# Selection and improvement of insect pathogenic fungi for the control of multi-resistant aphids

Submitted by Zoltán Erdős, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, August 2022.



I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

# Acknowledgements

An immense thank you to my PhD supervisors: Ben Raymond, Chris Bass and David Chandler. Support and guidance throughout the project from you all has been invaluable.

The presented work benefited from comments, discussion collaboration and encouragement of many, including Bartek Troczka, Vicky and Mark Mallott, Angela Hayward, Luke Lear, Christopher Mitchell, Daniela Farina, Daniel Padfield, Gillian Prince, David Studholme, Tatiana Dimitriu, Andy Matthews, Peter Halswell, Elitsa Penkova, Hugh White and M.D.Sharma.

Special thanks to Vicky and Mark for their supply of plants and aphids, the SERFS PhD office inhabitants for regular coffee breaks and encouragements. I would like to thank all of my peers and technicians working in the SERSF, DDM and ESI labs making it a joyful environment.

I would like to acknowledge AHDB for providing me with my studentship, the University of Exeter for providing lab and office space.

## Publications

The following published papers have arisen from this thesis:

- Erdos, Z., Chandler, D., Bass, C., & Raymond, B. (2021). Controlling insecticide resistant clones of the aphid, Myzus persicae, using the entomopathogenic fungus Akanthomyces muscarius: fitness cost of resistance under pathogen challenge. *Pest Manag Sci, 77*(11), 5286-5293. doi:10.1002/ps.6571
- Raymond, B., & Erdos, Z. (2022). Passage and the evolution of virulence in invertebrate pathogens: Fundamental and applied perspectives. *Journal of Invertebrate Pathology, 187*, 107692. doi:https://doi.org/10.1016/j.jip.2021.107692

I contributed to another published project:

- Egleton, M., Erdos, Z., Raymond, B., & Matthews, A. C. (2021). Relative efficacy of biological control and cultural management for control of mollusc pests in cool climate vineyards. *Biocontrol Science and Technology*, 1-14. doi:10.1080/09583157.2021.1882387

**Abstract**

The entomopathogenic fungus (EPF) *Akanthomyces muscarius* has been used as a biocontrol agent for the management of insect pests in agriculture. Nevertheless, the licensing and commercial success of aphid biocontrol agents has been a challenge. This project had three main aims: test if this fungus could be used as a management tool for the control of insecticide resistant aphids (*Myzus persicae*); to test whether we could apply evolutionary theory to improve the properties of this species as biocontrol agents and finally to produce a high quality genome of this fungus as a resource for future use.

While we expected that pesticide resistant insects might have a fitness cost that would increase susceptibility to fungus, this proved not to be the case. In contrast, some susceptible clones, particularly those subject to decades of laboratory rearing, showed enhanced susceptibility to a fungal pathogen, but not reduced reproductive fitness, an observation consistent with down-regulation of costly immune functions in culture. Nevertheless, changes in susceptibility were small and overall, fungal pathogen control is compatible with insecticides and should not increase the selection pressure for resistance of *M. persicae* to chemical insecticides.

Experimental passage has been used to successfully increase virulence of insect pathogens. Passage experiments with EPF are relatively unexplored. Here we adopted a theory based approach and selected for speed of kill, pathogen yield and infectivity at different scales of competition in *M. persicae*. The selection experiment resulted in small but significant increases in virulence when compared to the ancestor, which also resulted in increased virulence against another aphid host, *Brevicoryne brassicae.* In addition, we also observed

increases in spore production on solid media and changes in timing of sporulation in some treatments. Changes in sporulation were unrelated to virulence showing that it is possible to produce more virulent mutants with improved sporulation characteristics.

We also present a new population genomic resource for *A. muscarius* comprising a high-quality genome assembly together with resequenced genomes of 13 experimentally selected lineages of this fungi.

The work presented here benefits industrial partners and stakeholders by providing novel methodologies for strain improvement of EPF and efficacy data of EPF on aphid genotypes resistant to synthetic insecticides. Increased use of biopesticides containing EPF can lead to a reduction in the use of chemical pesticides, increased biodiversity and reduced impacts on pollinators and other beneficial insects and could facilitate integrated biocontrol of difficult to manage aphid pests.

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# Introduction

## Background

The standard method used for controlling insect crop pests for the past decades have been foliar sprays of chemical insecticides. The extensive use of insecticides have resulted in the development of resistance in a wide range of important agricultural pests against almost all classes of synthetic insecticides (Sparks & Nauen, 2015). The first study reporting on insecticide resistance was published more than a 100 years ago (Melander, 1914). Georghiou and Lagunes-Tejeda (1991) documented over 500 arthropod species that had developed resistance against insecticidal substances. The Arthropod Pesticide Resistance Database (APRD) located at Michigan State University, USA (https://www.pesticideresistance.org) is collecting information on both field and laboratory resistance in arthropods. Whalon et al. (2008) reviewed the information available at the APRD in August 2007 and reported 7747 resistance cases and 553 species with resistance to at least one insecticide. Currently, there are more than 16000 documented cases of insecticide resistance involving more than 600 arthropod species (Sparks et al., 2020).

The importance of insecticide resistance management have been recognized (A. W. A. Brown, 1958; McKenzie, 1996; Whalon et al., 2008), and significant effort has been made by regulators and the crop protection industry to develop resistance management strategies that minimizes the risk of the development of resistance (Nauen et al., 2012; Sparks & Nauen, 2015). The concept of integrated pest management (IPM) originates from the 1950s and aims to reduce insecticide resistance, non-target effects and other negative environmental consequences by integrating cultural, biological and chemical control measures to reduce

economic injury to crops (Stern et al., 1959). The additional use of natural enemies or biological agents can result in a more targeted treatment, and would help to reduce the amount of harmful chemicals needed to achieve a sufficient level of control (Hull et al., 1997). The ever-increasing costs, more stringent regulatory restrictions, time and complexity of developing new insecticides (Lamberth et al., 2013; Maienfisch & Stevenson, 2015; Sparks & Lorsbach, 2017) means that an effective insecticide resistance management (IRM) system is critical to preserve the utility and investment in present and future arthropod pest control (Sparks et al., 2020). Great emphasis should be placed on sustainable agricultural production with the global challenges of climate change and population growth (Parry, 2019).

Biopesticides include microorganisms (algae, bacteria, fungi, protozoa and viruses), pheromones, semiochemicals, macrobials, invertebrates (insects and nematodes) and plant extracts/botanicals (OECD, 1996). They represent only about 5% of the global market of plant protection products but have shown a strong long-term growth compared to synthetic chemicals (Marrone, 2019). The fastest growth in the near future is expected to be in Europe due to regulatory changes and the increasing demand for organic products (Marrone, 2014, 2019).

Microbial biopesticides based on entomopathogenic fungi (EPF) infect their hosts percutaneously, which makes EPF an ideal candidate for the control of sap-feeding insects that are difficult to treat with bacteria and viruses that infect via ingestion (Chandler, 2017). The use of EPF present minimal risk to non-target organisms which is a key characteristic to be considered in IPM systems (Lacey et al., 2015). Compatibility of EPF with other control agents, such as fungicides is an important quality to be considered for IPM. Studies aiming to improve EPF tolerance to fungicides using a combination of random mutagenesis and artificial

selection has been shown to increase resistance of fungal strains to broad-spectrum fungicides without a loss of virulence (Shinohara et al., 2013; M. Xie et al., 2018). Combining insecticides with EPF in an integrated pest management system (IPM) could have an additive effect (Pelizza et al., 2018; Pelizza et al., 2014). This would also alleviate some of the selection pressure for insecticide resistance in insect populations. Similarly, combinations of EPF and semi-refined botanical extract has been shown to have an additive effect when applied against *Aphis fabae* (Fernandez-Grandon et al., 2020). Combining biopesticides with different mode of action can potentially improve efficacy of individual components and also reduce the build-up of resistance.

Despite the number of available formulated products, bioinsecticides based on entomopathogenic fungi (EPF) have not taken a significant share of the insecticide market. The efficacy of these products is highly dependent on environmental factors, such as temperature, humidity and UV. This could lead to poor and unreliable performance compared to chemical pesticides. Products based on EPF exhibit a slower speed of kill and, due to the lack of residual effect need to be applied more frequently, which complicates the logistics of use (Jaronski, 2010b). Great effort and resources is being invested in improving the quality of control achieved by EPF ranging from application methods to genetic modification of organisms. Jaronski (2010b) captures the considerable effort made to improve EPF strains via novel spore delivery methods, UV protectants, humidity stabilizers and different growth substrates to increase sporulation, virulence and stress resistance. Studies aiming to improve EPF via genetic modification includes overexpression of virulence genes encoding catalytic enzymes (Fang et al., 2012), expression of genes encoding insect specific toxins (Lovett et al., 2019; Timofeev et al., 2019; M. Xie et al., 2015) and improving

tolerance to environmental conditions such as temperature, UV and oxidative stress (X. Q. Xie et al., 2010). Protoplast fusion has also been successfully used to create hybrid strains with improved biocontrol efficacy (Goettel et al., 2008). Strain enhancement using genetic engineering provides an exceptional tool to enhance tolerance against abiotic stresses and increase virulence. The use of transgenic fungi has been showing promising results (Bilgo et al., 2017; Lovett et al., 2019; Lovett & St Leger, 2018), however field application of such GM organisms would face regulatory issues in Europe and many other regions.

Experimental evolution in a controlled environment can be an effective tool for strain improvement and identification of the genetic background of variation in traits of interest. Such an approach has been widely used in microorganisms studying phenotypic and genotypic adaptation to selective pressures *in vitro* (Barrick & Lenski, 2013; Barrick et al., 2009; Segrè et al., 2006; Swamy & Zhou, 2019; Tenaillon et al., 2016). *In vivo* studies of adaptation are driven by complex selection pressures and host specific effects making it much harder to predict changes based on *in vitro* experiments (Hernandez & Koskella, 2019). There are no studies to date employing serial passaging combined with both phenotypic and genomic analysis using EPF. This approach provides a tool to identify genotypes associated with adaptations to specific selective pressures as seen in a number of other host-pathogen models (Cooper et al., 2020; Guidot et al., 2014).

The loss of effectiveness of synthetic chemicals and the growing concern about their negative effect on human health and the environment urges scientist to work on finding new alternatives as well as improving existing ones (Chandler et al., 2011). Furthermore, European legislators are implementing strict restrictions on the use of synthetic pesticides that fall under the newly implemented criteria for

human endocrine disruptors (European Commission, 2018d), which creates additional pressure on the farmers to reduce the use of chemical insecticides. This should increasingly push industry to find other alternatives for the control of aphid populations.

## Description of Study system

### Myzus persicae

*Myzus persicae* (Hempitera, Aphididae), commonly refered to as the peach-potato aphid, is one of the most economically important pest of agricultural and holticultural crops. It is distributed worldwide, with a broad host range and ability to adapt to new host plants. *M. persicae* exhibits a great range of genetic variation in terms of colour, life cycle, host-plant relationship and methods of insecticide resistance (Blackman & Eastop, 2000). Plant damage is caused via direct damage due to feeding and, more importantly by the transmission of plant pathogenic viruses (Brault et al., 2010). *M. persicae* can transmit more than 100 different plant viruses such as mosaic virus and black ring spot virus (Blackman & Eastop, 2000). The capacity of *M. persicae* to adapt to new host plants, the rapid life-cycle and the ability to evolve resistance to insecticides are some of the key factors that make *M. persicae* a high threat for growers worldwide.

Life cycle and infestation

The life cycle of *M. persicae* is dependent on the temperature during winter. The holocyclic life cycle of *M. persiace* predominantly occurs in colder climates due to the shortened photoperiod and low temperature, producing sexual forms. Virginoparae produce migrant gynoparae that overwinter on a host plant, mainly on species belonging to the *Prunus*. Gynoparae produce mating females that

mate on the host plant, resulting in eggs overwintering in diapause. Milder winter conditions allow for the anholocyclic life cycle to continue all year round. Viviparous parthenogenesis has been observed in mild conditions over the winter, allowing *M. persicae* to birth live offspring without the need for mating throughout the year (Blackman, 1974). A female can drop up to 50 nymphs in a lifetime, allowing for the production of large amounts of biomass in short period of time (Watson, 2000).

## Control methods

### Chemical control

The predominant control method for aphid pests currently relies on synthethic chemical insecticides. As a result, the peach-potato aphid is listed as number three among resistant arthropods ranked by number of unique compounds for which resistance has been reported (Sparks et al., 2020; Sparks & Nauen, 2015). In the UK, 62% of outdoor insecticide use in 2017 was targeting aphid pests on brassicas (Garthwaite, 2019). Pirimicarb and pyrethroid resistance is dominant in aphid populations in the UK.

### Alternative control

Alternative control measures against aphids include cultural control and biological control using parasitoids and pathogens. Cultural control consists of techniques preventing aphid access to crops or promoting refuge to aphid predators by means of managing the physical and biological environment of crops at establishment and during growth (Wratten et al., 2007). Cultural control methods, such as mulches, non-crop refuge, intercropping and pruning can have intrinsic benefits, do not impose high costs or cause environmental damage or health risks (Tilman et al., 2002).

Biological control can follow different strategies. Classical control is based on introducing natural enemies in a habitat they did not previously occupy. Augmenatation relies on mass-producing and realeasing naturally occuring enemies in sufficient numbers to control the target pest. This approach could take the form of inoculative or inundative release. The former aiming at establishing the natural enemy for the period of crop risk, the latter not expected to produce further generations. Conservation biocontrol involves habitat management to enchance natural enemies or their behaviour (Van Driesche & Bellows, 1996).

Aphids are exploited by numerous parasitoids, predators and entomopathogens. Hymenopteran (Aphidiidae, Aphelinidae) and Dipteran parasitoids are produced commercially and used in augmentative control with most success in protected crop production (van Lenteren, 2000). However, mass rearing of insect parasitoids is costly and can negatively impact the quality of natural enemies (Boivin et al., 2012). These challenges can drive up the costs for growers.

**Entomopathogenic fungi**

One of the major groups of eukaryotic microbes in terrestrial and aquatic ecosystems is the kindom Fungi. Species diversity within the kingdom Fungi is estimated to be between 1.5 and 5 million (Blackwell, 2011). With approximately 100,000 species described, the majority of fungal species are still unknown. Few if any organisms in terrestrial ecosystems exist in nature in the complete absence of fungi, and for this reason “fungi are essential players in the maintenance of ecosystem health” (Araujo & Hughes, 2016).

Coevolution with arthropods - known to be the most species rich groups of eukaryotes - have led to the development of a wide array of intimate interactions ranging from mutualistic endosymbiosis to parasitism (Vega et al., 2009). The

ability of fungi to cause disease to insects is one of them. Entomopathogenicity evolved independently and repeatedly in all major phyla of the fungi (J. B. Wang et al., 2016).

Entomopathogenic fungi (EPF) are important natural pathogens of arthropods. There are approximately 750 fungal species causing infection in insect and mite populations (Sinha et al., 2016). Within the kingdom Fungi, the phyla *Ascomycota* and *Entomophthoromycota* contains the largest number of entomopathogenic fungi (Araujo & Hughes, 2016; Humber, 2008).

Ascomycete; Hypocreales

The phylum *Ascomycota* forms the largest group in the kingdom Fungi with approximately 64,000 species described (Kirk et al., 2008). It is one of the most diverse and ubiquitous phyla of eukaryotes with species in numerous ecological niches in almost all terrestrial and aquatic environments. The majority of fungi within *Ascomycota* can be characterised by specialized sac-like structure (ascus) that produce meiotic spores (ascospores) (Schoch et al., 2009). The largest and most well studied group of Ascomycota entomopathogens belong to the order of Hypocreales. These pathogens evolved with a pattern of repeated transitions among different hosts of plants, fungi and insects. (Blackwell, 2011; J. B. Wang et al., 2016). The anamorphic Hypocrealen fungi are considered to be opportunistic. Showing a hemibiotrophic lifestyle, they are able to colonize the cadaver after host death and exhibit hyphal growth while shifting to a saphrophytic lifestyle (Araujo & Hughes, 2016; Hesketh et al., 2010). The well characterized genus of *Beauveria*, *Isaria*, *Akanthomyces* and *Metarhizium* have been used in commercial development as biopesticides, against a wide range of arthropods (Goettel et al., 2008). These fungi have four major propagule types that can be used when developing commercial products. Aerial conidia are the

primary infectious propagule found in a natural environment. These spores are produced on the fungal outgrowth of insect cadavers. Blastospores are proliferative stages within the insects and can also be produced in liquid fermentation. Microcycle conidia are produced on the ends of hyphal strands under certain conditions (S. Zhang et al., 2010). The major form of vegetative fungal proliferation is mycelium and its derivatives, that are the true producers of conidia (Jaronski, 2014). Faria and Wraight (2007) reported 171 commercial products based on fungal propagules. Most of these products are based on *B. bassiana* and *M. anisoplae* as their active ingredient. A more recent survey recorded at least 28 mycoinsecticides or mycoacaricides based on Hypocrealen fungi (Wraight et al., 2017b).

Akanthomyces spp.

*Akanthomyces* spp. previously *Lecanicillium*, has been reclassified in the family of anamorphic Cordycipitaceae and includes the insect pathogens *A. attenuatus*, *A. lecanii*, *A. dipterigenus* and *A. muscarius* (Table 1) (Gams & Zare, 2001; Kepler et al., 2017). These fungi demonstrate the classic pathway of pathogenesis found in entomopathogenic mitosporic fungi: adherence of conidia to the host cuticle; germination of conidia; penetration of the cuticle due to enzymatic hydrolysis and mechanical pressure; production of blastospores within the hemocoel; ramification of the mycelia and invasion of tissues causing death of the host; and finally, production of conidia on the surface of the cadaver (Askary et al., 1999; Goettel et al., 2008). There are commercial products available for growers based on *Akanthomyces* spp. with activity against a wide variety of pests worldwide (Faria & Wraight, 2007).

Akanthomyces muscarius

*A. muscarius* is widely distributed, with a preference to temperate climates. It has a broad host range and has been commercialized (Mycotal Koppert, NL) as a mycoinsecticide against whiteflies, thrips, aphids and mites (Faria & Wraight, 2007). It forms compact, white to (rarely) pale yellow or uncoloured colonies. The conidiogenous cells are generally longer than those of *A. lecanii* 15-35 x 1-1.7 µm, produced directly on prostrate hyphae or secondary branches. Conidia are produced in globose heads, they are ellipsoid to subcylindrical, irregular in size and shape, longer and narrower than in *A. lecanii* (2-6 × 1-1.8 µm). Octahedral crystals are commonly present. Optimum growth temperature is around 24 °C (depending on strain), with no growth at or above 33°C (Zare & Gams, 2003).

Table 1 Taxonomy of *Akanthomyces muscarius* (Petch) Spatafora (Kepler et al., 2017).

| | |
|---|---|
| **Kingdom** | Fungi |
| **Phylum** | Ascomycota |
| **Class** | Sordariomycetes |
| **Order** | Hypocreales |
| **Family** | Cordycipitaceae |
| **Genus** | Akanthomyces |
| **Species** | Muscarius |

Entomopathogenic and mycoparasitic activity of *A. muscarius* has been previously well described (Zare & Gams, 2003). Application as biological control agent is better suited to greenhouses as the fungi generally requires a high humidity level for infection. The process of infection involves numerous extracellular proteolytic, lipolytic and chitinolytic enzymes (Askary et al., 1999; Bidochka et al., 1999).

Entomopathogenic fungi as biocontrol agents

Entomopathogenic fungi (EPF) are diverse and ubiquitous pathogens of arthropods. Their potential as biological control agents have prompted vast amount of research (Goettel et al., 2008; Lacey et al., 2015; Pell et al., 2010; Roy et al., 2010; Vega et al., 2009). EPF are ideal candidates as successful control agents against sucking pests, such as aphids, that are hard to control with bacterial or viral microbial biocontrol agents (MCAs) that need to be ingested (Chandler, 2017). The most well studied group of EPF are the asexual forms of ascomycetes, due to the relative ease of in vitro mass-propagation of infective spores that can be formulated and applied as sprays (Jaronski, 2010a; Jaronski & Mascarin, 2017). These mycoinsecticides have been developed for inundative control programmes against aphids (i.e. Mycotal, BotaniGard, Naturalis-L). Unlike ascomycetes, entomophthoralean species are very difficult to mass-produce and have been used in conservation biocontrol and inoculative augmentation approaches (A. B. Jensen et al., 2008; Shah & Pell, 2003; Zhou, 2012). Several products are available for use in greenhouses against foliar pests in Europe (Wraight et al., 2017b). Many ascomycete species have been formulated into commercial products such as *Metarhizium* spp. several *Beauverai bassiana* strains and *Akanthomyces muscarius* (Petch) formerly *Lecanicillium muscarium* (Zare & Gams, 2003). These products are mass-produced in liquid or solid state frementation and formulated into emulsifiable suspensions or wettable powders (Copping, 2009).

Efficacy of these products are hindered mostly by environmental conditions. High humidity and a moderate temperature is needed for effective and consistent use of spore-based mycoinsecticides. These abiotic constraints make EPF based insecticides more viable in greenhouse crop production. Germination requires very high humidity. This can arise from the ambient environment and also from

niches on the host's body. This humid boundary layer can surround leaves, other parts of the host plant or the insect pest (Wraight et al., 2017b). The rapid development of most aphids species involve four nymphal stages before moulting to adult stage (Dixon, 1973) making the establishment of infection more difficult. Furthermore, infected adults continue to oviposit at nearly normal rates until death, which does not occur until 3-4 days after infection (Jandricic et al., 2014). A combination of these factors make aphids difficult to control with entomopathogenic fungi. Frequent, high-dose applications needed to achieve control also means a greater cost for growers.

Virulence

Virulence, most commonly referred to as the harm caused to the host by a pathogen during infection, is of importance to a wide range of disciplines in biological sciences. The conditions that influence changes in virulence are of interest for biologists in public health and disease management but also for biological control of invertebrate pests and vector species. A better understanding in the mechanisms shaping virulence and how to maintain or increase virulence through artificial selection in the laboratory is of key interest for invertebrate pathologists (Raymond & Erdos, 2022).

Life history trade-offs and correlates of virulence are common basis for selecting for virulence. Multiple life history traits may be related to virulence, and their response to selection for virulence at different scales in entomopathogenic fungi is unknown. The most common definition of virulence as 'harm done to hosts during infection' is also not helpful for these obligate killers. A more suitable definition for biocontrol applications is instantaneous mortality rate, widely used for virulence that captures both the probability of establishing infection (infectivity) and the speed of kill during infection (Day, 2002).

**Social evolution theory in the context of virulence**

A paradigm shift in the understanding of evolution of cooperation following the 1960s (Hamilton, 1964a, 1964b) lead to a major effort by biologists to explain social behaviour between living organisms. Costly behaviours that benefit other individuals can be difficult to explain. Hamilton proposed the idea of inclusive fitness, which consists of direct fitness effects that impact on the reproduction of the individual and indirect fitness effects that impact the reproduction of related individuals (West, Diggle, et al., 2007).

Cooperation can be favoured if the behaviour provides a direct fitness benefit to the individual that performs the behaviour and the costs are outweighed by the fitness benefits. This behaviour is termed mutualism and can be explained by shared interest or an enforcing mechanisms that rewards co-operators or punishes cheaters (West, Griffin, et al., 2007).

Altruistic cooperation, when an action provides benefit to another individual carrying the cooperative gene is termed kin selection (J. M. Smith, 1964). This could occur via kin discrimination, when cooperation is preferentially directed towards relatives, and limited dispersal, which is based on spatial proximity of the actors where cooperation is directed toward all neighbours who tend to be relatives. Kin selection favours cooperation when the degree of genetic relatedness is relatively high compared to the whole population (West, Diggle, et al., 2007).

Spiteful interactions, when a behaviour reduces the fitness of the actor and the recipient, can be more difficult to explain. Kin selection can provide an understanding for spite. Spiteful behaviours can be favoured when the recipient of the action has a lower relatedness to the actor than the population as a whole and the recipients can be specifically targeted by the action. The actor benefits

indirectly from the behaviour by reducing competitions for the actors relatives (West et al., 2006).

Hamilton proposed a simple mathematical model to show when altruistic behaviours will be selectively favoured. Altruism is favoured If $rb - c > 0$, where $r$ is the relatedness, $c$ is the costs incurred to the actor, and $b$ is the benefit to the recipient.

The well-established virulence-transmission trade-off hypothesis (Anderson & May, 1982; Ewald, 1983) argues that virulence is an unavoidable consequence of parasite transmission. The longer the host is alive the higher the chance for the parasite to be transmitted, which would lead to virulence attenuation over time. This model is further complicated by introducing mixed infection.

Social interaction between microbial parasites has been shown to have an important effect on the ability of the parasites to cause damage to their host. Transmission of pathogens could depend on the level of cooperation between multiple co-infecting strains. Alizon (2008) showed that more virulent strains can take over, when multiple infection is predicted to decrease overall virulence. Superinfection (Nowak & May, 1994) and coinfection (May & Nowak, 1995) models try to explain the effect of mixed strain infections on the virulence of pathogens. Mixed infection parasite virulence predict that lower within-host relatedness should select for higher virulence as co-infecting parasites compete for host resources (Frank, 1992), whereas high relatedness would lead to cooperating parasites and lower virulence (Buckling & Brockhurst, 2008). However, experimental support is limited and equivocal as some studies showed that more virulent strains are favoured within-host competition and others leading to the less virulent strains taking over. Arguments against multiple infections leading to higher virulence are based on the theory that low relatedness would

lead to a decrease in parasite cooperation (Alizon & van Baalen, 2008). Decrease in virulence can originate from the diverse modes of parasite competition within host. Garbutt et al. (2011) showed that multiple infections with the bacteria *B. thuringiensis* leads to increased antagonism between co-infecting strains and decreases virulence.

Experimental evolution using EPF is a relatively unexplored area compared to other biological control agents such as bacteria (Raymond & Bonsall, 2013; Raymond et al., 2007; Raymond et al., 2012) or nematodes (Shapiro- Ilan & Raymond, 2016). The few available studies exploiting spontaneous genetic changes and selection for the improvement of virulence report mixed results. Quesada-Moraga and Vey (2003) reported increased virulence of *B. bassiana* against *Locusta migratoria* following two passages through the host, suggesting that an interruption from artificial culturing is beneficial for maintining the level of virulence. In their study Valero-Jimenez et al. (2017) did not observe a significant change in virulence of *B. bassiana* towards *Anopheles coluzzii* after 10 consecutive selection cycles through mosquitos.

## Aims

The overall aim of this research project is to improve the performance of entomopathogenic fungi (EPF) for the control of *Myzus persicae*. Through applying selection methods based on social evolution theory to achieve increased virulence, we also get a better understanding on how different evolution of virulence models compare in a complex fungal pathogen of insects. Furthermore, we study potential fitness costs related to insecticide resistance under pathogen challenge, create genomic resources for *A. muscarius* by creating the first draft genome assembly for the species, attempt to functionally annotate the draft

genome and genomic variants obtained via the selection method. The aims for each chapter are further described below:

1. **Investigate the compatibility of entomopathogenic fungi (EPF) for the control of resistant aphids.** Insecticide resistant insects show increased susceptiblity, or suffer greater fitness costs under pathogen challange (Raymond et al., 2007). An increased fitness cost could be exploited to slow the evolution of insecticide resistance in *Myzus persicae* by using EPF in IPM systems. To study the fitness cost of resistance in *Myzus persicae*, clones with varying degree of insecticide resistance were screened in bioassays with the EPF *Akanthomyces muscarius*.
2. **Improvement of EPF virulence by applying social evolution theory in serial passaging.** Different theories of evolution of virulence predict different outcomes for competition of pathogens on different scales within host, between hosts and between populations. Does within host competition increase virulence (classical virulence evolution theory) or do high relatedness favour virulence (public goods production – social evolution theory)? We carried out an experimental evolution study with serial passaging to study how different scales of competition affect virulence and other key biocontrol traits, such as sporulation. The derived strains were than compared to the ancestor to identify changes resulting from passaging.
3. ***De Novo* genome assembly of *Akanthomyces muscarius* and analysis of *in vivo* gene expression.** Sequencing resources are scarce for the whole genus *Akanthomyces*. The only species with an available draft genome is *A. lecanii*. Here we create a high quality *de novo* genome assembly based on long- and short-read libraries, carry out variant discovery using re-sequenced libraries of derived strains and create a transcript library for fungi isolated from

agar plates and fungi isolated from aphid hosts. The transcripts are also used to study differential gene expression with the aim to identify genes involved in virulence towards aphids.

## I: Precision Sprayer design and calibration


### Abstract

The lack of commercially available low-cost laboratory spraying equipment for testing microbial biocontrol agents (MBA) makes it difficult for small microbiology laboratories to carry out MBA testing that resemble commercial use. This study presents an inexpensive, portable micro sprayer that can be calibrated utilizing laboratory consumables. The computer aided design files are made available so that it is freely modifiable and can be used for machine routing or 3D printing. Bioassay data was obtained by spraying *Myzus persicae* with spores of different species of entomopathogenic fungi. Observed variation in droplet deposition within tested pressure and volume settings, and spore deposition within sprayed concentrations were low. Bioassay results show reproducible mortality for the tested doses.

## Introduction

Entomopathogenic fungi (EPF) are an important resource for biological control of arthropods, with approximately 750 species able to infection in insects and mites (Sinha et al., 2016). Fungi belonging to the order of Hypocreales, such as *Beauveria bassiana, Akanthomyces longisporum* and *Metarhizium anisoplae* have been extensively studied and widely used as environmentally friendly biological control agents (Hesketh et al., 2008; Humber, 2008). Researchers in the field of biological control with EPF rely on robust results obtained via bioassays. A commonly used measure of efficacy when using infective propagules of microbial agents is lethal dose (LD) or lethal concentration (LC) (Finney, 1971). In order to obtain repeatable results when studying dose-time-mortality, it is imperative to conduct bioassays with a strict control of dosing method (Inglis et al., 2012). Infection of the insect host with fungal spores initiate via the cuticle (Ortiz-Urquiza & Keyhani, 2013), therefore the most common application of these microbial agents is topical. Depending on the size and number of insects and the target (habitat or insect surface), the method of application can vary greatly. The most frequently used method in applying fungal propagules on insect hosts is spraying (Inglis et al., 2012). There are a number of available devices including track- and handheld-sprayers designed for this purpose. The Potter spray tower is considered to be the standard of reference for such spraying techniques in the laboratory (Potter, 1952). However, this piece of equipment is stationary, takes up considerable laboratory space and is expensive. Hand-held sprayers are the cheapest option available, but they do not provide good control of the applied dose. Here, we provide a design for an appliance that could be readily made from inexpensive materials with relatively low cost. Similar setups are used frequently in laboratories working with microbial

biocontrol agents, but the lack of published designs makes it difficult to construct robust, reproducible equipment for small scale spraying.

## Methods

### Insect rearing

Insect stock cultures

Populations of *Myzus persicae* were reared in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) on Chinese cabbage plants at BBCH growth stage 13-15. Colonies were sub-cultured onto new plants or aphid cups (described below) as required by transferring 5-15 apterous adults onto fresh plants. Cultures were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle.

Production of known-age *Myzus persicae*

Known-age *M. persicae* populations were produced by confining cohorts of mature apterous virginoparae on detached Chinese cabbage leaves contained in aphid rearing cups consisting of two plastic cups with mesh vents containing water and an ozmotic bead (Osmocote Controlled Release Plant Food, Greenfingers, UK) (Figure 1). Alternatively, adult *M. persiace* were placed in clip cages on chinese cabbage plants (BBCH 13-15) in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK). Adults were removed after 48 hours and the nymphs were maintained until reaching adulthood. *M. persicae* became adults after 9 days ±1 days. Populations were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle.

## Fungal cultures

*In vitro* culture of strains of hypocrealean entomopathogenic fungi

Stock culture of *A. muscarius* 19.79[1], *B.bassiana* 433.99[1] and *B.bassiana* 1787.18[1] strain were obtained from Warwick Crop Centre, The University of Warwick (UK) and stored as conidia on porous plastic beads in cryotolerant plastic tubes (Pro-Lab Diagnostics, Bromborough, Wirral, UK) at -80 °C. A two-stage culturing system was used to provide material for experiments. Firstly, a culture was grown by removing a bead from cryopreservation and placing it on a Sabouraud dextrose agar (SDA) slope in 30 ml universal tube (Starlab, UK) and incubating it in complete darkness at 23±1 $^{o}$C for 10 days before transferring to cold storage at 4±2 $^{o}$C (this material is referred to as 'laboratory culture'). Secondly, 'working cultures' were grown from hyphal material taken from laboratory cultures and grown on SDA in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) in darkness at 23±1 °C for 10-14 days. Laboratory culture slopes were replaced every 3-4 months in order to minimize the risk of virulence attenuation of fungal strains caused by repeated subculture (Chandler, 1994).

Preparation of hypocrealean conidial suspensions

Conidial suspensions were made by agitating the mycelium of a 10-14 day old culture with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK) in 10ml of 0.01% Triton X-100. The suspensions were then passed through sterile cheesecloth to remove any hyphal fragments. Suspensions were enumerated using Fastread102 (Biosigma, IT) counting chambers and adjusted to the required concentration using 0.01% Triton X-100.

[1] Warwick Crop Centre Culture Collection strain number

## Micro-sprayer design and calibration

A portable micro-spray tower was designed following Mascarin et al. (2013) and Spence et al. (2020). The sprayer consists of an acrylic cylindrical tube, a top cap responsible for holding an artist airbrush (HP-SBS ECL3500 with standard nozzle, Iwata) securely in place with studded aluminium rods and a base cap acting as a sample tray. The airbrush is connected to a compressor (Powerjet Lite IS925, Iwata). The spray area allows space for a 90 mm Petri dish to be placed in the center (Figure 1). The top cap with the fastening, the base cap and the feet can be 3D printed or cut via a Computer Numeric Control (CNC) machine from a material of choice depending on the intended application. Here, we used 11.7 mm thickness PVC that can withstand repeated washing with 70% ethanol. The feet, base and top caps have 4 mm deep flanges connecting them to the acrylic tube. The parts are easily disassembled for cleaning and disinfection.

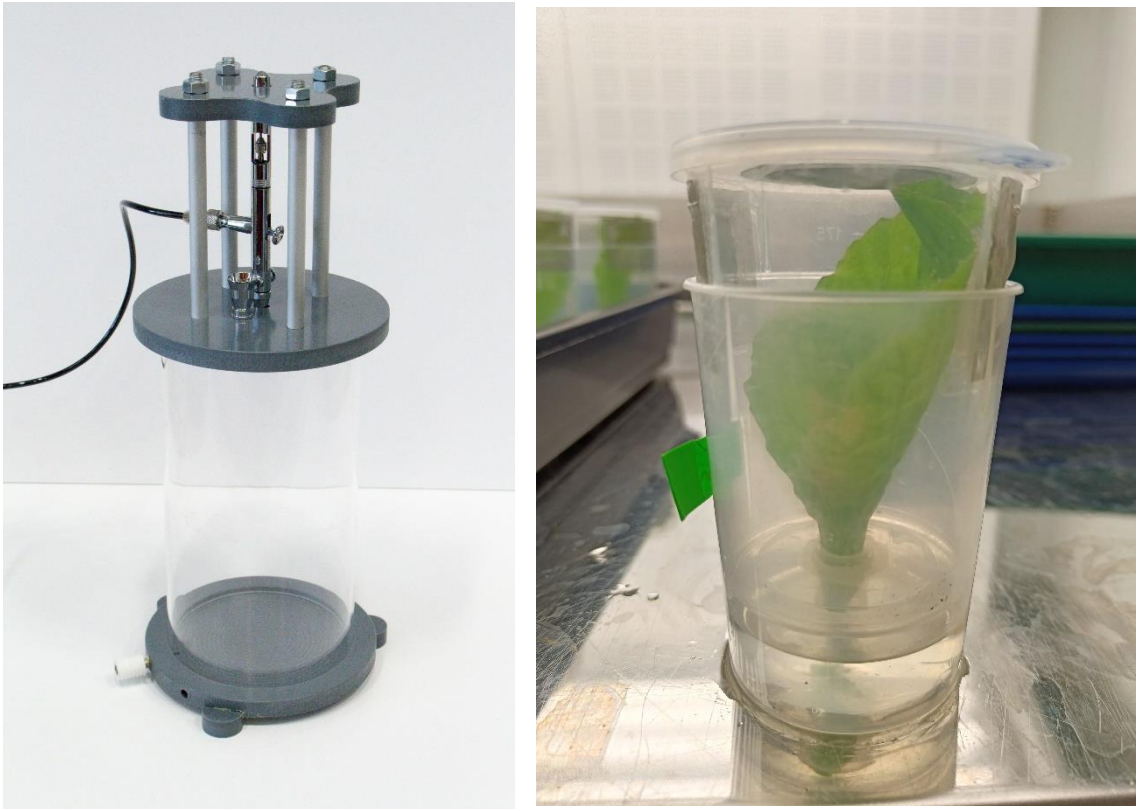


*Figure 1 Micro-sprayer assembly (left), Aphid rearing cup with a single leaf of Chinese cabbage (right).*

Calibration of the device was carried out by spraying deionized water on 9 cm diameter filter paper (Qualitative 415, VWR). In order to understand the relationship between pressure, volume and droplet deposition, filter papers were weighted directly before and after spraying. Droplet deposition (µl/cm$^2$) was calculated for all pressure and volume combinations of 8, 12, 16, 20, 24 PSI and 300, 400, 500, 750, 1000 µl resulting in 150 observations. A multiple linear model was used to describe the relationship between sprayed volume, pressure and liquid deposition on filterpaper.

The calibration was repeated with stain solution (Methyl blue, Sigma-Aldrich), for visual inspection of droplet deposition. Observations showed considerable fluctuations in pressure at 8 PSI, and that aphids were flushed out of the petri dish at 24 PSI, thus these two pressure settings were excluded from further

studies.

The relationship between fungal concentration and surface deposition of conidia was investigated using spore suspensions of *B.bassiana* 433.99, *B.bassiana* 1787.18 and *A. muscarius* 19.79 at different concentrations ranging from $4.6x10^6$ to $3.7x10^8$ conidia/ml. Spore suspensions were sprayed on to 90 mm diameter Petri dishes containing 4-5 glass coverslips (22 x 22 mm). The applied volume was 400 µl at 12 PSI according to the previous study. All concentrations were tested at least three times. Sprayed cover slips were transferred to 45 ml centrifuge tubes containing 5 ml 0.01% Triton-X-100 and vortexed for 1 minute to dislodge conidia. Recovered conidial suspensions were enumerated using Fastread 102 (Biosigma, IT) counting slides. Average number of conidia recorded from coverslips was used to quantify depositions rates (conidia/$mm^2$). A linear regression model was used to describe the relationship between conidial deposition and concentration.

A dose response bioassay was carried out using *A. muscarius* Ve6 (Mycotal, NL) and even-aged apterous adult *Myzus persicae*. 18-20 aphids were treated in a 55 mm Petri dish with a concentration in the range of $1x10^5$-$1x10^8$ conidia/ml. The seven tested concentration were replicated at least 3 times. Controls were treated with a sterile carrier (0.01% Triton-X-100). After treatment aphids were maintained on single leafs of Chinese cabbage (*Brassica pekinensis* var Wong Bok) in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated separately and observed for fungal outgrowth to confirm that death was associated with fungal infection.

## Results

Droplet deposition showed the least spread (2.62 ± 0.01 µl) with a volume of 400 µl sprayed at 12 PSI. A significant relationship between water deposition, volume and pressure was indicated by the multiple linear model ($F_{2, 147}$=5522; P<0.0001; adjusted $R^2$=0.989) (Figure 2).

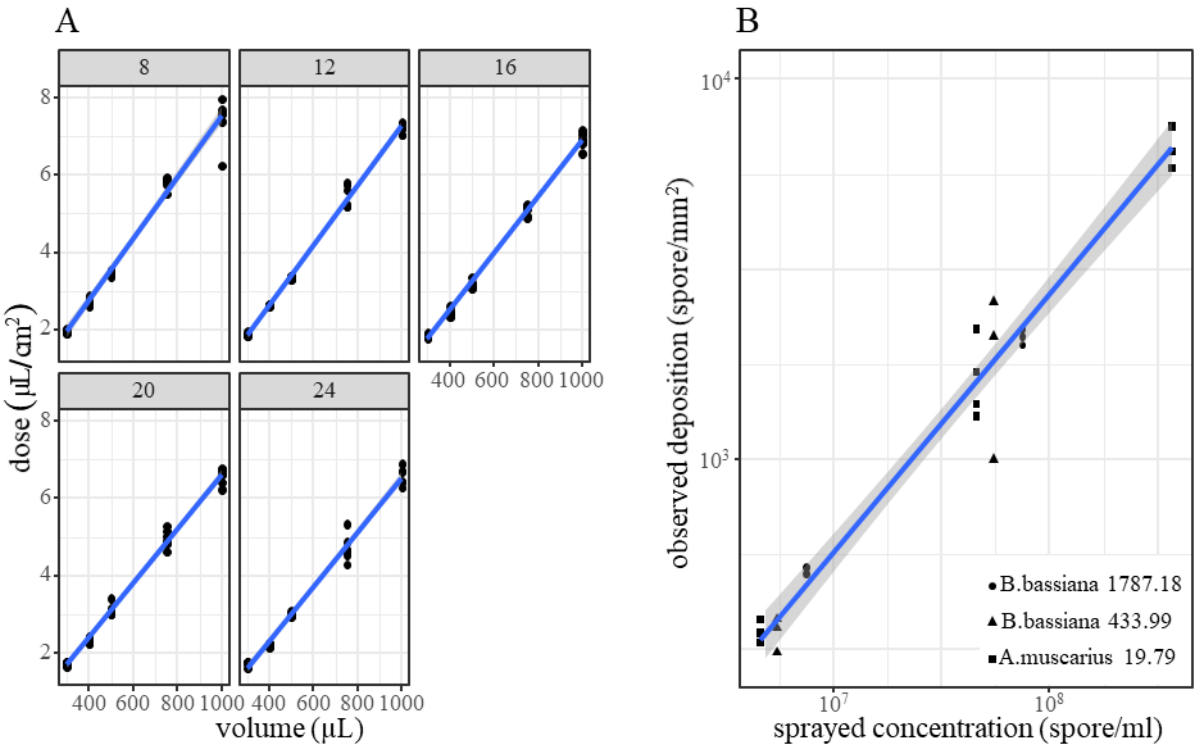



*Figure 2 Water deposition expressed as dose at different applied pressures and sample volumes. Panel headings (8-24) refer to application pressure in PSI (A). Observed conidia deposition at different applied concentrations (B).*

Visual inspection of spray patterns on the filter papers indicated that 400 µl sprayed at a pressure of 12 PSI provided the most even coverage.

The determination of spore deposition was limited to the range where spore counts could be accurately enumerated by the counting slides. There is a significant relationship between conidia deposition and applied concentration ($F_{1,}$

$_{22}$=449.3; P<0.0001; adjusted $R^2$=0.951). The results show that within concentration variation is low, which is key for dose-response testing of microbial propagules (Figure 2).

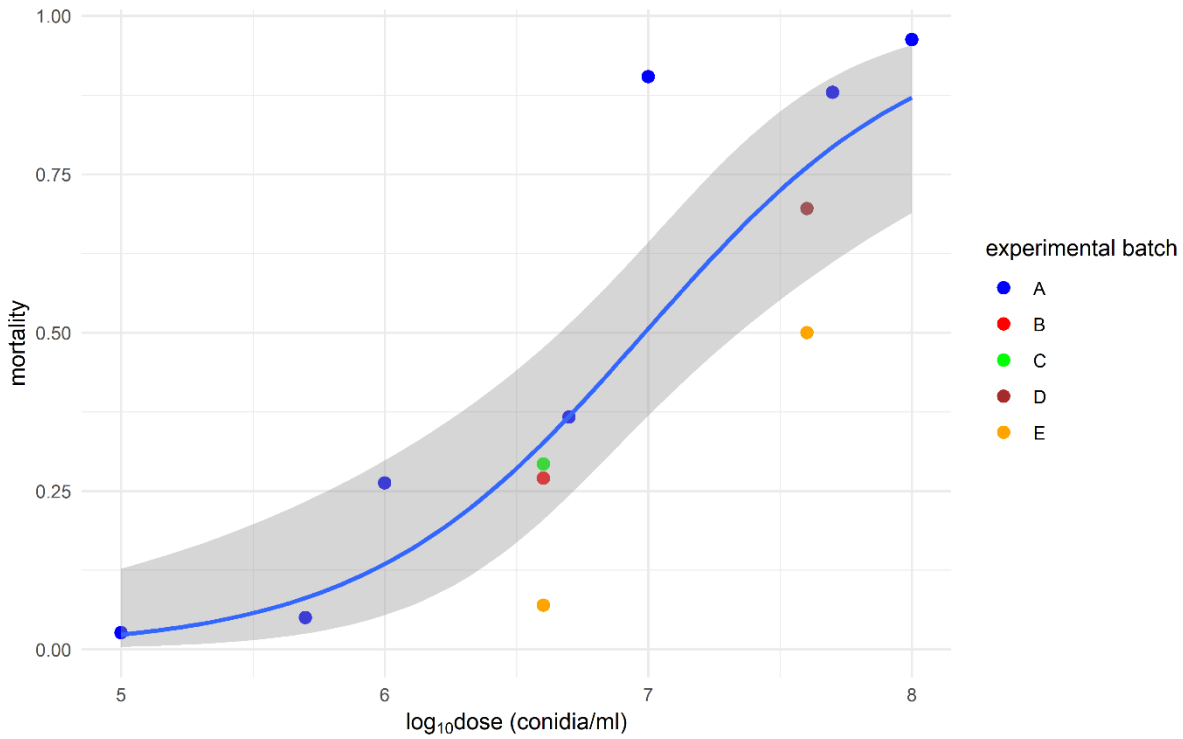



*Figure 3 Proportion mortality of M. persicae after 6 days of exposure to A. muscarius at different tested concentrations. Points represent pooled cumulative mortality recorded from individual aphid cups. Colours represent different aphid batches (blocks). A quasibinomial regression model (blue line) was fitted to predict the dose response. Grey area represents 95% confidence intervals.*

The dose-response bioassay resulted in repeatable mortality for doses spread in a wide range (Figure 3). The variation within doses could be attributed to the differences in aphid batches and the physiology of cabbage leafs cut from plants.

## Discussion

The described micro-sprayer provides a relatively cheap alternative to commonly used laboratory spraying equipment in foliar testing of microbial agents or pesticides. In comparison to the Potter spray tower, the advantages of the design includes inexpensive spare parts, portability, and usage in laboratories with restricted space. The most expensive parts of the presented setup are the compressor and the airbrush. The cost of the acrylic tube and the PVC sheet including the CNC machining of tower parts (four replicates) was under $600. These costs could be further reduced by material selection. The specifications of the tower (height, diameter) could easily be adjusted to suit individual preferences. The airbrush used here has numerous feed and nozzle attachements that can hold higher load volumes and produce different spray patterns (https://www.iwata-airbrush.com). However, the tower parts can fit, or can be modified to fit other similar airbrushes. Limitations related to the range of applied pressure was observed due to the low weight of aphids tested in our bioassays. Such constraints should not arise with heavier targets or applications conducted on leaf.

The design described here can be adjusted to accomodate the needs of most laboratories aiming to conduct microbial agent or pesticide testing without investing in expensive kit or restricting considerable space for stationary equipment. Further improvements were made by attaching a vacuum filter, to reduce contamination when opening the chamber. Alternatively the sprayer can be operated in a fumehood. The Computer Aided Design (CAD) files that can be used for 3D printing, CNC routing or modification to user requirements are available as supplementary materials (Erdos et al., 2020).

## II: Controlling insecticide resistant clones of the aphid, *Myzus persicae*, using the entomopathogenic fungus *Akanthomyces muscarius*: Fitness cost of resistance under pathogen challenge


### Abstract

Biological control is a cornerstone of Integrated Pest Management and could also play a key role in managing the evolution of insecticide resistance. Ecological theory predicts that the fitness cost of insecticide resistance can be increased under exposure to invertebrate natural enemies or pathogens and can therefore increase the value of integrating biological control into pest management. In this study of the peach potato aphid, *Myzus persicae*, we aimed to identify whether insecticide resistance affected fitness and vulnerability of different aphid clones to the entomopathogenic fungus *Akanthomyces muscarius*. Insecticide resistant clones were found to be slightly less susceptible to the pathogen than susceptible clones. However, this pattern could also be explained by the influence of length of laboratory culture, which was longer in susceptible clones and was positively correlated with susceptibility to fungi. Furthermore, resistance status did not affect aphid development time or intrinsic rate of increase of aphids. Finally, in a cage trial the application of fungus did not increase the competitive fitness of insecticide resistant clone 'O'. We found no fitness cost in reproductive rate or pathogen susceptibility associated with chemical resistance in *M. persicae*. In contrast, some susceptible clones, particularly those subject to decades of laboratory rearing, showed enhanced susceptibility to a fungal pathogen, but not reduced reproductive fitness, an observation consistent with down-regulation of costly immune functions in culture. Overall, fungal pathogen control is compatible

with insecticides and should not increase the selection pressure for resistance of *M*. *persicae* to chemical insecticides.

## Introduction

The development of a sustainable and more efficient crop production system is necessary to feed the rising human population. Pest management is a key issue for crop production with reports of up to 59% losses in yield of major crops worldwide due to herbivores, pathogens and weeds (Oerke, 2006). The intrinsic resistance in wild plants against pathogens and pests is lacking in domesticated crops, as conventional breeding programs can result in loss of resistance traits (Chen et al., 2015; Tamiru et al., 2015). Modern agricultural practices since the 1960s have relied on the intensive use of synthetic chemical pesticides to control crop pests, which helped increase yields significantly (Pretty, 2008). However, growing concerns towards their adverse effects on humans (Damalas & Eleftherohorinos, 2011; K.-H. Kim et al., 2017) and the environment (Geiger et al., 2010; Rundlöf et al., 2015; Tilman, 1999; Tilman et al., 2002) is prompting regulators to impose restrictions on their use.

Insecticide resistance provides a competitive advantage under insecticide selection pressure to those individuals that express the trait, however mutations conferring resistance can lead to evolutionary trade-offs that cause fitness penalties (Ffrench-Constant & Bass, 2017; Kraaijeveld et al., 2002). The mechanisms evolved to overcome the effects of chemical insecticides can have pleiotropic consequences on their fitness in particular environmental conditions (Foster et al., 2000), or when under attack by natural enemies (Foster et al., 2011; Foster et al., 2005) or pathogens (Pettis et al., 2013). This effect can be positive, for example when the resistance mechanism evolved to chemical insecticides also protects against a pathogen (*i.e.* cross-resistance). This effect can also be negative, when the increased resistance towards insecticides results in a 'fitness cost'. This fitness cost can manifest in a reduced ability in reproduction, survival

or can hinder the competitive ability of the resistant individual in the absence of the insecticide (Kliot & Ghanim, 2012). The loss of fitness may be even more severe in the presence of a pathogen (Kraaijeveld et al., 2012). A number of studies have shown increased fitness costs of resistance to the entomopathogenic bacterium *Bacillus thuringiensis* in *Plutella xylostella* (reduced egg fertility) and *Pectinophora gossypiella* (greater larval mortality) when under challenge from other pathogens (Gassmann et al., 2009), including baculovirus (Raymond et al., 2007) and nematodes (Gassmann et al., 2008). An enhanced fitness penalty - in which insecticide-resistant individuals had higher susceptibility to an entomopathogen than insecticide-susceptible individuals - could be exploited to reduce the frequency of resistant individuals in a population, thereby prolonging the effective 'shelf life' of the insecticide (Ambethgar, 2009; Gassmann et al., 2009). Thus, for Integrated Pest Management (IPM) programs that use both synthetics and biologicals, there may be resistance management benefits arising from using entomopathogens in rotation or in spatial mosaics with chemicals. However, we cannot assume that entomopathogens will always select against insecticide resistant genotypes- the consequences of resistance must be checked through experimentation.

Entomopathogenic fungi (EPF) have been used as a biological control agent in sustainable agriculture for decades. There are approximately 750 fungal species described with pathogenic activity against insects and mites (Sinha et al., 2016). Ascomycete fungi within the order Hypocreales, such as *Beauveria spp., Akanthomyces spp.* and *Metarhizium spp.* have been well-studied and used world wide as environmentally friendly biological pest control solutions (Hesketh et al., 2008; Humber, 2008). Host insects are infected by fungal propagules that penetrate the cuticle (Ortiz-Urquiza & Keyhani, 2013). This makes EPF ideal

candidates for the control of aphids and other sap-feeding insects that cannot be effectively controlled by microbial control agents that infect via ingestion. Fungal pathogens are becoming more established with an increasing number of strains being developed as mycoinsecticides and mycoacaricides to control greenhouse pests (Faria & Wraight, 2007; Wraight et al., 2017b). However, their potential as tools in resistance management is not yet fully explored.

*Myzus persicae* Sulzer (Hemiptera: Aphididae), the peach-potato aphid, is one of the most economically important crop pests in temperate regions of the world; methods for controlling this species rely almost solely on the application of chemical insecticides (Devonshire, 1998; Karagounis et al., 2006). The intensive use of insecticidal substances has led to the development of resistance to most classes of insecticides making *M. persicae* one of the most widely resistant insect species (Bass et al., 2014; Skinner et al., 2014). Resistance monitoring of *M. persicae* suggests that pirimicarb and pyrethroid resistance is dominant in aphid populations across the United Kingdom (Agriculture and Horticulture Development Board, 2019). Currently, there are seven distinct resistance mechanisms described in *M. persicae* (Bass et al., 2014). These include: the enhanced production of carboxylesterases conferring resistance to organophosphates (OP) and carbamates (Devonshire et al., 1983; Field et al., 1988); modification of the acetylcholinesterase enzyme (MACE) leading to insensitivity to dimethyl carbamates and pirimicarb (M. C. Andrews et al., 2004; Moores et al., 1994); target-site resistances due to mutation of the voltage-gated sodium channel, termed “knockdown resistance” (kdr) and “super-kdr” conferring resistance to pyrethroids (Eleftherianos et al., 2008; Martinez-Torres et al., 1999); mutation or duplication in a ligand-gated chloride channel responding to ɣ-aminobutyric acid (GABA) leading to resistance to cyclodienes (Ffrench-Constant

et al., 2000); overexpression of the P450 gene CYP6CY3 (Puinean et al., 2010) and mutation of the nicotinic acetylcholine receptor (nAChR) conferring resistance to neonicotinoids (Bass et al., 2011); and reduced penetration through the cuticle (Puinean et al., 2010).

The genetic basis for insecticide resistance involves genes associated with the increased production of metabolic enzymes linked with detoxification processes or alteration of insecticide receptor binding sites (Bass et al., 2014). The disruption caused by these genetic changes is often linked with fitness costs (*i.e.* traits that contribute to individual reproductive success) in this species, as in other insects (Ffrench-Constant & Bass, 2017; Kliot & Ghanim, 2012). For example, reported fitness costs for *M. persicae* include reduced overwintering capacity (Foster et al., 2000), reduced response to alarm pheromones (Foster et al., 2005; Foster et al., 1999) and an increased vulnerability to parasitoids (Foster et al., 2011). Further studies are needed to establish fitness costs of resistance in aphid individuals with a varying degree of resistance and individuals carrying a number of different resistance mechanisms (Ffrench-Constant & Bass, 2017).

The loss in effectiveness of chemicals due to the development of resistance and the growing concern about their negative effect on human health and the environment provides a strong incentive for stakeholders to work on finding alternatives as well as improving the efficacy of existing compounds (Chandler et al., 2011). Furthermore, recent regulatory bans and restrictions has led to few active substances being available for use to control aphids (European Commission, 2018a, 2018b, 2018c). Heavily relying on the few insecticides currently used for control can lead to an increase in selection pressure for resistance to evolve (Bielza et al., 2008). The ever-increasing costs, more stringent regulatory restrictions, and time and complexity of developing new

insecticides (Lamberth et al., 2013; Maienfisch & Stevenson, 2015; Sparks & Lorsbach, 2017) means that an effective insecticide resistance management (IRM) system is critical to preserve the utility and investment in present and future arthropod pest control (Sparks et al., 2020). Biological solutions can play an important part in IRM especially if pathogens such as fungi can enhance the fitness discrepancies between resistant and susceptible insects (Raymond et al., 2007).

In this study, we test whether resistance mechanisms evolved in response to insecticide use in *M. persicae* carry a fitness cost in terms of increased susceptibility to EPF. We use the pathogen *Akanthomyces muscarius*, (formerly *Lecanicillium muscarium* | *Verticillium lecanii*) Ve6 (Kepler et al., 2017), a biocontrol agent used in the whitefly mycoinsecticide Mycotal®, that has shown virulence against aphid pests in controlled conditions (Mohammed & Hatcher, 2016). We hypothesize that chemical resistance carries a fitness cost, therefore resistant aphids will be more susceptible to EPF. Firstly, we test the susceptibility of genetically distinct lineages of *M. persicae* with varying degree of chemical resistance against the fungus in a set of standardized bioassays. Secondly, we investigate the fitness of insecticide resistant and susceptible (hereafter referred to as “resistant” and “susceptible”, respectively) aphids under pathogen challenge in a competition assay. The *M. persicae* clones used in the study have been in captivity for different lengths of time. We therefore investigate whether a different degree of adaptation to laboratory conditions has an effect on aphid mortality and survival under pathogen challenge. Life-history data on development and fecundity of *M. persicae* lineages were collected to further characterize any differences in reproductive fitness associated with resistance.

## Methods

### Insect cultures

Populations of *Myzus persicae* were reared in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) on chinese cabbage, *Brassica pekinensis* plants at BBCH growth stage 13-15. Colonies were sub-cultured as required by transferring 5-15 apterous adults onto fresh plants. *M. persicae* individuals of defined age were produced by confining cohorts of mature apterous virginoparae in clip cages on chinese cabbage plants (BBCH 13-15) for 48 hours to produce progeny. Adults were then removed and the nymphs maintained for further nine days. Populations were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle. Rearing efficency is higher at 24±1°C, however, bioassays were carried out at 20±1°C, which is more representative for pest populations in the United Kingdom. Clones of *M. persicae* used in the study are shown in Table 2.

*Table 2 M. persicae clones used in the study. Resistance mechanisms: knockdown resistance (Kdr); super-knockdown resistance (sKdr); Overexpression of cytochrome P450 CYP6CY3 (CYP6CY3); Modified acetilcholinesterase enzyme (MACE); nicotinic acetylcholine receptor mutation (R81T).*

| Clone | Country of origin | Year of collection | Resistance mechanism |
|---|---|---|---|
| 4106A | UK | 2000 | - |
| 4255A | UK | 2000 | - |
| 5557 | Germany | 1967 | - |
| 1X | Italy | 1996 | - |

| Clone | Country of origin | Year of collection | Resistance mechanism |
|---|---|---|---|
| US1L | UK | 1974 | - |
| Chilpot | Chile | 2018 | - |
| 92H6 | Italy | 2010 | Kdr, sKdr, CYP6CY3 |
| 5191A | Greece | 2007 | MACE, CYP6CY3 |
| O | UK | 2007 | MACE, sKdr, CYP6CY3 |
| 5666A | France | 2009 | R81T, Kdr, sKdr, CYP6CY3 |
| 5444B | Italy | 2011 | R81T, Kdr, sKdr, CYP6CY3 |

**Fungal cultures**

Five grams of wettable powder formulation of Mycotal (Koppert, NL) containing *A. muscarius* spores was dissolved in 10 ml sterile water. 100 µl of the homogenized suspension was spread on Sabouraud dextrose agar (SDA) in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) and incubated in darkness at 23±1 °C for 10-14 days. Conidial suspensions were prepared by agitating mycelia with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK) in 10 ml of 0.01% V/V Triton X-100. The suspensions were then passed through sterile cheesecloth to remove any hyphal fragments. Suspensions were enumerated using Fastread102 counting chambers (Kova International, USA) and adjusted to the required concentration in 0.01% V/V Triton X-100. Applications for the competition assay were carried out by dissolving Mycotal in sterile water and directly applying according to the label rate.

**Laboratory bioassay of *M. persicae* susceptibility to *A. muscarius***

The median lethal concentration ($LC_{50}$) for *A. muscarius* was calculated as $4x10^6$ conidia/ml for the aphid clone O based on a dose response bioassay using seven doses and 18-20 insects per dose (Erdos et al., 2020). Furthermore, 20-22 even-aged adult apterous aphids were treated in a 55 mm Petri dish with the $LC_{50}$ of *A. muscarius* conidial suspension. Fungal suspensions were applied with a microspray tower consisting of an acrylic cylindrical tube, a top cap and artist airbrush (HP-SBS ECL3500 with standard nozzle, Iwata), similar to those in other EPF studies (Mascarin et al., 2013; Spence et al., 2020). Precise specifications and validation of the consistency of this set-up have been described previously (Erdos et al., 2020). The volume and pressure settings after calibration were determined as 400 µl and 83 kPa. Bioassays were carried out at $4x10^6$ and $4x10^7$ conidia/ml resulting in an applied dose of 615 and 1237 spores per $mm^2$, respectively. These doses correspond to the previously determined $LD_{50}$ and $LD_{90}$, respectively. Initial experiments used all the clones in Table 1, except for 'Chilpot'. After treatment, aphids were maintained on single leaves of Chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. The petiole of the leaves were placed in water with a nutrient bead (Osmocote Controlled Release Plant Food, Greenfingers, UK). Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated on SDA agar and observed for fungal outgrowth to confirm death attributed to fungal infection. Treatments were replicated at least 3 times. Controls were treated with sterile carrier (0.01% Triton-X-100).

**Laboratory bioassay: effect of adaptation to laboratory rearing**

The susceptible aphid clones used in this study have been maintained in the laboratory for more than double the time of resistant clones on average (Table 1). In order to empirically test whether time spent in laboratory rearing has an effect on the susceptibility of *M. persicae* to *A. muscarius*, a set of standardized bioassays were carried out using an additional recently captured susceptible aphid clone 'Chilpot' against our standard resistant clone 'O'. Bioassays were conducted as described above, using 20-22 even-aged adult apterous aphids and two previously described doses of *A. muscarius*. Treatments for each clone were replicated five times for the lower, four times for the higher dose.

**Competition assay**

A competition experiment was carried out in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) with whole Chinese cabbage plants in order to test whether the differences in pathogen susceptibility observed in bioassays translated to differences in competitive fitness over multiple generations in the presence of Mycotal®. Cages were set up with initial 15-15 even-aged apterous adults of susceptible (1X) and resistant (O) *M. persicae* genotypes. Treatment of Mycotal® were delivered with an artist airbrush (HP-SBS ECL3500 with standard nozzle, Iwata) at a 20 cm distance directly on the cabbage plant. The applied dose was 3 ml of suspension with a concentration of $2.7 \times 10^7$ spore/ml per plant and was applied three times at intervals of 7 days. Controls were treated with sterile carrier (0.01% Triton-X-100). The cages were maintained in a plant growth chamber at 20±1 °C, 70 % humidity and a 14:10 L:D regime. Duplicates were run simultaneously under the same conditions. After 22 days 2$^{nd}$ and instar nymphs were picked randomly from the plant and frozen at -80 °C individually in wells of

96 well plates. Frozen nymphs were used in high-throughput detection of a resistance mutation.

### High-throughput detection of knockdown resistance in *Myzus persicae* using allelic discriminating quantitative PCR

DNA extractions of sampled aphid nymphs were carried out by adding 50 µl of 300mM sucrose solution (0.3M sucrose, 0.3M NaCl, 60mM Tris-Cl pH 8) to each well, homogenizing using TissueLyser II (Qiagen) and boiling in a shallow water-bath for 9 minutes. The 96 well plates were centrifuged at 4000 rpm for 2 minutes and placed on ice for 5 minutes before transferring the supernatant DNA to a new 96 well PCR plate.

DNA samples were subjected to a high-throughput TaqMan assay (Anstead et al., 2004) to identify the frequency of resistant aphid clones. This is a PCR-based allelic discrimination assay utilizing fluorescent dye-labelled probes that specifically bind to the allele of the voltage-gated sodium channel gene with M918L (super-kdr) or the wild-type allele. The assays were run in 96 well plates with 12.5 µl reaction volumes. Each reaction contained 7.5 µl of TaqMan universal PCR master mix (Applied Biosystems, USA), 1.5 µl of fluorescent probe (M918L), 1.5 µl of sample DNA and 2 µl of nuclease free water.

### *Myzus persicae* development time and intrinsic rate of increase

Life history experiments tested whether resistance status affected reproductive fitness in *M. persicae* under controlled environment conditions. Apterous adults were placed individually on single leaves of Chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. The following day three of the newly produced nymphs were kept on the leaf, while the adult and the rest

of the progeny was removed. The time from birth to reproducing adult was recorded for the remaining three aphids. Development time was calculated for all aphid clones based on at least 4 replicate leaves.

The intrinsic rate of increase ($r_m$) was recorded the following 5-9 days counting the number of nymphs produced each day (per aphid) and calculated as described in Wyatt and White (1977)

$$r_m = (lnM_d \times c)/d$$

Where $M_d$ is the number of nymphs produced by the adult in the first *d* days of reproduction. The correction constant (c = 0.738) is an approximation of the total fecundity produced in the first days of reproduction.

**Statistical analysis**

Data analyses was carried out in R version 3.6.0 (R Core Team, 2019). Generalized linear models (GLM) with binomial error structure were used to analyze bioassay endpoint (day 7) data, survivorship analysis with Cox proportional hazard models ('Survival') were used to analyse mortality data collected daily (Therneau, 2020). Where model comparisons could not be made using F tests, due to the models have the same degrees of freedom, we compared explanatory power using the Aikaike Information Criterion (AIC) (Akaike, 1974). Frequency data from competition experiments were analyzed using Pearson's chi-squared test statistic. We tested the effect of adaptation to laboratory rearing (measured in years since capture) on susceptibility to *A. muscarius* using mixed model ANOVA and a set of standardized bioassays comparing the susceptibility of recently captured susceptbile and resistant clones. Aphid development data and intrinsic rate of increase was analyzed using

one-way ANOVA, comparisons were carried out using Tukey HSD with 95% confidence intervals.

## Results

### Standardized laboratory bioassay of *M. persicae* susceptibility to *A. muscarius*

No mortality due to fungal infection was observed prior to day three. Fungal treatment had a significant effect on aphid mortality on day four ($\chi^2 = 33.96$, *df* = 1, $p < 0.001$) and onwards. Mean control mortality after 7 days was 4.28% ± 1.54. Mortality of insecticide susceptible *M. persicae* was marginally higher at day 7 compared to resistant aphid clones ($\chi^2 = 4.9$, *df* = 1, $p = 0.028$, Figure 4B). We found no significant effect of individual clonal lineage on endpoint mortality when analyzed with GLM ($\chi^2 = 7.8$, *df* = 9, $p = 0.55$).

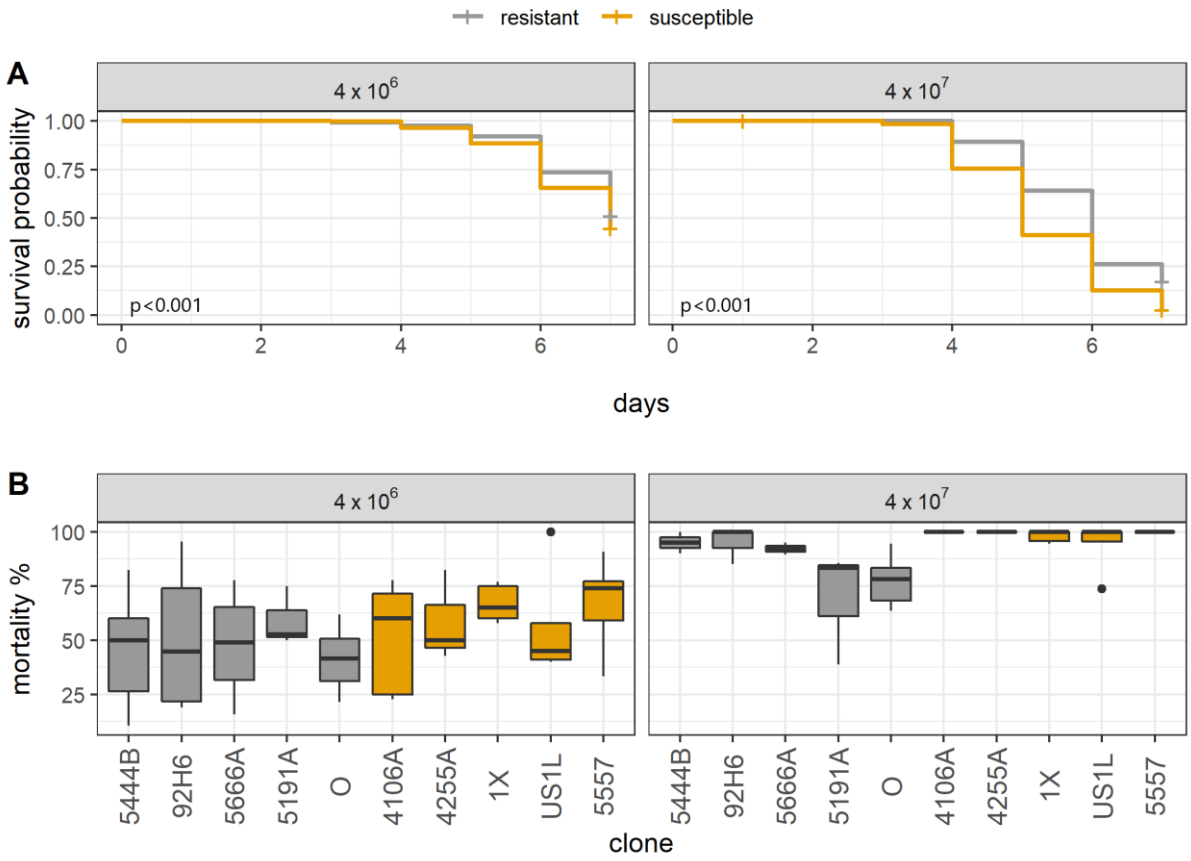



*Figure 4 Mean survivorship curves of insecticide resistant and susceptible aphids over 7 day after treatment with A. muscarius (A). Endpoint mortality of different*

*M. persicae clonal lineages after 7 days of exposure to A. muscarius (B). Panel headings indicate the concentration of fungal propagules (spores/ml). Boxplots showing median, first and third quartiles, whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are outliers. Clones are ordered according to year of collection.*

The insecticide susceptible *M. persicae* clones used in this experiment have been in laboratory rearing for longer than resistant aphids (Table 2). In order to understand whether the marginal difference seen in mortality is explained by adaptation to laboratory rearing, we evaluated whether number of years in laboratory rearing could explain the observed variation in mortality. The results indicate a possible negative effect of adaptation to laboratory rearing on aphid mortality in the bioassays ($\chi^2 = 3.3$, *df* = 1, p = 0.068). Based on the Akaike information criterion (AIC), the GLM with resistance as an independent variable explains variation slightly better than the same mixed model with time spent in laboratory rearing as an independent variable ($df_{resistance} = 75$, $AIC_{resistance} = 380.35$; $df_{time} = 75$, $AIC_{time} = 381.88$).

The survivorship analyses largely confirmed the results of the mixed models and showed a significant relationship between insecticide resistance and mortality due to EPF infection (Figure 4A), with resistant *M. persicae* having a lower risk of death (hazard ratio= 0.68) compared to susceptible aphids ($\chi^2 = 41.4$, *df* = 1, p < 0.001). Testing whether individual aphid genotype has an effect on mortality, we found clone O and 5191A to have a significantly lower hazard ratio compared to the other aphid clones (HR = 0.54; 0.51 respectively, likelihood ratio = 444.1, *df* = 10, p < 0.001). We found that time spent in laboratory rearing has an effect on aphid survival, with the more recently collected clones having lower hazard ratios ($\chi^2 = 20.8$, *df* = 1, p < 0.001).

In order to experimentally test the effect of laboratory adaptation on susceptibility to EPF we conducted standardised bioassays on a recently collected fully susceptible *M. persicae* (Chilpot) against a resistant genotype (O). We found no significant difference in endpoint mortality between the recently collected susceptible *M. persicae* and the resistant genotype ($\chi^2 = 0.043$, *df* = 1, p = 0.84, Figure 5). This finding is consistent with the hypothesis that adaptation to the laboratory rearing environment has a negative effect on the aphid's ability to survive infection by *A. muscarius.*

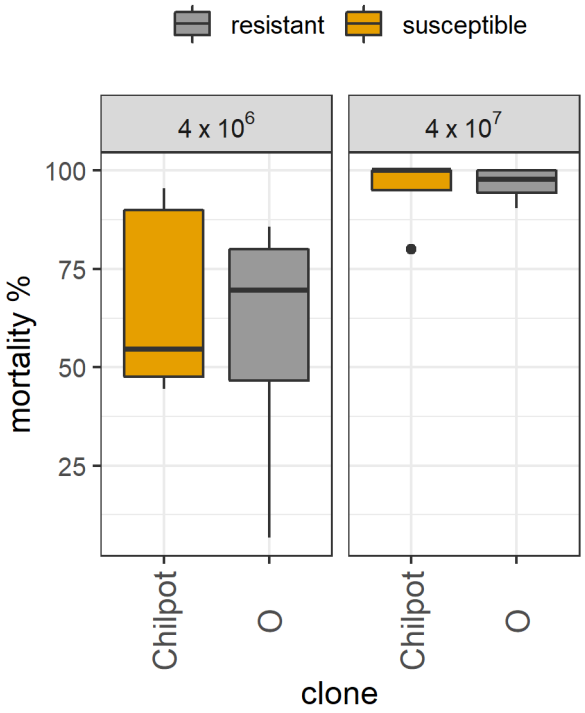



*Figure 5 Endpoint mortality of M. persicae clones Chilpot and O lineages after 7 days of exposure to A. muscarius. Panel headings indicate the concentration of fungal propagules (spores/ml). Boxplots showing median, first and third quartiles,*

*whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are outliers.*

**Competition assay**

TaqMan assays were effective at discriminating nymphs based on the prevalence of the M918L mutation (skdr). The proportion of resistant and susceptible clones of *M. persicae* changed significantly over the course of the experiment (Table 3) for both controls ($\chi^2$ = 139.4, *df* = 2, p < 0.001) and treatments ($\chi^2$ = 122.3, *df* = 2, p < 0.001). The resistant genotype clone O almost completely outcompeted the susceptible genotype 1X in all cages over three weeks regardless of fungal treatment. No significant difference in the proportions of resistant nymphs were found when comparing control and treated samples ($\chi^2$=6.4, *df* = 3, p = 0.094), suggesting that there is no significant fitness cost to infection with *A. muscarius*.

*Table 3 Proportion of resistant nymphs at endpoint of fitness competition experiment.*

| Treatment | Sample size | Proportion of resistant |
|---|---|---|
| Control 1 | 83 | 0.89 |
| Control 2 | 96 | 0.99 |
| Treatment | 93 | 0.97 |
| Treatment | 76 | 0.88 |

***Myzus persicae* clone life-history**

The development time of *M. persicae* clones used in this study were in the range of 5-9 days. We found no significant effect of resistance or adaptation to artifical rearing on development time ($F_{1,70}$ = 2.56, p = 0.11 and $F_{1,70}$ = 0.25, p =

0.62, respectively; Figure 6A). The intrinsic rate of increase ranged from 0.23 to 0.46 and similarly to development time, was not affected by insecticide resistance or adaptation to laboratory rearing in aphid clones ($F_{1,70} = 1.52$, $p = 0.22$ and $F_{1,70} = 0{,}015$, $p = 0.9$, respectively; Figure 6B). However, we found a significant difference in both development time ($F_{10,61} = 2.76$, $p < 0.01$) and intrinsic rate of increase ($F_{10,61} = 3.39$, $p < 0.01$) of different clones.

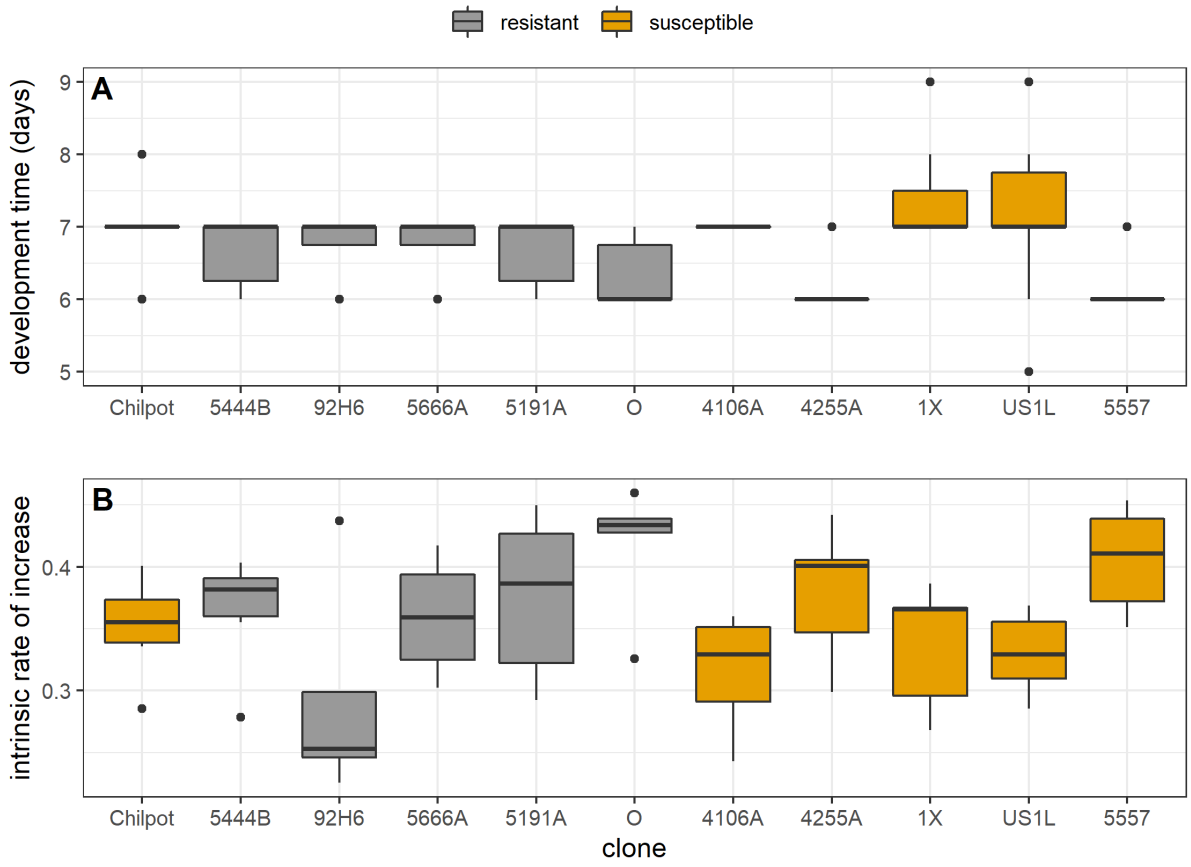



*Figure 6 Development time (A) and intrinsic rate of increase (B) of M. persicae clones used in the study. Clones are ordered according to year of capture.*

## Discussion

Our data reveal that both insecticide resistant and susceptible clones of *M. persicae* are vulnerable to *A. muscarius*, and control is not strongly compromised by pre-existing mechanisms of insecticide resistance. Contrary to our expectation, that insecticide resistant *M. persicae* would be more likely to succumb to fungal infection, we observed a marginally higher tolerance to entomopathogenic fungi in resistant aphids. The small effect size of resistance and the similarity in susceptibility of different *M. persicae* clones meant that there were no clear associations between fungal susceptibility and particular resistance mechanisms, suggesting that the observed slight cross-resistance between chemical resistance and tolerance to EPF is linked to minor polygenic effects, rather than a single effect of one major insecticide resistance gene. However, a wide range of secondary metabolites secreted by EPF might be impacted by the overexpression of cytochrome P450, glutathione S-transferase, nonspecific esterases and other detoxification enzymes that are overexpressed in resistant insects and can lead to improved tolerance to fungal entomopathogens (Butt et al., 2016; Serebrov et al., 2006).

Survivorship analysis revealed a similar relationship between insecticide resistance and susceptibility to EPF. We observed a small effect of cross-resistance akin to that detected via day seven mortality data described above. However, clones 5191A and O had significantly lower rates of death from fungal infection compared to the rest of the aphid clones. This effect was not obvious from only endpoint (day 7) mortality. Both clone 5191A and O were collected from the field in 2007 (tobacco and potato, respectively), carry the MACE mutation

which leads to insensitivity to pirimicarb (Bass et al., 2014), and overexpress the cytochrome P450 CYP6CY3, that metabolizes nicotine and detoxifies neonicotinoids (Bass et al., 2013). Clone O also carries a mutation in the voltage gated sodium channel that is essential for neural function (super-kdr).

Importantly the small differences shown in fungal susceptibility between resistant and susceptible clones did not mean that our focal insecticide resistant clone gained a fitness advantage in the presence of EPF over multiple generations. In our cage trials EPF application had no impact on the relative fitness of resistant clone O, showing that there is no substantive positive cross-resistance. The observed dominance of clone O in the competition experiment is most likely driven by the difference in life history traits of clones 1X and O (Figure 6). This difference in intrinsic rate of increase was only marginally significant for aphids on severed leaves (TukeyHSD, conf = 0.95, $p < 0.05$). Considering the timescale of the competition experiment, this small effect could have been augmented due to the multiple generations in this experiment and the increased power of competition experiments to identify fitness differences (Fry, 2003; Gassmann et al., 2009). Studying fitness cost under laboratory condition can be useful to identify specific traits with fitness deficits. However, these studies are limited as subtle fitness differences might only become apparent in field conditions, where hosts are exposed to environmental stress, compete for limited resources and are challenged by natural enemies (Ffrench-Constant & Bass, 2017; Kliot & Ghanim, 2012).

Years spent in artificial rearing had a significant effect on aphid survival when infected with entomopathogenic fungi. The insect immune defense is a costly evolved trait, which if not under selection can be reduced rapidly in artificial culture (McKean et al., 2008; Nystrand & Dowling, 2020; Schmid-Hempel, 2005).

The susceptible clones used in this study have been in artificial rearing for 21.4 years longer than resistant clones, on average. We did not observe a difference in susceptibility to EPF in the recently captured clone when tested against our standard resistant clone suggesting that the observed difference in susceptibility to EPF can be driven by adaptation to artificial rearing. In contrast, estimates of fitness from life-history experiments did not show that susceptible clones from long-term cultures had lower reproductive fitness, presumably because these traits are still under strong selection pressure in the laboratory.

Nevertheless, combining chemical pesticides with EPF to control aphids would still be beneficial in reducing the amount of chemicals used even if there is no fitness cost to exploit. The combination of biopesticides with different mode of action can potentially improve efficacy of individual components and also reduce the build-up of resistance. However, in order to combine a microbial biocontrol agent with chemical pesticides, first the effect of the chemical on different aspects of the biocontrol agent has to be evaluated *in vitro*. Several studies have investigated the inhibitory effect of chemicals on entomopathogenic fungi (Avery et al., 2013; Cuthbertson et al., 2008; Cuthbertson et al., 2012; Cuthbertson et al., 2005; Khun et al., 2020; Wei et al., 2004) and have shown that timing and order of application of the biocontrol agent and the chemical are paramount (Meyling et al., 2018; Rivero-Borja et al., 2018), while a range of different agrochemicals are potentially compatible with *A. muscarius* (previously *Lecanicillium muscarium*) and *Beauveria bassiana* spores (Cuthbertson et al., 2008; Cuthbertson et al., 2012; Cuthbertson et al., 2005).

*Akanthomyces muscarius* is currently available for commercial use for the control of whitefly and thrips in greenhouses. Our results show that regardless of pre-existing resistance mechanisms carried by *M. persicae*, all tested aphid

clones were found to be susceptible to treatment with *A. muscarius* Ve6 under controlled environmental conditions. The observed effect of cross-resistance between chemical insecticides and EPF is very small and unlikely to have a major impact on the prospect of biological control and is likely to be due to the confounding effect of length of laboratory culture. Furthermore, to test for any cost of resistance to insecticides in aphids, studies need to be carried out taking in to account the effect of adaptation to laboratory rearing.

## III: Experimental evolution as a tool for improving entomopathogenic fungi as biocontrol agents


### Abstract

Experimental passage has been used to successfully increase virulence of insect pathogens such as baculoviruses. Passage experiments with entomopathogenic fungi are relatively unexplored and showed mixed results at improving virulence. Here we adopted an approach based on social evolution and evolution of virulence theory using the fungal pathogen *Akanthomyces muscarius* and an aphid host *Myzus persicae,* selecting for speed of kill, pathogen yield and infectivity at different scales of competition. The speed of kill and yield selection treatments resulted in significant increases in virulence when compared to the ancestor. Gains in virulence were dose specific but also led to more rapid killing of an additional aphid species (*Brevicoryne brassica*) so increased virulence was not specific to the passage host. We also observed phenotypic change in sporulation, which is a key trait in microbial biocontrol. While maintaining sporulation throughout passaging has historically been challenging, we observed earlier sporulation and/or greater production of spores after selection in many lineages relative to the ancestor. Higher spore production did not trade-off against virulence and one lineage had both increased spore production and increased virulence. The current study contributes to a better understanding of the evolution of virulence of *A. muscarius* and highlights the potential of theory based experimental passage as a tool for improvement of biocontrol agents.

## Introduction

The negative impacts of intensive use of synthetic pesticides are apparent on human health, and the environment. Toxicity to vertebrates (Costa, 2017; Tang et al., 2018; Wenchao et al., 2018) and non-target organisms (Mahmood et al., 2016) are well known, as well as the negative impact on biodiversity (Habel et al., 2019; Riley et al., 2018). The evolution of resistance in natural populations (Singh et al., 2021) and the more stringent regulations imposed by legislators (European Commission, 2018a, 2018b, 2018c) accentuates the need of finding alternative pest management methods and improving existing practices to counteract the economic loss due to crop damage caused by pests and pathogens (Oerke, 2006). The use of insect pathogens in pest management systems are well documented (Lacey et al., 2015; Wraight et al., 2017a). Entomopathogenic fungi (EPF) have been used world-wide to control invertebrate pests both in greenhouses and on field, showing great potential in becoming a key tool in pest management systems.

Fungi possess numerous desirable traits that should promote their use as biological control agents. They pose minimal risks to non-target and beneficial organisms such as bees, earthworms and arthropod natural enemies including parasitic wasps and predatory beetles (Lacey et al., 2015). However, their short shelf life, inconsistent field performance, issues with quality control, lack of awareness within end users, and relatively higher costs compared with conventional pesticides have prevented adoption to mainstream use (Arthurs & Dara, 2018). Research towards improving EPF as biocontrol agents is diverse, ranging from: isolation of novel strains; improved spore application methods and

formulations; and genetic engineering (Fang et al., 2012; Jaronski, 2010a, 2010b; Jaronski & Mascarin, 2017; Lovett & St Leger, 2018). A relatively unexplored area of research with these insect pathogens is selection for virulence traits using experimental evolution. An increase in virulence could lead to lower doses needed for sufficient control, which could make the use of mycopesticides more cost-efficient.

Experimental evolution is a powerful tool in studying the factors that shape the virulence of pathogens. Carefully designed studies with conditions (such as environmental, genetic or demographic) imposed by researchers can lead to evolutionary changes in experimental populations that informs us about underlying evolutionary mechanisms (Kawecki et al., 2012). It can be used to study processes that influence the emergence of certain phenotypes and can test predictions based on theoretical work (Elena & Lenski, 2003; Rafaluk et al., 2015). The most effective study systems are those with short generation time and large population sizes (Elena & Lenski, 2003). Experimental evolution can be carried out in the form of serial passage experiments. With respect to symbiont or pathogens, this technique involves the sequential infection of the microbe from one host to another and the comparison of their evolved characteristics to the ancestral genotype (Ebert, 1998). *In vivo* experiments can be used to study coevolution of host and pathogen, or study one-sided selection by using naïve hosts (Rafaluk et al., 2015). Invertebrate pathologists use passage experiments in order to achieve increased pathogen virulence or modify host range. The results of simple passage experiments for improving the virulence of insect pathogens are not consistent (Chapuis et al., 2011; Valero-Jimenez et al., 2017) and would benefit from a theory based approach (Raymond & Erdos, 2022). Successful passage experiments with biocontrol agents to date have mostly used

viral pathogens of invertebrates, such as baculoviruses (Berling et al., 2009; Kolodny-Hirsch & Van Beek, 1997; Maleki-Milani, 1978). The success of simple passage of baculoviruses can be partly explained by the high genetic diversity found in natural populations (Shapiro et al., 1992; Thézé et al., 2014) but also because competitive ability within hosts may be correlated with desirable life history traits such as virulence in these pathogens (Raymond & Erdos, 2022).

The study of virulence evolution dates back to Pasteur's classic experiments on the attenuation of *B. anthracis* (Pasteur et al., 1881), which contributed to the establishment of "avirulence theory" (T. Smith, 1904), the hypothesis that pathogens will tend to evolve to be less virulent. In contrast, 'Classical' or 'trade-off' theories of virulence emphasize that pathogens need to gather host resources to replicate and achieve transmission (Alizon et al., 2009; Anderson & May, 1982; Gandon et al., 2001). Classical theory posits that selection should primarily act on transmission and should act to increase virulence only in so far as it increases transmission. Selection will also decrease virulence if it results in increased transmission rates due to longer duration of infection or reduced immobilization of hosts (Ebert & Bull, 2003). Such a trade-off would optimize transmission rates at intermediate virulence (Ewald, 1983). In classical models higher replication rate within host is also assumed to lead to increased virulence, with the faster growing genotype having a competitive advantage (S. P. Brown et al., 2002; Gandon et al., 2001).

For entomopathogens that are obligate killers such as *Akanthomyces muscarius* - the organism used in this study - speed of kill is an important component of virulence that is also a desirable biocontrol trait. Since death of hosts is essential for transmission, the normal trade-offs on intermediate virulence do not apply (Alizon & Michalakis, 2015). Nevertheless, there can still be trade-off in terms of

the timing of host death. For instance, trade-offs between pathogen yield and speed of kill have been observed in numerous pathogen-invertebrate systems (Bérénos et al., 2009; de Roode et al., 2008; K. H. Jensen et al., 2006; Redman et al., 2016): increasing speed of kill tends to reduce pathogen yield (the production of infectious propagules).

An alternative theoretical framework termed ‘kin selection’ or ‘social evolution theory’, originally developed for cooperative behaviour, can explain how different fitness components are favoured at different scales of selection (Buckling & Brockhurst, 2008; Hamilton, 1964a; West et al., 2006). Cooperative public goods benefit communities but are costly for individuals. Many virulence factors produced by micro-organisms behave in such a way. However, public goods virulence factors create the possibility of cheating, where an individual can gain a fitness advantage (such as increased replication rate) by freeloading on the products of others and not producing the virulence factors themselves (Raymond et al., 2012; West & Buckling, 2003). The production of public goods virulence factors might increase infectivity or pathogen yield but can be costly to produce and can reduce replication rate within host, which goes against classical virulence theories. Cry toxins produced by *Bacillus thuringiensis* and iron-scavenging siderophores produced by *Pseudomonas aeruginosa* are amongst public goods virulence factors that have been studied in insect models (Harrison et al., 2006; Raymond et al., 2012). Social cheating has been observed in both bacterial (Amanatidou et al., 2019; Diard et al., 2014) and in viral pathogens (Serrano et al., 2013; Simón et al., 2004) in a range of hosts.

The scale at which competition between pathogen genotypes happen can play a key role for the evolution of virulence. Within host competition is expected to

select for genotypes with high replication rate. Simple passage which selects for rapid within host replication can lead to increased virulence in many viral pathogens (Ebert, 1998), but can select for loss of costly virulence factors in social pathogens such as *B. thuringiensis* (Raymond et al., 2012). Competition between hosts can lead to selection for pathogen yield or increased infectious propagule production if virulence factors are involved in nutrient acquisition (siderophores) (Griffin et al., 2004). Competition between groups of hosts infected by populations of pathogens could select for maintanence of virulence factors as seen with Cry toxins, where the only function of the trait is to increase infectivity (Raymond & Crickmore, 2020).

Moreover, passaging for improved virulence can have other fitness implications that carry consequences for use in biological control. One evident example is the loss of sporulation following serial passage. Spore production was reduced in *Metarhizium anisoplea* following serial passage through ants (Hughes & Boomsma, 2006). The passage of *Ascosphaera apis* through the honey bee resulted in faster sporulation but lower total number of spores produced (Evison et al., 2015). Maintaining efficient sporulation is essential for the development of successful formulations used in biological control (Jaronski & Mascarin, 2017). For example, if gains in virulence trade-off against spore production then this will have little benefit for biocontrol manufacturers who may face increased production costs. Abiotic conditions, such as temperature, have long been known to be of importance for germination, growth and development of the fungal pathogen both *in vitro* and *in vivo* and have important implications for biological control (Benz, 1987; Yeo et al., 2003). Little is known about how selection for virulence in vivo affects the ability of the fungus to grow at different temperatures.

Besides sporulation, the ability of fungal strains to grow in artificial media is key for upscaling for industrial application.

*Akanthomyces* is a genus of fungi known to infect and kill arthropods (Gams & Van Zaayen, 1982; Kepler et al., 2017). The fungal infection cycle involves spore adhesion, germination, differentiation of infection structures, penetration of cuticle, colonization of hemocoel and sporulation following emergence from mycosed cadaver. This complex cycle of pathogenicity involves numerous virulence factors (Butt et al., 2016) and suggests that naïve passaging of the fungus through its host might not necessarily increase virulence. The aim of the present study is to improve the efficacy of *A. muscarius* as a biocontrol agent and further our understanding of how different selection regimes impact the virulence and life-history traits of *A. muscarius*. First, we carried out a passage experiment by selecting for different traits and altering the intensity of selection at different scales: selecting individual cadavers based on speed of kill, using between host pooling of cadavers to select for increased spore production of pathogens and between population competition based on infectivity. We hypothesized that changes in virulence would follow either classical trade-off or public goods models. For example, selection regimes focused on individual cadavers would produce virulent mutants with faster growth rates and reduced spore production, while selection based on yield or infectivity would be more likely to select for costly public good modes of virulence.

After selection we assayed changes in virulence and fungal life history, using in vitro growth and sporulation assays. Finally, we report on any changes in the thermal profile of the fungus due to serial passaging by carrying out growth assays at a range of temperatures in vitro.

## Methods

### Insect cultures

Populations of *Myzus persicae* and *Brevicoryne brassicae* were reared in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) on chinese cabbage, *Brassica pekinensis* plants at BBCH growth stage 13-15. Colonies were sub-cultured as required by transferring 5-15 apterous adults onto fresh plants. Individuals of defined age were produced by confining cohorts of mature apterous virginoparae in clip cages on chinese cabbage plants (BBCH 13-15) for 48 hours to produce progeny. Adults were than removed and the nymphs maintained for further nine days. Populations were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle.

### Fungal cultures

The ancestral strain of *A. muscarius* was isolated from Mycotal (Koppert, NL) and spread on Sabouraud dextrose agar (SDA) in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) and incubated in darkness at 23±1 °C for 10-14 days. During passage experiments fungi were propagated by plating surface sterilized aphid cadavers on SDA plates and incubating as described above. Conidial suspensions were prepared by agitating mycelia with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK) in 10 ml of 0.01% V/V Triton X-100. The suspensions were then passed through sterile cheesecloth to remove any hyphal fragments. Suspensions were enumerated using Fastread102 counting chambers (Kova International, USA) and adjusted to the required concentration in sterile water with 0.01% Triton X-100.

**Optimization of UV-C Mutagenesis**

Conidial suspensions were prepared by gently scraping the mycelium of a 10-14 day old culture with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK) in 12 ml of 0.01% Triton X-100. The suspensions were then passed through sterile cheesecloth to remove any hyphal fragments. UV treatments were carried out in a biological safety cabinet, utilizing the integrated disinfection UV lamp (36W) at a distance of 74 cm from the plates. The radiation of the lamp was stabilized by switching it on 30 min prior to exposure. Aliquots of 10 ml spore suspension were exposed to UV-C radiation for 0, 45, 60 and 90 seconds in 90 mm Petri dishes without lids. Treatments were replicated three times. 100 µl of conidial suspension were spread on SDA plates and incubated at 23 °C for 5 days. Control and 60 seconds treatments of the experiment were additionally repeated at another time point. Conidial lethality ratios were calculated as: (1 – (number of colonies on radiation-treated plates/ number of colonies on control plates)) x 100%. Conidial lethality rates increased with prolonged exposure time. Exposure for 45, 60 and 90 seconds resulted in mean conidial lethality of 33 ± 5.7, 50.4 ± 1.7 and 82.9 ± 1.7% respectively.

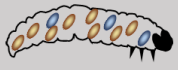
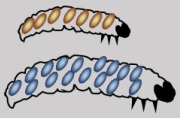
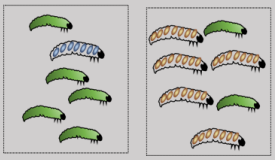
***In vivo* selection protocol**

Even-aged young adults of *M. persicae* were split into three treatments: between population selection for infectivity, between host selection for speed of kill and between host selection for yield (from here on referred to as: infectivity, speed and yield selection). All treatments were initially infected with the ancestor *A. muscarius* Ve6. The infectivity treatment had four replicates with four subpopulations in each replicate, while the speed and yield treatments were initiated with 8 replicates. Each subpopulation for the infectivity treatment

contained 13 adults resulting in 208 aphids per treatment. Each replicate for speed and yield treatments contained 13 adult aphids resulting in 104 aphids per treatment. The passage protocol for each treatment and their expected outcome are summarized in Table 4. The cohorts of 13 aphids were sprayed with a precision micro sprayer in a 55 mm petri dish as described in Erdos et al. (2020). After treatment, aphids were maintained on single leaves of Chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated on SDA agar to propagate inoculum for the next round of passage. Serial passaging was carried out as follows: The infectivity selection treatment was propagated from the first aphid cadaver from the most successful subpopulation (highest % mortality at 7 days past infection). The speed selection treatment was passaged from the first aphid to succumb to fungal infection in each replicate, while the yield selection treatment was propagated from the first 3-4 mycotized aphid cadavers mashed in sterile 0.01% V/V Triton X-100 in an Eppendorf. All sterilized aphid cadavers and mashed inocula were left to sporulate on SDA plates for 10-14 days at 23±1 °C (Figure 1). Fungal suspensions of selected strains were made as described above. Seven rounds of passage were carried out for all treatments. In order to increase genetic variation, passage rounds 2, 4 and 6 included a step of random mutagenesis before spraying the next cohort of aphids. Random mutagenesis was carried out by exposing fungal inoculum to UV irradiation in a biological safety cabinet for 60 seconds resulting in 50% germination, as determined previously. Dose was adjusted after mutagenesis to compensate for conidia mortality (Figure 7). To control for adaptation to media, the ancestor strain of *A.*

*muscarius* was plated on SDA and transferred to a new SDA plate after 10-14 days for 7 consecutive transfers with 3 individually evolving replicates.

*Table 4 Selective passage protocol with their respective expected outcome.*

| Treatment | Speed of kill (between host) | Yield (between-host) | Infectivity (between-population) |
|---|---|---|---|
| Selection method | first single cadaver | 3-4 pooled cadavers | Most successful subpop (% mortality), single cadaver |
| Expected outcome | higher pathogen growth rate | higher pathogen population size | increased infectivity |
| |  Replication rate  |  Yield  |  Infectivity  |

The concentration of fungal suspensions used in the serial passage experiment varied. The aim was to achieve mortality between 25-50 %, however counting spores for all selected lineages was not feasible for each round of infection, therefore a standardized dilution factor was used across each treatment based on one randomly subsampled lineage. This dilution factor varied between passage rounds to correct for changes seen in percentage mortality across the experiment.

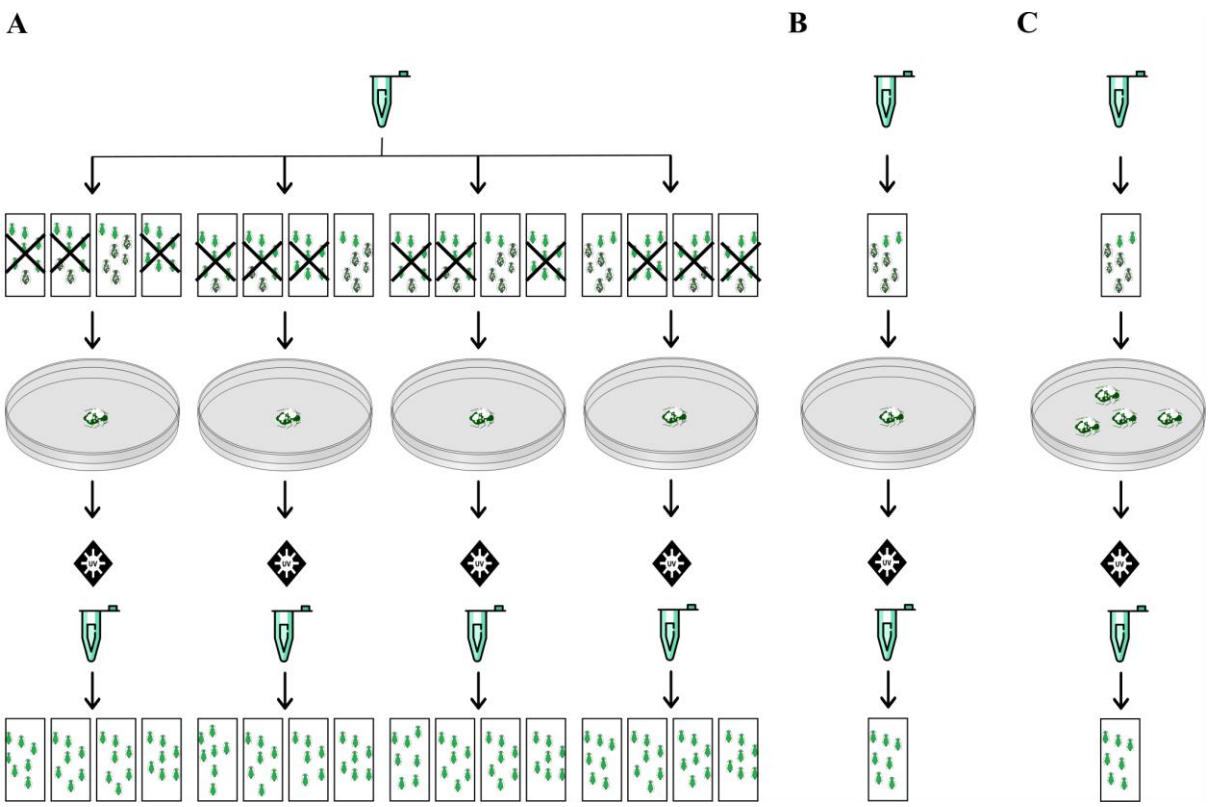



*Figure 7 Selective passage protocol*

*Rectangles represent individual bioassay chambers containing 11-13 adult apterous aphids on a single leaf of Chinese cabbage. Infectivity treatment (A): The first mycotized cadaver from the subpopulation with highest recorded mortality to propagate spores for next passage. Crossed out bioassay chambers are discarded from next round of passage. Speed treatment (B): The first mycotized cadaver is used to propagate the next round of infection. Yield treatment (C): The first 3-4 mycotized cadavers were pooled, homogenized and plated to propagate the next round of infection.*

**Endpoint assays - virulence**

Laboratory single dose bioassay of selected *A. muscarius* virulence towards *M. persicae*

18-20 adult apterous aphids were treated in a 55 mm Petri dish with a concentration of $3x10^6$ conidia/ml suspension of selected lines of *A. muscarius*. Fungal suspensions were applied with a microspray tower consisting of an acrylic cylindrical tube, a top cap and artist airbrush (HP-SBS ECL3500 with standard nozzle, Iwata), similar to those in other EPF studies; (Mascarin et al., 2013; Spence et al., 2020) precise specifications and validation of the consistency of this set-up have been described previously (Erdos et al., 2020). After treatment, aphids were maintained on single leaves of Chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated on SDA agar and observed for fungal outgrowth to confirm death attributed to fungal infection. Treatments were replicated 5 times. Controls were treated with sterile carrier (0.01% Triton-X-100). The bioassays were repeated in an additional aphid host, *Brevicoryne brassica* at $1x10^7$ conidia/ml and replicated 4 times.

Laboratory dose-response bioassay of selected *A. muscarius* virulence towards *M. persicae*

In order to further characterize the virulence of selected strains, concentrations of $1x10^6$, $1x10^7$ and $1x10^8$ conidia/ml suspension of the ancestral and derived strains BPI2, BHS3 and BHY3 were tested against *M. persicae*. Bioassays at these three doses were carried out as described above. Treatments were replicated 4 times and the whole experiment was repeated once. The high dose treatment for strains ancestor, BPI2 and BHY3 was repeated an additional time.

**Endpoint assays – fungal life history**

Mycelial growth rate

Radial growth of the ancestor and selected strains have been assessed by inoculating Sabouraud dextrose agar (SDA) in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK). Each Petri dish was split into four equal parts and inoculated in the centre of the quadrant with 7 µl of $3x10^6$ conidia/ml suspension. All plates were replicated three times and incubated at 22±1 °C in darkness for 8 days. Radial growth was measured for all sub-repetitions every day by two cardinal diameters drawn on the bottom of each plate. The thermal profile of selected lineages was also investigated by plating 10 µl of $1x10^6$ conidia/ml suspension on the centre of SDA plates and incubating at 15, 18, 22, 24, 28 ±1 °C in darkness for the following 11 days. All plates were replicated three times. Radial growth was measured every day (except day 9 and 10) by two cardinal diameters drawn on the bottom of each plate.

Spore production (pathogen yield)

Spore production of all fungal lines were assessed at 3 and 14 days post inoculation. SDA plates were inoculated by spreading 100 µl of $1x10^7$ conidia/ml suspension on each plate. The plates were incubated at 22±1 °C in darkness for 14 days. A sample of each plate was taken with a sterile cork borer after 3 days of incubation, suspended and vortexed in 1 ml of 0.01% V/V Triton X-100 in a 2 ml Eppendorf to recover spores. Spore production after 14 days of incubation was assessed by agitating mycelia with a 'L-shaped' spreader in 10 ml of 0.01% V/V Triton X-100. The samples were passed through sterile cheesecloth to remove any hyphal fragments and enumerated using Fastread102 counting chambers (Kova International, USA). The experiment was replicated at least three times using separate biological samples.

**Statistical analysis**

Data analyses was carried out in R version 3.6.0 (R Core Team, 2019). Conidial lethality data was analyzed using one-way ANOVA, comparisons were carried out using Tukey HSD with 95% confidence intervals. Generalized linear models (GLM) with binomial error structure and a logit link function were used to analyze bioassay endpoint (cumulative mortality) data with treatment and dose as fixed effects, experimental block as random effect. Pairwise comparisons were made using Tukey HSD with 95 % CIs. Survivorship analysis with Cox proportional hazard models ('Survival') were used to analyse mortality data collected daily (Therneau, 2020). Where model comparisons could not be made using F tests, due to the models having the same degrees of freedom, we compared explanatory power using the Aikaike Information Criterion (AIC) (Akaike, 1974). The relationship between colony radius and time was assumed to be linear. Colony growth rate was estimated from the slope of linear regression of colony radius on time. Spore counts of individual lineages were square root transformed and fitted to a linear model. Spore counts of selection treatments were square root transformed and fitted to a linear mixed effects model with lineage as a random effect.

# Results

## Selection

We carried out seven rounds of passage with *A. muscarius*, although all treatments received a standard inocula dilution at each round of infection, replicate level mortality varied between 25 and 80 % across the experiment as a whole. Not all replicates of the speed and yield treatments could be carried out until the final seventh round of passage due to extinction events. Four replicates went extinct in the speed treatment due to unsuccessful infection or infection not leading to host death within 7 days. Six replicates of the yield treatment were lost due to bacterial contamination following the pooling/homogenization of aphid cadavers (Figure 8). This contamination could not be cleared up with low concentration antibiotic SDA plates and was consistent in all lost replicates in this treatment. The infectivity treatment was the most consistent at producing mycotized cadavers.

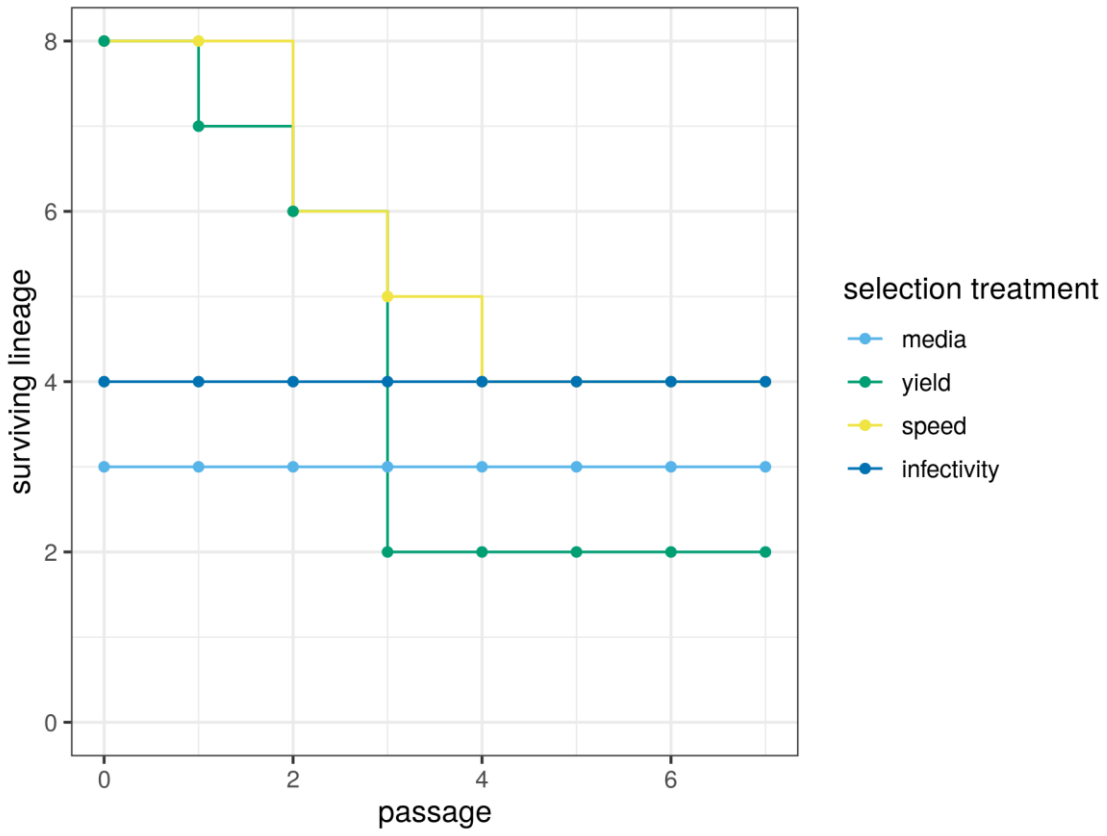



*Figure 8 Surviving lineages within each treatment by number of passage.*

**Endpoint assays - virulence**

Laboratory single dose bioassay of selected *A. muscarius* virulence towards *M. persicae*

No mortality due to fungal infection was observed prior to day four. Fungal treatment had a significant effect on aphid mortality on day four ($\chi^2 = 30.5$, $df = 5$, $p < 0.001$) and onwards. Mean control mortality after 7 days was 3.03% ± 2.9. The speed and yield treatments had a significant increase in virulence compared to the ancestor resulting in higher mortality after 7 days of exposure ($\chi^2 = 57.6$, $df = 5$, $p < 0.001$, Figure 9A). Survivorship analysis confirmed these results with significantly higher hazard ratios for the speed (1.84), yield (2.94) and infectivity

(1.78) treatments compared to the ancestor (1) (likelihood ratio = 159.5, df = 5, p < 0.001, Figure 9B).

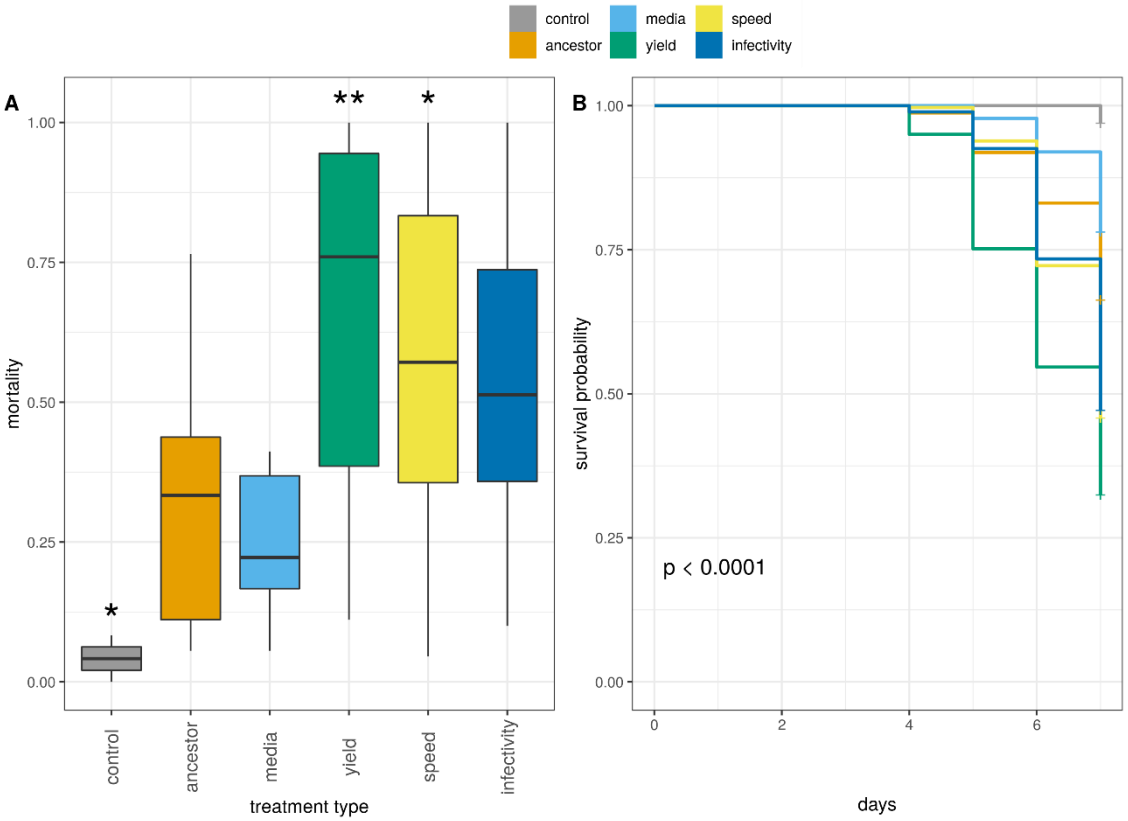



*Figure 9 Bioassays of the ancestor and the evolved lineages of A. muscarius after seven rounds of selection. Endpoint mortality of M. persicae after 7 days of exposure Boxplots showing median, first and third quartiles, whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are outliers (A). Mean survivorship curves of M. persicae after application of A. muscarius (B). Media: passage on SDA plates only. Significance levels denoted by asterisk are pairwise comparisons to the ancestor using Tukey HSD: **: p <= 0.01; *: p <= 0.05*

The response to selection was heterogeneous within all treatments. Lineages BPI2, BPI3 from the infectivity treatment, BHS4, BHS8 from the speed treatment

and BHY3 from the yield treatment achieved significantly higher mortality in aphids after 7 days of exposure ($\chi^2$ = 83.98, *df* = 12, p < 0.001, Figure 10). Survivorship analysis indicated that selected lineages BPI2, BPI3, BHS4, BHS8 and BHY3 had a significantly higher instantaneous risk of death, which aligns with results from endpoint mortality analysis (likelihood ratio = 301, df = 12, p < 0.001, Table 5).

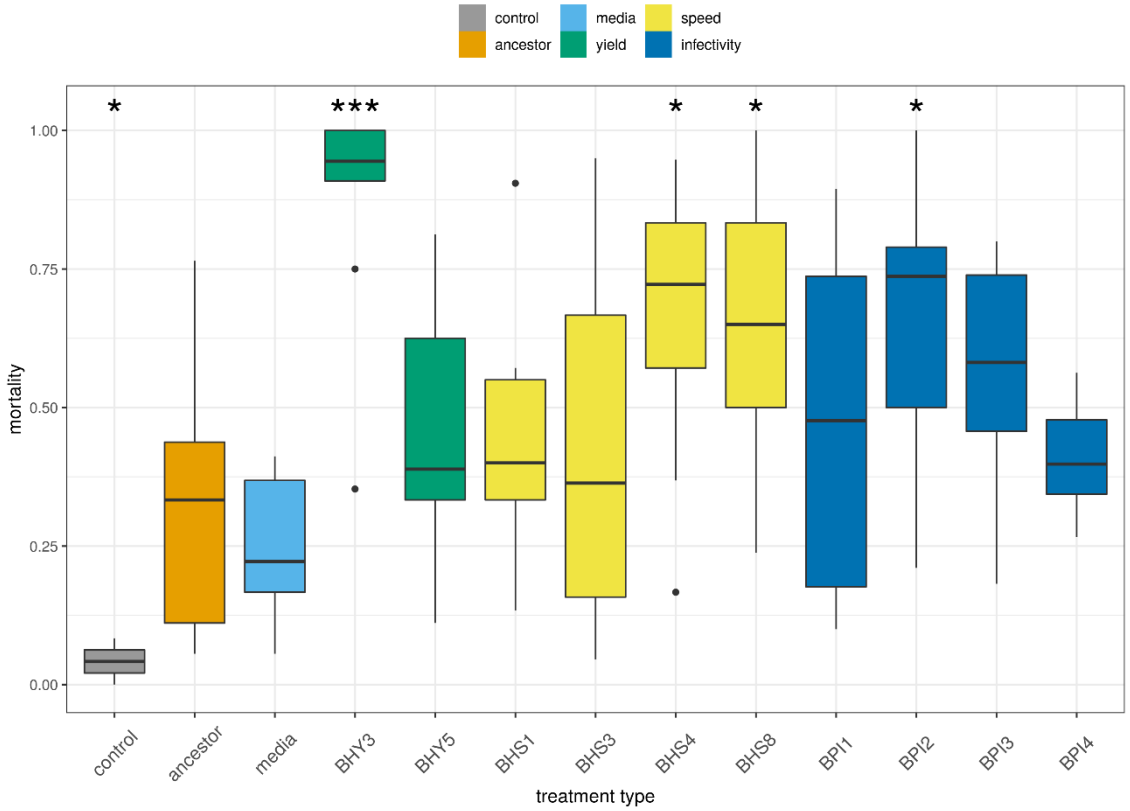



*Figure 10 Endpoint mortality of M. persicae after 7 days of exposure to selected lines of A. muscarius. Boxplots showing median, first and third quartiles, whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are*

*outliers. Media: passage on SDA plates only. Significance levels denoted by asterisk: ***: p <= 0.001; **: p <= 0.01; *: p <= 0.05*

*Table 5 Survivorship analysis of M. persicae infected with selected lines of A. muscarius*

| ***A. muscarius*** **line** | **treatment** | **hazard ratio (HR)** |
|---|---|---|
| control | sterile carrier | 0.07** |
| ancestor | ancestor | 1 |
| media | media | 0.59* |
| BPI1 | infectivity | 1.52* |
| BPI2 | infectivity | 2.3*** |
| BPI3 | infectivity | 2.16*** |
| BPI4 | infectivity | 1.22 |
| BHS1 | speed | 1.35 |
| BHS3 | speed | 1.35 |
| BHS4 | speed | 2.47*** |
| BHS8 | speed | 2.56*** |
| BHY3 | yield | 5.55*** |
| BHY5 | yield | 1.43 |

Laboratory dose-response bioassay of selected *A. muscarius* virulence towards *M. persicae*

The most promising strains BPI2, BHS3 and BHY3 belonging to their respective treatments were further tested at doses of $1x10^6$, $1x10^7$ and $1x10^8$ conidia/ml to characterize if there are any dose x virulence interactions. Dose had a significant

effect on aphid mortality after 7 days ($\chi^2 = 91.88$, *df* = 1, $p < 0.001$). We found no significant interaction between dose and selection treatment when looking at cumulative mortality ($\chi^2 = 1.80$, *df* = 3, $p = 0.62$) and there were no differences between any of the treatments ($\chi^2 = 1.98$, *df* = 3, $p = 0.58$). Survivorship analysis revealed a significantly higher instantanenous risk of death for aphids infected with lineage BPY3 (HR = 1.58). Although mortality at the high dose varied substantially in this set of assays, which was unexpected and reduced our ability to reject null hypotheses, we found a negative interaction between dose and lineage BHY3 suggesting dose specificity at the intermediate dose (likelihood ratio = 194.5, df = 7, $p < 0.001$, Figure 11).

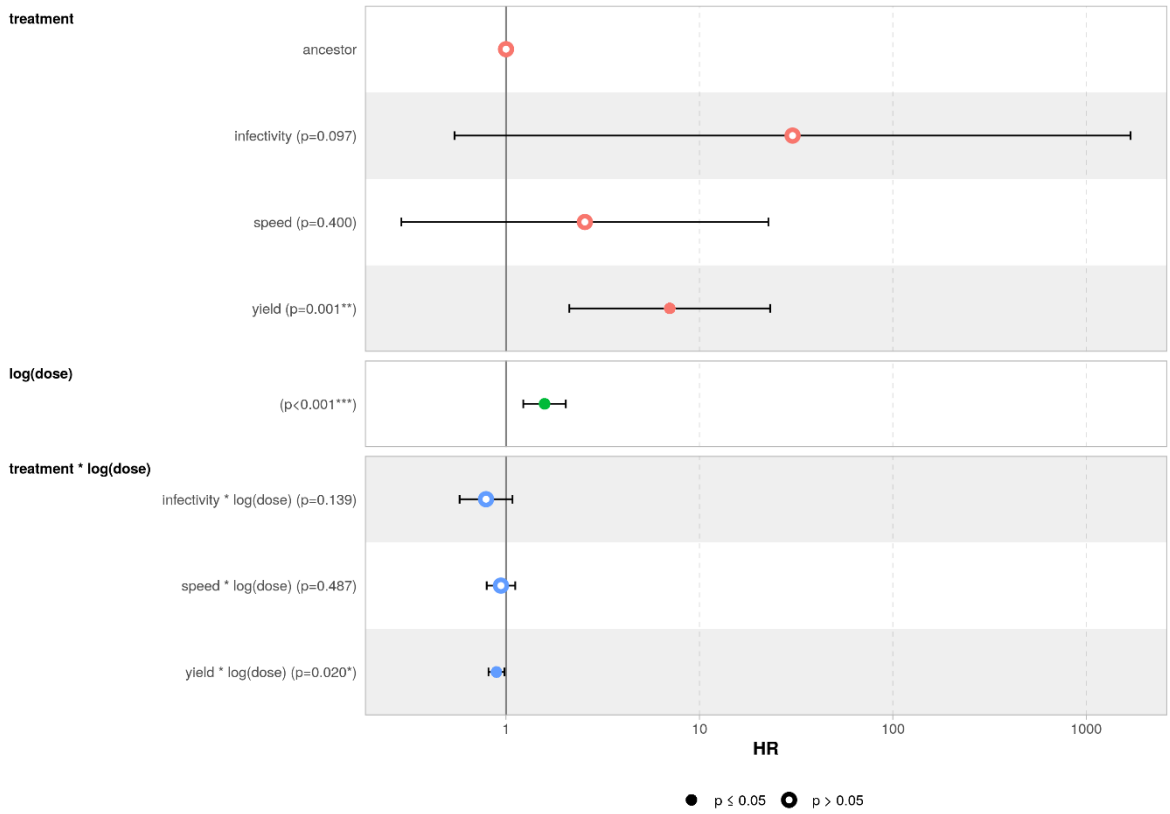



*Figure 11 Forest plots obtained with survivorship analysis using Cox proportional hazards. Instantaneous risk of death represented as hazard ratio (HR) with 95% confidence intervals on a logarithmic scale.*

Laboratory bioassay of selected *A. muscarius* virulence towards *B. brassicae*

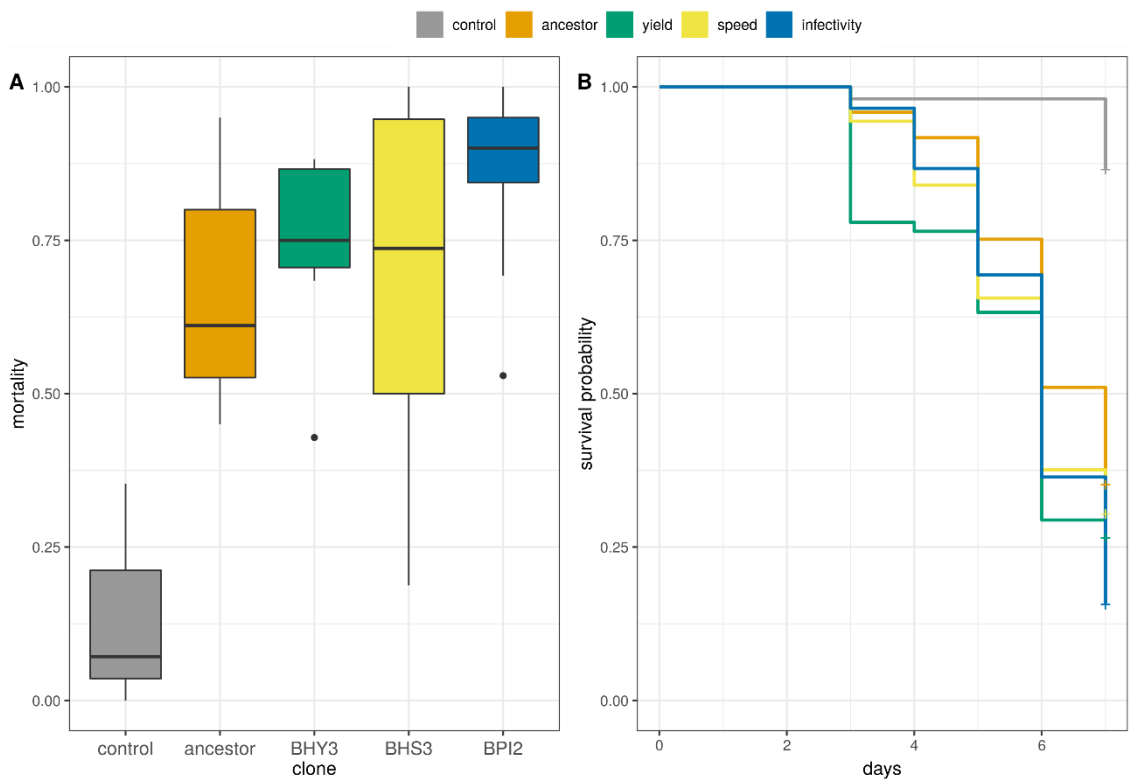



*Figure 12 Bioassays of the most promising evolved lineages in a second aphid host. Endpoint mortality of B. brassicae after 7 days of exposure to selected lines of A. muscarius (A). Boxplots showing median, first and third quartiles, whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are outliers. Mean survivorship curves of B. brassicae over 7 day after treatment with selected lines of A. Muscarius (B).*

This assay tested whether patterns of increased virulence in a subset of evolved lineages against selection host *M. persicae* could be repeated in a different aphid host *B. brassicae.* No mortality due to fungal infection was observed prior to day three. Fungal treatment had a significant effect on aphid mortality on day 3 ($\chi^2$ = 15.8, *df* = 4, $p < 0.001$) and onwards. Mean control mortality after 7 days was 13.7% ± 4.8. No difference was found between the endpoint mortality of the ancestor and the evolved lineages ($\chi^2$ = 2.7, *df* = 3, p = 0.44, Figure 12A).

However, survivorship analysis revealed a significantly higher risk of death for the infectivity lineage BPI2 (HR = 1.58) and the yield selected lineage BHY3 (HR = 1.50) compared to the ancestor (1) (likelihood ratio = 92.6, df = 4, $p < 0.001$, Figure 12B).

**Endpoint assays – life history**

Mycelial growth

The different selection treatments had a significant effect on mycelial growth at 22 °C *in vitro*, although effect sizes were small. Lineages BHS3 and BHS4 in the within host selection treatment had a lower mycelial growth, while BHS1 and BHS8 exhibited faster growth rate compared to the ancestor. Three strains from the between population treatment, BPI1 BPI2 and BPI4 had a faster growth rate, while BPI3 had a similar growth rate as the ancestor. The yield selection treatment resulted in slower growth rate for both lineages BHY3 and BHY5. The media treatment showed a marginally slower growth rate compared to the ancestor ($F_{11, 1161} = 210.95$, $p < 0.001$, Figure 13).

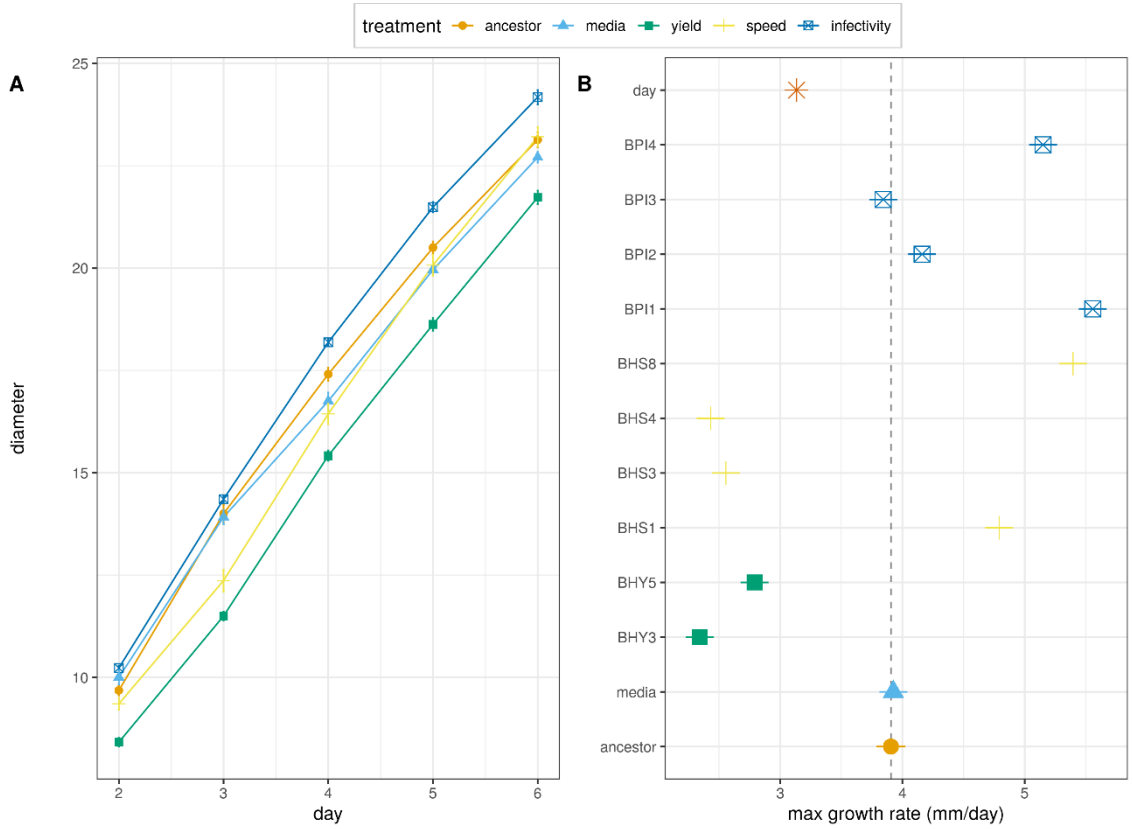



*Figure 13 Growth curves of selected lines of A. muscarius (A). Linear model estimates for maximal growth rate of individual replicates belonging to selection treatments (B).*

Thermal profile of selected lines

Selection treatment had a significant effect on mycelial growth rate at different temperatures ($F_{13, 1419} = 12.8$, $p < 0.001$). There was no evidence of adaptation to particular temperatures as we found no significant interaction between selection treatment and temperature ($F_{13, 1406} = 1.0$, $p = 0.43$). Lineages BHS3 and BHS4 in the speed treatment had a lower mycelial growth, while BHS1 and BHS8 exhibited similar growth rate when compared to the ancestor. Two lineages from the infectivity treatment, BPI1 and BPI2 had a faster growth rate, while BPI3 and BPI4 had a similar growth rate as the ancestor. Both lineages in the yield selection treatment resulted in a slower growth rate. These results broadly

correspond with the pattern shown in mycelial growth at a single temperature (Figure 14).

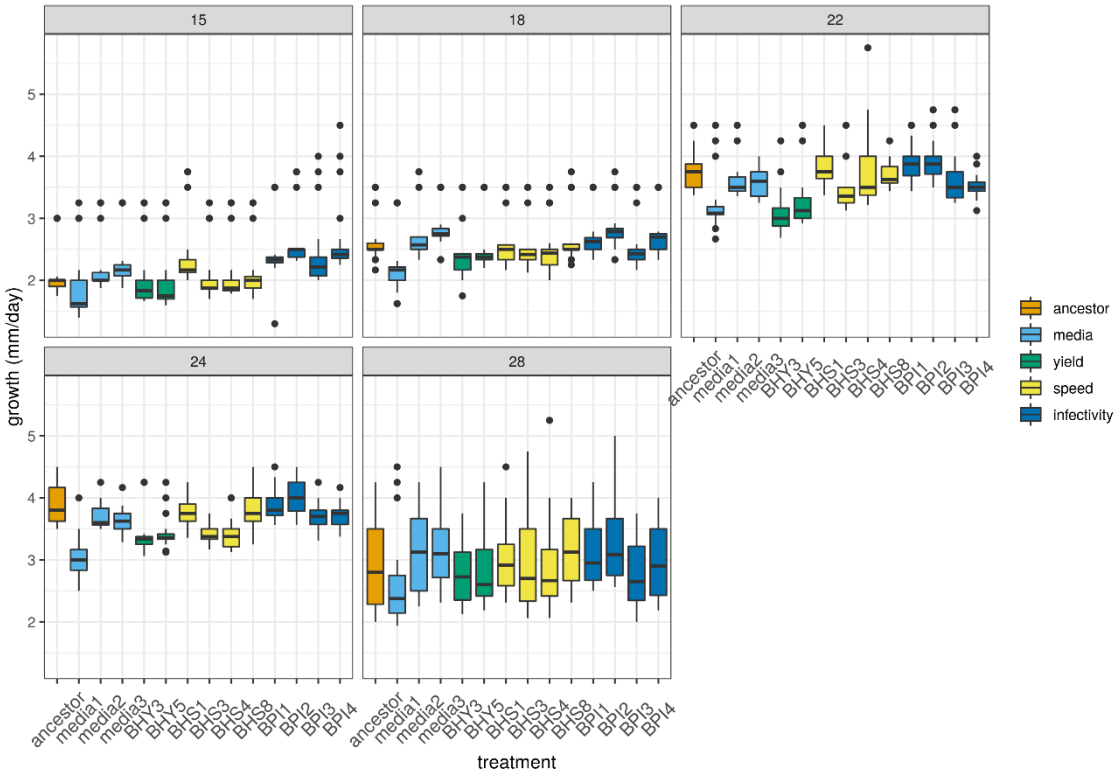



*Figure 14 Growth rate of selected lines of A. muscarius at different temperatures. Panel headings indicate temperature.*

Spore production and timing of sporulation

In these assays we tested how selection regimes affected both early sporulation and the total production of spores after two weeks of culture on solid media. Selection treatment had a significant effect on spore production 3 days post inoculation ($\chi^2 = 20.33$, $df = 4$, $p < 0.001$). Early spore production was significantly lower for lineages BHY3 and BHY5 in the yield treatment ($2.3 \times 10^4$ and $4 \times 10^4$ conidia/ml, respectively Figure 10A). All four lineages belonging to the infectivity treatment showed significantly higher spore production. The speed selection treatment resulted in a mixed outcome with lineages BHS1 producing significantly more spores ($1.3 \times 10^7$ conidia/ml) whereas BHS3 produced less spores (1.4 x

$10^5$ conidia/ml). Spore production was not statistically different between lineages media1, media3, BHS4, BHS8 and the ancestor (3.2 x $10^6$ conidia/ml) (Figure 15A, $F_{13, 43} = 13.15$, $p < 0.001$).

The effect of selection treatment on sporulation was even more pronounced at 14 days post inoculation ($\chi^2 = 31.02$, *df* = 4, $p < 0.001$). All lineages selected *in vitro* (media 1, 2 and 3) showed significantly lower sporulation, two lineages in the infectivity treatment (BPI1 and BPI4) and two lineages in the speed of kill treatment (BHS3 and BHS4) produced significantly more spores compared to the ancestor (Figure 15B, $F_{13, 50} = 11.28$, $p < 0.001$).

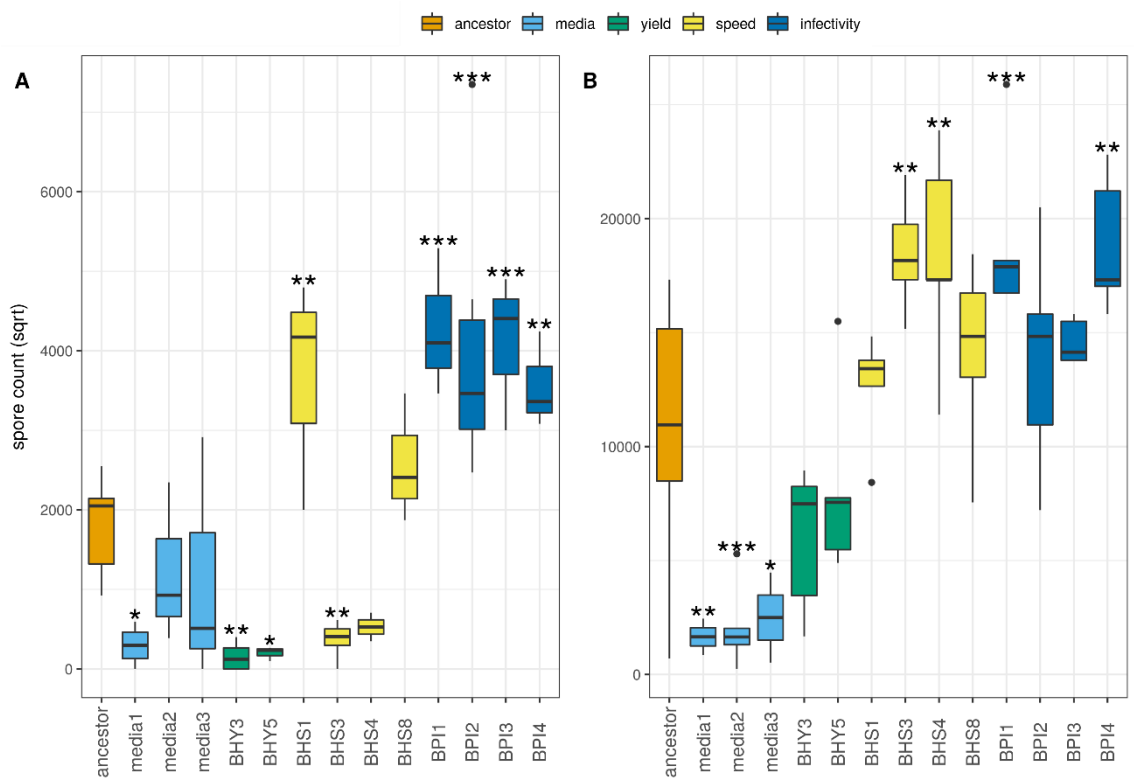


*Figure 15 Spore production of selected fungal lines after 3 days (A) and 14 days (B). Boxplots showing median, first and third quartiles, whiskers are 1.5 * interquartile range (IQR). Data beyond the end of the whiskers are outliers.*

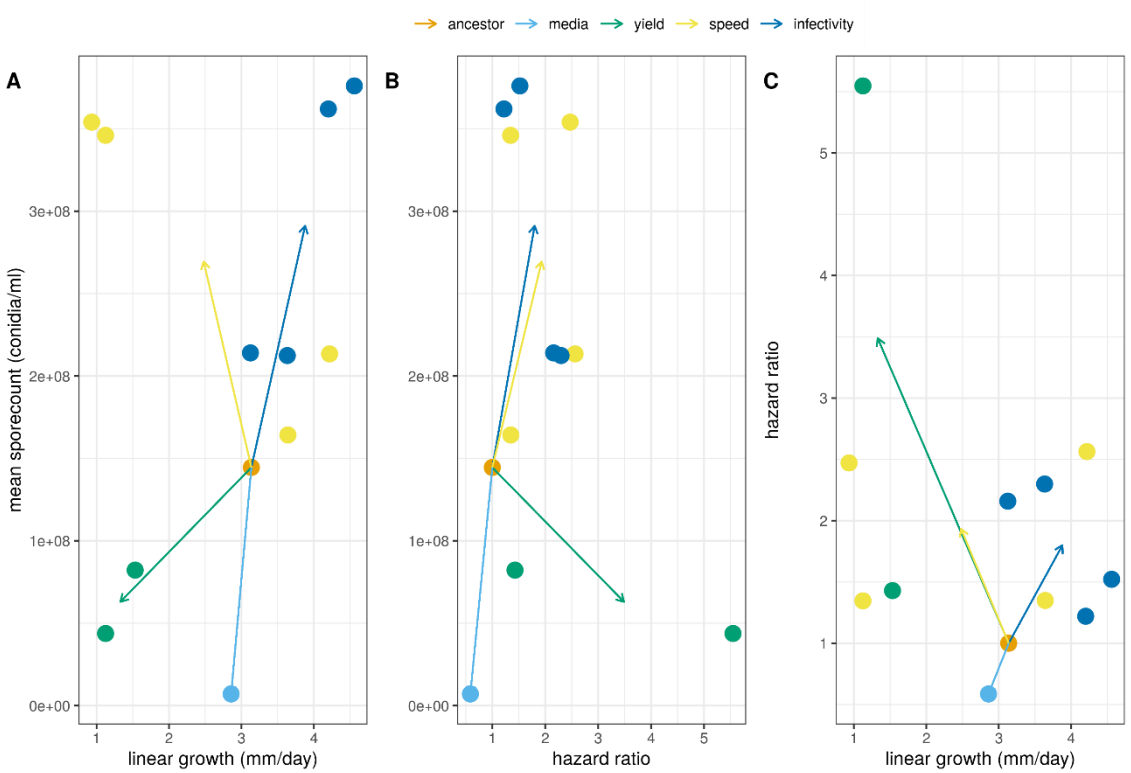



*Figure 16 Trends in virulence and life-history of fungal lineages belonging to different selection treatments. Points represent means per replicate. Arrows lead from ancestor to mean of selected lineages. Virulence is represented as the estimate of instantaneous hazard parameter from survivorship analyses.*

We found no significant correlation between virulence (measured as the instantaneous risk of death from survivorship analysis) and growth or sporulation. The speed and infectivity treatments could maintain or improve life history traits (linear growth, spore production) as well as being able to produce modest increase in virulence. In contrast the between host / yield selection regime could produce the biggest gains in virulence but generally reduced spore production

and growth rate. While we expected some attenuation of virulence in the *in vitro* controls, the biggest phenotypic change was a decreased spore production and we saw no evidence of any increases in growth rate on media.

Although it is hard to identify simple trade-offs with these life history data we did see the emergence of similar phenotypes within selection treatments. For example, yield selection and *in vitro* culture produced similar phenotypes, while 2/4 independent lineages in the infectivity and speed of kill selection regimes had very similar values for spore production, growth rate and virulence suggesting convergent evolution in phenotypic space (Figure 16).

## Discussion

Changes in fungal virulence and life-history did not correspond to the simple predictions of classical virulence or public goods models. We did not find evidence for strong trade-offs between virulence and life-history traits across treatments as seen in a number of other systems (de Roode et al., 2008; Evison et al., 2015; Hughes & Boomsma, 2006; K. H. Jensen et al., 2006). In addition, we did not see strong support for a social virulence model (public goods, tragedy of commons) as there were no consistent trade-off between virulence and growth rate, although, as we discuss below, there are potential social conflicts with respect to sporulation. The pathogenicity of EPF follows a complex infection cycle with multiple traits to potentially select upon (C. Wang & Wang, 2017). The variation in virulence and fitness of selected lines belonging to different selection treatments highlights the complexity of fungal virulence and points to a flexible genome that can evolve in different ways in response to differences in selection pressure and which suggest there are potentially multiple subtle trade-offs in fungal life-history. Nevertheless we did see improvement in either virulence or sporulation in a large proportion of lineages, emphasizing that applying evolutionary theory to serial passage experiments is certainly helpful in designing successful experiments for strain improvement. Treatments at different scales of selection led to a significant increase in virulence in at least one lineage belonging to each of the *in vivo* treatments.

Classical theory of virulence evolution would predict that within host competition favours higher replication rate of the pathogen, which in terms leads to higher virulence (S. P. Brown et al., 2002; de Roode & Altizer, 2010; de Roode et al.,

2005). However, we observed significant variation in growth rate *in vitro* of lineages belonging to the speed of kill selection treatment and no correlation to virulence. The two lineages that showed increased growth rate *in vitro* also showed faster sporulation, while lineages with slower growth rate *in vitro* produced more spores in total revealing potentially diverse trade-offs in life history. Growth rate of lineages in the yield treatment were significantly slower than the ancestor. A decrease of both growth rate and sporulation on artificial media suggests that the disruption of spatial structure in aphids lead to additional stress from natural microbiome of the host, reducing the effectiveness of antimicrobials produced by the fungi (Chao & Levin, 1981). Aphid symbionts have been shown to provide protection against a fungal entomopathogen (Parker et al., 2013). Between population selection for infectivity was expected to favour investment in social virulence factors. Therefore, we presumed increased investment in the production of such virulence factors to be costly and would decrease growth rate. Surprisingly, we observed no negative effect of infectivity treatment on growth rate. Conversely, two lineages out of four exhibited higher growth rate than that of the ancestor. Generally, the effect sizes of selection on radial growth were modest and as of yet we cannot predict whether this relates to changes in life history within hosts. *In vivo* growth rate would be more insightful in understanding interaction between virulence and pathogen replication, however the importance of *in vitro* growth should not be overlooked as effective large scale propagation of the fungi is required for biocontrol purposes (Jaronski, 2014). Replication rate in this pathogen could also be of less importance due to virulence being driven by toxins and secondary metabolites rather than use of host resources. This is supported by the lack of correlation between in vitro

growth and virulence observed across treatments and our result that the lineage with poorest growth kinetics *in vitro* is the most virulent.

Most treatments showed strong changes in terms of either timing of sporulation or total production of spores. While we did not see evidence for social conflicts in terms of virulence there is a body of work that argues that production of spores or persistent non-replicating cells is a form of cooperation and several aspects of our results do make sense in the light of this theory. The basis for this idea is that the production of a spore or resting stage can be cooperative- there is an individual level costs in terms of cessation of growth but potentially a group level benefit in terms of maximizing the conversion of local resources into resting stages before cells starve (Gardner et al., 2007; Ratcliff et al., 2013). When more genotypes are competing locally (ie when relatedness is lower) theory predicts an increase in cheating, which could manifest as a later switch to sporulation or the rise in frequency of non-sporulating mutants. Notably, in spore-forming bacteria (*Bacillus* spp) there are complex regulatory mechanisms that ensure a co-ordinated switch to sporulation (Garti-Levi et al., 2013; Schultz et al., 2009) or which lyse late-sporulating defectors (González-Pastor et al., 2003)

All lineages in the infectivity treatment exhibited faster early sporulation, with two lineages producing more spores in total. Fungi, similarly to bacteria produce a wealth of secreted metabolites, such as siderophores (Butt et al., 2016; Y. Li et al., 2016; Renshaw et al., 2002) that could act as public goods and play an important role in acquiring host resources (Y. Li et al., 2016; West & Buckling, 2003; West et al., 2006). The observed increase in spore production might be a result of a more efficient exploitation of resources which could be facilitated when there is reduced social conflict. Since infection produces population bottlenecks this commonly increases relatedness (van Leeuwen et al., 2015), we took care

to minimize social conflict in the speed of kill and infectivity treatments by passaging using single cadavers. The speed of kill selection treatment resulted in two of the surviving lineages showing faster sporulation, two lineages showing slower early sporulation. Interestingly, the two lineages that showed slower early sporulation ended up with higher total spore yield after 14 days, consistent with a rate / efficiency trade-off that can also operate when there are social conflicts (MaClean et al., 2010).

Conversely to our prediction that the yield treatment would favour pathogen population size (Griffin et al., 2004) we found that spore production was lowest in this treatment. This treatment produced the most virulent strain of the selection experiment. This could point to a trade-off between high virulence and pathogen fitness (Alizon et al., 2009) consistent with previous findings that serial passage of a fungal pathogen leads to an increase in virulence and decline in total spore production (Evison et al., 2015; Hughes & Boomsma, 2006). However, we found early spore production to be also lower in the yield treatment compared to the ancestor, suggesting that the change is not due to a shift towards faster sporulation (Evison et al., 2015) but rather to a diversion of resources to other traits. Since this treatment by necessity involved pooling of cadavers we would expect relatedness to be lower and the potential for social conflict over the timing of sporulation to be more intense. With the reduced spore production could have come increased investment of resources into the production of secondary metabolites and toxins that function to exploit the host leading to the observed increase in virulence (Alizon et al., 2009; de Roode et al., 2008). Similarly, the *in vitro* passaging treatment also lead to a loss of sporulation. Due to the lack of host bottleneck we expect relatedness to be lowest in the *in vitro* passaging treatment which would increase social conflict for timing of sporulation and

increase the fitness of late sporulating "cheats" (Gardner et al., 2007; Ratcliff et al., 2013). Surprisingly, selection in media did not produce increase growth rate in media, the most dramatic response were attenuation and reduced spore production.

Serial passage experiments with entomopathogenic fungi have not always been successful at increasing virulence. Passaging the opportunistic pathogen *Aspergillus flavus* through *Galleria mellonella* resulted in increased propagule production and shortened time to sporulation on cadavers as well as reduced growth on artificial media, but not increased virulence (Scully & Bidochka, 2005, 2006). The passage of *Beauveria bassiana* in *Anopheles coluzzi* also resulted in altered mycelial growth rate and sporulation on artificial media, increased virulence however was not observed (Valero-Jimenez et al., 2017). This could be explained by the lack of strong reproductive advantage for genotypes with higher virulence (Valero-Jimenez et al., 2017) and the low levels of underlying genetic variation in the initial infecting population (Scully & Bidochka, 2006). On the contrary, passaging the well-known entomopathogen *Metarhizium anisopliae* in the Asian blue tick *Rhipicephalus microplus* successfully increased the virulence of the fungus (Adames et al., 2011). Similarly, an increase in virulence of the heterothallic fungal parasite *Ascospaera apis* was observed after just three subsequent passages in the honey bee *Apis mellifera* (Evison et al., 2015). The authors also reported faster sporulation on cadavers and a decreased total spore production that suggests trade-offs between virulence and spore production (Hughes & Boomsma, 2006). This increase in virulence could be the result of relaxed selection on transmission due to the artificial transmission employed during the serial passage (Anderson & May, 1982). The different responses to

selection can also be explained by differences in selection pressure applied throughout the passage experiment and also the differences in the biology of the system.

The results presented here show that by applying evolutionary theory to selection in serial passaging can lead to robust increases in virulence. Clustering of lineages in the phenotypic space within treatments suggests that with more underlying mechanistic knowledge, theory based selection experiments could provide an improved method for targeted selection of desired phenotypes. With better understanding of genetic response to selection at different scales, there seems to be opportunities to improve virulence and spore-production traits at the same time as seen from a lineage (BHI1) belonging to the infectivity treatment. A strain with improved virulence and propagation potential would make biological control commercially more desirable for both producers and end users.

The modest increase in virulence observed in the present study of *A. muscarius* seems to be dose specific but not specific to the host, as the increase in virulence of the fungus was apparent in *Brevicoryne brassicae*, a different species to the one used in serial passaging. Changes in virulence were observed in terms of increase in percentage mortality, speed of kill or both. Passaging the fungi *in vitro* showed signs of attenuation compared with the ancestor, however the effect size is moderate and we did not have enough statistical power to differentiate between the treatments. Degeneration of fungi following serial passage *in vitro* has been well established with examples from many species of insect pathogens (Butt et al., 2006; Morrow et al., 1989; Safavi, 2012). Importantly we did not see a negative effect on virulence in any of the *in vivo* selection treatments.

Extinction events in the speed of kill selection regime can be seen as between population selection in action, emphasizing that it is challenging to minimize or control selection on different scales. Likewise, within and between host competition was still in operation for the infectivity treatment. Half of the replicates in the speed selection treatment were driven to extinction early on in passaging due to not inducing mortality in the host. This could be explained by the higher proportion of introduced mutations being deleterious (Eyre-Walker & Keightley, 2007) as 3 out of 4 replicates went extinct after a passage round with mutagenesis. An alternative explanation is that the inherent genetic variation in the starting ancestral population lead to a drift towards avirulence. Both of these potential reasons are enhanced by the strong bottleneck effect induced by the host on infecting populations (Scully & Bidochka, 2006; van Leeuwen et al., 2015). Replicates in the yield treatment went extinct due to bacterial contamination. The source of bacterial contamination likely originates from the natural microbiome of aphids as cadavers plated but not mashed did not exhibit bacterial growth. Disruption of the spatial structure could have reduced the efficacy of antimicrobials produced by the fungi, making it less competitive against the gut microbiome of the aphid (Chao & Levin, 1981; Fritts et al., 2021). This contamination outcompeted the fungus on agar plates making it impossible to collect spores for subsequent passage rounds.

Fungi in the speed and infectivity treatment showed similarities in their response to selection. Lineages tended to either increase virulence or spore production (with only one lineage increasing both and one lineage not responding to selection). These similarities highlight that we can only attempt to alter the importance of different scales of selection (between host, between population). The comparable response to selection in the speed and infectivity treatment could

originate from the use of a single cadaver in passaging. Host induced infection bottlenecks should lead to high relatedness emerging and different genotypes competing in different hosts for early infection (Scully & Bidochka, 2006). This could explain how, although less successful, the speed selection shows similar trends to the infectivity treatment in selecting for more efficient growth and sporulation with no loss in virulence.

The work presented here confirms that selective passaging can be used to increase the virulence of *A. muscarius* and highlights the potential in using targeted selective passage protocols to select for different fungal traits that are not necessarily virulence. Traits such as *in vitro* growth and sporulation are critical for economically successful application of EPF in pest management. An important aspect that is relatively unexplored in experimental evolution with entomopathogenic fungi is relatedness. Spore production and timing of sporulation seems to be closely linked to relatedness as treatments propagated from a single cadaver has shown improved sporulation compared to treatments with no bottleneck (*in vitro*) or pooled cadavers. This study is a step forward in understanding how selective forces shape the virulence of *A. muscarius* towards their insect hosts. In order to study evolutionary change in EPF, further studies should make use of next generation sequencing technologies and combine them with serial passage experiments to disentangle how selection acts on specific traits of the fungus. A better understanding of genome-wide host-pathogen interaction could provide a leap in the improvement of biocontrol strains.

## IV: *De Novo* genome assembly and population genomic analysis of *Akanthomyces muscarius*


### Abstract

The entomopathogenic fungus *Akanthomyces muscarius* is used as a biocontrol agent for the management of insect pests in agriculture. It also presents a potential model for studying host-pathogen interactions and the evolution of virulence in a laboratory setting. Here, we present a new population genomic resource for *A. muscarius* comprising a high-quality draft genome assembly together with resequenced genomes of 13 experimentally selected lineages of this fungus. We used long- and short-read sequencing to assemble a genome of 36.1 Mb containing 12231 genes, with 96.1 % completeness based on the core *Hypocrealen* gene set (Benchmarking Universal Single-Copy Orthologs). We leveraged this new genomic resource to predict biosynthetic gene clusters associated with secondary metabolite production, and to identify candidate genes involved in virulence by analysing transcriptional data from fungi grown *in vitro* and *in vivo*. We also identified variants in 13 derived lineages of fungi of *A. muscarius* previously selected for three different virulence traits. The first high-quality draft assembly and annotation of *A. muscarius* and associated population genetic and transcriptional resources presented in this study provide a powerful tool for further work with this commercially important species.

## Introduction

The extent to which selection pressure shapes the genomic architecture underpinning key traits of pathogens remains a key focus in evolutionary biology. For example, virulence of entomopathogenic fungi is a complex pathway, governed by multiple traits (Butt et al., 2016) making it difficult to disentangle the effect selection pressure has on the fitness of the fungi. Phenotypic changes followed by serial passage through hosts have shown a large variety of fitness effects in different experimental models using different selection pressures. Changes to virulence, growth rate and sporulation have all been described previously (Adames et al., 2011; Evison et al., 2015; Hughes & Boomsma, 2006). However, our knowledge of the underlying mechanisms of these adaptations are limited.

Mitosporic hypocrealen fungi, such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Akanthomyces* spp*.* are known for their entomogenous lifestyle and have been isolated from cadavers of insects and spiders. Numerous biocontrol products have been developed based on these fungi (Faria & Wraight, 2007). Species belonging to *Akanthomyces* can be found in soil, attached to leaf matter or leaf litter and are known to infect *Hemiptera* (*A. lecanii*), *Homoptera* (*A. muscarius*), *Coleoptera* (*A. neocoleopterorum*) and *Lepidoptera* (*A. pisillariiformis*) among other insect orders. The number of reports of endophytic activity of isolates have also been steadily increasing in the last few years (Nicoletti & Becchimanzi, 2020), highlighting the potential in using these fungi in agricultural pest management systems.

*Akanthomyces muscarius*, previously known as *Lecanicillium muscarium* (Gams & Zare, 2001) has been used commercially as a biological control agent for the management of aphids and whitefly. It follows the typical pathway of

pathogenesis of an entomopathogenic mitosporic fungi. Spores adhere to the cuticle of the host, where conidia germinate and penetrate the cuticle. This is followed by production of blastospores and secondary metabolites within the hemocoel and the invasion of host tissues, causing host death. The colonization of hemolymph involves different cell types, including yeast-like blastospores and hyphal bodies. EPF can counter the insect immune defense by a range of different strategies. Repression of proteases involved in phenoloxidase activation, collagen deposition to avoid immune stimulation, secretion of immune modulators and the modification of surface carbohydrate profile to evade granular cells have been previously observed and reported (Butt et al., 2016). Spores are released from the cadaver via conidiophores formed on the surface of the cuticle (Askary et al., 1999; C. Wang & Wang, 2017).

Prior to this study, we carried out a serial passage experiment (SPE) of *A. muscarius* through its host *Myzus persicae* with different selection pressures aimed at increasing the speed of kill, yield or infectivity of the fungus (see details in Chapter 3). We found multiple phenotypic changes in both virulence, growth and sporulation of fungal lineages belonging to different selection treatments. In this study, we supplement our knowledge of these derived lineages by creating genomic and transcriptomic resources in order to investigate the underlying changes in genotypes associated with the phenotypes resulting from the SPE.

The falling cost of generating next generation sequencing (NGS) datasets on populations of a pathogen provides an opportunity to recover genomic variants induced by artificial selection, such as single nucleotide polymorphisms (SNPs), insertions and deletion (indels) or other structural variants (SVs) in the form of inversions, translocation or duplications (Alkan et al., 2011). Using a reference-based approach by mapping short read data generated from NGS to a reference

genome to discover SNPs and short indels has strong potential to provide insight into the underlying processes and mechanisms governing the evolution of virulence in *A. muscarius*. The number and diversity of beneficial mutations that drives adaptation to the host could potentially occur via identical convergent mutations or different alternative pathways. Studying the underlying genetic change that drive adaptation requires good replication, complete genome sequences for unambiguous identification of mutations and complex biological systems for alternative solutions for adaptation. This approach has been used to study the number and diversity of mutations as well as genome evolution in *E. coli* adapting to new environments (Tenaillon et al., 2016; Tenaillon et al., 2012), molecular evolution in bacteria-phage host-parasite system (Paterson et al., 2010), the evolution of symbiont mediated protection against infections in a microbe-nematode model (King et al., 2016) and the genome evolution in response to accelerated development of *D. melanogaster* (Burke et al., 2010) amongst others.

Hypocrealen entomopathogenic fungi are known to produce a large number of secondary metabolites that play a role in host infection, competition and defense against predators (L. Zhang et al., 2020). These secondary metabolites interact with insect physiology and behaviour. Toxins and non-volatile compounds combat the host immune system (Gillespie & Claydon, 1989), whereas volatiles can act as repellents or attractants (Holighaus & Rohlfs, 2016). Genes associated with the production of these secondary metabolites are often organized in close proximity in contiguous genomic regions termed biosynthetic gene clusters (BCGs) (Keller et al., 2005). Tools based on next generation sequencing data, such as antiSMASH make it possible to rapidly identify BCGs in fungal genomes.

The use of such techniques has important consequences in strain discovery and strain improvement with biological control properties.

Here, we aim to better our understanding of the previously observed phenotypic changes in derived lineages of *A. muscarius* by generating a population genomic resource comprising a high-quality genome assembly together with resequenced genomes of the 13 previously selected lineages of fungi. We describe our draft genome for *A. muscarius* and a corresponding annotation that provides an important resource for future work in strain improvement for biological control. In addition, we carry out variant calling on strains of *A. muscarius* that went through a serial passage experiment as described in Chapter 3. We also created RNAseq libraries with the aim to study differential gene expression between fungi grown *in vitro* and *in vivo* to help identify genes involved in host exploitation. Furthermore, we attempt to identify BCGs present in the genome of *A. muscarius.*

## Methods

### Fungal cultures

The ancestral and derived strains of *A. muscarius* from a serial passage experiment with four treatments were spread on Sabouraud dextrose agar (SDA) in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) and incubated in darkness at 23±1 °C for 14 days. Cultures used are shown in Table 6.

*Table 6 A. muscarius lineages used in this study. For details on serial passage treatment see Chapter III.*

| ***A. muscarius* line** | **Serial passage treatment** |
|---|---|
| ANC | ancestor |
| media1 | media |
| media2 | media |
| media3 | media |
| BPI1 | infectivity |
| BPI2 | infectivity |
| BPI3 | infectivity |
| BPI4 | infectivity |
| BHS1 | speed |
| BHS3 | speed |
| BHS4 | speed |
| BHS8 | speed |

| *A. muscarius* line | Serial passage treatment |
|---|---|
| BHY3 | yield |
| BHY5 | yield |

**Insect cultures**

Populations of *Myzus persicae* were reared in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) on Chinese cabbage, *Brassica pekinensis* plants at BBCH growth stage 13-15. Colonies were sub-cultured as required by transferring 5-15 apterous adults onto fresh plants. Individuals of defined age were produced by confining cohorts of mature apterous virginoparae in clip cages on chinese cabbage plants (BBCH 13-15) for 48 hours to produce progeny. Adults were than removed and the nymphs maintained for a further nine days. Populations were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle.

**DNA extraction and library preparation**

To extract high quality genomic DNA of sufficient yield for long read sequencing we evaluated four commercial DNA extraction kits: Dneasy Blood & Tissue kit (Qiagen), PureLink™ Genomic DNA Mini Kit (Invitrogen), GeneJET Genomic DNA Purification Kit (Thermo Scientific) and Genomic-tip (Qiagen). Mycelia was harvested from 14 day old fungal cultures grown on SDA in 22±1 °C in darkness using a sterile scalpel. Harvested mycelia were homogenized in liquid nitrogen before proceeding with DNA extraction following manufacturer's instructions. The only modification to the recommended protocol was a prolonged lysis step

overnight and an additional centrifugation after lysis when using the Genomic-tip kit. The extracted DNA was quantified using a NanoDrop spectrophotometer 2000 (Thermo Scientific) and Qubit Fluorometer using the Qubit dsDNA BR assay kit (Thermo Scientific). DNA integrity was checked by agarose gel electrophoresis.

High molecular weight DNA was used to generate a long-read PacBio library which were sequenced using a single PacBio Sequel SMRT cell. Illumina libraries for the ancestor and evolved lines of *A. muscarius* were prepared using the Illumina NEBNext Ultra II FS Kit and sequenced on a single lane of Illumina NovaSeq SP (paired-end, 2x150 bp). All sequencing was carried out at the NERC Environmental Omics Facility (University of Liverpool, UK).

**RNA extraction and library preparation**

Total RNA was extracted from fungal mycelia cultured on SDA medium and infected *Myzus persicae* one day after host death using the ISOLATE II RNA Mini Kit (Bioline). Four biological replicates were used for both *in vitro* and *in vivo* extraction. 20 aphid cadavers were pooled for each replicate of *in vivo* RNA extraction. The integrity of RNA was determined by agarose gel electrophoresis, quantified using a NanoDrop spectrophotometer 2000 (Thermo Scientific) and Qubit Fluorometer using the Qubit RNA BR assay kit (Thermo Scientific). RNA was then sequenced to >68M paired end (PE) reads per replicate on an Illumina NovaSeq using a 150 bp PE read metric (Novogene, UK). The quality of reads was assessed using FastQC v0.11.8 (S. Andrews, 2010), and adapter sequences and low-quality reads removed using TrimGalore v0.6.5 (Krueger, 2012).

**Genome assembly**

The Illumina DNAseq raw Fastq files were trimmed for the presence of adapter sequences using Cutadapt v1.2.1 (Martin, 2011). The option -O 3 was used, so the 3' end of any read which matched the adapter sequence for 3 bp or more was trimmed. The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 15 bp after trimming were removed.

We tested four different assembly methods to perform the initial genome assemblies: Flye v2.9-b1768 (Kolmogorov et al., 2019) in both reduced coverage for initial disjointing assembly (-asm 50) and uneven coverage mode (-meta), MaSuRCA v4.0.5 (Zimin et al., 2017), Wengan v0.2 (Di Genova et al., 2021) and HASLR v0.8a1 (Haghshenas et al., 2020). We also tested a merged assembly of the HASLR and Flye assemblies to test whether this approach improved our genome assembly (Chakraborty et al., 2016).

All parameters were set as default except for genome size of 36 MB for the Flye assemblies. The obtained Flye assemblies were polished using PacBio reads using Racon v1.4.20 (1–4 iterations) (Vaser et al., 2017). Next, the polished sequences were additionally corrected using Illumina reads using POLCA (built-in MaSuRCA v4.0.5 polisher) (Zimin et al., 2017). PacBio read mapping to the genome assembly was performed with minimap2 v2.22 (H. Li, 2018), Illumina read mapping – with BWA v2.2.1 (H. Li, 2013).

Nx statistics for *A. muscarius* genome assemblies were calculated using QUAST v5.1.0rc1 (Gurevich et al., 2013). Nx is a specific length for which the subset of contigs of that length or longer covers at least x percent of the assembly (i.e., 50% for N50, 75% for N75, etc.). The completeness of the gene space in the assembled genome was assessed using the Benchmarking Universal Single-

Copy Orthologues (BUSCO) v5.2.2 pipeline using the hypocreales odb10 dataset with a total 4494 BUSCOs (Simão et al., 2015). The percentage of repetitive elements in genome assemblies was identified and masked using RepeatMasker v4.0.9 (Smit et al., 2013-2015). The best assembly was selected for *ab initio* annotation using the BRAKER2 v2.1.6 pipeline based on gene predictions from GeneMark-ES v4.68 (Lomsadze et al., 2005) and AUGUSTUS v3.3.0 (Stanke & Morgenstern, 2005) using the created RNAseq libraries as evidence. RNA-seq datasets were mapped against the repeat masked genome using HISAT2 v2.1.0 (D. Kim et al., 2015) followed by sorting and indexing with SAMtools -v1.10 (H. Li et al., 2009). BRAKER2 was run with the parameters *–softmasking—gff3*. Strand-specific RNA-seq alignments were split by forward and reverse strands and passed to BRAKER2 as separate BAM files to improve accuracy, as recommended in the BRAKER2 documentation. Functional annotation of the *de-novo* predicted gene models was performed based on homology searches against the NCBI nr and Interpro databases using Omicsbox v2.1.2. GO mapping was performed against the Gene Ontology database implemented in OmicsBox. Gene Ontology (GO) terms were assigned to the annotated sequences to predict the functions of the unique sequences. Identification of gene clusters involved in natural product biosynthesis was carried out using antiSMASH fungal version 6.1.1 (Blin et al., 2021).

**Transcriptome analysis and differential gene expression**

Clean RNAseq reads were aligned to the *A. muscarius* genome assembly using HISAT2 v2.1.0 (D. Kim et al., 2015), and gene expression estimated using the htseq-count tool implemented in the HTSeq v0.9.0 package (Anders et al., 2015). EdgeR v3.28.0 (Robinson et al., 2010) was used to normalize expected counts

for relative expression and effective library size with the Trimmed Mean of M-values (TMM) method and to identify significantly differentially-expressed genes using a corrected p-value threshold of $p < 0.05$ and a fold change >2.

**Variant calling**

Variant calling was carried out using the genome analysis toolkit (GATK) pipeline v4.1.0.0 to call both variant and reference bases from alignments (McKenna et al., 2010). Prepared Illumina libraries of the evolved and ancestral lineages were mapped to the assembled genome with BWA v2.2.1 (H. Li, 2013; Vasimuddin et al., 2019). Alignment pre-processing was carried out using Picard tools AddOrReplaceReadGroups, MarkDuplicates (see http://broadinstitute.github.io/picard/), followed by indexing with SAMtools v1.10 (Danecek et al., 2021). GATK HaplotypeCaller was used to call variants with the haploid setting, followed by CombineGVCFs, GenotypeGVCFs to perform joint genotyping. The minimum phred-scaled confidence threshold at which variants should be called was set at 10.0. Filtering of variants was carried using the parameters "QD < 2.0", "MQ < 40.0", "FS > 200.0" using VariantFiltration. Bases with a read depth of < 50 or > 8000 were removed, as well as sites not including the GATK PASS flag. Genetic differentiation was analysed using vcfR (Knaus & Grünwald, 2017). High quality variants that were fixed in more than half of the individually evolving replicates were considered to be convergent mutations (see example in Figure 1).

## Results

### DNA extraction

The most consistent DNA extraction of highest quality and yield was achieved with the Qiagen Genomic Tip kit (Table 7). The concentration of extracted DNA was in the range of 20-164 ng/µl with 1.82 ≤ A260/280 ≤ 1.9 and 1.68 ≤ A260/230 ≤ 3.11. Measured DNA concentrations with Qubit and NanoDrop showed a difference of less than 25 %. Gel electrophoresis confirmed intact bands of genomic DNA.

*Table 7 Summary statistics of genomic DNA extraction with different commercial DNA extraction kits. Values are mean+SD. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.*

| **Method** | **DNA concentration (ng/µL)** | **Yield (µg)** | **Purity ($A_{260}/A_{280}$)** | **Purity ($A_{260}/A_{230}$)** |
|---|---|---|---|---|
| Dneasy Blood & Tissue kit | 32.68±90.56 | 1.63±4.52 | 2.03±1.80 | 3.08±16.60 |
| PureLink™ Genomic DNA Mini Kit | 7.65±0.91 | 0.38±0.05 | 2.98±0.46 | 20.57±22.08 |

| | | | | |
|---|---|---|---|---|
| GeneJET Genomic DNA Purification Kit | 7.97±3.82 | 0.40±0.19 | 6.50±12.15 | 2.22±2.91 |
| Qiagen Genomic-tip | 93.57±52.43 | 8.34±4.80 | 1.87±0.02 | 2.20±0.29 |

**Genome features**

The PacBio library resulted in 5.9 million reads with an N50 of 26 kb. The Illumina libraries for the evolved and ancestral lines gave 72 million paired 150 bp reads on average. We performed a *de novo* genome assembly (see methods). Flye with –asm option and MaSurCA both assembled 12 contigs with an N50 of 4.8 Mb and 4.7 Mb, respectively. Wengan produced 16 contigs with a higher N50 of 5.3 Mb, while HASLR assembled 101 contig. All assembly methods produced a genome with a size in the range of 35.4 – 36.3 Mb. Based on assembly statistics and the higher average coverage observed for the Flye assembly we carried out further steps using this draft assembly. Polishing of the Flye_asm assembly with shortread data using Racon (1-4 iterations) or POLCA did not significantly improve the assembly statistics, however, based on the BUSCO scores we chose the POLCA corrected Flye_asm assembly for annotations. This final assembly resulted in a genome of 36.2 Mb with a 53 GC% and N50 of 4.86 Mb (Table 8). This is consistent with the 30-40 Mb genome size reported for most assemblies of filamentous ascomycete fungi, such as the close relative *Akanthomyces lecanii* (35.59 Mb, 53.1 % GC content, Table 9) (Shang et al., 2016). Detailed assembly properties are given in Table 8. Genome completeness was evaluated by BUSCO

analysis with 96.1% of the Hypocreales test gene set found to be present as complete single copies.

A total of 5.2 % of bases were identified as repetitive elements by RepeatMasker, that is comparable to *C. militaris* (3.04 %), *B. bassiana* (2.03 %) and *M. acridum* (1.52 %) (Xiao et al., 2012). These repeats belong to long interspersed nuclear elements (LINEs), long terminal repeat (LTR) retrotransposons and other DNA elements.

After softmasking repetitive elements we annotated the *A. muscarius* genome using the BRAKER2 pipeline. Strand-specific RNA-seq alignments split by forward and reverse strands as separate BAM files as well as the longest open reading frame from a *de novo* transcript assembly were fed to BRAKER2 to improve the accuracy of UTR models as recommended in the BRAKER2 documentation. Structural genome annotation using a workflow incorporating RNAseq data predicted 12231 genes, 42694 coding DNA sequences (CDS), with 25463 CDS resulting in annotations based on BLAST searches against the Hypocreales odb10 protein database (accessed November 2021).

*Table 8 Summary statistics of genomes obtained with different assemblers.*

| | MaSurCA | Flye-asm | Flye-meta | **FlyePOLCA** | HASLR | wengan | quickmerge |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| # contigs | 12 | 12 | 24 | **12** | 101 | 16 | 11 |
| Largest contig (Mb) | 7.41 | 7.45 | 7.41 | **7.45** | 6.01 | 7.39 | 7.45 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Total length (Mb) | 36.08 | 36.21 | 36.1 | **36.1** | 35.53 | 35.62 | 36.18 |
| GC (%) | 53.02 | 53.01 | 53.01 | **53.01** | 53.20 | 53.03 | 53.02 |
| N50 | 4.7 | 4.86 | 5.36 | **4.86** | 1.54 | 5.34 | 5.39 |
| N90 | 2.32 | 2.34 | 2.32 | **2.34** | 2.71 | 3.83 | 2.34 |
| L50 | 3 | 4 | 3 | **4** | 6 | 3 | 3 |
| L90 | 7 | 7 | 7 | **7** | 25 | 7 | 7 |
| Avg. coverage depth | 875 | 893 | 891 | **893** | 874 | 865 | 892 |

*Table 9 Genome assembly, gene prediction and annotation summaries of de novo sequenced insect pathogenic fungi, modified after Shang et al. (2016).*

| | ***Nomuraea rileyi*** | ***Isaria fumosorosea*** | ***Aschersonia aleyrodis*** | ***Akanthomyces lecanii*** | ***Beauveria brongniartii*** | ***Sporothrix insectorum*** | ***Ascosphaera apis*** | ***Akanthomyces muscarius*** |
|---|---|---|---|---|---|---|---|---|
| Sequenced strain | RCEF 4871 | ARSEF 2679 | RCEF 2490 | RCEF 1005 | RCEF 3172 | RCEF 264 | ARSEF 7405 | **Ve6** |
| Genome size (Mb) | 32 | 33.5 | 30.9 | 35.6 | 32.4 | 34.7 | 20.4 | **36.1** |
| Predicted genes | 8,764 | 10,060 | 8,461 | 11,030 | 9,595 | 9,496 | 6,442 | **12,231** |
| Contig No. | 1009 | 685 | 604 | 197 | 967 | 819 | 2863 | **12** |
| Coverage (×) | 107.34 | 86.99 | 76.05 | 81.94 | 73.46 | 70.37 | 100.88 | **893.00** |
| Repeat (%) | 6.15 | 3.84 | 0.41 | 0.95 | 2.87 | 0.74 | 1.36 | **5.2** |
| G+C content (%) | 49.92 | 53.65 | 53.9 | 53.1 | 51.8 | 54 | 47.67 | **53.01** |
| Completeness (%)* | 98.9 | 98.9 | 97.2 | 99.1 | 98.5 | 98.5 | 98.3 | **98.3** |
| NCBI Accession | AZHC00000000 | AZHB00000000 | AZGY00000000 | AZHF00000000 | AZHA00000000 | AZHD00000000 | AZGZ00000000 | **TBC** |

In the GO analysis, a total of 7126, 9903 and 4355 coding sequences (CDS) of *A. muscarius* were assigned to biological processes (BP), molecular functions (MF) and cellular components (CC) GO categories, respectively. The highest number of CDSs in BP was associated with transmembrane transport (16%), protein modification (8%) and regulation of transcription (7%). In MF, CDSs were predominantly assigned to hydrolase (17%), transferase (15%) and oxidoreductase activity (12%). Regarding cellular components, CDSs were abundant as nucleus (22%), membrane (14%) and integral component of

membranes (14%). The highest number of genes associated with enzyme codes belonged to hydrolases (1853) followed by transferases (1469) and oxidoreductases (1175).

The fungal version of antiSMASH predicted 44 BCGs for the *A. muscarius* genome. Non-ribosomal peptide synthetase (NRPS) were the most abundant cluster of natural product enzymes identified in the genome, followed by type 1 polyketide synthase (T1PKS) and terpene clusters (Table 10). Few clusters showed high similarity (>90 %) to previously described clusters: these are responsible for the biosynthesis of dimethyl coprogen, epichloenin A, clavaric acid and 1,3,6,8-tetrahydroxynaphthalene.

*Table 10 Summary of biosynthetic gene clusters predicted by antiSMASH. NRPS: Non-ribosomal peptide synthetase cluster; NRPS-like: NRPS-like fragment; T1PKS: Type I polyketide synthase; NAPAA: non-alpha poly-amino acids like e-Polylysin*

| BCG catergory | Number of identified clusters |
|---|---|
| NRPS | 18 |
| T1PKS | 8 |
| terpene | 6 |
| NRPS-like | 4 |
| NRPS,T1PKS | 3 |
| NRPS,T1PKS,NRPS-like | 1 |
| terpene,NRPS-like | 1 |
| NAPAA | 1 |
| T1PKS,indole | 1 |

**Variant calling & convergent evolution**

We identified 205 high-quality variants within the 14 samples (Figure 18). These comprised 58 insertion or deletions (indels) and 147 single nucleotide

polymorphisms (SNPs). The ancestral population already carried significant genetic variation at some sites (Figure 17), suggesting that random UV mutagenesis could potentially be dropped from the protocol.

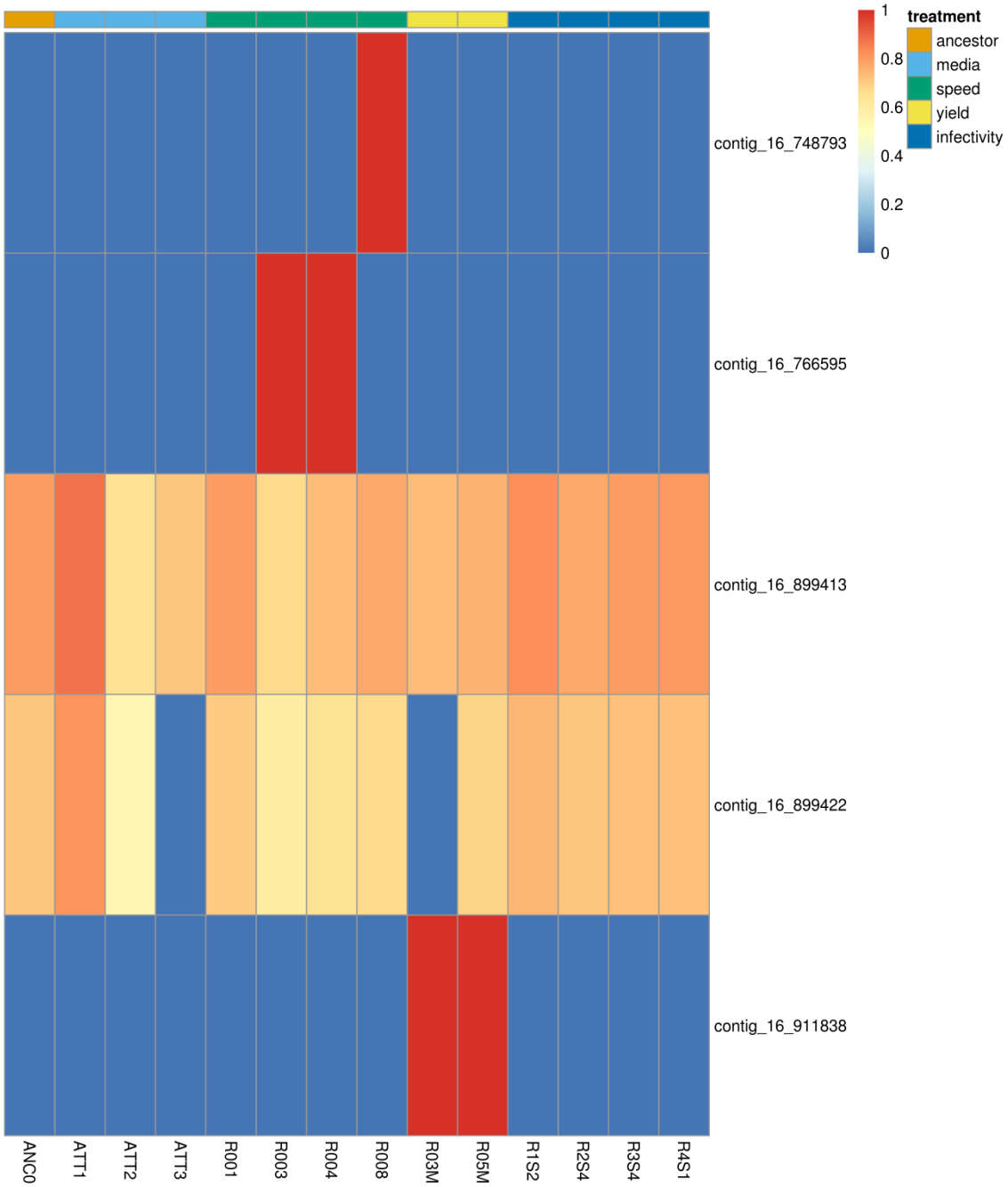



*Figure 17 Heatmap of allele frequencies for variants on contig 16. The colour of each cell in the heat-map indicates the estimated allele proportion in the population, based on the ratio of variant sequence reads versus total read depth*

*at that genomic site. Position 766595 and 911838 are examples of convergent mutations within corresponding selection treatment colour coded on the top bar.*

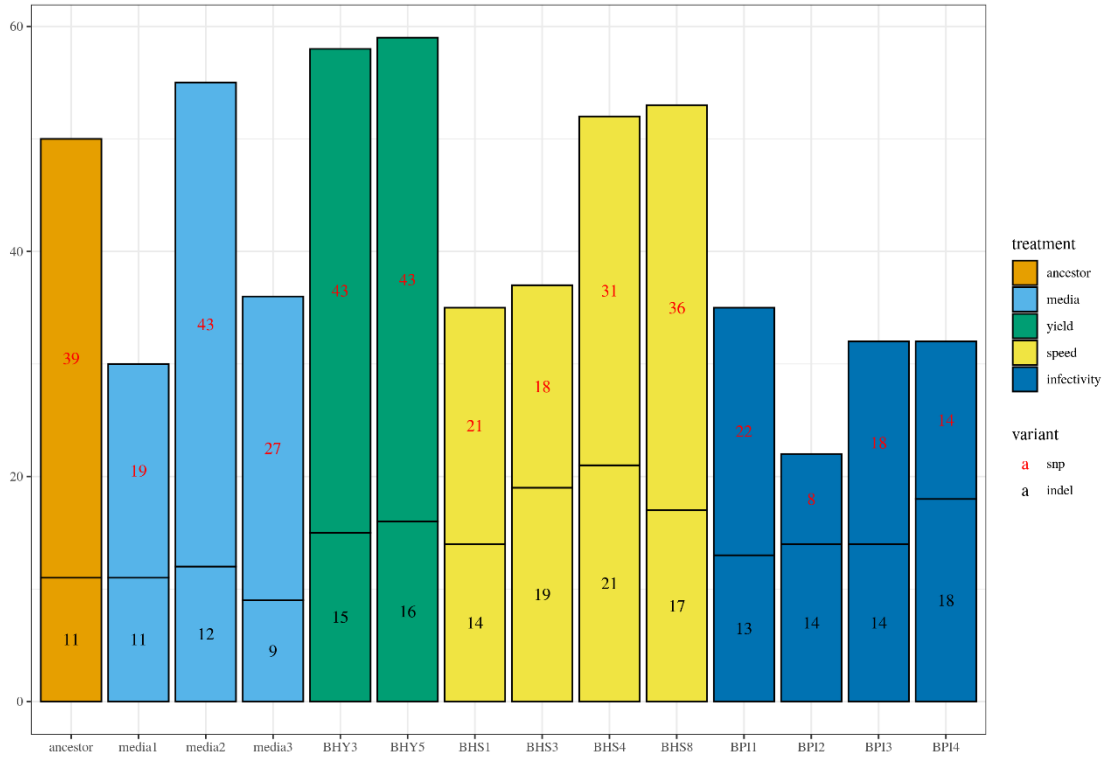



*Figure 18 Number of genomic variants per fungal lineage. Colours represent different selection treatments as described in detail in Chapter 3.*

Out of the total number of variants that passed filtering 45 SNPs and 18 INDELs show convergent evolution, ie were present in at least half of the replicates within each selection treatments. The highest number of unique SNPs showing convergent evolution were found in the speed selection treatment (14) followed by the yield (9) and infectivity (5) treatments. We identified only one SNP that was present in more than half of the replicates in the media treatment. Homology searches against the NCBI nr database using blastx shows that some of these variants fall in protein coding sequences (Table 11). These sequences include

heterokaryon incompatibility protein that play a role in vegetative and sexual recognition systems regulating self/nonself-recognition during vegetative growth (Wu et al., 1998). Fungal specific transcription factors, zinc finger protein (transcription regulation) that regulate gene expression (MacPherson et al., 2006). Arylsulfatase, an enzyme involved in sulfur acquisition (Cregut et al., 2013), cyclic nucleotide phosphodiesterase (PDE) that belongs to a group of enzymes degrading the phosphodiester bond in the second messenger molecules cAMP and cGMP and methionine permease involved in transmembrane activity.

*Table 11 Regions containing convergent variants and their best respective blast hits. Ref: reference allele, Alt: alternate allele, E-value: the number of alignments expected by chance with the calculated score or better, % iden: the highest percent identity for a set of aligned segments to the same subject sequence, accession: NCBI accession number, treatment: selective passage treatment described in Chapter III.*

| contig | position | Ref. | Alt. | type | description | scientific name | E-value | %. iden | accession | treatment |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 2759322 | T | C | SNP | Transcription factor | *Akanthomyces lecanii* RCEF 1005 | 0 | 98.64 | OAA72753.1 | yield |
| 3 | 1048102 | A | T | SNP | hypothetical protein BBO_04696 | *Beauveria brongniartii* RCEF 3172 | 5.00E-24 | 35.71 | OAA43553.1 | yield |
| 4 | 2881225 | C | A | SNP | Heterokaryon incompatibility | *Akanthomyces lecanii* RCEF 1005 | 0 | 98.52 | OAA77840.1 | speed |
| 8 | 1213799 | T | C | SNP | PRO1A C6 Zink-finger protein | *Cordyceps javanica* | 1.00E-132 | 65.37 | TQW08253.1 | yield |
| 8 | 1458577 | T | A | SNP | hypothetical protein LEL_01745 | *Akanthomyces lecanii* RCEF 1005 | 6.00E-133 | 99.45 | OAA82200.1 | speed |
| 16 | 766595 | A | C | SNP | arylsulfatase | *Akanthomyces lecanii* RCEF 1005 | 2.00E-39 | 100 | OAA71812.1 | speed |
| 16 | 911838 | T | A | SNP | fungal specific transcription factor | *Akanthomyces lecanii* RCEF 1005 | 2.00E-122 | 95.45 | OAA71766.1 | yield |
| 18 | 1176621 | T | † | INDEL | 3'5'-cyclic nucleotide phosphodiesterase | *Akanthomyces lecanii* RCEF 1005 | 0 | 91.52 | OAA74610.1 | speed |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 18 | 2006362 | GCT | G | INDEL | high affinity methionine permease | *Akanthomyces lecanii* RCEF 1005 | 2.00E-151 | 99.57 | OAA74361.1 | speed |
| 19 | 3592746 | G | A | SNP | hypothetical protein LLEC1_04806 | *Akanthomyces lecanii* | 3.00E-89 | 100 | OAQ98941.1 | speed |

†. Indel sequence TACCCGATATGGCAGC

## Integration with phenotypic data

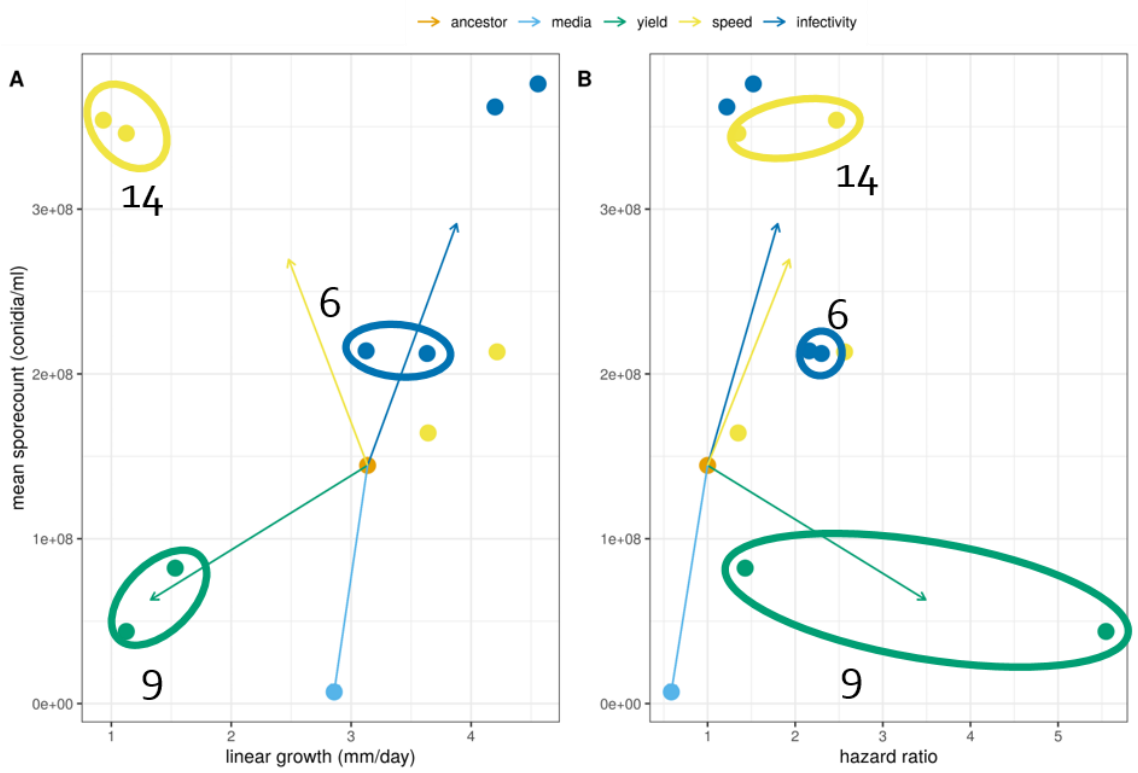



*Figure 19 Trends in phenotypic space of individually evolving fungal lineages belonging to different selection treatments redrawn from Chapter III. Points represent means per individually evolving replicate. Arrows lead from ancestor to mean of selected lineages. Virulence is represented as the estimate of instantaneous hazard parameter from survivorship analyses. Encircled clusters are colour coded according to treatment with a number representing the number of unique genomic variants identified belonging to the cluster.*

As mentioned above, our variant discovery highlights multiple unique genomic variants that show signs of convergent genomic evolution within selection

treatments. Furthermore, these genomic variants overlap with some of the phenotypic clusters belonging to different selection treatments identified in Chapter III (Figure 19). These convergent variants are spread across 9 contigs representing different chromosomes.

**Differential gene expression analysis**

Reads were counted by mapping clean reads of individual replicates to the *A. muscarius* genome assembly for the identification of differentially expressed genes (DEGs) between fungus re-isolated from infected aphids and agar plates. A total of 4469 DEGs (FDR < 0.05) were identified, with 1588 up-regulated in fungi infecting aphids and 2881 down-regulated, when compared to fungi isolated from agar plates. Focusing on two major expression patterns, genes strongly up- or down-regulated, we observed the most obviously upregulated functions *in vivo* relative to *in vitro* (> 2 fold) in lyases such as argininosuccinate lyase (ASL), catalysing the arginine biosynthetic pathway and Glutathione-dependent formaldehyde-activating enzyme (Gfa) that plays a role in formaldehyde catabolic processes. We found 11 genes associated with lipid metabolic pathway to be up-regulated. These include polyprenyl synthetase, fatty acid hydroxylases, Putative stearoyl-CoA desaturase and phosphatidylserine decarboxylase. Heat-labile enterotoxins were also highly up-regulated in infecting fungi. Cytochrome P450 (CYPs) domain containing proteins were found to be significantly down-regulated along with amino acid transporters and proteins involved in proteolysis and iron-binding. The number of significantly up- and down-regulated molecular functions are summarized in Figure 20.

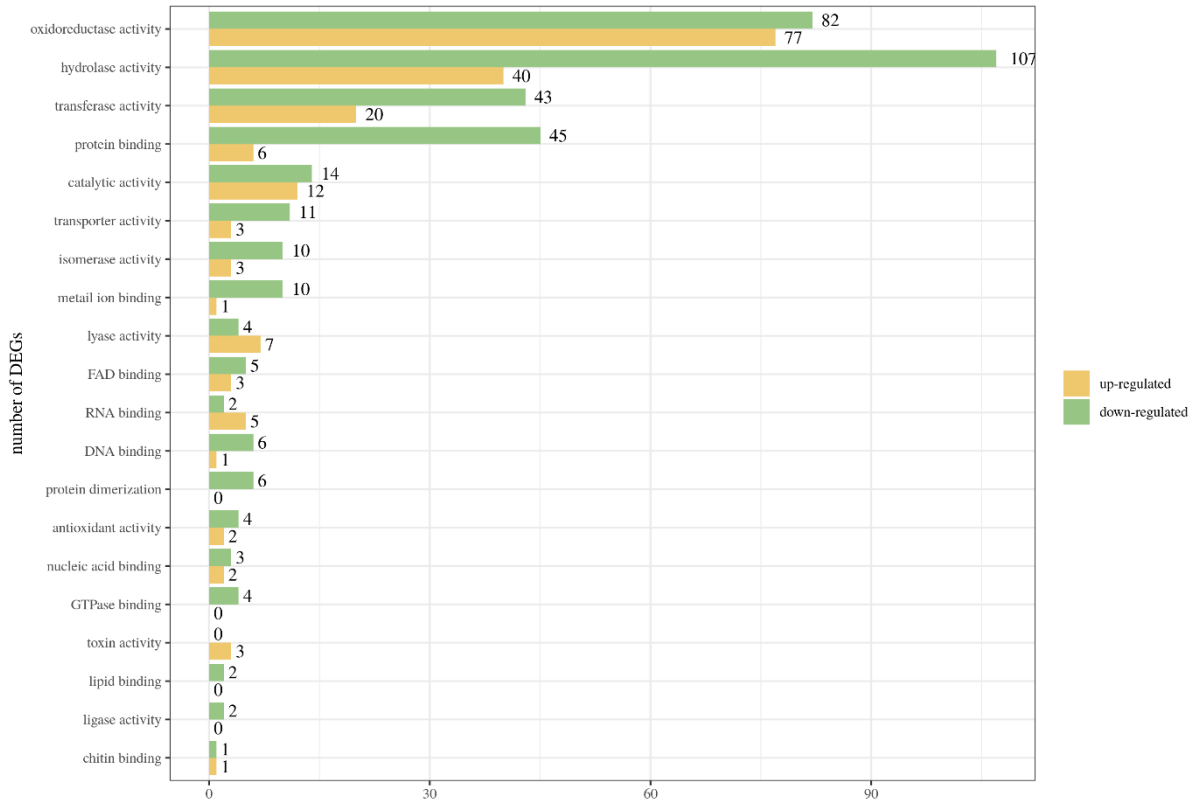



*Figure 20 Number of DEGs between A. muscarius isolated from M. persicae vs SDA agar plates with molecular function. Differentially expressed genes identified using a corrected p-value threshold of p < 0.05 and a fold change >2 for up-regulation or <-2 for down-regulation (see methods).*

## Discussion

The infection mechanisms of entomopathogenic fungi (EPF) have been thoroughly investigated in species commonly used in biological control applications, such as *Beauveria bassiana* and *Metarhizium* spp (St Leger & Wang, 2020; Valero-Jimenez et al., 2016; C. Wang & Wang, 2017). Other, less well-studied pathogens such as *Akanthomyces muscarius* lack the genomic resources for detailed study of molecular mechanisms undeprinning infection. Furthermore, the molecular and genetic response to increased selection pressure for virulence is not well understood, partly due to the scarcity of studies conducting serial passage experiments with EPF, but also due to the lack of targeted sequencing resources. In order to further our understanding of the mechanism of pathogenesis in *A. muscarius*, obtaining a high- quality genome assembly is critical. The only species in the genus *Akanthomyces* with an available genome assembly is *A. lecanii* (previously *Lecanicillium lecanii*) (Shang et al., 2016). In this study, we generated both genomic and transcriptomic resources and created a high-quality *de novo* genome assembly for *A. muscarius.*

A high proportion of sequences we report on correspond to hypothetical or unknown (not annotated) genes, however our analysis did identify genes related to virulence in *A. muscarius*. A range of gene products that show overexpression in the fungi infecting hosts include siderophores, metabolic components of the nitrogen pathway (argininosuccinate lyase), other metabolic kinases such as glucokinase and serine-type proteases (subtilisin) (Joshi et al., 1997; St. Leger et al., 1996). Siderophores, low-molecular weight, high-affinity iron-chelators are important for a range of biological functions such as virulence and stress resistance (Johnson, 2008). Argininosuccinate lyase has been found to be

essential for germination in *C. albicans* and *A. nidulans*, an important virulence factor (Jastrzębowska & Gabriel, 2015; Matsumoto et al., 2013). Subtilisin like proteases play a role in cuticle degradation (St. Leger et al., 1994).Cyclophilin-like proteins were also found to be up-regulated. Cyclophilin has previously been shown to be of importance for virulence in mammals and plants (Viaud et al., 2003). The high number of up-regulated genes in fungi *in vivo* associated with lipid metabolism is not surprising as lipids, alkenes, fatty acids and esters are some of the main components of the insect cuticle (Ali et al., 2009). Conidia germination was shown to be improved when coupled with secreted lipase in *N. rileyi*. Topical application of the enzyme also increased mortality of *S. litura* (Supakdamrongkul et al., 2010). Lipid assimilation, detoxification and hydrolisis has been identified as a key trait of EPF in overcoming the antimicrobial barrier of the insect cuticle, however our knowledge on the genetic mechanisms driving these function is scarce (Ortiz-Urquiza, 2021). The infection process of EPF relies on a complex array of enzymes activated at different times of the infective process. These include proteases, lipases, chitnases, galactosidases, catalases and glutaminases (Mondal et al., 2016). Our transcript analysis is capturing only one timepoint, the first day after host death. This explains why some functions such as hydrolases are mostly down-regulated whereas toxin activtiy is up-regulated. A time-series experiment collecting transciptomic data would provide insight into the timing of transitions between metabolic states during host infection.

Experimental evolution has been widely used to study adaptation to selective pressures *in vitro* over long term selection experiments in microbes such as *E.coli* (Barrick & Lenski, 2013; Barrick et al., 2009; Tenaillon et al., 2016) and yeast

(Segrè et al., 2006; Swamy & Zhou, 2019). *In vivo* passaging of microbes studying paralell or convergent evolution in a host provides a more complex selection pressure combined with possible bottleneck effects enforced by the host (van Leeuwen et al., 2015). This can make it difficult to identify genomic changes, especially due to low replication and difficulties of passaging. Host-pathogen interactions are context dependent, therefore predictions based on changes seen after *in vitro* selection cannot be expected to mimic nature or confer fitness benefits seen *in vitro*. This is has been shown in phage resistance evolution in a plant-bacteria-phage model system (Hernandez & Koskella, 2019).

Few *in vivo* studies were successful at showing convergent changes in pathogen genotype that follows adaptation to selection *in vivo*. One succesful example is from nasal infection of mice with the pathogenic bacteria *Streptococcus pneumoniae*, showing evidence for paralell evolution of a surface modulating mutation under strong selection pressure (Cooper et al., 2020). Similarly, serial passaging of the plant pathogen *R. solanacearum* in different host plants lead to paralell evolutionary change in a regulatory gene associated with fitness gains in independently evolving population on a certain host (Guidot et al., 2014). To date, the work presented here is the first to employ this approach to study the evolution of phenotype, genotype and their associations in an EPF passaged *in vivo*. We found convergent evolution in the phenotypic space by recording life-history traits and virulence of the evolved fungal lineages. Some of the lineages within these phenotypic clusters belong to different selection treatments, however we only found signs of convergent genomic evolution in lineages within selection treatments. This highlights the flexibility of fungi in both the genetic and physiological levels (St Leger & Wang, 2020), but also confirms that rigorous

selective passaging can be a powerful tool for studying host pathogen interactions as well as strain improvement. These convergent genomic changes were associated with transcription factors and genes involved in the regulation of gene expression as well as genes involved in metabolic pathways that could signal adaptation to the host.

Analysis of genomic variants has proven to be difficult due to the high number of uncharacterized coding sequences, however the few homolgy based matches indicate that these variants could play important roles in pathogenicty of the fungi. Each DEG and structural variant falling within CDS with unkown function might be involved in mechanisms of pathogenicity employed by the fungi when infecting aphids. The function of these genes should be further investigated via quantitative reverse transcription PCR (RT-qPCR) in order to confirm expression levels. This could be followed up with the generation of knockout mutants and pathogenicity assays. Methods for generating KO mutants of EPF have been described previously (Liu et al., 2013; Luo et al., 2015; Xu et al., 2014). Follow up studies (as described above) investigating the function of these variants could confirm the potential for selective passaging to be used for targeted selection of specific traits.

Our high quality genome assembly represents a powerful resource for future study including for the field of strain improvement and host-pathogen interactions using an organism with complex disease dynamics. In addition, the present study provides a plethora of new hypothesis to be explored in an experimental setting, in search for strain improvement and combating insect pests.

# General Discussion

Recent reduction in the number of available synthethic chemical pesticides due to more stringent regulation based on human and environmental safety, the evolution of resistance in target organisms, lack of new active substances in development and consumer concerns leave farmers and growers in need of effective alternative pest control solutions that can be combined in integrated pest management (IPM) (European Commission, 2018c; Garthwaite, 2019; Tilman, 1999; Tilman et al., 2002). Entomopathogenic fungi belonging to the order *Hypocreales* have been in agricultural use against invertebrate pests for decades. However, the potential of biopesticides based on EPF have not yet been fully unlocked. Applications can be unreliable due to high dependency on abiotic factors and slower speed of kill compared to synthetic chemicals, furthermore the lack of residual effect requires frequent multiple applications that increase the cost of use (Jaronski, 2010b). Improving the efficacy of these organisms as biocontrol agents for invertebrate crop pests will provide a sustainable and targeted tool for pest management. The aim of the current work was to investigate whether insecticide resistant *Myzus persicae* carry exploitable fitness costs that are only apparent under pathogen challenge. Such fitness cost would indirectly serve to slow the development of insecticide resistance in aphids. Furthermore, we explore how different selection regimes influence the virulence and other life history traits of the fungi, and whether using serial passaging combined with artificial selection can be an effective strain improvement tool for this invertebrate pathogen. In addition, we present the first *de novo* draft genome assembly and annotation for *A. muscarius* combined with transcriptomic sequencing resources.

## Fitness cost and resistance management

Insecticide resistance could lead to evolutionary trade-offs that result in fitness penalties when not under insecticide selection pressure (Ffrench-Constant & Bass, 2017). Fitness costs of insecticide resistance in *M. persicae* has been previously described (Foster et al., 2000; Foster et al., 2011; Foster et al., 2005; Foster et al., 1999). Here we show that in a laboratory setting, insecticide resistant *M. persicae* does not show impaired fitness in terms of susceptibility to *A. muscarius*, development time or intrinsic rate of increase. Contrary to our expectation, we found insecticide resistant aphids slightly more tolerant to EPF. However, our competition experiment shows that this effect, does not translate into a fitness advantage for the resistant clone over multiple generations. Studying fitness costs in laboratory setting can be used for targeted identification of specific traits related to loss of fitness. Nonetheless, slight differences in fitness might only be apparent in natural conditions where individuals are exposed to a complex combination of stressors. Environmental stress, presence of natural enemies and pathogens, qualities of the host plant and insect behaviour are all potentially influencing the insects success in natural environments (Kliot & Ghanim, 2012).

Furthermore, we highlight the potential confounding factor in time spent in artificial rearing. Maintaining a live aphid library of 170+ clones with different genetic background provides an irreplaceable resource to test a plethora of hypothesis in resistance development and more. However, careful consideration should be taken whether difference in length of time spent in artificial rearing needs to be accounted for when comparing the performance of clones, such as in pathogen bioassays. Immune functions are costly evolved traits that can be

lost or reduced in a laboratory setting (McKean et al., 2008; Nystrand & Dowling, 2020; Schmid-Hempel, 2005).

Combined use of chemical pesticides and EPF would be beneficial in reducing the amount of synthetics used and therefore slowing the evolution of resistance in insects, regardless of an apparent exploitable fitness cost in the host. Such combinations could allow for the use of sublethal doses of insecticides while achieveing sufficient levels of control (Pelizza et al., 2018; Rivero-Borja et al., 2018). However, previous studies have shown that thorough testing of application timing and combinations of fungal strain and synthetic pesticide is crucial for successful control (Cuthbertson et al., 2008; Cuthbertson et al., 2012; Meyling et al., 2018; Sain et al., 2019).

**Selective passage as strain improvement**

Benefits of using entomopathogenic fungi in IPM could be further augmented if their individual efficacy are improved. The development of EPF for better control of insect pests is taking numerous different routes simultaneously. Isolation of novel strains with inherently high virulence, and better maintenance of virulence factors in laboratory culture are one of the most obvious fields of research (Sharma et al., 2020). Novel isolates should be sent to culture collections to be maintained without further loss of virulence. The largest collection of entomopathogenic fungi is maintained at The Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) with more than 13000 isolates from 700+ taxa of fungi isolated from 1300 hosts. Improving spore formulation (Felizatti et al., 2021; Jaronski & Mascarin, 2017; Wraight et al., 2016) and delivery of spores (Farenhorst & Knols, 2010) are important to ensure that viable spores make contact with the insect cuticle. Formulations play

a key role in providing adequate shelf life for growers, easing the logistics and reducing the cost of use (Vega et al., 2009).

Increasing availability of genomic resources for EPF combined with genetic engineering opens the possibility for numerous ways of increasing fungal efficacy and cost-effectiveness by producing more stress resistant propagules and highly virulent strains. Reducing the speed of kill and/or the required lethal dose is often the aim of using genetic engineering (Lovett & St Leger, 2018). Successful implementation has been achieved by expressing endogenous proteins targeting the insect cuticle (Fan et al., 2007; Fang et al., 2005; St. Leger et al., 1996), neurotoxic peptides from predators and pathogens of insects (Pava-Ripoll et al., 2008; C. Wang & St Leger, 2007; M. Xie et al., 2015), proteins that target hormones and insect physiology (Zhao et al., 2014) and synthetic genes (Fan et al., 2007; Fan et al., 2010). Genetic engineering tools have mostly been developed for *Metarhizium* spp. and *Beauveria* spp. based on *Agrobacterium*-mediated transformation, and the use of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR associated protein 9). The potential in these tools is indisputable, currently it is the regulatory hurdles limiting their use instead of technical challenges (Lovett & St Leger, 2018).

Selective passaging for increased virulence is relatively unexplored in a fungi-insect model, with the few available studies showing mixed results (Raymond & Erdos, 2022). The work carried out in Chapter III shows, that integrating virulence theory into the design of passage experiments yield results not only in terms of increased virulence but can provide a better understanding in the underlying biology governing virulence and life history traits such as growth and sporulation that are crucial for development as inundative biocontrol agents.

The advantage of strain improvement using selective serial passage is that no previous mechanistic or genomic knowledge is required. As long as the fungi can successfully infect and kill the host, there is a basis for selection. Furthermore, strains selected with this method will not generally fall under genetically modified (GM) regulation therefore would potentially forego licensing that can be extremely lengthy and costly. However, the process of successfully producing derived strains with improved traits would take longer and will probably be more laborious than genetic engineering. Nevertheless, serial passaging in invertebrate pathogens have been proven invaluable in explaining the loss of virulence in vivo (Shapiro- Ilan & Raymond, 2016) or conditions where an intermediate level of virulence is favoured (Cornforth et al., 2015) and has been successful at increasing virulence in many pathogens of insects (Raymond & Erdos, 2022).

The work presented here is one of the few studies that employ serial passage through an insect host with an EPF and show increased virulence of the pathogen. This increase in virulence was modest, however repeatable in a different species of aphid, therefore not specific to the species used in the passage. Theoretical predictions of strong trade-offs between life history traits and virulence were not evident in the *A. muscarius – M. persicae* model. Our results suggest that there are multiple subtle trade-offs operating simultaneously. This seems a likely scenario considering the large genome size and complex pathogenicity and life cycle of this fungi (Butt et al., 2016; C. Wang & Wang, 2017). The lack of support for social virulence models are probably more surprising, considering the high number of secondary metabolites produced by *Hypocrealen* fungi that could act as public goods. However, there seems to be social conflict around spore production and timing of sporulation.

Spore production is potentially influenced by relatedness as passage with single cadavers (high relatedness) showed better sporulation compared to multiple cadavers or no host bottleneck (low relatedness). The timing of sporulation also varied significantly which might indicate conflict around switching to spore formation as opposed to mycelial growth (Ratcliff et al., 2013).

## Genome and sequencing resources

Serial passaging resulted in clusters of phenotypes within and between treatments with differences in their virulence, growth and sporulation. In order to further our understanding of the genomic background of these phenotypes we created the first draft genome assembly for *A. muscarius* and identified genomic variants within the derived lineages from Chapter III. Clustering of variants corresponding to the clusters identified in the phenotypic space of derived lineages indicates that such selective passage protocols can be used for targeted selection of specific traits and phenotypes given the underlying mechanistic knowledge.

We identified numerous differentially expressed genes in fungi isolated from one day old aphid cadavers compared to controls isolated from agar plates. The lack of functional validation and sequencing resources within the genus did not allow for a detailed annotation of our resources, however these resources combined with the above presents an extremely valuable resource for research of *A. muscarius*.

## Future work

- Identifying fitness costs in this model system should be carried out as field trials with whole plants in confinement.

- Follow up the yield treatment that showed the highest increase in virulence with different methods/antibiotics to counter bacterial growth
- Aphid nymphs have generally low susceptibility to EPF. Testing the selection protocol for increased virulence on aphid nymphs could help achieve better host population control.
- Test the importance of relatedness in a separate selection experiment directly, using relatedness as treatments.
- Is sporulation a social trait? Study the relative fitness of early/late sporulators in order to identify potential cheating in relation to resource use *in vivo*. Hypotheses regarding social cheating during spore formation could be tested with *in vivo* competition experiments using the derived fungal lineages and genetic markers based on the variants identified from sequencing of evolved lines.
- Investigate the function of genes highly overexpressed in infecting fungi and genes that exhibit sign of convergent evolution within selection treatments.

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