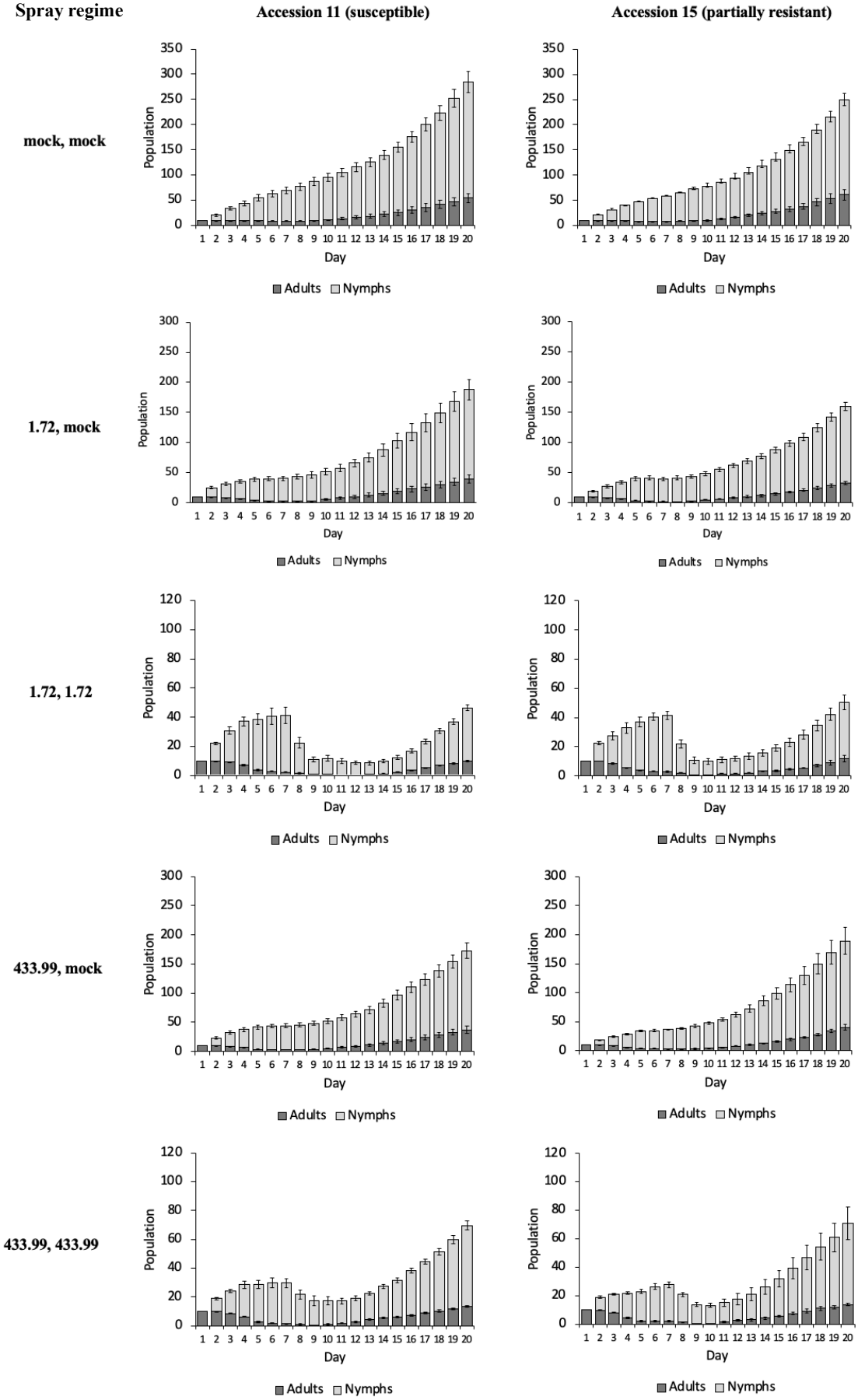


Figure 4.6: ***Brevicoryne brassicae*** **adult and nymph populations on** ***Brassica oleracea*** **accessions 11 (susceptible) and 15 (partially resistant) following two sprays, the first on assay day 1 and the second on assay day 5 with different regimes of mock, EPF isolate 1.72 and EPF isolate 433.99.**

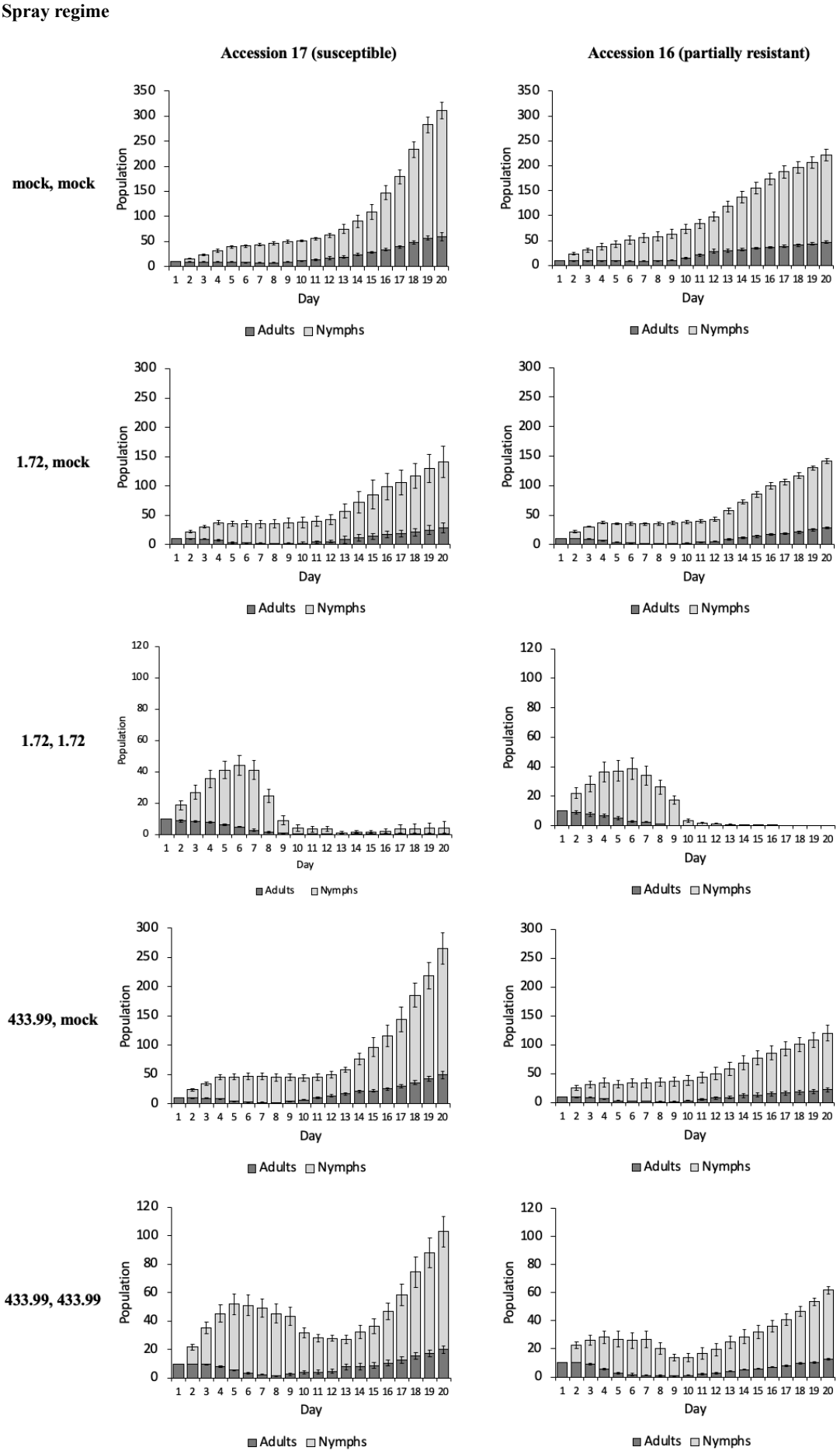


Figure 4.7: ***Brevicoryne brassicae*** **adult and nymph populations on** ***Brassica cretica*** **accessions 17 (susceptible) and 16 (partially resistant) following two sprays, the first on assay day 1 and the second on assay day 5 with different regimes of mock, EPF isolate 1.72 and EPF isolate 433.99.**

4.3.3.1. Evaluating the effects and interactions of combined partial varietal resistance/EPF deployment and upon *Brevicoryne brassicae* population development – GLM analysis

Through GLM analysis, replicate was identified as a significant factor influencing *B. brassicae* adults and nymph mortality on day 5 of aphid population monitoring bioassay procedure (day 5 total aphid population [Wald X^2 (2) = 25.462, p = 3.00×10^{-6}]. Despite this significance, no significant interactions between replicate and other experimental factors were identified on day 5 in this analysis (*Brassica* species x replicate [Wald X^2 (2) = 0.510, p = 0.775], EPF isolate x replicate [Wald X^2 (2) = 2.045, p = 0.360], resistant/susceptible x replicate [Wald X^2 (2) = 1.909, p = 0.385], spray regime x replicate [Wald X^2 (2) = 8.537, p = 0.074]). Replicate did not prove a significant factor influencing total aphid count on day 10 (Wald X^2 (2) = 4.74, p = 0.097) or day 20 (Wald X^2 (2) = 0.291, p = 0.864).

To account for between replicate variation on day 5, results were analysed using a GLMM approach, including replicate as a random effect factor. Three primary GLMMs were undertaken on overall aphid population size results on assay days 5, 10 and 20 to evaluate the effects and potential interactions of: (i) two EPF isolates; (ii) two *Brassica* species; (iii) whether accessions were previously identified as resistant or susceptible to *B. brassicae* (chapter 2); (iv) three spray regimes (Table 4.18). Owing to multiple comparisons, a Bonferroni correction was applied reducing significance threshold to 0.0167. Six secondary GLMMs were then undertaken, assessing adult and nymph populations on days 5, 10 and 20 in those cases where significant interaction effects were observed within primary analyses. Within each GLMM, all two-way, three-way, and four-way interactions between the four factors were evaluated.

<u>Primary GLMMs</u>: All three primary GLMMs for *B. brassicae* population gave significant improvements over a null model (p<0.001 in all three cases) (Table 4.18). Across all three GLMMs (for assay days 5, 10 and 20) 'Spray regime' was found to be a significant main effect (p=<0.001 in all cases) (Table 4.18). For the remaining three factors however, their significance as main effects was only noted for two of the three timepoints assessed – with 'EPF isolate' proving a significant main effect

on days 10 and 20 (p=<0.001 in both cases), '*Brassica* species' also proving a significant main effects on days 10 and 20 (p=<0.001) and 'resistant/susceptible' proving a significant main effect on days 5 and 20 (p=0.006, p=<0.001) (Table 4.18). At all three timepoints no significant four-way interaction was identified (Day 5 p=0.354, Day 10 p=0.642, Day 20 p=0.620). For all three days however a minimum of one three-way interaction of factors was noted; with '*Brassica* species x Resistant/Susceptible x spray regime' significant on day 5 (p=0.005), both 'EPF isolate x *Brassica* species x Spray regime' and '*Brassica* species x Resistant/Susceptible x spray regime' significant on day 10 (p=<0.001, p=0.001) and 'EPF isolate x *Brassica* species x spray regime' significant on day 20 (p=<0.001) (Table 4.18).

Secondary GLMMs: For the three-factor interaction term 'EPF isolate x *Brassica* species x spray regime', a significant interaction was noted for nymph population on day 10 (p=<0.001). On assay day 20 however, this interaction term proved significant for both adult and nymph populations (p=<0.001 in both cases). The three-factor interaction term '*Brassica* species x Resistant/Susceptible x spray regime' was significant on days 5 and 10 for both *B. brassicae* adult and nymph populations (day 5: p=0.004, p=0.010; day10: p=0.008, p=0.002 respectively) (Table 4.18).

Interaction plots were drawn for both interaction terms on the days for which they were found to be significant (Figures 4.8 and 4.9). Separate interaction plots were drawn for overall *B. brassicae* population, adult population, and nymph population. Pairwise simple contrast tests were also performed to identify which significant pairwise interactions were present underlying the overall significant interaction, with two sets of pairwise simple contrast tests undertaken for significant interaction (Tables 4.17, 4.18, 4.19 and 4.20).

4.3.3.2. Significant three-factor interaction 1: 'EPF isolate x *Brassica* species x spray regime'

For the interaction term 'EPF isolate x *Brassica* species x Spray regime' two sets of simple contrast tests were undertaken – the first evaluating comparisons between the

two EPF isolates (1.72, *A. dipterigenus* vs 433.99, *B. bassiana*) and the second between the two *Brassica* species (*B. oleracea* vs *B. cretica*) (Table 4.19).

Comparison of EPF isolates: With one exception, two sprays of 1.72 (*A. dipterigenus*) resulted in a significantly smaller *B. brassicae* population than those sprayed with 433.99 (*B. bassiana*) ($p=<0.05$) ([*B. brassicae* populations on *B. oleracea* treated with 1.72 and 433.99 respectively: Day 10 total population: ~15 vs ~18, Day 10 nymph population: ~14 vs ~16, Day 20 total population: ~45 vs ~70, Day 20 adult population: ~12 vs ~14, Day 20 nymph population: ~40 vs ~65] [*B. brassicae* populations on *B. cretica* treated with 1.72 and 433.99 respectively: Day 10 total population: ~2 vs ~22, Day 10 nymph population: ~2 vs ~20, Day 20 total population: 0 vs ~80, Day 20 adult population: 0 vs ~18, Day 20 nymph population: 0 vs ~70) (Table 4.19; Figure 4.8). This effect occurred (i) for the total *B. brassicae* population, the adult population, and the nymph population, and (ii) in both *Brassica* species (Table 4.19; Figure 4.8). The exception was for the day 10 adult population (owing to the finding of no significant three-way interaction for overall adult population within this GLMM, Table 4.18). Within this interaction *Brassica* species appears to act by altering the magnitude of difference between aphid mortality caused by 1.72 (*A. dipterigenus*) vs 433.99 (*B. brassicae*) (Figure 4.8), with t-statistics ranging from -1.333 to -2.654 for *B. oleracea* and -7.014 to -9.848 for *B. cretica* (Table 4.19). Note that this interaction effect was not observed for assay day 5, as readings for day 5 populations were taken prior to second spray being administered.

Comparison of *B. oleracea* and *B. cretica*: on day 10 for overall and nymph population (but not adult population) a significant difference was noted between mock, mock treatments, indicating a significant effect of plant accession upon *B. brassicae* population (*B. brassicae* population on *B. oleracea* and *B. cretica* respectively: Day 10 total population: ~98 vs ~70, Day 10 nymph population: ~82 vs ~55) (overall population $p=0.010$, nymph population $p=<0.001$), alongside a significant difference in population of the aphid cohort sprayed twice with 1.72 (*A. dipterigenus*) for overall and nymph population (*B. brassicae* populations following two 1.72 treatments on *B. oleracea* and *B. cretica* respectively: Day 10 total population: ~15 vs ~2, Day 10 nymph population: ~14 vs ~2) (Table 4.20).

Interaction plots indicated that in all cases, significant differences were explained by *B. cretica* causing a reduction in the *B. brassicae* population (either overall population or nymphs) relative to *B. oleracea* following two mock sprays or two sprays of 1.72 (*A. dipterigenus*). On day 20, the aphid population (total, adults, nymphs) treated with two sprays of 1.72 (*A. dipterigenus*) was significantly smaller on *B. cretica* compared to *B. oleracea* (Day 20 total population: ~45 vs 0, Day 20 adult population: ~12 vs 0, Day 20 nymph population: ~40 vs 0) ($p=7.55x10^{-7}$ and $p=1.20x10^{-6}$) (total population, $p=2.22x10^{-16}$; adult population, $p=4.41x10^{-12}$; nymph population $p=2.22x10^{-16}$).

4.3.3.3. Significant three-factor interaction 2: '*Brassica* species x resistant/susceptible x spray regime'

For the interaction term '*Brassica* species x Resistant/Susceptible x Spray regime' two sets of simple contrast tests were undertaken: (i) partially Resistant vs. Susceptible accessions of *B. oleracea* and *B. cretica* (accession 11 vs 15; 16 vs 17) (Table 4.21) and (ii) *B. oleracea* vs. *B. cretica* (Table 4.22).

<u>Partial resistance vs. susceptible accessions</u>: On day 5, a significant reduction in overall, adult and nymph *B. brassicae* populations occurred in *B. brassicae* cohorts subject to either a mock, EPF or an EPF, EPF spray regime (irrespective of EPF isolate) on partially resistant *B. cretica* compared to susceptible *B. cretica* ([*B. brassicae* population subject to a EPF, mock spray regime on partially resistant and susceptible *B. cretica* accessions: total population: ~33 vs 45, nymph population: ~32 vs ~ 44] [*B. brassicae* population subject to a EPF, EPF spray regime on partially resistant and susceptible *B. cretica* accessions: total population: ~30 vs ~45, nymph population: ~28 vs ~43) (Table 4.21; Figure 4.9). There was significant difference in *B. brassicae* populations between resistant and susceptible *B. oleracea* accessions subjected to any of the three spray regimes (Figure 4.9; Table 4.21).

On day 10 a broadly similar trend in results was observed – with simple contrast tests revealing that significant differences between resistant and susceptible accessions were restricted to *B. cretica* accessions (with one exception, a significant difference between resistant and susceptible *B. oleracea* accessions following a mock, mock spray regime, with a total population of ~83 *B. brassicae* on the

partially resistant accession relative to ~108 on the more susceptible accession) (Table 4.19). The most consistent findings were significant differences in *B. brassicae* population between partially resistant and susceptible *B. cretica* accessions following both an EPF, EPF spray regime or a mock, mock spray regime (Table 4.22). A significant difference between *B. cretica* resistant and susceptible accessions following an EPF, mock spray regime was only observed within adult *B. brassicae* population on day 10 (with ~ 5 adults on the more partially resistant *B. cretica* accession relative to ~12 adults on the more susceptible accession) (Table 4.21). For spray regimes including a minimum of one EPF treatment (EPF, mock and EPF, EPF) and for which simple contrast tests revealed a significant difference in population, interaction plots revealed that this difference resulted from resistant accessions leading to significantly reduced *B. brassicae* populations relative to susceptible accessions (*B. brassicae* population on *B. cretica* partially resistant and susceptible accessions respectively under an EPF, EPF spray regime: Day 10 total population: ~5 vs ~14, Day 10 adult population: 0 vs ~1, Day 10 nymph population: ~5 vs ~13) (Table 4.21; Figure 4.9). However, where a significant difference was noted in *B. cretica* resistant/susceptible performance following a mock, mock treatment – interaction plots indicated this finding resulted from the susceptible accession significantly reducing *B. brassicae* population relative to the resistant accession (*B. brassicae* population on *B. cretica* partially resistant and susceptible accessions respectively under a mock, mock spray regime: Day 10 total population: ~85 vs ~60, Day 10 adult population: ~22 vs ~15, Day 10 nymph population: ~63 vs ~45) (Table 4.21; Figure 4.9) – a therefore contrary finding to the hypothesis that resistant accessions would universally lead to reduced *B. brassicae* populations.

<u>*B. oleracea* vs *B. cretica*:</u> Within the second set of simple contrast analyses on day 5 the most consistent significant contrast effect was between the susceptible *B. oleracea* (11) and *B. cretica* accessions (17) (Table 4.22). For overall population and nymph population this difference was evident on mock, mock sprayed and EPF, EPF sprayed cohorts (irrespective of EPF isolate) (Table 4.20). The results of interaction plots indicated that, following a mock, mock treatment, the susceptible *B. cretica* accession (17) significantly limits overall and nymph population size (~38 *B. brassicae* overall and ~32 nymphs) relative to the susceptible *B. oleracea* accession (15) (~57 *B. brassicae* overall and 54 nymph) (Figure 4.9). This trend was found to

be reversed in EPF, EPF treated cohorts (however on day 5 this cohort had only received the first EPF spray), with the susceptible *B. oleracea* accession (11) leading to a small but significant (p=<0.05) reduction in overall and nymph population (~35 *B. brassicae* overall and~33 nymphs) under this spray regime relative to the susceptible *B. cretica* accession (~45 *B. brassicae* overall and ~43 nymphs) (Figure 4.9). The trend for adult population was slightly different with differences being noted in the EPF, mock and EPF, EPF treated cohort (which at this experimental day can be considered equivalent owing to only the first spray having been applied at this time). In both cohorts, interaction plots revealed this difference stemmed from a significantly smaller *B. brassicae* adult population post spray on the susceptible *B. oleracea* accession (11) (~2-3 adults) relative to the susceptible *B. cretica* accession (17) (~4 adults) (Figure 4.9) although the effect size is small. This smaller adult population post EPF spray on accession 11 likely accounts for the observed lower nymph population on accession 11 relative to 17, with fewer surviving adults meaning fewer adults to reproduce between assay days 1 and 5.

On day 10 the contrasts underlying the interactions had notably shifted relative to day 5. For overall and nymph population, significant contrast differences were identified in both the mock, mock treated cohort on susceptible *B. oleracea* vs. *B. cretica* accessions (11 and 17) and in the EPF, EPF treated cohorts on resistant *B. oleracea* vs. *B. cretica* accession (15 and 16) (Table 4.22). Evaluation of the interaction plots (Figure 4.9) revealed in both cases this difference stemmed from a significant reduction in overall and nymph population on *B. cretica* accessions relative to *B. oleracea* accessions - indicating that in the absence of EPF treatment the susceptible *B. cretica* accession (17) had significantly reduced overall and nymph population relative to the susceptible *B. oleracea* accession (11). Similarly on the resistant *B. cretica* accession (16), following EPF treatment, the overall and nymph *B. brassicae* populations were significantly smaller relative to those on the resistant *B. oleracea* accession (15) (Figure 4.9; Table 4.22). For adult *B. brassicae* populations, this same effect of resistant *B. cretica* accession reducing population relative to the resistant *B. oleracea* accession following two EPF sprays was also observed (p=0.001) (Table 4.22). In contrast however, for adult populations on resistant *B. oleracea* and *B. cretica* accessions following a mock, mock treatment a different trend was observed, with the resistant *B. oleracea* accession in this case

leading to a significantly reduced adult *B. brassicae* population relative to on the resistant *B. cretica* accession (p=0.001). Similarly, for adult populations on susceptible *B. oleracea* and *B. cretica* accessions the susceptible *B. oleracea* accession led to a significantly reduced *B. brassicae* adult population relative to the susceptible *B. cretica* accession (p=0.041) (Figure 4.9; Table 4.22).

Table 4.18: Statistical output from three GLMMs assessing the main effects and interactions of EPF isolate, *Brassica* species, whether accessions were Resistant or Susceptible and spray regime (2x mock, 1x EPF 1x mock, or 2x EPF, 0x mock) upon overall *Brevicoryne brassicae* population (adults + nymphs) on days 5, 10 and 20 of a 20-day monitoring period. Populations started at a fixed point of 10 adult *B. brassicae* on day 1, within these being sprayed immediately with either a mock treatment of LC70 of 1.72 or 433.99. On bioassay day 5, mixed aphid population was then sprayed in-situ with either mock treatment, or EPF isolate 172 or 433.99. In all cases, spray regime was found to be the most significant main effect (P<0.001). Two 3-way interactions were noted at two of the three observed timepoints. Underlined numbers were significant findings while red numbers were not considered owing to no significant differences observed in primary analyses.

GLMM analysis		Statistic	Primary analyses			Secondary/supplementary analyses					
			Day5 Total	Day10 Total	Day20 Total	Day5 Adults	Day5 Nymphs	Day10 Adults	Day10 Nymphs	Day20 Adults	Day20 Nymphs
Model vs null		Sig. (P)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		F	3.859	25.641	27.075	18.501	2.72	12.11	23.35	16.61	27.75
		df	22, 95	22, 95	22, 95	22, 95	22, 95	22, 95	22, 95	22, 95	22, 95
Factors (Main effect)	EPF isolate	Sig. (P)	0.051	<0.001	<0.001	0.004	0.097	0.005	<0.001	<0.001	<0.001
		F	3.898	33.613	40.968	8.847	2.818	8.324	29.712	19.779	41.528
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1,95	1,95
	Brassica species	Sig. (P)	0.604	<0.001	<0.001	0.128	0.404	0.192	<0.001	<0.001	0.001
		F	0.271	25.408	14.983	2.363	0.704	1.723	34.444	13.839	12.227
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	Resistant/Susceptible	Sig. (P)	0.006	0.074	<0.001	0.031	0.013	0.726	0.129	0.005	<0.001
		F	7.804	3.266	22.775	4.802	6.44	0.123	2.347	8.393	28.493
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	Spray regime	Sig. (P)	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001
		F	19.101	226.682	231.458	201.087	7.534	101.18	196.666	102.158	225.255
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
2-factor interaction terms	EPF isolate x *Brassica* species	Sig. (P)	0.437	<0.001	<0.001	0.042	0.327	0.393	<0.001	0.001	<0.001
		F	0.608	15.258	19.07	4.256	0.972	0.735	13.255	11.647	18.35
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	EPF isolate x Resistant/Susceptible	Sig. (P)	0.121	0.61	0.876	0.447	0.102	0.350	0.608	0.802	0.780
		F	2.443	0.262	0.025	0.584	2.719	0.883	0.265	0.063	0.078
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	EPF isolate x Spray regime	Sig. (P)	0.106	<0.001	<0.001	0.008	0.188	0.009	<0.001	<0.001	<0.001
		F	2.295	25.25	34.267	5.031	1.699	4.924	22.97	12.968	33.872
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
	Brassica species x Resistant/Susceptible	Sig. (P)	0.539	0.613	<0.001	0.002	0.844	0.312	0.998	<0.001	<0.001
		F	0.381	0.257	15.522	9.871	0.039	1.034	0.001	13.196	16.277
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	Brassica species x spray regime	Sig. (P)	0.005	0.12	<0.001	0.326	0.005	0.461	0.09	0.004	<0.001
		F	5.517	2.166	14.662	1.133	5.602	0.781	2.47	5.876	14.399
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
	Resistant/susceptible x Spray regime	Sig. (P)	0.05	0.034	0.284	0.059	0.084	<0.001	0.097	0.067	0.365
		F	3.103	3.497	1.275	2.921	2.541	8.765	2.391	2.777	1.019
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
3-factor interaction terms	EPF isolate x *Brassica* species x Resistant/Susceptible	Sig. (P)	0.819	0.266	0.62	0.940	0.809	0.482	0.355	0.458	0.653
		F	0.052	1.252	0.248	0.006	0.058	0.498	0.862	0.555	0.203
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95
	EPF isolate x *Brassica* species x spray regime	Sig. (P)	0.521	<0.001	<0.001	0.082	0.540	0.697	<0.001	<0.001	<0.001
		F	0.657	11.806	21.962	2.572	0.619	0.363	11.143	10.285	20.126
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
	EPF isolate x Resistant/Susceptible x spray regime	Sig. (P)	0.387	0.816	0.752	0.259	0.405	0.343	0.812	0.764	0.744
		F	0.959	0.204	0.287	1.372	0.911	1.081	0.209	0.27	0.297
		df	0.204	2, 95	2, 95	1.081	0.209	2, 95	2, 95	2, 95	2, 95
	Brassica species x Resistant/Susceptible x spray regime	Sig. (P)	0.005	0.001	0.107	0.004	0.010	0.008	0.002	0.518	0.046
		F	5.51	7.697	2.285	5.831	4.804	5.116	6.501	0.663	3.174
		df	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95	2, 95
4-factor interaction term	EPF isolate x *Brassica* species x Resistant/Susceptible x spray regime	Sig. (P)	0.354	0.642	0.62	0.420	0.414	0.482	0.697	0.458	0.653
		F	1.048	0.446	0.248	0.876	0.891	0.498	0.362	0.555	0.203
		df	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95	1, 95

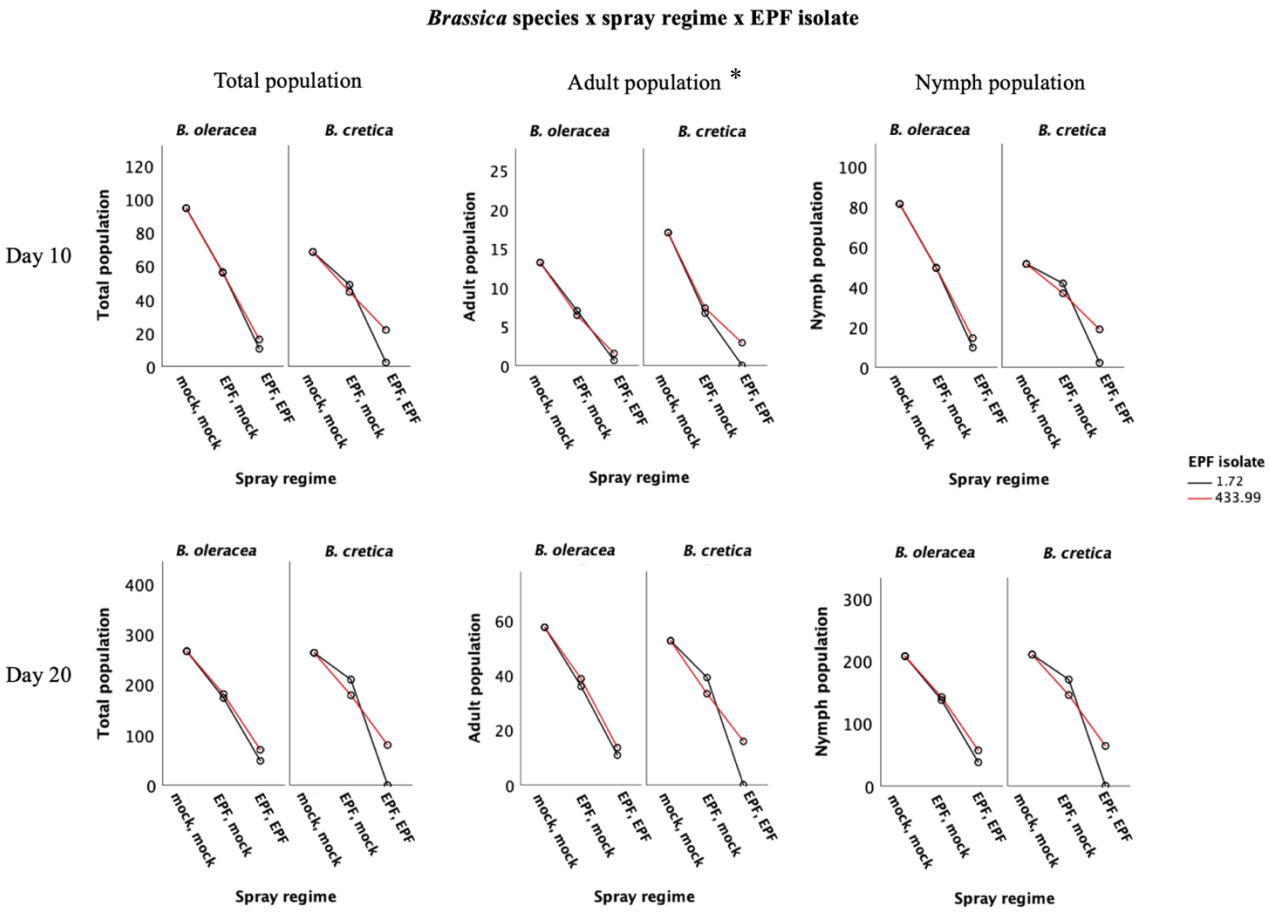


Figure 4.8: Interaction plots between EPF isolate, *Brassica* species and spray regime on population development assay days 10 and 20. GLMM analyses revealed a significant 3-way interaction for overall *Brevicoryne brassicae* population between these factors on these assessment days 10 and 20 (p=<0.001 in both cases) thus interaction plots were plotted to evaluate the nature of these interactions. Within this interaction, *Brassica* species appears to be moderating the interaction between EPF isolate and the regime with which EPF are sprayed – with EPF isolate 1.72 when sprayed twice appearing to result in a significantly reduced total *B. brassicae* population on *Brassica cretica* relative to *Brassica oleracea* accessions on both days 10 and 20 of assessment.

*GLMM analysis identified no significant three-way interaction between '*Brassica* species x spray regime x EPF isolate' for adult *B. brassicae* population alone on assay day 10.

Table 4.19: Interaction analysis simple contrasts tests result for the three-factor interaction term 'EPF isolate x *Brassica* species x spray regime', evaluating this interaction from the context of a contrast between EPF isolates 1.72 (*Akanthomyces dipterigenus*) and 433.99 (*Beauveria bassiana*) for effects on overall, adult and nymph *Brevicoryne brassicae* populations on assessment days 10 and 20. These tests demonstrated significant contrasts stemmed notably from difference between EPF isolates 1.72 and 433.99 following EPF, mock and EPF, EPF spray regimes on *Brassica cretica* and *Brassica oleracea* accessions. *Brassica* species appears to moderate this effect, with notably smaller *B. brassicae* population both overall and for adults and nymphs resulting from spraying aphids on *B. cretica* accessions relative to *B. oleracea* accessions (Figure 4.8).

Assay Day	Population	*Brassica* species	Spray regime	EPF isolate: Pairwise Contrasts	t	df	Adj. Sig.
10	**Overall population**	***B. oleracea***	**mock, mock**	**-**	**0**	**94**	**~1.000**
			EPF, mock	**1.72 vs 433.99**	**0.06**	**94**	**0.952**
			EPF, EPF	**1.72 vs 433.99**	**-2.408**	**94**	**0.018**
		B. cretica	**mock, mock**	**-**	**0**	**94**	**~1.000**
			EPF, mock	**1.72 vs 433.99**	**0.661**	**94**	**0.510**
			EPF, EPF	**1.72 vs 433.99**	**-7.284**	**94**	**9.83×10^{-11}**
	Adult population	*B. oleracea*	mock, mock	-	-5.74×10^{-14}	94	~1.000
			EPF, mock	1.72 vs 433.99	0.453	94	0.652
			EPF, EPF	1.72 vs 433.99	-1.832	94	0.07
		B. cretica	mock, mock	-	-1.36×10^{-14}	94	1.000
			EPF, mock	1.72 vs 433.99	-0.435	94	0.665
			EPF, EPF	1.72 vs 433.99	-4.444	94	2.42×10^{-5}
	Nymph population	*B. oleracea*	mock, mock	-	0	94	~1.000
			EPF, mock	1.72 vs 433.99	-0.026	94	0.979
			EPF, EPF	1.72 vs 433.99	-2.236	94	0.028
		B. cretica	mock, mock	-	6.21×10^{-15}	94	~1.000
			EPF, mock	1.72 vs 433.99	0.885	94	0.378
			EPF, EPF	1.72 vs 433.99	-7.014	94	3.51×10^{-10}
20	**Overall population**	***B. oleracea***	**mock,mock**	**-**	**-4.46×10^{-14}**	**94**	**~1.000**
			EPF, mock	**1.72 vs 433.99**	**-0.34**	**94**	**0.735**
			EPF, EPF	**1.72 vs 433.99**	**-2.654**	**94**	**0.009**
		B. cretica	**mock,mock**	**-**	**-6.50×10^{-15}**	**94**	**~1.000**
			EPF, mock	**1.72 vs 433.99**	**1.169**	**94**	**0.245**
			EPF, EPF	**1.72 vs 433.99**	**-9.626**	**94**	**1.11×10^{-15}**
	Adult population	*B. oleracea*	mock, mock	-	-2.89×10^{-14}	94	~1.000
			EPF, mock	1.72 vs 433.99	-0.54	94	0.591
			EPF, EPF	1.72 vs 433.99	-1.333	94	0.186
		B. cretica	mock, mock	-	-1.94×10^{-14}	94	~1.000
			EPF, mock	1.72 vs 433.99	1.087	94	0.28
			EPF, EPF	1.72 vs 433.99	-7.892	94	5.39×10^{-12}
	Nymph population	*B. oleracea*	mock, mock	-	2.28×10^{-14}	94	~1.000
			EPF, mock	1.72 vs 433.99	-0.288	94	0.774
			EPF, EPF	1.72 vs 433.99	-2.95	94	0.004
		B. cretica	mock, mock	-	2.14×10^{-14}	94	~1.000
			EPF, mock	1.72 vs 433.99	1.215	94	0.228
			EPF, EPF	1.72 vs 433.99	-9.848	94	4.44×10^{-16}

Table 4.20: Interaction analysis simple contrasts tests result for the three-factor interaction term 'EPF isolate x *Brassica* species x spray regime' evaluating this interaction from the context of a contrast between *B. oleracea* and *B. cretica* accessions for overall, adult and nymph *Brevicoryne brassicae* populations on assessment days 10 and 20. These tests demonstrated significant contrasts also resulted from between *Brassica* species differences following EPF, mock and EPF, EPF spray regimes on *Brassica cretica* and *Brassica oleracea* accessions. *Brassica* species appears to moderate this effect, with notably smaller *B. brassicae* population both overall and for adults and nymphs resulting from spraying aphids on *B. cretica* accessions relative to *B. oleracea* accessions (Figure 4.8).

Assay Day	Population	EPF isolate	Spray regime	*Brassica* species: Pairwise Contrasts	t	df	Adj. Sig.
10	**Overall population**	**1.72**	**mock,mock**	***B. oleracea* vs *B. cretica***	**2.634**	**94**	**0.010**
			EPF, mock	***B. oleracea* vs *B. cretica***	**1.109**	**94**	**0.270**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**5.302**	**94**	**7.55×10^{-7}**
		433.99	**mock,mock**	***B. oleracea* vs *B. cretica***	**2.634**	**94**	**0.010**
			EPF, mock	***B. oleracea* vs *B. cretica***	**1.69**	**94**	**0.094**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**-1.793**	**94**	**0.076**
	Adult population	1.72	mock, mock	*B. oleracea* vs *B. cretica*	-1.809	94	0.074
			EPF, mock	*B. oleracea* vs *B. cretica*	0.224	94	0.823
			EPF, EPF	*B. oleracea* vs *B. cretica*	2.301	94	0.024
		433.99	mock, mock	*B. oleracea* vs *B. cretica*	-1.809	94	0.074
			EPF, mock	*B. oleracea* vs *B. cretica*	-0.657	94	0.513
			EPF, EPF	*B. oleracea* vs *B. cretica*	-1.787	94	0.077
	Nymph population	**1.72**	mock, mock	***B. oleracea* vs *B. cretica***	3.687	94	<0.001
			EPF, mock	***B. oleracea* vs *B. cretica***	1.291	94	0.200
			EPF, EPF	***B. oleracea* vs *B. cretica***	5.192	94	1.20×10^{-6}
		433.99	mock, mock	***B. oleracea* vs *B. cretica***	3.687	94	<0.001
			EPF, mock	***B. oleracea* vs *B. cretica***	2.168	94	0.033
			EPF, EPF	***B. oleracea* vs *B. cretica***	-1.511	94	0.134
20	**Overall population**	**1.72**	**mock,mock**	***B. oleracea* vs *B. cretica***	**0.089**	**94**	**0.929**
			EPF, mock	***B. oleracea* vs *B. cretica***	**-1.451**	**94**	**0.150**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**9.992**	**94**	**2.22×10^{-16}**
		433.99	**mock,mock**	***B. oleracea* vs *B. cretica***	**0.089**	**94**	**0.929**
			EPF, mock	***B. oleracea* vs *B. cretica***	**0.092**	**94**	**0.927**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**-0.896**	**94**	**0.372**
	Adult population	**1.72**	mock, mock	***B. oleracea* vs *B. cretica***	0.687	94	0.494
			EPF, mock	***B. oleracea* vs *B. cretica***	-0.606	94	0.546
			EPF, EPF	***B. oleracea* vs *B. cretica***	7.933	94	4.41×10^{-12}
		433.99	mock, mock	***B. oleracea* vs *B. cretica***	0.687	94	0.494
			EPF, mock	***B. oleracea* vs *B. cretica***	1.04	94	0.301
			EPF, EPF	***B. oleracea* vs *B. cretica***	-0.877	94	0.383
	Nymph population	**1.72**	mock, mock	***B. oleracea* vs *B. cretica***	-0.099	94	0.921
			EPF, mock	***B. oleracea* vs *B. cretica***	-1.717	94	0.089
			EPF, EPF	***B. oleracea* vs *B. cretica***	9.988	94	2.22×10^{-16}
		433.99	mock, mock	***B. oleracea* vs *B. cretica***	-0.099	94	0.921
			EPF, mock	***B. oleracea* vs *B. cretica***	-0.177	94	0.860
			EPF, EPF	***B. oleracea* vs *B. cretica***	-0.871	94	0.386

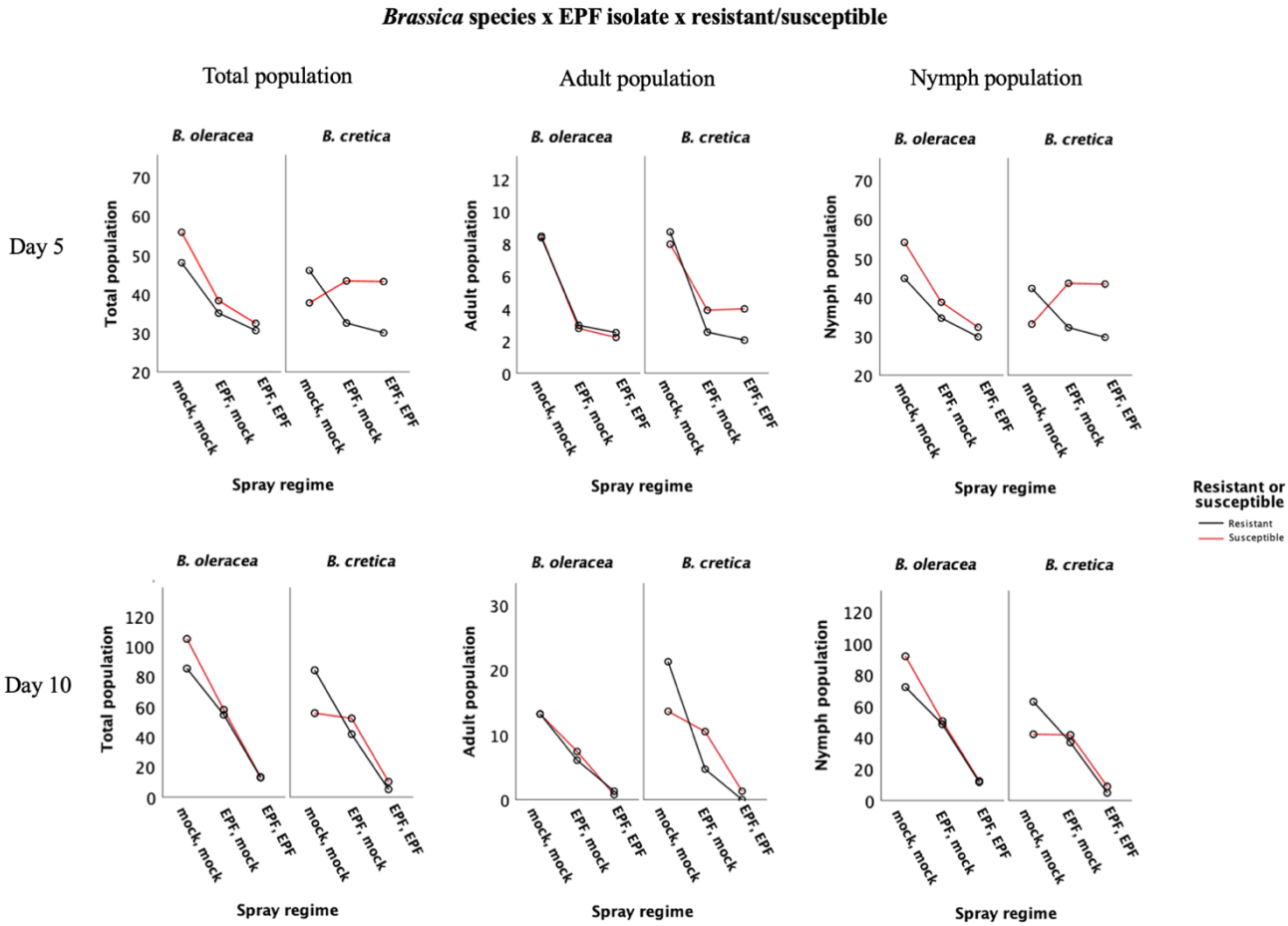


Figure 4.9: Interaction plots between *Brassica* species, spray regime and whether *Brassica* accessions were identified as partially resistant or susceptible to *Brevicoryne brassicae* on population development assay days 5 and 10. GLMM analyses revealed a significant 3-way interaction for overall *B. brassicae* population between these factors on these assessment days (p=0.005 and 0.001 respectively), thus interaction plots were plotted to evaluate the nature of these interactions.

Table 4.21: Interaction analysis simple contrasts tests result for the three-factor interaction term '*Brassica* species x resistant/susceptible x spray regime' for overall, adult and nymph *Brevicoryne brassicae* populations on assessment days 5 and 10. These tests demonstrated significant contrasts stemmed almost exclusively between partially resistant and susceptible accessions of *Brassica cretica* – with consistently significant differences noted between resistant and susceptible *B. cretica* accessions following an EPF, EPF spray regime.

Assay Day	Population	*Brassica* species	Spray regime	Partially resistant/susceptible: Pairwise Contrasts	t	df	Adj. Sig.
5	**Overall population**	***B. oleracea***	**mock, mock**	**Resistant vs susceptible**	**1.63**	**94**	**0.106**
			EPF, mock	**Resistant vs susceptible**	**0.812**	**94**	**0.419**
			EPF, EPF	**Resistant vs susceptible**	**0.521**	**94**	**0.604**
		B. cretica	**mock, mock**	**Resistant vs susceptible**	**-1.792**	**94**	**0.076**
			EPF, mock	**Resistant vs susceptible**	**2.537**	**94**	**0.013**
			EPF, EPF	**Resistant vs susceptible**	**3.146**	**94**	**0.002**
	Adult population	*B. oleracea*	mock, mock	Resistant vs susceptible	0.132	94	0.895
			EPF, mock	Resistant vs susceptible	-0.408	94	0.684
			EPF, EPF	Resistant vs susceptible	-0.706	94	0.482
		B. cretica	mock, mock	Resistant vs susceptible	-0.894	94	0.373
			EPF, mock	Resistant vs susceptible	2.538	94	0.013
			EPF, EPF	Resistant vs susceptible	3.787	94	<0.001
	Nymph population	*B. oleracea*	mock, mock	Resistant vs susceptible	1.691	94	0.094
			EPF, mock	Resistant vs susceptible	0.875	94	0.384
			EPF, EPF	Resistant vs susceptible	0.594	94	0.554
		B. cretica	mock, mock	Resistant vs susceptible	-1.79	94	0.077
			EPF, mock	Resistant vs susceptible	2.253	94	0.027
			EPF, EPF	Resistant vs susceptible	2.763	94	0.007
10	**Overall population**	***B. oleracea***	**mock, mock**	**Resistant vs susceptible**	**1.819**	**94**	**0.072**
			EPF, mock	**Resistant vs susceptible**	**0.476**	**94**	**0.635**
			EPF, EPF	**Resistant vs susceptible**	**0.113**	**94**	**0.91**
		B. cretica	**mock, mock**	**Resistant vs susceptible**	**-3.001**	**94**	**0.003**
			EPF, mock	**Resistant vs susceptible**	**1.621**	**94**	**0.108**
			EPF, EPF	**Resistant vs susceptible**	**2.364**	**94**	**0.02**
	Adult population	*B. oleracea*	mock, mock	Resistant vs susceptible	-0.018	94	0.985
			EPF, mock	Resistant vs susceptible	1.052	94	0.295
			EPF, EPF	Resistant vs susceptible	-1.116	94	0.267
		B. cretica	mock, mock	Resistant vs susceptible	-3.13	94	0.002
			EPF, mock	Resistant vs susceptible	3.98	94	<0.001
			EPF, EPF	Resistant vs susceptible	2.535	94	0.013
	Nymph population	*B. oleracea*	mock, mock	Resistant vs susceptible	2.14	94	0.035
			EPF, mock	Resistant vs susceptible	0.325	94	0.746
			EPF, EPF	Resistant vs susceptible	0.33	94	0.742
		B. cretica	mock, mock	Resistant vs susceptible	-2.884	94	0.005
			EPF, mock	Resistant vs susceptible	0.862	94	0.391
			EPF, EPF	Resistant vs susceptible	2.066	94	0.042

Table 4.22: Interaction analysis simple contrasts tests result for the three-factor interaction term '*Brassica* species x resistant/susceptible x spray regime' for overall, adult and nymph *Brevicoryne brassicae* populations on assessment days 5 and 10. These tests demonstrated significant contrasts stemmed almost exclusively between partially resistant and susceptible accessions of *Brassica cretica* – with consistently significant differences noted between resistant and susceptible *B. cretica* accessions following an EPF, EPF spray regime.

Assay Day	Population	Partially Resistant/ Susceptible	Spray regime	*Brassica* species: Pairwise Contrasts	t	df	Adj. Sig.
5	**Overall population**	**Susceptible**	**mock, mock**	***B. oleracea* vs *B. cretica***	**4.11**	**94**	**8.44×10^{-5}**
			EPF, mock	***B. oleracea* vs *B. cretica***	**-1.198**	**94**	**0.234**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**-2.691**	**94**	**0.008**
		Resistant	**mock, mock**	***B. oleracea* vs *B. cretica***	**0.435**	**94**	**0.665**
			EPF, mock	***B. oleracea* vs *B. cretica***	**0.663**	**94**	**0.509**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**0.204**	**94**	**0.839**
	Adult population	Susceptible	mock, mock	*B. oleracea* vs *B. cretica*	0.668	94	0.506
			EPF, mock	*B. oleracea* vs *B. cretica*	-2.292	94	0.024
			EPF, EPF	*B. oleracea* vs *B. cretica*	-3.717	94	<0.001
		Resistant	mock, mock	*B. oleracea* vs *B. cretica*	-0.413	94	0.681
			EPF, mock	*B. oleracea* vs *B. cretica*	0.794	94	0.429
			EPF, EPF	*B. oleracea* vs *B. cretica*	0.999	94	0.320
	Nymph population	Susceptible	mock, mock	*B. oleracea* vs *B. cretica*	4.26	94	4.85×10^{-5}
			EPF, mock	*B. oleracea* vs *B. cretica*	-0.929	94	0.356
			EPF, EPF	*B. oleracea* vs *B. cretica*	-2.306	94	0.023
		Resistant	mock, mock	*B. oleracea* vs *B. cretica*	0.526	94	0.600
			EPF, mock	*B. oleracea* vs *B. cretica*	0.569	94	0.571
			EPF, EPF	*B. oleracea* vs *B. cretica*	0.109	94	0.913
10	**Overall population**	**Susceptible**	**mock, mock**	***B. oleracea* vs *B. cretica***	**5.22**	**94**	**1.07×10^{-6}**
			EPF, mock	***B. oleracea* vs *B. cretica***	**0.853**	**94**	**0.396**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**1.327**	**94**	**0.188**
		Resistant	**mock, mock**	***B. oleracea* vs *B. cretica***	**0.111**	**94**	**0.912**
			EPF, mock	***B. oleracea* vs *B. cretica***	**1.883**	**94**	**0.063**
			EPF, EPF	***B. oleracea* vs *B. cretica***	**3.677**	**94**	**<0.001**
	Adult population	Susceptible	mock, mock	*B. oleracea* vs *B. cretica*	-0.235	94	0.815
			EPF, mock	*B. oleracea* vs *B. cretica*	-2.077	94	0.041
			EPF, EPF	*B. oleracea* vs *B. cretica*	-0.923	94	0.358
		Resistant	mock, mock	*B. oleracea* vs *B. cretica*	-3.312	94	0.001
			EPF, mock	*B. oleracea* vs *B. cretica*	1.097	94	0.275
			EPF, EPF	*B. oleracea* vs *B. cretica*	3.355	94	0.001
	Nymph population	Susceptible	mock, mock	*B. oleracea* vs *B. cretica*	6.289	94	1.00×10^{-8}
			EPF, mock	*B. oleracea* vs *B. cretica*	1.562	94	0.122
			EPF, EPF	*B. oleracea* vs *B. cretica*	1.655	94	0.101
		Resistant	mock, mock	*B. oleracea* vs *B. cretica*	1.076	94	0.285
			EPF, mock	*B. oleracea* vs *B. cretica*	1.905	94	0.060
			EPF, EPF	*B. oleracea* vs *B. cretica*	3.468	94	0.001

4.4. Discussion

In this chapter, four *Brassica* accessions identified in Chapter 2 (a partially resistant and susceptible accession of *B. oleracea* and *B. cretica*) and two EPF isolates (*A. dipterigenus* 1.72 and *B. bassiana* 433.99) selected in Chapter 3 were tested together against *B. brassicae*. The aim was to determine whether a combination of partial host plant resistance and entomopathogenic fungi resulted in significantly improved control of *B. brassicae*, either at an individual mortality or population development level, compared to using EPF on aphid-susceptible *Brassica* accessions. Two bioassay experiments were undertaken. The first experiment (4.2.2) was designed to quantify the effects of host plant resistance on EPF virulence. The second experiment (4.2.3) quantified the effects of combining host plant resistance and EPF on aphid population development. In both experiments, the EPF were applied at their respective LC_{70} concentrations, with this being a pragmatic choice. In principle, the LC_{50} is likely to be the concentration which may be most sensitive to the effects of host plant resistance, but it would also require a larger aphid sample size, limiting therefore the number of treatments that could be simultaneously assessed.

4.4.1. 'Susceptibility window' hypothesis for aphid nymph EPF susceptibility

Based on the evidence from several studies (James *et al.*, 2003; Kim and Roberts, 2012; Jandricic *et al.*, 2014) that aphid nymph intermoult period is a major limiting factor for the virulence of hypocrealean EPF (alongside supporting evidence in chapter 3), it can be hypothesised that for each nymph life stage there exists a 'susceptibility window' for successful EPF infection, i.e. the period when the time needed for EPF conidia to infect is shorter than the time until the next moulting event. There is also a 'pivot point' when the time needed to infect is equal to time to the next moulting event. After this point, any conidia applied to the insect will have insufficient time to successfully infect before being shed on the nymph cuticle (Figure 4.10).

The results from Chapters 2 and 3 gave indications about how partial host plant resistance and the particular EPF isolate could affect the aphid nymph susceptibility window to EPF infection, resulting in potentially significant impacts upon EPF virulence and *B. brassicae* population development. In Chapter 3 it was identified

that EPF isolate 1.72 (*A. dipterigenus*) was significantly more virulent to *B. brassicae* nymphs than isolate 433.99 (*B. bassiana*). Isolate 1.72 (*A. dipterigenus*) was also found to germinate and grow (on artificial media) much more rapidly than isolate 433.99 (*B. bassiana*). This suggests that the high virulence of isolate 1.72 (*A. dipterigenus*) to *B. brassicae* nymphs may result in part from it being able to more rapidly infect *B. brassicae* nymphs, i.e., the susceptibility window is for isolate 1.72 (*A. dipterigenus*) is intrinsically longer than for 433.99 (*B. bassiana*) (Figure 4.10).

In Chapter 2 (section 2.3.2.1) it was found that the partially resistant *B. cretica* accession (16) significantly increased the total time to aphid development by approximately 48 hours compared to the susceptible *B. cretica* accession (17) as well as the *B. oleracea* accessions 11 and 15. In retrospect the observation period used in the experiment (= once every 24 hours) may have been too long to statistically discriminate differences in intermoult period caused by plant genotype for instars 1 - 3. The partially resistant *B. oleracea* accession (15) caused a significant increase in instar 4 duration compared to the susceptible *B. oleracea* accession (11) (by approximately 8 hours), raising the prospect that further instar durations may also have been affected. Thus, it is possible that host plant resistance increases the susceptibility window for fungal infection (Figure 4.10). It is also possible that different combinations of *Brassica* accessions and EPF will result in different susceptibility windows (Figure 4.10).

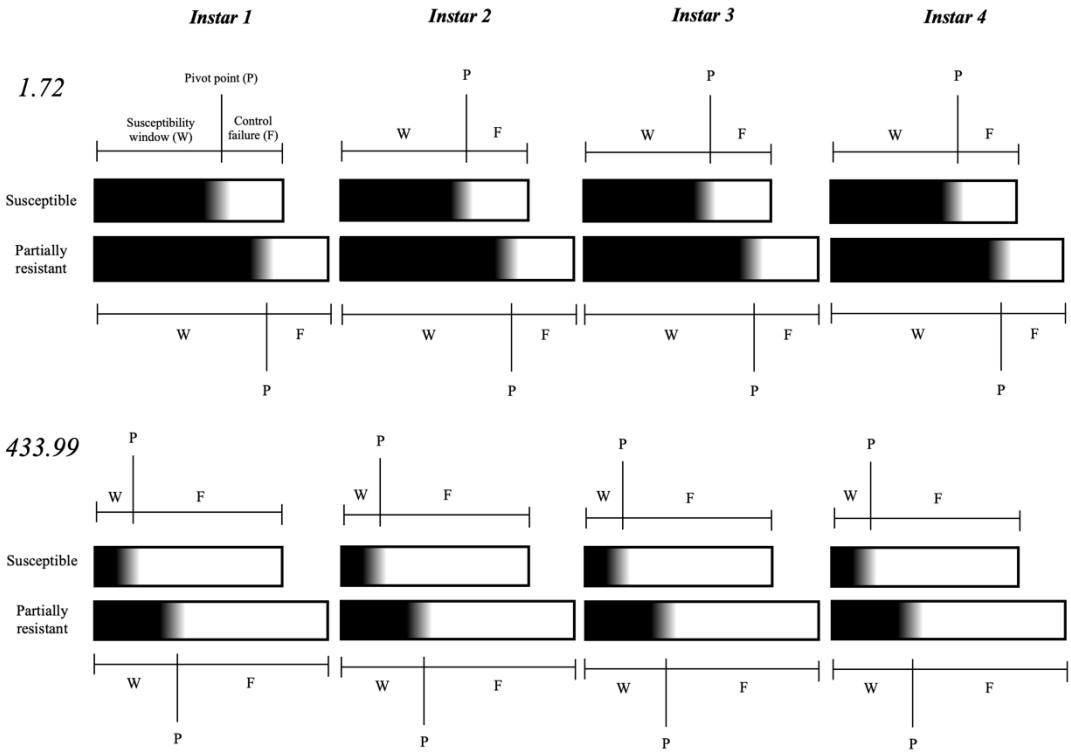


Figure 4.10: Concept diagram demonstrating the 'susceptibility window' for aphid nymphs to infection by contrasting EPF isolates and on plant accessions demonstrating different levels of partial host plant resistance and thus with different degrees of effect upon nymph intermoult periods. It was hypothesised that the faster virulence of isolate 1.72 (*Akanthomyces dipterigenus*) demonstrated in Chapter 3 stems from it infecting *B. brassicae* nymphs more quickly thus shifting the 'pivot point' between control success and failure to the right and therefore extending the nymph susceptibility window for successful infection relative to the slower and less virulent EPF isolate 433.99 (*Beauveria bassiana*). Owing to evidence in Chapter 2 demonstrating that accession 16 significantly extends the development time of *B. brassicae* nymphs relative to accession 17, it was hypothesised that this results from a cumulative increase in the duration of each instar phase, therefore extending the intermoult period. As the pivot point is likely determined primarily by the EPF isolate and is therefore relatively fixed, it can be assumed that partial resistance induced extensions of the intermoult period extend primarily the nymph susceptibility window.

The results from Chapter 3 showed that, while nymphs are more challenging to control using EPF, they are nevertheless more important targets than adult aphids because their control is likely to have a far more pronounced effect upon aphid population development by preventing reproduction of the next generation. EPF infection of adult aphids was found not to reduce their reproductive rate or total reproduction prior to mortality. Thus, on a per-aphid basis, the control of adults is likely to have much less of an effect upon *B. brassicae* population development. Unfortunately, the results of Chapter 2 suggest that EPF virulent to nymphs may be rare, with only one of the ten EPF isolates evaluated in this present study, 1.72 (*A.*

dipterigenus), resulting in high levels of nymph mortality. The relative rarity of EPF isolates virulent to aphid nymphs in combination with the small population-level impact of adult control may help explain the lack of success of EPF as aphid control agents to date. However, this could be remedied if partial host plant resistance could be deployed to increase the susceptibility of nymphs to EPF isolates.

While aphid nymphs are thus the main focus of this study, the data also provide indications that partial resistance could influence adult aphid EPF susceptibility. In Chapter 2 it was found that *B. cretica* caused a significant reduction in the average weight of adult aphids, which could affect EPF virulence by its effects on lethal dose, since in general smaller individuals require smaller doses (Prior *et al.*, 1995). There is also a possibility that morphological differences between the plant accessions (e.g., leaf hairs or cuticular waxes) could make the leaf surface microclimate more /less suitable for fungal infection (Fargues *et al.,* 2005; Jaronski, 2010).

Within these assays two EPF isolates were evaluated; 1.72 (*A. dipterigenus*), a likely aphid specialist isolate which was highly virulent to adult and nymph *B. brassicae,* and 433.99 (*B. bassiana*), a generalist isolate which only demonstrated significant virulence to *B. brassicae* adults. While the results from Chapters 2 and 3 suggested that partial host plant resistance might impact positively on 433.99 (*B. bassiana*) by increasing the aphid development time, it was not clear whether this would also apply to 1.72 (*A. dipterigenus*).

4.4.2. Adult *Brevicoryne brassicae*

In experiment 4.2.2, a 2-factor interaction between 'EPF isolate x *Brassica* species' had a significant effect on *B. brassicae* adult survival. This interaction resulted primarily from EPF isolate 1.72 (*A. dipterigenus*) causing lower adult survival on *B. oleracea* accessions relative to *B. cretica*, although the effect size was small. This was unexpected, as the experiments on the effects of *Brassica* accessions upon adult aphid development, reproduction and weight in Chapter 2 indicated that *B. cretica* (at a species level) was more resistant than *B. oleracea*. This is supported by studies against a range of other insect pests and plant pathogens in the literature (Ellis *et al.*, 1999; Happstadius *et al.*, 2008; Taylor *et al.*, 2017). One potential reason for this

finding may be due to physical phenotypic differences between these accessions. Both the partially resistant (16) and susceptible (17) accessions of *B. cretica* share an identical phenotype, with thick waxy flat leaves (Figure 4.11). In contrast *B. oleracea* accessions have less thick and waxy leaves, with the leaves of the more susceptible accession (11) demonstrating notably frilled/wavy leaf margins leading to a significantly more cupped leaf structure (Figure 4.11) (Gladman, A.K. observation). While not evaluated within this study, such differences in leaf phenotype could result in differences between leaf surface humidity owing to a potential microclimate effect upon temperature and/or humidity, with such an effect potentially resulting in environmental conditions closer to calculated EPF optima on *B. oleracea* accessions (Fargues *et al.*, 2005; Jaronski, 2010; Pincebourde and Woods, 2012). While this species-level effect is potentially also a factor within all further assays, the disagreement in the significance of this interaction between Cox and GLMM models indicates that the overall influence of this factor is likely to be small and thus likely to be outweighed by more significant effects. Nonetheless, these results do highlight a further component of the complex tritrophic interaction between host plants, aphid pests and insect pathogens – with leaf and plant phenotype and its relationship to the leaf surface microclimate being a potential influencer of EPF aphid treatment outcomes (Cuthbertson *et al.*, 2005; Ulusoy and Ölmez-Bayhan, 2006).

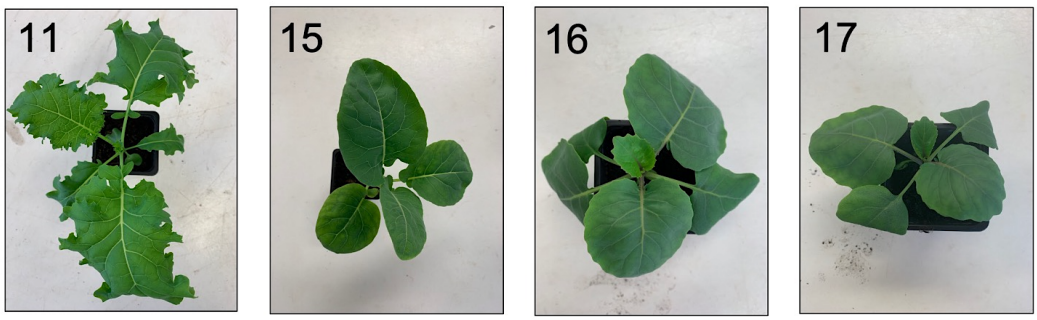


Figure 4.11: Variable phenotypes of the four *Brassica* accessions evaluated in combined experiments with entomopathogenic fungi against *Brevicoryne brassicae*. Labelled numbers correspond with the plant accession experimental numbers described in Table 2.2 – 11 and 15 being *B. oleracea* accessions and 16 and 17 being *B. cretica*. Plants shown at 5 weeks old (7 days for germination, 4 weeks growth in 20 ± 2 °C, 60% RH, L:D 16:8 h conditions) immediately prior to bioassay commencement.

4.4.3. *Brevicoryne brassicae* nymphs

Within the *B. brassicae* nymph assay of Experiment 4.2.2, a 3-factor interaction between 'EPF isolate x *Brassica* species x resistant/susceptible' had a significant effect on nymph survival. This interaction resulted from a decrease in *B. brassicae* nymph survival following treatment with 433.99 (*B. bassiana*) on more partially resistant *Brassica* accessions. This effect occurred (i) within *B. oleracea* (i.e., accession 15 vs. 11, where survival was ~35% lower on the partially resistant accession) and (ii) across *Brassica* species (i.e. *B. cretica* 16 and 17 *vs. B. oleracea* 11, where survival was ~50% lower on both *B. cretica* accessions relative to the more susceptible *B. oleracea* accession). Thus, it is likely that partial host plant resistance 'activated' the virulence of 433.99 (*B. bassiana*) to aphid nymphs. These findings are supported by published evidence that partial host plant resistance in bird cherry (*Prunus padus*) to bird cherry-oat aphids (*R. padi*), and in and oilseed rape (*B. napus*) to turnip aphids (*Lipaphis erysimi*), both operate in-part through effects on nymph development rate (Simon *et al.*, 1991; Amjad and Peters, 1992). A possible mechanism underlying these results is that through their effect on nymph intermoult period, partially resistant accessions (particularly accession 16, *B. cretica*) extended nymph intermoult periods (a maximum extension of intermoult period of 1.3 days was seen in Chapter 2, for instar 4 duration between accession 16, *B. cretica* and 2, *B. villosa* subsp. *tinei*), possibly allowing 433.99 (*B. bassiana*) conidia more time to germinate and infect nymphs and thereby acting by increasing the length of the nymph susceptibility window to EPF (Figure 4.12) (Butt and Goettel, 2000; Kim and Roberts, 2012).

In this assay EPF were applied to a first instar cohort ranging from 0-24hrs old, with a median age therefore of 12hrs. Owing to the likely slower speed of infectivity of isolate 433.99 (*B. bassiana*) than 1.72 (*A. dipterigenus*) (as suggested by the results of Chapter 3), the susceptibility window for isolate 433.99 (*B. bassiana*) nymph infection was always likely to be significantly shorter. From the results of this study, it is hypothesised that *B. oleracea* accession 15, through its partial resistance, resulted in a small increase in the intermoult period of first instars relative to accession 11, increasing the susceptibility to conidia of 433.99 (*B. bassiana*) (Figure 4.12). Interestingly, *B. oleracea* accession 11 still resulted in notably higher

mortality relative to the control treatment, suggesting that instar length was sufficient for some fungal infection.

The results of this experiment - along with slower germination of 433.99 (*B. bassiana*) relative to 1.72 (*A. dipterigenus*) (Chapter 3) - suggest that (i) aphid intermoult period at 20°C is likely an important limiter of 433.99 (*B. bassiana*) efficacy against *B. brassicae* nymphs, as the susceptibility window for infection is too short for EPF success and (ii) *Brassica* accessions that enable infection might do so by extending the intermoult period. While an increase in EPF virulence on partially or fully pest resistant crops has previously been noted by several authors (Tkaczuk *et al.*, 2007; Hatting *et al.*, 2010), this study marks both the first time that this effect has been linked directly with a partial resistance effect upon aphid biology and the first time such a phenomenon has been investigated for *B. brassicae* on *Brassica* plants. Hatting *et al.* (2010) did however note that plant resistance may also elicit a higher level of aphid activity and movement, with this leading potentially to great secondary conidial acquisition providing a further mechanism through which

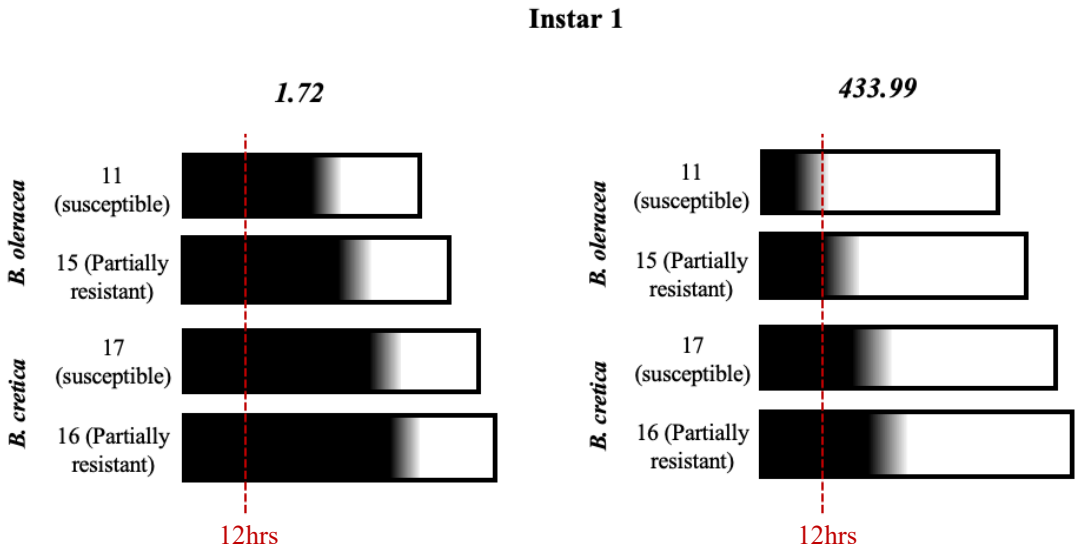


Figure 4.12: Concept diagram outlining the susceptibility window EPF to aphid nymphs subject to each EPF isolate x *Brassica* accession combination in experiment 4.2.2 and how this may relate to the observed virulence. In this experiment EPF conidia were applied to a fixed age cohort ranging in age from 1-24hrs, thus with a median age of ~12hrs as indicated in this figure with a red dashed line. The results of this study suggest that when applied to first instar nymphs of this age, isolate 1.72 (*Akanthomyces dipterigenus*) is highly virulent regardless of *Brassica* partial resistance. Isolate 433.99 (*Beauveria bassiana*) however, owing to its likely slower rate of nymph infectivity, is able to benefit from the effect of partial resistance extending the nymph susceptibility window, with this permitting significant 'activation' of 433.99 (*B. bassiana*) virulence on accessions 15, 16 and 17 relative to accession 11.

partial resistance and EPF integration may interact to improve EPF virulence (Hatting *et al.*, 2010).

Why was no difference observed in isolate 1.72 (A. dipterigenus) virulence on partially resistant vs. susceptible Brassica accessions?

In Chapter 3, isolate 1.72 (*A. dipterigenus*) caused significantly higher *B. brassicae* nymph mortality than 433.99 (*B. bassiana*) - which may be related isolate 1.72 (*A. dipterigenus*) being an aphid specialist (Safavi *et al.*, 2002; Faria and Wraight, 2007) and having faster rates of germination and growth as demonstrated in Chapter 3. These findings suggest a hypothesis that at 20°C, isolate 1.72 (*A. dipterigenus*) has a pivot point significantly later in the instar phase and thus a relatively longer susceptibility window than isolate 433.99 (*B. bassiana*) (Figure 4.10; 4.12). While no improvement for 1.72 (*A.* dipterigenus) virulence was noted in experiment 4.2.2, partial host plant resistance could still be beneficial for this EPF under field conditions, which are often suboptimal for EPF conidial germination and growth (Yeo *et al.*, 2003; Kendon *et al.*, 2021). This could occur, for example, if falling temperatures reduce the germination rate of isolate 1.72 (*A. dipterigenus*) relative to their effects on aphid development rate (Dampc *et al.* 2021) – thus effectively shifting the pivot point to the left and reducing the susceptibility window.

Why was no difference observed in the virulence of EPF isolate 433.99 (B. bassiana) against aphids feeding on partially resistant vs. susceptible accessions of B. cretica?

In experiment 4.2.2, isolate 433.99 (*B. bassiana*) caused the same high level of mortality for aphid nymphs feeding on *B. cretica* accession 16 (partially resistant) and accession 17 (susceptible) (~15-20%). It can be hypothesised that, through their effects upon *B. brassicae* nymphs, both *B. cretica* accessions elicited longer intermoult periods and thus extended equally the window of nymph susceptibility to 433.99 (*B. bassiana*) conidia (Figure 4.12). In Chapter 2, accession 16 (partially resistant) however was noted to significantly extend overall *B. brassicae* nymph time to adulthood (by ~2 days) relative to accession 17 (susceptible); thus, it was predicted that accession 16 would elicit significantly higher 433.99 (*B. bassiana*) virulence relative to accession 17. The results of this present bioassay suggest

therefore either an additional mechanism by which *B. cretica* accessions can increase EPF virulence to nymphs, or a more pronounced slowing of aphid intermoult period for nymphs on *B. cretica* accession 17 in this assay than was suggested in Chapter 2 section 2.3.2.1. With respect to further mechanisms, there is evidence in Chapter 2 that at a species level *B. cretica* accessions may demonstrate greater partial resistance than *B. oleracea* – with accessions 16 and 17 among the top performing accessions for their effects upon all evaluated aphid life history traits. Thus, while the mechanism of action of *B. cretica* partial resistance on EPF has yet to be fully ascertained, the observations made in this present assay do highlight possible further effects of plant resistance upon EPF virulence warranting further evaluation.

4.4.4. Can the integration of EPF and partial host plant resistance significantly limit *Brevicoryne brassicae* population development?

Having identified a significant interaction between partial host plant resistance and EPF virulence, resulting in a significant observable increase in *B. brassicae* nymph mortality following treatment with EPF isolate 433.99 (*B. bassiana*), a logical next step was to determine whether such an interaction could elicit a significant difference in *B. brassicae* population development. The key measure of success for conventional and IPM crop protection regimes is whether they are able to maintain pest populations below an economic damage threshold (Wearing, 1988). With this in mind, experiment 4.2.3 investigated the effects of combining host plant resistance and EPF on aphid population development, which was done by spraying EPF on two occasions (day 1 and day 5), with day 1 spray targeting fixed-age 10-day old adults, and day 5 spray targeting a mixed cohort including those adults which survived day 1 spraying (thus fixed-age 14-days old) and nymphs born by fixed age adults on days 1-5. No significant 2, 3 or 4-factor interactions were identified including both intraspecific resistance ('Resistant or Susceptible') and EPF isolate. Two 3-factor significant interactions were however identified:

- *Interaction 1: 'EPF isolate x Brassica species x spray regime'*: this indicates that the interspecific variation in partial resistance and its interaction with different EPF isolates and application regimes significantly influenced *B. brassicae* population development.

- *Interaction 2: 'Brassica species x resistant or susceptible x spray regime'*: this indicates that, irrespective of the particular EPF isolate, differences in partial resistance between and within *Brassica* species also elicited significant differences in aphid population size in ways that were dependent upon EPF spray regime (but irrespective of particular EPF isolate).

The focus of experiment 4.2.3 on population development made the interpretation of the results more complex. Within Chapter 2, accessions were screened for partial resistance by measuring their population development over a 2-week period from a fixed starting of 3 nymphs, with indications in Chapter 2 that this methodology may have been most sensitive to partially resistant accessions which acted primarily by significantly increasing *B. brassicae* nymph development time. Population development however is influenced also by variables such as adult reproduction rate, weight, (which were both evaluated in Chapter 2) and survival time (Dixon *et al.*, 1982; Wiktelius and Pettersson, 1985; Grüber and Dixon, 1988). The accessions categorised as 'partially resistant' did not consistently show the most significant effects on these metrics. Thus, there may be some inconsistency in categorising accessions as either 'partially resistant' or 'susceptible' with respect to aphid population development. For this reason, interactions (such as interaction 2) which include the binary 'resistant or susceptible' factor must be interpreted with caution. It should be remembered that the *Brassica* accessions in this study fall on a continuum for their partial resistance effects upon *B. brassicae* development time and other metrics.

In Chapter 2, the most consistent differences in *B. brassicae* biology were noted between plant species, with *B. cretica* accessions 16 and 17 significantly limiting adult reproduction, weight, and development rate (particularly for accession 16) relative to *B. oleracea* accessions. In some respects, labelling *B. cretica* 16 as 'partially resistant' and *B. cretica* 17 as 'susceptible' was something of a false choice, with both eliciting more significant effects on *B. brassicae* biology relative to the majority of *B. oleracea* accessions (with accessions 16 and 17 ranked first and second on the partial resistance continuum of these four accessions). Owing to this, it was predicted that *B. cretica* accessions overall would have a more significant effect on *B. brassicae* population development than *B. oleracea* accessions, and thus had

the potential to interact with EPF to more significantly influence *B. brassicae* population. Owing to these issues, interaction 1 was considered primary, with interspecific differences in resistance likely having greater biological significance.

4.4.5. Interaction 1: 'EPF isolate x *Brassica* species x spray regime'

Through analysis of this interaction, two important findings were identified. These are discussed separately:

(i) In experiment 4.2.3, two spray applications of EPF isolate 1.72 (*A. dipterigenus*) resulted in a significantly smaller *B. brassicae* populations compared to aphids sprayed twice with 433.99 (*B. bassiana*). This effect was more pronounced on *B. cretica* relative to *B. oleracea*.

As no significant differences were identified in the day 10 or 20 population of aphid cohorts receiving only one EPF spray (on experimental day 1 which targeted adults alone), this finding likely stemmed from the significantly higher nymph virulence of isolate 1.72 (*A. dipterigenus*) relative to 433.99 (*B. bassiana*). Very little is known about the relative susceptibility of aphid adults vs. nymphs to hypocrealean EPF, but the available evidence indicates that these EPF in general perform relatively poorly against aphid nymphs (Kim and Roberts, 2012; Jandricic *et al.*, 2014). The exception appears to be 1.72 (*A. dipterigenus*). Despite being a purported aphid specialist, beyond this thesis only a handful of studies have looked at the virulence of isolate 1.72 (*A. dipterigenus*) against nymphs (Safavi *et al.*, 2002; Faria and Wraight, 2007). To the best of our knowledge, this project is the first study to demonstrate how a significant difference in virulence to aphid nymphs between two EPF isolates (1.72 and 433.99) can influence aphid population development. In the context of the proposed 'susceptibility window' hypothesis, this result possibly stemmed from the faster germination and growth of 1.72 (*A. dipterigenus*), relative to 433.99 (*B. bassiana*) (Figure 4.10). On day 5, at the point where the second spray was applied, nymphs in the mixed age cohort ranged from 0-96hrs old with a median age therefore of ~48hrs. It is likely that in this population, nymphs were on average closer to moulting, thus conidia would have less time to infect prior to moulting. While the faster rate of infectivity of isolate 1.72 (*A. dipterigenus*) could likely still allow for a significant number of lethal infections, the nymph susceptibility window

for isolate 433.99 (*B. bassiana*) may not have been sufficient for infection (Figure 4.13)

Aphids feeding on *B. cretica* showed greater susceptibility to two sprays of 1.72 (*A. dipterigenus*) than aphids feeding on *B. oleracea*. While this may be associated with the negative effects of *B. cretica* on aphid weight and reproduction, it could also be related to the difference in aphid development rate on *B. cretica* vs. *B. oleracea.* Owing to the older average age of nymphs sprayed in experiment 4.2.3 (relative to 4.2.2), it is possible that a larger proportion of nymphs were sprayed at a timepoint closer to the next nymph moulting event, in which case an increase of intermoult period *by B. cretica* would aid fungal infection (Figure 4.13).

The results of chapter 2 nonetheless demonstrate significant further effects of *B. cretica* partial resistance relative to *B. oleracea*. From a population development perspective, it may be important that partially resistant accessions limit multiple aspects of aphid biology simultaneously. Further research is necessary to identify which effect of partial resistance may be the most important for limiting aphid populations. The results of this assay suggest that there would be significant value in running a new, larger *Brassica* partial resistance screening programme to identify *B. oleracea* accessions with partial resistance effects equivalent to *B. cretica*, with such accessions both more likely to demonstrate favourable interactions with isolate 1.72 (*A. dipterigenus*) and possibly also 433.99 (*B. bassiana*), and potentially of significant value for plant breeding programmes.

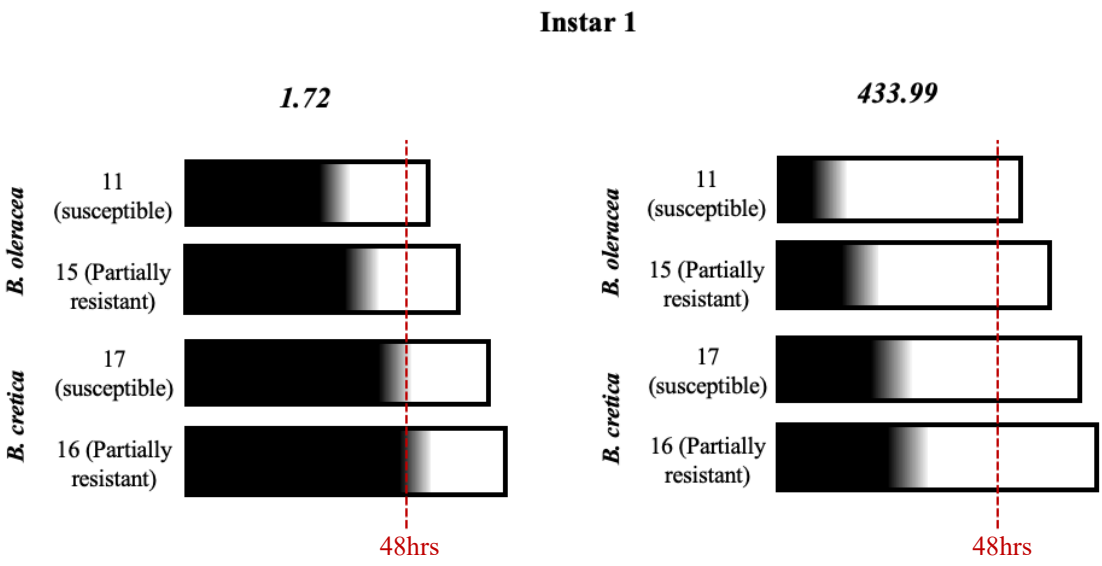


Figure 4.13: Concept diagram outlining the likely susceptibility window for EPF to aphid nymphs subject to each EPF isolate x *Brassica* accession combination and how this may relate to the virulence of each isolate in experiment 4.2.3 and observed differences in *Brevicoryne brassicae* population development. In this experiment, the second spray of EPF conidia on assay day 5 was applied to a mixed adult and nymph cohort, with nymphs ranging in age from 0-96hrs, thus with a median age of ~48hrs as indicated in this figure with a red dashed line. While in this experiment it is likely the proportion of nymphs at each age relative to the nymph susceptibility window for successful EPF infection (as dictated by both the level of *Brassica* partial resistance and the particular EPF isolate) that dictates virulence and thus likely *B. brassicae* population development, consideration of just the median age of nymphs allows for the results of this study to be contextualised more simply, with such an approach potentially explaining why interspecific partial resistance only appeared to improve the performance of EPF isolate 1.72 (*Akanthomyces dipterigenus*) and why isolate 1.72 overall appeared to result in a more significant effect upon *B. brassicae* population relative to isolate 433.99 (*Beauveria bassiana*).

(ii) In experiment 4.2.2. host plant resistance significantly increased the virulence of EPF isolate 433.99 (*B. bassiana*) to *B. brassicae* nymphs. However, in experiment 4.2.3 no significant difference in *B. brassicae* population was noted when 433.99 (*B. bassiana*) was used with *B. oleracea* or *B. cretica* accessions.

This observation may stem from the differences in the nymph cohorts that were sprayed between assays 1 and 2 as described previously. It is possible that the majority of the nymph population in experiment 4.2.3 were closer to their next moulting event relative to 4.2.2. As was suggested by the results of 4.2.2 and Chapter 3 section 3.3.9, the likely slower rate of infectivity of isolate 433.99 (*B. bassiana*) means that the nymph susceptibility window for 433.99 (*B. bassiana*) is

smaller than for 1.72 (*A. dipterigenus*). The results of this study therefore suggest that when 433.99 (*B. bassiana*) is applied to a population with a higher proportion of individuals closer to moulting, the levels of partial resistance between *B. cretica* and *B. oleracea* are unable to extend the intermoult period sufficiently for infection (Figure 4.13).

There may exist further mechanisms underlying these differences in population development. In all prior experiments undertaken on nymphs in this thesis, EPF have been assessed on 1-day old first instars, with this instar phase selected owing to literature indications that earlier instars pose the most significant control challenge (Kim and Roberts, 2012). However, despite evidence that the duration of *B. brassicae* instar phases 1-3 are roughly equal (Hughes, 1963) there remains the possibility that instar phases 2 and 3 (of which some of the 0-96hr old nymphs will be) may simply be more challenging for EPF control than instar phase 1. Further research is thus necessary evaluating the virulence of EPF to nymphs of each instar, with evidence in the literature to suggest significant differences in gene expression between instar phases which may elicit differences in aphid immunity (de Vos *et al.*, 2010; Ayyanath *et al.*, 2015). It is also possible that the effect of partial resistance on different aspects of aphid nymph biology such as size and weight may also be important, with this influencing whether nymphs receive a lethal dose or not (Prior *et al.*, 1995).

4.4.6. Interaction 2: '*Brassica* species x resistant or susceptible x spray regime'

While the results of this study were primarily considered in the context of interaction 1, several further insights into this overall interactive system were identified through consideration of interaction 2. The primary finding of this second interaction was that on the partially resistant *B. cretica* accession (16), on day 5 following an EPF single spray of adult aphids there was a significant decrease in overall, adult and nymph populations relative to EPF sprayed cohorts on the susceptible *B. cretica* accession (17) - indicating that the resistant *B. cretica* may have both increased the susceptibility of adult *B. brassicae* to EPF and limited reproduction of surviving adults. The first effect was unexpected, with all previous assays indicating significantly high adult mortality following EPF treatment regardless of *Brassica* accession and its level of partial resistance. This finding suggests that while *Brassica*

cretica may not have resulted in significantly lower survival in the first assay, from a population development context *B. cretica* may induce sufficient differences in *B. brassicae* EPF susceptibility and reproduction to result in a significantly reduced population. This finding was further identified on day 10 of the assay, being reflected in significantly lower overall, adult and nymph populations. This finding therefore warrants further evaluation of how partial resistance effects on adult EPF susceptibility and reproduction may impact aphid population development.

4.4.7. Conclusion and future work

The results of these two assays demonstrate the potential benefits of integrating partial host plant resistance and entomopathogenic fungi. Within the first assay, partial resistance significantly increased the virulence of a generalist EPF isolate (433.99, *B. bassiana*) to a fixed-age cohort of *B. brassicae* nymphs, with ~45-50% greater nymph mortality on partially resistant *Brassica* accessions. Partial resistance therefore permitted a relatively nymph avirulent generalist EPF isolate (433.99, *B. bassiana*) to match the nymph virulence of an aphid-specialist EPF isolate (1.72, *A. dipterigenus*).

While this improvement in isolate 433.99 (*B. bassiana*) performance was not reflected in population development within the second assay, the second assay in this chapter nonetheless confirmed that partial resistance can lead to a significantly reduced *B. brassicae* population when combined with 1.72 (*A. dipterigenus*). This isolate significantly reduced aphid populations relative to the generalist as 433.99 (*B. bassiana*).

Based on the results of this study, significant further research is warranted evaluating both the proposed 'susceptibility window' hypothesis as well as the combined deployment of partial host plant resistance with EPF and the nature of their interactions:

- With respect to the proposed 'susceptibility window' hypothesis:
  - Firstly, while there is evidence that EPF isolate 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) differ in their nymph virulence and rate of germination/growth, this needs corroborating on living nymph cuticles,

with an experiment treating nymphs with EPF and directly monitoring the infection process over time.

- The duration of nymph instar phases on a range of plant accessions needs to be known with greater precision. To gather data at a sufficiently fine scale will likely require a new methodology, with video recordings of nymphs during their entire development process which can then be analysed to determine the exact time to moult likely the optimal method to assess this. Owing to likely variation between individual nymphs, it would also be beneficial to expand the replication.
- With such detailed information about EPF time to infection and intermoult periods, an assay evaluating fixed-age nymph virulence when treated at a significantly shorter time scale (e.g., at hourly intervals over a 24hr period) would then allow for more precise determination of whether such a susceptibility window and pivot point for EPF virulence exist for each instar phase and, if so, to characterise them and how they differ dependent upon the level of partial resistance, EPF isolate and environmental conditions.

- There is significant scope for further research within the *Brassica* partial resistance x EPF x *B. brassicae* system.
  - Firstly, using this existing system of EPF isolates and *Brassica* accessions, there would be value in repeating the first assay to see if adults and nymphs could be killed at a lower EPF dose of isolate 1.72 (*A. dipterigenus*), since this would be valuable for field control (Fournier and Brodeur, 2000).
  - The population development assay could be developed further, starting with a fixed age nymph cohort of *B. brassicae* on the different *Brassica* accessions and evaluating how a single spray with EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*), done on different days, affects population development. This would allow for the interaction between EPF and partial resistance and its relationship with population development to be better explored.
  - To fully evaluate the interaction between partial host plant resistance and EPF, the work described in this chapter must be taken as a proof of

concept and a starting point rather than a conclusion. Beyond this project, to evaluate this interaction further and fully will require the identification of a wider range of partially resistant *Brassica* accessions, preferably with partial resistance affecting *B. brassicae* development rate, reproduction, and fitness in different ways. This could allow the relative important of each of these effects to be investigated. There would also be benefit in screening and identifying a wider range of EPF such that EPF with different levels of virulence to adult and nymph *B. brassicae* and with different speeds of infectivity can be evaluated.

5. General Discussion

Despite international policy commitments to promote the use of IPM (European Parliament, 2009), farmers and growers in the UK and elsewhere still rely heavily on synthetic chemical insecticides for outdoor crops, and the use of alternative methods has yet to become mainstream (van Lenteren, 2000; Chandler *et al.*, 2008). A major impediment to IPM adoption has been a lack of a holistic science of IPM, with little mechanistic understanding of how different plant protection methods work together, particularly biologically based controls (Stenberg, 2017). This means that IPM systems used in commercial practice are often developed on an *ad hoc* basis, with the success of any strategy reliant more upon intuition than scientific evidence. Research into how different IPM elements interact is therefore urgently needed in order to identify beneficial synergistic (i.e., mutually reinforcing) effects and to eliminate antagonistic IPM pairings (Stenberg, 2017; Deguine *et al.*, 2021). Owing to the wide variety of IPM tools available to growers, studying the multifactorial interactions between all elements is likely to be intractable. However, a manageable starting point is to investigate the combined effects of just two IPM elements. In his conceptual framework for IPM, Stenberg (2017) highlighted seven key interactions which if characterised could allow for significant improvements in IPM strategies (Stenberg, 2017). Of these, the effects of combining heritable plant resistance and biological controls can be considered to be particularly important because of the potential for bidirectional interactions (Stenberg, 2017).

The aim of this study was to investigate the effects of combining partially resistant *Brassica* accessions and EPF against the cabbage aphid (*B. brassicae*). While partial resistance and EPF have individually both shown potential for vegetable *Brassica* protection from aphid pests (Ellis *et al.*, 1998; Prince and Chandler, 2020), to date neither have been deployed commercially (Cowger and Mundt, 2002; Stall *et al.*, 2009; Caffier *et al.*, 2016; Pilet-Nayal *et al.*, 2017). This project sought to: (i) Identify partially resistant *Brassica* accessions and characterise their effects on *B. brassicae;* (ii) identify EPF virulent to *B. brassicae* adults and nymphs; and (iii) determine and quantify any interactions between partial resistance and EPF on *B. brassicae* survival and population development. The research produced several novel findings and has implications for the development of future, holistic IPM systems, particularly with respect to plant breeding for partial pest resistance.

Brassica partial antibiosis resistance to *B. brassicae* was identified in this study, with the most resistant accessions limiting *B. brassicae* populations (over a 2-week period) by approximately one third relative to the most susceptible accessions. *Brassica* accessions partially resistant to *B. brassicae* have been identified before (Singh *et al.,* 1994; Ellis *et al.*, 1995, 1996 1998, 2000; Ellis and Kift, 2003; Munthali, 2009), but this is the first study to characterise their effects on a range of aphid life history traits (time to development, adult weight, and reproduction). Partial resistance acts by limiting the pest's realised biotic potential (i.e. its maximum reproductive capacity at a given set of conditions and resources) (Gatehouse, 2002; Dogimont *et al.*, 2010). Quantifying the effects of partially resistant plants on the individual life history traits that contribute to biotic potential is an important first step towards understanding their mechanism of action. Significant differences were observed in *B. brassicae* development rate (overall, and instar 4), weight during development (highest weight reached and mean relative growth rate) and reproduction (daily/gross over an 8-day period, intrinsic rate of increase and population doubling time) on different *Brassica* accessions. Overall time to development was the main explanatory variable for partial resistance, accounting for 54% of the observed experimental variation. *Brassica cretica* accessions showed the most significant overall effects, suggesting that *B. cretica* may be a useful source of partial *B. brassicae* resistance as a wild crop relative (Ramsey and Ellis, 1994; Shuhang *et al.*, 2016). These results agree with other studies which have previously demonstrated that that partial crop resistance can have significant effects upon aphid development rate, reproduction, and longevity in a range of different pest/crop combinations (e.g., pea aphid, *Macrosiphum pisi*, on *Pisum sativum* L., and bird cherry-oat aphid, *Rhopalosiphum padi* L., on *Triticum monococcum* L. and *Triticum aestivum* L.) (Harrington, 1941; Greenslade *et al.,* 2016; Singh *et al.*, 2020; Simon *et al.*, 2021). Two *B. oleracea* accessions (11 and 15) and two *B. cretica* accessions (16 and 17) were selected for combined assessment with EPF. These accessions showed significant differences in their effects on *B. brassicae* both within and between each species. Within-species effects occurred on *B. brassicae* development time, while between-species effects occurred on development time, aphid weight and reproduction.

Ten EPF isolates from five species were evaluated for their virulence to *B. brassicae* adults and nymphs. The optimal temperatures for growth (23.6-25.2ºC) and germination (24.4-25.0ºC) were estimated for all isolates using a non-linear model (Ratkowsky *et al.*, 1983; Zwietering *et al.*, 1991; Omuse *et al.*, 2021). UK EPF isolates tended to have lower optimal temperatures compared to isolates from elsewhere, which may demonstrate adaptation to local (i.e., UK) conditions, as has been suggested in other studies of EPF isolated from warmer vs. cooler regions (Bidochka *et al.,* 1998; Scholte *et al.*, 2004; Kryukov *et al.*, 2012; Klingen *et al.*, 2015). All ten isolates were pathogenic to adult *B. brassicae* when treated with 2ml of a 1 x 10^7 conidia ml^{-1} solution (resulting in ~50-95% mortality) with isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) giving the highest overall mortality (~90-95%). With one exception, nymphs were much less susceptible to EPF infection than adult aphids. Isolate 1.72 (*A. dipterigenus*) however caused 100% nymph mortality, whereas the other isolates gave <30% mortality. These findings confirm that under favourable environmental conditions EPF can be highly virulent against aphid adults (Hall and Burges, 1979; Fournier and Brodeur, 2000; Derakhshan *et al.*, 2007; Farag, 2008; Asi *et al.*, 2009ab; Akbari *et al.*, 2013; Jandricic *et al.*, 2014; Lee *et al.*, 2015; Chandler, 2017; Ramanujam *et al.*, 2017; Soleymadzade *et al.*, 2019; Prince and Chandler, 2020; Gebreyohans *et al.*, 2021) and support the proposal that aphid nymphs pose a significantly greater challenge for EPF based control (Butt and Goettel, 2000; Kim and Roberts, 2012; Jandricic *et al.*, 2014). This low susceptibility has been suggested to stem from EPF conidia being removed during aphid moulting, with the relationship between nymph intermoult period and the speed at which conidia can germinate, grow, and penetrate the cuticle being an important determinant of EPF efficacy (Kim and Roberts, 2012).

EPF isolate 1.72 (*A. dipterigenus*) was previously sold as the bio-aphicide - Vertalec®, however surprisingly few studies have been undertaken to specifically evaluate its virulence of this EPF isolate to aphid nymphs (Milner, 1997; Askary *et al.*, 1998; Safavi *et al.*, 2002; Faria and Wraight, 2007). 1.72 (*A. dipterigenus*) demonstrated far greater virulence to nymphs than 433.99 (*B. bassiana*), which is sold as the biopesticide Botanigard®, which required ~100x, ~200x and ~2000x the concentration of 1.72 (*A. dipterigenus*) to produce 50, 70 and 90% nymph mortality respectively. Artificial media studies indicated that 1.72 (*A. dipterigenus*) virulence

to nymphs may stem partly from it germinating and growing faster than 433.99 (*B. bassiana*), suggesting that a key adaptation for aphid specialist isolates may be rapid growth and germination to circumvent aphid defence by moulting. EPF isolates 1.72 (*A. dipterigenus*) and 433.99 (*B. bassiana*) were selected for combined assessment with partially resistant *Brassica* accessions.

The effects of combining partial host plant resistance and EPF were evaluated in two different laboratory bioassay experiments using EPF applied at their respective LC_{70} concentrations. Experiment 1 (a simple, speed of kill assay) measured the survival separately of aphid adults and nymphs feeding on different *Brassica* accessions and treated with a single spray of EPF. Experiment 2 measured the development of an aphid population produced by 10-day old adults feeding on different *Brassica* accessions and given either one or two consecutive EPF sprays. Statistically significant interactions occurred between factors depending on the experiment:

- In experiment 1, there was a 2-factor interaction between '*EPF isolate x Brassica species'*, resulting primarily from isolate 1.72 (*A. dipterigenus*) causing lower adult survival on *B. oleracea* accessions relative to *B. cretica*. There was also a 3-factor interaction between '*EPF isolate x Brassica species x resistant/susceptible*', caused by a decrease in *B. brassicae* nymph survival following treatment with isolate 433.99 (*B. bassiana*) on more partially resistant *Brassica* accessions.
- The results of experiment 2 were more complex, reflecting the nature of the experiment. There were no statistically significant 2, 3 or 4-factor interactions including both 'intraspecific resistance' and 'EPF isolate'. There was however a 3-factor interaction between *'EPF isolate x Brassica species x spray regime'*, i.e., interspecific variation in partial resistance and its interaction with EPF isolate and spray regime significantly influenced *B. brassicae* population development. A second, 3-factor interaction occurred between *'Brassica species x resistant or susceptible x spray regime'*, i.e., irrespective of the particular EPF isolate, differences in partial resistance between and within *Brassica* species elicited significant differences in aphid

population development in ways that were dependent upon EPF spray regime.

Thus, interactions can occur between host plant genotype and EPF depending on the context in which they are used, including the presence of other experimental factors. This has important implications for biocontrol of *B. brassicae* using host plant resistance and EPF, and may have wider implications for other IPM systems:

IPM systems for aphids must target the most important pest life stages.

- It is clear from this study that the primary targets for host plant resistance and EPF are aphid nymphs rather than adults, suggesting that future work should focus on nymph control. Although adult aphids were susceptible to all the EPF isolates tested in this study, there was no effect of fungal treatment on their reproduction. Most EPF isolates had low virulence to nymphs, meaning that they would be unable to prevent aphid population increase. The exception was isolate 1.72 (*A. dipterigenus*), which had high virulence to aphid nymphs regardless of the host plant genotype.

Selection of partially resistance Brassica genotypes for plant breeding programmes should consider the use of EPF (and other biocontrol agents) in IPM.

- In the Brassica accession screen done in Chapter 2, aphids showed the lowest population development when feeding on *B. cretica* compared to *B. oleracea*.
- In the simple speed of kill bioassay done in Chapter 4, use of partial plant resistance enabled EPF 433.99 (*B. bassiana*) to kill significantly more aphid nymphs, which in contrast were far less susceptible to this pathogen when feeding on aphid-susceptible plants. The size of the effect was such that isolate 433.99 (*B.* bassiana) caused the same levels of nymph mortality as 1.72 (*A. dipterigenus*). Thus, in this case, host plant resistance acted as an 'enabler/activator' for using an EPF biopesticide that might otherwise be discounted. However, in the more complex population development experiment of Chapter 4, using a mixed-age aphid group, host plant resistance did not result in an improvement in 433.99 (*B. bassiana*)

virulence. This indicates that aphid population makeup is an important variable for the 'enhancement' of 433.99 (*B. bassiana*) nymph virulence.

- In the same experiment, two spray applications of 1.72 (*A. dipterigenus*) resulted in a significantly smaller *B. brassicae* population compared to aphids sprayed twice with 433.99 (*B. bassiana*). This effect was more pronounced on *B. cretica* relative to *B. oleracea*. This finding indicates that against more complex nymph cohorts even the virulence of otherwise significantly nymph-virulent isolates can fall, through the combination of isolate 1.72 (*A. dipterigenus*) with more partially resistant *Brassica* accessions however, some of this loss of virulence can be mitigated.
- Taken as a whole, the results of this study show that partial plant resistance can impact positively on the virulence of EPF, depending on the age profile of the target aphid population and the fungal isolate. Thus, in principle, it would be possible to breed new *Brassica* crop lines that improve aphid biocontrol with EPF, provided that other factors (aphid population age structure, EPF spray application regime) are considered as well. This helps us move forwards to Stenberg's holistic science of IPM (Stenberg, 2017).

Partial host plant resistance to aphids may make nymphs more susceptible to EPF by increasing the 'susceptibility window' for fungal infection.

- The experiments in this study suggest a potential mechanism underlying the interaction between partial host plant resistance to *B. brassicae* and EPF infection. For each aphid nymphal instar, it can be hypothesised that there is a window of opportunity for successful EPF infection, with the size of this window being a product of both the rate of nymph development and the speed of EPF infection. Host plant resistance can slow down the rate of nymph development between instars, increasing the time-period for which they are susceptible to fungal infection.

For use in the field, the results of this study indicate that the best (i.e., the most robust and reliable) control is likely to be achieved when combining plant accessions with high levels of partial resistance with EPF with the fastest speed of infectivity to nymphs. The application timing for EPF in this system must be carefully considered

too, to give the best level of population control. If EPF are applied under favourable conditions and at a sufficiently high dose, it is likely that this combination would allow for high levels of mortality for nymphs within the 'window of opportunity'.

5.1. Future work

This research has demonstrated the potential value of combining EPF and partial host plant resistance for aphid control and is starting to develop a new understanding of interactions between host plant resistance and microbial control agents in IPM. Further work is required and can be divided into two categories: (i) follow-on research within the *Brassica x* EPF x *Brevicoryne*' system and (ii) further research within a broader IPM context.

5.1.1. Further research within the *Brassica*-EPF-*Brevicoryne brassicae* system

The results of this study suggest that improved methods could be used to identify more *Brassica* accessions with partial resistance to aphid pests and EPF isolates with greater virulence.

5.1.1.1. *Brassica* partial antibiosis resistance screening

In this study, partial plant resistance appeared to act primarily by increasing juvenile aphid development times. The ability to reduce aphid reproduction would also be a highly desirable crop trait for IPM. This could be done by running an additional, parallel screen of candidate accessions using as the starting aphid population a fixed aged cohort of adult *B. brassicae* instead of nymphs. It may also be worth extending the duration of the screen.

In this present study, candidate *Brassica* accessions were pre-selected for screening based on (i) gene expression data on the JA signalling pathway for accessions from DFFSs (diversity fixed foundation sets), and (ii) the results of previous research which had identified some partially resistant accessions to *B. brassicae* (Ellis *et al.*, 1998). The selection was done using the best information available at the time and succeeded in allowing for the rapid identification of partially *B. brassicae* resistant accessions to evaluate in combination with EPF. There does however exist significant potential to improve upon this pre-selection approach to make it more targeted and efficient. While there remains a significant lack of information and

research surrounding *Brassica* resistance to *B. brassicae* and other *Brassica* pests, perhaps the most promising avenue to improve this approach is to further utilise our advancing knowledge of plant-aphid interactions including gene expression data on plant anti-aphid defence pathways to guide pre-selections. Within this present study, transcriptomic preselection used data on pre-infestation upregulation of JA pathway genes. However, as knowledge of plant-aphid defence continues to grow, further gene expression targets are likely to be identified. New research on *Brassica* transcriptomic responses to aphid feeding should therefore be done as a priority. Until such targets are identified, the best approach for future screening is to undertake a classical large, randomised screen utilising different, complementary methods to identify accessions that affect different aspects of aphid biotic potential, particularly intermoult period and reproduction. This would be a significant time investment but would be highly valuable if it was paired with transcriptomics and association mapping, as has been deployed to great success previously to identify QTLs underlying disease resistance in *Brassica* crops (Jestin *et al.*, 2010; Kaur *et al.,* 2020; Dakouri *et al.*, 2021). Through knowledge of the effects of a large number of *Brassica* accessions on different aspects of aphid biology, it should be possible to identify QTLs associated with discrete partial resistance traits. The identification of such QTLs is essential if partial resistance is to be fully characterised, its biochemical basis understood and the process of breeding into elite commercial varieties initiated (Tanksley and Nelson, 1996).

5.1.1.2. Identification of aphid virulent EPF

The priority for EPF control of aphid pests is to target the nymphs, since although aphid adults appear susceptible to many EPF isolates there is no effect of infection on reproduction and hence little impact upon population development. Targeted screening of EPF against aphid nymphs should reduce the time spent evaluating EPF isolates to identify commercially viable biocontrol agents.

It is likely that two classes of EPF will be identified; (i) generalists which offer a low to mid level of control against aphid nymphs; and (ii) a small subset of aphid specialists which can be expected to have greater virulence. The choice of whether to develop generalist or specialist EPF isolates into biopesticides for aphid control is likely to be a significant decision in the future and will depend on the size of the

market. At the moment, biopesticide manufacturers want generalist EPF isolates, i.e., ones that can kill a range of pest species, in order to maximise their economic potential. However, *a priori*, use of EPF with narrow host ranges are likely to be better for the environment since there will be less harm to non-target insect species. Given the increasing problems with conventional pesticides for aphid control, it is possible that the market will open up in future for aphid specific EPF products. Alternatively, the evidence from the present study suggests that generalist EPF virulence to aphid nymphs can be significantly improved through their combined deployment with partially resistant *Brassica* accessions. The level of control provided by such generalist EPF may fall short of that offered by specialist EPF isolates such as 1.72 (*A. dipterigenus*), particularly against mixed age cohorts of aphids. The best strategy would be to use partial plant resistance with an aphid specialist EPF, where interactions with even moderate levels of partial resistance are likely to significantly limit aphid population development. For generalist EPF, higher levels of host plant partial resistance are likely to be necessary.

This suggests there will be a trade-off in future 'holistic' aphid IPM strategies. Selection of generalist EPF isolates is likely to be quicker, easier, and commercially more attractive to biopesticide companies, but will require integration with high levels of partial crop resistance, which itself is a difficult process. Conversely if an aphid specialist EPF is required, greater time input is likely to be necessary to identify a 'winning' EPF isolate, but it is likely to benefit significantly from more moderate levels of partial plant resistance, and there will be added benefits if combined with more pronounced levels of partial resistance. For this reason, careful consideration must be given to whether the economic advantages of developing generalist EPF outweigh the benefits of using more pest-specialist isolates. This also points to the need to develop biopesticides in partnership with plant breeding and other IPM elements as part of a holistic approach.

5.1.1.3. Further evaluation of the interaction between partial resistance and EPF

Within this present study, it was shown that host plant resistance could 'activate' the virulence of generalist EPF isolates to aphid nymphs and improve the virulence of specialist EPF isolates when faced with more challenging mixed nymph cohorts.

This led to the development of a 'susceptibility window' hypothesis for successful EPF infection. To develop this research further, a wider range of *Brassica* accessions and EPF isolates will be needed, demonstrating more nuanced differences in partial resistance, nymph virulence and speeds of infection. Research should focus initially on characterising the effects of these *Brassica* accessions upon the duration of each instar, and the speed with which EPF can infect nymphs – with this providing detail on the likely nymph 'susceptibility window' for each combination. Fixed-age nymph colonies can be established and their susceptibility to EPF at different numbers of hours/days predicted through knowledge of the likely 'window of opportunity' for EPF infection. The effect of different plant accessions on nymph fitness and adult reproduction should then be evaluated, followed by research to measure effects upon population development. In accessions in which the nymph susceptibility windows for EPF infection are roughly equal but the effects on adult reproduction and/or fitness are different, the rate of population development following a single EPF application will provide useful information as to the importance of adult reproduction and fitness partial resistance effects relative to effects on nymph development rate. Finally, it would be useful to quantify the effects of repeated EPF spray applications, as would be done in the field. The time of spray application would be based on predictions of when most nymphs are likely to be within the next 'susceptibility window' for infection. Through these types of hypothesis-driven experiments, new information can be gained to inform the inclusion of 'partial resistance + EPF' into broader IPM systems.

5.1.2. Further research into IPM interactions

Within the context of the seven key interactions outlined by Stenberg (2017), this study could serve as a blueprint for the evaluation of further interactions between heritable resistance and other forms of biological control (such as parasitoids). Additionally, introducing a further plant resistance or biological control variable into this existing system would warrant investigation. For example, EPF and parasitoids interact both indirectly, by EPF removing potential parasitoid hosts, and directly owing to the potential for EPF to infect parasitoids (Silva *et al.,* 2014; Jensen *et al.,* 2020).

Heritable plant resistance and biological control (and their potential interaction) form just a subcomponent of a larger and more interconnected IPM strategy, with its component interactions. A different route to progress this research may be to take this study as a framework upon which further two-factor interactions can be assessed, such as the interaction between 'biological control and biorational volatiles' or the interaction between 'heritable plant resistance and plant vaccination'. Through research investigating different pairwise interactions between biological IPM tools, a solid foundation of knowledge between different IPM tools could be built.

5.2. Concluding remarks

This study has shown that, for the control of aphids on *Brassica* crops with EPF, it is nymphs which pose the most significant challenge. It is likely, therefore, that other pest insects with short juvenile instar phases are also likely to pose challenges for the success of EPF. By combining EPF with partially resistant *Brassica* accessions however, the results of this study reveal that it is possible to 'activate' otherwise avirulent EPF isolates, and also to improve EPF isolates with higher intrinsic levels of nymph virulence. It is hypothesised that these EPF improvements stem primarily from the ability of partial host plant resistance to slow the rate of nymph development, likely increasing the nymph intermoult period and resulting therefore in a longer nymph EPF susceptibility window. At a population development level, there are also indications that partial resistance effects upon fitness and adult reproduction rate may contribute to reduced populations. In the short term, the findings of this research demonstrate the value in screening for partial host plant resistance to insect pests, with such resistance directly limiting the rate of population development and potentially increasing the susceptibility of individuals to different IPM tools.

This study is among the first to demonstrate the value of combining heritable plant resistance with microbial control and is likely the first to demonstrate the ability of partial resistance to activate/improve the efficacy of EPF to aphid nymphs. It is hoped that by confirming the significant improvements in EPF virulence and population level control which can be gained through this approach, this study can be used as a blueprint for further research into IPM interactions.

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