University of São Paulo
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Virulence of *Metarhizium anisopliae* and *Metarhizium rileyi* propagules: molecular investigation during infection on different insect hosts

Isabella Alice Gotti

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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UNIVERSITY OF COPENHAGEN
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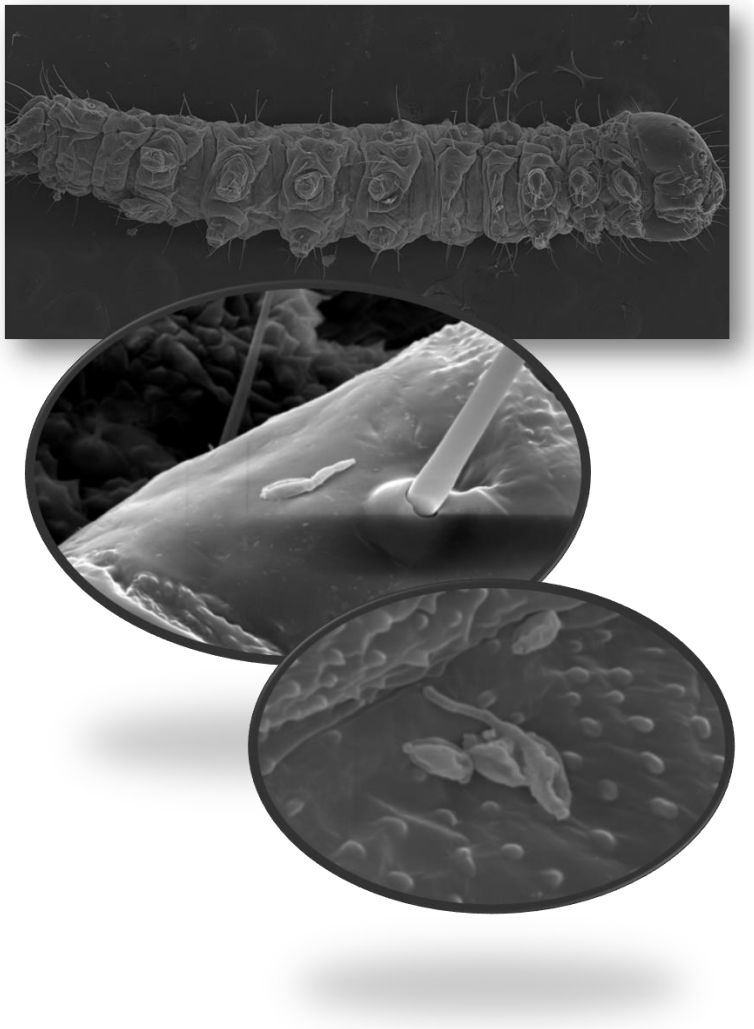

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To my father, Ivan Gotti,

who supported me, cheered all the victories, suffered the distance, and on the last mile of this journey, couldn´t wait to see me as a doctor.

You gave me strength until the end.
We will always have reasons to celebrate.

This Thesis is for you!

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Take the risk,

or lose the chance.

(Marko Halilovic)

SUMMARY

RESUMO

Virulência de propágulos de *Metarhizium anisopliae* e *Metarhizium rileyi*: investigação molecular durante a infecção a diferentes hospedeiros

Os entomopatógenos são uma ferramenta valiosa para o manejo de pragas em culturas agrícolas e podem ser usados em combinação com inseticidas para melhorar seu desempenho e reduzir a ocorrência de resistência a ingredientes ativos. O gênero *Metarhizium* (Ascomycota: Hypocreales) é abundante, distribuído globalmente e mais conhecido por infectar e matar diferentes artrópodes. Algumas espécies de *Metarhizium* possuem uma ampla gama de hospedeiros (generalistas), enquanto outras apresentam especificidade para certas famílias de insetos (especialistas). Essa abrangência na capacidade de causar doença a insetos pode ser utilizada para testar hipóteses sobre especiação e especificidade aos hospedeiros. Neste trabalho foram investigadas as espécies *M. anisopliae* e *M. rilyei*, e seus propágulos produzidos *in vitro*: blastosporos e conídios aéreos. Muitos estudos mostraram que as sequencias gênicas podem fornecer informações cruciais sobre como esses organismos se reproduzem e persistem em diferentes ambientes. Usando *Metarhizium* spp. como modelo, o primeiro capítulo da tese apresenta brevemente o processo de infecção por fungos entomopatogênicos e os benefícios do uso de conídios e blastosporos para o controle biológico de pragas no campo. Mostra também as principais questões e hipóteses que constroem este trabalho e apresentam os avanços alcançados. O segundo capítulo é uma revisão bibliográfica, destacando os avanços recentes em genômica e biologia molecular de fungos entomopatogênicos, especialmente nas interações inseto-fungo. O terceiro capítulo aborda os fatores biológicos e genéticos que permitem que os blastosporos infectem insetos e os tornem potencialmente eficazes para o controle biológico no campo. Enquanto a espécie generalista *M. anisopliae* em condições de alta osmolaridade produz um maior número de blastosporos com menor volume celular, *M. rileyi,* especialista em lepidópteros, produz uma menor quantidade de propágulos com maior volume celular. Também comparamos a virulência de blastosporos e conídios dessas duas espécies de *Metarhizium* para a praga *Spodoptera frugiperda*. Descobrimos que conídios e blastosporos de *M. anisopliae* são infectivos, mas matam menos insetos quando comparados a conídios e blastosporos de *M. rileyi*, sendo os conídios de *M. rileyi* mais virulentos. Usando transcriptômica comparativa durante a penetração de propágulos na cutícula de lagartas, no terceiro capítulo revelamos que os blastosporos de *M. rileyi* expressam mais genes relacionados à virulência contra *S. frugiperda* do que *M. anisopliae*. Em contraste, os conídios de ambos os fungos expressam mais fatores de estresse oxidativo do que os blastosporos. Esses resultados destacam que os blastosporos usam um mecanismo de virulência diferente dos conídios, o que pode ser explorado em novas estratégias de controle biológico. Por fim, no quarto capítulo investigamos a plasticidade fenotípica de blastosporos de *M. anisopliae* entre diferentes espécies de insetos. Determinamos que os blastosporos de *M. anisopliae* são altamente virulentos para *Tenebrio molitor*, e a porcentagem de formação de apressórios nas asas membranosas deste inseto foi três vezes maior do que nas asas dos outros insetos. Nós demonstramos uma clara diferença no padrão de expressão gênica durante o processo infectivo em *Gryllus assimillis*, *Spodoptera frugiperda*, *Apis mellifera,* e *Tenebrio molitor*. Isso significa que o transcriptoma e a virulência de *M. anisopliae* mudam consideravelmente de acordo com o inseto, com diferença significativa para *G. assimillis*. Tais diferenças estão associadas à expressão de enzimas como proteases, cutinases, lipases e peptidases, que podem estar relacionadas à degradação de compostos específicos da asa de cada inseto; hidrofobinas e destruxinas que estão associadas à virulência e metabolismo secundário. Os perfis de expressão gênica de propágulos fúngicos durante a penetração em diferentes asas de insetos foram caracterizados com o objetivo de aumentar nossa compreensão do processo fúngico de patogenicidade e virulência de *M. anisopliae* a insetos. Os resultados aqui obtidos podem potencialmente ajudar a encontrar genes candidatos cuja manipulação pode facilitar a descoberta de agentes de controle biológico mais eficazes.

Palavras-chave: Fungos entomopatogênicos, Virulência, Transcriptoma, Propágulos

ABSTRACT

Virulence of *Metarhizium anisopliae* and *Metarhizium rileyi* propagules: molecular investigation during infection on different insect hosts.

Insect pathogens are a valuable tool to manage crop pests. They can be used in combination with insecticides to improve their performance and reduce the occurrence of resistance to commercially active ingredients The genus *Metarhizium* (Ascomycota: Hypocreales) is highly abundant, globally distributed, and best known for infecting and killing many different arthropods. Some *Metarhizium* species have a broad host range (generalists). In contrast, others show specificity for certain insect families (specialists) and can be used to test hypotheses regarding speciation and host specificity. In this work two *Metarhizium* species were investigated, *M. anisopliae* and *M. rilyei,* and their propagules produced *in vitro*: blastospores and aerial conidia. Many studies have shown that sequence data can provide crucial information about how these organisms reproduce and persist in different environments. Using *Metarhizium spp*. as a model, the first chapter briefly present the entomopathogenic fungi infection process and the benefits of using conidia and blastospores for biological pest control in the field. It also shows the main questions and hypotheses that build this work and introduce the advances achieved. The second chapter, a review article, highlights recent advances in genomics and molecular biology of entomopathogenic fungi to further investigate the gene-for-gene relationships in insect-fungus interactions. In the third chapter we investigated the biological and genetic factors that allow blastospores to infect insects and make them potentially effective for biological control in the field. While the generalist *M. anisopliae* produces under high osmolarity conditions smaller blastospores in more significant number, the Lepidopteran specialist *M. rileyi* produces fewer propagules with a higher cell volume. We also compared the virulence of blastospores and conidia of these two *Metarhizium* species towards the economically important caterpillar pest *Spodoptera frugiperda*. We found that conidia and blastospores from *M. anisopliae* were both infectious but killed fewer insects compared with *M. rileyi* conidia and blastospores, where *M. rileyi* conidia had the highest virulence. Using comparative transcriptomics during propagule penetration on insect cuticles, this third chapter also shows that *M. rileyi* blastospores expresses more virulence-related genes against *S. frugiperda* than *M. anisopliae*. In contrast, conidia of both fungi express more virulence-related oxidative stress factors than blastospores. These results highlight that blastospores use a different virulence mechanism than conidia, which may be explored in new biological control strategies. Finally, chapter four investigated the phenotypical plasticity of *M. anisopliae* blastospores among different insect species. We determined that *M. anisopliae* blastospores are highly virulent for *Tenebrio molitor,* and the percentage of appressorium formation in the membranous wings of this insect was three times higher than in the wings of the other insects. We showed a clear difference in the gene expression pattern in the blastospores of *M. anisopliae* during the infective process in *Gryllus assimillis*, *Spodoptera frugiperda*, *Apis mellifera,* and *Tenebrio molitor*. This implies that *M. anisopliae* transcriptome and virulence change remarkably according to the insect, with the most significant differences for *G. assimillis*. These differences are associated with the expression of enzymes such as proteases, cutinases, lipases, and peptidases which might be related to the degradation of specific compounds of each insect wing; hydrophobins and destruxins which are associated with virulence and secondary metabolites. The gene expression profiles of fungal propagules during penetration on different insect cuticles were characterized to increase our understanding of the fungal process of insect pathogenicity. The results obtained here can potentially help to find gene candidates whose manipulation can ultimately lead to discovering more effective biological control agents.

Keywords: Entomopathogenic fungi, Virulence, Trancriptome, Propagules

SAMMENDRAG

Virulens af *Metarhizium anisopliae* og *Metarhizium rileyi* propagules: molekylær undersøgelse under infektion på forskellige insektværter.

Biologiske bekæmpelsesmidler er en af grundpillerne i integreret skadedyrsbekæmpelse (IPM), et lovende værktøj til at reducere skadedyrspåvirkningen. Mikrobielle patogener, såsom insektpatogener, er et værdifuldt værktøj til at håndtere skadedyr og kan bruges i kombination med insekticider for at forbedre deres ydeevne og reducere forekomsten af resistens over for kommercielt aktive ingredienser. Denne afhandling fokuserer på at undersøge en insektpatogen svampeslægt, *Metarhizium* (Ascomycota: Hypocreales) og de mekanismer, der bruges til at inficere insektværter. Denne slægt er meget divers, globalt distribueret og bedst kendt for at inficere og dræbe mange forskellige leddyr. Nogle *Metarhizium*-arter har et bredt værtsspektrum(generalister). I modsætning hertil viser andre specificitet for visse insektfamilier (specialister) og kan bruges til at teste hypoteser vedrørende artsdannelse og værtsspecificitet. Dette arbejde undersøgte to *Metarhizium*-arter, *M. anisopliae* og *M. rileyi*, og deres vækstformer produceret *in vitro*: blastosporer og luftkonidier. Mange undersøgelser har vist, at sekvensdata kan give afgørende information om, hvordan disse organismer formerer sig og forbliver i forskellige miljøer. Men få organiserer informationen i en tidslinje af begivenheder i samme slægt. Ved at bruge *Metarhizium* spp. som model præsenterer første kapitel kort den entomopatogene svampeinfektionsproces og fordelene ved at bruge konidier og blastosporer til biologisk skadedyrsbekæmpelse i marken. Den viser også de vigtigste spørgsmål og hypoteser, som dette arbejde bygger på og introducerer de opnåede fremskridt. Det andet kapitel er en oversigtsartikel som fremhæver de seneste fremskridt inden for genomik og molekylærbiologi af entomopatogene svampe for yderligere at undersøge gen-for-gen-relationerne i insekt-svamp-interaktioner. Tredje kapitel diskuterer de biologiske og genetiske faktorer, der tillader blastosporer at inficere insekter og gøre dem effektive til biologisk bekæmpelse i marken. Mens generalisten *M. anisopliae* producerer et højere antal og mindre blastosporer, producerer Lepidoptera-specialisten *M. rileyi* færre blastosporer med et højere cellevolumen under forhold med høj osmolaritet. Vi sammenlignede også virulensen af blastosporer og konidier af disse to *Metarhizium*-arter mod det økonomisk vigtige skadedyr *Spodoptera frugiperda*. Vi fandt, at conidier og blastosporer fra *M. anisopliae* begge var infektiøse, men dræbte færre insekter sammenlignet med *M. rileyi* conidia og blastosporer, hvor *M. rileyi* conidia havde den højeste virulens. Ved at bruge komparativ transkriptomics under penetration af insektkutikula viser dette tredje kapitel også, at *M. rileyi* blastosporer udtrykker flere virulensrelaterede gener mod *S. frugiperda* end *M. anisopliae*. I modsætning hertil udtrykker konidier af begge svampe flere virulens-relaterede oxidative stressfaktorer end blastosporer. Disse resultater fremhæver, at blastosporer bruger en anden virulensmekanisme end konidier, som kan udforskes i nye biologiske kontrolstrategier. Endelig undersøgte fjerde kapitel den fænotypiske plasticitet af *M. anisopliae* blastosporer blandt forskellige insektarter. Vi fandt, at *M. anisopliae* blastosporer er meget virulente for *Tenebrio molitor*, og procentdelen af appressoriumdannelse på membranøse vinger på dette insekt var tre gange højere end i vingerne på de andre insekter. Vi viste en klar forskel i genekspressionsmønsteret i blastosporerne af *M. anisopliae* under infektionsprocessen i forskellige insekter, med de mest signifikante forskelle fundet for *G. assimillis*. Forskellene er forbundet med ekspressionen af enzymer såsom proteaser, cutinaser, lipaser og peptidaser, som kan være relateret til nedbrydningen af specifikke forbindelser i hver insektvinge. Også hydrofobiner og destruxiner, som er forbundet med virulens og sekundære metabolitter viste variation in ekspression mellem arter. Genekspressionsprofilerne for blastosporer under gennemtrængning på forskellige insekter blev karakteriseret med det formål at øge vores forståelse af insektpatogenicitet. De her opnåede resultater kan potentielt hjælpe med at finde genkandidater, hvis manipulation i sidste ende kan føre til opdagelsen af mere effektive biologiske kontrolmidler.

Nøgleord: Entomopatogene svampe, virulens, trancriptome, propaguler.

1. GENERAL INTRODUCTION

1.1. About the thesis

Fungal entomopathogens are important agents for the biological control of agricultural pests. They exhibit both sexual and asexual reproduction and produce a variety of infective propagules (Vega et al., 2012).

This thesis investigates insect-pathogenic fungi of the genus *Metarhizium* (Ascomycota: Hypocreales). They are highly abundant, globally distributed, and best known for infecting and killing many arthropods. Besides that, most *Metarhizium* species are saprophytes, rhizosphere colonizers, and beneficial root endophytes, which can switch between these lifestyles. Regarding pathogenicity, some *Metarhizium* species have a broad host range (generalists). In contrast, others show specificity for certain insect families (specialists) and can be used as model to understand speciation and host specificity.

With this, I investigated two *Metarhizium* species, *M. anisopliae* and *M. rilyei*, and two propagules produced *in vitro*, blastospores and conidia. The gene expression profiles of fungal propagules during penetration on different insect cuticles were characterized aiming to increase our understanding of the fungal process of insect pathogenicity. The results obtained here can potentially help to find gene candidates whose manipulation can ultimately lead to discovering more effective biological control agents.

This general introduction is split into four parts. First, I briefly present the entomopathogenic fungi infection process and the benefits of using conidia and blastospores as infection methods for biological pest control in the field. Then, I present the objects of this study, including investigations of biology, phylogeny, and host range, comparing *M. anisopliae* with *M. rileyi*. Finally, I also show the main questions and hypotheses that build this work and the advances achieved.

1.2. The entomopathogenic fungi propagules and their infection process

Entomopathogenic propagules must penetrate the insect cuticle using physical and enzymatic tools to colonize the host successfully. Therefore, they must deal with different environmental setbacks, insect tegument composition, and natural defense that modulate them genetically and phenotypically.

Entomopathogenic fungi have different structures in the natural lifecycle such as conidia, blastospores, mycelium, and resistance spores, some can be used for pest control (Leite et al., 2003). In nature, conidia have the function of reproduction and dissemination in the environment; blastospores act in the dissemination in the host hemolymph; mycelium migrates out of the host and allows fungal conidiogenesis; and resistance spores enable the fungus to survive in the soil (Leite et al., 2003). Regarding inundative strategies for biological pest control, conidia, and blastospores can be applied to overwhelm many factors that keep a pathogen in non-epizootic equilibrium with its host (Jaronski, 2010).

In a generic model for the infection process of entomopathogenic fungi, conidia adhere to the insect cuticle, where they germinate and form the germ tube and the appressorium (Figure 1 A). The penetrating hyphae break the cuticle towards the hemocoel, where the blastospores or hyphal bodies are formed (Figure 1 B). Fungal growth continues through the insect's hemocoel, invading organs, disrupting metabolic processes, and enabling the production of toxic metabolites, causing the insect's death (Vega et al., 2012).

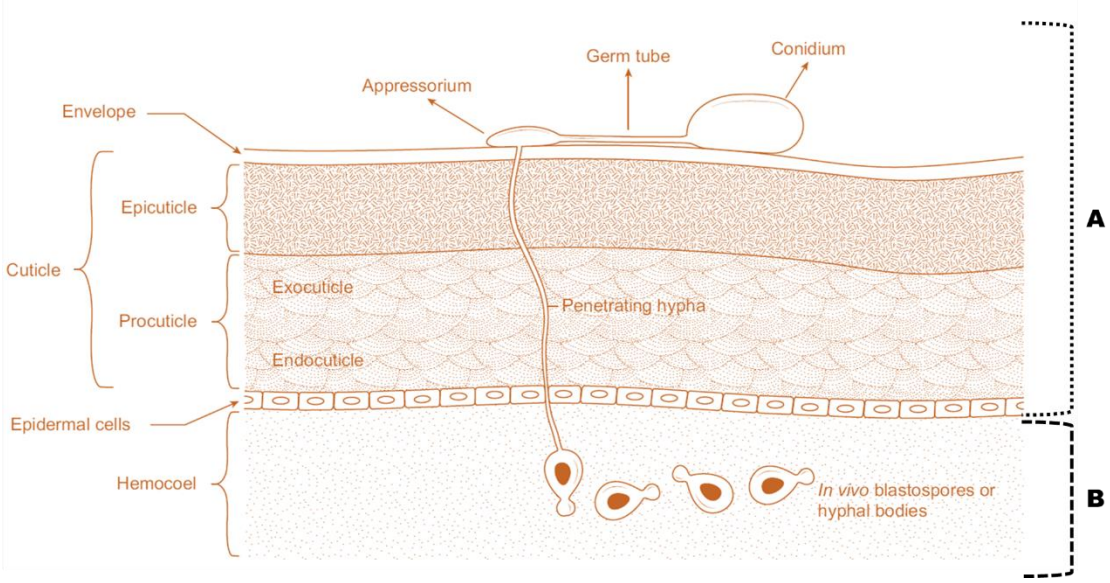


Figure 1. A generic model of the infection process for fungal entomopathogens. **(A)** Adhesion and penetration on insect cuticle. **(B)** Blastospores and hyphal bodies formation before fungal growth inside the insect body. Adapted from Vega *et al.* (2012). Fungal Entomopathogens in: **Insect Pathology (Second Edition)**. San Diego: Academic Press, 2012. p.185.

The surface of aerial conidia of most hypocrealean entomopathogenic fungi is covered with a rodlet layer composed of hydrophobin proteins that confer a hydrophobic charge to them (Holder and Keyhani, 2005), facilitating the passive attachment to hydrophobic surfaces like insect cuticles, for example. In contrast, blastospores have no hydrophobins, which confers the ability to adhere better to hydrophilic surfaces - especially in aquatic environments (Holder *et al.*, 2007; Greenfield *et al.*, 2014).

Many virulence factors influence fungal pathogenicity in insect hosts (Butt *et al.*, 2016). The potency of these virulence determinants depends on the pathogen specificity and the correct orchestration of virulence genes by a complex signaling apparatus (Butt *et al.*, 2016). Adhesins and other adhesion molecules are key pathogenicity determinants since the firm adhesion of spores to the host surface is an attribute of virulent fungal strains (Altre *et al.*, 1999). Typically, the more spores that adhere to the host cuticle, the faster the fungus will kill its host; thus, poor adhesion is a feature of hypo-virulent isolates (Jackson and Jaronski, 2009). Spore attachment is a two-step process: the first step is mediated by preformed physio-chemical properties of the spores, e.g., hydrophobic and electrostatic forces (Boucias *et al.*, 1988; Holder and Keyhani, 2005), and the second step involves the secretion of enzymes and mucilage. Hydrolytic enzymes degrade the cuticle, release nutrients, and facilitate penetration, while mucilage is often secreted to enhance binding to the host cuticle (Sosa-Gomez *et al.*, 1997).

Regarding the gene expression involved in the adhesion to insect cuticle, this process for the *M. anisopliae* conidia is mediated by an adhesin-like protein, known as MAD1, that is also shown to be involved in conidial germination and formation of blastospores (Wang and St Leger, 2007). Several other cell wall proteins are essential in adhesion and stress management (Butt et al., 2016). CWP10 is a non-hydrophobic cell wall protein that enhances conidial hydrophobicity and adhesion, while CP15 helps conidia cope with thermal and oxidative stresses (Li *et al.*, 2010; Ying and Feng, 2011).

Following spore attachment to the cuticle, germ tube formation is a critical event that depends on environmental and nutritional optimal conditions. Temperature, humidity, nutritional, chemical, and physical factors can influence germination (Vega *et al.*, 2012). For some entomopathogenic fungi, carbon and nitrogen sources are required to form a germ tube; however, only having nutritional conditions cannot guarantee successful germination.

Some components of the insect cuticle could have inhibitory effects on conidia, and fungi or bacteria on the cuticle might also inhibit germination (Sosa-Gomez *et al.*, 1997; Priyatno and Ibrahim, 2002).

For most entomopathogenic fungi, the differentiation of appressoria is a prerequisite for infection; it may be produced directly from conidia or at the end of germ tubes, or even laterally from hyphae (Butt et al., 2016). According to Srisukchayakul *et al.* (2005), *Metarhizium rileyi* does not produce appressoria but can penetrate directly (Srisukchayakul *et al.*, 2005).

The physicochemical and nutritional cues at the host surface influence both differentiation of infection structures and specificity (Lin *et al.*, 2011). Butt *et al.* (2016) state that cuticular physicochemical cues influence appressorium differentiation and phenotype. For example, conidia of *Metarhizium acridum* can germinate and differentiate appressoria on *Schistocerca gregaria* cuticle but fail to germinate on a nonhost like a Lepidoptera species (Butt *et al.*, 2016). Interestingly, blastospores of *Metarhizium anisopliae* could well germinate and differentiate appressoria on *Tenebrio molitor* membranous wings but fail to differentiate appressoria on *Apis mellifera*, as demonstrated in more detail in Chapter 4.

The insect cuticle surface is a harsh environment for entomopathogenic fungi; conidia are exposed to harmful UV, fluctuating humidity and temperature, antagonistic microbes, and innate host defenses (Butt *et al.*, 2016). To deal with many variables, they have evolved various mechanisms to cope with these biotic and abiotic stresses, often involving genes linked with virulence, heat shock, osmotic stress, cell wall integrity, and signal transduction (Ortiz-Urquiza and Keyhani, 2013). The Heat shock proteins (HSPs) and specific cell wall proteins, help manage environmental stress (Liao *et al.*, 2014). Besides formed enzymes that degrade fungistatic fatty acids in the insect cuticle, many other protein families are up-regulated, such as the cytochrome P450 monooxygenase (Lin *et al.*, 2011).

The next step is breaching the insect cuticle by mechanical pressure and producing cuticle-degrading enzymes. In this way, the fungus can obtain nutrition and eventually colonize the host. For the *Metarhizium* genus, the perilipin-like protein (MPL1) is involved in generating the appressorial turgor pressure required to breach through the cuticle (Wang and St Leger, 2007). The enzymes involved in cuticle degradation include proteases, chitinases, and lipases, which are needed to degrade proteins, chitin, and lipids, respectively (Schrank and Vainstein, 2010).

Once the fungus has gained access to the hemocoel, it utilizes insect nutrients for growth and reproduction. Fungal isolates that grow profusely in the hemolymph kill the insect by consuming the host nutrients and physically damaging the tissues, disrupting the host's physiology. Eventually, the hypha emerges from within the insect and sporulates at the end of this pathogenicity cycle, producing aerial conidia (Vega *et al.*, 2012).

Blastospores can be produced in liquid media and infect hosts (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015; Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015; Morales-Reyes *et al.*, 2018; Iwanicki, N. S. A. *et al.*, 2020). It is possible to produce large amounts of blastospores by liquid fermentation in a short time (<4 days), in a small space, and with less manual labor than the solid fermentation method (Iwanicki, N. S. A. *et al.*, 2020).

Blastospores differ from conidia in several ways. They are hydrophilic spores and usually are considered more virulent against susceptible hosts. They germinate faster than conidia (2-8 hrs versus 12–24 hrs), an attribute that could be regarded as a virulence determinant (Butt *et al.*, 2016). This rapid germination rate of blastospores produced in liquid culture- is best suitable as the primary infective structure in commercial bioinsecticides (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015).

The benefits of blastospores may overcome possible disadvantages in relation to aerial conidia, such as their expected lower tolerance to abiotic stresses since they are vegetative cells (Bernardo *et al.*, 2018). A suitable formulation may protect blastospores against abiotic stresses (Kim *et al.*, 2013) and increase their efficiency in bioproducts. In

addition, dehydrated blastospores of some entomopathogenic fungi remained viable for long periods when refrigerated and unrefrigerated, thereby possibly extending their shelf-life (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015; Bernardo *et al.*, 2018).

Even though blastospores have numerous advantages over aerial conidia, such as greater insect infectivity (Jackson, 1997; Lohse *et al.*, 2015; Alkhaibari *et al.*, 2016) and a cheaper and more efficient fermentation process (Jaronski and Mascarin, 2017), they lack vital qualities that impede their overall commercial development: desiccation tolerance and good shelf life. A reasonable shelf life must be guaranteed for a biocontrol agent to become a successful product (Dietsch *et al.*, 2021).

1.3. The *Metarhizium* genus: phylogeny, biology, and host ranges

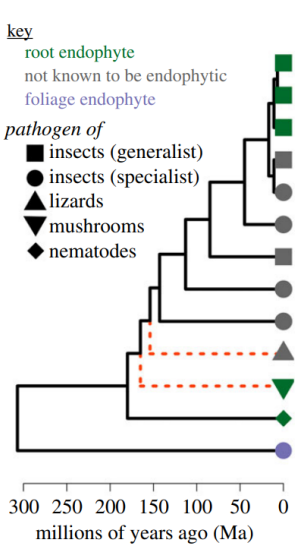
The *Metarhizium* (Ascomycota: Hypocreales) genus is a highly abundant group of fungi (St. Leger and Wang, 2020). It comprises entomopathogenic fungi employed for biological control of crop pests and vector-borne diseases since it was first described (Sbaraini *et al.*, 2016). However, several *Metarhizium* species also have multifunctional lifestyles and ecological niches, including insect pathogens, plant symbionts, and soil saprophytes (Brunner-Mendoza *et al.*, 2019). Being so diverse, they provide many independently evolved models of adaptation and response to different environments and insect hosts (St. Leger and Wang, 2020).

According to St. Leger and Wang (2020), these adaptions clarify the evolution and strategies of host selectivity and the reasons for the selection of sexuality or clonality. In this context, comparative genomic studies of *Metarhizium* species can understand differences in metabolism, host range, and root colonization, revealing which proteins and gene families are responsible for this rapid evolution of ecological interactions (Hu *et al.*, 2014). On chapter 3, this thesis exemplifies how the accuracy of comparative transcriptomic analysis can improve the studies of different lifestyles comparing the protein families expressed between two *Metarhizium* species with varied ranges of host: the generalist M. anisopliae and the Lepidoptera specialist *M. rileyi*.

Regarding the specialization of the ability to infect insects, *Metarhizium* strains show a continuum process ranging from the inability of the pathogen to infect many hosts, passing through the lower performance in most hosts, and then the ability to kill a panel of insects (St. Leger and Wang, 2020). For example, multi-host generalist strains of *M. robertsii* and *M. brunneum* have greater nutritional versatility than specialists, colonize plant roots, and are facultative entomopathogens. St. Leger and Wang (2020) assume that specialized strains probably depend on insect hosts for reproduction. Thus, the ability of a generalist strain to attack diverse insects does not rule out adaptations to exploit nutrients on cuticles of frequently met hosts.

Figure 2 shows a phylogenomic tree with the estimated divergence time for sequenced *Metarhizium* species and related fungi. It also includes orange dashed lines with the saprophyte *M. marquandii* and the lizard pathogen *M. viride*. According to Rehner and Kepler (2017), they have not been sequenced, but their branch points on the phylogeny are estimated from a multi-gene phylogeny (Rehner and Kepler, 2017). Also, in Figure 2, the genome size, the spore size, and the total number of genes can be found to the right of the tree.

Aiming to show examples of activities that likely contributed to the evolution of diverse lifestyle options, St. Leger and Wang (2020) provide in Figure 2 the number of secondary metabolite (SM) gene clusters and copy numbers of genes encoding proteases (subtilisins and trypsins) and carbohydrate-degrading glycoside hydrolases (GH), specifically GH18 (chitinases).



	genome size (Mb)	spore length × width (μm)	genes	subtilisins	trypsins	GHs	GH18	SM clusters
M. anisopliae	38.5	5.02 × 2.02	10 891	56	26	148	20	63
M. robertsii	41.7	4.69 × 2.34	11 689	63	36	154	24	64
M. brunneum	37.1	4.48 × 2.09	10 689	60	32	140	16	64
M. guizhouense	43.2	7.19 × 2.99	11 787	58	29	162	28	66
M. majus	42.1	11.76 × 2.42	11 535	56	25	147	20	58
M. acridum	39.4	4.2 × 2.55	9849	43	15	126	14	40
M. frigidum	50.4	ND	12 347	69	42	156	24	52
M. album	30.5	4.35 × 1.59	8472	28	12	108	12	34
M. rileyi	31.8	ND	8945	25	9	121	18	30
M. viride	ND	ND	ND	ND	ND	ND	ND	ND
M. marquandii	ND	ND	ND	ND	ND	ND	ND	ND
P. chlamydosporia	43.8	ND	14 204	31	9	200	18	48
E. festucae	35.2	ND	12 199	18	4	98	11	38

Figure 2. A phylogenomic tree with the estimated time of divergence and genomic information for sequenced *Metarhizium* species and related fungi. St. Leger and Wang (2020), *Metarhizium*: jack of all trades, master of many. **Open Biol. 10**: 200307. https://doi.org/10.1098/rsob.200307.

Among the several *Metarhizium* species used as biological control agents, the *Metarhizium anisopliae* (Metschn.) Sorokin deserves special attention. Besides the capability of association with plant roots, this species can infect a broad range of arachnid and insect hosts, from agricultural pests to vectors of human disease. The *M. anisopliae* is also one of the most studied and applied biological control agents worldwide, having been successfully used since the 1970s in Brazil to control spittlebugs in sugarcane and pastures (Parra, 2014). The pioneering immunologist Elie Metchnickoff initiated trials of this fungus against the wheat cockchafer *Anisoplia austriaca* in 1879, making it one of the first organisms seriously investigated for use against agricultural pests (Wasuwan *et al.*, 2021).

The other fungal species of this thesis is the *Metarhizium rileyi* (Farlow) Kepler, S.A. Rehner & Humber. Previously known as *Nomuraea rileyi*, it is pathogenic to Lepidoptera insect species, infecting specially noctuids like *Anticarsia gemmatalis* and *Spodoptera frugiperda*, which are critical pests of important crops (Alves, 1998). *M. rileyi* is a dimorphic fungus with yeast-like hyphal bodies and a proper filamentous growth phase, with several isolates from different locations worldwide (Fronza *et al.*, 2017).

According to Fronza *et al.* (2017), the genetic diversity of *M. rileyi* and its characteristics during the penetration through insect cuticles are the leading of differences in the pathogenicity of this fungus. Advances in its research have contributed to our current knowledge of different aspects of this pathogen and how it interacts with its host insects. However, further research is needed to clarify still unknown points, e. g., its enzymatic capacity to attack different cuticle composts, which provide other nutritional qualities.

The potential of this species as an entomopathogen is indisputable; therefore, obtaining more significant knowledge about the aspects mentioned above, whose peculiarities need to be understood and respected, can elevate its status in pest control. Aiming this, in Chapter 3, we investigated and highlighted the differences between *M. anisopliae* and *M. rileyi* propagules, comparing their mode of infection to caterpillars. Table 1 summarizes some of the main differences among the species of this thesis.

Table 1. Summary of the main reported differences between *Metarhizium anisopliae* and *Metarhizium rileyi* species. Information obtained from Mongkolsamrit *et al.* (2020). Revisiting *Metarhizium* and the description of new species from Thailand. **Studies in Mycology**. Volume 95, March 2020, Pages 171-251(Mongkolsamrit *et al.*, 2020).

	M. anisopliae	***M. rileyi***
Colony color	Greyish green	Light Green
Spore morphology	Cylindrical to ellipsoid	Oval-cylindrical
Spore length (µm)	5.0 – 7.0	2.5 - 3.5
Blastospores/Yeast cells	Only in liquid media	Solid and liquid media
Host range	Generalist	Lepidoptera specialist
Ability to epizootic	No	Yes
Commercial production	Yes	No

In this thesis, *Metarhizium* species are studied not only because of their long-recognized utility as pest control agents but also because of their exciting biology that informs fungal associations with many other organisms. Many of the proposed uses of *Metarhizium* have required extensive ecological studies to demonstrate efficacy and safety. According to St. Leger and Wang (2020), such analyses are complicated as entomopathogenic fungi are very heterogeneous and occupy the same wide range of habitats as their hosts, with near ubiquity in soil and plants.

A great deal of the biodiversity among insect pathogens has been explored at deep taxonomic levels with the genomic sequencing of some important entomopathogenic fungi such as *Metarhizium*, *Beauveria,* and *Cordyceps*. These genomes have helped to elucidate the genetic basis of the entomopathogenic lifestyle, showing that the convergent evolution of entomopathogenicity has occurred via the repeated evolution of an 'entomopathogenicity toolkit' with increased numbers of enzymes that degrade insect cuticles and lineage-specific suites of insect-induced toxins (St. Leger and Wang, 2020).

In Chapter 2, this thesis starts collecting and organizing scientific information on the evolution of multifunctional lifestyles of the *Metarhizium* genus. This chapter aims to bring an overview of the genomic perspectives on the evolution of fungal host-specificity of *Metarhizium* spp. before presenting the approach and the insights obtained so far.

1.4. Main questions and hypotheses

This thesis's central questions and hypotheses were based on published and unpublished observations of previous studies of the Insect Pathology and Microbial Control Laboratory staff and collaborators at the University of São Paulo (ESALQ/USP). The topics represent the line of reasoning developed here and lead us to the main discussion regarding specialization and modes of action of different fungal propagules during their penetration on insect's tegument. Thus, below, I present the question, the observations and hypotheses raised for each.

Are blastospores produced in liquid culture media more virulent than aerial conidia?

Blastospores are usually considered more virulent than conidia as they form germ tubes and penetrate the host integument faster than conidia (Alkhaibari *et al.*, 2016; Bernardo *et al.*, 2018). For pest control purposes, blastospores can be produced and formulated as sprayable products and outperform in virulence the traditional solid-grown conidia against various target pests (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015; Alkhaibari *et al.*,

2016; Bernardo *et al.*, 2018; Iwanicki *et al.*, 2018; Iwanicki, *et al.*, 2020). Besides that, blastospores' virulence can be markedly affected by culture conditions and media composition against arthropod targets (Mascarin, Jackson, Kobori, Behle, Dunlap, *et al.*, 2015; Mascarin *et al.*, 2021).

Thus, regarding many studies referencing blastospores as being equally or more virulent than aerial conidia for many fungal species and different insect pests, this thesis hypothesizes that blastospores of *M. anisopliae* and *M. rileyi* are more virulent than conidia to insect hosts.

Does the osmotic stress in liquid culture media improve the blastospore production of more virulent propagules on Metarhizium species?

Adapting to environmental changes is crucial for fungal viability (Saito and Posas, 2012). These adaptations could be morphological, since they can modulate their phenotype in stress conditions; genetically since they contain complex signaling pathway networks to handle these stresses; and behaviorally, since they can modulate their behavior by being more virulent, for example (Babazadeh *et al.*, 2013; Lovett and St. Leger, 2015; Mascarin, Jackson, Kobori, Behle, Dunlap, *et al.,* 2015; Treusch *et al.*, 2015; Song *et al.*, 2016).

Mascarin *et al.* (2015) reported that high glucose concentrations on blastospore production by submerged cultures of Beauveria bassiana increased the media's osmotic pressure and led to the production of significantly smaller blastospores compared to blastospores produced in media with lower glucose concentrations. They also reported that blastospores of isolate ESALQ1432 produced in media containing 140 g glucose L^{-1} showed greater virulence toward whitefly nymphs (*Bemisia tabaci*) than blastospores produced in media containing 40 g glucose L^{-1}.

Regarding the evidence of the osmotic stress impact on fungal species, especially on the Hypocreales entomopathogenic fungi *B. bassiana*, this thesis also hypothesizes that if *B. bassiana* responds to osmotic stress changes in liquid fermentation, producing smaller and more virulent blastospores, *M. anisopliae* and *M. rileyi*, also Hypocreales entomopathogenic fungi, could respond equally, creating smaller and more virulent propagules.

Comparing propagules: do blastospores and conidia act the same way on the insect cuticle during the penetration?

Regarding adhesion and germination on insect cuticles, both conidia produced in solid substrates and blastospores produced under liquid fermentation can penetrate insect cuticles using mechanical pressure and enzymatic action (Vega *et al.*, 1999; Alkhaibari *et al.*, 2016; Bernardo *et al.*, 2018; De Paula *et al.*, 2021). Compared to aerial conidia, blastospores are more virulent against susceptible hosts (Mascarin, Jackson, Kobori, Behle, Dunlap, *et al.*, 2015; Alkhaibari *et al.*, 2016; Bernardo *et al.*, 2018; De Paula *et al.*, 2021), although they are less tolerant to desiccation (Iwanicki *et al.*, 2018). This makes blastopores an attractive but tricky alternative to conidia for biological control, although it is unclear exactly why blastospores are more virulent.

Iwanicki *et al.* (2020) revealed significant transcriptomic differences between the metabolism of blastospores and hyphae. Their results showed a clear molecular distinction between the blastospore and mycelial phases. While the main physiological processes associated with up-regulated genes in blastospores obtained by liquid fermentation were oxidative stress, amino acid metabolism, respiration processes, transmembrane transport, and production of secondary metabolites, the up-regulated genes in hyphae were associated with increased growth, metabolism, and cell wall re-organization (Iwanicki *et al.*, 2020). These findings illustrate essential aspects of fungal morphogenesis in *M. anisopliae* and highlight the main metabolic activities of each propagule under in vitro growth conditions; however, we still don't know how exactly they act when applied to insect tegument.

Thus, starting from the knowledge of the transcriptomic profile of blastospores during their production in liquid fermentation and knowing that they germinate and penetrate faster than conidia, we hypothesize that their transcriptomic profile must change during cuticle penetration, being different from conidia and adapting themselves to colonize the host.

Comparing fungal species: do Metarhizium anisopliae and Metarhizium rileyi act the same way on the insect cuticle during the penetration?

The *Metarhizium* genus has been subdivided into several species according to the sequences of several genes. Some of these species have a broad host range (generalists). In contrast, others show specificity for certain insect families (specialists) and can be used to test hypotheses regarding speciation and host specificity (Wang *et al.*, 2016). Besides the host range, *Metarhizium* species differ in the way they colonize hosts (Kershaw *et al.*, 1999). Generalists and specialists fungi have mechanisms for evading host immunity; however, generalists are less able to subvert immune responses specific to certain insects. The lack of adaptations may have selected the generalists for the rapid killing of hosts before the host can mount an enfeebling immune response (Wang *et al.*, 2012; Hu *et al.*, 2014).

Regarding the fungi specialization and their adaptations to evade insect' hosts, and knowing that M. anisopliae is a generalist fungus, while *M. rileyi* is a specialist in Lepidoptera species, we hypothesize that even belonging to the same genus, *M. anisopliae*, and *M. rileyi* use different mechanisms to penetrate *Spodoptera frugiperda* (Lepidoptera: Noctuidae) caterpillars.

As a generalist, how can Metarhizium anisopliae be pathogenic to different insect species? Can this fungal species adapt its phenotype and gene responses to penetrate and cause diseases in various insects?

Fungal adaptive responses may be mediated by epigenetic mechanisms, allowing short-term specialization while maintaining the broad host range potential (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013). Evidence increasingly suggests that a significant factor driving the co-evolutionary arms race between the pathogen and the host occurs on the cuticular surface – also involving mediation of the insect cuticle microbiome (Shang *et al.*, 2022; Hong *et al.*, 2023). Although considerable progress has been made in recent years, much regarding the molecular determinants mediating these interactions in the pathogen and the host remains unknown (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013).

Considering the ability of adaptive responses of fungal entomopathogens our final hypothesis is that *M. anisopliae*, a generalist fungus, can also modulate its phenotype and gene responses to adapt and cause disease to different orders of insects.

1.5. Approach

The rapid advance of genome technologies has revolutionized our understanding of microorganisms. In our field of studies, the 'omics' like genomics, transcriptomics, and proteomics have revealed the evolutionary line of fungal adaptation to insect hosts and to different host ranges. They've also revealed the evolutionary relationships between insect and non-insect pathogens as the functions of several pathogenicity genes.

Many studies have shown that sequence data can provide crucial information about how these organisms reproduce and persist in different environments. However, few organized the information in a timeline of events in the same genus. Using *Metarhizium spp.* as a model, the second chapter highlights recent advances in genomics and

molecular biology of entomopathogenic fungi to investigate the gene-for-gene relationships in insect-fungus interactions.

Chapter 3 discusses the biological and genetic factors that allow blastospores to infect insects and make them potentially effective for biological control in the field. While the generalist *M. anisopliae* produces a higher number and smaller blastospores, the Lepidoptera specialist *M. rileyi* produces fewer propagules with a higher cell volume under high osmolarity conditions. We also compared the virulence of blastospores and conidia of these two *Metarhizium* species towards the economically important caterpillar pest *Spodoptera frugiperda*. We found that conidia and blastospores from *M. anisopliae* were both infectious but killed fewer insects compared with *M. rileyi* conidia and blastospores, where *M. rileyi* conidia had the highest virulence.

Using comparative transcriptomics during propagule penetration on insect cuticles, chapter three also shows that *M. rileyi* expresses more virulence-related genes against *S. frugiperda* than *M. anisopliae*. In contrast, conidia of both fungi express more virulence-related oxidative stress factors than blastospores. These results highlight that blastospores use different virulence mechanisms than conidia, which may be explored in new biological control strategies.

Finally, chapter four investigated the phenotypical plasticity of *M. anisopliae* blastospores among different insect species. This chapter shows that M. anisopliae blastospores are highly virulent for *T. molitor*, and the percentage of appressorium formation in the membranous wings of this insect was three times higher than in the wings of the other insects. We showed a clear difference in the gene expression pattern in the blastospores of *M. anisopliae* during the infective process in different insects, with the most significant differences found for *G. assimilalis*. The differences are associated with the expression of enzymes such as proteases, cutinases, lipases, and peptidases which might be related to the degradation of specific compounds of each insect wing; hydrophobins, and destruxins which are associated with virulence and secondary metabolites.

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2. GENOMIC PERSPECTIVES ON THE EVOLUTION OF FUNGAL HOST-SPECIFICITY OF *Metarhizium* spp.

ABSTRACT

Entomopathogenic fungi play a pivotal role in regulating insect populations in nature, and representative species have been developed as promising environmentally friendly mycoinsecticides. The rapid advance of genome technologies has revolutionized our understanding of these microorganisms. Studies revealed the evolutionary line of fungal adaptation to insect hosts and different host ranges, the evolutionary relationships between insect and non-insect pathogens, and the functions of several pathogenicity genes. However, some questions regarding the specialization and the genes evolved in pathogens adaptations to new host remains unclear. Using *Metarhizium* spp. as a model, this review highlights recent molecular biology studies investigating genes and their roles in fungus-host interactions contributing to fungal virulence in insects. Sequence data can provide crucial information on how these organisms reproduce and persist in different environments. Relative to our advanced insights into the mechanisms of fungal host-specificity, future studies will be necessary to unravel the gene-for-gene relationships in interactive models. Besides that, molecular techniques could allow the design of multiple pathogens with different strategies to be used for diverse ecosystems.

Keywords: *Metarhizium*; genome technologies; evolution; pathogenicity

2.1. Introduction

Fungi are the most typical pathogens of insects and regulators of pest populations. They are well suited for development as biopesticides due to simple production in vitro and their mechanism of infection pests in the field. Compared with other microbial products, fungi infect insects by direct penetration while bacteria and viruses must be ingested to cause diseases (Lovett and St Leger, 2017).

Entomopathogenic fungi are very heterogeneous. Besides colonizing insects, they occupy many habitats and can be found in association with soil and plants (Wang *et al.*, 2016; Brunner-Mendoza *et al.*, 2019; St. Leger and Wang, 2020). The interactions between fungi, hosts, and the environment are diverse and dynamic. This could complicate studies between different fungi because their interactions may be necessarily disparate. As a solution, researchers usually pick a couple of related fungal species for a thorough investigation of host-pathogen interactions and make comparisons with other related species.

Most of what we know about interactions between fungi and their hosts has been determined with the experimentally tractable Hypocrealean Ascomycete *Metarhizium* (family Clavicipitaceae) and *Beauveria* (family Cordycipitaceae) (Wang *et al.*, 2016). These fungi, commonly applied as bioinsecticides, can colonize plants, and be used as plant endophytes to improve agricultural production while reducing the potential of crop pests (Wang *et al.*, 2016; Brunner-Mendoza *et al.*, 2019; St. Leger and Wang, 2020). In the soil, *Metarhizium* species and *B. bassiana* can infect, kill insects, and form an endophytic relationship with a plant. This relationship transfers insect-derived nitrogen

directly to the plant, and this endophytic association could increase overall plant productivity (Behie and Bidochka, 2014).

The genus *Metarhizium*, for example, contains species with a narrow host range and or broad host range (Wang *et al.*, 2016; St. Leger and Wang, 2020; Kiruthiga *et al.*, 2022). These species could be pathogens of arthropods, saprophytes, and root colonizers. Consistent with their wide lifestyle options, researchers demonstrated that *Metarhizium* spp. exhibit a flexible metabolism according to its host (Wang and St Leger, 2007; Wang *et al.*, 2011; Wang *et al.*, 2012; St. Leger and Wang, 2020; Yan-Li *et al.*, 2022). Understanding the biology, the metabolism, and modes of infection of these fungi may expand their biotechnological potential but requires in-depth studies of fungal host-specificity and evolution.

In this case, comparative genomics offers a way forward to understanding themes of fungal biology from specific components involved in insect pathology. It allows broad and narrow host-range path comparisons and facilitate identifying fungal fitness traits and the selective forces that act upon them (Wang *et al.,* 2014). Therefore, the genomics may improve our understanding of how and why entomopathogenic fungi interact with insects and other components of their environments.

These evolutionary questions are crucial for biocontrol agents and address fundamental, yet poorly understood, issues by asking: When organisms adapt to a new host, do they do so because of changes in a few or many genes? Which ones? Are the same genes or networks involved in independent cases of environmental adaptation and insect pathogenicity?

This review highlights recent advances in the genomics and molecular biology of entomopathogenic fungi, focusing in *Metarhizium* species. It aims to group pulverized information and build a line of reasoning to understand the specialization and the pathogenicity factors involved in *Metarhizium*-hosts interactions.

2.2. *Metarhizium* spp.: evolution of multifunctional lifestyles

The genus *Metarhizium* (Ascomycota: Hypocreales) comprises entomopathogenic fungi employed for the biological control of crop pests and vector-borne diseases since it was first described (Sbaraini *et al.*, 2016). The first microbial control study using an entomopathogenic fungus to control pests was carried out by the Russian researcher Ilya Mestchnikoff, who studied *M. anisopliae* on *Anisoplia austriaca* and then on *Cleonus puctventris*, sugar beet pest (Alves, 1998).

M. anisopliae (Metschn.) Sorokin is one of the best-known entomopathogenic fungi with more than 200 species among the orders Orthoptera, Dermaptera, Hemiptera, Lepidoptera, Diptera, Hymenoptera, Coleoptera, Homoptera, and others (Alves, 1998). It is widely distributed in nature and is found in soils surviving for long periods (Bidochka *et al.*, 2011).

The first known species of *Metarhizium* was isolated from infected insects in Ukraine in the late 1870s (Oliveira *et al.*, 2018). This fact made researchers believe, for many years, that the mode of infection of these fungi was only penetrating directly into the insect cuticle; through mechanical pressure and cuticle-degrading (Beys-Da-Silva *et al.*, 2014). However, recent studies have demonstrated that several *Metarhizium* spp show different abilities to form associations with insects, plant roots, and the rhizosphere (Behie and Bidochka, 2014; Wang *et al.*, 2016; Brunner-Mendoza *et al.*, 2019). Although best known for its insecticidal properties, the recognized ability of *Metarhizium* to colonize crop plants and translocate insect-derived nitrogen (Behie and Bidochka, 2014) has increased interest in this genus as a promotor of plant growth and crop yield, in addition to a biocontrol agent.

This unique multifunctional lifestyle of *Metarhizium* makes it an economically and ecologically important regulator of soil insect communities, as well as various plants' health, growth, productivity, and microbiome composition. This reflects on the current research on this genus, which focuses on its regulatory abilities to formulate environmentally friendly alternatives to chemical pesticides and fertilizers (Stone and Bidochka, 2020).

With broad insect host ranges, *Metarhizium* spp. may also be applied for more extensive control of various insects. For instance, commercial formulations of *M. anisopliae* have been tested and utilized for the management of insects from several orders, especially for the control of spittlebug pests of sugarcane in Brazil – which is considered one of the most successful biological control programs in the world (Iwanicki *et al.*, 2019). As a result, *M. anisopliae* is the widest entomopathogen commercialized in Brazil, with the highest number of product registrations and an estimated 20,000 km² of land treated annually (Iwanicki *et al.*, 2019).

Investigating the evolution of the genus, the comparative genome analysis performed by Hu *et al.* (2014) places the *Metarhizium* genus as a monophyletic lineage that diverged from the clavicipetacean plant pathogens and endophytes about 231 million years ago (MYA). According to the same authors, the close physical proximity of the plant-associated ancestor of *M. album* to plant-sap-sucking hemipteran bugs probably facilitated the switch from plant endophytic to entomopathogenic; thus, the hemipteran-specific *M. album* would be a basal species of the *Metarhizium* clade with an estimated divergence time about 117 MYA (Hu *et al.*, 2014) (Figure 3).

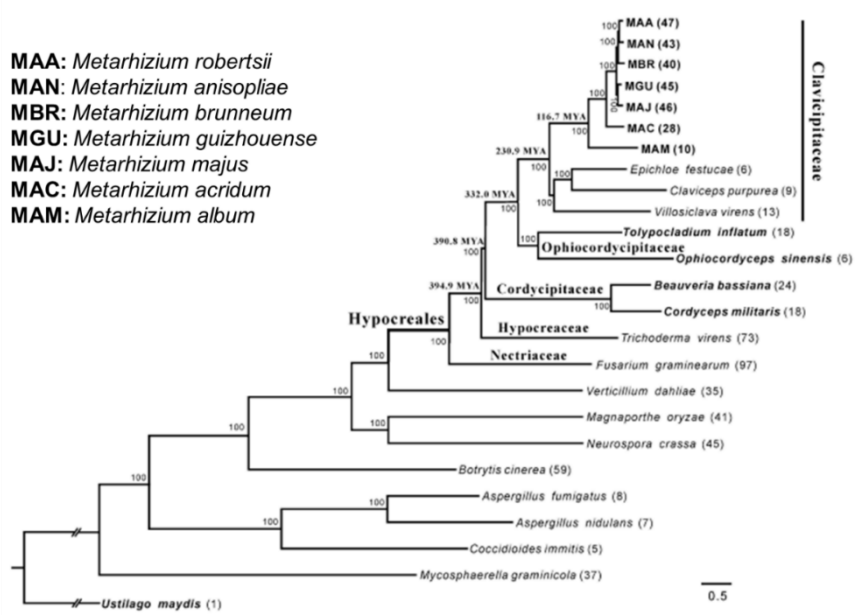

Figure 3. Phylogenetic tree. Species of insect pathogenic fungi are highlighted in bold. The numbers in parenthesis are the HET domain-containing proteins encoded in each genome, indicating that the basal Clavicipitacean fungi resemble MAM in containing comparatively few HET proteins. MYA, million years ago. Hu *et al.* (2014). Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. **PNAS: 111, 16796–16801.** https://doi.org/10.1073/pnas.1412662111.

Today we know that *Metarhizium* species can also transfer nitrogen to plants and this plays an essential role in the ecological cycling of insect nitrogen back to plant communities (Behie & Bidochka, 2014). Besides that, many studies reported that this association could also provide direct and indirect control of pests (Mantzoukas *et al.*, 2014; R Jaber and Ownley, 2017; Vega, 2018). According to Vega (2018), using entomopathogenic fungi as endophytes provides a novel alternative for managing insect pests and works as biofertilizers.

Like other insect pathogenic fungi, an essential infection step by *Metarhizium* spp. is penetration through the host insect's cuticle (Butt *et al.*, 2016). Considering that pathogenicity in insects evolved from the ability to associate with plants we can infer that the evolutionary process that transformed *Metarhizium* spp. in entomopathogen must have involved specific adaptations breaching this barrier.

Although capable of plant root colonization and killing insects, *Metarhizium* is not an obligate plant symbiont nor an obligate insect pathogen (St. Leger and Wang, 2020). Therefore, this genus exhibits extraordinary transcriptional plasticity, modulating its transcriptome to allow physiological adaptation to diverse and dynamic environments (Stone and Bidochka, 2020).

Evolutionary and comparative analyses of filamentous ascomycetes were also performed by Shang *et al.* (2016). The authors analyzed 33 fungal pathogens that infect insects, plants, and mammals. The data indicated that both convergent and divergent evolutions of protein families occurred in insect pathogens, which resulted in similar or altered abilities in these fungi to utilize different substrates (Shang *et al.*, 2016). According to the authors, the results puzzle out the evolutionary patterns of fungal pathogenicity and the protein families that correlate to fungal pathotype formations.

From the evolutionary aspect, we can understand that the results of Hu *et al.* (2014) and Shang *et al.* (2016) corroborate each other. Entomopathogenic fungi's capacity to infect insects is delivered from their relationship with plants, and this is clear. However, fundamental questions remain, including whether generalization or specialization for particular hosts is an ancestral condition, whether we can identify the existence of transitional forms, and the underlying molecular mechanisms driving speciation in this case. Seeking answers to these doubts, the genomic basis for speciation has been improved, but many points in host shifting remains unclear as many questions are being made.

2.3. Fungal-pathogen speciation and host adaptation

Host-pathogen interactions are, scientifically accepted, the major driving force for diversification (Hu *et al.*, 2014). The speciation may be understood as an important component of this biological diversification and is increasingly viewed as a continuous process rather than an event (Gavrilets *et al.*, 2000). Nevertheless, many challenges to our understanding of host-pathogen interactions remain e.g., the synthesis and integration of concepts and the findings across different ecological scales, and the failure to identify transitional species, hindering the progress in understanding genomic divergence patterns along the speciation (Hu *et al.*, 2014; Näpflin *et al.*, 2019).

The genus *Metarhizium* has been subdivided into several species according to the sequences of several genes. Some of these species have a broad host range (generalists). In contrast, others show specificity for certain insect families (specialists) and can be used to test hypotheses regarding speciation and host specificity (Wang *et al.*, 2016). Besides the host range, *Metarhizium* species differ in the way they colonize hosts (Kershaw *et al.*, 1999)

Generalists and specialists' fungi have mechanisms for evading host immunity; however, generalists are less able to subvert immune responses specific to certain insects. A lack of adaptations may have been selected for the rapid killing of hosts before the host can mount an enfeebling immune response (Wang *et al.*, 2012; Hu *et al.*, 2014).

An example of how fungi can be entomopathogenic is by producing enzymes that penetrate the insect's cuticle. Among these enzymes are proteases, lipases, and aminopeptidases. In recent years the degradation machinery of entomopathogenic fungi has attracted much attention, especially in *Metarhizium* spp. In these species, the chitinolytic system probably has two principal biological functions: First, the chitin-degrading enzymes act on the cell wall remodeling, which is necessary for hyphal vegetative growth. Second, the infection of arthropod hosts requires prior

chitin hydrolysis of the exoskeleton (Oliveira *et al.*, 2018). Furthermore, this could justify the ability of *M. anisopliae* to differentiate into specialized cell types during its infection cycle.

The strategies for infection, virulence, and suppression of host defenses used by generalists tend to target features standard in many insects. Frequently they involve overpowering the host by using numerous enzymes and toxins with adverse effects on many diverse species (St. Leger and Wang, 2020). However, specialists do not produce large amount of toxins and may take longer to kill than generalists, striking a constant balance between virulence and evading/ resisting to host immunity, thus exhibiting a more sophisticated form of pathogenicity (St. Leger and Wang, 2020).

Looking more in depth to the fungal propagules, the switch between conidia to hyphae and the formation of infection structures (i.e., appressorium and blastospore), are processes that require chitin degradation (Schrank and Vainstein, 2010). As chitin is present in the exoskeleton of several arthropods, enzymes involved in chitin degradation and assimilation are predicted to play essential roles in host-entomopathogen interactions (Vega *et al.*, 1999; Mantilla *et al.*, 2012; Butt *et al.*, 2016).

Generalists, like *M. robertsii*, typically kill hosts quickly via toxins and grow saprophytically in the cadaver. In contrast, specialist *M. acridum* causes systemic infection of host tissues before the host dies (Wang *et al.*, 2016). According to Wang *et al.* (2016), this may reflect greater adaptation by the specialists to subverting or evading their hosts' immune systems, so they do not need to kill quickly.

This hypothesis could be related to the development of appressoria; a entomopathogenic fungi structure that seems to play an important role in the early stages of infection. In *M. anisopliae,* for example, a generalist specie, enzymes are produced during the formation of appressoria (Clarkson and Charnley, 1996). However, *M. rileyi,* a specialist one, may not form appressoria (Srisukchayakul *et al.*, 2005) and, supporting the hypothesis of Wang *et al.* (2016), may have any other adaptation to subverting or evading the immune systems of their hosts. It means that, besides host range, generalist, and specialist *Metarhizium* species differ in the way they colonize hosts.

On the past, a generally accepted infection model of *M. rileyi* indicated that this specie did not form appressoria, penetrating directly into the host tegument (Srisukchayakul *et al.*, 2005). However, as appressorium formation is a conserved characteristic of pathogenic fungi, including plant pathogens (Shi *et al.*, 2023), and today we can explain that *M. rileyi* also form appressoria to penetrate insect cuticles and why was it known like that. Through morphogenesis analysis, Wang *et al.* (2021) demonstrated, for the first time, that *M. rileyi* forms appressorium to invade the host through cuticle penetration. Its infection could elicit both cellular and humoral immune reactions of *Spodoptera litura* larvae. The authors also found that the Mrpmk1 gene is essential for appressorium differentiation and mycelium reemerging in the insect (Wang *et al.*, 2021). During the infection, *M. rileyi* triggered both cellular and humoral immunity of *S. litura* producing stage-specifically oxalic acid and F-actin arrangement, besides morphological changes. According to Wang *et al.* (2021), this behavior may play roles in nutrient acquisition and mycelium reemerging.

Comparative genomic analyses performed by Hu *et al.* (2014) revealed directional speciation of seven *Metarhizium* species from specialists with narrow host ranges (i.e., *M. album* and *M. acridum* specific to hemipterans and acridids, respectively) to transitional species with intermediate host ranges (*M. majus* and *M. guizhouense* both have host ranges limited to two insect orders), and then to generalists (i.e., *M. anisopliae*, *M. robertsii*, and *M. brunneum*) (Figure 4).

An analysis of three *Fusarium* species also suggested a transition from specialist to generalist coupled with genome and protein family expansions (Cuomo *et al.*, 2007; Ma *et al.*, 2010). Wang *et al.* (2011) commented that are factors missing from specialists that limit their ability to cause disease in multiple insects, as demonstrated by an

increase in host range following the transfer of genes from generalist strains to the specialist *M. acridum* (Wang *et al.*, 2011).

According to Hu *et al.* (2014), this host adaptation was coupled with a complex interplay between an array of genomic features that worked together to drive fungal speciation and provide a roadmap for identifying the variation underlying adaptation and speciation. Besides that, the authors affirm that specialization in *Metarhizium* is also associated with the retention of sexuality and rapid evolution of existing protein sequences. In contrast, generalization is associated with protein family expansion, loss of genome-defense mechanisms, genome restructuring, horizontal gene transfer, and loss of sexuality (Hu *et al.*, 2014) (Figure 5).

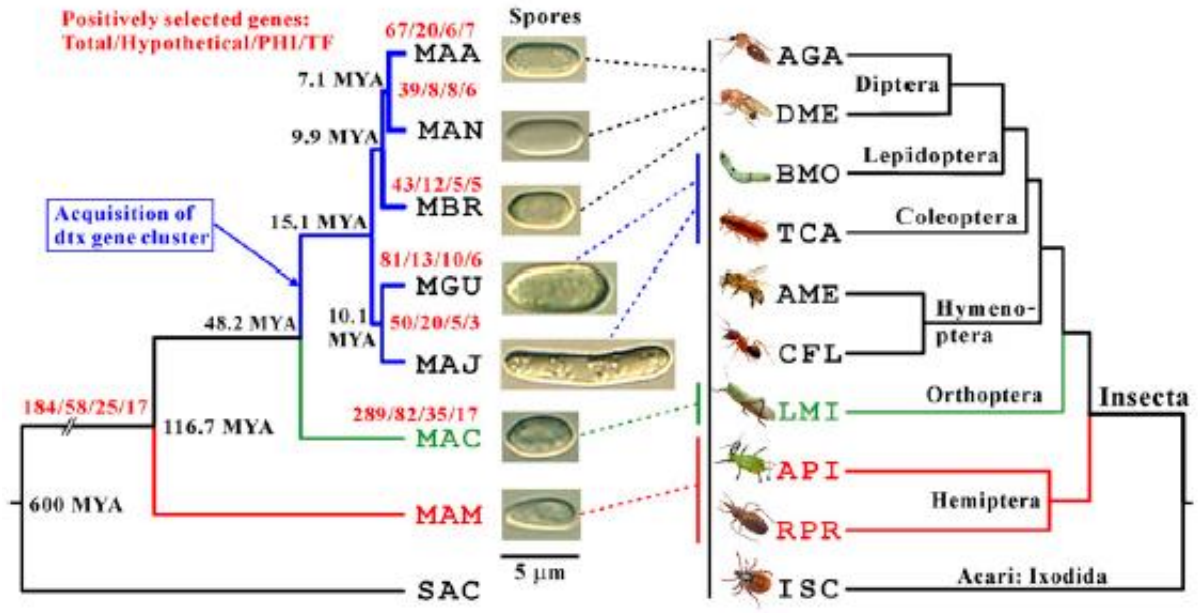

Figure 4. Reconstructed phylogeny of *Metarhizium* species showing their insect host ranges and divergence time. MAA, *Metarhizium robertsii*; MAN, *Metarhizium anisopliae*; MBR, *Metarhizium brunneum*; MGU, *Metarhizium guizhouense*; MAJ, *Metarhizium majus*; MAC, *Metarhizium acridum*; MAM, *Metarhizium album*; SAC, *Saccharomyces cerevisiae*. Hu *et al.* (2014). Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation

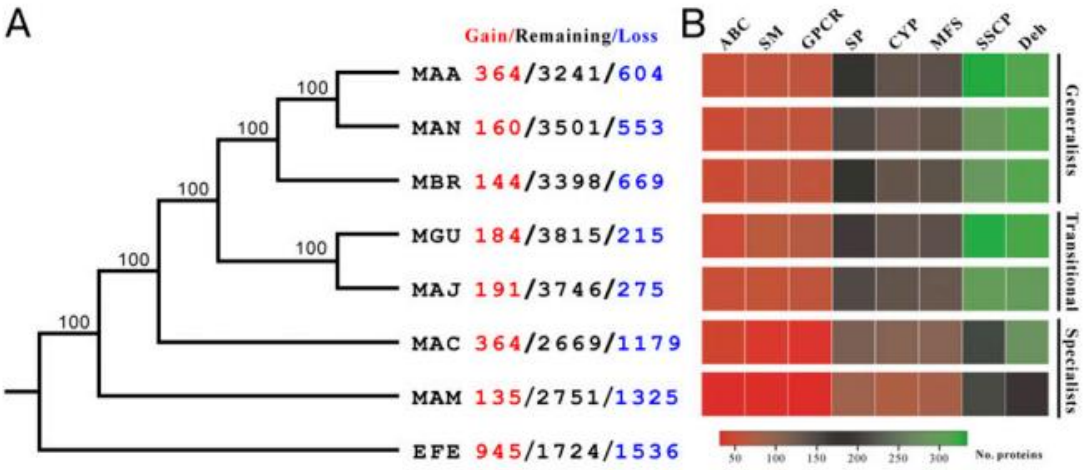

Figure 5. (A) Gain and loss analysis of protein families across *Metarhizium* lineages. MAA, *Metarhizium robertsii*; MAN, *Metarhizium anisopliae*; MBR, *Metarhizium brunneum*; MGU, *Metarhizium guizhouense*; MAJ, *Metarhizium majus*; MAC, *Metarhizium acridum*; MAM, *Metarhizium album*; (B) Heat-mapping analysis of protein families involved in virulence and detoxification. ABC, ATP-binding cassette superfamily transporters; SM, core proteins involved in secondary metabolism; GPCR, G protein-coupled receptors; SP, secreted proteases; CYP, cytochrome P450s; MFS, major facilitator superfamily transporter; SSCP, small secreted cysteine-rich proteins; Deh, dehydrogenases. Hu et al. (2014). Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation

In the same thought, an analysis by Xu *et al.* (2016) indicated that the genomes of the generalist species of *Metarhizium* encode more gene clusters than the specialists. This study demonstrated that there are 12 clusters conserved in the genomes of seven species of *Metarhizium* spp (Xu *et al.*, 2016). With these results, the authors suggested that both conserved and divergent evolutions may have occurred in different *Metarhizium species* to produce bioactive secondary metabolites.

Xu *et al.* (2016) also quantified the secondary metabolites produced for different *Metarhizium* species. The results indicated that the level of destruxins varied significantly, and the generalists produced the highest amounts, while specialists did not produce it. Destruxins were the first toxins studied in fungi and have been well characterized by many pathogenicity studies, including for *Metarhizium* spp. (Golo *et al.*, 2014) . If we consider that enzymes like destruxins can be produced by plant pathogens (Wang *et al.*, 2012), results found by Xu *et al.* (2016) could suggest that the *Metarhizium* ancestor contained this generic cluster, and specialists have lost it.

So far, we know that generalist species with a broad host range have diverged from specialist species with narrow host ranges 30–50 MYA. However, Stone & Bidochka (2020) highlight that the evolutionary history of this divergence remains unclear and proposed two different scenarios for insect host range divergence of *Metarhizium spp*. Using the example of the destruxin (DTX) gene cluster, which produces non-selectively insecticidal toxins, the generalists-first scenario means that *Metarhizium spp*. with a broad host range may have carried the DTX gene cluster, which was subsequently lost in the lineage(s) leading to specialist species. On the other hand, if *Metarhizium* evolved first with a narrow host range that lacked the broadly insecticidal gene cluster, the lineage(s) leading to generalist species may have laterally acquired DTX genes (Stone and Bidochka, 2020).

Shang *et al.* (2016) performed substrate utilization tests and revealed growth feature differences among insect pathogens when compared with plant pathogens. Besides that, the authors demonstrated that the growth of *M. rileyi* was poor on pectin, xylose, and even sucrose, which contrasted with *M. acridum* and *M. robertsii*. It was also found that *M. rileyi* had lost the ability to grow on xylose (Shang *et al.*, 2016). The intriguing question in these findings is how

a specific fungus could have lost a generalist ability (like growing on xylose). In accordance with Xu *et al.* (2016), the authors justified that host-adaptive convergent evolution is coupled with divergent evolutions, resulting in altered protein family features within or between fungal pathotypic groups.

The large secretomes of generalist insect pathogens probably reflect the many habitats to which they must adapt, including the cuticle and the hemolymph, as well as additional environmental habitats in the soil and with plants. These complex lifestyles are reflected in the transcriptomes; many different genes are induced during adaptation to host cuticle, hemolymph, or root exudate (Wang *et al.*, 2016).

Zhang *et al.* (2019) reported that the emergence of broad host range entomopathogens occurred by horizontal gene transfer. According to the authors, the broad-host-range entomopathogen *M. robertsii* has 18 genes that are derived via horizontal gene transfer (HGT). The necessity of degrading insect cuticles served as a significant selective pressure to retain these genes, as 12 are up-regulated during penetration; 6 were confirmed to have a role in penetration, and their collective actions are indispensable for infection (Zhang *et al.*, 2019) (Figure 6).

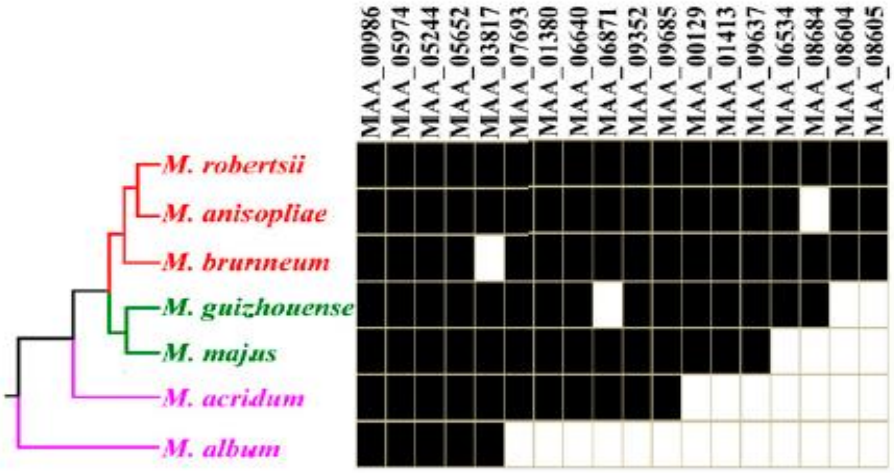

Figure 6. Distribution of homologs of the *M. robertsii* HGT genes in other *Metarhizium* species. Pink branches indicate specialists; green branches indicate species with intermediate host range; red branches indicate generalists. Zhang *et al.* (2019) Horizontal gene transfer allowed the emergence of broad host range entomopathogens.

2.4. What to expect from genomics to improve the efficacy of *Metarhizium* spp.

Entomopathogenic fungi are very heterogeneous. For example, they occupy a wide range of habitats such as soil and plants (Wang *et al.*, 2016). Because of their mode of infection through the cuticle and their potential as contact insecticides, biocontrol researchers have made a substantial effort to investigate the pathogenicity machinery and explain why some species are more capable of controlling pest insects and witch genes and metabolites are responsible for it.

The rapid advance of genome technologies has revolutionized our understanding of entomopathogenic fungi and has identified the functions of several pathogenicity genes of *Metarhizium* species (Gao *et al.*, 2011; Sbaraini *et al.*, 2016; Wang *et al.*, 2016; Zhang *et al.*, 2019). We can say for sure that evolutionary genomics has recently entered a new era in the study of host-pathogen interactions. A variety of novel genomic techniques has transformed the identification, detection and classification of both hosts and pathogens. These advances promote a greater resolution

that helps decipher their underlying dynamics and provides novel insights into their environmental context (Näpflin *et al.*, 2019).

Recently, progress has also been made in determining the factors that influence the distribution, population structure, and nutritional characteristics of *Metarhizium*. Studies with these fungi, which employs a vast array of metabolites to aid insect infection, have revealed the mechanisms and importance of a portion of its genes and metabolites for virulence. However much remains unknown and may hinder the development of these fungi as biocontrol agents (Wang and St Leger, 2007; Schrank and Vainstein, 2010; Wang *et al.*, 2012; Wang *et al.*, 2014; Wang *et al.*, 2016; Augustyniuk-Kram, 2018; Mukherjee and Vilcinskas, 2018).

Understanding these fungi and expanding their biotechnological potential requires in-depth studies of their germination and infection of insects. In this case, comparative genomics and metabolomics investigation have facilitated identifying fungal fitness traits and the selective forces that act upon them to improve our understanding of how and why entomopathogenic fungi interact with insects and other components of their environments.

Sequence data can also provide crucial information on how these organisms reproduce and persist in different environments. Besides that, genomic studies offer a way forward by disentangling common themes of fungal biology from specific components involved in insect pathology. However, genomic-based studies about host-specificity evolution are generally inconclusive, demonstrating the need for more research. Researchers usually pick a couple of related fungal species for a thorough study of host-pathogen interactions, and this is a limiting factor for more reviews and conclusions.

Molecular biology methods have elucidated pathogenic processes in several fungal biocontrol agents including the important and commonly applied entomopathogenic fungi, *Metarhizium anisopliae* (St. Leger and Wang, 2010). In addition to understanding ecological aspects and the interactions between pathogens and hosts, the knowledge obtained may be applied in the development of new strains of modified microorganisms, increasing it insecticidal potential in the field.

The development of recombinant DNA techniques, for example, has made it possible to significantly improve the insecticidal efficacy of viruses, bacteria and fungi (St. Leger and Wang, 2010). These advances have been achieved by combining new knowledge derived from basic studies of the molecular biology and genomics of these pathogen. According to St. Leger and Wang (2010), the increasing public acceptance of genetically modified crops expressing Bacillus thuringiensis toxins, the field application of genetically modified microorganisms for biological control of pests should have a bright future if care is taken to ensure social acceptance through rigorous risk benefit analysis (St. Leger and Wang, 2010).

2.5. Final remarks

In this review, we could comprehend that molecular biology studies have revealed the genes that function in fungal interactions with insect hosts at different infection stages. First, we have a brief introduction of the *Metarhizium* spp. multiple lifestyles and could understand how multiple and important the representants of this genus are. We also had an overview of the speciation and host-adaptation that drove generalists to specialists in the *Metarhizium* genus. Analyzing our outstanding level of understanding the fungus-plant and fungus-insect interaction mechanisms, future efforts will be needed to investigate the function of genes and proteins in fungus-insect interactions. Besides that, more in-deep studies are needed regarding the mechanisms involved in regulating the propagules during fungal colonization of the insect.

The genomic resources are essential for answering fundamental questions in entomopathogenic fungi model systems. Furthermore, the precision and malleability of molecular techniques could allow the design of multiple pathogens with different strategies to be used for diverse ecosystems. Thinking on the future, field application of genetically modified insecticidal microorganism should have a bright future if our understanding of their unique biology continues to grow, and care is taken to ensure social acceptance through rigorous and transparent risk and benefit analyses.

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3. BLASTOSPORES FROM *Metarhizium anisopliae* AND *Metarhizium rileyi* ARE NOT ALWAYS AS VIRULENT AS CONIDIA ARE TOWARDS *Spodoptera frugiperda* CATERPILLARS AND USE DIFFERENT INFECTION MECHANISMS

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ABSTRACT

Infective conidia from entomopathogenic fungi are widely used to control insect pests. Many entomopathogenic fungi also produce yeast-like cells called blastospores under specific liquid culture conditions that can directly infect insects. However, little is known about the biological and genetic factors that allow blastospores to infect insects and making them potentially effective for biological control in the field. Here, we show that while the generalist *Metarhizium anisopliae* produces a higher number and smaller blastospores, the Lepidoptera specialist M. rileyi produces fewer propagules with a higher cell volume under high osmolarity conditions. We compared the virulence of blastospores and conidia of these two *Metarhizium* species towards the economically important caterpillar pest *Spodoptera frugiperda*. Conidia and blastospores from *M. anisopliae* were equally infectious, but acted slower, and killed fewer insects than *M. rileyi* conidia and blastospores, where *M. rielyi* conidia had the highest virulence. Using comparative transcriptomics during propagule penetration on insect cuticles, we show that *M. rileyi* blastospores expresses more virulence-related genes against *S. frugiperda* than *M. anisopliae*. In contrast, conidia of both fungi express more virulence-related oxidative stress factors than blastospores. Our results highlight that blastospores use a different virulence mechanism than conidia, which may be explored in new biological control strategies.

Keywords: Blastospores; conidia; cuticle penetration; liquid fermentation; transcriptome; virulence

3.1. Introduction

Biological control agents are one of the pillars of integrated pest management (IPM), which is a promising tool for reducing the impact of pests. Microbial pathogens, such as insect pathogens, are a valuable tool to manage crop pests and can be used in combination with insecticides to improve their performance and reduce the occurrence of resistance to commercially active ingredients (Ambethgar, 2009).

Fungi are common pathogens of insects and natural regulators of pest-insect populations (St. Leger and Wang, 2020). A notable example is fungi from the genus *Metarhizium* (Ascomycota: Hypocreales), which are employed for the biological control of crop pests and vector-borne diseases (Sbaraini *et al.*, 2016; St. Leger and Wang, 2020). Several *Metarhizium* species have multifunctional lifestyles and occur in several ecological niches, including as insect

pathogens, plant-root symbionts, and soil saprophytes (Brunner-Mendoza *et al.*, 2019). The species *M. anisopliae* (Metsch.) Sorokin, which in Brazil is used to control sugarcane pests, is one of the best examples of using an entomopathogenic fungus as pest control (Parra, 2014).

Entomopathogenic fungi are produced primarily *in vitro* on solid medium or under liquid fermentation to obtain different structures such as conidia, blastospores, or mycelium. Except for conidia, the blastospores and mycelium are produced using liquid culture media (Wang *et al.*, 2017). The natural life cycle of entomopathogenic fungi involves different fungal structures and cell types. Fungal conidia ensure dissemination in the environment and transmission to new insect, plant, and soil niches, whereas blastospores are naturally formed when these fungi proliferate inside the host hemolymph. Blastospores thus naturally differ from conidia in several ways because they are hydrophilic, germinate faster than conidia (2-8 hrs versus 12–24 hrs), and are often more virulent against susceptible hosts (De Paula *et al.*, 2021). The rapid germination rate of blastospores produced under liquid culture conditions could be regarded as a virulence determinant and is a desirable trait for use as an infective structure in commercially produced entomopathogens (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015; Butt *et al.*, 2016).

Much progress has been made in the production and formulation technologies, which coupled with knowledge concerning the target insect's biology, make entomopathogenic fungi attractive components of integrated pest management practices (Mantilla *et al.*, 2012). It is possible to produce large amounts of blastospores in a small space by liquid fermentation in a short time (<4 days) (Maldonado-Blanco *et al.*, 2014; Lohse *et al.*, 2015; Iwanicki *et al.*, 2018; Iwanicki, N. S. A. *et al.*, 2020). Furthermore, compared with solid fermentation methods, liquid fermentation requires less manual labor and does not rely on a high value substrate such as rice grains making liquid fermentation potentially more cost effective in a commercial production setting (Iwanicki *et al.*, 2018).

The blastospores of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) produced in high osmolarity liquid fermentation are smaller and more virulent for pest insects than blastospores produced without osmotic stress (Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015). A better understanding of the biological and genetic factors behind blastospore production and virulence could help enable this propagule being used as an active ingredient of commercially produced biological control products.

Comparative genomics has facilitated identifying fungal fitness traits and the selective forces that act upon them to improve our understanding of how and why entomopathogenic fungi interact with insects and other components of their environments (Wang *et al.*, 2016). These technologies have helped to identify the functions of several pathogenicity genes of entomopathogenic fungi (Gao *et al.*, 2011; Sbaraini *et al.*, 2016; Wang *et al.*, 2017). However, in-depth studies of germination and infection of blastospores and conidia in insects are needed to better understand these fungi and expand their biotechnological potential. Genome studies of entomopathogenic fungi have helped us understand the potential possessed by these fungi both as insect pathogens and as microbial biocatalysts.

The fungus *Metarhizium rileyi* (Farlow) Kepler, S.A. Rehner & Humber, previously known as *Nomuraea rileyi*, is pathogenic to Lepidoptera, infecting noctuids like *Anticarsia gemmatalis* and *Spodoptera frugiperda*, which are critical pests of important crops (Fronza *et al.*, 2017; Grijalba *et al.*, 2018). It is a dimorphic fungus with yeast-like hyphal bodies and a filamentous growth phase (Fronza *et al.*, 2017). Much less is known about *M. rileyi* compared with *M. anisopliae*, but recent advances in *M. rileyi* research have contributed to our knowledge of different aspects of this pathogen and this species' potential as an entomopathogen is indisputable (Fronza *et al.*, 2017; Zhong *et al.*, 2017; Grijalba *et al.*, 2018; Yan-Li *et al.*, 2022).

The *Spodoptera frugiperda* (J.E. Smith, 1797), known in English as fall armyworm, is a polyphagous pest reported to infest 353 host plant species in North and Central America (Montezano *et al.*, 2018). It prefers wild and

cultivated grasses, maize, rice, sorghum, millet and sugarcane, cultures that are overspread in Brazil, reinforcing the importance of finding new strategies of its control.

Here, we first compare the virulence of *M. anisopliae* and *M. rileyi* conidia and blastospores against *Spodoptera frugiperda* larvae. Second, we use comparative transcriptome analysis of *M. anisopliae* and *M. rileyi* during infection of *S. frugiperda* cuticle to identify virulence-related genes expressed specifically during cuticle penetration. We intent to compare the virulence of *M. anisopliae* and *M. rileyi* spores against *S. frugiperda* larvae. Besides that, we characterize the gene expression profiles of fungal propagules grown on caterpillars' cuticle. These findings can help us to understand the forces that act upon entomopathogen fitness traits and may underpin both our understanding of the natural roles of these fungi in nature and the development of new mycoinsecticides further.

3.2. Materials and Methods

3.2.1. Fungal isolates and insect rearing

Two isolates of *Metarhizium anisopliae* (ESALQE9 and ESALQ4676) and two isolates of *Metarhizium rileyi* (ESALQ4946 and ESALQ5611) were selected from the entomopathogen collection "Prof. Sergio Batista Alves" of the Insect Pathology and Microbial Control Laboratory - University of São Paulo, Piracicaba, Brazil (ESALQ/USP). The fungi were cultured in Petri dishes containing Potato Dextrose Agar (PDA) for *M. anisopliae*, and Sabouraud Maltose Agar, supplemented with yeast extract (modified SMAY) for *M. rileyi*. Fungal cultures were incubated in B.O.D. (Biological Oxygen Demand) at 26°C for ten days before harvesting the conidia for the assays.

The *Spodoptera frugiperda* eggs were purchased from specialized breeders and kept under consistent laboratory conditions (temperature 27 ± 1°C, photoperiod 14:10 L:D, relative humidity 70 ± 10%). Hatched caterpillars were transferred to plastic pots and fed on corn leaves (*Zea mays*) until the second instar (4-5 days after the hatching of the eggs).

3.2.2. Blastospore and conidia production

Suspensions of *M. anisopliae* and *M. rileyi* conidia were obtained by washing sporulated fungus plates with 10 mL of 0.01% aqueous solution of Tween® 80. These suspensions were used to inoculate a pre-culture liquid medium in the standard concentration of $5x10^6$ conidia/mL for *M. anisopliae*, and $5x10^7$ conidia/mL for *M. rileyi* production. The liquid culture medium was a basal medium supplemented with 45 g/L yeast extract and 80 g/L or 200 g/L of carbon source to create osmotic stress. The carbon source (20% w/v) was autoclaved separately from the salt solution and added before the fungus inoculation. The basal media contained, per liter of distilled water: KH_2PO_4: 2.0g; $CaCl_2$: 0.4g; $MgSO_4$: 0.3g; $FeSO_4$: 0.05g; $CoCl_2$: 37mg; $MnSO_4$: 16mg; $ZnSO_4$: 14mg; Thiamine, Riboflavin, Pantothenate, Niacin, Pyridoxamine, Thioctic Acid: 500µg each; and Folic Acid, Biotin, Vitamin B12: 50µg each (Jackson, 1997; Jackson and Jaronski, 2009).

Fungal cultures were grown in 250 mL "baffled flasks" with 50 mL of the medium containing the fungal inoculum in each flask. Cultures were kept in an orbital shaker (350 rpm, 28°C) and shaken daily by hand to minimize mycelial growth on the flask's wall. The concentration of 80 g carbon source L^{-1} was considered the standard treatment. Two shake flasks (replicates) per experimental treatment were used for each experiment, and all experiments were

independently repeated three times (n=6). Blastospore concentration was determined microscopically using a Neubauer chamber.

To determine the effect of the osmotic stress on cell size and morphology, the length and width of 50 random blastospores from each treatment flask were measured using a Leica Biosystems light microscope and image system (Leica DM4B; LAS Version 4.1.0) at 400x magnification. Subsequently, the cell size was indirectly inferred by the volume in cubic micrometers (μm^3) of an ellipsoid given by the equation 1, where *a* is the length and *b* is the width (Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015).

$$V\ (\mu m^3) = \frac{4}{3} \times (\pi \times a \times b^2) \quad (1)$$

Fungal conidia were produced in Petri dishes (90×10 mm) with a solid medium according to the fungi species. PDA medium was used for *M. anisopliae* and Modified SMAY for *M. rileyi*. The plates were incubated for 15 days at 27 ± 1 °C with relative humidity (RH) higher than 90%.

The mean blastospore yield produced by each fungus in each osmotic level was analyzed by generalized linear models (GLM) with quasipoisson distribution and compared by Analysis of Variance (ANOVA) with chi-square test. Pairwise comparisons between treatments were carried out using the general linear hypothesis (function glht) in the multcomp R package (Hothorn *et al.*, 2023). Data overdispersion were checked by the half-normal plots (function hnp) implemented at hnp R package (Moral *et al.*, 2022). The blastospore volume obtained by each fungus in both osmotic levels were analyzed by linear models with normal distribution compared by ANOVA with F Test followed by a Tukey HSD post hoc test with $p< 0.05$ level of probability.

3.2.3. Virulence bioassay

Blastospores produced in liquid media under different osmotic stress conditions and conidia produced in solid media were used for the virulence tests. To avoid the influence of possible metabolites dispersed in the medium, after the development of the fungus in its respective liquid culture media, blastospores were separated from the media before spray application on the *S. frugiperda* caterpillars. Blastospores were collected by filtration through 2 layers of lens cleaning cloth (Whatman nº 105) and washed twice with a potassium buffer saline solution (composition per liter: NaCl: 8.0g; KCl: 0.2g; Na_2HPO_4: 1.44g; KH_2PO_4: 0.24g; pH adjusted to 6.0). During this process, the medium was centrifuged at 5,000 rpm for 10 min at each wash. Fresh conidia were harvested with a microbiological loop and suspended in 10 mL of 0.01% (v/v) Tween 80®. All suspensions were vortexed to ensure the samples' homogeneity, and concentrations were determined microscopically using a Neubauer chamber. The suspensions were adjusted to a concentration of $5x10^7$ propagules/mL dose, for blastospores and for conidia respectively.

Using a Potter Spray Tower (Burkard Manufacturing Co Ltd), 90 mm Petri dishes containing 16 second-instar caterpillars received a volume of 2 mL of a fungal suspension for each treatment. The treatments consisted of fresh blastospores of two different carbon source levels, fresh aerial conidia, and a control group that received only 0.05% aqueous solution of Tween® 80. After 24h, the caterpillars were placed individually into wells of plastic trays, and they were fed with corn leaves during the evaluation period. Caterpillar mortalities were evaluated daily until day 7. Dead larvae were surface disinfected by soaking in 70% ethanol, transferred to humid chambers, and kept at 28°C

to confirm if the mortality was caused by the fungus. Confirmed mortality was analyzed by visualizing morphologically the fungal sporulation.

The second-instar caterpillars was used to test the virulence of fungi for two reasons: 1) 1° instar are highly susceptible and naturally experience high mortality rates (Islam *et al.*, 2023); 2) in practical terms, caterpillars larger than the third instar are less susceptible and have a habit of seeking refuge in the whorls of corn plants, where the probability of fungal infection decreases (Pannuti *et al.*, 2016).

Survival analysis was performed with censored data for dead larvae until day 7 using a parametric model for survival data with Weibull distribution (survival r-package) (Jacobson *et al.*, 2022). The survival curves were compared by a log-likelihood ratio test at $p<0.05$. The insect confirmed mortality (mycosis) by each fungus were compared between the treatments using generalized linear models (GLM) and quasipoisson distribution. Data were analyzed by ANOVA with F-test and pairwise comparisons were carried out using the function glht in the multcomp r-package (Hothorn *et al.*, 2023) to compare treatments. Data overdispersion were checked by the function hnp implemented at hnp r-package (Moral *et al.*, 2022). The Lethal Times (LT50 and LT95) were calculated using a probit analysis in the ecotox r-package (R).

3.2.4. Microscopy of propagule germination and penetration on insect cuticle

One isolate of each fungal species was selected for the penetration studies, RNA extraction and transcriptomic studies. The *M. anisopliae* ESALQ E9 was selected for being a commercial generalist isolate, having registered product in Brazil for spittlebugs on sugarcane and pasture. The other one, the *M. rileyi* ESALQ 5611, was selected for being isolated from *S. frugiperda* caterpillars as a guarantee of its specificity.

Scanning Electron Microscopy (SEM) was used to determine if fungal propagules would form appressoria structures on insect cuticles and to determine the best time point for RNA extractions. Suspensions of *M. anisopliae* and *M. rileyi* propagules were sprayed on *Spodoptera frugiperda* caterpillars, and different time points after spraying were selected. Petri dishes containing five third-instar caterpillars received a volume of 2 mL of a fungal solution of concentration $1x10^7$ propagules mL with a Potter Spray Tower. The treatments were new blastospores produced with 200 g L of glucose and new aerial conidia from each *M. anisopliae* and *M. rileyi* isolate. After the caterpillars' spraying with blastospores and conidia, the samples were collected and prepared for analysis by SEM techniques. Analyzed times were 2h, 4h, 6h, and 8h after spraying for blastospores and 12h, 16h, 24h, and 32h after spraying for aerial conidia.

Caterpillars were anesthetized by cold shock (refrigerator at 4°C for 5 minutes) and subsequently fixed in 4% paraformaldehyde in phosphate buffer solution (PBS), for 48 hours, at 4°C. This was followed by dehydration in an increasing series of acetone (70%, 80%, 90%, 95%, and twice in 100%, lasting 10 minutes for each batch). After drying at an Automated Critical Point Dryer (Leica EM CPD300), they were glued in aluminum stubs using double-sided tape until samples were metalized with gold in the Sample Sputter Coater (Balzers SCD 050). The specimens were examined and photographed using a JEOL JSM-IT300 scanning electron microscope operated at 15.0 kV.

3.2.5. RNA extraction and sequencing

Before RNA extractions, *S. frugiperda* caterpillars were inoculated with *M. anisopliae* (ESALQE9) or *M. rileyi* (ESALQ5611) by soaking them in suspensions of conidia or blastospores of each isolate. Each replicate consisted of seven 4th instar caterpillars. Five replicates were used, totalizing 20 samples. Fungal suspensions were prepared at a concentration of $5x10^7$ propagules mL for each treatment.

After inoculation, *S. caterpillars* were fed with corn leaves and kept under controlled temperature and humidity conditions for 6 hours for blastospores treatments and 24 hours for conidia treatments (time at which appressoria are formed according to SEM analysis). After that, the digestive tube of the caterpillars was removed on ice, and the cuticle was used for RNA extraction. The RNA was extracted using TRIZOL® (Invitrogen Life Technologies). The protocol was carried out according to the manufacturer's recommendations (Invitrogen). After extraction, the total RNA was treated with RQ1 RNAse-free DNase Promega according to the usage information (Corporation).

Total RNA was quantified in the fluorometer (Qubit, Invitrogen), and the concentration and quality of the samples were evaluated by analysis in spectrophotometer NanoDrop ND-1000 (Wilmington, USA). The RNA integrity was assessed on agarose-formaldehyde gel 1%. Samples were sequenced with Illumina HiSeq 2500 technology, which yielded at least 20 million 150-bp paired-end reads per library. Library preparation and sequencing were performed by the NGS Soluções Genômicas in Piracicaba-SP, Brazil.

3.2.6. RNA-Seq reads

The quality of the raw reads before and after quality and adaptor trimming was assessed using the FastQC program. Illumina adapters and low-quality sequences were removed using TrimGalore, AfterQC, and Trimmomatic V0.32 with the following options: LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 HEADCROP:7 MINLEN:36 (Iwanicki, N. S. *et al.*, 2020). To compile the FastQC reports and identify patterns between the samples of each treatment, the MultiQC program was used before and after read trimming.

To align reads to the genome, the reference genomes of *M. anisopliae* and *M. rileyi* were downloaded from the National Center for Biotechnology Information (NCBI) search database. The GenBank assembly accession GCA_013305495.1 (JEF-290 strain), was chosen for *M. anisopliae* and the GenBank assembly accession GCA_007866325.1 (Cep018-CH2) for *M. rileyi*. The annotation files were downloaded as well. Filtered reads were mapped to reference genomes using the HISAT2 software following standard settings (available at https://github.com/DaehwanKimLab/hisat2). RNA-seq reads are available at the National Library of Medicine (NCBI) with the BioProject accession number: PRJNA976674.

Differential Gene Expression analysis

Before running the Differential Gene Expression analysis, the gene count matrixes were obtained with the python script provided by John Hopkins University, Center for computational biology (available at http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual#deseq). The gene count matrixes were used as input files for the differential expression analysis conducted using the DESeq2 package from the statistical software R (2023.03.0+386 version).

Before running the DESeq2 package, pre-filtering and sample-to-sample distances were assessed for all treatments using Principal Component Analysis (PCA) plots. Rows with reading counts <5 were excluded, and genes with adjusted p-values <0.1 were considered differentially expressed. Diagnostic plots (MA-plot and Volcano-plot)

were prepared and analyzed for each treatment. Heat maps of differentially expressed genes were made using the heatmap.2 packages from the R software.

The InterPro database was used to annotate protein sequences and better identify protein domains of the differential expressed genes (Blum *et al.*, 2021). *Metarhizium anisopliae* and *Metarhizium rileyi* protein sequences were downloaded from NCBI and used as input on the InterProScan (Jones *et al.*, 2014). Then, only mRNA was filtered from the output for reference in the gene-protein analysis.

3.3. Results

3.3.1. Blastospores production in different levels of osmotic stress

Growth of *M. anisopliae* at a high carbon source concentration of 200 g/L resulted in higher blastospore yield of both isolates compared to the production of fungus under the standard concentration of 80 g/L (z=2.055, p=0.039917; Figure 7A). A comparison of the two *M. anisopliae* isolates showed that the isolate ESALQE9 responds better to the osmotic stress in liquid fermentation, resulting in higher yields. The blastospore production by the isolate ESALQ 4676 did not differ between the different sugar levels (z=-1.363, p=0.1729, Figure 7A). The high carbon source concentration resulted in smaller *M. anisopliae* cells than those produced under the standard concentration for both isolates (p<0.0001). When grown with 80g carbon source L^{-1}, both isolates showed the same pattern with a mean blastospore volume of 1050µ3 compared to 730µ3 when produced under 200g carbon source L^{-1} (Figure 7C; E).

Metarhizium rileyi blastospores production under different concentrations of carbon source showed a different pattern under the same osmotic stress conditions. While *M. anisopliae* total cell volume reduces under osmotic stress conditions during liquid fermentation, *M. rileyi* develops reversely. The high carbon source concentration did not increase the blastospore production, on the other hand, blastospores production was higher in low sugar concentration for the isolate ESALQ 4946 (z=7.456, p<0.001; Figure 1B) and for the isolate ESALQ 5611 (z=12.44, p<0.001). Also, the higher carbon source concentration did not result in smaller blastospores of *M. rileyi* (Figure 7B). A clear pattern of blastospores sizes was not observed, however isolate ESALQ 4676 produced smaller blastopores in lower carbon source concentration (p<0.001). Blastopores produced by the isolate ESALQ 5611 did not present size differences regardless of the sugar concentration (p=0.2232).

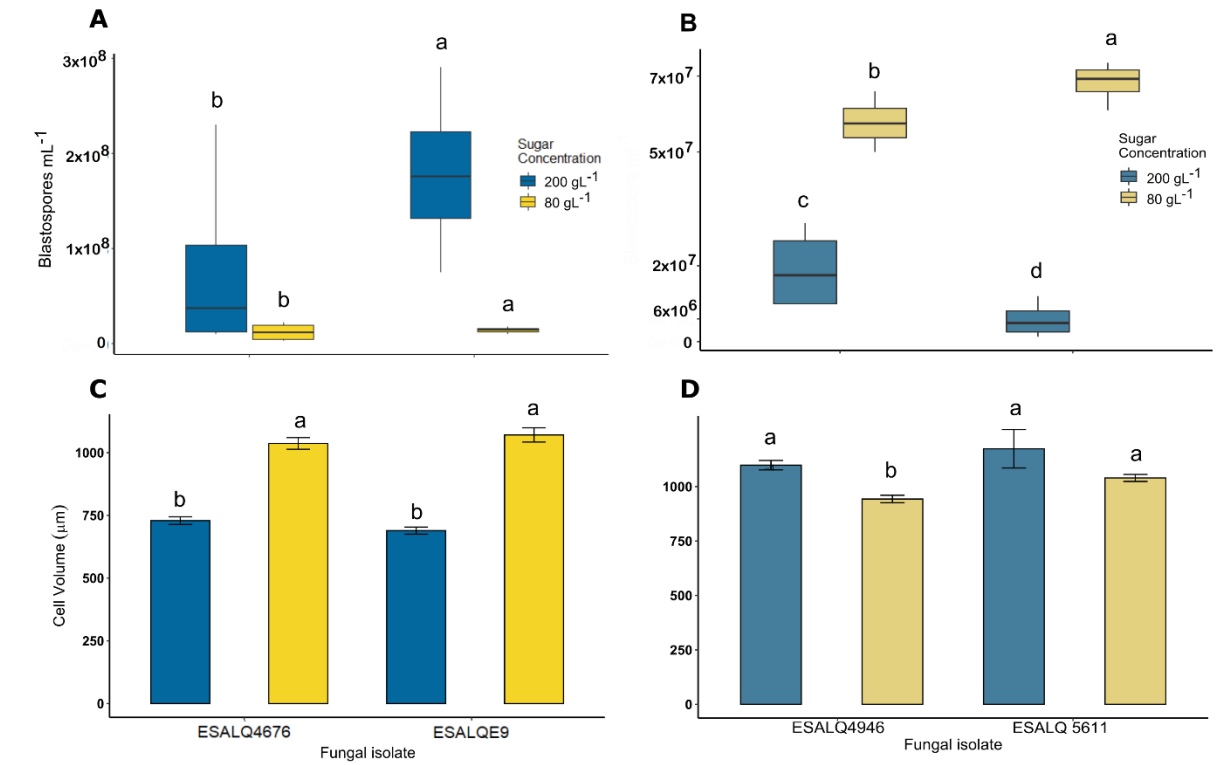


Figure 7. Blastospore production of *M. anisopliae* and *M. rileyi* in liquid fermentation under different levels of carbon source (80g L^{-1} and 200g L^{-1}). (A) Blastospore production (yield per mL^{-1}) of two isolates each of *M. anisopliae*. (B) Cell volume (µ3) of two isolates each of *M. anisopliae* (C) Blastospore production (yield per mL^{-1}) of two isolates each of *M. rileyi*. (D) Cell volume (µ3) of two isolates each of *M. rileyi*. Experiments were independently repeated three times. Means (±SD) followed by non-corresponding letters are significantly different (Tukey's test, $p<0.05$).

3.3.2. Virulence bioassay

The *M. anisopliae* isolate (ESALQ E9), a generalist fungus isolated from the soil, acted slowly, and killed less than 50% of the insects in 7 days with all the propagules at concentration of 5 $\times 10^7$ mL^{-1}. This result is strongly supported by the Median Lethal Time calculated for the propagules (LT50). *M. anisopliae* conidia have an estimated LT50 of 7.5 days, compared to blastospores produced under 80g carbon source L^{-1} of 18.5 days, and 20.6 days for blastospores produced under 200g carbon source L^{-1} (Table 2).

On the other hand, *M. rileyi* (ESALQ 5611), a specialist fungus isolated from a *Spodoptera frugiperda* caterpillar, killed 50% of the evaluated insects within 3 – 6 days, with all the propagules at concentration of 5 $\times 10^7$ mL^{-1}. Conidia attained the highest LT50 value (2.57 days), while the lowest LT50 value was attributed to blastospores produced under 80g carbon source L^{-1} (6.28 days). Comparing blastospores types, the propagules produced under 200g carbon source L^{-1} were more efficient in killing *S. frugiperda* caterpillars (4.84 days) (Table 3).

The survival of the *S. frugiperda* caterpillars that received the control treatment and the *M. anisopliae* propagules was different ($\chi^2 = 25.98$, $p<0.001$). There was no difference in caterpillar mortality between the *M. anisopliae* blastospores and conidia applied to the caterpillars ($p<0.83735$). Comparing fungal species, *M. anisopliae* propagules were less virulent to *S. frugiperda* caterpillars (mortality rate was less than 25% for all treatments) and resulted in less

than 5% mycosed insects irrespective of infective propagule (Figure 8B) with significant difference between the propagules and the control (F=4.5292, p=0.01037). Therefore, despite producing more blastospores and smaller cells, the osmotic stress did not produce more virulent propagules (p>0.583).

Survival of *S. frugiperda* differed between the control and the *M. rileyi* propagules (χ^2= 177.46, p<0.001). Conidia of *M. rileyi* were more virulent than both blastospores (p<0.001), even those produced under high concentration of the carbon source. Blastospores produced using 200g carbon source L^{-1} were more virulent than those produced on the standard carbon source concentration (p<0.001). Blastospores produced using 200g carbon source L^{-1} also resulted in more mycosed insects than the other propagules and the control (Figure 8C,D).

Table 2. Estimated median lethal time (LT50), 95% lethal time (LT95), lower confidence interval (lci) and upper confidence interval (uci) of the *Spodoptera frugiperda* caterpillars after being sprayed with *Metarhizium anisopliae* propagules. Time is measured in days.

	Letal Time 50 (LT50)			**Letal Time 95 (LT95)**		
Fungal propagule	**Estimated**	**lci***	**uci***	**Estimated**	**lci**	**uci**
Conidia	17.5	11.6	47.8	162	55.8	2306
Blastospores 80g L^{-1}	18.5	11.8	58.3	245	71.1	6394
Blastospores 200g L^{-1}	20.6	12.1	99.4	530	106	71405

*lci - lower confidence interval 95% and uci = upper confidence interval 95%

Table 3. Estimated median lethal time (LT50), 95% lethal time (LT95), lower confidence interval (lci) and upper confidence interval (uci) of the *Spodoptera frugiperda* caterpillars after being sprayed with *Metarhizium rileyi* propagules. Time is measured in days.

	Letal Time 50 (LT50)			**Letal Time 95 (LT95)**		
Fungal propagule	**Estimated**	**lci***	**uci***	**Estimated**	**lci**	**uci**
Conidia	2.57	1.97	3.12	10.2	7.23	20.2
Blastospores 80g L^{-1}	6.28	5.84	6.88	18.4	15.3	27.3
Blastospores 200g L^{-1}	4.84	3.99	6.12	13.3	9.06	39.4

*lci - lower confidence interval 95% and uci = upper confidence interval 95%

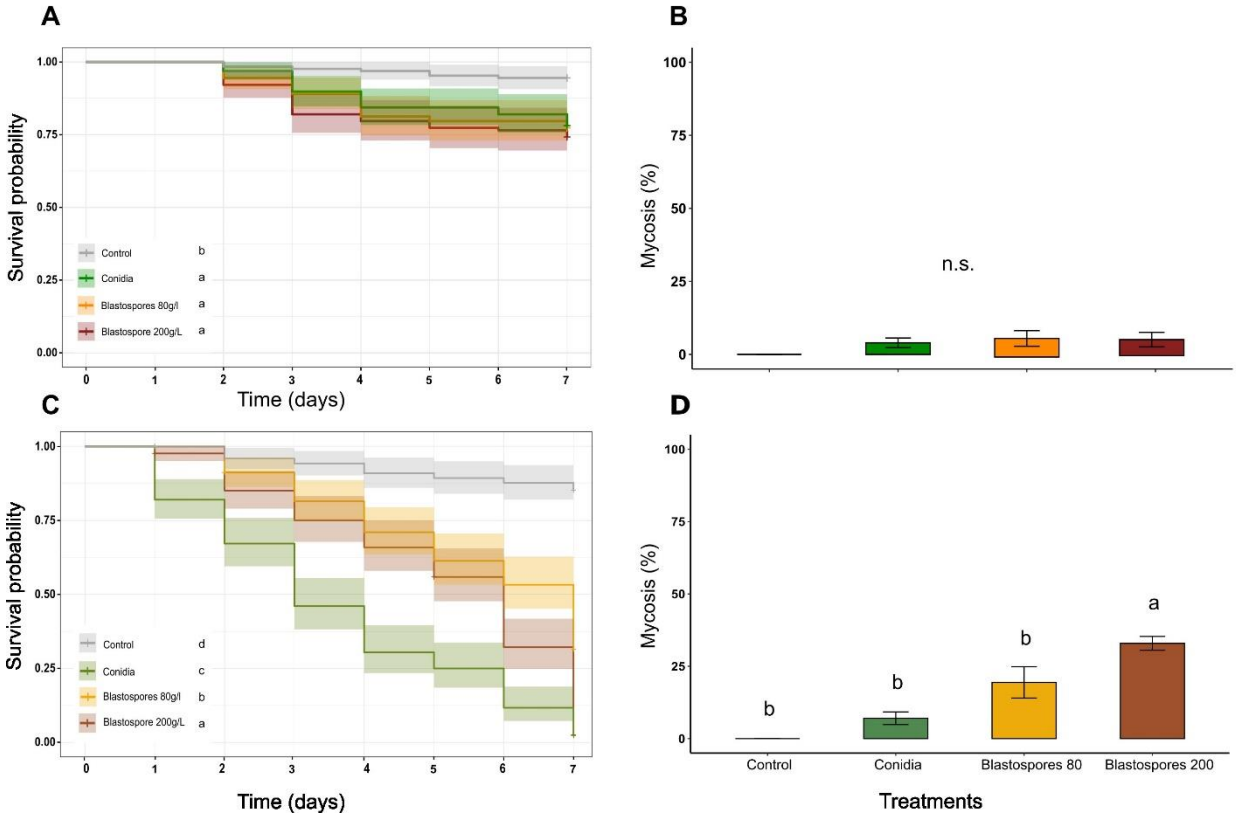


Figure 8. Survival probability and the percentage of mycosed cadavers of *Spodoptera frugiperda* caterpillars treated with *Metarhizium* spp. conidia and blastospores produced in two sugar source concentrations (80 and 200g/L^{-1}). (A) Survival of *S. frugiperda* caterpillars exposed to conidia and blastospores of *M. anisopliae* for 7 days (B)Percentage of *S. frugiperda* cadavers colonized by *M. anisopliae*. (C) Survival of *S. frugiperda* caterpillars exposed to conidia and blastospores of *M. rileyi* for 7 days (D) Percentage of *S. frugiperda* cadavers colonized by *M. anisopliae*. The letters in front of the captions and above the bars indicate the statistical difference between the treatments: equal letters represent treatments that did not differ from each other, while different letters indicate a statistical difference at a 0.05% probability level.

3.3.3. Propagules germination and penetration through the insect cuticle

Image analysis revealed that blastospores germinate between 2 and 4 hours after spraying and penetrate between 6 and 8 hours after spraying. The exact penetration was not possible to see. Comparing the images of 6 hours, when both blastospore and germ tube are turgid, with the images of the collapsed blastospore at 8 hours after spraying it is possible to infer that the blastospores penetrated sometime between 6 and 8 hours after spraying (Figure 9A).

Furthermore, conidia images showed that germination occurred 16 hours after spraying and estimated penetration between 24 and 32 hours after spraying. A similar aspect is observed in blastospores: by 24 hours, conidia and germ tube seem to be turgid. At 32 hours, the conidia seemed to be collapsed, letting us infer those penetrations occurred (Figure 9B).

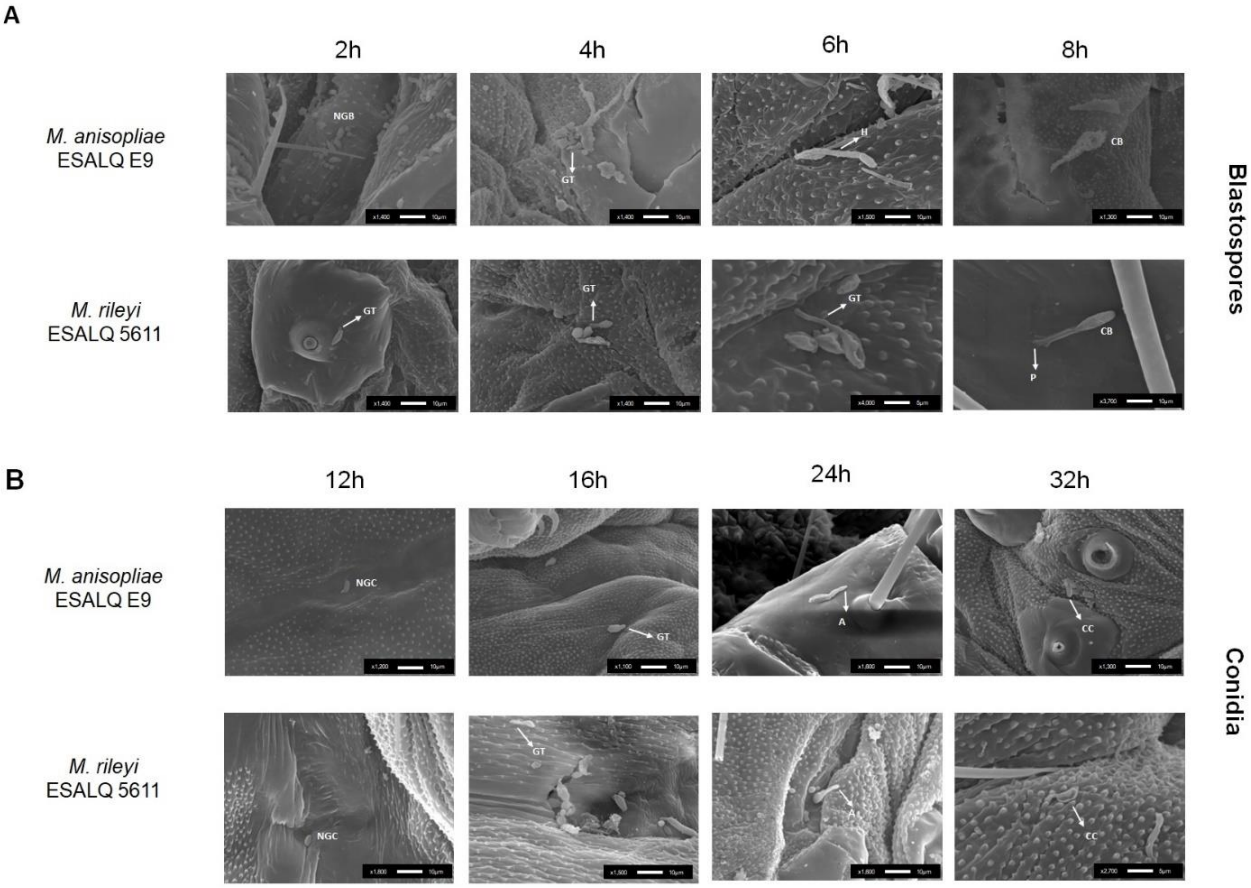


Figure 9. Scanning Electron Microscopy micrographs of *Metarhizium spp.* propagules during germination on *Spodoptera frugiperda* integument for Blastospores. 2, 4, 6, and 8h post exposure for Blastospores (A), and 12, 16, 24, and 32h for conidia (B). NGB: non-geminated blastospores; NGC: non-germinated conidia; GT: germ tube; H: hyphae; CB: collapsed blastospore post infection; P: propagule penetration; A: appressorium; CC: collapsed conidia post infection.

3.3.4. RNAseq sequencing and Differential Gene Expression analysis

Based on analysis of SEM pictures, replicate samples were collected for RNAseq analysis 24 hours post exposure for *Metarhizium anisopliae* and 8 hours post exposure for *Metarhizium rileyi*. Analyzing the number of mapped reads in each sample, we found that less than 1% of the reads were uniquely mapped to the respective fungi's genome (Appendix A). When the same samples were mapped with the *Spodoptera frugiperda* reference genome, it was possible to observe almost 70% of uniquely mapped reads, confirming that the low alignment to the fungal genomes is because of reduced amount of fungal RNA in the samples, compared to the large amount of insect RNA (Appendix B).

The Principal Componet Analysis of the *M. anisopliae* and *M. rileyi* propagules during penetration on *S. frugiperda* caterpillars showed a good separation between blastospores and conidia for both fungi (Figure 10). The PCA plots largely separate treatments and explained 58% of sample variation in *M. anisopliae* (Figure 10A) and 33% in *M. rileyi* (Figure 10B), respectively. For *M. anisopliae*, reads mapped to 4.870 genes in the reference genome with 319 genes being differentially expressed between blastospores and conidia (FDR adjusted p<0.1, Log2 Fold change (*FC*)>2 or <-2). Out of these 319 genes, 36 genes were up-regulated in blastospores, and 283 were up-regulated in conidia (Figure

5A). For *M. rileyi*, reads mapped to 6.128 genes in the reference genome with 101 genes being differentially expressed between blastospores and conidia (FDR adjusted $p<0.1$, Log2FC>2 or <-2). Out of these differentially expressed genes, 39 genes were up-regulated in blastospores, and 62 were up-regulated in conidia (Figure 11B).

Protein domain analysis of differentially expressed genes revealed 194 Protein Families (PFAM) terms among up-regulated genes in *M. anisopliae* conidia, compared to 19 in *M. anisopliae* blastospores. For *M. rileyi*, we found 22 up-regulated PFAM terms in blastospores and 28 in conidia (Figure 11C). Conidia from *M. anisopliae* and *M. rileyi* share 19 PFAM terms related to virulence factors. In contrast, blastospores from both species only share one PFAM term corresponding to a Heat shock protein (HSP) (Figure 11C). All the treatments share only one PFAM: the Cytochrome P450, an oxidative stress factor (Figure 11C, Appendix C).

Up-regulated genes of *M. anisopliae* and *M. rileyi* propagules were grouped by PFAM annotation (Figure 11D). Analyzing the amount of PFAM terms presented in different functions, it was observed that, in general, conidia of both fungi expressed more virulence and oxidative stress factors than blastospores. *M. rileyi* blastospores expressed more of these factors against *S. frugiperda* than *M. anisopliae* blastospores does. To better analyze the virulence of *M. anisopliae* and *M. rileyi* propagules against *S. frugiperda* caterpillars, we focused only on PFAMs related to virulence and oxidative stress factors (Figure 12). In this analysis *M. anisopliae* conidia have 72 unique virulence-related elements compared to 11 in *M. rileyi*. Conidia from both species share ten genes from all those virulence-related factors, while blastospores share no one.

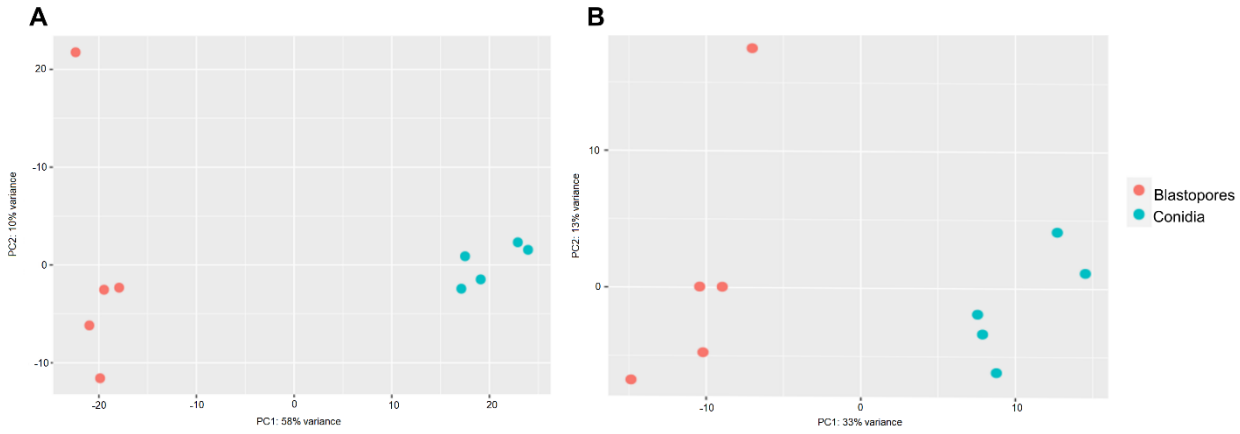


Figure 10. Principal Component Analysis of regularized-logarithmic (rlog) transformed gene counts of *Metarhizium anisopliae* and *M. rileyi* propagules during penetration on *Spodoptera frugiperda* caterpillars. (A) *M. anisopliae* samples. (B) *M. rileyi* samples. Orange and green dots represent blastospores and conidia samples, respectively.

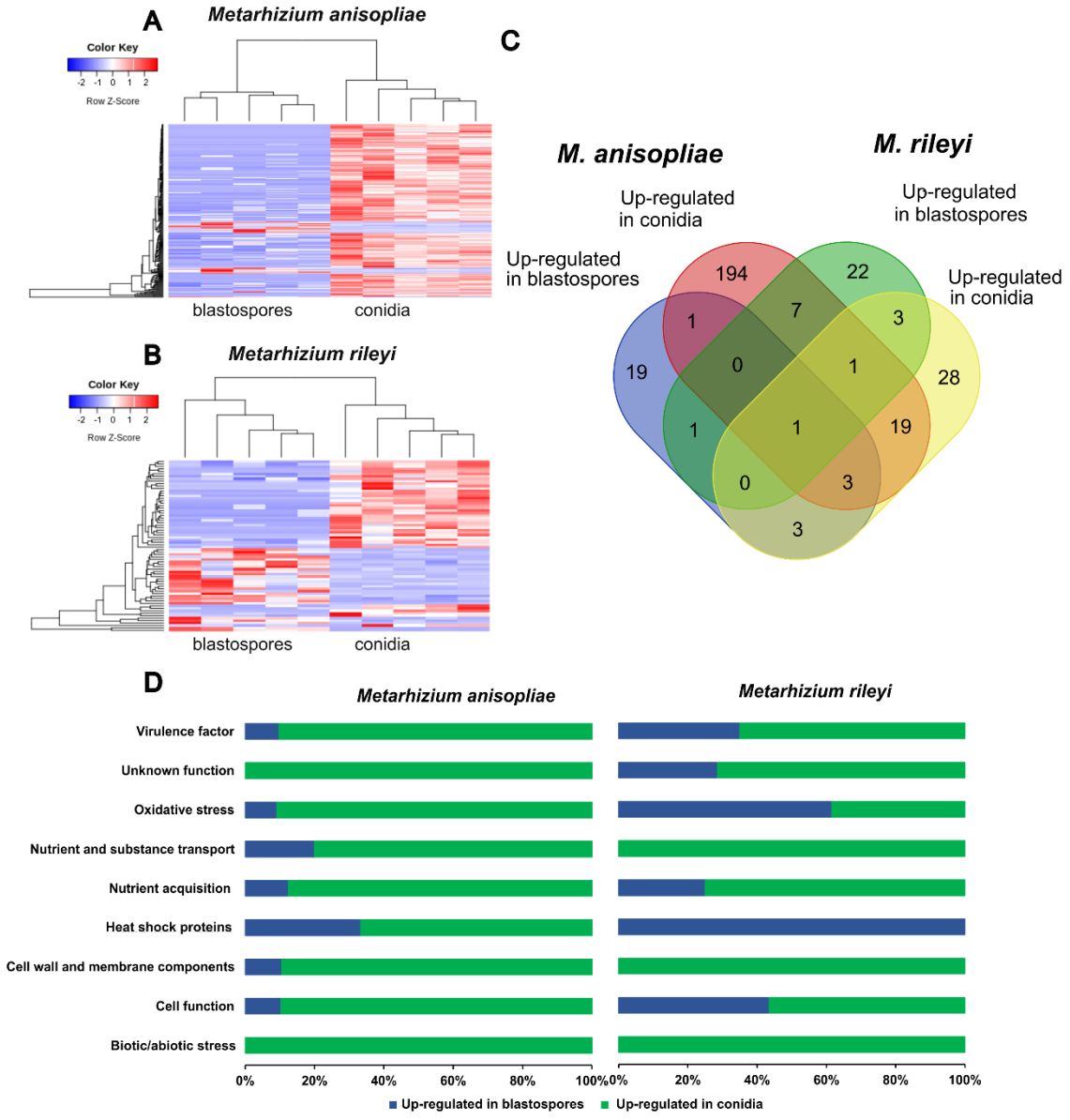
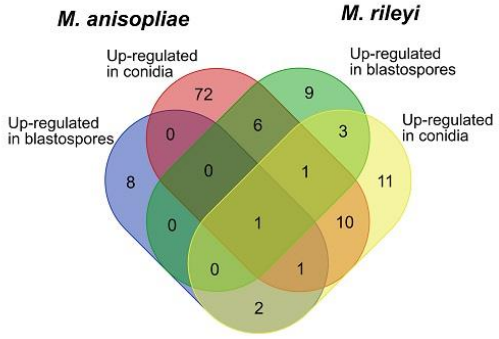


Figure 11. Differential gene expression between blastospores and conidia on *Spodptera frugiperda*. (A) Heatmap of the 319 differentially expressed genes (Padj<0.1) in *Metarhizium anisopliae* blastospores compared to conidia during penetration on *S. caterpillars*. (B) Heatmap of the 101 differentially expressed genes (Padj<0.1) in *Metarhizium rileyi* blastospores compared to conidia during penetration on *S. frugiperda* caterpillars. (C) Venn Diagram of up-regulated PFAM terms expressed in *M. anisopliae* and *M. rileyi* propagules during penetration on *S. frugiperda* cuticle. (D) Percentage of PFAM terms upregulated in *M. anisopliae* and *M. rileyi* propagules during penetration on *S. frugiperda* caterpillar's cuticle (Log2FC<2; p<0.001).

A



B

PFAM	Protein Name	Function
Shared by all structures		
PF00067	Cytochrome P450 4F5	Oxidative stress
Shared by *M.anisopliae* and *M. rileyi* conidia		
PF01425	Amidase signature domain	Virulence factor
PF03576	Peptidase S58, DmpA	Virulence factor
PF13193	AMP-binding enzyme, C-terminal domain	Virulence factor
PF00171	Aldehyde dehydrogenase domain	Virulence factor
PF02629	CoA-binding	Virulence factor
PF00903	Glyoxalase/fosfomycin resistance/dioxygenase domain	Virulence factor
PF11327	Egh16-like virulence factor	Virulence factor
PF00125	Histone H2A/H2B/H3	Virulence factor
PF02353	Mycolic acid cyclopropane synthetase	Virulence factor
PF07732	Multicopper oxidase, N-termianl	Virulence factor
Shared by *M. rileyi* blastospores and conidia		
PF00155	Aminotransferase, class I/class II	Oxidative stress
PF13499	EF-hand domain	Virulence factor
PF13641	Glycosyltransferase-like family 2	Oxidative stress

Figure 12. (A) Venn Diagram of Virulence up-regulated protein families expressed in *Metahrizium anisopliae* and *Metarhizium rileyi* propagules during penetration on *Spodoptera frugiperda* cuticle. (B) Up-regulated Protein Families related to virulence factors and oxidative stress found in *M. anisopliae* and *M. rileyi* propagules during penetration on *S. frugiperda* cuticle.

3.4. Discussion

The present study focused on investigating the impact of glucose concentration on *Metarhizium* blastospores production in liquid culture and the virulence of blastospores compared to conidia against *Spodoptera frugiperda* caterpillars. We also used comparative transcriptomics to identify genes and protein families that are actively expressed during the infection process of insects *in vivo* to investigate why blastospores seem more virulent than conidia.

A previous study found that blastospores of different isolates of *B. bassiana* cultivated in media with glucose concentrations greater than 220 g L^{-1} were as much as 53% smaller in volume compared to blastospores produced in media with glucose concentrations of 40–100 g L^{-1} (Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015). We observed a reduction in the *Metarhizium anisopliae* blastospores volume produced under osmotic stress. Fewer blastospores were also produced under osmotic stress during liquid fermentation of *Beauveria bassiana*, which has been suggested to be an osmoadaptation attributed to the concentration of solutes in the cytoplasm (Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015). In the yeast (*Saccharomyces cerevisiae*), the dynamics of osmostress-induced cell volume loss is a consequence of osmotic adaptation to restore optimal diffusion rates for biochemical and biological cell processes (Morales-Reyes *et al.*, 2018). In contrast we observed that *Metarhizium rileyi* have more mycelia and hyphal bodies under osmotic stress conditions, which imply that it is not possible to generalize for all entomopathogenic fungi studied to date.

Changes in nutrition, pH, and temperature can affect fungal morphogenesis and phenotype of fungal cells (Klein and Tebbets, 2007). The dimorphism between yeast-like growth and mycelial formation in the entomopathogenic fungus *M. rileyi* under *in vitro* conditions is impacted by specific nutritional conditions (Boucias *et al.*, 2016). During *in vivo* replication of *M. rileyi*, a tightly orchestrated development program involving specific chemical quorum sensing chemicals mediate the transition from hyphal bodies to mycelia (Boucias *et al.*, 2016). Such a correlated switch in fungal cell phenotype is likely correlated with cell population density and indicates the production of autoinducers mediating controls in the developmental programs that modulate either cell behavior or phenotype (Albuquerque and Casadevall, 2012).

Entomopathogenic fungi are very heterogeneous, and their growth patterns differ extensively between species. *Beauveria bassiana* (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015; Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015) and *M. anisopliae* studied here supported our hypothesis of higher blastospores production under high osmotic stress conditions, but in contrast the *M. rileyi* isolates did not. More studies are needed to understand the role of osmotic stress in inducing yeast-like growth and in the formation of blastospores in liquid cultures of *M. rileyi*.

The germination and penetration time-point of *M. anisopliae* and *M. rileyi* propagules were also studied here. Our results are in concordance with previous studies (Vega *et al.*, 2012; De Paula *et al.*, 2021; Kiruthiga *et al.*, 2022), and showed the time to germination, appressoria formation, and penetration found in our SEM analysis revealed that blastospores germinate and penetrate between 2 and 9 hours. In contrast, conidia germinate and penetrate between 16 and 32 hours after spraying.

Entomopathogenic fungi infect susceptible hosts via direct cuticle penetration, with the initial contact between the fungal propagule and the insect epicuticle potentially determining the outcome of the interaction (Ortiz-Urquiza and Keyhani, 2013). Thus, the cuticle is the first and most significant barrier to entomopathogenic fungi and an important immune component in insects. Dynamic interactions at the cuticle surface influence the pathogens' ability to infect hosts and entomopathogenic fungi evolved mechanisms for adhesion and recognition of the host surface. These specific adaptations include the production of specialized infectious cellular structures (e.g., appressoria or penetrant tubes); hydrolytic, assimilatory, and/or detoxifying enzymes (e.g., lipase/esterases, catalases, cytochrome P450s, proteases, and chitinases); and secondary and other metabolites that facilitate infection [30].

Delayed penetration can prolong the exposure of fungal propagules to biotic and abiotic factors harmful to the pathogen (Lovett and St. Leger, 2015). Furthermore, delaying entry to the hemocoel allows the host to mobilize its cellular and humoral defenses (Dubovskiy *et al.*, 2013). Thus, successful propagules must not only be able to cope with the physical environment on the host surface but also perform against insect innate immune defenses (Dubovskiy *et al.*, 2013). The most external surface of the insect cuticle, the epicuticle, is formed by a thin lipid layer making it hydrophobic, which facilitates the attachment of the hydrophobic conidia (Pedrini, 2018). However, many studies have shown that the hydrophilic blastospores, can germinate, penetrate, and kill insects faster than conidia (Vega *et al.*, 1999; De Paula *et al.*, 2021; Kiruthiga *et al.*, 2022).

Despite winning the battle against the insect cuticle by germinating faster than conidia, we did not observe that blastospores are more virulent compared to conidia against *S. frugiperda* for *M. anisopliae* and *M. rileyi*. Comparing the genes expressed during *M. anisopliae* and *M. rileyi* propagules penetration and its virulence for *S. frugiperda* caterpillars, the higher mortality caused by the conidia of both fungi correlate with a high number of virulence-related protein family domains (PFAMs) expressed. Interestingly, we only found one shared PFAM between blastospores of *M. anisopliae* and *M. rileyi*. The only PFAM shared between blastospores, and conidia of both fungi is the cytochrome P450, an oxidative stress factor. During propagule attachment to the cuticle, entomopathogenic fungi must cope with harmful environmental conditions such as solar radiation and fluctuating humidity and temperature, antagonistic microorganisms, and antifungal compounds released by insects (Ortiz-Urquiza and Keyhani, 2013). Thus, the infectious conidia have evolved various mechanisms to cope with these biotic and abiotic stressors, often involving genes linked with virulence and oxidative stress (Ortiz-Urquiza and Keyhani, 2013; Lovett and St. Leger, 2015).

Our finding that the total gene-expression profiles of conidia and blastospores differ fundamentally may be linked to difference in past selection pressures of these two propagules, being adapted to the external environment and proliferation to the internal body cavity of insects, respectively. Differences in transcriptomic profiles of conidia and blastospores during cuticle infection also highlight that despite both propagules being infectious, different mechanisms

are underlying their respective infection mechanism, which in turn likely explains the faster germination rate of blastospores.

Selection for virulence in entomopathogenic fungi are likely governed by two opposing pressures being exerted: (a) specialization towards specific insect host species or populations, or (b) to maintain a broad host range increasing the number of available hosts. Here we studied two species that seem to act differently in the environment: *M. anisopliae*, widely known for its broad host range, and *M. rileyi*, a specialist to Lepidoptera species, especially Noctuid. The virulence tests performed here shows that specialization result in greater virulence to insects; demonstrated by the higher virulence of *M. rileyi* to *S. frugiperda* caterpillars. However, comparative transcriptomics during propagules penetration on insect cuticles showed that this confirmed virulence may be not directly linked to a greater number of virulence related expressed PFAM terms. In general, *M. anisopliae* propagules were less virulent to *S. frugiperda* caterpillars but expressed more virulence related PFAM terms compared to *M. rileyi*. Fungal adaptive responses may explain these results as they can be mediated by epigenetic mechanisms allowing short-term specialization while maintaining the broad host range potential (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013). Evidence increasingly suggests that a significant factor driving the co-evolutionary arms race between the pathogen and the host occurs on the cuticular surface – also involving mediation of the insect cuticle microbiome (Shang *et al.*, 2022; Hong *et al.*, 2023).

Although considerable progress has been made in recent years, much regarding the molecular determinants mediating these interactions in the pathogen and the host remains uncovered (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013). Studies comparing broad host-range fungi such as *M. anisopliae* with more specialist fungi like *M. rileyi* should be particularly useful for delineating the factors involving infection of different fungal propagules and the effective host range of entomopathogenic fungi.

3.5. Conclusion

The virulence tests performed here shows that *M. rileyi*, a lepidoptera specialist fungi, were more virulent to *S. frugiperda* caterpillars compared to *M. anisopliae*, a generalist one. Comparative transcriptomics during propagule penetration on insect cuticles shows that, in general, *M. anisopliae* expresses more virulence and oxidative stress factors compared to *M. rileyi*. However, analyzing differences between propagules, *M. rileyi* blastospores expresses more virulence-related genes against *S. frugiperda* than *M. anisopliae*. In contrast, conidia of both fungi express more virulence-related oxidative stress factors than blastospores. Our results highlight that blastospores use a different virulence mechanism than conidia and increase our understanding of the fungal process of insect pathogenicity. On the future, we potentially may find gene candidates whose manipulation could lead to more effective insect biological control agents that may be explored in new biological control strategies.

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Appendix

APPENDIX A. Summary of RNA-Seq read filtering and mapping. Values represent each of the five biological replicates for each treatment. Samples 1 – 10 and 21 - 40 were mapped with the *M. anisopliae* reference genome: GCA_013305495.1. Samples 11 – 20 were mapped with the *M. rileyi* reference genome: GCA_007866325.1.

Sample	Treatment	Number of Input Reads	Uniquely mapped Reads (%)	Reads mapped to too many loci (%)	Unmapped reads (%)
1	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	17582304	0.14%	0,36%	99,03%
2	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	15741262	0.15%	0,30%	99,07%
3	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	14721168	0.10%	0,24%	99,26%
4	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	17926588	0.15%	0,28%	99,11%
5	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	13467826	0.14%	0,35%	98,91%
6	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	15694999	0.57%	0,32%	98,66%
7	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	15941969	0.63%	0,55%	98,09%
8	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16652007	0.36%	0,30%	98,89%
9	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16245901	0.55%	0,91%	97,35%
10	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16604615	0.36%	0,65%	98,12%
11	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	17243904	0.32%	0,00%	99,67%
12	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	15728103	0.19%	0,00%	99,80%
13	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	16375249	0.23%	0,00%	99,75%
14	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	15457153	0.24%	0,00%	99,75%
15	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	17595338	0.20%	0,00%	99,79%
16	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	17249224	0.49%	0,00%	99,49%
17	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	15896404	0.40%	0,00%	99,59%
18	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	17481701	0.95%	0,00%	99,04%
19	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	14985625	1.12%	0,00%	98,85%
20	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	14836469	0.25%	0,00%	99,74%

APPENDIX B. Summary of RNA-Seq read filtering and mapping with *Spodoptera frugiperda* reference genome. Values represent each of the five biological replicates for each treatment. All the samples were mapped with the *S. frugiperda* reference genome: GCA_019297735.1

Sample	Treatment	Number of Input Reads	Uniquely mapped Reads (%)	Reads mapped to too many loci (%)	Unmapped reads (%)
1	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	17546491	68,42%	0,06%	23,59%
2	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	15707692	66,08%	0,15%	25,91%
3	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	14700702	66,56%	0,11%	26,32%
4	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	17887751	66,43%	0,11%	25,96%
5	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* blastospores	13441206	67,80%	0,13%	23,98%
6	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	15590528	67,48%	0,05%	25,75%
7	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	15827454	64,15%	0,06%	28,21%
8	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16581026	62,82%	0,06%	30,52%
9	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16126915	67,18%	0,06%	21,76%
10	*Spodoptera frugiperda* cuticle + *Metarhizium anisopliae* conidia	16526741	66,13%	0,08%	22,96%
11	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	17187484	68,97%	0,12%	21,31%
12	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	15696812	71,16%	0,15%	21,56%
13	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	16335133	75,19%	0,09%	17,07%
14	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	15418547	72,16%	0,09%	20,43%
15	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* blastospores	17557909	70,08%	0,15%	20,43%
16	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	17161999	68,81%	0,09%	24,35%
17	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	15831745	70,15%	0,13%	20,28%
18	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	17313507	66,22%	0,10%	23,39%
19	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	14814037	68,07%	0,12%	21,03%
20	*Spodoptera frugiperda* cuticle + *Metarhizium rileyi* conidia	14797306	72,48%	0,13%	21,25%

APPENDIX C. Up-regulated Protein families and their general functions expressed in *Metahrizium anisopliae* and *Metarhizium rileyi* propagules during penetration on *Spodoptera frugiperda* cuticle.

	Total	PFAM	Protein Name	Function
Shared by all structures	1	PF00067	Cytochrome P450 4F5	Oxidative stress
Shared by *M. anisopliae* blastospores and conidi	1	PF00172	Zn(2)-C6 fungal-type DNA-binding domain	Cell function
Shared by *M. rileyi* blastospores and conidia	3	PF00155	Aminotransferase, class I/classII	Oxidative stress
		PF13499	EF-hand domain	Virulence factor
		PF13641	Glycosyltransferase like family 2	Oxidative stress
Shared by *M. anisopliae* and *M. rileyi* blastospor	1	PF00011	Heat shock protein	Heat shock
Shared by *M. anisopliae* and *M. rileyi* conidia	19	PF00171	Aldehyde dehydrogenase domain	Virulence factor
		PF01198	Ribosomal protein L31e	Cell function
		PF02629	CoA-binding	Virulence factor
		PF00903	Glyoxalase/fosfomycin resistance/dioxygenase doma	Virulence factor
		PF11327	Egh16-like virulence factor	Virulence factor
		PF01775	Ribosomal protein 50S-L18Ae/60S-L20/60S-L18A	Cell function
		PF01425	Amidase signature domain	Virulence factor
		PF02353	Mycolic acid cyclopropane synthetase	Virulence factor
		PF01042	Endoribonuclease L-PSP	Cell function
		PF00096	Zinc finger C2H2-type	Cell function
		PF01384	Phosphate transporter	Nutrient and substance transport
		PF05368	NmrA-like domain	Cell function
		PF13193	AMP-binding enzyme, C-terminal domain	Virulence factor
		PF00125	Histone H2A/H2B/H3	Virulence factor
		PF00153	Mitochondrial substrate/solute carrier	Cell function
		PF07732	Multicopper oxidase, N-termianl	Virulence factor
		PF03576	Peptidase S58, DmpA	Virulence factor
		PF02136	Nuclear transport factor 2	Nutrient and substance transport
		PF13561	Enoyl-(Acyl carrier protein) reductase	Cell function

4. HOST-SPECIFIC PATTERNS OF VIRULENCE AND GENE EXPRESSION PROFILES OF A BROAD HOST-RANGE ENTOMOPATHOGENIC FUNGUS

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ABSTRACT

Generalist pathogens with a broad host range encounter many different host environments. Such generalist pathogens are often highly versatile and adjust their expressed phenotype to the host being infected. The fungal genus *Metarhizium* occupy various ecological niches, including plant symbionts, soil saprophytes, and insect pathogens with applications in biological control of pests. The species *M. anisopliae* is highly diverse combining the capability of association with plant roots with infection of a broad range of arachnid and insect hosts, from agricultural pests to vectors of human disease. It is also one of the most studied and applied biological control agents worldwide. Here we investigate the phenotypic plasticity and differential gene expression in *M. anisopliae* blastospores during infection of different insect hosts. The virulence of *M. anisopliae* blastospores was evaluated against *Tenebrio molitor, Spodoptera frugiperda*, *Gryllus assimilis*, and *Apis mellifera*. Furthermore, the percentage of appressorium formation on their membranous wings was determined and the transcriptome profile during its penetration on the hosts was also analyzed. Our findings reveal that *M. anisopliae* blastospores exhibit high virulence against *Tenebrio molitor*, with significantly higher appressorium formation in its wings compared to other the other tested insects. We showed a clear difference in the gene expression pattern in the blastospores of M. anisopliae during the infective process in *Gryllus assimillis*, *Spodoptera frugiperda*, *Apis mellifera*, and *Tenebrio molitor*. This implies that *M. anisopliae* transcriptome and virulence change remarkably according to the insect, with the most significant differences for *G. assimillis*. These differences are associated with the expression of enzymes involved in the degradation of specific compounds present in each insect wing, as well as hydrophobins, destruxins, and secondary metabolites associated with virulence. The study emphasizes the differences in gene expression among the four insect orders and highlights the virulence-related genes specific to each infective process.

Keywords: Adaptive responses; blastospores; gene expression; fungal infection; phenotypical plasticity.

4.1. Introduction

Pathogenic organisms with a broad host range (generalists) infect different hosts that exert diverging selection pressures on the pathogen (Schmid-Hempel, 2011). Furthermore, individual genotype-specific host variation also influences the pathogen and the outcome of host-pathogen interactions (Salvaudon *et al.*, 2007). Generalist pathogens, therefore, often target host body compartments that are conserved across species or genotypes (Agosta *et al.*, 2010). Although targeting conserved host traits supports infection across divergent host species, broad host range pathogens also need to tailor the expression of virulence-associated traits to the specific host species and host genotype being infected (Honaas *et al.*, 2013). Many generalist pathogens are, therefore, versatile, showing considerable phenotypic flexibility (Agosta *et al.*, 2010).

Metarhizium (Ascomycota: Hypocreales) is a highly abundant genus of fungi with several identities (St. Leger and Wang, 2020). It comprises entomopathogenic species employed for biological control of crop pests and vector-borne diseases since it was first described (Sbaraini *et al.*, 2016). However, several *Metarhizium* species also have multifunctional lifestyles and other ecological niches, including plant symbionts and soil saprophytes (Brunner-Mendoza *et al.*, 2019). Being diverse, they provide many independently evolved models of adaptation and response to different environments and insect hosts (St. Leger and Wang, 2020).

Regarding the specialization of the ability to infect insects, *Metarhizium* strains show a continuum ranging from the inability to infect many hosts, over lower performance in most hosts, to the ability to kill a panel of insects (St. Leger and Wang, 2020). For example, multi-host generalist strains of *M. robertsii, M. brunneum,* and *M. anisopliae* have greater nutritional versatility than specialists, colonize plant roots, and are facultative entomopathogens. The ability of a generalist isolate to attack diverse insects does, however, not rule out local adaptation to exploit nutrients on cuticles of frequently met hosts. Generalist *Metarhizium* isolates often exhibit considerable variation in insect virulence (St. Leger and Wang, 2020).

Among the several *Metarhizium* species able to be used as biological control agents, the *Metarhizium anisopliae* (Metschn.) Sorokin deserves special attention. Besides the capability of association with plant roots, this species can infect a broad range of arachnid and insect hosts, from agricultural pests to vectors of human disease. The *M. anisopliae* is also one of the most studied and applied biological control agents worldwide, being none of the first organisms seriously investigated for use against agricultural pests (Wasuwan *et al.*, 2021).

Different structures of entomopathogenic fungi are infectious to insects and can thus be used for pest control, e.g., blastospores, mycelium, and resistance spores (Leite *et al.*, 2003). Regarding inundative strategies for biological pest control, conidia, and blastospores can be applied to overwhelm many factors that keep a pathogen in non-epizootic equilibrium with its host (Jaronski, 2010). Blastospores can be produced in liquid media and used directly to infect insect hosts (Mascarin, Jackson, Kobori, Behle, Dunlap*, et al.*, 2015; Iwanicki *et al.*, 2020). It is possible to produce large amounts of blastospores by liquid fermentation in a short time (<4 days), in a small space, and with less manual labor than the solid fermentation method (Iwanicki *et al.*, 2020). These structures differ from conidia in several ways. They are hydrophilic spores and usually are considered more virulent against susceptible hosts. They germinate faster than conidia (2-8 hrs versus 12–24 hrs), an attribute that could be regarded as a virulence determinant (Butt *et al.*, 2016). This rapid germination rate of blastospores produced in liquid culture is best suitable as the primary infective structure in commercial bioinsecticides (Mascarin, Jackson, Kobori, Behle and Delalibera, 2015)

For infection to be successful, the fungal propagule must adhere and penetrate the insect cuticle, a polymer network composed of chitin and other proteins (Vega *et al.*, 2012). The surface of aerial conidia of most hypocrealean entomopathogenic fungi is covered with a rodlet layer composed of hydrophobin proteins that confer a hydrophobic charge to them (Holder and Keyhani, 2005), facilitating the passive attachment to hydrophobic surfaces like insect cuticles, for example. In contrast, blastospores have no hydrophobins, which confers the ability to adhere better to hydrophilic surfaces - especially in aquatic environments (Holder *et al.*, 2007; Greenfield *et al.*, 2014).

Following propagule attachment to the cuticle, germ tube formation is a critical event that depends on environmental and nutritional optimal conditions. Temperature, humidity, nutritional, chemical, and physical factors can influence germination (Vega *et al.*, 2012). For some entomopathogenic fungi, carbon and nitrogen sources are also required to form a germ tube (Sosa-Gomez *et al.*, 1997; Priyatno and Ibrahim, 2002).

The differentiation of germ tubes into appressoria is also a prerequisite for infection. Appressoria may be produced directly from conidia or at the end of germ tubes, or even laterally from hyphae (Butt *et al.*, 2016). The

physicochemical and nutritional cues at the host surface influence both differentiation of infection structures and specificity (Lin *et al.*, 2011). Butt *et al.* (2016) state that cuticular physicochemical cues influence appressorium differentiation and phenotype. For example, conidia of *Metarhizium acridum* can germinate and differentiate into appressoria on *Schistocerca gregaria* cuticle but fail to germinate on a nonhost like a Lepidoptera species (Butt *et al.*, 2016). Evidence increasingly suggests that a significant factor driving the co-evolutionary arms race between the pathogen and the host occurs on the cuticular surface – also involving mediation of the insect cuticle microbiome (Butt *et al.*, 2016; Shang *et al.*, 2022; Hong *et al.*, 2023).

Fungal adaptive responses may be mediated by epigenetic mechanisms, allowing short-term specialization while maintaining the broad host range potential (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013). Evidence increasingly suggests that a significant factor driving the co-evolutionary arms race between the pathogen and the host occurs on the cuticular surface – also involving mediation of the insect cuticle microbiome (Shang *et al.*, 2022; Hong *et al.*, 2023). Although considerable progress has been made in recent years, much regarding the molecular determinants mediating these interactions in the pathogen and the host remains unknown (Vilcinskas, 2010; Ortiz-Urquiza and Keyhani, 2013). In this context, comparative genomic studies can understand differences in metabolism, host range, and root colonization, revealing which proteins and gene families are responsible for this rapid evolution of ecological interactions (Hu *et al.*, 2014).

Considering the ability of adaptive responses of fungal entomopathogens, we hypothesize that *M. anisopliae*, a generalist fungus, can also modulate its phenotype and gene responses to adapt and cause disease to different orders of insects. Thus, this paper investigates the phenotypic plasticity and the differential gene expression of *M. anisopliae* blastospores during the infection of different insect orders.

4.2. Material and Methods

4.2.1. Fungal isolate and blastospores production

The *Metarhizium anisopliae* commercial isolate ESALQ E9 was chosen for the tests. It is held in the entomopathogen collection "Prof. Sergio Batista Alves" of the Laboratory of Insect Pathology and Microbial Control of ESALQ/USP, Piracicaba, São Paulo, Brazil. The fungus was cultured in Petri dishes containing Potato Dextrose Agar medium and incubated in B.O.D. (Biological Oxygen Demand) for 15 days at 27 ± 1 °C and relative humidity (RH) higher than 90%.

For the blastospore production, suspensions of *M. anisopliae* conidia were obtained by washing the spore-containing fungus plate with 10mL of 0.01% aqueous solution of Tween® 80. This suspension was used for inoculation in a preculture liquid medium at 5 x10^6 conidia/mL. The medium composition was a basal salt solution with trace metals and vitamins (Jackson, 1997; Jackson and Jaronski, 2009) supplemented with 80 g glucose L^{-1} and 45g L yeast extract. Glucose (20% w/v) was autoclaved separately from the salt solution and added before the inoculation of the fungus. The basal salt solution contained, per liter of distilled water: KH_2PO_4, 2.0g; $CaCl_2$, 0.4g; $MgSO_4$, 0.3g; $FeSO_4$, 0.05g; $CoCl_2$, 37mg; $MnSO_4$, 16mg; $ZnSO_4$, 14mg; thiamine, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500µg each; and folic acid, biotin, vitamin B12, 50µg each.

The liquid culture medium was filled into baffled flasks" type (250mL), adding 45mL of the medium and 5mL of the fungal inoculum to each one. The flasks were kept for three days in an orbital shaker (350 rpm, 28°C) and

were shaken daily to minimize mycelial growth on the wall of the vials. Four shake flasks per experimental treatment were used for each experiment described in the sections below.

Blastospores were separated from the medium after the fungus's growth in its respective liquid culture media to avoid the influence of possible metabolites dispersed in the medium. They were collected by filtration through 2 layers of lens cleaning cloth (Whatman nº 105) and washed twice with a potassium buffer saline solution with the following composition per liter: NaCl 8.0g, KCl 0.2g, Na_2HPO_4 1.44g, KH_2PO_4 0.24g, and pH adjusted to 6.0. During this process, the medium was centrifuged at 5,000 rpm for 10 min at each wash.

Before each experiment, all the suspensions were vortexed to ensure the samples' homogeneity, and concentrations were determined microscopically using a Neubauer chamber.

4.2.2. Insects used in bioassays

Four agricultural and economically important insect orders were chosen for the study: Coleoptera; Lepidoptera; Orthoptera, and Hymenoptera. The insect species chosen were *Tenebrio molitor, Spodoptera frugiperda*, *Gryllus assimilis*, and *Apis mellifera,* respectively. Insects were purchased from selected breeders and kept in laboratory conditions until their use.

The tissue used for the fungal germination and transcriptome studies was the membranous wings of each insect species. The wings were chosen instead of the insect integument first because of the possibility of a lower volume of insect RNA on the samples after the extraction, and second, aiming for greater standardization of the penetration surface since insects have different levels of integument rigidity.

For the RNA extraction, the wings were removed by freezing the adults and then removing them using scissors and a scalpel. Before use in each study, wings were superficially washed with alcohol 70%. Adult *Tenebrio molitor*, *Gryllus assimilis*, and *Apis mellifera* were used on the bioassays and virulence tests. As an exception, caterpillars were used for *Spodoptera frugiperda*, considering that this is the target stage for control.

4.2.3. Virulence tests

Virulence tests were performed on the various hosts to check if there was a relationship between the phenotypic plasticity and gene expression of *M. anisopliae* blastospores during the infection of various insect hosts.

Experiments were conducted individually for each insect species and repeated twice. The *Metarhizium anisopliae* (ESALQ E9) propagules were produced in batches using the abovementioned methodology. For each experiment, suspensions of $1x10^7$ blastospores and $1x10^7$ conidia were prepared using 0.05 % of Tween® 80 for delivery on the tegument of the insects using a micropipette. A suspension of 0.05% of Tween® 80 was always used as a control. The mortality of each experiment was evaluated daily for ten days. Dead insects were kept in humid chambers on B.O.D at 28°C to confirm if the mortality was caused by the fungus (visualizing fungal sporulation). The peculiarity of each experiment is described below.

Survival analysis for all experiments was performed equally with censored data for dead larvae until day 10 using a parametric model for survival data with Weibull distribution (survival r-package) (Jacobson *et al.*, 2022). The survival curves were compared by a log-likelihood ratio test at $p<0.05$. The insect confirmed mortalities (mycosis) by each fungus were compared between the treatments using generalized linear models (GLM) and quasipoisson

distribution. Data were analyzed by ANOVA with F-test, and pairwise comparisons were carried out using the function glht in the multcomp package (Hothorn *et al.*, 2023) to compare treatments. Data overdispersion was checked by the function hnp implemented at hnp package (Moral *et al.*, 2022).

Tenebrio molitor

3µL of blastospores, conidia or control suspensions were applied in *Tenebrio molitor* larvae (between 1 - 1.5cm length). Each treatment consisted of 3 replicates of 24 insects separated individually into plastic trays containing 1cm³ of potato. Insects were fed daily.

Spodoptera frugiperda

3µL of blastospores, conidia or control suspensions were applied in *Spodoptera frugiperda* caterpillars (3rd instar). Each treatment consisted of 3 replicates of 20 insects separated individually into plastic trays containing 1cm³ of lettuce. Insects were fed daily.

Gryllus assimilis

5µL of blastospores, conidia or control suspensions were applied in *Gryllus assimilis* juveniles (between 15 – 20mm in size). Each treatment consisted of 3 replicates of 20 insects separated individually into plastic cups containing 2g of chicken feed and 1cm³ of cucumber. Insects were fed daily.

Apis mellifera

3µL of blastospores, conidia, or control suspensions were applied in *Apis mellifera* adults. Each treatment consisted of 3 replicates of 20 insects separated into plastic cups. Insects were fed daily with 1mL of water-diluted honey (1:1).

4.2.4. Fungal germination and appressoria formation

To determine the best concentration and time-point for RNA extraction of wings, they were inoculated with *M. anisopliae* blastospores. Four fungal suspension concentrations ($5x10^6$; $1x10^7$; $5x10^7$, and $1x10^8$ blastospores/mL) and two post-infection times (4 and 6 hours) were selected. The technique was inspired by (Wang and St Leger, 2005), who studied appressoria formation on insect cuticles.

After dissecting and surface sterilizing the wings, they were inoculated with a different volume of the same blastospore suspension according to the size of each species: *Tenebrio molitor*: 20 µL/wing; *Spodoptera frugiperda*: 20µL/wing; *Gryllus assimilis*: 50 µL/wing; and *Apis mellifera*: 5 µL/wing. The wings were placed on microscope slides inside a Petri dish with humid paper. Treatments had five replicates of 4 wings each.

The plates were kept in B.O.D (Biological Oxygen Demand) incubator (27 ± 1 °C) and then observed in a light microscope at 400x magnification. The criteria used to choose the best fungal suspension concentration and the best time-point for RNA extraction were: 1) The amount of blastospores: must cover the tissue homogeneously without overlapping propagules – to avoid non-germination; 2) Germ-tube: must be at least the double size of the propagule and appear homogeneously germinated; 3) Appressoria formation: must be present in at least one treatment to guarantee that penetration was occurring.

To better investigate the phenotypical changes of *M. anisopliae* blastospores during penetration in different insect wings, the percentage of appressoria formation was also analyzed by counting 100 germinated blastospores with and without appressoria in each replicate of 4 wings, totaling 2000 germinated blastospores in each treatment.

The mean appressoria yield formed in each insect species was analyzed by generalized linear models (GLM) with quasipoisson distribution and compared by ANOVA with the chi-square test. Pairwise comparisons between treatments were carried out using the function glht in the multcomp package (Hothorn *et al.*, 2023). Data overdispersion was checked by the function hnp implemented at hnp package (Moral *et al.*, 2022).

4.2.5. RNA extraction and sequencing

Before inoculation with blastospores suspension, the membranous wings were prepared as described before and placed in Petri dishes. Each wing received a 5 x 107 mL blastospores suspension by micro pipetting. After that, they were kept in B.O.D (Biological Oxygen Demand) incubator for 6 hours (defined by the preview step result) at 27 ± 1 °C and relative humidity (RH) higher than 90%.

Treatments comprised the four species described above, with five replicates each, totaling 20 samples. Due to the different wing sizes of insect species, it was necessary to adjust the wings amount to fit in the microtube sample. Thus, each treatment had a different number of wings per replicate which received a volume of the same blastospore suspension as follows: *Spodoptera frugiperda*: 20 wings; 20μL/wing; *Tenebrio molitor*: 20 wings; 20 μL/wing; *Gryllus assimilis*: 8 wings; 50 μL/wing; and *Apis mellifera*: 35 wings; 5 μL/wing.

The RNA was extracted using TRIzol® (Invitrogen Life Technologies). The protocol was carried out according to the manufacturer's recommendations (Invitrogen). After extraction, the total RNA was treated with RQ1 RNAse-free DNase Promega according to the usage information (Promega Corporation).

Total RNA was quantified in the fluorometer (Qubit, Invitrogen), and the concentration and quality of the samples were evaluated by analysis in spectrophotometer NanoDrop ND-1000 (Wilmington, USA). The RNA integrity was assessed on agarose-formaldehyde gel 1%. Samples were sequenced with Illumina HiSeq 2500 technology, which yielded at least 20 million 150-bp paired-end reads per library. Library preparation and sequencing were performed by the NGS Soluções Genômicas in Piracicaba-SP, Brazil. The data presented in this study are openly available in the National Library of Medicine (NCBI), BioProject accession number PRJNA979911.

4.2.6. RNAseq data handling and differential gene expression analysis

The quality of the raw reads before and after quality and adaptor trimming was assessed using the FastQC program. Illumina adapters and low-quality sequences were removed using TrimGalore, AfterQC, and Trimmomatic V0.32 with the following options: LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 HEADCROP:7 MINLEN:36. To compile the FastQC reports and identify patterns between the samples of each treatment, the MultiQC program was used before and after trimming.

To align reads to the genome, the reference genome of *Metarhizium anisopliae* (GenBank assembly accession GCA_013305495 - JEF-290 strain) was downloaded from the National Center for Biotechnology Information (NCBI)

search database. The annotation files were downloaded as well. Filtered reads were mapped to reference genomes using the HISAT2 software following standard settings (available at https://github.com/DaehwanKimLab/hisat2).

Before running the Differential Gene Expression analysis, the gene count matrixes were obtained with the python script: prepDE.py, provided by John Hopkins University, Center for computational biology, CCB (available at http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual" \l "deseq"). The gene count matrixes were used as input files for the differential expression analysis conducted using the DESeq2 package from the statistical software R.

Before running the DESeq2 package, pre-filtering and sample-to-sample distances were assessed for all treatments using PCA plots. Rows with reading counts <5 were excluded, and genes with adjusted *p-values* <0.1, and -2.0 < Lo2FC > 2.0, were considered differentially expressed. Diagnostic plots (MA-plot and Volcano-plot) were prepared and analyzed for each treatment. Heat maps of differentially expressed genes were made using the heatmap.2 packages from the R software.

The InterPro database was used to annotate protein sequences and identify protein domains of the differential expressed genes (Blum *et al.*, 2021). *Metarhizium anisopliae* JEF-290 protein sequences were downloaded from NCBI and used as input on the InterProScan (Jones *et al.*, 2014). Differential expressed genes from each pairwise combination was used to identify the unique differentially expressed genes (that is uniquely differentially expressed on a single host) and analyzed using an enrichment analysis. Enrichment analyses of PFAM domains, were performed based on the hypergeometric distribution using the PHYPER function in R and significantly enriched PFAM domains assigned based on $p<0.001$.

4.2.7. Gene expression per host

A conservative approach to designate differential expression were used. Only genes with false discovery rate (FDR) adjusted p-values < 0.0005 and log2 fold change (FC) > 3, for up-regulated genes and log2FC < -3, for down-regulated genes were considered differentially expressed. Venn diagrams were made using the web application "InteractiVenn" (Heberle *et al.*, 2015) while heatmaps were made using the ggplot2 package in in the statistical software R (R Core Development, 2015).

4.3. Results

4.3.1. Virulence tests

The virulence tests of *Metahrizium anisopliae* (ESALQ E9 isolate) propagules towards *Gryllus assimillis*, *Spodoptera frugiperda*, *Tenebrio molitor,* and *Apis mellifera* showed different mortality levels among the insect species. However, the same pattern could be observed in all of them: blastospores were always significantly more virulent and killed faster than conidia ($p<0.05$). Both blastospores and conidia caused high mortality in the *T. molitor* and *A. mellifera* populations but neither blastospores nor conidia were virulent *G. assimillis* and *S. frugiperda* (Figure 13).

M. anisopliae blastospores killed almost 50% of the total *T. molitor* population four days post-infection, while conidia took nine days to kill the same amount. Blastospores were significantly more virulent compared to conidia and control (Figure 13A, $X^2= 56.8$, $p<0.0001$) and resulted in 80% mycosed insects of blastospores with significant difference between the blastospores, conidia, and control (Figure 13B, $F=43.2$ $p<0.0001$)

M. anisopliae blastospores and conidia killed almost 50% of the *Apis mellifera* population between 3- and 4-days post-infection. The natural mortality was also high in the control, however, the blastospores treatment remained significantly different compared to conidia and the control (Figure 13C, X^2= 22.3, p<0.0001), even though it did not result in more mycosed insects (Figure 13D, F= 27, p<0.0001).

Despite the low virulence to *Gryllus assimillis*, considering the mortality time, rate, and mycosis, *M. anisopliae* blastospores were significantly more virulent than conidia as they killed more insects, quickly (Figure 13E, X^2= 18.1, p<0.0001), and resulted in more confirmed mortality compared to the conidia and control (Figure 13F, F=2.6, p=0152).

The same pattern was observed for *Spodoptera frugiperda* caterpillars, where both *M. anisopliae* propagules were low virulent. However, even killing only 25% of the insect population, blastospores were significantly different compared to conidia and the control (Figure 13G, X^2= 3.1, p=0.033) and resulted in more mycosed insects (Figure 13H, F=3.22, p=0.111).

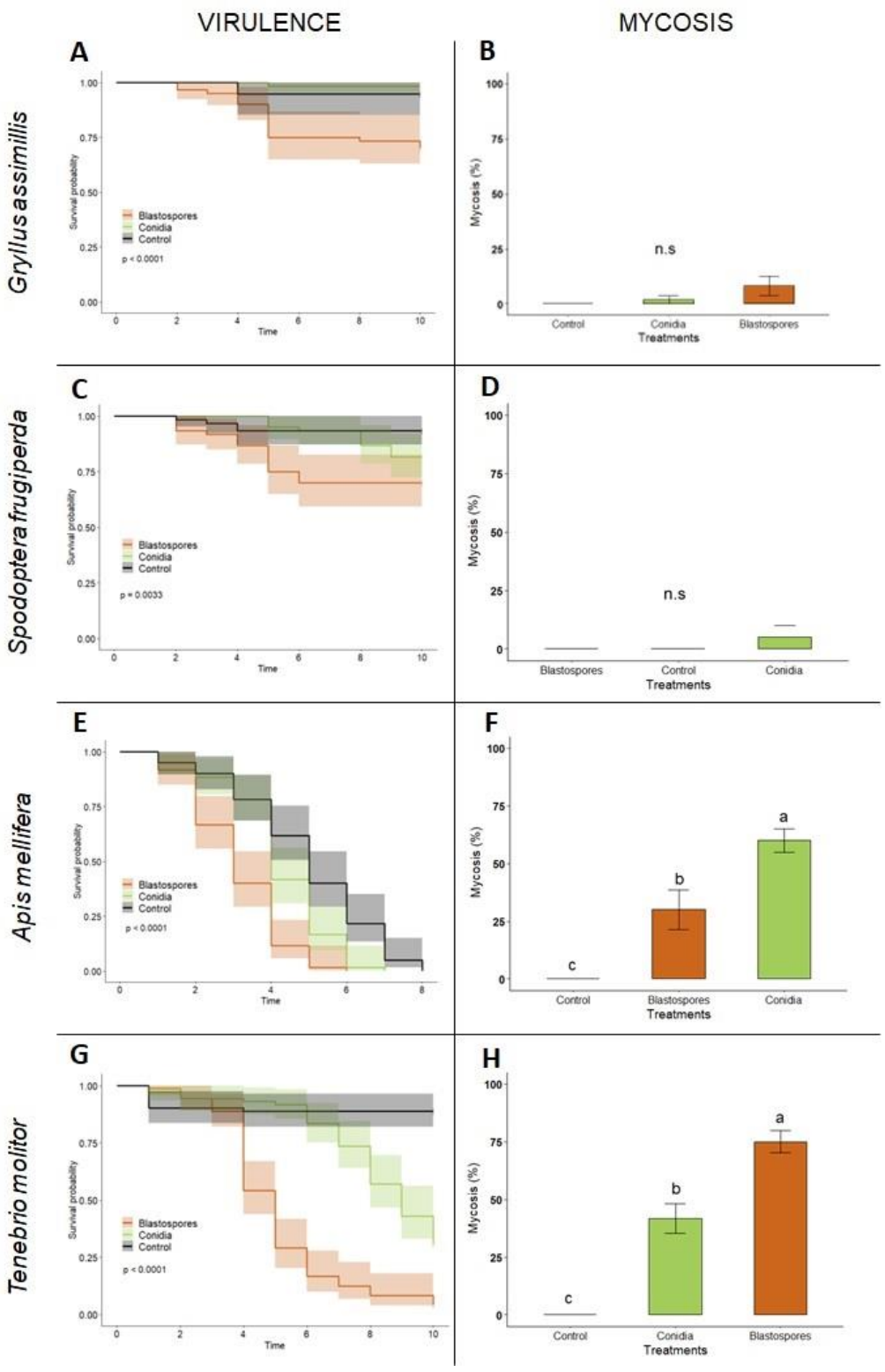


Figure 13. Survival probability and the percentage of mycosed cadavers of different insects treated with *Metarhizium* spp. blastospores and conidia. (A) Survival of *Gryllus assimilis* juveniles exposed to conidia and blastospores of *M. anisopliae*; (B) Percentage of *G. assimilis* cadavers colonized by *M. anisopliae* propagules. (C) Survival of *Spodoptera frugiperda* caterpillars exposed to conidia and blastospores of *M. anisopliae*; (D) Percentage of *S. frugiperda* cadavers colonized by *M. anisopliae* propagules. (E) Survival of *Apis mellifera* adults exposed to conidia and blastospores of *M. anisopliae*; (F) Percentage of *A. mellifera* cadavers colonized by *M. anisopliae* propagules. (G) Survival of *Tenebrio molitor* larvae exposed to conidia and blastospores of *M. anisopliae*; (F) Percentage of *T. molitor* cadavers colonized by *M. anisopliae* propagules. The letters above the bars indicate the statistical difference between the treatments: equal letters represent treatments that did not differ from each other, while different letters indicate a statistical difference at a 0.05% probability level.

4.3.2. Fungal germination and appressoria formation

The light microscopy observation of the *Metarhizium anisopliae* germination upon insect wings allowed us to decide the best volume of fungal suspension for infection and the best time-point for RNA extraction. The best concentration for wings infection was $5x.10^7$ propagules mL since the blastospores didn´t germinate well in the concentration of $1x10^8$ propagules mL. It was observed that the higher the concentration, the worse the blastospore distribution, impairing its germination.

The best time-point was 6 hours post-infection (Figure 14B). At this time, the germination rate was visually higher and more homogeneous compared to 4 hours post-infection (Figure 14A). Another observation was that *M. anisopliae* germinate differently in different insect hosts. The germination time and appressoria formation have different patterns in different insect hosts, e.g., blastospores upon *Tenebrio molito*r wings germinated faster and formed more appressoria compared to *Spodoptera frugiperda* and *Gryllus assimilis* wings (Figure 14A). This fact is likely coupled to the differential gene expression on the different hosts and may be explained by transcriptomic studies during the penetration in each one. Despite the differences among germination in different insect species, the time-point for RNA extraction was standardized for 6 hours precisely to understand these gene expression differences.

The appressoria formation during the blastospore germination on wings significantly differed among species (p<0.001; Figure 15). The greater amount was observed in *Tenebrio molitor*, when 27.2% of the germinated blastospores formed appressorium. The second one was *Spodoptera frugiperda* with 8.2% and *Grillus assimillis* with 6.8% in third place. Germinated blastospores presented only hyphae, and none formed appressorium on *Apis mellifera*.

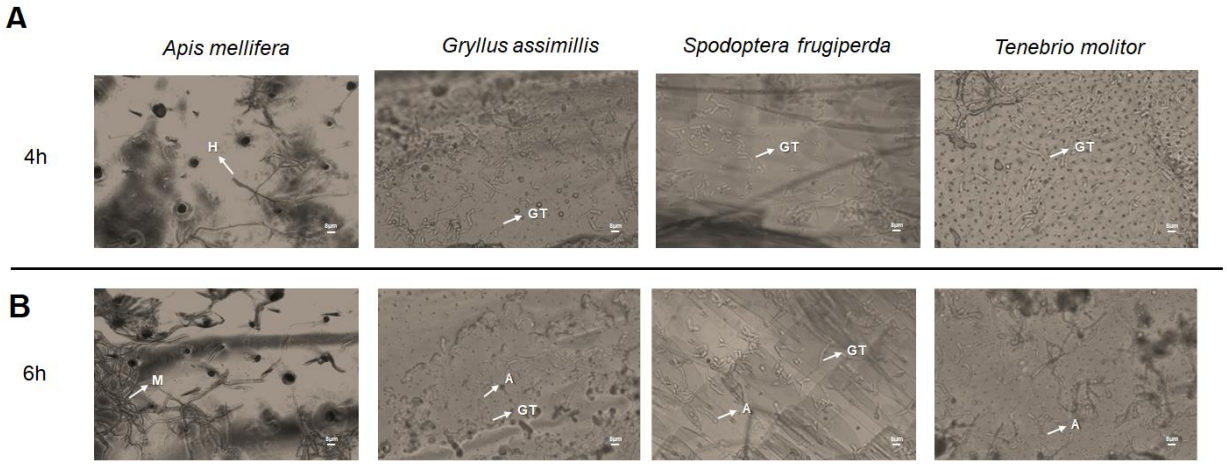


Figure 14. Photomicrographs of different concentrations of *Metarhizium anisopliae* blastospores after 4- (A) and 6-hours (B) post-infection at *Apis mellifera*, *Gryllus assimilis*, *Spodoptera frugiperda* and *Tenebrio molitor* membranous wings. Light microscope at 400x magnification. H: Hyphae; GT: Germ Tube; A: Apressorium

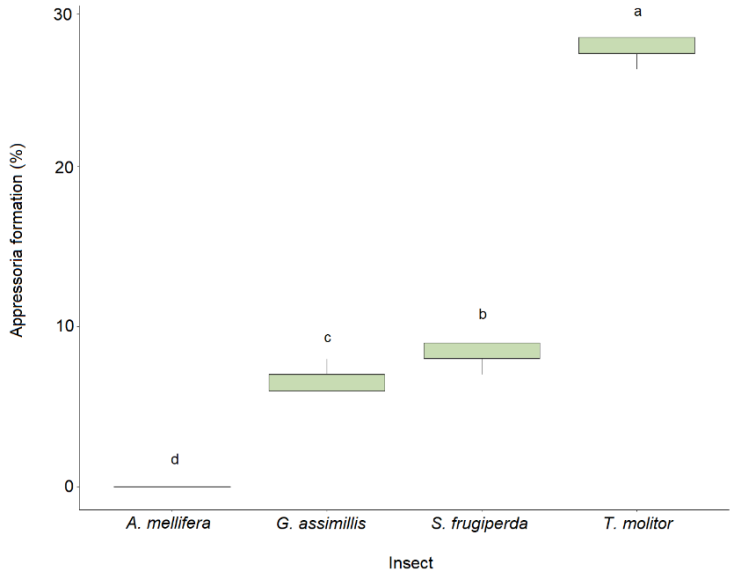


Figure 15. *Metarhizium anisopliae* appressoria formation (%) during its penetration on *Apis mellifera*, *Gryllus assimilis*, *Spodoptera frugiperda* and *Tenebrio molitor* membranous wings. Means (±SD) followed by non-corresponding letters are significantly different (Tukey's test, $p<0.05$).

4.3.3. RNAseq analysis

A Principal Component Analysis (PCA) of the *M. anisopliae* blastospores during penetration on different insect wings explained 54% of sample variation and showed good separation between treatments (Figure 16). The diagnostic plots were made for its Differential Gene Expression where it is possible to see the down and up-regulated genes in pairwise combinations among all the insect species (Figure 17). Analyzing those MA plots, we see that expressed genes were more different when species were compared to *Gryllus assimilis* with a high amount of down and up-regulated genes (Figure 17A, B, C) and more similar when species were compared to *Spodoptera frugiperda* and *Tenebrio molitor*, with less down and up-regulated genes (Figure 17D, E, F).

A total of 10103 genes were expressed in *M. anisopliae* blastospores during penetration on different insect wings. From those, 1988 genes mainly were differential expressed (p-adj<0.001; $-2.0 < Log_2FC > 2.0$). The heatmap on Figure 19A shows that *M. anisopliae* blastospores express a different pattern of genes during it penetration on *Gryllus assimilis* wings compared to other species.

Differential expressed genes were filtered manually from each pairwise combination, and the unique differentially expressed ones were submitted to the enrichment analysis. A total of 1261 Protein Families (PFAMs) were selected as uniquely expressed during *M. anisopliae* blastospore penetration on *Gryllus assimilis*, *Spodoptera frugiperda*, *Apis mellifera* and *Tenebrio molitor*. Based on literature review, the PFAMs were classified, according to their primary function, into four groups: cell function; Cell wall and membrane components; Nutrient and substance transport, and Virulence Factor (Figure 19B). From those, only 19 protein families were found to be enriched (*phypher*>0.001) among the differentially expressed genes on the four insect species. (Figure 19C).

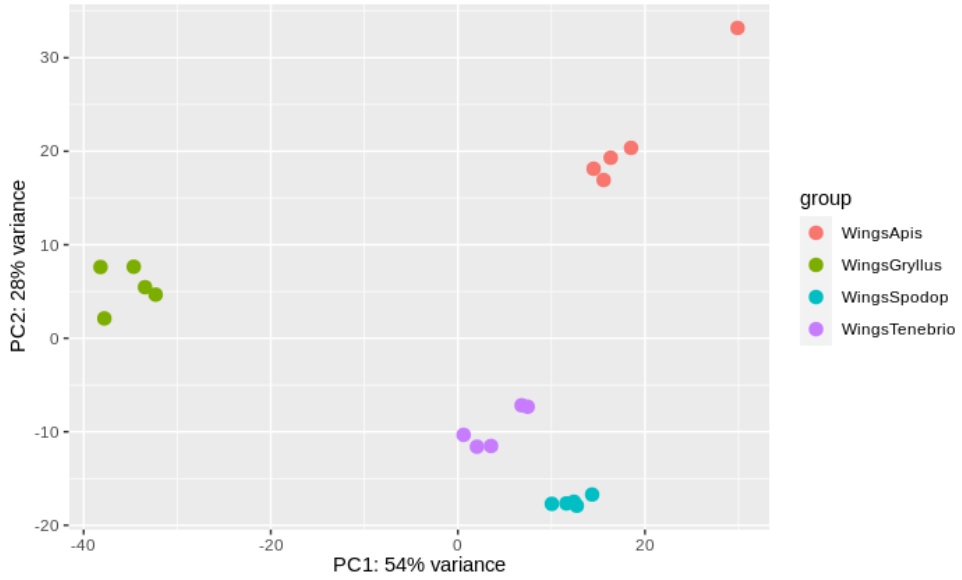


Figure 16. Principal component analysis of regularized-logarithmic (rlog) transformed gene counts of *M. anisopliae* blastospores samples during penetration on *Apis mellifera*, *Gryllus assimilis*, *Spodoptera frugiperda* and *Tenebrio mollitor* wings.

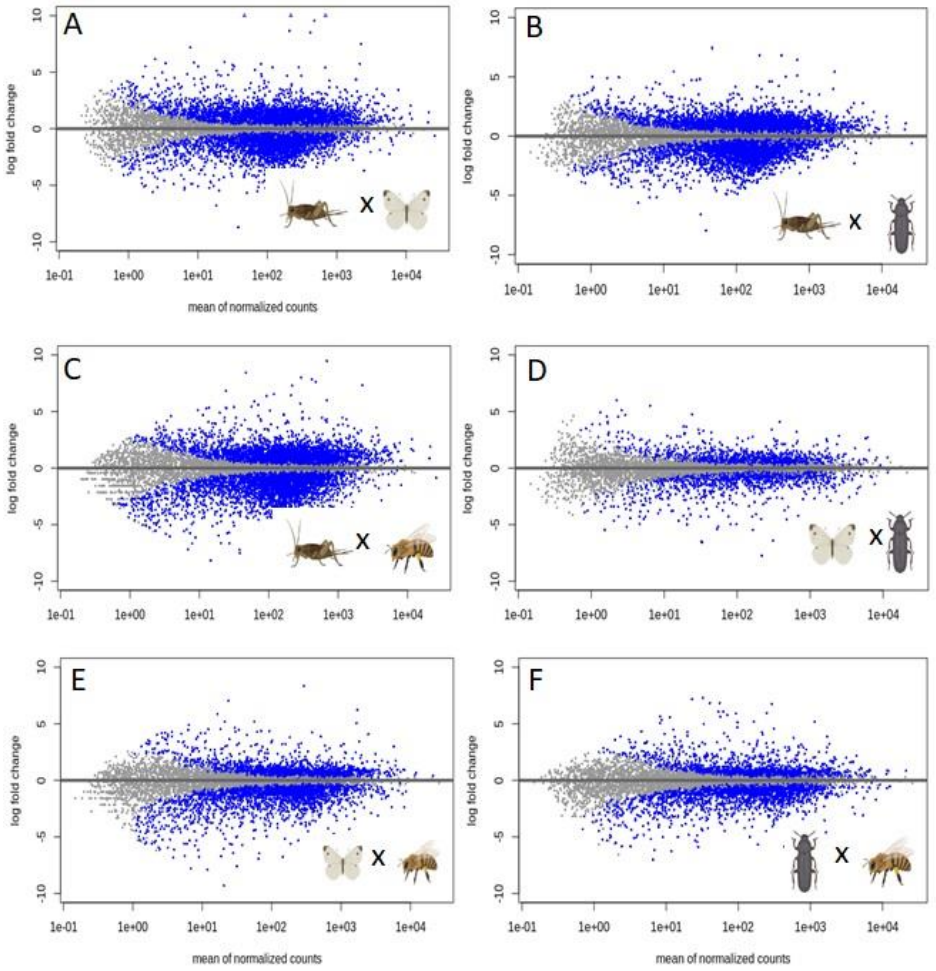


Figure 17. MA plots of differentially expressed genes in *M. anisopliae* blastospores during germination on different insect wings. Each group comprise five biological replicates. The X-axis and Y-axis in the MA plot denote the mean normalized counts and log2 fold changes between the two groups, respectively. All differentially expressed genes with adjusted p-value below 0.1 are shown in blue. (A) *Gryllus assimillis* x *Spodoptera frugiperda*; (B) *Gryllus assimillis* x *Tenebrio molitor*; (C) *Gryllus assimillis* x *Apis melífera*; (D) *Spodoptera frugiperda* x *Tenebrio molitor*; (E) *Spodoptera frugiperda* x *Apis melífera*; (F) *Tenebrio molitor* x *Apis melífera*

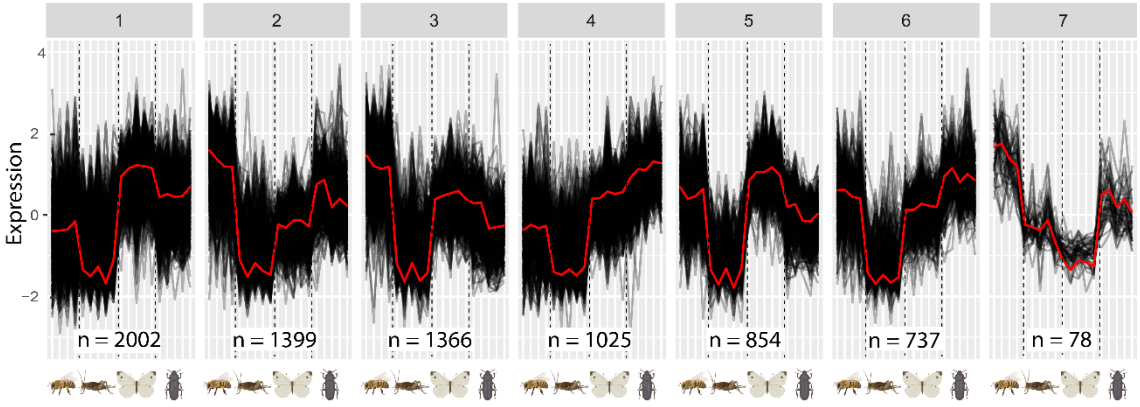
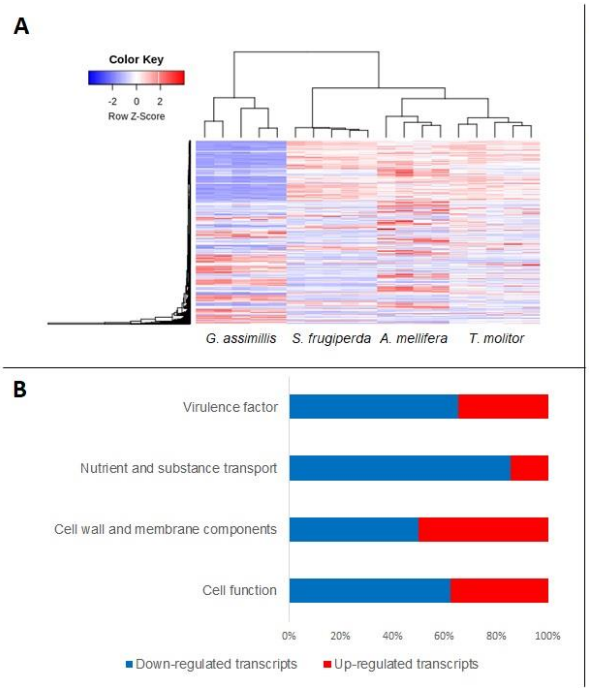


Figure 18. Gene co-expression network modules. In total 7461 genes were clustered into seven modules showing host-specific gene expression patterns. At the top the number refers to each of the seven idnetified modules numbered 1 -7. For each module individual gene expression is shown as black lines, and the median expression shown in red. The number of genes in each modules is shown just above the x-axis. Stipled lines separate the 19 RNAseq samples used in this analysis into host insect species

shown in the x-axeses: *Apis mellifera, Gryllus assimilis, Spodoptera frugiperda,* and *Tenebrio molitor* (A single RNAseq sample from *A. Mellifera* was excluded from this analysis because extremely high variance).



C

PFAM	Description	Up-regulated transcripts	Total transcripts	*phyper*
Cell function				
PF00668	Condensation domain	60	85	8,99E-28
PF00168	C2 domain	14	17	7,06E-09
PF00271	Helicase conserved C-terminal domain	40	96	1,09E-08
PF07728	AAA domain	8	8	7,21E-07
PF00806	Pumilio-family RNA binding repeat	16	27	9,72E-07
PF02854	MIF4G domain, mRNA translation	5	6	0,000749337
PF00575	S1 RNA binding domain	6	9	0,001300907
Cell wall and membrane components				
PF02463	RecF/RecN/SMC N terminal domain	6	6	2,48E-05
Nutrient and substance transport				
PF01061	ABC-2 type transporter	12	22	7,16E-05
PF01369	Sec7 domain	4	4	0,000853064
PF06422	CDR ABC transporter	6	9	0,001300907
Virulence factor				
PF00550	Phosphopantetheine binding ACP domain	53	101	3,55E-16
PF00501	AMP-dependent synthetase/ligase	54	109	4,71E-15
PF00176	SNF2-related domain	18	23	2,02E-10
PF08501	Shikimate dehydrogenase substrate binding dom	4	4	0,000853064
PF00612	IQ calmodulin-binding motif	5	6	0,000749337
PF00225	Kinesin motor domain	7	11	0,000734424
PF00107	Zinc-binding dehydrogenase	16	42	0,000948547
PF09286	Pro-kumamolisin	6	9	0,001300907

Figure 19. (A) Heatmap of the 1988 genes differentially expressed in *Metarhizium anisopliae* blastospores during penetration on different insect wings. Adjusted p-value below 0.001 and Log_2FoldChange between -2 and 2. (B) Percentage of *M. anisopliae* Protein Families (PFAMs), uniquely expressed, during penetrations on *Gryllus assimillis*, *Spodoptera frugiperda*, *Apis mellifera* and *Tenebrio molitor*, according to its functions. (C) Detailed table of *M. anisopliae* enriched Protein Families (PFAMs), during penetrations on *Gryllus assimillis*, *Spodoptera frugiperda*, *Apis mellifera* and *Tenebrio molitor*, according to its functions (phyper>0.001).

4.3.4. Gene expression per host

To clarify the main differences in blastospore gene expression during infection on insects' wings, we determined the *M. anisopliae* E9 genes that are uniquely differentially expressed (Log2FC > 3.0 or <-3, and *P-adj* < 0.0005) between hosts. Additionally, we focused on *T. molitor* since this insect showed high mortality in virulence assays.

Tenebrio molitor (Tm)

In *M. anisopliae* blastospores 52 genes were up regulated in Tm compared with *A. mellifera* (Am), 33 genes compared with *S. frugiperda* (Sf), and 147 compared to *G. assimillis* (Ga) (Figure 20A). Of these, only two genes shared up-regulation in Tm compared with Am, Sf, and Ga (Figure 20A, C). These genes are a Tyrosinase-like protein associated in fungi with melanin biosynthesis and virulence and a Dibenzothiophene desulfurization enzyme associated with desulfurization processes for sulfur assimilation or to oxidize the sulfur residues within organosulfur compounds as part of a xenobiotic response. Furthermore, we determined the percentage of genes associated with virulence and secondary metabolites in blastospores uniquely up regulated between Tm and each insect. We found that between Tm and Ga, 1.5% of the genes associated with virulence include the adhesin MAD1 and Tripeptidyl-peptidase sed2. Between Tm and Sf we found 15% of genes associated with virulence, such as a Hydrophobin-like protein MPG1 and destruxins, while between Tm and Am we found 9% of genes associated with virulence which include lipases and secreted aspartic proteases.

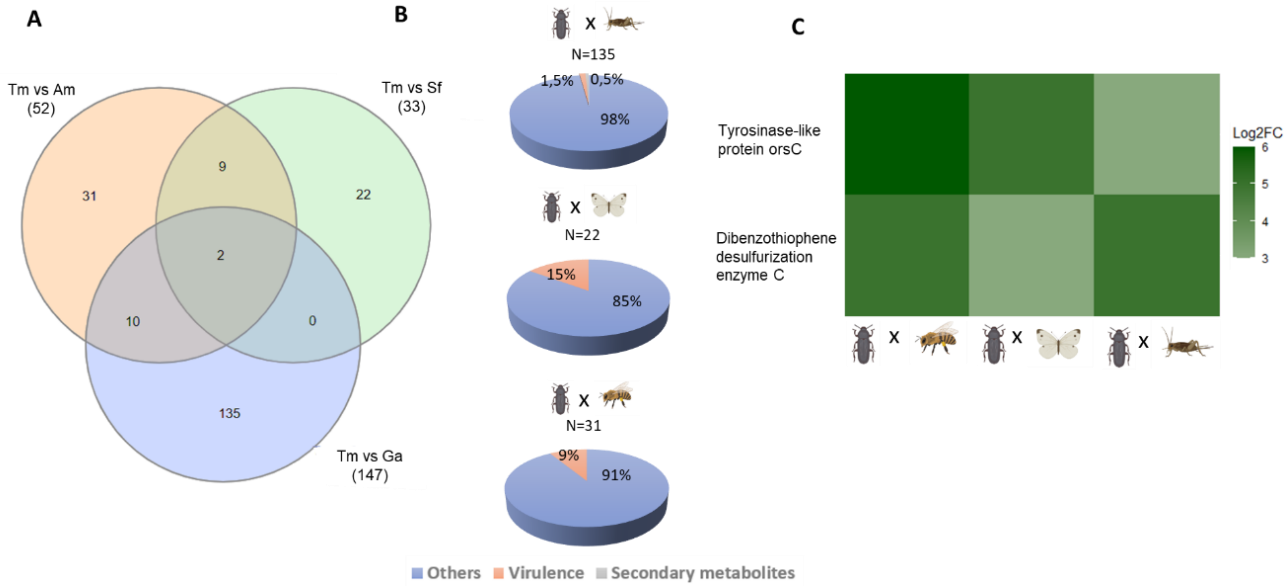


Figure 20. (A) Venn diagrams showing differentially-expressed genes of *Metarhizium anisopliae* (ESALQ E9), up-regulated in blastospores during infection on wings between *Tenebrio molitor* and *Apis mellifera* (Tm vs Am), *T. molitor* and *Spodoptera frugiperda* (Tm vs Sf) and *T.molitor* and *Gryllus assimillis* (Tm vs Ga). $Log_2FC > 3$, *FDR-p-value* < 0.0005. (B) Percentage of differentially expressed up-regulated genes associated with virulence, secondary metabolites, and others in blastospores during infection on wings between *T. molitor* and other insects. (C) Heatmap of the only two up-regulated genes differentially expressed in common in blastospores during infection on wings between *Tenebrio molitor* and other insects.

Spodoptera frugiperda

During infection on *S. frugiperda* wings, we found six genes in blastospores that shared up-regulation in Sf compared with Am, Tm, and Ga. Interestingly, 50% of these genes are associated with enzymes that degrade specific substrate such as two cuticle degrading proteases, which might be associated with specific cuticle compounds of Sf wings and a streptogrisin protease associated with the degradation of chitin-linked proteins. The latter was highly expressed in Sf compared to Ga ($Log_2FC = 5.6$) and Tm ($Log_2FC = 5.9$). Compared only with Am *M. anisopliae* blastospore up-regulates in Sf wings three lipases, fatty acid hydrolase and fatty-acid binding protein, and a secreted aspartic protease while compared with Tm we one glycosyltransferase and six hypothetical protein up-regulated. On the other hand, we determined 172 genes up regulated in Sf compared with Ga, including trypsin, tyrosinase, peptidase sed 2, and several hypothetical proteins and genes associated with DNA activity (Appendix D).

Apis mellifera

In *A. mellifera* we found 53 shared up-regulated genes in blastospores compared with Sf, Tm and Ga. Most of these genes are associated with secondary metabolites, including two nonribosomal peptide synthases, two polyketide synthases, one aurovertin biosynthesis transcription factor, several substrate transporters, and hypothetical proteins. While only compared with Tm, we found six up-regulated genes, including adhesion, hyphal regulator, and hypothetical proteins. Conversely, we found 54 genes up-regulated only compared to Sf such as hydrophobin, aquaporin, KP4 killer toxin, and several substrate transporter and hypothetical proteins. As observed for the other hosts, compared with Ga we found a large number of up-regulated genes (n=251) in Am which includes two aurovertin biosynthesis cluster transcription factor, autophagy-related proteins, cell wall proteins, cuticle degrading protease, indole diterpene prenyltransferase, cytochrome P450 monooxygenases, serine protease, three nonribosomal peptide synthetase, stress response protein NST1, peptidases (Appendix E, F).

Gryllus assimillis

In *G. assimillis* we found 22 shared up-regulated genes compared with Sf, Tm, and Am. These genes include lipase, endo-chitosanase, amidase, substrate transporters and several hypothetical proteins. Compared only with Tm we found ten genes up-regulated, such as monooxygenases and glucosidases, and compared only with Sf we found 55 genes up-regulated, including hydrophobin MPG1, thioredoxin, dextrusin, and several hypothetical proteins. Conversely, we found compared only with Am 56 up-regulated genes, including glutathione transferases, lipase, secreted aspartic protease, superoxide dismutase, and several transporters and hypothetical proteins (Appendix G, H).

4.4. Discussion

In this study, we aim to determine the plasticity of the fungus *M. anisopliae* in infecting insects of different Orders and to which extent phenotypic observations are related to different gene expression patterns on insects' wings. We first evaluated the virulence of *M. anisopliae* blastospores against insects of four Orders and determined the percentage of appressorium formation in the membranous wings of each insect. Then, we analyzed the transcriptome of blastospores during the first hours of infection using membranous insect wings as a model. To identify the shared protein families expressed in *M. anisopliae* during the blastospores infection process, we made an enrichment analysis of PFAM associated with up-regulated genes in all pairwise comparisons. Finally, we showed the main differences in gene expression between the four Orders of insects and emphasized those virulence-related genes characteristic of the infective process on each insect. We determined that *M. anisopliae* blastospores are highly virulent for *T. molitor,* and the percentage of appressorium formation in the membranous wings of this insect was three times higher than in the wings of the other insects. The principal component analysis revealed distinct expression profiles for each of the insects. This implies that *M. anisopliae* transcriptome changes remarkably according to the insect, with the most significant differences found for *G. assimillis*. We found that *M. anisopliae* recruits protein families associated with ABC nutrient transporters, RNA and DNA activity, and dehydrogenases during the infection process, regardless of insect order. On the other hand, the pairwise comparison showed genes differentially expressed associated with enzymes such as proteases, cutinases, lipases, and peptidases which might be related to the degradation of specific compounds of each insect wing; hydrophobins and destruxins, which are associated with virulence and secondary metabolites. The greater virulence of *Metarhizium* blastospores than conidia has been demonstrated for insects and ticks (Alkhaibari *et al.*, 2016; Wassermann *et al.*, 2016; Alkhaibari *et al.*, 2017) and, in most cases, is associated with faster germination of the former. We showed that the virulence of conidia and blastospores of *M. anisopliae* varied among different host being blastospores more virulent than conidia, regardless of insect species. In our studies, the *T. molitor* was highly susceptible to *M. anisopliae* blastospores, with mortality and mycosis of 100% and 75%, respectively. The greater susceptibility of this insect to the fungus *M. anisopliae* is already known. *T. mollitor* is highly adapted to dry environments and feeds on dry grains (Howard, 1955), whereas the natural habitat of *Metarhizium* is the soil and humid environments (Zimmermann, 2007; Riguetti Zanardo Botelho *et al.*, 2019; St. Leger and Wang, 2020). Because they live in a different ecological niche, *T. mollitor* is a species susceptible to soil pathogens and widely used as bait in experiments to isolate entomopathogenic soil fungi, mainly those of the genus *Metarhizium* (Steinwender *et al.*, 2014; Iwanicki *et al.*, 2019; Riguetti Zanardo Botelho *et al.*, 2019). Besides the natural susceptibility of the *T. mollitor*, we showed that blastospores germinate faster on *T. mollitor* than on *S. frugiperda* and *G. assimilis* wings and formed three times more appressoria than on other insects' wings.

Appressoria are specialized adhesion structures produced by fungi that help it to overcome the host cuticle barriers(Chethana *et al.*, 2021). Appressoria formation is associated with virulence in entomopathogenic fungi (Ortiz-Urquiza and Keyhani, 2013) and phytopathogenic fungi (O'connell *et al.*, 2012; Chethana *et al.*, 2021). In the appressorium region, enzymes such as lipases, cutinases, and proteases are secreted by the fungus to degrade the integument (Ortiz-Urquiza and Keyhani, 2013). We found in *T. mollitor,* compared to *G. assimilalis*, a high expression of the enzyme Tripeptidyl- peptidase Sed2. Tripeptidyl peptidase belongs to the sedolisin family, a subtilisin serine protease sub-family. In *Aspergillus fumigatus*, SedB, SedC, SedD, and SedA are known to degrade the proteins and provide nutrition to fungus during infection (Reichard *et al.*, 2006), while *M. anisopliae* requires the subtilisin-like Pr1 for peptide degradation during pathogenesis (Gao *et al.*, 2011). Here we propose that blastospores secrete peptidase Sed2 during the initial infection phase to degrade proteins from *T. mollitor* wings and use them as nutrition. Besides Sed2 we found up-regulated lipases and secreted aspartic proteases in blastospores during *T. mollitor* infection compared to *A. mellifera* infection, which might be associated with lipid and peptide degradation from the waxy layer and insect cuticle. In addition, the Dibenzothiophene desulfurization enzyme was highly expressed in blastospores during infection on *T. mollitor* compared to other insects. This enzyme is associated with desulfurization processes for sulfur assimilation or oxidizing the sulfur residues within organosulfur compounds as part of a xenobiotic response (Linder, 2018). We speculate that *T. molitor* hindwing composition might have sulfur-containing compounds, which explain the high expression of that enzyme during fungus germination on wings.

Besides Dibenzothiophene desulfurization enzyme, blastospores up-regulate tyrosinase-like protein orsC during infection on *T. mollitor* wings. The tyrosinase-like protein orsC orthologous in *Aspergillus niger* is part of the gene cluster that mediates the biosynthesis of orsellinic acid (Sanchez *et al.*, 2010). Orsellinic acid is a secondary metabolite from the group of nonreduced polyketide, and it is a precursor of the toxin oosporein in *Beauveria bassiana* (Feng *et al.*, 2015). Koczyk et al. (2015) demonstrated that the capability of higher fungi to biosynthesize the simplest precursor molecule, orsellinic acid, is highlighted as an ancestral trait underlying the biosynthesis of aromatic compounds (Koczyk *et al.*, 2015). In this sense, *M. anisopliae* blastospores produce an aromatic compound derived from orsellinic acid during infection on *T. mollitor* wings, which might be associated with virulence as is the mycotoxin oosporein in *Beauveria bassiana*. In addition, the mycotoxin destruxin was upregulated in *T. mollitor* compared to *S. frugiperda.* In insects, destruxin harmful effects include, within others, the inhibition of Malpighian tubule fluid secretion and suppression of insect defense responses. The early expression of destruxin by *M. anisopliae* during the initial phase of infection on *T. mollitor* suggests a constitutive response of the fungus to deal with the insect immune response or avoid competition with other pathogens.

In addition to appressorium, *Metarhizium* produces an adhesin called MAD1, responsible for providing adherence to the insect's tegument and MAD2, accountable for the adherence of the fungus to plant roots (Wang and St Leger, 2007). In our study, MAD1 adhesin was highly expressed in *T. mollitor* compared to *G. assimilalis*, which may be related to greater adherence of blastospores to the membranous wings of *T. mollitor* compared to the latter. Therefore, we argued that the greater virulence observed for *T. molitor* might be related to the expression of specific enzymes for the degradation of the wing cuticle and the expression of secondary metabolites that may play a role during the initial stages of infection for instance, to avoid competition during host colonization.

The gene expression profile on *T. mollitor* wing was like that of the *A. mellifera,* which includes up-regulation of secondary metabolites associated with nonribosomal peptide synthases, polyketide synthases, and aurovertin biosynthesis transcription factor. On the other hand, the gene expression profile of *M. anisopliae* during infection on *S.*

frugiperda was pronounced in the up-regulation of genes associated with enzymes that degrade specific substrates, such as two cuticle degrading proteases and a streptogrisin protease associated with the degradation of chitin-linked proteins.

4.5. Conclusion

We determined that *M. anisopliae* blastospores are highly virulent for *T. molitor,* and the percentage of appressorium formation in the membranous wings of this insect was three times higher than in the wings of the other insects. The Principal Component Analysis revealed distinct expression profiles for each of the insect. This implies that *M. anisopliae* transcriptome changes remarkably according to the insect with the most significant differences found for *G. assimillis*. We found that *M. anisopliae* recruits protein families associated to ABC nutrient transporters, RNA and DNA activity, and dehydrogenases during infection process regardless of insect order. On the other hand, pairwise comparison showed genes differentially expressed associated with enzymes such as proteases, cutinases, lipases, and peptidases which might be related to the degradation of specific compounds of each insect wing; hydrophobins and destruxins, which are associated with virulence and secondary metabolites.

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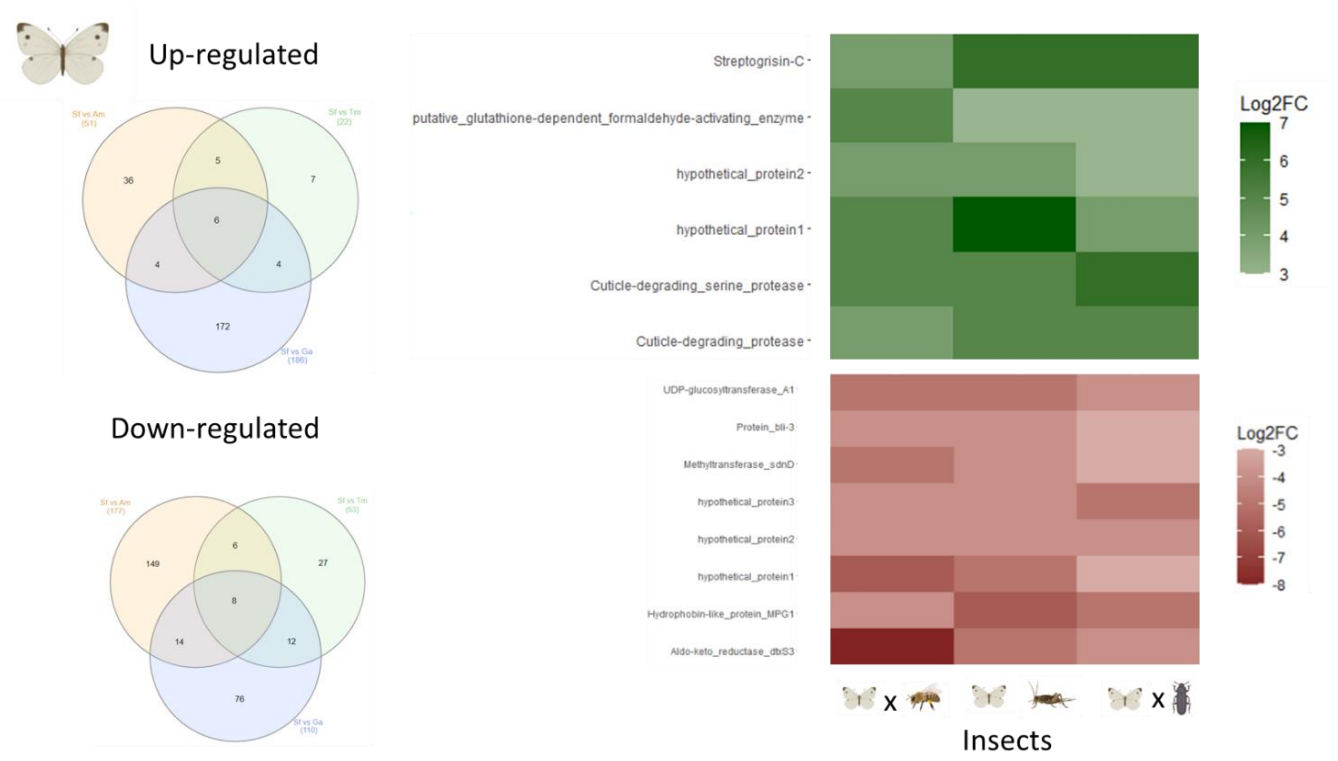
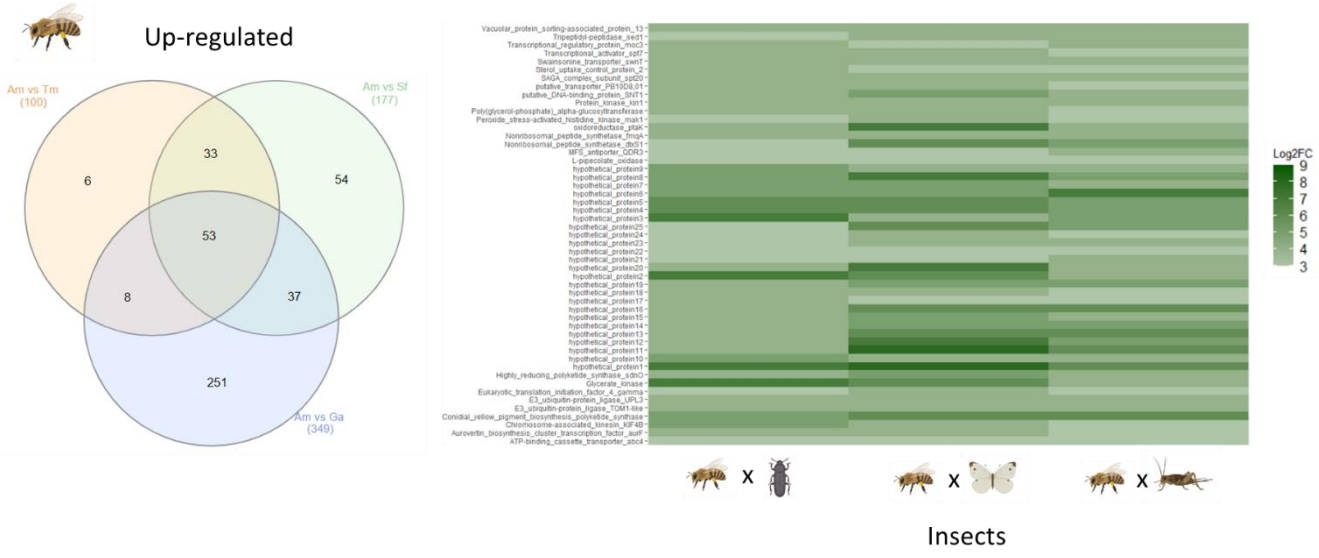
Appendix

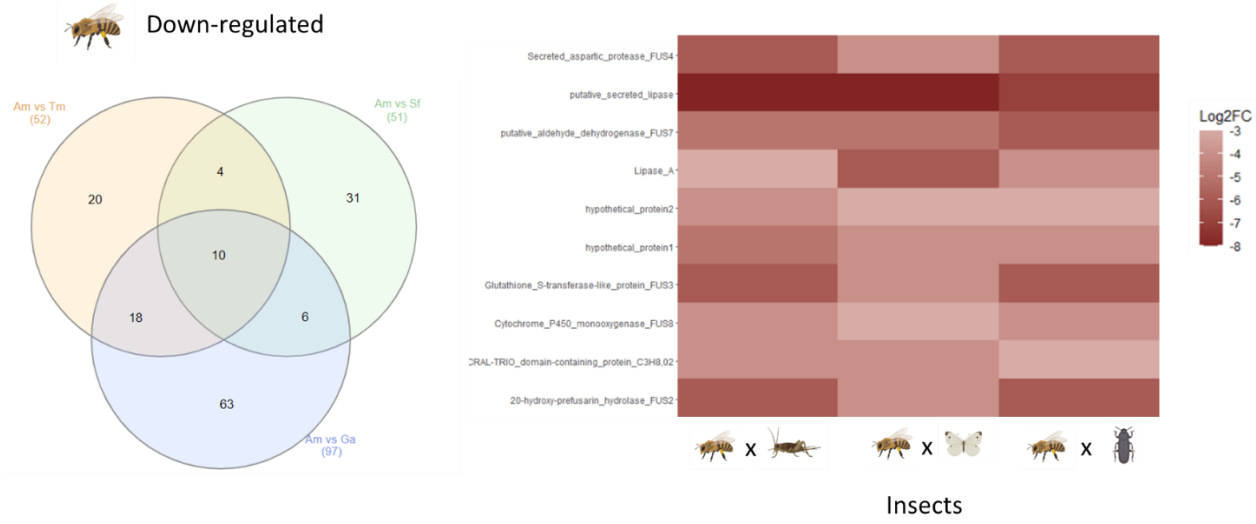
APPENDIX D. Venn diagrams showing differentially expressed genes ($Log_2FC > 3$, FDR-p-value < 0.0005) of *Metarhizium anisopliae* ESALQ E9: up and down-regulated in blastospores during infection on wings between *Spodoptera frugiperda* and *Apis mellifera*, *S. frugiperda* and *Gryllus assimillis* and *S. frugiperda* and *Tenebrio mollitor* and heatmap of shared genes between the three hosts.



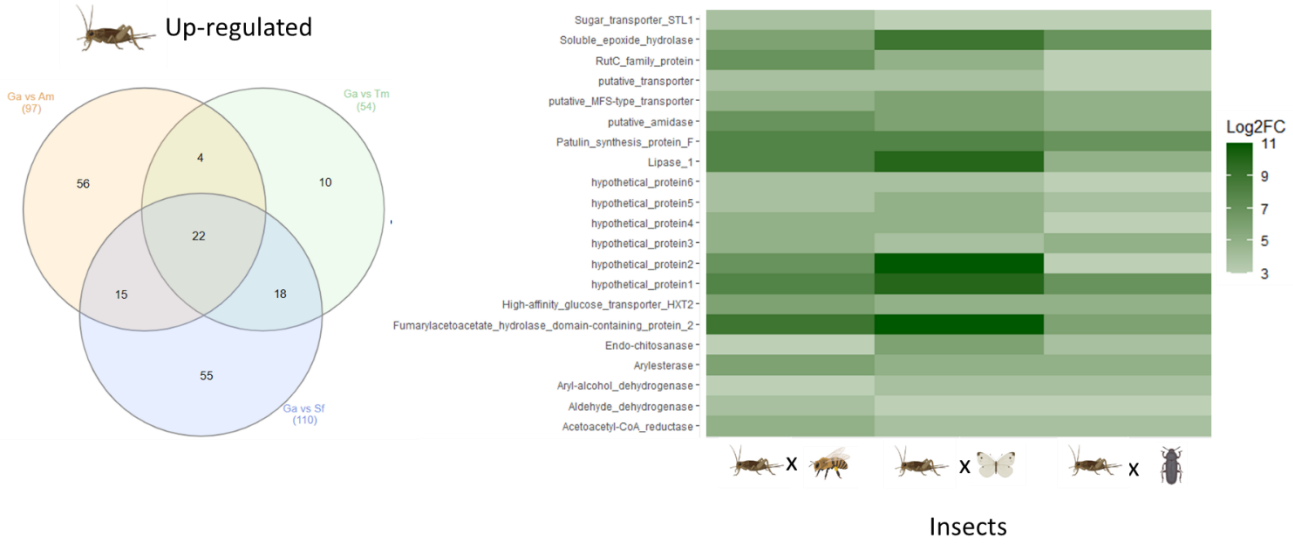
APPENDIX E. Venn diagrams showing differentially expressed genes ($Log_2FC > 3$, FDR-p-value < 0.0005) of *Metarhizium anisopliae* E9: up-regulated in blastospores during infection on wings between *A. mellifera* and *S. frugiperda*, *A. mellifera* and *G. assimillis* and *A. mellifera* and *T. mollitor* and heatmap of shared genes between the three hosts.



APPENDIX F. Venn diagrams showing differentially expressed genes ($Log_2FC > 3$, FDR-p-value < 0.0005) of *Metarhizium anisopliae* E9: down-regulated in blastospores during infection on wings between *A. mellifera* and *S. frugiperda*, *A. mellifera* and *G. assimillis* and *A. mellifera* and *T. mollitor* and heatmap of shared genes between the three hosts.



APPENDIX G. Venn diagrams showing differentially expressed genes ($Log_2FC > 3$, FDR-p-value < 0.0005) of *Metarhizium anisopliae* E9: up-regulated in blastospores during infection on wings between *G. assimillis* and *A. mellifera, G. assimillis* and *S. frugiperda* and *G. assimillis* and *T. mollitor* and heatmap of shared genes between the three hosts.



APPENDIX H. Venn diagrams showing differentially expressed genes ($Log_2FC > 3$, FDR-p-value < 0.0005) of *Metarhizium anisopliae* E9: down-regulated in blastospores during infection on wings between *G. assimillis* and *A. mellifera, G. assimillis* and *S. frugiperda* and *G. assimillis* and *T. mollitor* and heatmap of shared genes between the three hosts.