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Niche adaptations and plasticity within the fungal entomopathogenic genus *Metarhizium* in Brazil

Joel da Cruz Couceiro

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

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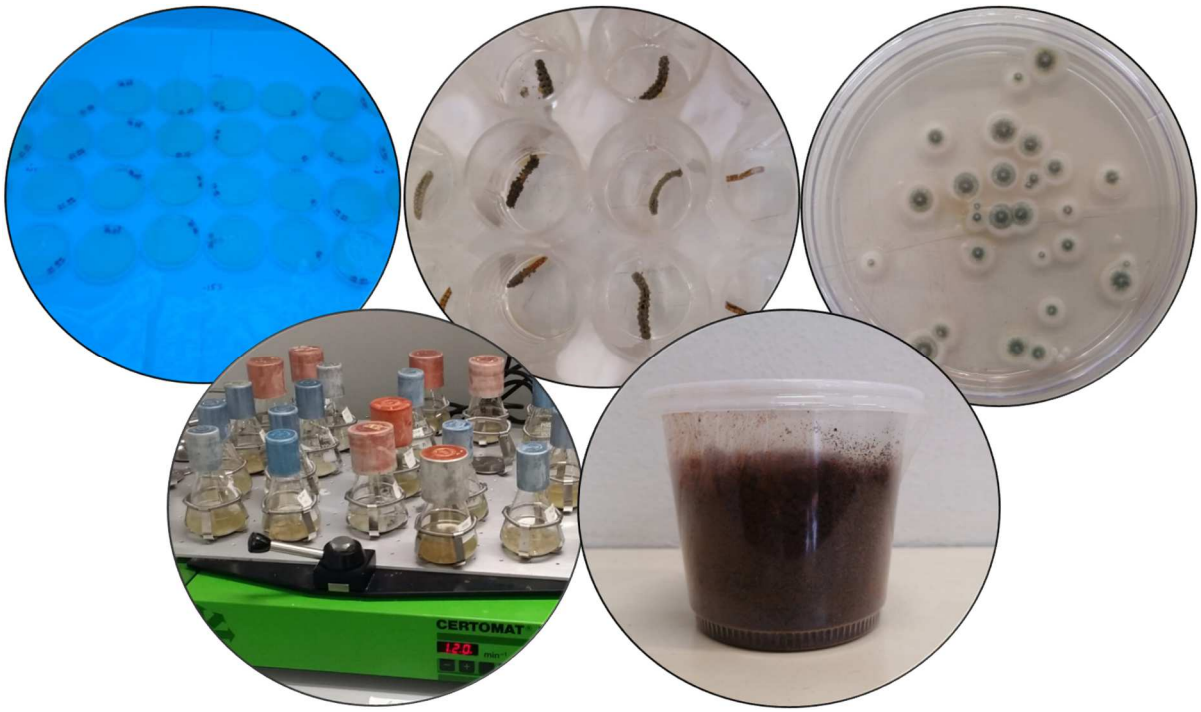

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1. *Metarhizium* 2. Radiação UV-B 3. Temperatura 4. Genômica 5. Expressão gênica 6. Virulência 7. Microbiota do solo I. Título

To my parents, João and Telma, my biggest supporters

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SUMMARY

RESUMO

Adaptações a nichos e plasticidade no gênero de fungos entomopatogênicos *Metarhizium* no Brasil

O gênero de fungos entomopatogênicos *Metarhizium* tem distribuição mundial. A abundância natural e distribuição de *Metarhizium* spp. podem ser afetadas por diversos fatores. No Brasil, *Metarhizium anisopliae* subclado Mani 2 causa a maioria das infecções de insetos acima do solo, enquanto *Metarhizium robertsii* é a espécie de *Metarhizium* mais abundante no solo e provavelmente mais associada a raízes de plantas. O principal objetivo destes estudos foi avaliar os efeitos de fatores bióticos e abióticos em isolados selecionados de *Metarhizium anisopliae* subclado Mani 2, *Metarhizium robertsii* e *Metarhizium brunneum*, espécie rara no Brasil. O primeiro aspecto estudado foi a influência de radiação UV-B e altas temperaturas na viabilidade de conídios e atividade de micélio. Foi identificada considerável variabilidade intra- e interespecífica a esses fatores abióticos. Exposição à radiação UV-B por 8 h reduziu drasticamente a sobrevivência de conídios. Isolados de *M. robertsii* apresentaram crescimento micelial ótimo a 33 °C, mas conídios que não toleraram a temperatura mais alta testada (40 °C), enquanto isolados de *M. anisopliae* apresentaram crescimento micelial ótimo a 25 °C e conídios foramm capazes de tolerar incubação a 40 °C. O estudo demonstrou que isolados coespecíficos respondem diferentemente a fatores abióticos. O segundo aspecto estudado foram características genômicas e a expressão gênica relativa de seis genes-alvo em dois substratos (solução de exsudato de raiz e suspensão de cutícula de inseto). O sequenciamento de genomas, do gene de adesão a insetos *Mad1* e gene de adesão a plantas *Mad2* de doze isolados brasileiros e de dois isolados de referência de *M. robertsii* revelaram variabilidade genotípica relacionada à origem geográfica dos isolados. Variabilidade em expressão gênica foi mais acentuada para *Mad2*, protease *Pr1A* e hidrofobina *Hyd1*, possivelmente relacionada ao estilo de vida de cada espécie. O terceiro aspecto foi a interação dos isolados com um inseto hospedeiro, avaliado através de bioensaios de virulência. Dois métodos de inoculação foram testados, representando exposição a conídios aéreos no ambiente acima do solo e exposição a conídios no ambiente do solo. A mortalidade e esporulação em cadáveres variaram dependendo do isolado. Na maioria dos casos, isolados de *M. brunneum* e *M. robertsii* apresentaram menor esporulação quando a contaminação ocorreu no solo comparado ao método de pulverização (acima do solo). O último aspecto estudado foi a interação dos doze isolados com a microbiota nativa do solo, avaliando a persistência dos isolados durante 120 dias através de unidades formadoras de colônia por grama de solo. As densidades dos isolados reduziram ao longo do tempo, e embora tenha ocorrido variabilidade entre as repetições no tempo, as densidades entre isolados não diferiram dentro de cada repetição. Os resultados obtidos dos quatro estudos indicam que isolados de *M. robertsii* estão mais adaptados a desenvolver micélio no solo, onde podem associar-se a plantas na rizosfera, enquanto isolados de *M. anisopliae* Mani 2 estão mais adaptados a desenvolver conídios no ambiente acima do solo. Os resultados enfatizam a importância de se avaliar aspectos ecológicos de isolados fúngicos para se fazer seleção de isolados e incluir esses entomopatógenos em programas de controle biológico.

Palavras-chave: *Metarhizium*, Radiação UV-B, Temperatura, Genômica, Expressão gênica, Virulência, Microbiota do solo

ABSTRACT

Niche adaptations and plasticity within the fungal entomopathogenic genus *Metarhizium* in Brazil

The entomopathogenic fungal genus *Metarhizium* has a worldwide distribution. Natural abundance and distribution of *Metarhizium* spp. can be explained by diverse factors. In Brazil, *Metarhizium anisopliae* subclade Mani 2 causes most of the infections of insects above-ground, while *Metarhizium robertsii* is the most abundant *Metarhizium* species in the soil and is probably more associated with plant roots. The main objective of this PhD studies was to evaluate the effects of biotic and abiotic factors on selected isolates of *Metarhizium anisopliae* subclade Mani 2, *Metarhizium robertsii* and *Metarhizium brunneum*, a rare species in Brazil. The first aspect studied was the influence of UV-B radiation and high temperatures on conidial viability and mycelia activity. Considerable intra- and interspecific variability was found in tolerance to these abiotic factors. Exposure to UV-B radiation for 8 h harshly reduced survival of conidia. Mycelia of *M. robertsii* isolates showed optimum growth at 33 °C, but their conidia could not tolerate the highest temperature tested (40 °C), while mycelia of *M. anisopliae* isolates had optimum growth at 25 °C and their conidia were the most tolerant to incubation at 40 °C. It was demonstrated that conspecific isolates responded differently to abiotic factors. The second aspect studied was genomic features and relative gene expression of six target genes in two substrates (root exudate solution and insect cuticle suspension). Sequencing of the genomes, insect adhesin gene *Mad1* and plant adhesin gene *Mad2* of the twelve Brazilian isolates and two well-known reference isolates of *M. robertsii* revealed genotypic variability related to the geographic origin of the isolates. Gene expression variability was more pronounced for *Mad2*, protease *Pr1A* and hydrophobin *hyd1*, possibly relating to the lifestyles of each species. The third aspect was the interaction of the isolates with an insect host, evaluated through virulence bioassays. Two methods of inoculation were tested, representing exposure to aerial conidia in the above-ground environment and exposure to conidia in the soil environment. Mortality and sporulation on cadavers varied depending on the isolate. In most cases, isolates of *M. brunneum* and *M. robertsii* showed lower sporulation when contamination occurred in the soil compared to the above-ground spraying method. The last aspect studied was the interaction of the twelve isolates with native soil microbiota, evaluating their persistence for 120 days through assessment of colony forming units per gram of soil. The densities of the isolates decreased over time, and although there was variability between replicates in time, within each replicate the densities among isolates did not differ. The results obtained in all four studies indicate that isolates of *M. robertsii* are more adapted to develop mycelia in the soil, where they can associate with plants in the rhizosphere, while isolates of *M. anisopliae* Mani 2 are more adapted to develop conidia in the above-ground environment. The results emphasize the importance of evaluating ecological aspects of fungal isolates in order to conduct isolate selection and to include these entomopathogens in biological control programs.

Keywords: *Metarhizium*, UV-B radiation, Temperature, Genomics, Gene expression, Virulence, Soil microbiota

SAMMENDRAG

Nichetilpasninger og –plasticitet hos den insektpatogene svampeslægt *Metarhizium* i Brasilien

Insektpatogene svampe fra slægten *Metarhizium* er udbredt kloden rundt. Naturlig forekomst og udbredelse af *Metarhizium* spp. kan påvirkes af mange faktorer. I Brasilien forårsager *Metarhizium anisopliae* undergruppe Mani 2 de fleste infektioner i insekter over jorden, mens *Metarhizium robertsii* er den mest udbredte art i jordbunden, hvor den sandsynligvis associerer med planterødder. Hovedformålet med dette ph.d. projekt var at undersøge effekterne af både biotiske og abiotiske faktorer på udvalgte isolater af *M. anisopliae* undergruppe Mani 2, *M. robertsii* og *Metarhizium brunneum*, en sjældent forekommende art i Brasilien. Den første undersøgelse fokuserede på betydningen af UV-B lys og høje temperaturer på svampekonidiers overlevelse og på mycelieaktivitet. Der blev fundet tydelig intra- og interspecifik variation i tolerance overfor disse faktorer. Eksponering for UV-B lys i 8 timer gav kraftig reduktion i konidiernes overlevelse. Myceliet af isolaterne fra *M. robertsii* viste optimal vækst ved 33 °C, men deres konidier kunne ikke tolerere den højeste temperatur (40 °C). Myceliet af isolater fra *M. anisopliae* havde optimal vækst ved 25 °C, og konidierne af disse isolater var mest tolerante overfor den høje temperatur ved 40 °C. Det blev demonstreret, at isolater af samme art responderede forskelligt ved eksponering af de abiotiske faktorer. Den anden undersøgelse fokuserede på analyse af isolaternes genomer og på deres genekspression af seks målgener i to substrater (opløsning af rodeksudat og insektkutikula). Sekvensering af genomerne samt genet for insekt-adhesin *Mad1* og plante-adhesin *Mad2* af de tolv brasilianske isolater samt to referenceisolater af *M. robertsii* viste genotypisk variation, som relaterede til isolaternes geografiske oprindelse. Variationen i genekspression var mest tydelig for *Mad2*, protease *Pr1A* og hydrophobin *hyd1*, muligvis relateret til arternes nichetilpasninger. Den tredje undersøgelse fokuserede på interaktionen mellem isolaterne og en insektvært, som blev gennemført ved to typer af bioassays. To forskellige metoder til eksponering af svampene blev anvendt, henholdsvis repræsenterende eksponering for luftbårne konidier over jorden og konidier i jordbunden. Relativ mortalitet og sporulering fra kadavere var isolatafhængige. I de fleste tilfælde viste isolater af *M. brunneum* og *M. robertsii* lavere sporulering, når insekterne blev inficeret i jord end i fri luft. Det fjerde og sidste studium fokuserede på hvordan de tolv isolater blev påvirket af inkubering i jordbunden, som blev undersøgt over en periode på 120 dage ved at kvantificere antallet af kolonier pr. gram jord. Tætheden af alle isolater faldt over tid, og selvom der var variation mellem de tre eksperimentelle gentagelser af forsøget, var der ikke forskel på isolaterne indenfor de respektive gentagelser. Resultaterne af de fire undersøgelser tyder på at isolater af *M. robertsii* er mere tilpasset til at udvikle mycelium i jordbunden, hvor de kan associere med planterødder, mens isolater af *M. anisopliae* undergruppe Mani 2 er mere tilpasset til at udvikle konidier i miljøet over jorden. Resultaterne understreger vigtigheden af at vurdere de økologisk relevante egenskaber hos insektpatogene svampeisolater for at gennemføre den bedst mulige udvælgelse af isolater til anvendelse i biologisk bekæmpelsesprogrammer.

Nøgleord: *Metarhizium*; UV-B stråling; temperatur; genom-analyse; genekspression; virulens; jordbundens mikrobiota

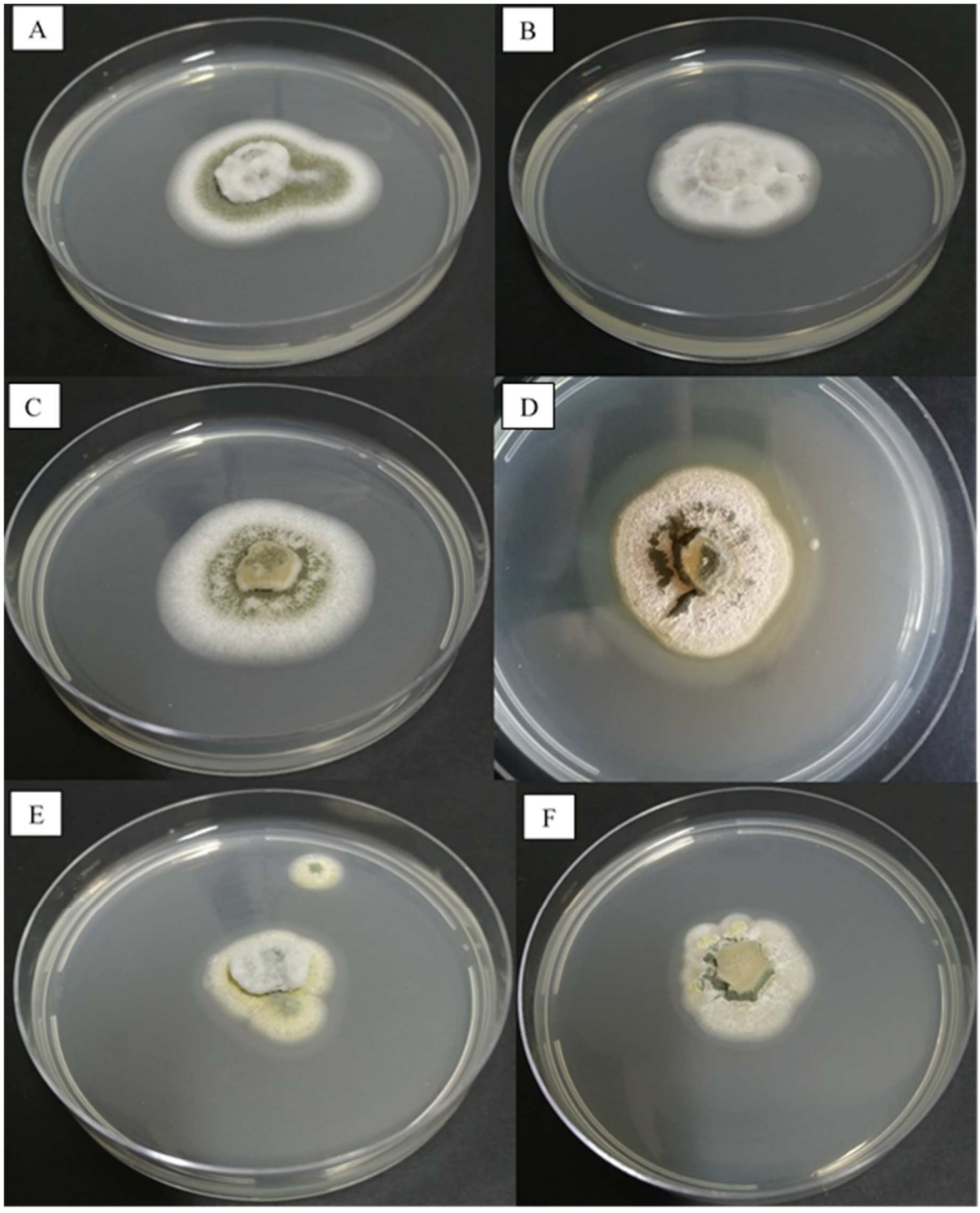
1. INTRODUCTION

1.1. The genus *Metarhizium*

Metarhizium Sorokin (Ascomycota: Hypocreales) is an important genus of fungi, mostly known for its role as an entomopathogen (St. Leger and Wang, 2020). Among *Metarhizium* species specialists and generalists strategies are described, and overall the members can infect more than 200 insect species from seven orders (Brunner-Mendoza et al., 2019; Zimmermann, 2007).

The type species of the genus, *Metarhizium anisopliae*, was described in 1879 by Metschnikoff, as *Entomophthora anisopliae*, and reclassified by Sorokin in 1883 (Zimmermann, 2007). Initially, the classification of species was based on morphological characters (Zimmermann, 2007), but in recent years, molecular features have also been included, using a multilocus analysis of informative genes (e.g., EF-1α, RPB1, RPB2 and β-tubulin) (Bischoff et al., 2009; Brunner-Mendoza et al., 2019). Currently, more than 50 species have been described (Mongkolsamrit et al., 2020).

Phenotypic aspects of *Metarhizium* colonies can vary depending on the species and on the artificial medium used for growth, but generally, in a simple medium such as potato dextrose agar (PDA), they produce white mycelia and dark green or light green conidia (Brunner-Mendoza et al., 2019; Kamp and Bidochka, 2002). The morphology of the colonies of the isolates used in the present study is shown in Figure 1. For this purpose, the isolates were cultivated in PDA for 10-14 days at 25 ± 1 °C.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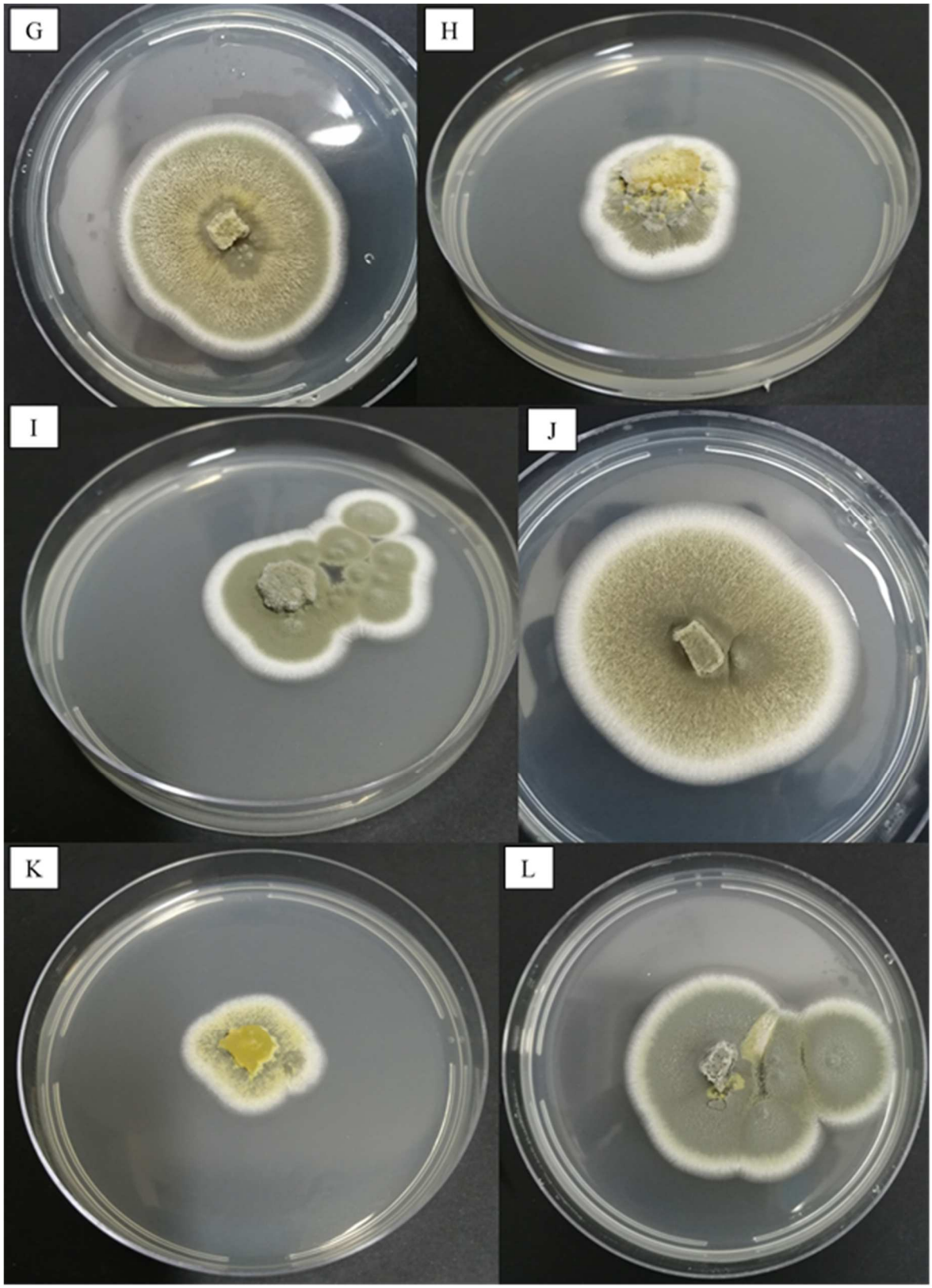

Figure 1. Morphology of colonies grown in Potato Dextrose Agar medium. (A) *Metarhizium robertsii* ESALQ 1426; (B) *M. robertsii* ESALQ 1635; (C) *M. robertsii* ESALQ 5168; (D) *M. brunneum* ESALQ 5022; (E) *M. brunneum* ESALQ 5181; (F) *M. brunneum* ESALQ 5286; (G) *M. anisopliae* ESALQ 43; (H) *M. anisopliae* ESALQ 1076; (I) *M. anisopliae* ESALQ 1116; (J) *M. anisopliae* ESALQ 1175; (K) *M. anisopliae* ESALQ 1604; (L) *M. anisopliae* ESALQ 1641.

1.2. *Metarhizium* as an entomopathogen

Many fungal species can infect insects, causing enzootic and epizootic diseases in natural environments (Lacey et al., 2015). The first step of the infection process is adherence to the cuticle of the host, in which the genes *Mad1* and *hyd1* have an essential role (Sevim et al., 2012; St. Leger et al., 1992; St. Leger and Wang, 2020; Wang and St. Leger, 2007). Germination follows, with the formation of appressoria and secretion of enzymes (mainly proteases, lipases and chitinases) to degrade the insect cuticle and allow penetration; during this step, the major protease secreted is the subtilisin-like serine protease Pr1A (St. Leger et al., 1989; Zimmermann, 2007). In the haemocoel, propagules differentiate as blastospores, a yeast-like stage to invade other tissues and consume nutrients; secondary metabolites, e.g., destruxins, are also produced in the step. When the host dies, the fungus grows saprophytically inside the cadaver, and then conidiophores emerge outside, where new infective conidia are produced (St. Leger and Wang, 2020; Vega et al., 2012; Zimmermann, 2007).

Characteristics such as penetration through the cuticle and low risk to non-hosts make hypocrealean entomopathogenic fungi suitable for biological control programs (Lacey et al., 2015; Zimmermann, 2007). *Metarhizium* spp., *Beauveria* spp. and *Cordyceps fumosorosea* (= *Isaria fumosorosea*) are among the main fungal species developed as formulated products worldwide due to the ease of mass production (Lacey et al., 2015; Vega et al., 2009). In Brazil, studies with *Metarhizium anisopliae* s.l. received more attention in the late 1960s, intensifying this fungus application in the 1970s (Li et al., 2010). There are currently 61 products based on *Metarhizium anisopliae* registered in the Ministry of Agriculture, Livestock, and Supply (MAPA), mainly used to control spittlebugs in sugar cane crops and pastures (Agrofit, 2021).

1.3. *Metarhizium* as an endophyte

Hypocrealean fungi can also act as endophytes, i.e., fungi or bacteria that live inside plant tissues without causing symptoms of disease (Behie et al., 2015; Bruck, 2005; Vega, 2008; Vega et al., 2008; Wilson, 1995; Wyrebek et al., 2011). In this interaction, the fungus can supply nitrogen derived from insects to the plant and, in return, obtain carbohydrates (Barelli et al., 2016; Behie et al., 2012; Behie and Bidochka, 2014). Entomopathogenic fungi have also been reported as plant growth promoters: switchgrass *Panicum virgatum* inoculated with *Metarhizium robertsii* J.F. Bisch., Rehner & Humber showed larger roots with higher hair density and a greater number of emerged lateral roots (Sasan and Bidochka, 2012). Another benefit of this interaction

is that fungi still have entomopathogenic activity even as an endophyte: the number of the two-spotted spider mite *Tetranychus urticae* in strawberry plants inoculated with *Beauveria bassiana* or *M. robertsii* was significantly lower compared to non-inoculated plants (Canassa et al., 2020).

Analysis of genome sequences of *Metarhizium* species revealed that the genus is closer to plant-associated fungi (either endophytes or pathogens) than to animal-pathogenic fungi, indicating that these entomopathogens evolved from endophytic fungi and only subsequently developed the ability to infect insects (Barelli et al., 2016; Gao et al., 2011). The emergence of this new adaptation probably occurred by horizontal gene transfer and/or mutations and it was of great importance because it allowed the fungi to access another source of nitrogen (Barelli et al., 2016). Phylogenetic analyses have shown that the evolution of the gene *Mad2* is more congruent with the phylogeny of the gene *EF-1a* than the evolution of the gene *Mad1*, indicating that the divergence within the genus *Metarhizium* was more influenced by the association with plants than by host insects (Wyrebek and Bidochka, 2013).

1.4. Diversity and distribution of *Metarhizium* spp. in Brazil

Metarhizium spp. have a worldwide distribution, present in tropical and temperate regions (St. Leger and Wang, 2020). These fungi naturally occur in soils of forest and agricultural areas, being very important in these ecosystems, where they are among the main groups of insect pathogens (Lacey et al., 2015).

A survey of isolates in soils of five biomes across Brazil (Amazon, Caatinga, Atlantic Forest, Cerrado and Pampa) revealed that *Metarhizium* species are widely distributed throughout the country, with *M. robertsii* being the most abundant species and the only one found in all biomes studied (Botelho et al., 2019). Despite this abundance in soils, isolates of *M. robertsii* are rarely observed to cause natural infections of terrestrial insects in Brazil, and it is suggested that this species may be adapted for activity and survival in the below-ground environment, potentially associating with plant roots (Botelho et al., 2019; Iwanicki et al., 2019; Rezende et al., 2015). *Metarhizium humberi* Luz, Rocha & Delalibera was identified as the second most abundant species in Brazilian soil, followed by *M. anisopliae* (Botelho et al., 2019), which, based on sequence haplotypes, can be divided into three subclades, Mani 1, Mani 2 and Mani 3 (Rezende et al., 2015). These authors reported that isolates of Mani 2 accounted for almost all isolates obtained from infected insects and had a wide distribution in Brazil. However, only one isolate belonging to this subclade was found in soil by Botelho et al. (2019), indicating that *M. anisopliae* Mani 2 is adapted to activity and survival in the above-ground environment, where it associates with insect

hosts. On the other hand, *Metarhizium brunneum* Petch has a very low occurrence in Brazil where it shows similar habitat association as *M. robertsii*, indicating that it is possibly adapted to the soil environment and plant associations as well (Botelho et al., 2019; Iwanicki et al., 2019).

Other species previously reported in Brazil include *Metarhizium acridum* (Driver & Milner) Bischoff, Rehner & Humber, *Metarhizium pingshaense* Q.T. Chen & H.L. Guo, *Metarhizium alvesii* Lopes, Faria, Montalva & Humber, *Metarhizium pemphigi* (Driver & R.J. Milner) Kepler, S.A. Rehner & Humber, *Metarhizium majus* (J.R. Johnst.) J.F Bisch., Rehner & Humber and *Metarhizium lepidiotae* (Driver & Milner) J.F. Bisch., Rehner & Humber (Botelho et al., 2019; Lopes et al., 2017, 2014, 2013a, 2013b; Luz et al., 2019; Rezende et al., 2015; Rocha et al., 2013).

1.5. Factors that affect the distribution of *Metarhizium* spp.

The community composition in each geographical region varies depending on the habitat, presence and diversity of susceptible hosts and the prevailing abiotic conditions (Brunner-Mendoza et al., 2019). Among the latter, ultraviolet light, high temperatures and relative humidity are relevant (Zimmermann, 2007). UV-B radiation can be very harmful to fungal isolates, with effects of exposure including reduced development and death (Fernandes et al., 2015). Regarding temperature, *Metarhizium* spp. are considered mesophilic, with an optimum range between 25 and 30 °C (Walstad et al., 1970); high temperatures can reduce growth, virulence, and germination (Ekesi et al., 1999; Hallsworth and Magan, 1999). High relative humidity is required for conidial germination (Zimmermann, 2007). Walstad, Anderson & Stambaugh (1970) demonstrated that conidia of *M. anisopliae* only germinated with relative humidity above 90% and that the time for sporulation increased as moisture decreased.

In a natural environment, fungal propagules are subjected to several trophic interactions, e.g., competition with other microorganisms present in the soil. Walstad, Anderson & Stambaugh (1970) reported that spores of *M. anisopliae* and *B. bassiana* could not germinate on different non-sterilized substrates. Still, germination occurred when the spores transferred to a sterile medium, indicating that the inhibition was due to the presence of other microorganisms. The authors also reported that both fungi presented antibiosis capacity against saprophytic fungi commonly found in soil, such as *Aspergillus* sp., *Cladosporium* sp. and *Fusarium* spp. Sharma, Gupta & Yadava (1999) reported that the growth of *B. bassiana* and *M. anisopliae* was inhibited in the presence of *Penicillium* sp., *Aspergillus niger*, *Trichoderma viride* and *Rhizoctonia bataticola*, but also inhibited the growth of the latter two species. These studies demonstrate that entomopathogenic

fungi can affect and be affected by other microorganisms in the soil. The development of these fungi will depend on the competitors present and the influence of abiotic factors (Jaronski, 2007).

1.6. Objectives and hypothesis

Considering the distribution of *Metarhizium* spp. in Brazil and the apparent differences in the ecology of the species in natural environments, the overall objective of this study was to investigate the influence of biotic and abiotic factors on the distribution patterns of *Metarhizium anisopliae*, *Metarhizium robertsii* and *Metarhizium brunneum* in Brazil.

The objective of the **first chapter** was to compare *in vitro* tolerance of selected isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* to UV-B radiation and elevated temperatures. Isolates of *M. anisopliae* Mani 2 have a presumed niche of exploring insect resources above-ground, therefore being more exposed to abiotic factors than in the soil. Regions of lower latitude are subjected to higher temperatures and stronger solar irradiation. Considering this, the hypotheses were: (i) conidia of isolates of *M. anisopliae* would be more tolerant to UV-B radiation and to high temperatures than conidia of isolates of *M. brunneum* and *M. robertsii*; (ii) isolates of *M. anisopliae* would show increased radial mycelial growth at higher temperatures compared to isolates of *M. brunneum* and *M. robertsii*; and (iii) isolates from lower latitudes would be more tolerant to UV-B radiation and high temperatures than isolates from higher latitudes.

In the **second chapter**, the first objective was to compare the genomes of the 12 Brazilian isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* to obtain information on their phylogenomic relationships and on the abundance of selected protein families. Two well-known reference isolates of *M. robertsii* were also included in the analysis. Insect adhesin *Mad1* and plant adhesin *Mad2* genes have an essential role in interacting with host surfaces. Thus the second objective was to assess the genotypic variation in their coding sequences. The third objective was to evaluate the relative expression of six target genes after inoculating the isolates in a root exudate solution or an insect cuticle suspension. The hypotheses were: (i) *M. anisopliae* Mani 2 would show higher expression of pathogenicity-related genes than *M. brunneum* and *M. robertsii*; (ii) *M. brunneum* and *M. robertsii* would show higher expression of a gene related to plant-association than *M. anisopliae* Mani 2; and (iii) the expression of constitutive genes would be similar in the three species.

In **chapter three**, the first objective was to compare the virulence of the selected isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* against *Tenebrio molitor* larvae through two application methods, representing exposure to aerial conidia in the above-ground environment (application using a Potter spray tower) and exposure to conidia in the soil environment (application to non-sterile soil and assessment by insect bait method). Considering the presumed niches for the three *Metarhizium* species, it was hypothesized that: (i) isolates of *M. anisopliae* Mani 2 would be most virulent and show increased sporulation when applied above-ground; and (ii) isolates of *M. brunneum* and *M. robertsii* would be more virulent and show increased sporulation when applied in the soil. The second objective was to test the virulence of mixtures of selected pairwise isolate combinations, based on the premise that concomitant infections are common in natural environments and likely occur with insects.

The objective of the **fourth chapter** was to evaluate the persistence over time of the isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* in a non-sterile sandy clay soil contained in pots placed in a greenhouse. The hypothesis was that isolates of *M. brunneum* and *M. robertsii* would show a more prolonged survival indicated by a higher density of colony-forming units by the end of the experimental time compared to isolates of *M. anisopliae* Mani 2.

1.7. Obtained results and future perspectives

The first study showed that there was considerable intra- and interspecific variability in conidial tolerance to UV-B radiation and high temperatures, and in mycelial growth in different temperature regimes. Mycelia of *M. robertsii* isolates grew the most at a higher temperature (33 ºC), compared to *M. anisopliae* and *M. brunneum* isolates, which had optimum growth at 25 °C. Conidia of *M. anisopliae* isolates could better tolerate a high temperature (40 °C). A correlation between tolerance to these abiotic factors and latitude of origin was not observed, thus the hypotheses were not confirmed (Couceiro et al., 2021). Despite this, the data showed the effects of UV-B radiation and high temperatures on twelve isolates representing three species of *Metarhizium*, and that isolates of the same species can respond differently to these abiotic factors.

In the second study (Manuscript 2), the genome sizes obtained for the twelve Brazilian isolates of *Metarhizium* spp. were similar to data from a previous report (Hu et al., 2014). The analysis revealed interesting differences regarding *M. robertsii*. Brazilian isolates clustered separately from the North American reference isolates, indicating a degree of genotypic variability between isolates from different geographic locations. The phylogenies of adhesins *Mad1* and

Mad2 also revealed intraspecific divergence, for both *M. robertsii* and *M. brunneum*, with isolates clustering by geographic origin. Gene expression data showed intra- and interspecific differences between the groups in both conditions tested (root exudate and insect cuticle), especially for genes *Mad2* (plant adhesin), *Pr1A* (protease) and *hyd1* (hydrophobin). The study provided new information about the genomes of three species of *Metarhizium* and indicated that intraspecific genotypic and gene expression differences might be related to adaptation to living in different habitats. It also emphasizes the importance of selecting a diverse group of isolates for genomic studies to capture natural variability within fungal species.

In the third study (Manuscript 3), all twelve isolates were found to be pathogenic to larvae of *T. molitor*. However, the hypotheses tested were not confirmed due to the intra- and interspecific variability in both virulence and sporulation. Application of mixtures of isolates resulted in mortalities and median lethal times (LT_{50}) comparable to single applications in most cases, indicating independence between the isolates; one mixture showed slightly higher LT_{50} and lower mortality, indicating antagonism between the isolates. As previously stated by Seid et al. (2019), the efficiency of combinations depends on isolate selection. The application of mixtures did not increase the percentage of mycosis in cadavers. The results also showed that the commercial isolate caused lower larval mortality and sporulation in both methods of application. This emphasizes the importance of isolate selection during the development of a biopesticide based on fungal entomopathogens.

In the fourth study (Chapter 4), the density of isolates in non-sterile sandy clay soil, measured as colony forming units per gram of soil, varied between the three replicates in time. The abundance of CFUs decreased over time for all isolates, reaching its lowest point at the end of the experimental time. Within each replicate, the isolates did not differ, indicating they have similar ability to compete with the native soil microbiota.

Overall, the studies demonstrated that regarding ***M. anisopliae*** **Mani 2**: the highest temperature was not optimal for mycelial growth, but in most cases, their conidia tolerated high temperature or UV-B radiation better than conidia of *M. robertsii* and *M. brunneum* (Chapter 1); the group of *M. anisopliae* Mani 2 isolates highly expressed the gene *Pr1A*, mainly involved in fungal penetration of host cuticle (Chapter 2); these results indicate a selection pressure for development of conidia in *M. anisopliae* Mani 2, consistent with its presumed lifestyle in the above-ground environment, where this subclade is more subjected to adverse effects of abiotic conditions and can explore insects as a resource. Regarding ***M. robertsii***: mycelia had optimum growth at the highest temperature tested, but their conidia did not tolerate high temperature or UV-B radiation (Chapter 1); both groups of isolates (ESALQ and ARSEF) exhibited higher relative expression of

the gene *hyd1*, involved in processes such as adhesion and sporulation (being highly expressed in mycelia and mycelia with conidiophores) (Chapter 2); isolates had a generally lower percentage of sporulation in cadavers from the insect bait method (exposure below-ground) compared to sporulation in cadavers from the spray tower method (Chapter 3). These results indicate a selection pressure for the development of mycelia in this species, consistent with its presumed lifestyle in the below-ground environment, where isolates would associate with plant roots via mycelia. Regarding ***M. brunneum***: conidia could not tolerate high temperature or UV-B radiation (Chapter 1); sporulation in cadavers was generally lower inoculating the isolates in the soil (exposure below-ground) (Chapter 3); considering the presumed niche that *M. brunneum* is more adapted to the below-ground environment, these results indicate that there is also a selection pressure for this species to develop mycelia in the soil, similarly to *M. robertsii*.

The four studies increase the knowledge about the ecology of three *Metarhizium* species, regarding different adaptations to living below- or above-ground. The results reinforce the need for comprehensive isolate selection and manipulation of their natural habitats to enhance biological control programs' quality. Future studies can focus on further questions, such as:

- Laboratory experiments using conidial formulations with ultraviolet radiation protectants;
- Identification of other relevant differences in the genomes of the sequenced isolates (e.g., genes and proteins not evaluated in Chapter 2);
- Sequencing other isolates from distinct geographic regions to compare their genomes with genomes already available;
- Evaluate virulence of the isolates in semi-field and/or field conditions;
- Field experiments to evaluate the potential of the isolates as endophytes.

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2. UV-B RADIATION TOLERANCE AND TEMPERATURE-DEPENDENT ACTIVITY WITHIN THE ENTOMOPATHOGENIC FUNGAL GENUS *Metarhizium* IN BRAZIL

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ABSTRACT

Metarhizium comprises a phylogenetically diverse genus of entomopathogenic fungi. In Brazil, *Metarhizium anisopliae* s.str. subclade Mani 2 is predominantly isolated from insects, while *M. robertsii* and *M. brunneum* mostly occur in the soil environment. Solar radiation and high temperatures are important abiotic factors that can be detrimental to fungal propagules. We hypothesized that among 12 Brazilian isolates of *Metarhizium* spp., *M. anisopliae* Mani 2 (n=6), being adapted to abiotic conditions of the phylloplane, is more tolerant to UV light and high temperatures than *M. robertsii* (n=3) and *M. brunneum* (n=3). Inoculum of each isolate was exposed to UV-B for up to 8 h and viability evaluated 48 h later. After 8 h under UV-B, most of the isolates had germination rates below 5%. Discs of mycelia were incubated at different temperatures, and diameter of colonies were recorded for 12 days. Mycelia of *M. robertsii* isolates grew faster at 33 °C, while *M. anisopliae* and *M. brunneum* grew most at 25 °C. Dry conidia were incubated at 20, 25 or 40 °C for 12 days, and then viabilities

were examined. At 40 °C, conidia of five *M. anisopliae* isolates were the most tolerant. In the three experiments, considerable intra- and inter-specific variability was detected. The results indicate that conclusions about tolerance to these abiotic factors should be made only at the isolate level.

Keywords: entomopathogenic fungus; *Metarhizium*; UV-B radiation; temperature; tolerance

2.1. Introduction

Several species within the fungal entomopathogenic genus *Metarhizium* Sorokin (Ascomycota: Hypocreales) occur worldwide in soils of natural and agricultural ecosystems (Jaronski, 2007; Lacey et al., 2015). *Metarhizium* is one of the main genera causing disease in insects (Hesketh et al., 2010). Mainly known for their action against insects, some species of this genus are also able to associate intimately with different plants as endophytes (Bruck, 2005; Hu and St. Leger, 2002; Wyrebek et al., 2011), in a relationship where the fungus can supply their plant hosts with nitrogen derived from insects (Barelli et al., 2016; Behie et al., 2012, 2017; Behie and Bidochka, 2014) as well as promote plant growth (Jaber and Enkerli, 2016a, 2016b; Sasan and Bidochka, 2012).

In agroecosystems, *Metarhizium* communities are diverse and relatively complex (e.g., Steinwender et al., 2014), and the different species exhibit patterns of spatial distribution. In Brazil, a large diversity of *Metarhizium* spp. has been documented in soils of both natural and agricultural habitats (Botelho et al., 2019; Rezende et al., 2015). Still, there is currently limited knowledge of the characteristics of the isolates of these species of Brazilian origin.

In soils of the Cerrado biome in Brazil, Rocha et al. (2013) found a great abundance of *M. anisopliae* s.l., and by sequencing the 5' EF-1α region of isolates from many areas of Brazil, Rezende et al. (2015) reported *M. anisopliae* s.str. haplotypes grouped in the subclades Mani 1, Mani 2 and Mani 3. *Metarhizium robertsii* Bischoff, Rehner & Humber is the most widespread and abundant species in Brazil, easily isolated from soils of many biomes (Botelho et al., 2019), while in temperate regions such as Denmark, *M. robertsii* has been recovered in low abundance in an experimental agricultural field (Steinwender et al., 2015, 2014). In contrast, *Metarhizium brunneum* Petch was recovered as the most prevalent species in agricultural soils in Denmark, while it has a limited occurrence in Brazil (Brunner-Mendoza et al., 2019; Kepler et al., 2015; Steinwender et al., 2014).

Even though the three species, *M. robertsii, M. brunneum,* and *M. anisopliae*, seemingly occupy overlapping niches, acting as entomopathogens and being found in soils worldwide

(Lacey et al., 2015), there is a notable ecological difference between their distribution in Brazil. Generally, only isolates of *M. anisopliae* subclade Mani 2 are obtained as natural infections of insects collected in field crops in Brazil (Rezende et al., 2015), evidencing that members of this subclade are predominantly adapted to explore insects as a resource. On the other hand, isolates of *M. robertsii* can infect and kill different taxa of insects in laboratory conditions, but this is rarely observed in field conditions in Brazil (Lopes et al., 2013). Frequently, *M. robertsii* establishes associations with plant roots, indicating that this species is well adapted to the soil and rhizosphere environment (Rezende et al., 2015; Sasan and Bidochka, 2012). Similarly, *M. brunneum* occurs predominantly in soils and is associated with roots (Steinwender et al., 2014, 2015), and at least in temperate regions, it is generally not found infecting insects above ground naturally (Meyling et al., 2011).

Metarhizium spp. are commonly found in cultivated areas and are therefore considered to be adapted to agricultural habitats (Meyling and Eilenberg, 2007; Vega et al., 2012). Abiotic factors (e.g., solar radiation, temperature, humidity, wind) can significantly influence the development, survival, and distribution of entomopathogenic fungi in the environment (Inglis et al., 2001). Agricultural habitats have limited canopy cover and are therefore considered frequently exposed to relatively high solar radiation and temperature fluctuations (Bidochka et al., 2001; Vega et al., 2012). Intense exposure to solar radiation can be detrimental to any organism (Solomon, 2008). Four hours of exposure to UV-B light can be enough to considerably reduce colony development and conidial viability, as well as delay conidial germination and cause conidial inactivation of *Metarhizium* spp. isolates (Braga et al., 2001a, 2001b, 2001c). Temperature is also an important factor affecting fungal propagules. Walstad et al. (1970) reported that the optimum temperature range for isolates of *M. anisopliae* s.l. was 25–30 °C, but most isolates could germinate and sporulate between 15 and 35 °C. Temperatures outside the latter range can decrease growth rates and reduce the virulence of *Metarhizium* isolates (Acheampong et al., 2020; Ekesi et al., 1999; Inglis et al., 2001; Thomas and Jenkins, 1997; Tumuhaise et al., 2018).

Fungal propagules present above ground, such as in phylloplanes, are more exposed to the harmful effects of solar radiation and high temperatures than propagules in the soil environment. Therefore, it should be expected that natural selection will favor fungi that are more tolerant to these abiotic factors above ground as adaptive traits. Consequently, phylloplane-inhabiting fungal isolates are expected to exhibit higher survival rates than those inhabiting the soil after the same exposure time to UV light and high temperatures.

The adverse effects of these abiotic factors on entomopathogenic fungal isolates are considered significant obstacles to their application as biopesticides due to a reduced probability

of establishing epizootics (Braga et al., 2001a). Moreover, tolerance to UV radiation or high temperatures may be responsible for the niche differentiation between the three *Metarhizium* species mentioned, i.e., *M. anisopliae* subclade Mani2 acting mostly as an entomopathogen above ground, and *M. brunneum* and *M. robertsii* primarily associated with plant roots below ground.

Based on these assumptions, we selected isolates of the *M. anisopliae* subclade Mani 2, *M. robertsii,* and *M. brunneum* from different hosts and habitats in Brazil to evaluate their relative conidial survival after exposure to UV-B radiation, and conidial germination and mycelial growth rates under different temperature regimes. Information about these responses is useful for expanding the knowledge about the ecology of *Metarhizium* species and understanding how particular fungal isolates can survive under unfavorable abiotic conditions that can affect their abundance and distribution. It was expected that isolates of *M. anisopliae* subclade Mani 2 would exhibit the highest tolerance to UV-B radiation and elevated temperatures since the members of this subclade are most frequently found infecting insects above ground, thereby being more exposed to these abiotic factors. In contrast, *M. robertsii* and *M. brunneum* isolates, more often recovered from the soil environment and less exposed than *M. anisopliae*, would exhibit the lowest tolerance to UV-B and show the most activity at relatively low temperatures compared to *M. anisopliae*. Furthermore, isolates from lower latitudes of Brazil, where solar irradiation is stronger, and temperatures are usually high, should also be more tolerant to UV-B radiation and high temperatures than those from higher latitudes.

2.2. Materials and methods

2.2.1. Fungal isolates

Twelve isolates of *Metarhizium* spp. (six of *M. anisopliae* subclade Mani 2, three of *M. robertsii,* and three of *M. brunneum*) were selected from the Entomopathogen Collection "Prof. Sérgio Batista Alves", of the Laboratory of Pathology and Microbial Control of Insects, at the Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, State of São Paulo, Brazil. Information about the collection site and origin of the isolates is shown in Table 1. Isolates ESALQ 1426 and ESALQ 1635 (*M. robertsii*) and all isolates of *M. anisopliae* were identified by Rezende et al. (2015). Isolate ESALQ 5168 (*M. robertsii*) and all isolates of *M. brunneum* were identified by Iwanicki (2015) and Iwanicki et al. (2019). Identification in all studies were based on sequencing of the 5' EF-1α region.

Table 1. List of isolates used in the study, all deposited in the Entomopathogen Collection "Prof. Sérgio Batista Alves", ESALQ/USP, Piracicaba, State of São Paulo, Brazil.

Species	Isolate code	Origin	Collection site (City, State)	Latitude
Metarhizium anisopliae s.str. subclade Mani 2	ESALQ 43	Hemiptera: Cercopidae	Flexeiras, Alagoas	9°16' S
	ESALQ 1116	Coleoptera: Scarabaeidae	Piracicaba, São Paulo	22°43' S
	ESALQ 1641	Hemiptera: Cercopidae	Boca da Mata, Alagoas	9°38' S
	ESALQ 1076	Meadow soil	Arapongas, Paraná	23°25' S
	ESALQ 1175	Meadow soil	Córrego Rico, São Paulo	21°15' S
	ESALQ 1604	Biotech G, Biotech® Controle Biológico (commercial isolate)	Unknown	Unknown
Metarhizium robertsii	ESALQ 1426	Soybean soil	Londrina, Paraná	23°18' S
	ESALQ 1635	Native forest soil	Delmiro Gouveia, Alagoas	9°23' S
	ESALQ 5168	Coleoptera: Scarabaeidae	Iracemápolis, São Paulo	22°34' S
Metarhizium brunneum	ESALQ 5022	Sugarcane soil	Iracemápolis, São Paulo	22°34' S
	ESALQ 5286	Sugarcane soil	Iracemápolis, São Paulo	22°34' S
	ESALQ 5181	Sugarcane root	Iracemápolis, São Paulo	22°34' S

2.2.2. Effect of UV-B Radiation Exposure

The isolates were grown in Petri dishes containing culture medium PDAY — Potato Dextrose Agar (Difco Laboratories, Sparks, MD, USA) enriched with yeast extract (2.5 g L^{-1}) (KASVI, São José dos Pinhais, PR, Brazil) — and held in B.O.D. incubator (Biological Oxygen Demand) for 10 days (25 ± 1 °C, 12 h photophase). After this period, conidia were harvested to prepare suspensions of each isolate (concentration: 10^6 conidia mL^{-1}) using sterile distilled water plus 0.05% Tween 80. Aliquots of 150 μL were inoculated covering the four central quadrants of Rodac Petri dishes (Replicate Organism Detection and Counting, 60 × 10 mm; J Prolab, São José dos Pinhais, PR, Brazil) containing 5 mL of PDAY plus 0.1% v/v Derosal 500 SC (Carbendazim, Bayer CropScience, SP, Brazil), a fungicide that has fungistatic properties at low concentrations. The plates were kept open in a laminar flow cabinet until all the liquid evaporated.

The experiment was conducted in a wooden box with four fluorescent lamps UVB-313EL (Q-Lab Corporation, USA), with peak irradiation corresponding to a wavelength of 313 nm (equivalent to UV-B light) and mean irradiation values of 659.54 mW m^{-2} or 2.38 kJ m^{-2}. Before the exposure experiment, the lamps were turned on for 30 minutes to generate a stable irradiation level. The plates were then placed in the box and covered with an acetate sheet to prevent exposure to wavelengths below 290 nm, which includes UV-C (280 nm). The temperature inside the box was 27 ± 1 °C during the experimental exposures. The experiment was performed using a randomized complete block design, in which all treatments were repeated three times at the same conditions. In each of the replicates (blocks), separate conidial suspensions of each isolate were prepared and used for inoculation in five plates of each isolate

corresponding to each of the five exposure times (0, 2, 4, 6, and 8 hours), totalizing 12 isolates × 5 exposure time (hours) × 3 blocks (replicates) = 180 observations. Plates representing the control were not exposed to UV-B (time = 0h), while the other plates were exposed for 2, 4, 6 or 8 h (irradiation doses corresponding to 4.76, 9.52, 14.28 and 19.04 kJ m^{-2}, respectively). Every 2 h, the respective plate of each isolate was transferred to an incubator (25 ± 1 °C, 12 h photophase). The incubation time was 24 h for control plates (otherwise germination tubes grow and it becomes impossible to count germinated conidia) and 48 h for exposed plates to allow DNA repair and germination of conidia. Viabilities were then evaluated, counting germinated and non-germinated conidia under a light microscope and at least 200 conidia per plate; a propagule was considered germinated when the length of its germ tube was equal to or higher than its diameter.

2.2.3. Effects of temperature

2.2.3.1. Mycelial growth

The isolates were cultivated in culture medium PDA (Potato Dextrose Agar; Difco Laboratories, Sparks, MD, USA) and held in B.O.D. incubator (25 ± 1 °C, 12 h photophase) for three days. After this period, mycelial discs (Ø = 1 cm) were made using a cork borer and transferred to the center of new Petri dishes (Ø = 9 cm) containing PDA. There were five plates (replicates) per treatment, consisting of 12 isolates at each of the five temperature regimes (5 plates x 5 temperatures = 25 plates per isolate). The plates were sealed with parafilm and incubated in B.O.D., in the dark, in five temperature regimes: 15 °C constant, 20 °C constant, 25 °C constant, 33 °C constant, and 33 °C for 8 h and 20 °C for 16 h. A completely randomized design was used. Two orthogonal axes were drawn at the bottom of the plates to serve as a reference. The diameter of the colonies was measured daily for 12 days using a ruler. The bioassay was repeated three times.

2.2.3.2. Survival of conidia

Eleven isolates were cultivated on PDAY and placed in B.O.D. incubator (25 ± 1 °C, 12 h photophase), while the isolate ESALQ 1635 (*M. robertsii*), which exhibited poor sporulation on PDAY, was grown only on PDA. After 10 days, conidia were harvested with a spatula, dried in a desiccator with silica (relative humidity—RH <20%; water activity—a_w ≤0.3) and placed in

Eppendorf tubes, which were vacuum packed to avoid interference of humidity; each tube contained 0.1 g of pure conidia. There were three Eppendorf tubes (replicates) per treatment, consisting of 12 isolates for each temperature regime (3 tubes x 3 temperatures = 9 tubes per isolate). A completely randomized design was used. The tubes were placed in B.O.D. incubators in the dark at 20 °C, 25 °C or 40 °C. After 12 days, the tubes were placed inside a laminar flow cabinet and kept open for 30 min to allow slow hydration of conidia and avoid imbibition damage. Later, viabilities were evaluated according to the protocol of Oliveira et al. (2015). Briefly, suspensions of each tube (concentration: 10^6 conidia mL^{-1}) were prepared, and aliquots of 150 μL were inoculated in Rodac Petri dishes containing PDA with an antibiotic (Pentabiótico: 500 mg L^{-1}; composed of benzathine benzylpenicillin, procaine benzylpenicillin, benzylpenicillin potassium, dihydrostreptomycin base, and streptomycin base) and a fungistatic (0.1% v/v Derosal 500 SC), covering the four central quadrants; plates remained open in a laminar flow cabinet until all the liquid evaporated. The plates were then closed and incubated in B.O.D. (25 ± 1 °C, 12 h photophase) for 24 h, after which viabilities were analyzed by counting germinated conidia.

2.2.4. Statistical analyses

For analyses of the three experiments' data, generalized linear models (GLMs) were used, allowing analysis of the normal and proportional responses, as long as the distribution is part of the exponential family (Nelder and Wedderburn, 1972).

All models were selected using the half-normal plot (Moral et al., 2017), and a likelihood-ratio test allowed us to compare similarities between isolates in different conditions (Demétrio et al., 2014; Fatoretto et al., 2018). In the UV-B experiment, the quasi-binomial model was proposed and allowed to capture any overdispersion present in the data (Demétrio et al., 2014). For the experiment of temperature affecting the survival of conidia, the theory of combined models was used, particularly the beta-binomial model (Molenberghs et al., 2017). These models can capture the data's overdispersion and allow the addition of the random effects that model the correlation within individuals. The model was adjusted using the GAMLSS package (Rigby and Stasinopoulos, 2005). Modeling of mycelial growth data was performed considering normal data, the dependence of the parcels (plates) inside the same B.O.D. incubator, and between parcels, which were the measurements over time of the same plate. A model was designed for each temperature, considering measurements from the 4^{th} day. All analyses were performed using the software R (R Core Team, 2020).

2.3. Results

2.3.1. Effect of UV-B on conidial germination of isolates

Due to considerable variability in the data, the binomial model did not present a goodness-of-fit. The best fitted model was a quasi-binomial model with a quadratic linear predictor for exposure time. In this model, conidial viability is described by separate curves, in which each fungus has distinct initial proportions (different intercepts) and different slopes (decay form) for each time (Figure 1).

Germination of control plates (no UV-B exposure) ranged between 97 and 100%. UV-B light had little effect on conidia germination after 2 h of exposure, and germination rates were still above 70% for all isolates after 4 h. After 6 h of exposure, most of the isolates started to show a substantial decline in conidial germination. By the end of the experimental time (8 h of exposure), nine isolates exhibited germination below 5% (Figure 1). Isolates *M. robertsii* ESALQ 1426, *M. brunneum* ESALQ 5181 and ESALQ 5286, and *M. anisopliae* ESALQ 1175 and ESALQ 1641 reached complete inactivation of conidia at 8 h, while isolate *M. robertsii* ESALQ 1635 was the most tolerant to UV-B radiation, being the only isolate to retain more than 50% of germination after 8 h.

Since some isolates exhibited similar curves, likelihood-ratio tests were conducted to identify possible similarities between their viability. Six groups were defined ($\alpha = 0.05$), in order of UV-B tolerance (highest to lowest): (i) *M. robertsii* ESALQ 1635; (ii) *M. anisopliae* ESALQ 1076; (iii) *M. anisopliae* ESALQ 1116; (iv) *M. anisopliae* isolates ESALQ 43, ESALQ 1175 and ESALQ 1604, *M. robertsii* isolates ESALQ 1426 and ESALQ 5168, and *M. brunneum* isolate ESALQ 5022; (v) *M. brunneum* isolates ESALQ 5181 and ESALQ 5286; and (vi) *M. anisopliae* ESALQ 1641 (Figure 1).

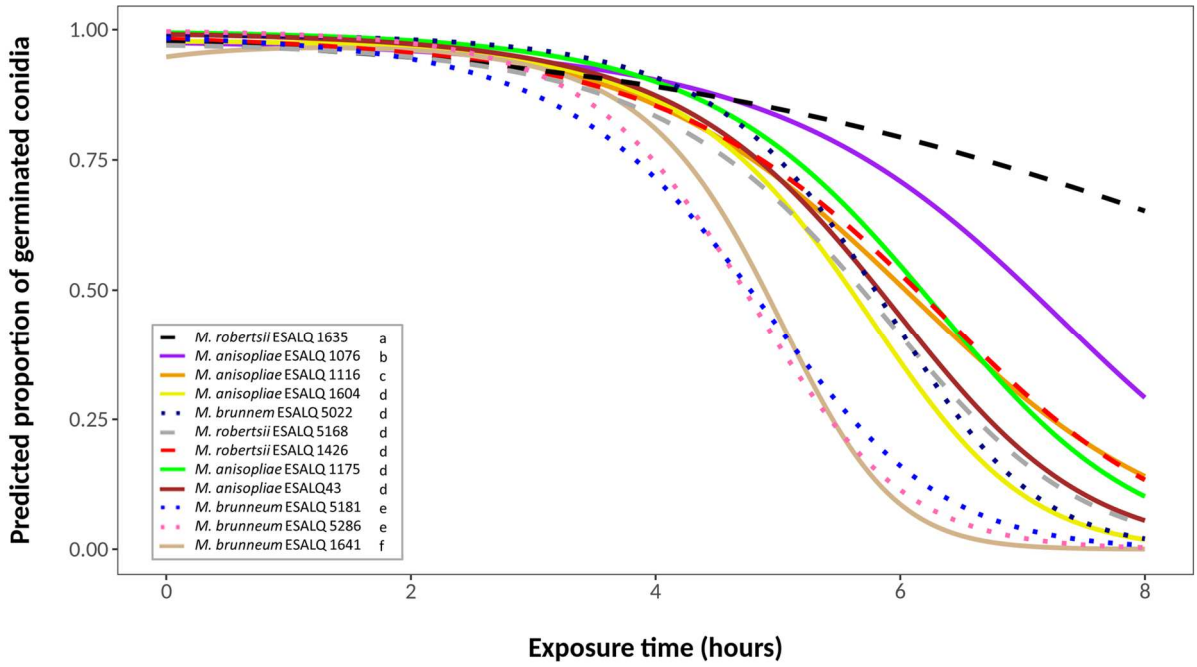

Figure 2. Predicted proportion of germinated conidia of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after UV-B exposure for 2, 4, 6 or 8 h, adopting a quasi-binomial model with logit link function (α = 0.05%).

2.3.2. Effect of temperature on mycelial growth of isolates

The selected model for the analysis of mycelial growth of the 12 isolates at 15 °C was a quadratic regression model; the hypothesis that it well represents the data was accepted after performing the lack of fit test, considering the isolate as a factor and measurement day as a quadratic term (P = 0.4058; Figure 2). Through a likelihood-ratio test to compare the slope (growth rate) and the intercept between curves, isolates were grouped according to their growth rate and/or intercept. The highest growth rates were achieved by isolates *M. brunneum* ESALQ 5181 and *M. robertsii* ESALQ 1426 (Group 1), followed by *M. brunneum* ESALQ 5022 (Group 2), *M. robertsii* ESALQ 5168 (Group 3), and ESALQ 1635 (Group 4). The six *M. anisopliae* isolates had the lowest growth rates at this temperature, although, comparing the curves, *M. brunneum* ESALQ 5286 was grouped with three of them (ESALQ 43, ESALQ 1641, and ESALQ 1116).

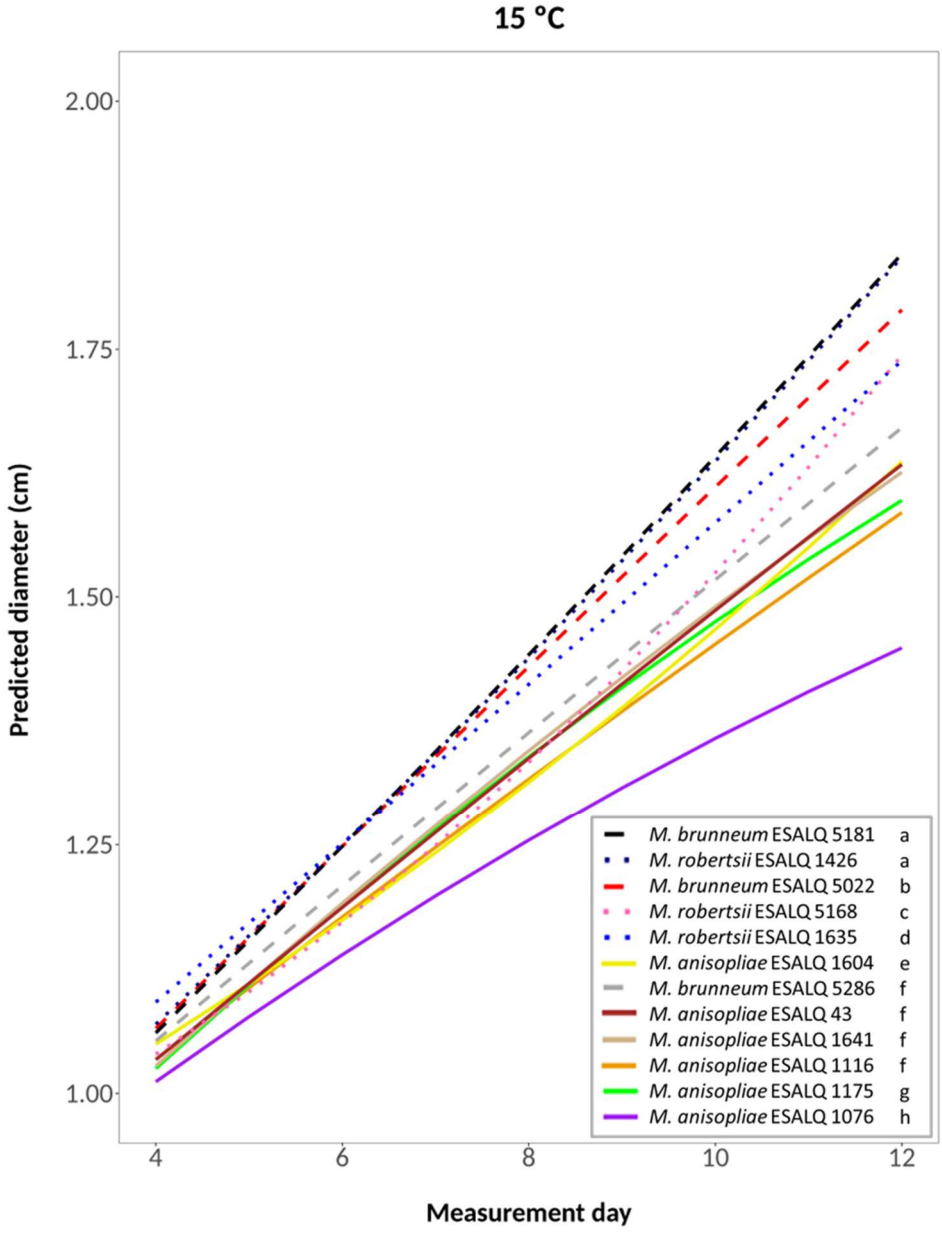

Figure 3. Predicted diameter of colonies of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after 12 days at 15 °C constant, adopting a quadratic model (α = 0.05%).

A fifth-degree polynomial model was selected for the temperature 20 °C constant (hypothesis accepted against a whole model: P = 0.8095; Figure 3). At this temperature, *M. robertsii* ESALQ 1426 and ESALQ 5168 had the highest growth rates, being grouped. Six other isolates were also grouped: *M. brunneum* ESALQ 5286 and *M. anisopliae* ESALQ 1641, ESALQ 1604, ESALQ 43, ESALQ 1175 and ESALQ 1116. The other isolates were considered individual groups. Growth was lowest for *M. anisopliae* ESALQ 1076 and *M. brunneum* ESALQ 5181.

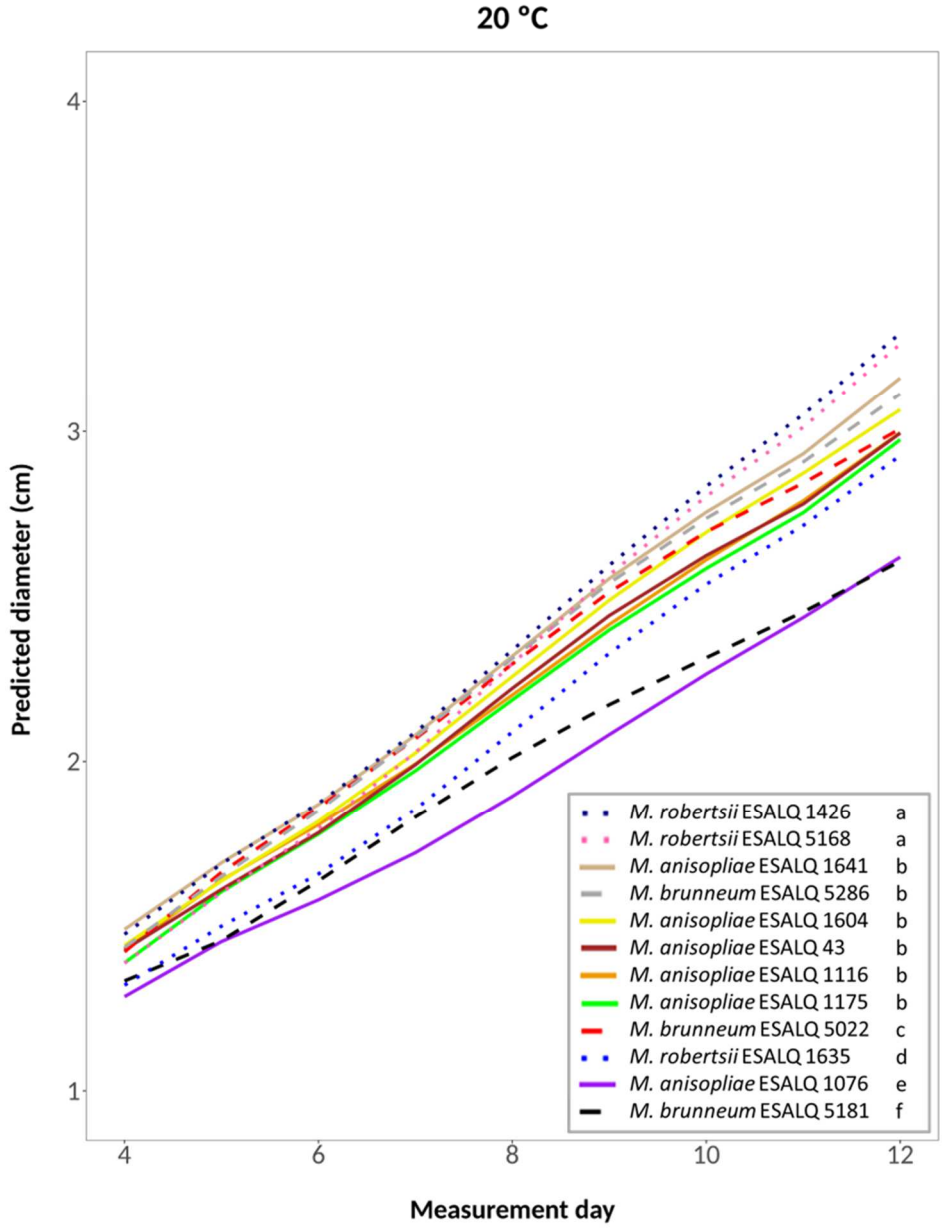

Figure 4. Predicted diameter of colonies of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after 12 days at 20 °C constant, adopting a fifth-degree polynomial model (α = 0.05%).

A cubic model was selected for the treatments at 25 °C constant, at 33 °C constant and the one alternating 20 and 33 °C (hypotheses accepted against a whole model, respectively: P = 0.2309, Figure 4; P = 0.3961, Figure 5; P = 0.3904, Appendix A). For these treatments, the three *M. robertsii* isolates had the fastest growth, with ESALQ 1426 and ESALQ 5168 being grouped with the highest rates under these three temperature conditions. Similarly, the three temperature regimes resulted in five isolates (*M. brunneum* ESALQ 5022, ESALQ 5181 and ESALQ 5286, and *M. anisopliae* ESALQ 1076 and ESALQ 1604) consistently showing the lowest growth rates (Figures 4-5, Appendix A).

In summary, the three *M. robertsii* isolates achieved higher growth at 33 °C, while isolates of *M. brunneum* and *M. anisopliae* had optimal growth rates at 25 °C. For all 12 isolates, mycelial growth was slower at 15 °C. A comparison of growth rates for each isolate is shown in Appendix B.

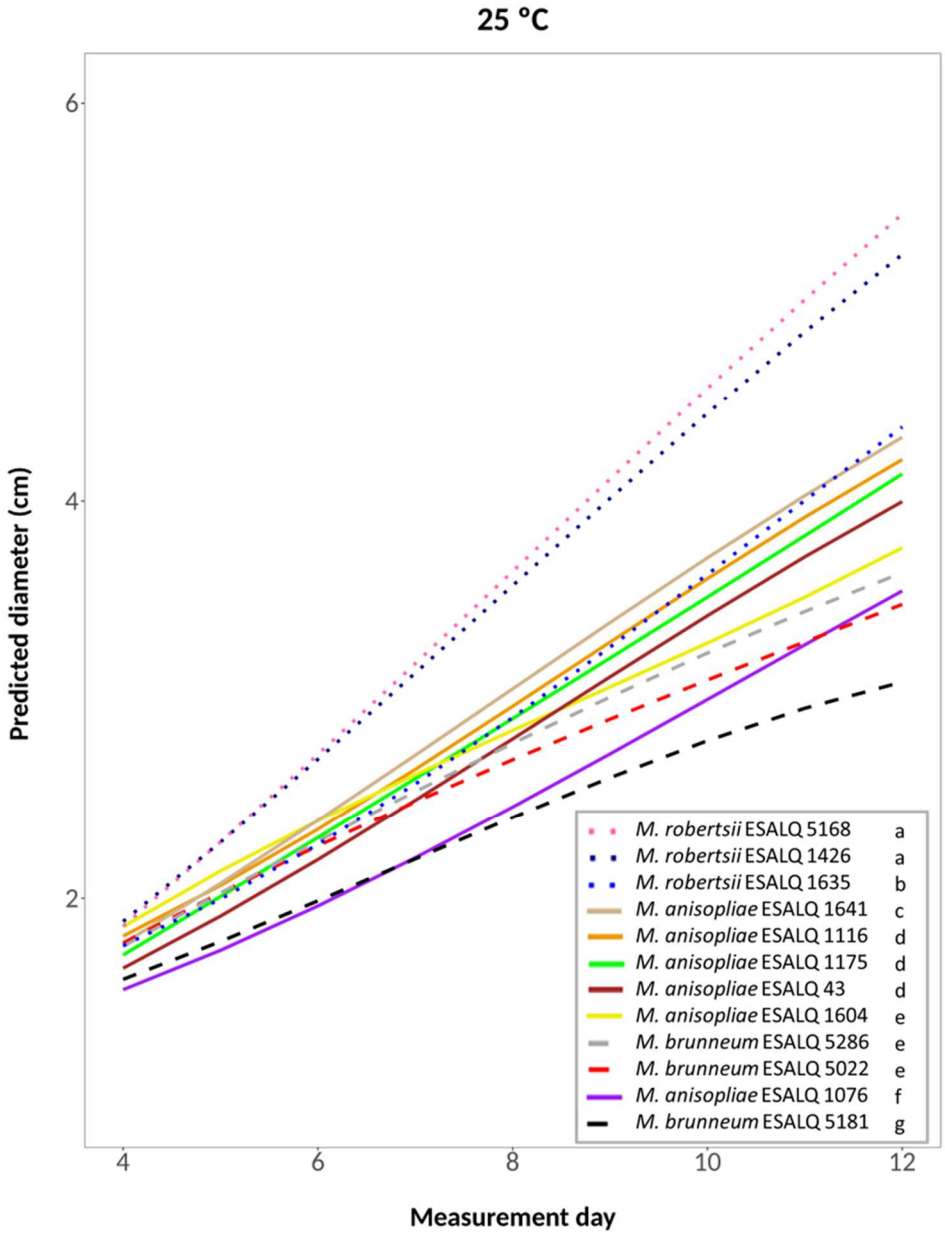

Figure 5. Predicted diameter of colonies of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after 12 days at 25 °C constant, adopting a cubic model (α = 0.05%).

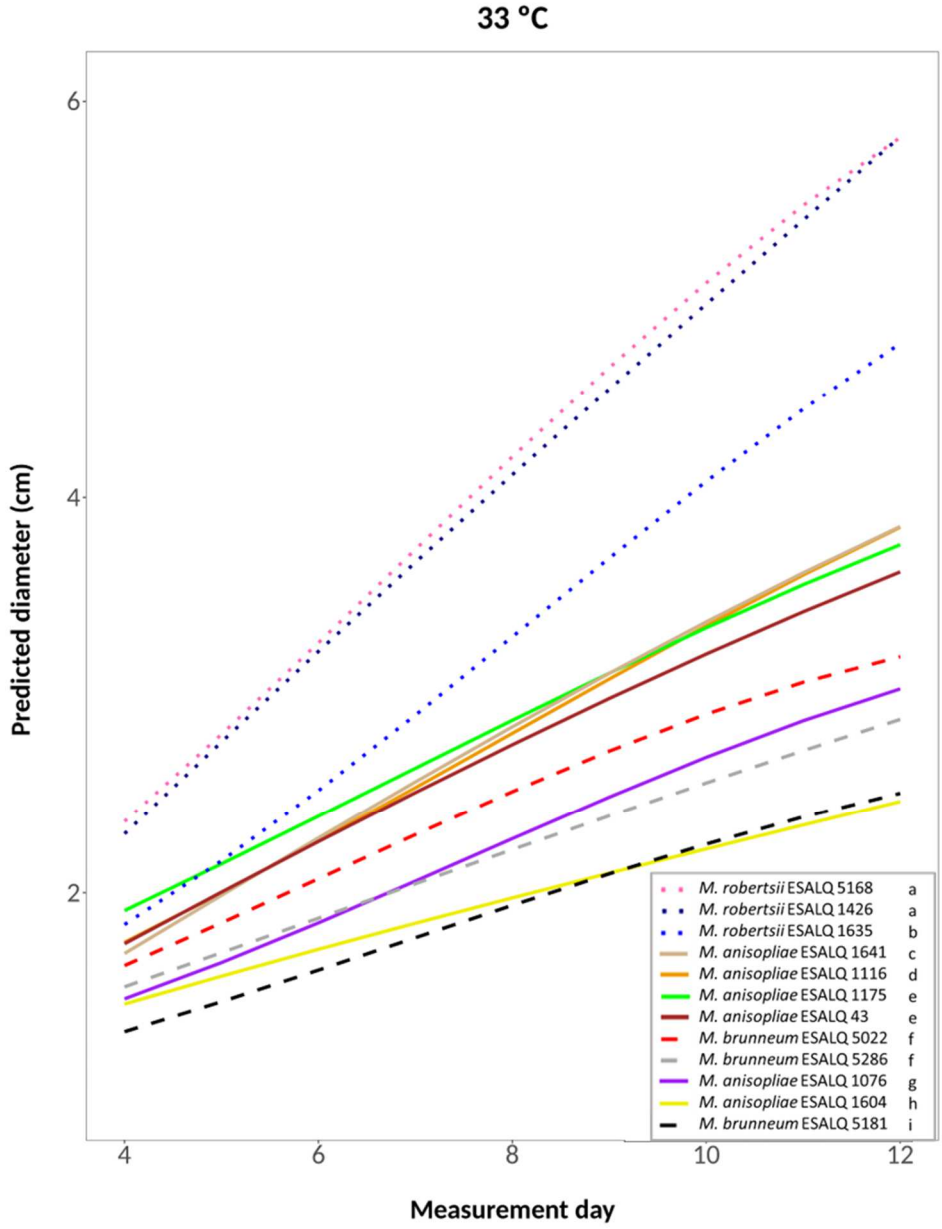

Figure 6. Predicted diameter of colonies of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after 12 days at 33 °C constant, adopting a cubic model (α = 0.05%).

2.3.3. Temperature affecting the survival of conidia

The beta-binomial model, with the parameters of the distribution location (mean) and scale (variance), brought a plausible realization of the data of temperature effects on conidial survival (Table 2). All 12 isolates maintained high viabilities at 20 and 25 °C, but the same did not occur at 40 °C: conidia of all *M. brunneum* and *M. robertsii* isolates exhibited very low proportions of survival, while the conidia of the *M. anisopliae* isolates showed higher tolerance.

Table 2. Survival of conidia (percentage) of *Metarhizium robertsii* (*Mr*), *M. brunneum* (*Mb*), and *M. anisopliae* s.str. (*Ma*) after 12 days of incubation at 20, 25 or 40 °C, adopting a beta-binomial distribution (α = 0.05%). Different letters in the same column indicate significant differences between isolates.

Conidial survival at 20 °C		**Conidial survival at 25 °C**		**Conidial survival at 40 °C**	
Species/Isolate	**%**	**Species/Isolate**	**%**	**Species/Isolate**	**%**
Mb ESALQ 5286	94.5 a	*Ma* ESALQ 43	94.3 a	*Ma* ESALQ 1175	66.6 a
Ma ESALQ 43	94.1 a	*Mb* ESALQ 5286	93.7 a	*Ma* ESALQ 1641	61.1 a
Ma ESALQ 1604	93.5 a	*Mb* ESALQ 5022	92.7 a	*Ma* ESALQ 1076	44.1 b
Mb ESALQ 5181	93.4 a	*Mb* ESALQ 5181	90.7 a	*Ma* ESALQ 43	34.4 b
Ma ESALQ 1076	91.7 a	*Ma* ESALQ 1175	89.6 a	*Ma* ESALQ 1116	26.6 b
Mr ESALQ 1426	91.3 a	*Ma* ESALQ 1116	89.5 a	*Ma* ESALQ 1604	8.9 c
Ma ESALQ 1641	91.3 a	*Mr* ESALQ 1426	89.2 a	*Mr* ESALQ 1635	5.1 c
Mb ESALQ 5022	90.8 a	*Ma* ESALQ 1604	89.1 a	*Mb* ESALQ 5286	4.7 c
Ma ESALQ 1175	90.6 a	*Mr* ESALQ 1635	89.0 a	*Mb* ESALQ 5022	3.8 c
Mr ESALQ 1635	90.4 a	*Ma* ESALQ 1076	89.0 a	*Mr* ESALQ 5168	1.8 d
Ma ESALQ 1116	89.8 a	*Mr* ESALQ 5168	86.9 a	*Mr* ESALQ 1426	1.7 d
Mr ESALQ 5168	84.5 b	*Ma* ESALQ 1641	86.4 a	*Mb* ESALQ 5181	0.5 d

A likelihood-ratio test was conducted for each temperature to check for similarities between isolates and according to the experimental design. The isolates were separated into two groups for the treatments at 20 °C (hypothesis accepted, $P = 0.4628$): one composed of the isolate *M. robertsii* ESALQ 5168, with a slightly lower predicted proportion of germination than the other 11 isolates. At 25 °C, no differences were found among the 12 isolates (hypothesis accepted, $P = 0.6220$). At 40 °C, it was possible to separate the isolates into four groups (hypothesis accepted, $P = 0.0652$) (in order of high to low tolerance): (i) *M. anisopliae* ESALQ 1175 and ESALQ 1641; (ii) *M. anisopliae* ESALQ 1076, ESALQ 43 and ESALQ 1116; (iii) *M. anisopliae* ESALQ 1604, *M. robertsii* ESALQ 1635, *M. brunneum* ESALQ 5286 and ESALQ 5022; and (iv) *M. robertsii* ESALQ 5168 and ESALQ 1426, *M. brunneum* ESALQ 5181.

In order to give an overview of all results, Table 3 contains a summary of the performance of each of the 12 isolates across all the parameters tested and shows that isolates of the same species can exhibit very different responses depending on environmental variables.

Table 3. Performance of each isolate of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. in three experiments involving exposure to UV-B radiation for up to 8 h, mycelial growth after 12 days under different temperature regimes (15 °C constant, 20 °C constant, 25 °C constant, 33 °C constant, and 20 °C for 16 h + 33 °C for 8 h), and conidial survival after 12 days at three temperatures (20 °C, 25 °C and 40 °C). In each column, groupings are delimited by closed cells (α = 0.05%). Tolerance order decreases from the top to the bottom of the table. Isolates of *M. robertsii* are colored in gray, *M. brunneum* in blue and *M. anisopliae* in green.

UV-B tolerance	Mycelial Growth 15 °C constant	Mycelial Growth 20 °C constant	Mycelial Growth 25 °C constant	Mycelial Growth 33 °C constant	Mycelial Growth 20 °C/16 h + 33 °C/8 h	Conidial Survival 20 °C	Conidial Survival 25 °C	Conidial Survival 40 °C
ESALQ 1635	ESALQ 5181	ESALQ 1426	ESALQ 5168	ESALQ 5168	ESALQ 5168	ESALQ 5286	ESALQ 43	ESALQ 1175
ESALQ 1076	ESALQ 1426	ESALQ 5168	ESALQ 1426	ESALQ 1426	ESALQ 1426	ESALQ 43	ESALQ 5286	ESALQ 1641
ESALQ 1116	ESALQ 5022	ESALQ 1641	ESALQ 1635	ESALQ 1635	ESALQ 1635	ESALQ 1604	ESALQ 5022	ESALQ 1076
ESALQ 1604	ESALQ 5168	ESALQ 5286	ESALQ 1641	ESALQ 1641	ESALQ 1175	ESALQ 5181	ESALQ 5181	ESALQ 43
ESALQ 5022	ESALQ 1635	ESALQ 1604	ESALQ 1116	ESALQ 1116	ESALQ 1641	ESALQ 1076	ESALQ 1175	ESALQ 1116
ESALQ 5168	ESALQ 1604	ESALQ 43	ESALQ 1175	ESALQ 1175	ESALQ 1116	ESALQ 1426	ESALQ 1116	ESALQ 1604
ESALQ 1426	ESALQ 5286	ESALQ 1116	ESALQ 43	ESALQ 43	ESALQ 43	ESALQ 1641	ESALQ 1426	ESALQ 1635
ESALQ 1175	ESALQ 43	ESALQ 1175	ESALQ 1604	ESALQ 5022	ESALQ 5022	ESALQ 5022	ESALQ 1604	ESALQ 5286
ESALQ 43	ESALQ 1641	ESALQ 5022	ESALQ 5286	ESALQ 5286	ESALQ 5286	ESALQ 1175	ESALQ 1635	ESALQ 5022
ESALQ 5181	ESALQ 1116	ESALQ 1635	ESALQ 5022	ESALQ 1076	ESALQ 1076	ESALQ 1635	ESALQ 1076	ESALQ 5168
ESALQ 5286	ESALQ 1175	ESALQ 1076	ESALQ 1076	ESALQ 1604	ESALQ 1604	ESALQ 1116	ESALQ 5168	ESALQ 1426
ESALQ 1641	ESALQ 1076	ESALQ 5181	ESALQ 5181	ESALQ 5181	ESALQ 5181	ESALQ 5168	ESALQ 1641	ESALQ 5181

2.4. Discussion

Solar radiation, especially of the UV-B type, is very harmful to fungal propagules, significantly affecting their survival and efficacy against insects in the environment (Acheampong et al., 2020; Fernandes et al., 2015; Ignoffo and Garcia, 1992; Inglis et al., 2001). Previous reports (e.g. Braga et al., 2001a, 2001c; Fernández-Bravo et al., 2017) showed that 2 h of exposure to UV-B light (irradiances of 920 or 1200 mW m^{-2}, corresponding to total doses of 6.6 or 8.6 kJ m^{-2}, respectively) was enough to substantially reduce conidial culturability (in some cases, more than 50%) of *Metarhizium* spp. isolates, including some of Brazilian origin. In the present study, it was only after 6 h of exposure (total dose of 14.28 kJ m^{-2}) that the germination rates were reduced for

almost all 12 isolates of *Metarhizium* spp. from Brazil, with five of them becoming completely inactivated after 8 h of exposure (total dose of 19.04 kJ m^{-2}).

Considerable intra- and inter-specific variability in UV-B tolerance was found among the 12 isolates. Similar observations were reported by Fargues et al. (1996), who compared the survival of conidia of isolates of *Metarhizium flavoviride*, *Beauveria bassiana*, *M. anisopliae* s.l. and *Cordyceps fumosorosea* (= *Isaria fumosorosea*, *Paecilomyces fumosoroseus*) and found different degrees of tolerance between and within species (e.g., for *B. bassiana* isolates, survival ranged from 0 to 100%). Furthermore, Fernandes et al. (2007) found a large range of resilience to UV-B radiation testing 59 *Beauveria* spp. isolates, while Huang and Feng (2009) reported variable tolerances among 20 *B. bassiana* isolates, based on UV-B lethal doses (LD_{50}, LD_{75}, and LD_{95}; J cm^{-2}) after irradiation.

When investigating the association between the latitude of origin of *Beauveria* spp. isolates (most of them from Brazil) and their UV tolerance, Fernandes et al. (2007) reported a significant inverse correlation, i.e., the most UV tolerant isolates were from regions of lower latitude, where solar irradiation is more intense than at higher latitudes. Braga et al. (2001c), working with isolates of *M. anisopliae* s.l., reported a similar correlation and stated that natural selection for UV-B tolerance must have occurred with these isolates. In the present study, no apparent latitudinal gradient in UV-B tolerance could be seen, as the isolate most susceptible to UV-B radiation (ESALQ 1641) was obtained in a region of lower latitude, while the second (ESALQ 1076) and third (ESALQ 1116) most tolerant isolates were collected in areas of higher latitudes. Likewise, Fargues et al. (1996) did not find a correlation between the geographic origin of *B. bassiana* isolates and UV-B tolerance.

Our results also indicate no apparent relationship between UV-B tolerance and the substrate of isolation or fungal species. Isolates obtained from different substrates (i.e., insect, soil or root) or of varying *Metarhizium* species were grouped by the similarity of their survival curves (Figure 1), while others originating from the same type of substrate had angles, even if they were of the same species (e.g., *M. anisopliae* ESALQ 1076 and ESALQ 1175, both from meadow soil). This finding is in accordance with reports that used *Metarhizium* spp. and *Beauveria* spp., and a positive correlation involving UV-B tolerance and isolation substrate or fungal species has yet to be found (Fernandes et al., 2007; Fernández-Bravo et al., 2016, 2017).

One *M. robertsii* isolate (ESALQ 1635) was by far the most UV-B tolerant, and some isolates of the three species had similar conidial survival curves. This indicates that tolerance to UV-B radiation was not selected at the species level among those considered adapted for the above-ground environment. Evidence suggests that a difference in habitat (e.g., phylloplane

versus soil) does not influence the ability of isolates to resist elevated levels of UV radiation (Fernández-Bravo et al., 2016, 2017).

Many reports about the effects of temperature on *Metarhizium* spp. isolates state that the optimal temperature for growth ranges between 25 and 30 °C (Acheampong et al., 2020; Dimbi et al., 2004; Ekesi et al., 1999; Zimmermann, 2007). Our data for *M. anisopliae* and *M. brunneum* isolates corroborate this and show that the best temperature for their mycelial growth *in vitro* was 25 °C. At temperatures higher than 30 °C, isolates can grow, but there is a decrease in growth rate, and generally, growth ceases at 35 °C (Acheampong et al., 2020; Ouedraogo et al., 1997). These authors reported an isolate of *M. anisopliae* s.l. with an optimum growth temperature of 28–32 °C. Welling et al. (1994) studied an isolate of *M. flavoviride* that achieved the highest growth at 30–34 °C. Similarly, our three *M. robertsii* isolates had the highest growth rates at 33 °C, indicating higher thermotolerance of the mycelium than for the *M. anisopliae* and *M. brunneum* isolates.

Overall, the three isolates of *M. robertsii* showed relatively high growth rates, the three isolates of *M. brunneum* had the lowest growth rates, and the six *M. anisopliae* isolates were intermediate. However, considerable intraspecific variability was found for the isolates of all three species. Ouedraogo et al. (1997) also reported within-species variability between isolates of *M. anisopliae* s.l. and *M. flavoviride*.

An association between thermotolerance and habitat of origin was reported for species such as *M. anisopliae* s.l., *M. flavoviride* and *M. rileyi* (= *Nomuraea rileyi*), with isolates from tropical or subtropical regions being more tolerant and having higher growth rates at higher temperatures than isolates from temperate areas (Fargues et al., 1992; Vidal et al., 1997). The performance of the three *M. brunneum* isolates under different temperatures in this study, the high prevalence of this species in temperate zones such as North America and Europe (where temperatures are cooler), and their low occurrence in South America (Rezende et al., 2015; Steinwender et al., 2014) might indicate that this species is not adapted to tolerate high temperatures. Our isolates were obtained from tropical and subtropical regions in Brazil, and an association between thermotolerance and latitude of origin could not be found in our study (e.g., *M. robertsii* ESALQ 1426, from south Brazil, was more tolerant to 33 °C than isolates from northeast Brazil). Other studies also reported the absence of a relationship between thermotolerance and isolate geographic origin (*Metarhizium* spp. and *B. bassiana*) (De Croos and Bidochka, 1999; Devi et al., 2005; Rangel et al., 2005). Furthermore, no association was found between tolerance and substrate of isolation (e.g., *M. robertsii* ESALQ 1426, from soil, and *M. robertsii* ESALQ 5168, from insects, had the highest growth rates and grouped at all temperatures except at 15 °C).

Exposure time to certain temperatures is essential when considering the tolerance of an isolate. Welling et al. (1994) reported that two out of three *Metarhizium* spp. isolates showed higher growth in a temperature cycle of 16 h/25 °C + 8 h/34 °C compared to 34 °C constant, and all of them were able to grow at temperatures above 40 °C when the temperature cycle included a period of 16 h at 25 °C, although with a decrease in growth rate compared to treatments with lower maximal temperatures (e.g., 25 °C constant or cycle of 16 h/25 °C + 8 h/30 °C). In our study, we used a cycle of 8 h at 33 °C and 16 h at 20 °C, and even though the three isolates of *M. brunneum* and two of the *M. anisopliae* isolates did not grow well at 33 °C constant, growth was numerically higher than with alternating temperatures.

The viabilities of dried conidia were not significantly affected by treatments at 20 or 25 °C, varying in germination rates between 84 and 95% after 12 days, with only a slight reduction. On the other hand, there was an apparent effect of high temperature, as dried conidia of all 12 isolates showed a significant decrease in survival at 40 °C. This lower viability can be explained by the fact that high temperatures can retard and inhibit conidial germination and cause growth termination (Inglis et al., 2001; Walstad et al., 1970). Conidia of two isolates of *M. anisopliae* s.l. submitted to a range of temperatures (-20 to 37 °C) survived for longer periods in the lower range (Daoust and Roberts, 1983), and dried conidia of an isolate of *M. acridum* (reported as *M. flavoviride* by the authors) also had a higher germination percentage after being stored in colder conditions compared to storage at higher temperatures (Moore et al., 1996). Clerk and Madelin (1965) evaluated the conidial survival of an isolate of *M. anisopliae* s.l. at 8, 18 and 25 °C under different RH conditions, and treatments at 25 °C affected conidia the most. A reduction in the temperature of storage can increase the survival of conidia (Clerk and Madelin, 1965; Walstad et al., 1970).

In agreement with other studies (Fargues et al., 1997; Rangel et al., 2005), and similar to what we found about UV-B radiation, there was no apparent correlation observed between conidial thermotolerance and latitude or substrate of isolation. Our hypothesis relating this tolerance to fungal species was not fully met since ESALQ 1604 (*M. anisopliae*) exhibited similar conidial survival to ESALQ 1635 (*M. robertsii*), and ESALQ 5022 and ESALQ 5181 (*M. brunneum*). However, it is worth noting that the other five *M. anisopliae* isolates had significantly higher conidial survival proportions at 40 °C. Rangel et al. (2005) found some variability in wet and dry heat tolerance of conidia among isolates of *Metarhizium* species exposed to 40 and 45 °C, with *M. acridum* isolates exhibiting a significantly higher tolerance compared to isolates of *M. anisopliae* s.l., *M. robertsii* or *M. brunneum*.

Although our experimental time was relatively short, it was enough to reduce conidial viabilities of some isolates below 5%, evidencing their susceptibility to high temperatures, an undesirable characteristic for biological control purposes in field conditions, especially in tropical countries (such as Brazil) where the air temperatures can reach above 40 °C in the summer. Conidial survival at such high temperatures can be increased by drying and developing formulations with oils (Moore et al., 1996; Paixão et al., 2017).

Mycelia comprises a mass of hyphae related to the absorption of nutrients, vegetative growth, and plant associations. In contrast, conidia are asexual, infective spores capable of persisting in the environment in the absence of a host, forming reservoirs of inoculum (Behie et al., 2012; Evans and Hywel-Jones, 1997; Hesketh et al., 2010; Vega et al., 2012). Because of this, conidia are more subjected to adverse environmental conditions than mycelia; thus, they should exhibit increased tolerance to abiotic factors to improve their survival. Our data showed that mycelial growth rates of the three isolates of *M. robertsii* were higher at the highest temperature tested, but their conidia did not tolerate high temperature or UV-B radiation. On the other hand, the opposite result was obtained for most isolates of *M. anisopliae*, which produced conidia that showed increased tolerance to the stressful conditions tested, but the same could not be concluded for their mycelial growth *in vitro*. The fact that mycelia of *M. robertsii* isolates had optimum growth at 33 °C, while their conidia were not tolerant to storage at 40 °C (all three isolates) or UV-B radiation (ESALQ 1426 and ESALQ 5168), indicates a stronger selection pressure for mycelial growth rather than survival of conidia. This trait may have an essential role in their association with plant roots, where the active hyphae are the structure forming the association in the rhizosphere (Behie and Bidochka, 2014; Sasan and Bidochka, 2012). In contrast, conidia of *M. anisopliae* isolates could better tolerate the adverse experimental conditions, suggesting that adaptation for unfavorable conditions of these propagules is selected for, as they are important for survival in the environment and for infecting new hosts.

Although we found some variation in tolerance to UV-B radiation and high temperatures between isolates of the same species, similarities could also be seen, e.g., the three isolates of *M. robertsii* growing faster at 33 °C or conidia of isolates of *M. anisopliae* better tolerating incubation at 40 °C. The present study shows that isolates of each species represent biological plasticity and that isolates of the same species can exhibit very different responses to environmental variables, thus not making it possible to generalize results to the species level. A similar conclusion was reported by Canassa et al. (2020) concerning the ability of *Metarhizium* spp. isolates to interact with plants. Our study provides new insights into the ecology of entomopathogenic fungi and their adaptations for variation of important environmental abiotic

factors that can impact the fungi abundance and distribution patterns. Knowledge of such adaptations can provide the foundation for future selection of candidate isolates with high environmental resilience for the development of biological control agents.

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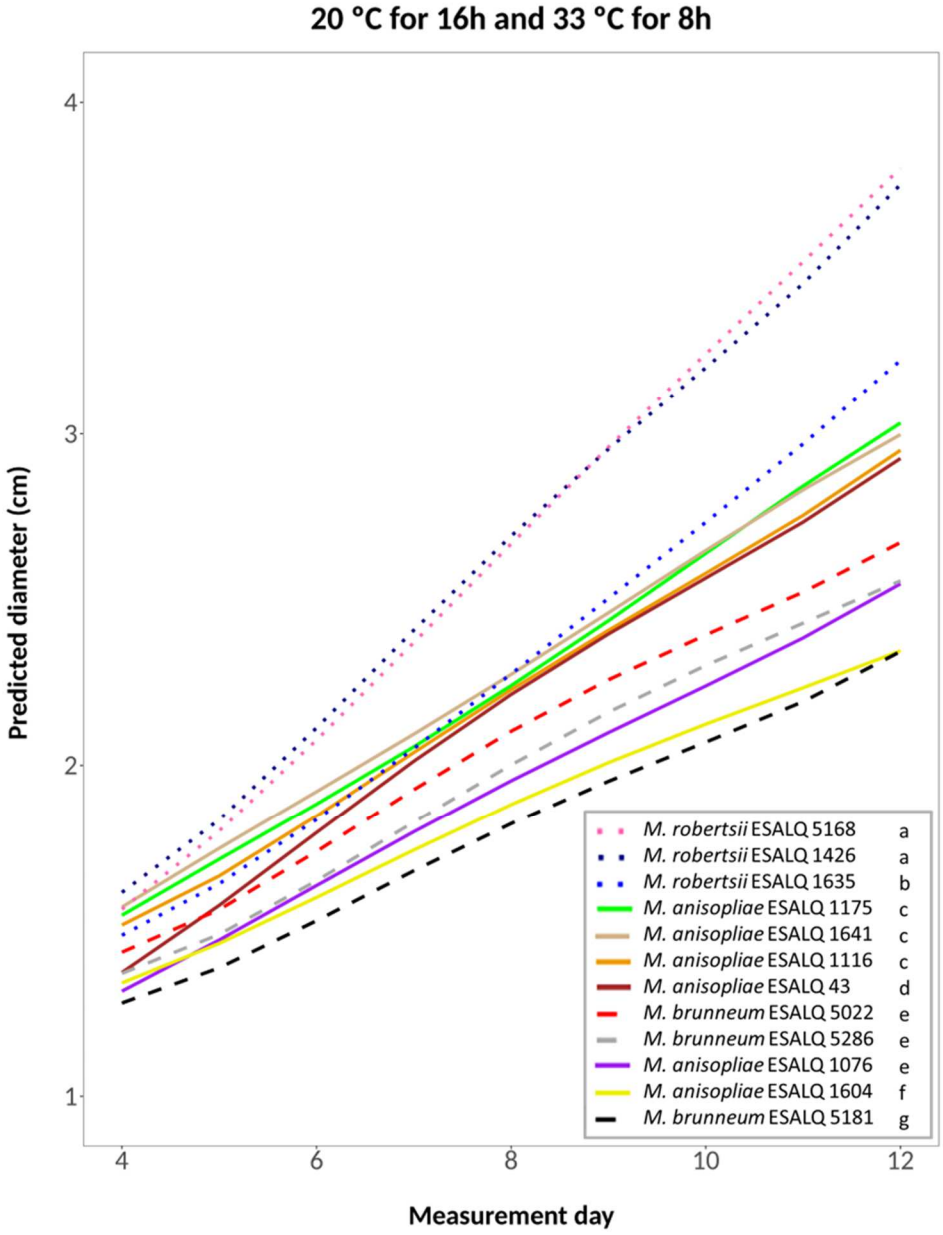
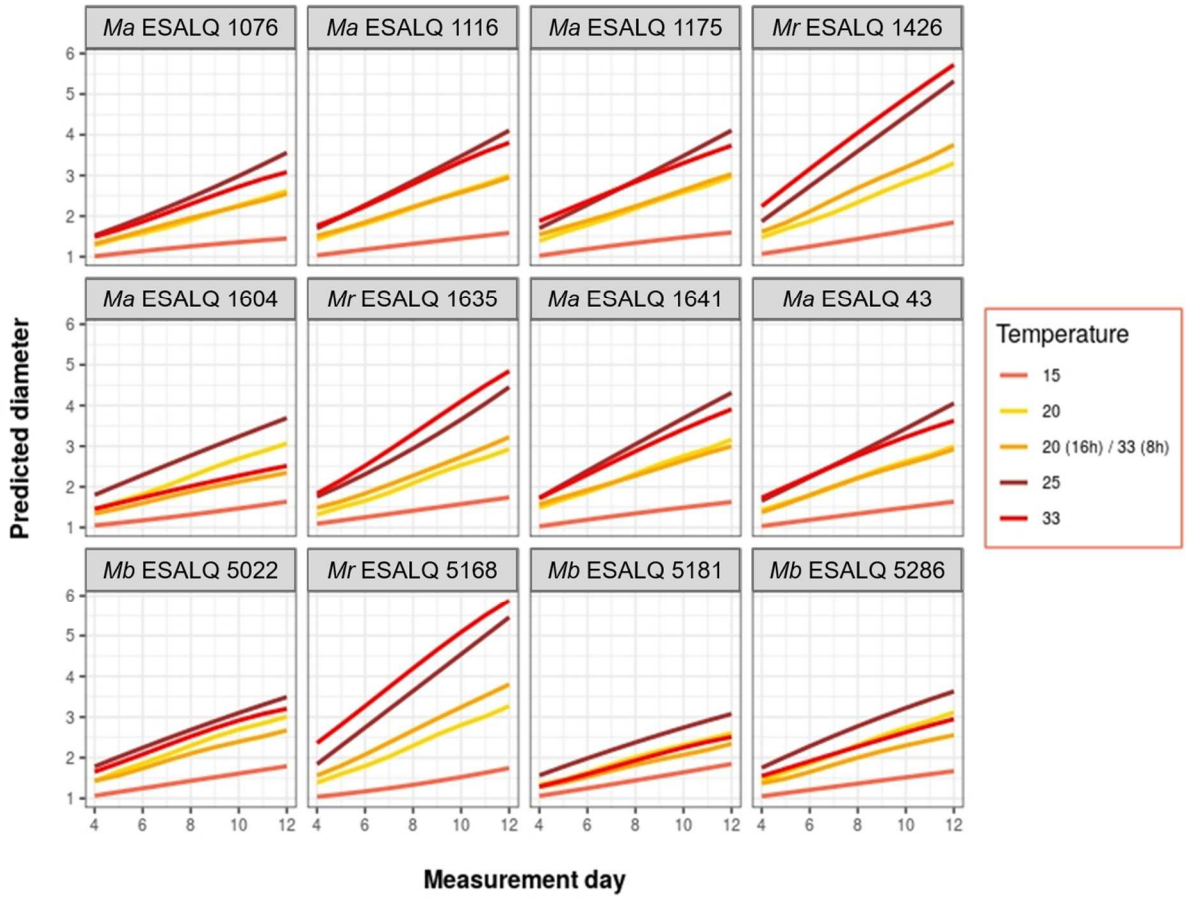
APPENDICES

APPENDIX A - Predicted diameter of colonies of isolates of *Metarhizium robertsii*, *M. brunneum* and *M. anisopliae* s.str. Mani 2 after 12 days in a cycle of 20 °C for 16 h and 33 °C for 8 h, adopting a cubic model (α = 0.05%).



APPENDIX B - Comparison of the predicted diameters of colonies of isolates of *Metarhizium robertsii* (*Mr*), *M. brunneum* (*Mb*) and *M. anisopliae* s.str. (*Ma*) after 12 days in 5 different temperature conditions (15 °C constant, 20 °C constant, 25 °C constant, 33 °C constant, cycle of 20 °C for 16 h and 33 °C for 8 h).



3. COMPARATIVE GENE EXPRESSION AND GENOMICS REFLECT GEOGRAPHICAL DIVERGENCE IN THE ENTOMOPATHOGENIC FUNGAL GENUS *Metarhizium*

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ABSTRACT

Several species within the fungal genus *Metarhizium* can both infect insects and colonize plant roots. In Brazil, a specific subgroup within *Metarhizium anisopliae* s.str. named "subclade Mani 2" is frequently observed infecting above-ground insects, whereas sympatric *M. robertsii* and *M. brunneum* predominantly occur in the soil environment. Genotypic variability within the genus may be linked to adaptations to these different habitats. Here, we present a comparative analysis of the complete genomes and the adhesin genes *Mad1* and *Mad2* of 14 *Metarhizium* isolates representing *M. anisopliae* Mani 2 (n=6), *M. robertsii* (n=5) and *M. brunneum* (n=3). In addition, the relative gene expression of six selected target genes was compared in root exudate solution and insect cuticle suspension. We hypothesized that *M. anisopliae* Mani 2 is adapted to insect-pathogenicity in the above-ground environment, reflected by higher relative expression of pathogenicity-related genes. In contrast, *M. robertsii* and *M. brunneum* are adapted to the soil environment, hence having a higher expression of genes related to plant associations. Phylogenomic and adhesin phylogenetic trees revealed species differences but also intraspecific variability associated with the geographic origin of isolates. Differences in relative gene expression were observed, with one pathogenicity-related gene (Pr1) being higher expressed in *M. anisopliae*. The *Mad1* gene was more conserved than *Mad2* and similarly expressed in exudate solution, while *Mad2* was highly expressed by all Brazilian isolates in both exudate and cuticle conditions. The variabilities observed correlated with different habitats and lifestyles, demonstrating the importance of selecting a diverse collection of isolates in genomic and gene expression studies.

Keywords: *Metarhizium*; phylogenomic; adhesin; *Mad1*; *Mad2*; gene expression

3.1. Introduction

Entomopathogenic fungi in the genus *Metarhizium* (Ascomycota: Hypocreales) can naturally infect and kill insects and are widely used as biological control agents (St. Leger and Wang, 2020). These fungi infect their hosts through the cuticle and present low risk to natural enemies, other non-host arthropods and vertebrates (Zimmermann, 2007a, 2007b).

Besides infecting insects, many entomopathogenic fungi of the order Hypocreales can associate intimately with plants in the rhizosphere or inside tissues as endophytes (Vega, 2008; Vega et al., 2008). The plant association of *Metarhizium* spp. was first reported for *Metarhizium anisopliae* (Metsch.) Sorokin by Hu & St. Leger (2002), who coined *M. anisopliae* as rhizosphere competent, and later other species of *Metarhizium* were reported to possess the ability to colonize plants (Behie et al., 2015; Wyrebek et al., 2011). During interaction with plant roots, it has been demonstrated that *Metarhizium* can supply nitrogen derived from infected insects in the soil to the plants and, in return, obtain carbohydrates exuded from the plant roots (Barelli et al., 2016; Behie et al., 2017, 2012; Behie and Bidochka, 2014). The fungus can also promote plant growth (Jaber and Enkerli, 2016a, 2016b; Sasan and Bidochka, 2012) and reduce above-ground herbivore populations feeding on fungal colonized plants (Canassa et al., 2020).

The fungal species *M. anisopliae*, *Metarhizium robertsii* Bischoff, Rehner & Humber and *Metarhizium brunneum* Petch can generally be found in soils of natural and managed ecosystems worldwide (Lacey et al., 2015). Although the three species can function as both entomopathogens and plant associates, an essential distinction regarding their ecological role in Brazil is emerging. While *M. anisopliae* is very abundant in the Cerrado biome in Brazil (Rocha et al., 2013), phylogenetic data of the 5' EF-1α region of isolates from the various areas of Brazil showed that *M. anisopliae* s.str. can be split into three subclades, namely Mani 1, Mani 2 and Mani 3, with isolates of Mani 2 usually being the only representatives infecting insects under field conditions, and Mani 1 and Mani 3 only found in the soil (Rezende et al., 2015). This indicates that Mani 2 isolates are mainly adapted for utilization of insects as resources. Overall, *M. robertsii* is the most prevalent *Metarhizium* species in Brazil, widely present in native and agricultural soils of different biomes throughout the country (Botelho et al., 2019). Under laboratory conditions, isolates of *M. robertsii* can effectively kill various species of insects, yet natural infections of insects by *M. robertsii* are rarely observed in Brazil (Lopes et al., 2013). Instead, *M. robertsii* is frequently reported to form associations with plant roots, indicating that isolates of this species are primarily adapted to

activities in the soil environment, including root colonization (Rezende et al., 2015; Sasan and Bidochka, 2012). While *M. brunneum* is abundant in agricultural soils in temperate regions of Europe, the species is rarely isolated in Brazil (Botelho et al., 2019; Brunner-Mendoza et al., 2019; Steinwender et al., 2014, 2015). Similar to *M. robertsii*, *M. brunneum* is mainly associated with the soil environment and, at least in temperate regions, is rarely observed infecting insects above ground in field conditions (Meyling et al., 2011; Steinwender et al., 2014, 2015).

The dual lifestyle of *Metarhizium* spp. exploiting both insects and plants as host organisms is possible partly because of adhesion proteins specific to different hosts. Wang, Hu & St. Leger (2005) detected a high frequency of two expressed sequence tags (ESTs), later categorized as the genes *Mad1* (*Metarhizium* adhesin-like protein 1) and *Mad2* (*Metarhizium* adhesin-like protein 2), when an isolate of *M. anisopliae* was grown in culture medium containing hemolymph of *Manduca sexta* or bean root exudate, respectively. Experiments with knockout mutants of *M. anisopliae* and mutant yeasts verified the role of these two genes in the adhesion of conidia to either insect or plant host surfaces and, in consequence, the ability of these fungi to infect insects or to associate with plants (Wang and St. Leger, 2007).

While genes (and their encoded proteins) involved in the endophytic ability of *Metarhizium* species are still mostly unknown (Branine et al., 2019), the functions of proteins relevant in the insect pathogenicity process, such as subtilisins and hydrophobins, are described (Goettel et al., 1989; Sevim et al., 2012; Small and Bidochka, 2005; St. Leger et al., 1988a). Pr1A, a subtilisin-like serine protease member of the family Pr1 (Bagga et al., 2004; Freimoser et al., 2003), is the main protein produced during penetration of the host cuticle and it is a determinant factor of pathogenicity (Goettel et al., 1989; St. Leger et al., 1988a, 1989). The gene *Pr1A* is upregulated under nutrient-deprived conditions and downregulated in nutrient-rich conditions, such as in the insect hemolymph (Small and Bidochka, 2005; St. Leger et al., 1988b, 1989).

Hydrophobins are amphiphile proteins exclusive to the kingdom fungi, with an important role related to surface forces (Bayry et al., 2012; Linder et al., 2005). They form rodlet layers which cover fungal aerial structures, such as conidia, rendering their surface hydrophobic, and in *Metarhizium* spp., these proteins have a role in adherence to the host cuticle, together with MAD1 (St. Leger et al., 1992b; St. Leger and Wang, 2020).

Studies of gene expression and genomics allow us to understand how genes are regulated depending on the growth conditions of an organism and to identify relevant intra- and interspecific variations in the genomes of individuals. A comparative genomic study showed that *M. robertsii* (reported as *M. anisopliae* at the time) encodes more subtilisins, trypsins, chitinases, dehydrogenases and cytochrome P450s than *Metarhizium acridum*, which was related to their

presumed lifestyles: *M. robertsii* is a generalist species with a broad host range, thus the higher number of these proteins in the genome allows this species to deal with multiple types of hosts (Gao et al., 2011). On the other hand, the genome of *M. acridum* contains a reduced number of proteins involved in cuticle degradation and detoxification due to its limited host range as a specialist pathogen of locusts (Gao et al., 2011). Investigating differential gene expression of *M. robertsii* under several conditions, Barelli, Padilla-Guerrero & Bidochka (2011) reported that *M. robertsii* up- or downregulated the expression of *Mad1*, *Mad2* and other selected genes according to the composition, temperature and pH of the culture medium.

Genomic studies have spearheaded the identification of relevant protein families (and their quantity) in the genus *Metarhizium* (St. Leger and Wang, 2020). Suitable examples include: cytochrome P450s, which are involved in the general metabolism of organisms, including detoxification, and are also related to the production of secondary metabolites and pathogenicity in fungi (Chen et al., 2014); MRT, a raffinose transporter fundamental for the rhizosphere competency of *M. robertsii* (Fang and St. Leger, 2010); and the modular enzymes polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Dutta et al., 2014; Miller and Gulick, 2016), involved in the production of secondary metabolites which may act during infection in hosts or against other microorganisms (Molnár et al., 2010; Zimmermann, 2007a).

However, species of the genus *Metarhizium* exhibit significant genotypic variation, and even isolates of the same species show some degree of genetic variability, potentially linked to their adaptation to different habitats (Bidochka et al., 2001). Nevertheless, much of the current knowledge about genomic variation and gene expression within and between *Metarhizium* species are based on studies of a limited number of isolates (*M. robertsii* ARSEF 23 and ARSEF 2575, *M. anisopliae* ARSEF 549, *M. acridum* CQMa 102). Therefore, it may not be straightforward to extrapolate results obtained from a single isolate to the population level or conclude about adaptations of particular species. For example, Wyrebek & Bidochka (2013) showed that *Mad2* had diverged more than *Mad1* among *Metarhizium* spp. when studying 14 isolates mostly of North American origin. The authors suggested that this divergence represented selection for plant association rather than for insect hosts as a main adaptive trait within individual species. If so, we should expect *M. anisopliae* Mani 2, which potentially is more adapted to explore insect resources, to have more variation in *Mad1*, while Brazilian isolates of *M. brunneum* and *M. robertsii*, which are considered to be more adapted to the soil/rhizosphere environment, would exhibit higher variation in *Mad2*, similar to what Wyrebek & Bidochka (2013) reported. Although these authors found low intraspecific variability for both *Mad1* and *Mad2* in *M. brunneum* and *M. robertsii*, a limited number of isolates was tested (two and five, respectively), and all but one had the same

origin (Ontario, Canada). The higher variation found for *Mad1* and *Mad2* in *M. guizhouense* (two isolates from Ontario and one from China) indicates that sampling from different locations may reveal intraspecific variation not yet reported (Wyrebek and Bidochka, 2013).

In the present study, we therefore selected twelve isolates of Brazilian origin belonging to *M. anisopliae* Mani 2 (n=6), *M. brunneum* (n=3) and *M. robertsii* (n=3), and compared their genomes and specific gene sequences against two well-described reference isolates of *M. robertsii* from the USA. In addition, the expression of selected genes for the 14 isolates was evaluated. First, we sequenced the genomes of all isolates to provide comparative information on their genetic distances and abundance of selected protein families between and within the three species. Then, because of their relevance in the adhesion of conidia to host surfaces, we sanger-sequenced the coding sequences of genes *Mad1* and *Mad2* to verify their genetic sequences and assess the variation among all isolates through the construction of gene phylogenies. Finally, the variation in expression of six target genes was evaluated after inoculating the isolates into a root exudate solution or an insect cuticle suspension. We hypothesized that: (i) pathogenicity-related genes would be highly expressed in *M. anisopliae* Mani 2 compared to *M. brunneum* and *M. robertsii* since isolates of Mani 2 are more frequently isolated from insects in field conditions; (ii) the expression of an endophytism-related gene would be higher in *M. brunneum* and *M. robertsii* than in *M. anisopliae* Mani 2, as they are usually associated to plants in the soil; and (iii) the expression of constitutive genes would be similar in the three species. Knowledge of variation in genome composition and gene expression provides insight into the adaptation of entomopathogenic fungi to their environment, identification of potential constraints in the application of fungal isolates for biological control, as well as for the identification of target genes that could be used for future bioengineering approaches.

3.2. Materials and methods

3.2.1. Fungal isolates

Fourteen isolates of *Metarhizium* spp. (six of *M. anisopliae* subclade Mani 2, five of *M. robertsii* and three of *M. brunneum*) were selected: 12 from the Entomopathogen Collection "Prof. Sérgio Batista Alves", of the Laboratory of Pathology and Microbial Control of Insects (Luiz de Queiroz College of Agriculture – University of São Paulo, Piracicaba, SP, Brazil), and two from the USDA Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA) (Table 1, Figure 1).

Table 1. List of isolates used in the study. ESALQ isolates were obtained from the Entomopathogen Collection "Prof. Sérgio Batista Alves" (ESALQ/USP, Piracicaba, São Paulo, Brazil), and ARSEF isolates from the USDA Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA).

Species	Isolate code	Origin	Collection site (state, country)
Metarhizium anisopliae s.str. subclade Mani 2	ESALQ 43	Hemiptera: Cercopidae	Alagoas, Brazil
	ESALQ 1116	Coleoptera: Scarabaeidae	São Paulo, Brazil
	ESALQ 1641	Hemiptera: Cercopidae	Alagoas, Brazil
	ESALQ 1076	Meadow soil	Paraná, Brazil
	ESALQ 1175	Meadow soil	São Paulo, Brazil
	ESALQ 1604	Biotech G, Biotech® Controle Biológico (commercial isolate)	-
Metarhizium brunneum	ESALQ 5022	Sugarcane soil	São Paulo, Brazil
	ESALQ 5286	Sugarcane soil	São Paulo, Brazil
	ESALQ 5181	Sugarcane root	São Paulo, Brazil
Metarhizium robertsii	ESALQ 1426	Soybean soil	Paraná, Brazil
	ESALQ 1635	Native forest soil	Alagoas, Brazil
	ESALQ 5168	Coleoptera: Scarabaeidae	São Paulo, Brazil
	ARSEF 23	Coleoptera: Elateridae	North Carolina, USA
	ARSEF 2575	Coleoptera: Curculionidae	South Carolina, USA

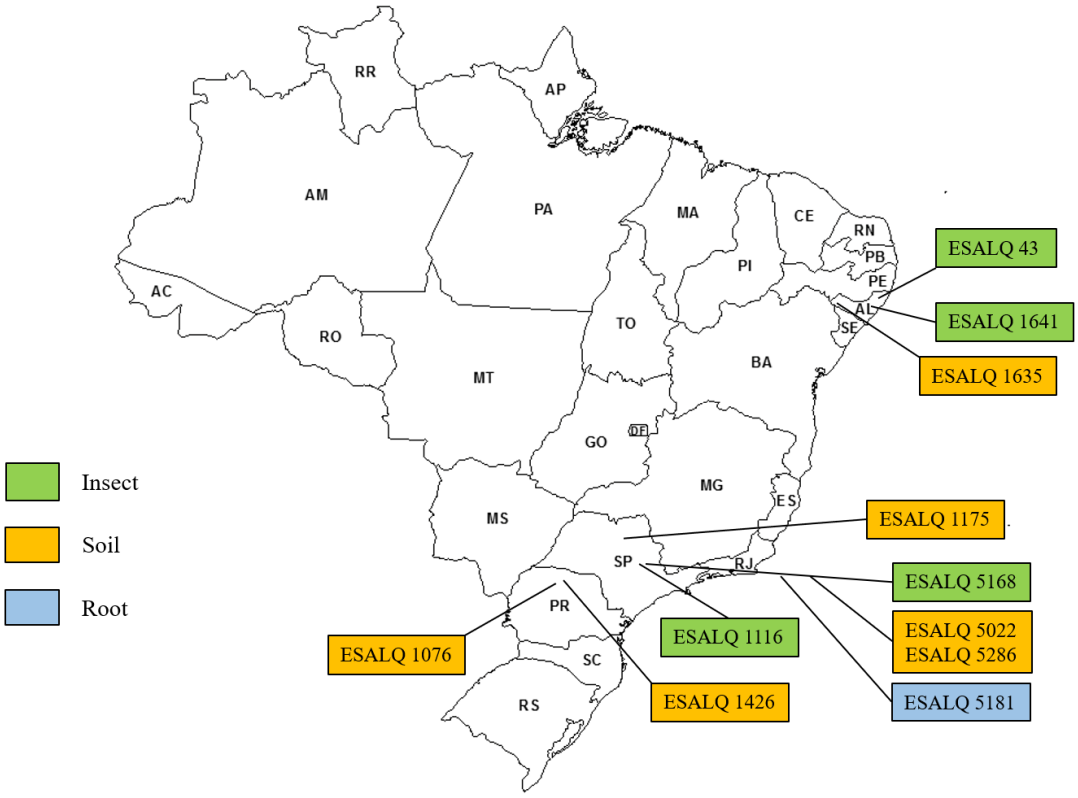

Figure 1. Location of the collection sites in Brazil of ESALQ isolates of *Metarhizium anisopliae* s.str. Mani 2 (ESALQ 43, ESALQ 1076, ESALQ 1116, ESALQ 1175 and ESALQ 1641), *M. brunneum* (ESALQ 5022, ESALQ 5181 and ESALQ 5286) and *M. robertsii* (ESALQ 1426, ESALQ 1635 and ESALQ 5168) used in the present study. Green boxes indicate isolates obtained from insect hosts, orange boxes indicate isolates obtained from soil samples, and the blue box indicates isolate obtained from root sample. Isolate ESALQ 1604 (*M. anisopliae*) is a commercial isolate with an unknown collection site, thus it is not represented in the map.

3.2.2. Whole-Genome Re-sequencing (WGR)

3.2.2.1. DNA extraction and sequencing

Isolates were cultivated in culture media Sabouraud Dextrose Agar (SDA) for 7–10 days, and then conidia were harvested and inoculated in Erlenmeyer flasks containing 20 mL of YPD broth (0.2% yeast extract, 2% peptone, 3% dextrose), which were incubated on a rotary shaker for three days (170 rpm, 20-23 °C). The resulting fungal material was vacuum filtered and lyophilized overnight.

The fresh, dried material was crushed into a powder and put in Eppendorf tubes, in which 500 µL of CTAB buffer, 1 µL of 2-mercaptoethanol, 3 µL of RNase (diluted to 10 mg ml^{-1}) and 3 µL of proteinase K were added per sample, then the tubes were placed in a heat block at 60 °C for 1 h. Next, 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) were added to each

sample, shaking the tubes slightly and centrifuging at 11,000 rpm and 4 °C for 10 min. Approximately 400 μL (2 x 200 μL) of the aqueous upper phase were transferred to new tubes, in which 500 μL of chloroform/isoamyl alcohol (24:1) were added, followed by centrifugation at 11,000 rpm and 4 °C for 10 min. After this, 300 μL of isopropanol were added to the samples, which were mixed and put in a freezer (-20 °C) overnight. The following day, samples were centrifuged at 11,000 rpm and 4 °C for 10 min before supernatants were discarded, and the pellets were washed with 500 μL of absolute ethanol and centrifuged at 11,000 rpm and 4 °C for 10 min. The ethanol was discarded, and the samples were washed twice with 70% ethanol. The tubes were air-dried, and lastly, DNA was dissolved in 40–50 μL of 1xTE buffer (pre-heated at 60 °C). The material was quantified on a Qubit 4 (Thermo Fisher Scientific) and sent to BGI Europe A/S (Copenhagen, Denmark), which performed short insert fragment library preparation and PE150 sequencing on BIGSEQ (DNBseq technology), as well as quality control (removing adaptors and low-quality readings). Raw reads are deposited in the Sequence Read Archives, under the BioProject PRJNA746571, and their accession numbers are shown in Appendix A.

3.2.2.2. Mapping of the reads

The reference genomes selected for mapping were ARSEF 23 for *M. robertsii* (GenBank accession number ADNJ00000000), ARSEF 549 for *M. anisopliae* (GenBank accession number AZNF00000000) and ARSEF 3297 for *M. brunneum* (GenBank accession number AZNG00000000). Alignment of reads to the reference genomes was performed using BWA version 0.7.17, applying the algorithm BWA-MEM on paired-end mode (Li, 2013; Li and Durbin, 2009). SAMtools version 1.11 was used to sort and remove duplicate reads (Li et al., 2009), and the quality of mapped reads was assessed with Qualimap version 2.2.2 (Okonechnikov et al., 2016). A consensus sequence for each isolate was obtained using RGAAT version 1.0 (Liu et al., 2018), and their sizes were determined by assembly-stats version 1.0.1 (https://github.com/sanger-pathogens/assembly-stats). Estimation of genome completeness was performed using BUSCO version 4.1.3 (Seppey et al., 2019), selecting the dataset for Hypocreales version odb10.2019-11-20 as a reference.

3.2.2.3. Annotation

Genomes were annotated with AUGUSTUS version 3.3.3, trained with the reference genomes aforementioned (Hoff and Stanke, 2013; Stanke and Morgenstern, 2005). Functional

annotation of proteins was performed using InterProScan 5.46-81.0, running the Pfam analysis (Mitchell et al., 2019).

3.2.2.4. Assessment of orthologues and phylogenomic analysis

Orthology inference was performed using OrthoFinder version 2.3.12 (Emms and Kelly, 2019). A maximum-likelihood phylogenomic tree was constructed with the sequences of the orthogroups, utilizing the software IQ-TREE version 2.0.3 (Nguyen et al., 2015) implementing the substitution model JTT+F+R5, determined as the best-fit by ModelFinder (option -m MFP) (Kalyaanamoorthy et al., 2017), based on AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion), and with branch supports using the ultrafast bootstrap (Hoang et al., 2018) with 1,000 bootstrap replicates. The *M. acridum* isolate CQMa 102 was used as an outgroup. FigTree version 1.4.4 was used for editing the tree.

3.2.3. Coding sequences of genes *Mad1* and *Mad2*

Fungal pellets were obtained similarly to described under 2.2.1. Small pellets of each isolate were put in Eppendorf tubes and crushed into a powder. Next, 500 µL of CTAB mixture (500 µL of CTAB buffer + 1 µL of 2-mercaptoethanol per sample; pre-heated in a water bath at 60 °C) were added to the tubes, which were shaken and placed in a heat block at 60 °C for 1 h (slightly shaking them each 15 min). After this, 500 µL of chloroform/isoamyl alcohol (24:1) were added, and the tubes were centrifuged at 11,000 rpm and 4 °C for 10 min. Then, 350 µL of the aqueous upper phase of each sample were transferred to new Eppendorf tubes containing 1 µL of RNase (stock: 100 mg mL^{-1}) and incubated in a heating cabinet at 37 °C for 30 min, after which 210 µL of isopropanol were added, with the samples being mixed and placed in a freezer (-20 °C) overnight. The following day, the samples were centrifuged at 11,000 rpm and 4 °C for 10 min so that the DNA would stick to the bottom of the tubes. The supernatants were discarded, and 500 µL of 70% ethanol were added to the samples, which were again centrifuged at 11,000 rpm and 4 °C for 10 min. The ethanol was discarded, the material was rinsed twice with 70% ethanol, and the tubes were air-dried. Lastly, 100 µL of 1xTE buffer were added to dissolve the DNA. Quantification of the material was performed on 0.8% agarose gel and on mySPEC (VWR).

For all 14 isolates, polymerase chain reactions (PCR) were performed for the coding sequences (CDS) of insect adhesin gene *Mad1* and plant adhesin gene *Mad2*. Primers were

designed using CLC Main Workbench 8.1 (QIAGEN). Reactions were conducted in a thermocycler (T100, Bio-Rad Laboratories) and performed in volumes of 51 μL (*Mad2*) or 56 μL (*Mad1*), consisting of: 37.5 μL of Milli-Q water, 5 μL of 10X DreamTaq buffer (Fisher Scientific), 2 μL of dNTP 2.5 μM, 0.5 μL of DreamTaq DNA polymerase (Fisher Scientific), 2.5 μL of each forward and reverse primers (TAG Copenhagen A/S) and 1 μL of genomic DNA (ca. 25 ng). For *Mad1*, Mad1_F3 (5'-CCTGACATCCAACAACACACT) and Mad1_R3 (5'-CGCCGCAGCTCAATTCAT) were used as end primers in PCR reactions, while Mad1_F4i (5'-AGCAGACCACTCCCAGCAA) and Mad1_R4i (5'-AGGCAGAATAACAGTCGTAGGT) were used as internal primers for sanger sequencing. A one-step PCR was performed with the following conditions: 10 min at 94 °C for initial denaturation; 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C and extension for 3 min at 72 °C; and 10 min at 72 °C for the final extension. For *Mad2*, primers used were Mad2_F2 (5'-CGTCCACTCTTTTTCACATT) and Mad2_R2 (5'-GGATATATGCTGTGCGGT), and PCR conditions were: 1 min at 94 °C for initial denaturation; 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C and extension for 3 min at 72 °C; and 10 min at 72 °C for the final extension. The presence of PCR products was evaluated on 0.8% agarose gel. Samples were purified using illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to the manufacturer's instructions and sequenced by Macrogen Inc. (Amsterdam, the Netherlands).

CLC Main Workbench 8.1 was used to edit and assemble the sequences using the default settings. The sequences were deposited in GenBank and their accession numbers are shown in Appendix A. For each gene, multiple alignments of the 14 CDS sequences were constructed in CLC Main Workbench 8.1, including the reference sequence of *M. robertsii* ARSEF 2575 [(accession numbers DQ338437 (*Mad1*) and DQ338439 (*Mad2*)]. Maximum likelihood phylogenetic trees were constructed with MEGA X (Kumar et al., 2018), implementing the nucleotide substitution model HKY+G for both genes, based on the AIC score of the optimal substitution model, with 1,000 bootstrap replicates. The phylogenies also included nine other isolates representing five species of *Metarhizium* (accession numbers can be found in Appendix B).

3.2.4. Analysis of *in vitro* gene expression

3.2.4.1. Preparation of fungal growth substrates

Wheat seeds were surface sterilized in a 4% sodium hypochlorite solution for 2 h, washed three times in sterile distilled water and placed in Petri dishes containing YPD agar (0.2% yeast extract, 1% peptone, 2% dextrose, 1.5% agar) to germinate. Four days later, germinated seeds were transferred to Schott glass bottles (Sigma-Aldrich) filled with 50 mL of sterile distilled water and placed in a rotary shaker at 60 rpm and 20-23 °C for 4 days. After this period, the material was passed through a filter membrane (Puradisc FP 30 Cellulose Acetate Syringe Filter 0.2 μm, GE Healthcare Life Sciences) and freeze-dried. The resulting dried root exudate was diluted in sterile distilled water to produce a 1% v/v solution and then stocked at -4 °C. The sterility of the solution was assessed by plating five samples (200 μL) in Sabouraud Dextrose Agar and incubating at 26 °C for 7 days.

Insect cuticle was obtained by cutting off the wings, hind legs and pronotum of freeze-killed locusts (*Locusta migratoria*). The material was crushed under liquid nitrogen, mixed with sterile distilled water to produce a 1% w/v suspension and stocked at -4 °C.

3.2.4.2. Sample preparation and RNA extraction

Isolates were grown in Petri dishes containing one-quarter strength Sabouraud dextrose agar plus yeast extract (SDAY/4: 0.25% peptone, 0.25% yeast extract, 1% dextrose, 2% agar) for 10 days (26 °C, 12 h photophase), after which inoculum of each isolate was prepared (2 mL; $1x10^8$ conidia mL^{-1}) and applied in Erlenmeyer flasks containing 200 mL of YPD broth. Culture flasks were placed on a rotary shaker for 4 days at 180 rpm and 20-23 °C. The resulting fungal material was vacuum filtered and divided into portions of 1 g wet weight, which were inoculated in flasks containing 50 mL of the substrates (root exudate solution or insect cuticle suspension), and then placed on a rotary shaker at 120 rpm and 20-23 °C. There was one flask per treatment (isolate plus substrate). After 24 h, cultures were vacuum filtered. The fungal material was collected for RNA extraction, performed using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions with the following changes: (i) after crushing the material, 450 μL of buffer RLT and 5 μL of 2-mercaptoethanol were added and samples were shaken; (ii) 500 μL of 25:24:1 phenol/chloroform/isoamyl alcohol were added to the samples, which were shaken and centrifuged at 14,000 rpm and 4 °C for 5 min; (iii) after transferring 450 μL of the supernatants to new tubes, 450 μL of 24:1 chloroform/isoamyl alcohol were added to the

samples, and they were centrifuged again at 14,000 rpm and 4 °C for 5 min; (iv) 400 μL of the supernatants were transferred to the lilac spin columns of the kit, and the procedure continued according to the manufacturer's instructions. Quantification of RNA was performed on mySPEC. To conduct reverse transcriptions, tubes of each isolate containing 200 ng μL^{-1} of RNA were prepared (total volume: 10 μL), and then 0.8 μL of 100mM Oligo-dT solution (5'-TTT TTT TTT TTT TTT TTT-3') and 0.8 μL of 100mM random hexamers (5'-NNN NNN-Wobble-3') were added. The samples were incubated at 70 °C for 5 min and put on ice right after. Next, a master mix (total volume = 4.2 μL) composed of 3 μL of M-MLV RT-Buffer 5X (Promega), 0.8 μL of 10 mM dNTPs and 0.4 μL of M-MLV Revertase (Promega) were added to each tube. Reactions were vortexed and incubated at 42 °C for 1 h and then at 72 °C for 15 min.

All procedures were repeated three times as independent replicates.

3.2.4.3. Relative quantification of gene expression

For each combination of fungal isolate + gene + substrate, nine qPCR reactions (three for each flask of each independent replicate) were performed. Table 2 contains the list of target and reference genes evaluated, primers used and annealing temperatures. Target genes were selected for their known relevance for either insect or plant association, based on Pava-Ripoll et al. (2011) and Barelli, Padilla-Guerrero & Bidochka (2011). Genes *18S rRNA*, *gpd* and *try* were selected as reference genes, based on Fang & Bidochka (2006). Primers for *18S rRNA* and EST AJ274118 were obtained from Barelli, Padilla-Guerrero & Bidochka (2011), primers for *gpd* and *try* were obtained from Fang & Bidochka (2006), and the remaining primers were designed using CLC Main Workbench 8.1 or the Primer-BLAST Tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/); in this case, before designing the primers, sequences corresponding to *Pr1A*, *hyd1* and *rib* were downloaded from GenBank (accession codes presented in Appendix C) and aligned, using the default settings of CLC Main Workbench 8.1, to obtain consensus sequences for each gene. Reactions were performed in volumes of 25 μL, consisting of 9.5 μL of nuclease-free water, 12.50 μL of SYBR Green, 0.5 μL of each primer and 2 μL of cDNA. qPCR conditions were: 95 °C for 15 min; 40 cycles of 95 °C for 30 s, annealing temperature (depending on the target gene) for 30 s and 72 °C for 1 min. An additional cycle of 95 °C for 1 min, 50 °C for 30 s, and 95 °C for 30 s was performed to generate dissociation curves to address the specificity of the primers.

Gene expression was analyzed using relative quantification, which quantifies the expression of a target gene in a particular condition relative to its expression in another

condition. Expression results are thus shown as fold changes between conditions for a given gene. Data were combined for isolates of the same species before the analysis (due to large genomic and expressional differences, *M. robertsii* isolates were separated into two groups ESALQ, from Brazil, and ARSEF, from the USA). Data for the target genes were normalized against the geometric mean of the values obtained for the three reference genes. Analyses were performed applying the ΔΔCt method and the linear regression test (pcr_test('lm'); α = 5%) from the "pcr" package (Ahmed and Kim, 2018) in R (R Core Team, 2021). Comparisons between species were relative to the species group with the lowest expression level for a given target gene.

Table 2. Target and reference genes used to evaluate differential expression among the selected isolates of Metarhizium spp., with their respective forward and reverse primers, annealing temperatures (Ta; °C) and amplicon sizes (bp) for qPCR reactions.

Target genes			
Gene or EST	**Primers (5' – 3')**	**Ta (°C)**	**Amplicon size (bp)**
Metarhizium adhesin-like protein 1 (*Mad1*; insect adhesin)	F: GGGTCATCTACCCCAAGCAG R: GAGGTGCTAGGCAGTGTGAG	60	142
Metarhizium adhesin-like protein 2 (*Mad2*; plant adhesin)	F: GTCAAGCTTCCCCTTGGCAT R: CAGTCGCAAGGGTGGACATA	56	160
Subtilisin-like protease (*Pr1A)*	F: CCATTGGTAGCAAAAGCTACGG R: CGGGTCTTGGAGTCACTGG	60	136
Ribosomal protein (*rib*)	F: GCCTACCTGCGAGTTTCCTT R: CTTGGAGACACCGAACTGCT	60	183
Hydrophobin-like protein (*hyd1*)	F: GCAACAAAGTGGCGCAAAAC R: GTCTGCGAGGTGCATTTGTC	60	121
[1] EST AJ274118 – unknown product (constitutive)	F: GGGGGTTTGATTATGTGGTTGGTATTAGCA R: TAACTTCAGTCGTGCGTGCCATTTCTAC	57	100
Reference genes			
[1] *18S rRNA*	F: AGGCCCGGGTAATCTTGT R: GACCTTGTTACGACTTTTACTTCCTCT	60	266
[2] Glyceraldehyde 3-phosphate dehydrogenase (*gpd*)	F: GACTGCCCGCATTGAGAAG R: AGATGGAGGAGTTGGTGTTG	60	149
[2] Tryptophan biosynthesis enzyme (*try*)	F: TTGCAATGCATGTTTGATGTC R: CAAAGAGTGGTATCGAGTTAC	60	147

[1] Primer sequences obtained from Barelli, Padilla-Guerrero & Bidochka (2011)
[2] Primer sequences obtained from Fang & Bidochka (2006)

3.2.5. Phylogenies of *Pr1A*, *hyd1* and *rib*

Consensus sequences for *Pr1A*, *hyd1* and *rib* were obtained in the same manner described in section 2.4.3. Then, BLAST searches were performed against the orthogroups defined by OrthoFinder (section 2.2.4.), and matching sequences with > 90% of similarity were retrieved. Multiple alignments for each gene were performed using the algorithm ClustalW with default options in MEGA X (Kumar et al., 2018), and phylogenetic trees were constructed implementing the amino acid substitutions models WAG (*Pr1A*), Dayhoff (*hyd1*) and LG (*rib*), based on AIC and BIC, with 1,000 bootstrap replicates.

3.2.6. Genetic diversity

Estimates of genetic diversity were calculated for *Mad1*, *Mad2*, *Pr1A*, *hyd1* and *rib*. For each gene, the amino acid sequences of the 14 isolates were aligned using ClustalW with default options, and the overall mean genetic distances were calculated using the "Distance" module of MEGA X (Kumar et al., 2018), implementing the Poisson correction model and treating gaps as complete deletions, with 1,000 bootstrap replicates.

3.3. Results

3.3.1. WGR

3.3.1.1. General features and phylogenomic tree

Genome sizes varied among the species: 38.5 Mb for *M. anisopliae*, 37.0 Mb for *M. brunneum,* and 41.6 Mb for *M. robertsii*. According to BUSCO, genomes were estimated to be, on average, 98% complete. The BUSCO output for each isolate is shown in Appendix D.

A total of 153,128 genes were identified across the 14 isolates, with 153,011 (99.9%) being assigned to 11,999 orthogroups; of these, 9,648 contained genes from all 14 isolates, and 9,497 consisted entirely of single-copy genes. The number of predicted genes was 10,854–10,859 for *M. anisopliae*, 11,203–11,223 for *M. robertsii,* and 10,636–10,641 for *M. brunneum*. Appendix E contains general statistics for each isolate obtained from OrthoFinder.

A phylogenomic analysis confirmed that isolates of each species grouped in the same clade (Figure 2). It is worth noting that, within *M. robertsii*, isolate ESALQ 1635 formed a separate

branch from ESALQ 1426 and ESALQ 5168, and the ESALQ and ARSEF isolates clustered separately. The tree also shows that *M. anisopliae* and *M. robertsii* are sister groups.

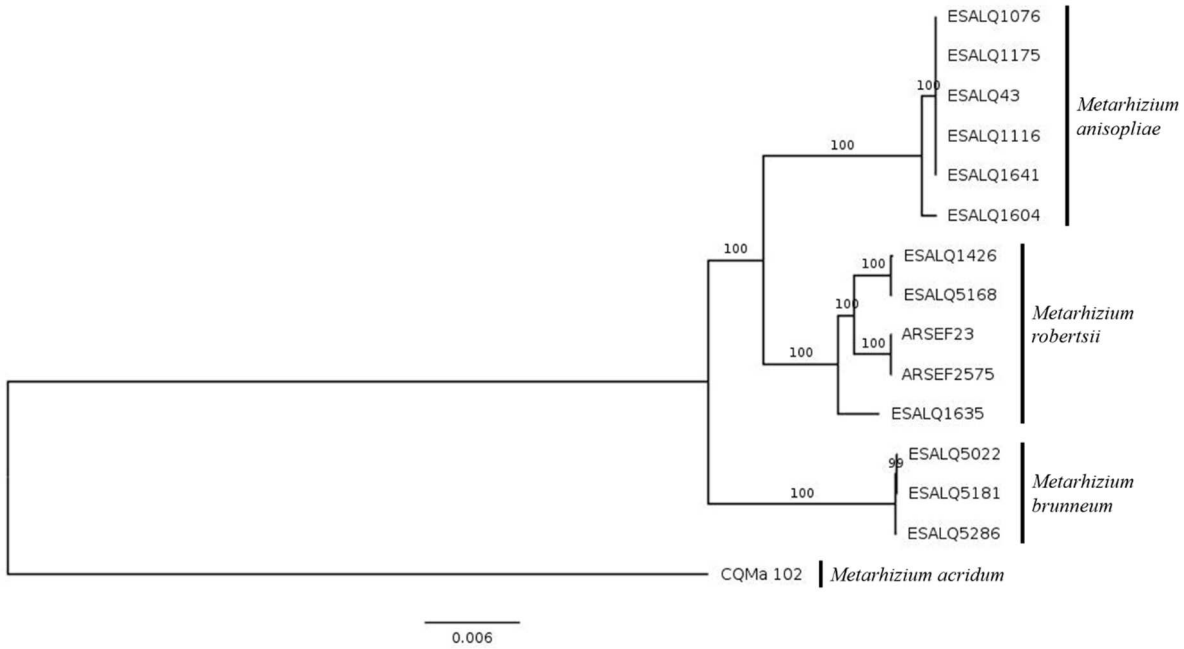

Figure 2. Phylogenomic tree based on a concatenated multiple alignments of 7,848 amino acid sequences, showing the relationship between fourteen isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. robertsii* and *M. brunneum* (Table 1). *Metarhizium acridum* CQMa 102 was used as an outgroup. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.

3.3.1.2. Relevant protein families

The number of cytochrome P450s was higher in isolates of *M. robertsii* (128 or 129) compared to isolates of *M. anisopliae* (122) and *M. brunneum* (126) (Appendix F). Our analysis also showed that isolates of *M. anisopliae* had a higher number of oligosaccharide transporters (70) compared to *M. brunneum* (68) and *M. robertsii* (67–69) (Appendix F).

Proteins involved in fungal pathogenicity to insects include hydrophobins, subtilisins and trypsins. Out of these three protein families, isolates of *M. robertsii* had the highest numbers of subtilisins (52) and trypsins (30-33). Few hydrophobins were identified (3-4), with isolates of *M. anisopliae* having one protein more than isolates of *M. brunneum* and *M. robertsii* (Appendix F).

The numbers of domains of NRPS and PKS were conserved within species, while variability was found between species (Appendix G). This was also the case of the domains of the NRPS destruxin synthetase dtxs1, involved in the biosynthesis of destruxins (Appendix H).

3.3.2. Coding sequences of *Mad1* and *Mad2*

The six isolates of *M. anisopliae* had the shortest CDS of *Mad1*, 2,097 bp (698 a.a.), and they all shared the same sequence, except for a single bp difference in ESALQ 1604. The length of CDS of *Mad1* for the three isolates of *M. brunneum* was 2,136 bp (711 a.a.), and no bp differences were found. For *M. robertsii*, the length of CDS of *Mad1* was 2,154 bp (717 a.a.) for ESALQ 1635, ARSEF 23 and ARSEF 2575, and 2,169 bp (722 a.a.) for ESALQ 1426 and ESALQ 5168. The sequence of ESALQ 1635 was more similar to the sequences of the two ARSEF isolates (8 bp differences to ARSEF isolates vs 11 bp differences + 15 deletions to ESALQ 1426/5168). The maximum-likelihood phylogenetic tree (Figure 3) shows that all 14 isolates used in the present study grouped with their respective species, although in separate branches (in the case of *M. brunneum* and *M. robertsii*). A BLAST search against the NCBI database confirmed that the translated sequences matched the protein MAD1 (queries cover = 99%, percentages of identity > 99.31%, E-values = 0.0).

The lengths of *Mad2* were comparable among species: 921 bp (306 a.a.) for *M. anisopliae* and *M. robertsii*, and 915 bp (304 a.a.) for *M. brunneum*. Isolates of *M. anisopliae* and *M. brunneum* from Brazil had the same intraspecific sequences, but the two *M. brunneum* isolates studied by Wyrebek & Bidochka (2013) clustered far from their Brazilian conspecifics. Regarding *M. robertsii*, a single bp difference was found between the Brazilian isolates ESALQ 1635 and ESALQ 1426/ESALQ 5168, while ARSEF 23 and ARSEF 2575 shared the same sequence and clustered in a separate distant clade with other North American isolates studied by Wyrebek & Bidochka (2013). The phylogenetic tree shows that isolates of *M. brunneum* clustered in separate clades, represented by either Brazilian or North American isolates (Figure 4). The same was observed for *M. robertsii*. Translated sequences were confirmed to match the protein MAD2 after conducting a BLAST search against the NCBI database (queries cover = 99%, percentages of identity >98%, E-values = 0.0).

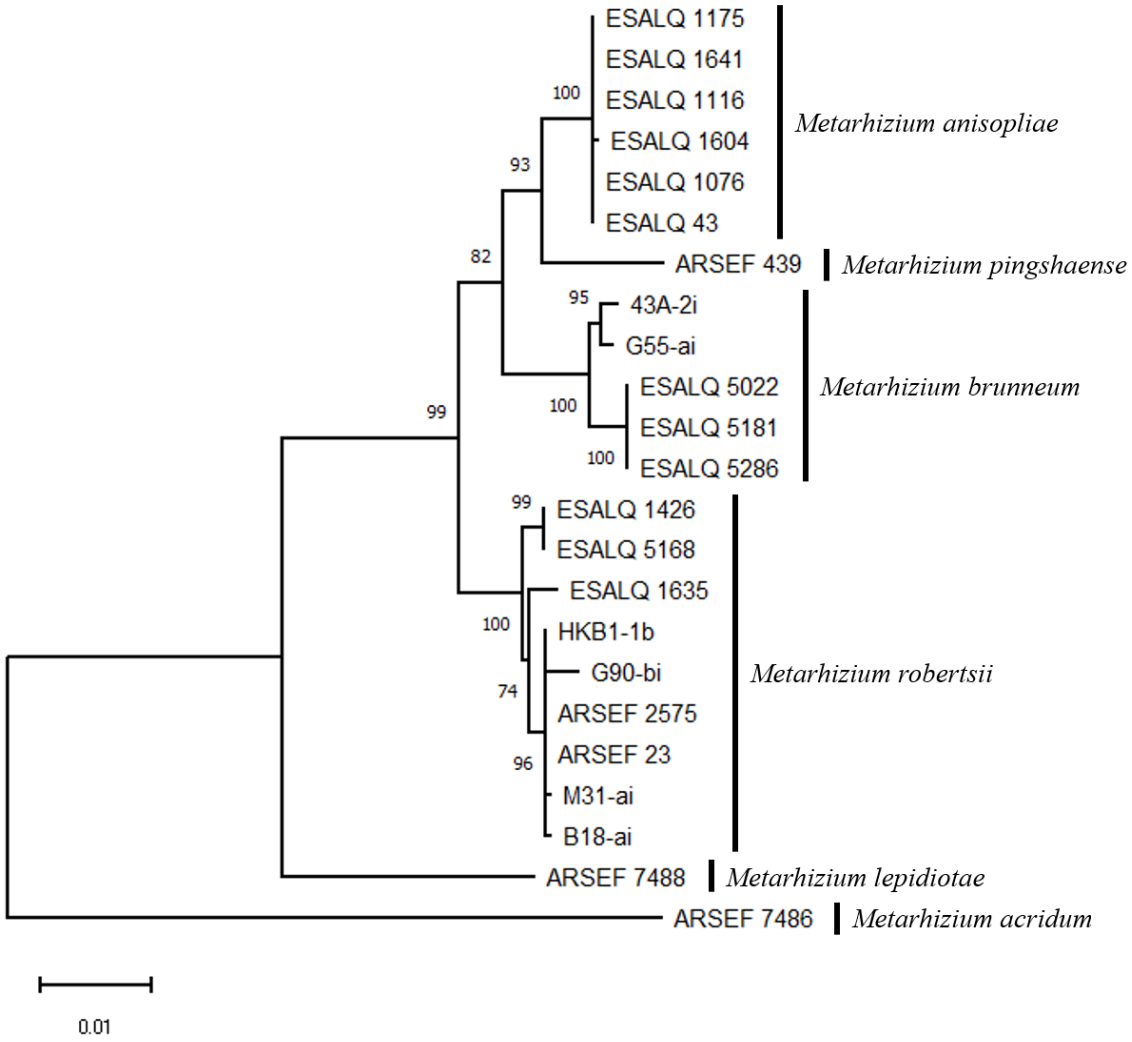

Figure 3. Maximum-likelihood phylogenetic tree of coding sequences of *Mad1* for *Metarhizium* species. It includes 14 isolates from this study (ESALQ and ARSEF; GenBank accession numbers in Appendix A and 9 obtained from GenBank [from Wyrebek & Bidochka (2013); accession numbers in Appendix B]. Alignment had a length of 2,211 bp. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.

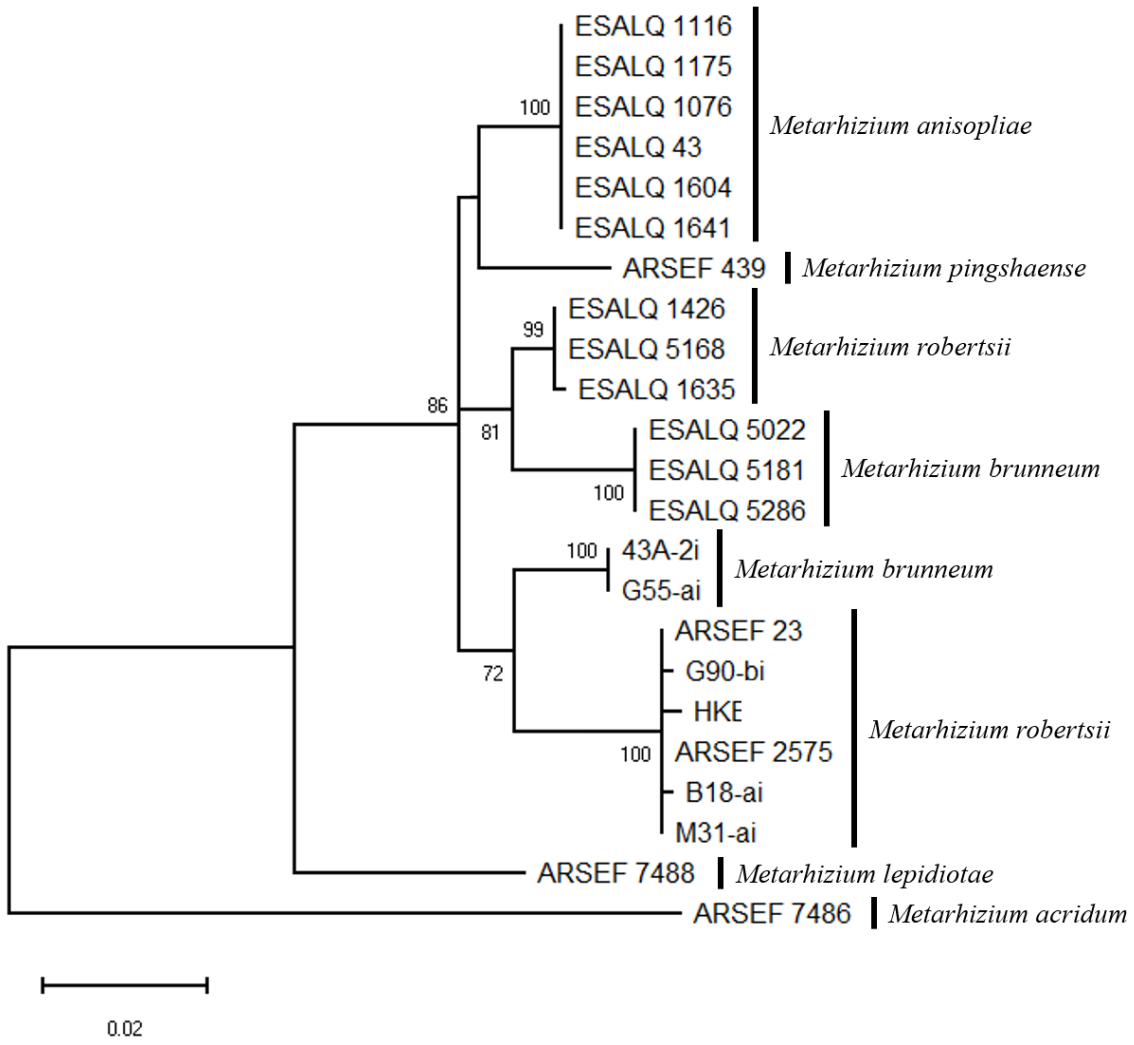

Figure 4. Maximum likelihood phylogenetic tree of coding sequences of *Mad2* for *Metarhizium* species. It includes 14 isolates from this study (ESALQ and ARSEF; GenBank accession numbers in Appendix A) and 9 obtained from GenBank [from Wyrebek & Bidochka (2013); accession codes in Appendix B]. Alignment had a length of 930 bp. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.

3.3.3. Phylogenies of *Pr1A*, *hyd1* and *rib*

The sequences of the three genes *Pr1A*, *hyd1* and *rib* were conserved intraspecifically, while interspecific variation was detected. Phylogenies of *Pr1A* and *hyd1* showed that each species was represented on their own branch, with *M. brunneum* being more closely related to *M. anisopliae* for *Pr1A*, and to *M. robertsii* for *hyd1* (Appendices I and J, respectively). In the case of *rib*, isolates of *M. anisopliae* clustered with isolates of *M. robertsii*, while isolates of *M. brunneum* were in a separate branch (Appendix K).

3.3.4. Genetic distance analysis

Comparing the two adhesin genes, the genetic distance for *Mad1* was lower than for *Mad2*, indicating that the degree of divergence is smaller in the insect adhesin. Overall, the lowest genetic distance estimated was of the gene *rib*, which encodes a ribosomal protein, and the degree of divergence was the highest for the gene *hyd1*. Table 3 shows the genetic distances calculated for the five target genes based on amino acid substitutions per site.

Table 3. Estimated genetic distance (number of amino acid substitutions per site) for five target genes among 14 isolates of *Metarhizium* representing three species (*Metarhizium anisopliae* s.str. Mani 2, *M. brunneum*, and *M. robertsii*). Standard error estimates (S.E.) were obtained by 1,000 bootstrap replicates.

Gene	Genetic distance (S.E.)
Mad1	0.012 (0.003)
Mad2	0.030 (0.007)
Pr1A	0.031 (0.006)
hyd1	0.342 (0.048)
rib	0.006 (0.003)

3.3.5. *In vitro* gene expression

No differences between the species were found in the relative expression of *Mad1* in root exudate solution ($P > 0.05$ for all pairwise comparisons; Figure 5A). Analysis in cuticle condition showed that the group of two *M. robertsii* ARSEF isolates had significantly higher relative expression compared to the group of six *M. anisopliae* isolates (2.49-fold, $P = 0.011$), while there were no significant differences regarding the other comparisons ($P > 0.05$ for all pairwise comparisons; Appendix L).

Expression of *Mad2* was similar for the groups of *M. anisopliae* and *M. robertsii* ESALQ isolates in both exudate (Figure 5B; $P = 0.306$) and cuticle conditions (Appendix M; $P = 0.433$), while being significantly higher than the expression in the groups of *M. brunneum* and *M. robertsii* ARSEF isolates (>200-fold, $P < 0.001$ for pairwise comparisons between these species and *M. anisopliae*/*M. robertsii* ESALQ in both conditions); between groups of *M. brunneum* and *M. robertsii* ARSEF isolates, the former showed higher expression than the latter (>40-fold, $P < 0.001$ in both conditions).

The group of six isolates of *M. anisopliae* had significantly higher expression of *Pr1A* compared to the other three groups of *Metarhizium* spp. in both conditions (>2,000-fold, $P <$

0.001 for all pairwise comparisons). Analysis of gene expression in root exudate solution showed no differences between groups of *M. brunneum*, *M. robertsii* ESALQ and *M. robertsii* ARSEF isolates ($P > 0.05$ for all pairwise comparisons; Figure 5C). In contrast, in insect cuticle suspension, the group of *M. brunneum* isolates exhibited significantly higher expression than *M. robertsii* ESALQ ($P = 0.021$; Appendix N, while no difference was found between groups of *M. robertsii* ESALQ and ARSEF ($P = 0.113$) or between groups of *M. robertsii* ARSEF and *M. brunneum* ($P = 0.501$).

In both root exudate and insect cuticle conditions, the ARSEF and ESALQ groups of *M. robertsii* showed higher expression of *hyd1* compared to groups of *M. anisopliae* and *M. brunneum* (>1,500-fold, $P < 0.001$ for all pairwise comparisons; Figure 5D for expression in root exudate, Appendix O for insect cuticle). Between the two *M. robertsii* groups, gene expression was similar (root exudate: $P = 0.999$; insect cuticle: $P = 0.539$), as it was between the groups of *M. anisopliae* and *M. brunneum* (root exudate: $P = 0.275$; insect cuticle: $P = 0.786$).

Variation was limited for the gene *rib*, although the group of *M. anisopliae* isolates had significantly higher expression than the group of *M. brunneum* isolates in root exudate solution (3.18-fold, $P = 0.036$), while the other groups did not differ from each other ($P > 0.05$ for all pairwise comparisons; Figure 5E). In cuticle condition, significant differences were found between *M. brunneum* and both groups of *M. robertsii* (vs *M. robertsii* ESALQ: $P = 0.023$; vs *M. robertsii* ARSEF), whereas other pairwise comparisons did not reveal differences ($P > 0.05$ for all pairwise comparisons; Appendix P).

Expression of the EST AJ274118 in root exudate solution was higher for the group of *M. brunneum* isolates compared to the group of *M. robertsii* ARSEF isolates (3.45-fold, $P = 0.012$), while there were no differences between the other species ($P > 0.05$ for all pairwise comparisons; Figure 5F). Analysis of expression in insect cuticle condition showed no differences between the groups ($P > 0.05$ for all pairwise comparisons; Appendix Q).

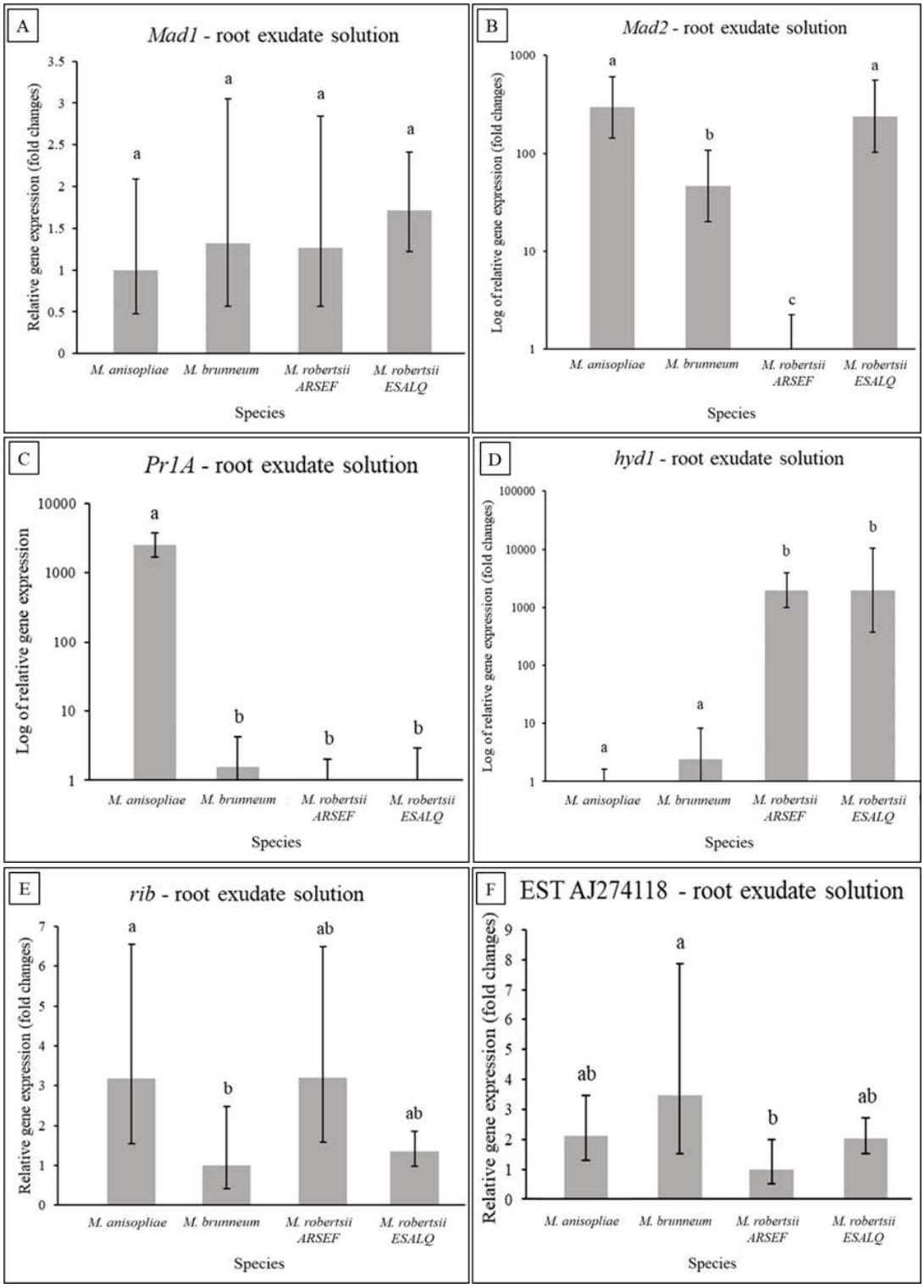

Figure 5. Relative gene expression (in fold changes) of six target genes for isolates of *Metarhizium anisopliae* s.str. Mani 2 (n=6), *M. brunneum* (n=3) and *M. robertsii* (ESALQ, n=3, and ARSEF, n=2) in 1% v/v root exudate solution using the double delta Ct method. (A) Gene *Mad1*, expressions are relative to that of *M. anisopliae*; (B) gene *Mad2*, expressions are relative to that of *M. robertsii* ARSEF; (C) gene *Pr1A*, expressions are relative to that of *M. robertsii* ESALQ; (D) gene *hyd1*, expressions are relative to that of *M. anisopliae*; (E) gene *rib*, expressions are relative to that of *M. brunneum*; (F) EST AJ274118, expressions are relative to that of *M. robertsii* ARSEF. For each gene, different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$]. Plot bars represent the standard deviation.

3.4. Discussion

Genomic studies usually focus on just one isolate of a determined species (Gao et al., 2011; Hu et al., 2014; Pattemore et al., 2014). Although the data generated by such an approach are relevant to improve the knowledge about the genome composition of the particular species, it is not sufficient to account for potential intraspecific variability. Our results demonstrate the existence of intraspecific differences in the number of genes in *Metarhizium*, and it is worth noting that ESALQ isolates of *M. robertsii* had up to 10-20 more genes than ARSEF isolates of *M. robertsii* (Appendix E). The phylogenomic tree (Figure 2) also reflects variability not previously reported, especially for *M. robertsii*. The isolates of this species formed a monophyletic group; however, isolate ESALQ 1635 clustered separately from the other isolates of *M. robertsii*, which formed two sister groups, one composed of ARSEF isolates and the other composed of ESALQ 1426 and ESALQ 5168. This indicates a degree of genotypic variability between isolates from different geographic locations and shows the importance of studying multiple isolates from different environments to assess the natural genomic variability to make inferences at the species-level. The interspecific relationships in the tree were in accordance with Hu et al. (2014), who reported *M. brunneum* as an ancestral species compared to *M. anisopliae* and *M. robertsii*, which form sister groups.

Isolates of *M. robertsii* had the largest genome (41.6 Mb), and isolates of *M. brunneum* the shortest (37 Mb), and this was reflected in the number of genes, in which case isolates of *M. robertsii* also possessed more protein-coding genes than the other two species (Appendix E). The genome sizes matched those reported by Hu et al. (2014), who sequenced isolates of seven species of *Metarhizium*, including *M. robertsii*, *M. anisopliae* and *M. brunneum*. However, we found fewer genes than these authors, possibly due to the methods used for mapping and assembly of the genomes.

Hu et al. (2014) stated that, due to their lifestyle, generalist species of *Metarhizium* show an expansion of protein families related to pathogenicity and detoxification compared to specialists. The three species of our study are considered generalists. We found that the number of the selected proteins families (e.g., trypsins, cytochrome P450s) were similar to the ones reported by the authors mentioned above, supporting their findings of this expansion across multiple isolates.

We found minor variations in the number of proteins encoded by each species. However, these differences, especially regarding pathogenicity-related proteins or domains of PKSs and NRPSs, do not necessarily translate into lower or higher virulence for a species of entomopathogenic fungi (Chapter 3). Our analysis of *Mad1* showed variation in the sequence

length not only between species but also intraspecifically. Intraspecific variation was seen within *M. robertsii* isolates, where ESALQ 1426 and ESALQ 5168 (from Brazil) had insertions in *Mad1* and *Mad2* sequences compared to ESALQ 1635 (Brazil), ARSEF 23 and ARSEF 2575 (USA). Wyrebek & Bidochka (2013) did not find this variation in the size of MAD1 between American and Canadian isolates of *M. robertsii*, however, they reported intraspecific variability for *Metarhizium guizhouense*. While the protein of Ontario isolates was composed of 706 a.a., a Chinese isolate had a protein of 717 a.a.; the authors also reported interspecific variability in the size of MAD1. The length of *Mad2* in the present study was conserved within species, but not interspecifically, since the sequences of *M. brunneum* isolates were 6 bp (2 a.a.) shorter. This contrasts with a previous report which found a conserved length of MAD2 for species of the PARB clade (Wyrebek and Bidochka, 2013), which includes the same three species of our study.

Our phylogenetic tree of *Mad1* showed that isolates of the same species clustered together in the same clade, but they were on separate branches in each clade, according to their continental origin. All six isolates of *M. anisopliae* had the same sequence of *Mad1*, except for a single bp in ESALQ 1604, thus contradicting our prediction that isolates of this species would show higher variation in this gene because they are frequently found associated with insects. The phylogeny of *Mad2* presented Brazilian isolates of *M. brunneum* and *M. robertsii* in a single clade, while North American isolates of both species were in another clade. No divergence was observed within the Brazilian *M. anisopliae* group. These results indicate some degree of geographic divergence, reflecting adaptations needed to survive in different habitats with contrasting environmental conditions. This divergence was less pronounced in the phylogeny of *Mad1*, suggesting that this gene is more conserved than *Mad2* (Wyrebek and Bidochka, 2013). The analysis of genetic distances supports this assumption, showing that *Mad1* diverged less than *Mad2*. This higher divergence of *Mad2* may be related to the hypothesis that plant association has been an important factor in the evolution of *Metarhizium* (Wyrebek and Bidochka, 2013) and that adaptation to new environments is dependent on the habitat and interaction with plants rather than insects (St. Leger and Wang, 2020; Wang et al., 2011). At present, we cannot conclude on potential comparable geographical variation of *Mad2* within *M. anisopliae* based on the current isolate sampling.

Interspecific differences regarding pathogenicity factors probably occur because of differential gene expression rather than due to the number of genes possessed by each species (Leão et al., 2015). The relative expression of *Mad1* in root exudate solution was similar for the four species groups. Although there were significant differences in gene expression between *M. anisopliae* and *M. robertsii* ARSEF in insect cuticle suspension, this difference was limited. These

differences might be related to the fact that this gene is primarily conserved in the genus *Metarhizium* (Wyrebek and Bidochka, 2013), although sequence variations exist. On the other hand, there was high variability in the relative expression of *Mad2* between species and intraspecifically, as relative expression by ESALQ isolates of *M. robertsii* was much higher than in ARSEF isolates. Wyrebek & Bidochka (2013) reported high genetic variation in *Mad2* comparing several *Metarhizium* species, and our phylogenetic tree of the CDS of *Mad2* showed that ARSEF and ESALQ isolates grouped in separate clades according to their geographic location. Brazil is one of the richest countries in biodiversity (Mittermeier et al., 2005), with very diverse microbial communities (including *Metarhizium*), especially in the Amazon (Botelho et al., 2019). Considering that fungal populations in the soil can be affected by biotic factors, e.g., interacting with other microorganisms and with plant roots (Bidochka et al., 2001; Jaronski, 2007), the higher relative expression of *Mad2* in Brazilian isolates of *M. robertsii* may reflect adaptations to enhance competitive abilities for associating with roots in the rhizosphere. Therefore, our analyses showed that all species groups from Brazil upregulated the plant adhesion potential similarly, which contrasts the posed expectation that only *M. robertsii* and *M. brunneum* would do this.

We found interspecific variation in the relative expression of the insect pathogenicity-related genes *Pr1A* and *hyd1* in both exudate solution and cuticle suspension. These two genes are expressed under nutrient-deprived conditions, e.g., formation of appressoria, and it has been previously demonstrated that *hyd1*/*ssgA* is expressed coordinately with *Pr1* (St. Leger et al., 1989, 1992a, 1992b). Besides functioning during adhesion (*hyd1*) or penetration (*Pr1A*) to the cuticle, these genes also have a role in other processes, e.g., sporulation (Sevim et al., 2012; Small and Bidochka, 2005). Although we expected that *M. anisopliae* would exhibit the highest relative expression of both genes, this happened only for *Pr1A*. In contrast, relative expression of *hyd1* was much higher in both groups of *M. robertsii*. In the phylogenies of both genes, *M. anisopliae* and *M. robertsii* grouped in different clades, and the genetic distances estimated show some degree of divergence, especially for *hyd1*. This may indicate that, at least *in vitro*, *M. anisopliae* and *M. robertsii* have developed differential strategies, reflecting their lifestyles: most of the infections above ground are caused by isolates of *M. anisopliae* Mani 2 (Iwanicki et al., 2019; Rezende et al., 2015), so it seems reasonable that this species invests in expressing more *Pr1A* than the other species groups studied when exposed to a substrate. On the other hand, studies by Fang & Bidochka (2006) and Sevim et al. (2012) reported that, although *hyd1* is expressed in most developmental stages, its expression levels vary; *hyd1* is highly expressed in mycelia and mycelia with conidiophores (but weakly in swollen and germinated conidia), which would be consistent with the presumed niche of *M. robertsii* as a species better adapted to below-ground lifestyle in an

environment. Here, the fungus can form associations with plant roots and potentially transfer insect-derived nitrogen to the plant via mycelia (Behie et al., 2017, 2012; Rezende et al., 2015; Steinwender et al., 2015), which could explain why *M. robertsii* would invest in expressing more *hyd1* than *M. anisopliae*.

Contrary to our hypothesis that expression of constitutive genes would be similar in all groups, there was also interspecific variation in the relative expression of *rib* in both substrates, and of EST AJ274118 in root exudate solution. However, it is worth noting that the differences were relatively minor, and this observation could be related to the nature of their encoded products (a ribosomal protein and a constitutive product), which may not show significant variability between species. The genetic distance analysis showed that *rib* is a very conserved gene among the species, and the phylogeny demonstrated a very close relationship between the isolates.

Our study provided new information about the diversification of genomes representing three species of the genus *Metarhizium*. The detailed phylogenies of adhesins *Mad1* and *Mad2* and documented variability in the relative expression of the six selected genes demonstrate further both genotypic and functional divergence within *M. robertsii*. The intraspecific variation between isolates from different geographical origins may be related to adaptations to differential environmental factors. Our data emphasize the importance of conducting gene expression coupled with genomic analyses on a diversity of fungal isolates to capture the natural variability within this group of entomopathogenic fungi.

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APPENDICES

APPENDIX A - Accession numbers of the sequences obtained in the present study. Genomes were deposited in the Sequence Read Archives (SRA), under the bioproject PRJNA746571, and genes *Mad1* and *Mad2* were deposited in GenBank.

Species	Isolate	Genome SRA accession number	*Mad1* GenBank accession number	*Mad2* GenBank accession number
M. robertsii	ARSEF 23	SRR15182435	MZ611894	MZ611908
	ARSEF 2575	SRR15182434	MZ611895	MZ611909
	ESALQ 1426	SRR15182431	MZ611882	MZ611896
	ESALQ 1635	SRR15182430	MZ611883	MZ611897
	ESALQ 5168	SRR15182429	MZ611884	MZ611898
M. brunneum	ESALQ 5022	SRR15182422	MZ611885	MZ611899
	ESALQ 5181	SRR15182433	MZ611886	MZ611900
	ESALQ 5286	SRR15182432	MZ611887	MZ611901
M. anisopliae	ESALQ 43	SRR15182428	MZ611888	MZ611902
	ESALQ 1076	SRR15182425	MZ611889	MZ611903
	ESALQ 1116	SRR15182427	MZ611890	MZ611904
	ESALQ 1175	SRR15182424	MZ611891	MZ611905
	ESALQ 1604	SRR15182423	MZ611892	MZ611906
	ESALQ 1641	SRR15182426	MZ611893	MZ611907

APPENDIX B - List of *Metarhizium* spp. isolates included in the phylogenetic trees of coding sequences of *Mad1* and *Mad2*, with their respective accession numbers in GenBank.

Species	Isolate	Location	*Mad1* GenBank accession number	*Mad2* GenBank accession number
M. brunneum	43A-2i	Canada	KC484642	KC484629
	G55-ai		KC484643	KC484630
M. robertsii	G90-bi		KC484640	KC484627
	B18-ai		KC484638	KC484625
	HKB1-1b		KC484637	KC484624
	M31-ai		KC484639	KC484626
M. pingshaense	ARSEF 439	Australia	KC484641	KC484628
M. lepidiotae	ARSEF 7488		KC484648	KC484635
M. acridum	ARSEF 7486	Niger	KC484649	KC484636

APPENDIX C - GenBank accession numbers for the sequences used to align and to obtain consensus sequences for the genes *Pr1A*, *hyd1* and *rib*.

Gene	GenBank accession number		
Pr1A	FJ659159.1	FJ659160.1	FJ659161.1
	FJ659162.1	FJ659163.1	FJ659166.1
	FJ659169.1	FJ659175.1	FJ659180.1
	FJ659181.1		FJ659183.1
hyd1	M85281.1	HM560700.1	AJ274156.1
rib	CN809270.1	XM_007821686.1	XM_014686727.1

APPENDIX D- Number of BUSCO matches of the selected isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. BUSCO was run on protein mode, selecting the dataset Hypocreales odb10.2019-11-20. A total of 4,494 BUSCO groups were searched. S = complete and single-copy BUSCOs; D = complete and duplicate BUSCOs; C = complete BUSCOs (C = S + D); F = fragmented BUSCOs; M = missing BUSCOs.

Species	**Isolates**	**S (% S)**	**D (% D)**	**C (% C)**	**F (% F)**	**M (% M)**
Metarhizium anisopliae s.str. Mani 2	ESALQ 43	4386 (97.6)	19 (0.4)	4405 (98)	34 (0.8)	55 (1.2)
	ESALQ 1076	4386 (97.6)	19 (0.4)	4405 (98)	34 (0.8)	55 (1.2)
	ESALQ 1116	4386 (97.6)	19 (0.4)	4405 (98)	34 (0.8)	55 (1.2)
	ESALQ 1175	4386 (97.6)	19 (0.4)	4405 (98)	34 (0.8)	55 (1.2)
	ESALQ 1604	4385 (97.6)	19 (0.4)	4404 (98)	34 (0.8)	56 (1.2)
	ESALQ 1641	4386 (97.6)	19 (0.4)	4405 (98)	34 (0.8)	55 (1.2)
Metarhizium brunneum	ESALQ 5022	4394 (97.8)	13 (0.3)	4407 (98.1)	28 (0.6)	59 (1.3)
	ESALQ 5181	4394 (97.8)	13 (0.3)	4407 (98.1)	28 (0.6)	59 (1.3)
	ESALQ 5286	4393 (97.8)	14 (0.3)	4407 (98.1)	28 (0.6)	59 (1.3)
Metarhizium robertsii	ARSEF 23	4381 (97.5)	24 (0.5)	4405 (98)	28 (0.6)	61 (1.4)
	ARSEF 2575	4381 (97.5)	24 (0.5)	4405 (98)	28 (0.6)	61 (1.4)
	ESALQ 1426	4383 (97.5)	24 (0.5)	4407 (98)	30 (0.7)	57 (1.3)
	ESALQ 1635	4386 (97.6)	23 (0.5)	4409 (98.1)	26 (0.6)	59 (1.3)
	ESALQ 5168	4383 (97.5)	24 (0.5)	4407 (98)	29 (0.6)	58 (1.4)

APPENDIX E - General statistics of orthology inference using the protein sequences of the selected isolates of *Metarhizium anisopliae* s.str. Mani2 (gray), *M. brunneum* (orange) and *M. robertsii* (blue).

Species	Isolate code	Number of genes	Number of genes in orthogroups	Percentage of genes in orthogroups	Number of orthogroups containing isolate	Percentage of orthogroups containing isolate	Number of Pfam entries
Metarhizium anisopliae	ESALQ 43	10,858	10,855	100	10,694	89.1	12,855
	ESALQ 1076	10,857	10,854	100	10,693	89.1	12,854
	ESALQ 1116	10,859	10,856	100	10,695	89.1	12,855
	ESALQ 1175	10,859	10,856	100	10,694	89.1	12,855
	ESALQ 1604	10,854	10,832	99.8	10,668	88.9	12,853
	ESALQ 1641	10,857	10,854	100	10,693	89.1	12,853
Metarhizium brunneum	ESALQ 5022	10,636	10,631	100	10,502	87.5	12,636
	ESALQ 5181	10,641	10,637	100	10,509	87.6	12,631
	ESALQ 5286	10,640	10,635	100	10,508	87.6	12,632
Metarhizium robertsii	ARSEF 23	11,203	11,199	100	11,016	91.8	13,133
	ARSEF 2575	11,206	11,202	100	11,017	91.8	13,136
	ESALQ 1426	11,223	11,213	99.9	11,025	91.9	13,120
	ESALQ 1635	11,221	11,181	99.6	10,968	91.4	13,073
	ESALQ 5168	11,214	11,206	99.9	11,019	91.8	13,122

APPENDIX F - Number of proteins (from selected protein families) involved in pathogenicity, detoxification and rhizosphere competency identified in isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. Data was obtained after running orthologues sequences against the Pfam database.

Species	Isolate code	Substilase (PF00082)	Hydrophobin (PF01185 and PF06766)	Trypsin (PF00089)	Cytochrome P450 (PF00067)	Sugar (oligosaccharide) transporter (PF00083)
Metarhizium anisopliae	ESALQ 43	48	4	24	122	70
	ESALQ 1076	48	4	24	122	70
	ESALQ 1116	48	4	24	122	70
	ESALQ 1175	48	4	24	122	70
	ESALQ 1604	47	4	25	120	70
	ESALQ 1641	48	4	24	122	70
Metarhizium brunneum	ESALQ 5022	50	3	28	126	68
	ESALQ 5181	50	3	28	126	68
	ESALQ 5286	50	3	28	126	68
Metarhizium robertsii	ARSEF 23	52	3	33	129	67
	ARSEF 2575	52	3	33	129	67
	ESALQ 1426	52	3	33	129	69
	ESALQ 1635	52	3	30	128	68
	ESALQ 5168	52	3	33	129	69

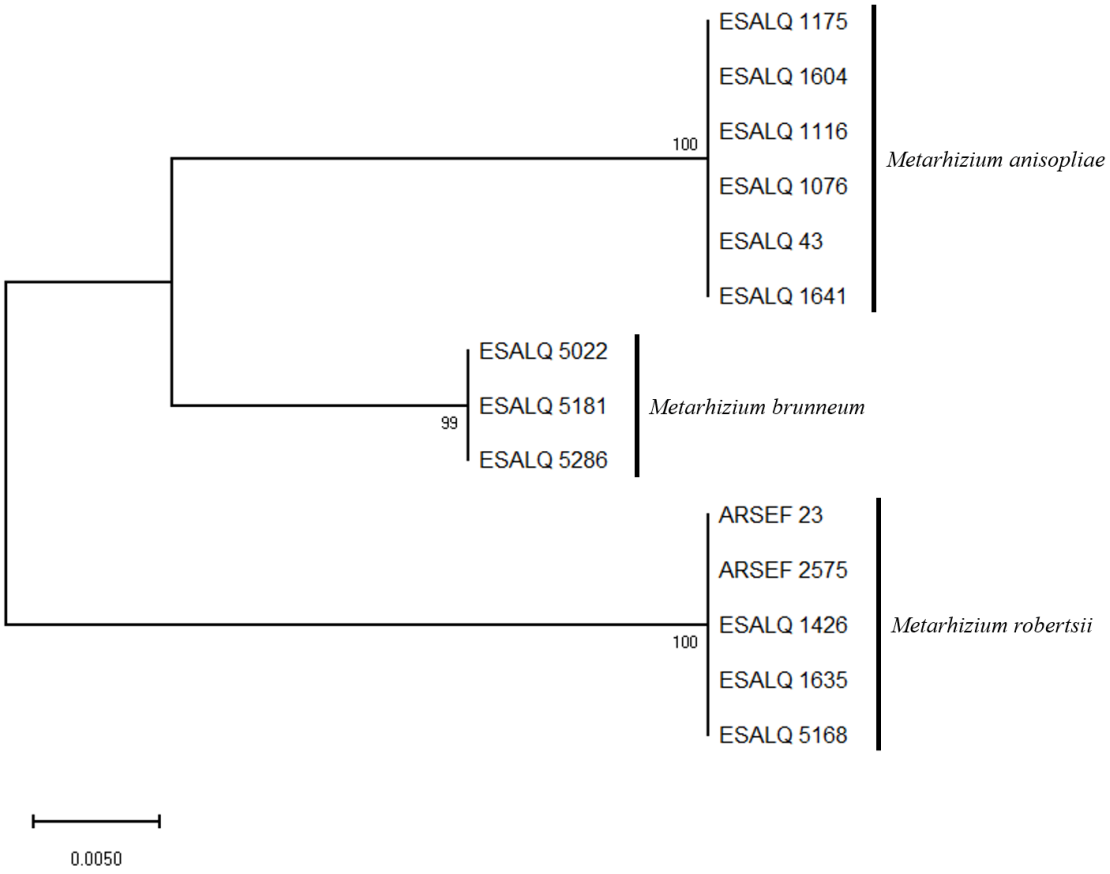
APPENDIX G - Number of protein domains of non-ribosomal peptide synthases (NRPS), polyketide synthases (PKS) and NRPS-PKS hybrids involved in the production of secondary metabolites identified in isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. Data was obtained after running orthologues sequences against the Pfam database.

Species	Isolate code	Starter unit acyl transferase (PF16073)	β-ketoacyl synthase N-and C- terminal (PF00109 / PF02801)	Ketoacyl-synthetase (PF16197)	Acyl transferase (PF00698)	Polyketide synthase dehydratase (PF14765)	PP-binding (PF00550)	Thioesterase (PF00975)
Metarhizium anisopliae	ESALQ 43	14	35 / 34	21	34	28	108	9
	ESALQ 1076	14	35 / 34	21	34	28	108	9
	ESALQ 1116	14	35 / 34	21	34	28	108	9
	ESALQ 1175	14	35 / 34	21	34	28	108	9
	ESALQ 1604	14	36 / 34	22	34	29	108	9
	ESALQ 1641	14	35 / 34	21	34	28	108	9
Metarhizium brunneum	ESALQ 5022	13	38 / 38	23	37	29	115	10
	ESALQ 5181	13	38 / 38	23	37	29	115	10
	ESALQ 5286	13	38 / 38	23	37	29	115	10
Metarhizium robertsii	ARSEF 23	14	37 / 35	23	36	29	101	10
	ARSEF 2575	14	37 / 35	23	36	29	101	10
	ESALQ 1426	14	37 / 35	24	36	27	104	9
	ESALQ 1635	13	37 / 35	23	36	28	101	9
	ESALQ 5168	14	37 / 35	24	36	28	104	9

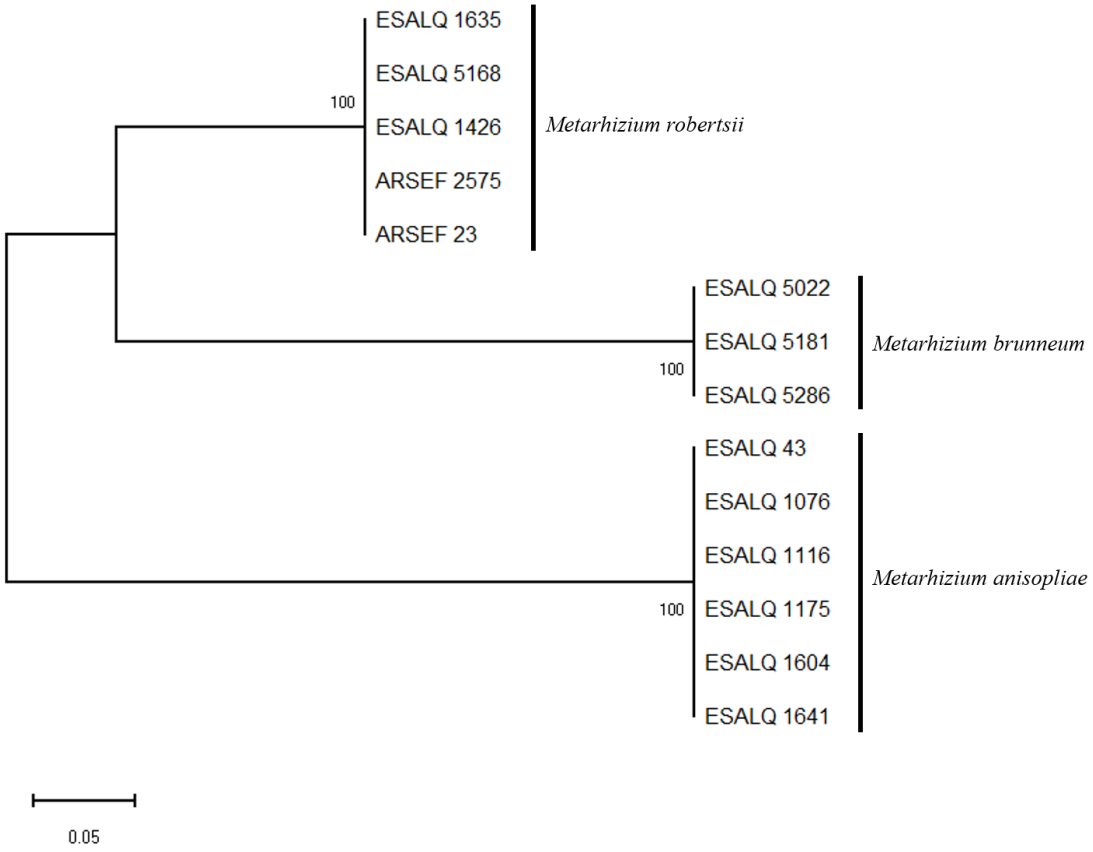
APPENDIX H - Number of protein domains of the non-ribosomal peptide synthetase dtxS1 (destruxin synthetase), involved in the production of destruxin, identified in isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. Data was obtained after running orthologues sequences against the Pfam database.

Species	Isolate code	AMP-binding (PF00501)	Condensation (PF00668)	Methyltransferase type 11 (PF08241)	PP-binding (PF00550)
Metarhizium anisopliae	ESALQ 43	111	86	13	108
	ESALQ 1076	111	86	13	108
	ESALQ 1116	111	86	13	108
	ESALQ 1175	111	86	13	108
	ESALQ 1604	111	86	12	108
	ESALQ 1641	111	86	13	108
Metarhizium brunneum	ESALQ 5022	113	89	10	115
	ESALQ 5181	112	89	10	115
	ESALQ 5286	113	89	10	115
Metarhizium robertsii	ARSEF 23	104	84	11	101
	ARSEF 2575	104	84	11	101
	ESALQ 1426	106	83	10	104
	ESALQ 1635	104	83	13	101
	ESALQ 5168	106	84	10	104

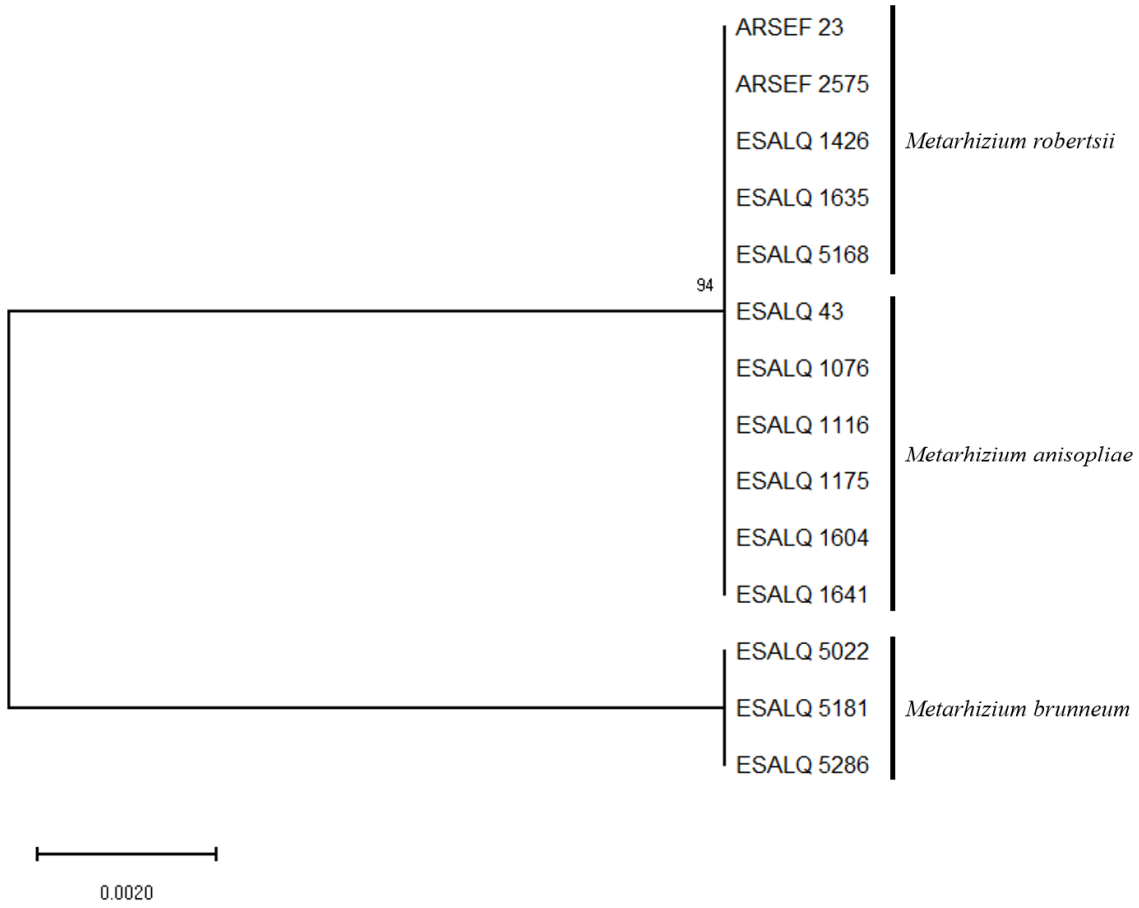
APPENDIX I - Maximum likelihood phylogenetic tree of the gene *Pr1A* for isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*, based on sequences of orthogroups identified between the three species. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.



APPENDIX J - Maximum likelihood phylogenetic tree of the gene *hyd1* for isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*, based on sequences of orthogroups identified between the three species. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.



APPENDIX K - Maximum likelihood phylogenetic tree of the gene *rib* for isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*, based on sequences of orthogroups identified between the three species. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.



APPENDIX L - Relative gene expression (with confidence interval, CI), in fold changes, of *Mad1* for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to that of *M. anisopliae*. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression (CI)	
Mad1	Insect cuticle suspension	*M. anisopliae*	1.00 (0.64 – 1.54)	a
		M. brunneum	1.35 (0.57 – 3.19)	ab
		M. robertsii ESALQ	1.41 (0.93 – 2.13)	ab
		M. robertsii ARSEF	2.49 (2.23 – 2.78)	b

APPENDIX M - Relative gene expression (with confidence interval, CI), in fold changes, of *Mad2* for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to that of *M. robertsii* ARSEF. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression (CI)	
Mad2	Insect cuticle suspension	*M. robertsii* ARSEF	1.00 (0.90 – 1.10)	a
		M. brunneum	77.17 (34.51 – 172.52)	b
		M. anisopliae	293.04 (139.13 – 617.23)	c
		M. robertsii ESALQ	417.79 (265.63 – 657.12)	c

APPENDIX N - Relative gene expression (with confidence interval, CI), in fold changes, of *Pr1A* for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to that of *M. robertsii* ESALQ. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression (CI)	
Pr1A	Insect cuticle suspension	*M. robertsii* ESALQ	1.00 (0.62 – 1.59)	a
		M. robertsii ARSEF	2.18 (2.00 – 2.39)	ab
		M. brunneum	2.99 (1.22 – 7.31)	b
		M. anisopliae	3,476.29 (1,811.82 – 6,669.83)	c

APPENDIX O - Relative gene expression (with confidence interval, CI), in fold changes, of *hyd1* for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to that of *M. brunneum*. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression (CI)	
hyd1	Insect cuticle suspension	*M. brunneum*	1.00 (0.64 – 1.54)	a
		M. anisopliae	1.26 (0.48 – 3.30)	a
		M. robertsii ESALQ	1,738.14 (174.47 – 17,315.93)	b
		M. robertsii ARSEF	3,480.30 (1,635.66 – 7,405.25)	b

APPENDIX P - Relative gene expression (with confidence interval, CI), in fold changes, of *rib* for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to those of *M. brunneum*. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression	
rib	Insect cuticle suspension	*M. brunneum*	1.00 (0.59 – 1.67)	a
		M. anisopliae	1.79 (0.68 – 4.72)	ab
		M. robertsii ESALQ	5.64 (3.56 – 8.94)	b
		M. robertsii ARSEF	7.55 (6.19 – 9.19)	b

APPENDIX Q - Relative gene expression (with confidence interval, CI), in fold changes, of EST AJ274118 for *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* (ESALQ and ARSEF) in 1% w/v insect cuticle suspension, using the double delta Ct method. Expressions are relative to those of *M. robertsii* ARSEF. Different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$].

Gene	Condition	Species	Relative expression	
EST AJ2714118	Insect cuticle suspension	*M. robertsii* ARSEF	1.00 (0.44 – 2.25)	a
		M. robertsii ESALQ	1.17 (0.79 – 1.73)	a
		M. anisopliae	1.66 (1.12 – 2.44)	a
		M. brunneum	1.82 (1.19 – 2.78)	a

4. EFFECT OF INOCULATION METHODS OF SINGLE AND COMBINED APPLICATIONS OF *Metarhizium* SPP. TO *Tenebrio molitor* LARVAE

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ABSTRACT

The genus *Metarhizium* comprises important species of entomopathogenic fungi that possess great potential for use in biological control programs. Concomitant infections naturally occur in the environment and possibly affect infection success of insects by the fungi involved. In Brazil, isolates of the subclade Mani 2 of *Metarhizium anisopliae* s.str. are responsible for most natural infections of insects above-ground, while isolates of *M. brunneum* and *M. robertsii* are generally associated with the soil environment where they may associate with plant roots. In this study, the virulence of six isolates of *M. anisopliae* s.str. subclade Mani 2, three of *M. brunneum* and three of *M. robertsii* was evaluated against *Tenebrio molitor* larvae. Using a standard spray application method for above-ground exposure of insects, we tested the hypothesis that isolates of *M. anisopliae* Mani 2 would be most virulent and exhibit highest sporulation in cadavers, whereas application in non-sterile soil would result in higher infection and sporulation by *M. brunneum* and *M. robertsii*. The effects of mixed infection of selected isolates from different species were also evaluated. All isolates were pathogenic to the host, although there was intra- and interspecific variability in virulence using both application methods. Sporulation also varied between isolates and species, although it was generally lower for *M. brunneum* and *M. robertsii* than for *M. anisopliae* in the soil. Median lethal times were similar for the isolates using both methods of application, spraying directly on the insects and insect baiting of soil (total amount of conidia applied: $2x10^8$ and $4x10^7$, respectively). Co-infections did not result in additive or synergistic effects; rather in one case an antagonism effect was observed. The results reinforce the importance of isolate selection for use of entomopathogens as biocontrol agents.

Keywords: *Metarhizium*; virulence; sporulation; co-infections

4.1. Introduction

Entomopathogenic fungi comprise a group of microorganisms capable of infecting insects, naturally causing enzootic and epizootic diseases and helping to control pest populations (Alves, 1998). Because of its importance as a natural regulator of insect populations, the genus *Metarhizium* Sorokin (Ascomycota: Hypocreales) is one of the best-known genera of fungal entomopathogens (Lacey et al., 2015). It comprises several specialist and generalist species whose life-cycles include a pathogenic stage inside an insect host (Hesketh et al., 2010; Jaronski, 2007), parasitizing more than 200 species from at least seven orders of insects (Zimmermann, 2007a).

The infection process caused by these pathogens starts when conidia adhere to the host's cuticle through hydrophobic interactions and surface-associated proteins (Vega et al., 2012; Wang and St. Leger, 2007; Zimmermann, 2007a). The propagules germinate and form the appressorium, and through mechanical pressure and enzymatic degradation, the fungus penetrates the cuticle. Inside the insect, the pathogen's vegetative growth occurs when it consumes nutrients, damages tissues, and produces toxins, eventually leading to host death. After an incubation period, conidiophores emerge and produce new conidia for dispersal in the environment (Vega et al., 2012; Zimmermann, 2007a).

In the absence of a suitable host, fungal propagules can survive in the soil, which acts as a reservoir of inoculum (Hesketh et al., 2010; Vega et al., 2012); in grasslands, the concentration can reach up to 10^6 conidia g^{-1} of soil (Scheepmaker and Butt, 2010). Assessment of fungal presence in the soil can be performed by techniques such as insect baiting, in which insects susceptible to infection, e.g., *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and *Galleria mellonella* L. (Lepidoptera: Pyralidae), are added to soil samples, and subsequent cadavers can be recovered and incubated in suitable conditions for fungal sporulation (Hesketh et al., 2010; Meyling and Eilenberg, 2007; Vega et al., 2012).

Unlike other entomopathogenic microorganisms, fungal entomopathogens infect their hosts through the cuticle and represent low risk to natural enemies, other non-arthropod hosts and vertebrates (Zimmermann, 2007a, 2007b). These characteristics make them great candidates for development as biopesticides (Lacey et al., 2015; St. Leger and Wang, 2020). *Metarhizium* has been integrated into biological control programs in Brazil since the 1970s when *Metarhizium anisopliae* (Metsch.) Sorokin was first used to control the spittlebug *Mahanarva fimbriolata* Stål (Hemiptera: Cercopidae) (Li et al., 2010). This turns to be one of the world's most extensive biological control programs, with *M. anisopliae* being applied in 2 million ha of sugarcane (Mascarin et al., 2019; Parra, 2014).

Metarhizium anisopliae, *Metarhizium brunneum* Petch and *Metarhizium robertsii* Bischoff, Rehner & Humber are three species of soil-inhabiting, generalist entomopathogens capable of infecting a diverse array of insect species (Hu et al., 2014; St. Leger and Wang, 2020). Despite the similarities of their ecological niches, there is an essential difference regarding their distribution in Brazilian biomes. *Metarhizium anisopliae* s.str. can be split into three subclades based on data of the 5' EF-1α region (Mani 1, Mani 2 and Mani 3), and only isolates of subclade Mani 2 are commonly isolated from naturally infected insects in field conditions, indicating an adaptation to obtain resources from insects (Rezende et al., 2015). In contrast, isolates of *M. brunneum* and *M. robertsii* are rarely observed causing infections in insects in the above-ground environment, although they can do so in laboratory conditions. Instead, these species are commonly associated with plant roots, indicating they are more adapted to the rhizosphere environment (Iwanicki et al., 2019; Lopes et al., 2013; Rezende et al., 2015; Sasan and Bidochka, 2012; Steinwender et al., 2015).

Most studies about entomopathogenic fungi and their hosts evaluate single infections of a pathogen infecting a host species at a time. However, there is evidence that concomitant infections, i.e., infections involving at least two species or genotypes of pathogens/parasites, occur in several host-pathogen systems and may be the rule in natural systems (Cox, 2001; Pedersen and Fenton, 2007; Rigaud et al., 2010). Diverse communities of pathogens and parasites naturally occur in these environments (Lafferty et al., 2008; Meyling and Hajek, 2010), and concomitant infections likely occur in insect hosts as well (Thomas et al., 2003). Effects of pathogen co-infections may depend on exposure level, the environmental conditions or the order in which the propagules are acquired. Effects of co-infections can be synergistic (when the effect of a combination is more pronounced than that of each pathogen alone), antagonistic (when the effect of a combination is less pronounced than that of each pathogen alone) or independent (when the pathogens do not interact in a combination) (Inglis et al., 1999; Meyling et al., 2018; Staves and Knell, 2010; Thomas et al., 2003; Wang et al., 2002).

Based on this, in this chapter, the mortality of selected isolates of *M. anisopliae* s.str. subclade Mani 2, *M. brunneum* and *M. robertsii* against *T. molitor* larvae was investigated through two methods, representing exposure to aerial conidia in the above-ground environment and exposure to conidia in the soil environment. We hypothesized that isolates of *M. anisopliae* are more adapted to interactions with insects in the phylloplane than *M. robertsii* and *M. brunneum* isolates, thus they would show relatively higher virulence and sporulation in the hosts when applied above-ground, whereas isolates of *M. robertsii* and *M. brunneum*, more adapted to the soil environment, would show relatively higher virulence and sporulation below-ground. Based on the premise mentioned above that concomitant infections likely occur in insects in natural

environments, mixtures of isolates were also tested, evaluating differences in their virulence against the host.

4.2. Materials and methods

4.2.1. Fungal isolates

Twelve isolates of *Metarhizium* spp. (six of *M. anisopliae* subclade Mani 2, three of *M. robertsii* and three of *M. brunneum*) were selected from the Entomopathogen Collection "Prof. Sérgio Batista Alves", of the Laboratory of Pathology and Microbial Control of Insects, at the Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (Piracicaba, SP, Brazil). Table 1 contains information about the collection site and origin of the isolates.

Table 1. List of isolates used in the study, selected from the Entomopathogen Collection "Prof. Sérgio Batista Alves", ESALQ/USP, Piracicaba, São Paulo, Brazil.

Species	Isolate code	Origin	Collection site (city, state)
Metarhizium anisopliae s.str. subclade Mani 2	ESALQ 43	Hemiptera: Cercopidae	Flexeiras, Alagoas
	ESALQ 1116	Coleoptera: Scarabaeidae	Piracicaba, São Paulo
	ESALQ 1641	Hemiptera: Cercopidae	Boca da Mata, Alagoas
	ESALQ 1076	Meadow soil	Arapongas, Paraná
	ESALQ 1175	Meadow soil	Córrego Rico, São Paulo
	ESALQ 1604	Biotech G, Biotech® Controle Biológico (commercial isolate)	Unknown
Metarhizium brunneum	ESALQ 5022	Sugarcane soil	Iracemápolis, São Paulo
	ESALQ 5286	Sugarcane soil	Iracemápolis, São Paulo
	ESALQ 5181	Sugarcane root	Iracemápolis, São Paulo
Metarhizium robertsii	ESALQ 1426	Soybean soil	Londrina, Paraná
	ESALQ 1635	Native forest soil	Delmiro Gouveia, Alagoas
	ESALQ 5168	Coleoptera: Scarabaeidae	Iracemápolis, São Paulo

4.2.2. Insect rearing

Larvae of the yellow mealworm *Tenebrio molitor* were obtained from a rearing maintained in a climate room (28 ± 2°C, 70 ± 10% R.H.), fed with a diet consisting of 50% wheat bran and 50% quail feed, with the addition of carrot slices. The length of the insects ranged between 1.0 and 1.5 cm. Before use in experiments, the larvae were washed with sterile distilled water and dried on a paper towel.

4.2.3. Virulence bioassays

For experiments with isolates applied separately, there were 13 treatments: application of conidial suspensions of 1-6) *M. anisopliae* subclade Mani 2 isolates (n=6); 7-9) *M. robertsii* isolates (n=3); 10-12) *M. brunneum* isolates (n=3); and 13) control.

The most virulent isolate of each species was selected for tests with combinations of isolates, based on the experiments with single isolate applications. These experiments included four treatments with application of combinations of conidial suspensions of isolates: 1) *M. anisopliae* subclade Mani 2 + *M. robertsii*; 2) *M. anisopliae* subclade Mani 2 + *M. brunneum*; 3) *M. robertsii* + *M. brunneum*; 4) control.

Each bioassay was repeated three times.

4.2.4. Exposure above-ground

The isolates were cultivated in Petri dishes (Ø = 9 cm) containing Potato Dextrose Agar (PDA; Difco Laboratories, Sparks, MD, USA) and held in B.O.D. incubator (25 ± 1 °C, 12h photophase) for 7–10 days. Then, using a spatula, conidia were harvested to prepare suspensions of each isolate using sterile distilled water plus 0.05% Tween 80, and concentrations were adjusted to 10^8 conidia mL^{-1}; this concentration was selected based on a previous test in the laboratory.

Larvae were put in Petri dishes (Ø = 12 cm) and sprayed in a Potter spray tower (15 psi) with 2 mL of fungal suspensions. Larvae in the control treatment were sprayed with sterile distilled water plus 0.05% Tween 80. There were five replicates (Petri dishes) per treatment, with ten larvae in each replicate dish. After pulverization, the Petri dishes with exposed larvae were transferred to a B.O.D. incubator (25 ± 1 °C, 12h photophase) and provided with carrot daily. Evaluations of mortality were carried out daily for ten days.

Isolates ESALQ 5168 (*M. robertsii*), ESALQ 5022 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae*) were selected for the application of combinations. The three mixture combinations of fungal isolates were prepared by combining equal volumes of the fixed conidial suspensions at 10^8 conidia mL^{-1}, then using a vortex to ensure proper mixing. The application followed as described above.

4.2.5. Exposure below-ground

The top layer of a sandy clay soil was collected from the experimental area of the Department of Entomology and Acarology of ESALQ, sieved and stored in plastic bags (25 x 35 x 0.010 cm) for one day at room temperature. Each bag contained 200 g of non-sterile soil.

Procedures for fungal growth were the same as described in 2.3.1, and fungal suspensions were prepared with an adjusted concentration of 10^8 conidia mL^{-1}. For each isolate (except ESALQ 1635 and ESALQ 5168, see below), 5 mL of the suspensions were applied in a Schott glass bottle containing 50 g of hydrated, sterile parboiled rice. Bottles were shaken and placed in an incubator for ca. ten days (25 ± 1 °C, 12h photophase) for conidial production. After this period, the rice was washed with 100 mL of sterile distilled water plus 0.05% Tween 80 and filtered through a sieve, resulting in conidial suspensions whose concentrations were determined ($2x10^6$ conidia mL^{-1}) and applied at $2x10^5$ conidia g^{-1} of soil; this concentration was selected based on a review paper by Scheepmaker & Butt (2010), which report a natural background level of up to 10^6 CFU g^{-1} of soil for *M. anisopliae*. Isolates ESALQ 1635 and ESALQ 5168, which had poor sporulation on rice in bottles, were produced in polypropylene bags (10 mL of suspensions applied in 300 g of hydrated, sterile parboiled rice, and after conidial production, the rice was washed with 300 mL of sterile distilled water).

Twenty milliliters of the suspensions were applied in the plastic bags containing the soil. The content was shaken inside the bag to mix the suspension with the substrate, which was then placed in plastic cups (300 ml), leaving a space left between the soil surface and the pot lid. One day after inoculation, ten *T. molitor* larvae were placed in each cup, walking freely. The cups were maintained in B.O.D. incubator (25 °C, 0:24h L:D) for 21 days. To increase the contact of the insects with the soil, the cups were inverted daily during the first week. The control treatment contained soil inoculated with sterile distilled water plus 0.05% Tween 80. There were 5 cups per treatment. The soil was moistened with sterile distilled water when necessary. Evaluations of insect mortality were carried out from the fifth day, every three days, for 21 days. Dead larvae were removed from the cups.

Isolates ESALQ 1426 (*M. robertsii*), ESALQ 5286 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae*) were selected for the application of mixtures. The mixtures were prepared by combining equal volumes of the different conidial suspensions, to reach a final concentration of $2x10^5$ conidia g^{-1} of soil, then using a vortex to ensure proper mixing. The application followed as described above.

4.2.6. Sporulation on cadavers

Dead larvae from both bioassays were surface sterilized first in 70% alcohol, and later in 1% sodium hypochlorite, washed in distilled water, then placed in moist chambers inside B.O.D. incubator (25 ± 1 °C, 12h photophase) for seven days to confirm the fungal emergence from the cadavers. Identification of Metarhizium spp. was based on morphological criteria of appearance of green conidial clusters characteristic for the genus (Humber, 2012).

4.2.7. Statistical analyses

Data for the three replicates of each bioassay were combined after an ANOVA confirmed that the output of each isolate did not differ across the replicates ($P > 0.05$). Kaplan-Meier survival estimates were obtained using the function "survfit()" from the package "survival" (Therneau, 2021; Therneau and Grambsch, 2000), and the curves were generated using the function "ggsurvplot()" from the package "survminer" (Kassambara et al., 2021). Survival curves were compared through a log-rank test using the function "survdiff()" from the package "survival"; when significant differences were detected, pairwise log-rank tests were performed using the function "pairwise_survdiff()" from the package "survminer".

Generalized linear models (GLMs) were used for the analysis of sporulation in cadavers. The response variable was expressed as percentage of mycosed larvae (the number of sporulating larvae divided by the number of larvae exposed to the fungal treatments). The models which best described the data were selected based on Akaike's Information Criterion and using half-normal plots (Moral et al., 2017). Estimated means for each treatment were compared using a Tukey HSD test with FDR-adjusted P-values ($\alpha = 0.05\%$), using the function "glht" from the package "multcomp" (Hothorn et al., 2008). Median lethal times (LT_{50}s) were estimated using the package "flexsurv" (Jackson, 2016). All analyses were conducted in R (R CORE TEAM, 2019).

4.3. Results

4.3.1. Exposure above-ground

Survival of *T. molitor* larvae significantly differed among treatments (Kaplan-Meier: $\chi^2 = 367$, d.f. = 12, $P < 0.001$), with the control showing higher survival probability than each of the 12 isolates (log-rank: $P < 0.001$ for all pairwise comparisons between the control and any isolate; Figure 1). Isolate ESALQ 1604 (*M. anisopliae*) was the least virulent (log-rank: $P < 0.001$ for all

pairwise comparisons against any isolate) and it was the only one that did not achieve at least 50% of mortality by the end of the evaluation time. Survival was the lowest when isolates ESALQ 1116, ESALQ 1175, ESALQ 1641 (*M. anisopliae*), ESALQ 5168 (*M. robertsii*), ESALQ 5022 and ESALQ 5181 (*M. brunneum*) were applied, resulting in ≥75% mortalities and LT_{50} values varying between 6.6 and 7.4 days (Table 2). Based on this result, isolates ESALQ 1116, ESALQ 5168 and ESALQ 5022 were selected for the application of mixtures of isolates.

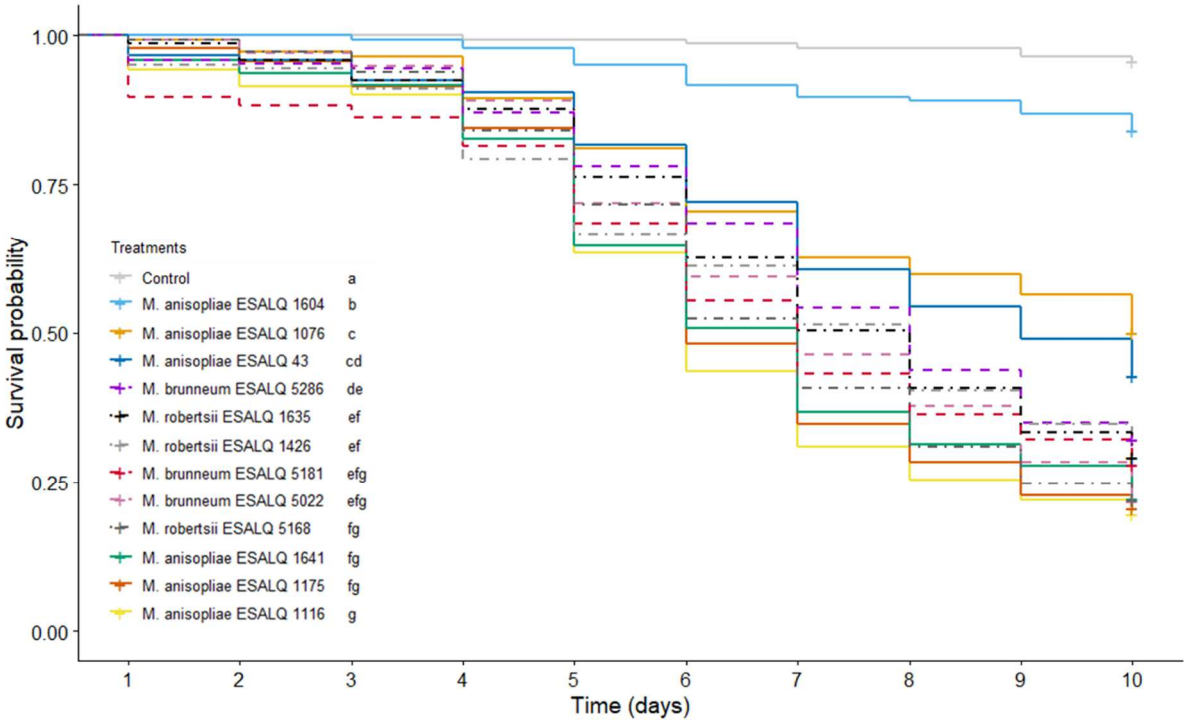

Figure 1. Daily cumulative survival probability (Kaplan-Meier) of *Tenebrio molitor* larvae inoculated through a Potter spray tower with fungal suspensions (10^8 conidia mL^{-1}) of isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. According to a log-rank test, treatments followed by different letters are significantly different ($\alpha = 0.05\%$).

Table 2. Median lethal time (LT_{50}; in days) of *Tenebrio molitor* larvae inoculated through a Potter spray tower with fungal suspensions (10^8 conidia mL^{-1}) of isolates of *Metarhizium anisopliae* s.str. Mani 2 (*Ma*), *M. brunneum* (*Mb*) and *M. robertsii* (*Mr*).

Treatment	LT_{50} (days)	LCL[1]	UCL[2]
Ma ESALQ 1604	> 10	-	-
Ma ESALQ 1076	9.9	8.9	10.9
Ma ESALQ 43	9.1	8.3	10.0
Mb ESALQ 5286	8.1	7.4	8.8
Mr ESALQ 1635	7.8	7.2	8.5
Mr ESALQ 1426	7.7	7.0	8.4
Mb ESALQ 5181	7.4	6.8	8.1
Mb ESALQ 5022	7.3	6.7	7.9
Mr ESALQ 5168	7.0	6.5	7.6
Ma ESALQ 1641	7.0	6.4	7.6
Ma ESALQ 1175	6.8	6.2	7.3
Ma ESALQ 1116	6.6	6.1	7.2

[1]LCL = lower confidence limit (95%)
[2]UCL = upper confidence limit (95%)

The binomial-normal model was selected for larval sporulation data. Isolates ESALQ 1604 (*M. anisopliae*) and ESALQ 5181 (*M. brunneum*) did not differ in terms of sporulation in cadavers (less than 25% of larval sporulation), and exhibited significantly lower mean percentages of sporulation than isolates ESALQ 1175, ESALQ 1641 (*M. anisopliae*), ESALQ 5022, ESALQ 5286 (*M. brunneum*) and ESALQ 1426 (*M. robertsii*). The other comparisons between isolates were not significantly different (Figure 2). Dead control larvae did not show any sign of sporulation.

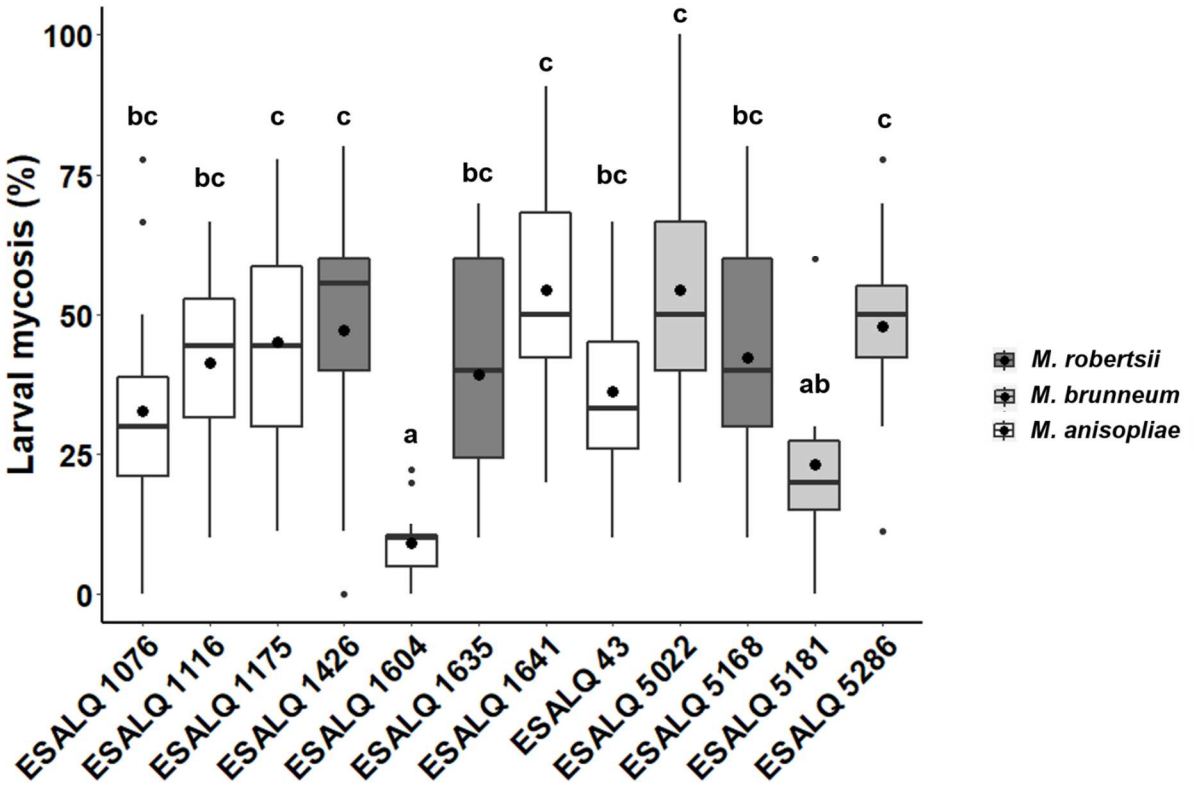

Figure 2. Percentage of sporulation of *Tenebrio molitor* larvae inoculated through a Potter spray tower with fungal suspensions (10^8 conidia mL^{-1}) of isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. According to a Tukey HSD test, means followed by different letters are significantly different (α = 0.05%). Boxes show the median, 25th and 75th percentiles, dots inside the boxes represent the mean percentage for each isolate, and dots outside the boxes represent outliers.

When mixtures of two isolates were applied, there was also an effect of treatment in the survival of *T. molitor* (Kaplan-Meier: χ^2 = 172, d.f. = 3, P < 0.001), with fungal treatments resulting in lower survival probability than the control (log-rank: P < 0.001 for all pairwise comparisons between the control and any combination of isolates; Figure 3). Virulence was similar between the mixtures ESALQ 5168+5022 (*M. robertsii* + *M. brunneum*) and ESALQ 5168+1116 (*M. robertsii* + *M. anisopliae*) (P = 0.097), and between ESALQ 5168+1116 and ESALQ 5022+1116 (P = 0.592), whereas mixture ESALQ 5022+1116 was significantly more virulent than ESALQ 5168+5022 (P = 0.02). The LT_{50} values varied between 7.3 and 8.5 days (Table 3). Larval sporulation data fitted a binomial-normal model, with the three mixtures showing similar mean sporulation (Figure 4). Sporulation was not observed in dead control larvae.

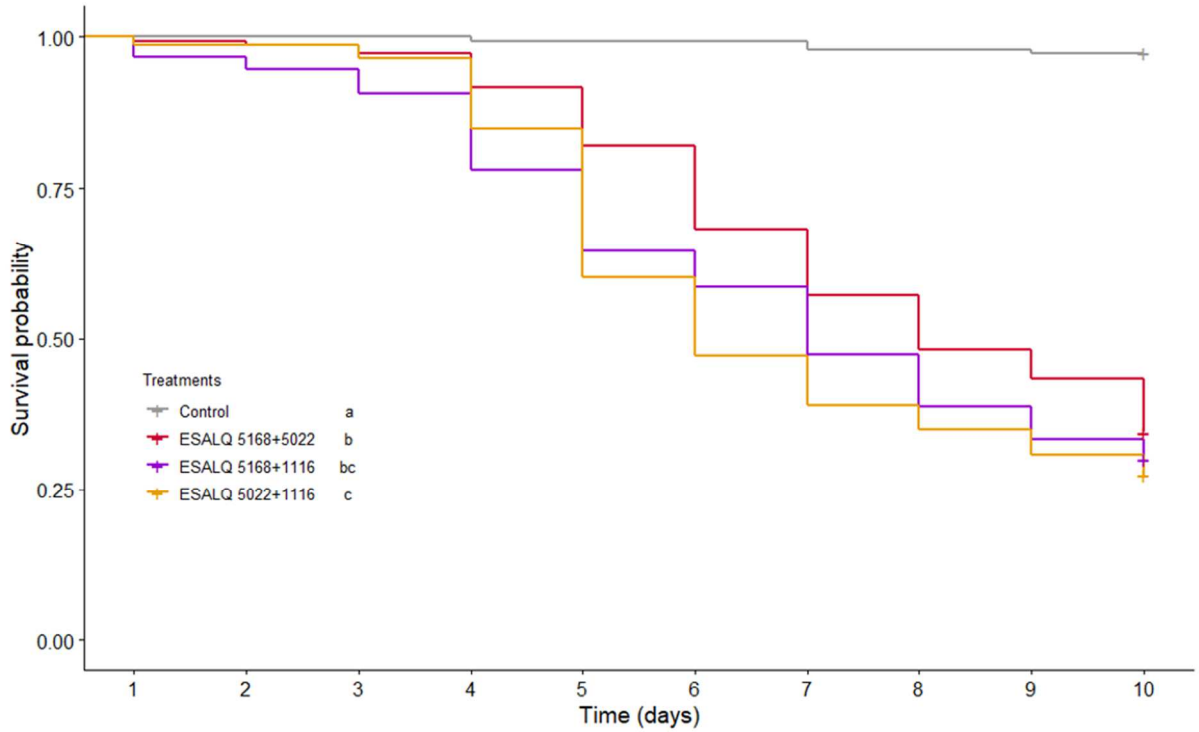

Figure 3. Daily cumulative survival probability (Kaplan-Meier) of *Tenebrio molitor* larvae after being inoculated through a Potter spray tower with mixtures of fungal suspensions (10^8 conidia mL^{-1}) of isolates ESALQ 5168 (*Metarhizium robertsii*), ESALQ 5022 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2). According to a log-rank test, treatments followed by different letters are significantly different (α = 0.05%).

Table 3. Median lethal time (LT_{50}; in days) of *Tenebrio molitor* larvae after being inoculated through a Potter spray tower with mixtures of fungal suspensions (10^8 conidia mL^{-1}) of isolates ESALQ 5168 (*Metarhizium robertsii*; *Mr*), ESALQ 5022 (*M. brunneum*; *Mb*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2; *Ma*). LT_{50} of the three isolates are presented for comparisons (from Table 2).

Treatment		LT_{50} (days)	LCL[1]	UCL[2]
Mr + *Mb*	ESALQ 5168 + ESALQ 5022	8.5	7.8	9.2
Mr + *Ma*	ESALQ 5168 + ESALQ 1116	7.6	7.0	8.3
Mb + *Ma*	ESALQ 5022 + ESALQ 1116	7.3	6.7	7.9
Mb	ESALQ 5022	7.3	6.7	7.9
Mr	ESALQ 5168	7.0	6.5	7.6
Ma	ESALQ 1116	6.6	6.1	7.2

[1]LCL = lower confidence limit (95%)
[2]UCL = upper confidence limit (95%)

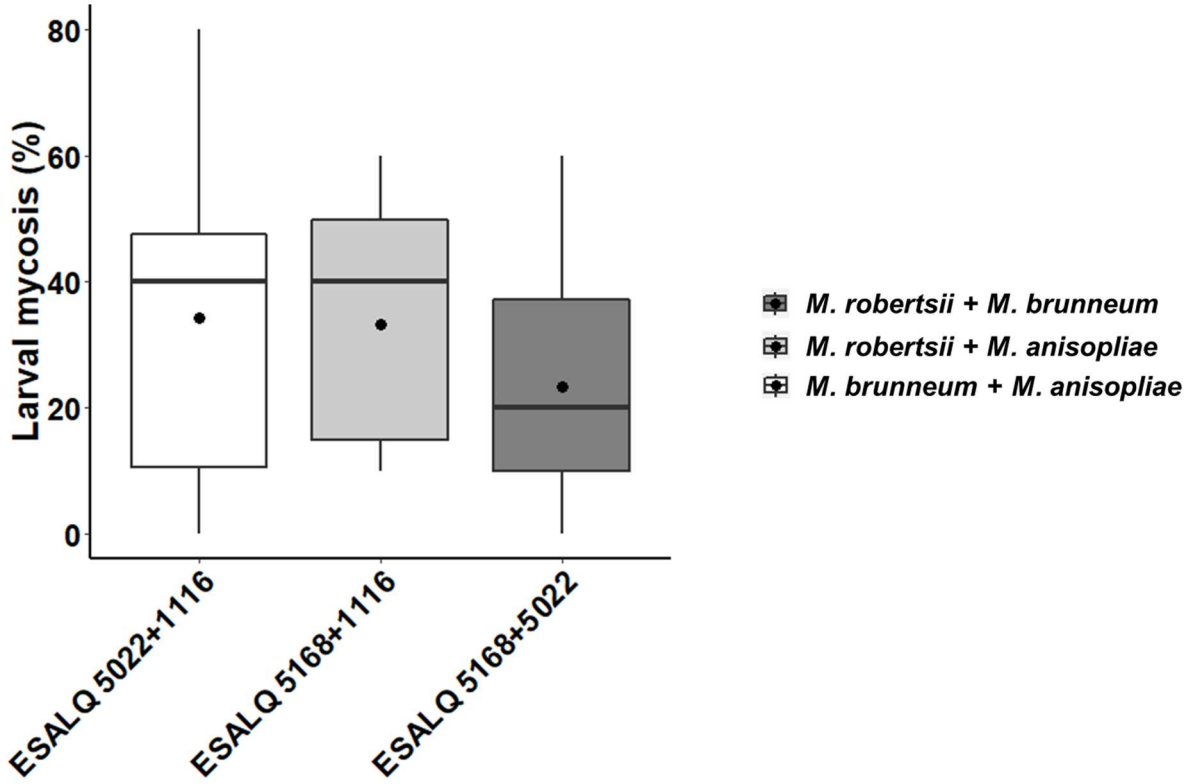

Figure 4. Percentage of sporulation of *Tenebrio molitor* larvae inoculated through a Potter spray tower with mixtures of fungal suspensions (10^8 conidia mL^{-1}) of isolates ESALQ 5168 (*Metarhizium robertsii*), ESALQ 5022 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2). According to a Tukey HSD test, means were not significantly different ($\alpha = 0.05\%$). Boxes show the median, 25th and 75th percentiles, and dots inside the boxes represent the mean percentage for each isolate.

4.3.2. Exposure below-ground

Survival of *T. molitor* larvae exposed to individual *Metarhizium* spp. isolates in non-sterile soil was significantly affected by treatments (Kaplan-Meier: $\chi^2 = 1{,}119$, d.f. = 12, $P < 0.001$). Control larvae had a significantly higher survival probability than all isolates (log-rank: $P < 0.001$ for all pairwise comparisons between control and any isolate; Figure 5). Among fungal treatments, isolate ESALQ 1604 (*M. anisopliae*) was the least virulent (log-rank: $P < 0.001$ for all pairwise comparisons against any isolate) and was the only one not to achieve 100% of larval mortality by the end of the experimental time. There were significant differences among lethal times. Isolate ESALQ 1116 (*M. anisopliae*) was the most virulent (log-rank: $P < 0.02$ for all pairwise comparisons against any isolate), with a LT_{50} of 7.2 days (Table 4), followed by ESALQ 1426 (*M. robertsii*; LT_{50} = 7.5 days). Based on the results, isolates ESALQ 1116, ESALQ 1426 and ESALQ 5286 were selected for application of mixtures.

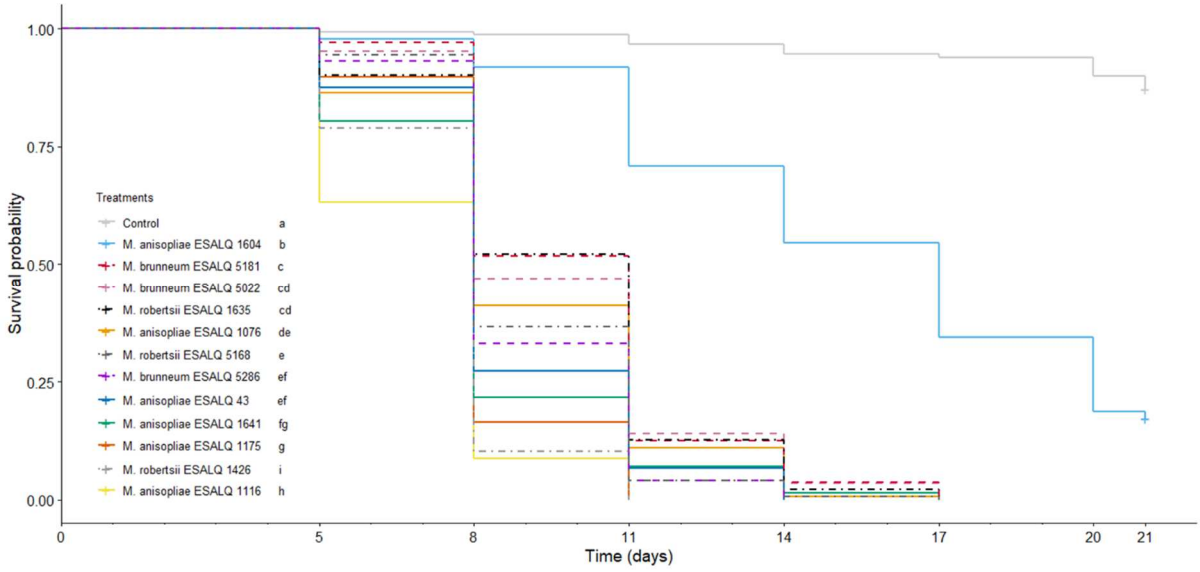

Figure 5. Cumulative survival probability (Kaplan-Meier) of *Tenebrio molitor* larvae after exposure to soil inoculated with fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. According to a log-rank test, treatments followed by different letters are significantly different ($\alpha = 0.05\%$).

Table 4. Median lethal time (LT_{50}; in days) of *Tenebrio molitor* larvae after exposure to soil inoculated with fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates of *Metarhizium anisopliae* s.str. Mani 2 (*Ma*), *M. brunneum* (*Mb*) and *M. robertsii* (*Mr*).

Treatment		LT_{50} (days)	LCL[1]	UCL[2]
Ma	ESALQ 1604	16.5	15.8	17.3
Mb	ESALQ 5181	10.0	9.6	10.4
Mb	ESALQ 5022	10.0	9.6	10.4
Mr	ESALQ 1635	9.8	9.5	10.3
Ma	ESALQ 1076	9.4	9.0	9.8
Mr	ESALQ 5168	9.0	8.6	9.3
Mb	ESALQ 5286	8.8	8.4	9.1
Ma	ESALQ 43	8.7	8.4	9.1
Ma	ESALQ 1641	8.7	8.4	9.1
Ma	ESALQ 1175	7.9	7.6	8.2
Mr	ESALQ 1426	7.5	7.2	7.8
Ma	ESALQ 1116	7.2	6.9	7.5

[1]LCL = lower confidence limit (95%)
[2]UCL = upper confidence limit (95%)

A binomial-normal model was selected as the best-fit for larval sporulation data. Sporulation was not observed in dead control larvae. Mean sporulation was significantly lower for isolates ESALQ 1604 (*M. anisopliae*), ESALQ 5181 (*M. brunneum*) and ESALQ 5168 (*M. robertsii*), achieving less than 20% of sporulation. Except for these three isolates and ESALQ 1635, the other isolates had higher mean sporulation (above 40%), which did not differ among them (Figure 6).

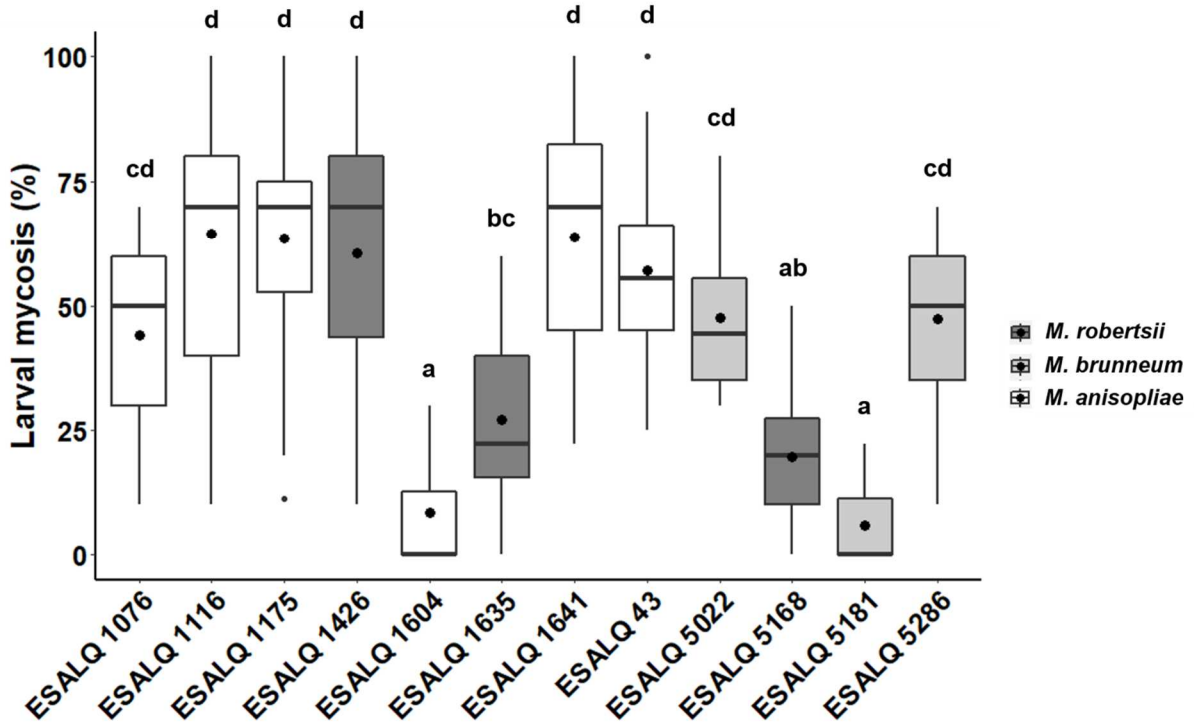

Figure 6. Percentage of sporulation of *Tenebrio molitor* larvae after exposure to soil inoculated with fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii*. According to a Tukey HSD test, means followed by different letters are significantly different (α = 0.05%). Boxes show the median, 25th and 75th percentiles, dots inside the boxes represent the mean percentage for each isolate, and dots outside the boxes represent outliers.

Application of mixtures of two fungal isolates in soil resulted in differences between the groups tested (Kaplan-Meier: $\chi^2 = 476$, d.f. = 3, $P < 0.001$), in which control larvae had higher survival probability than the mixtures (log-rank: $P < 0.001$ for all pairwise comparisons between the control and any combination of isolates; Figure 7). The three mixtures did not differ and achieved 100% mortality by day 14 (log-rank: $P = 0.85$ for pairwise comparisons between fungal treatments), with median lethal times of ca. 7 days (Table 5). Larval sporulation data fitted a binomial-normal model. There were no differences between the three mixtures, with mean percentages of sporulation varying between 50 and 65% (Figure 8).

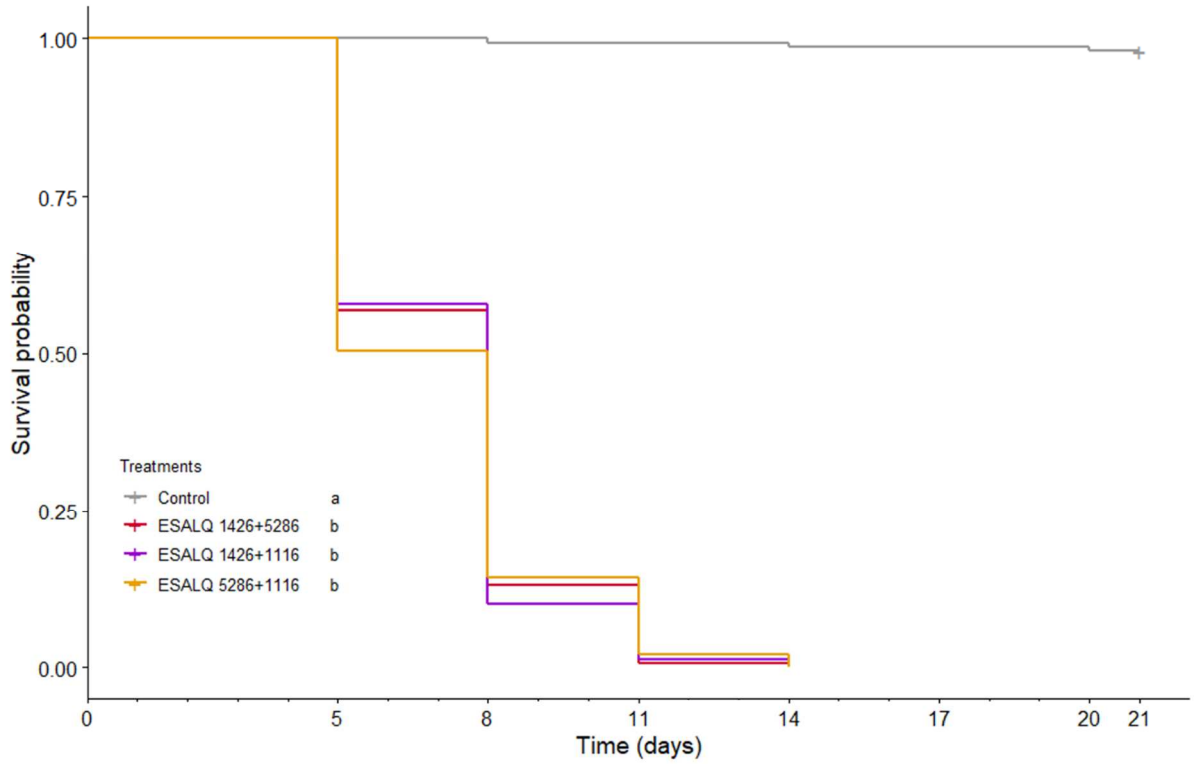

Figure 7. Cumulative survival probability (Kaplan-Meier) of *Tenebrio molitor* larvae after exposure to soil inoculated with mixtures of fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates ESALQ 1426 (*Metarhizium robertsii*), ESALQ 5286 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2). According to a log-rank test, treatments followed by different letters are significantly different ($\alpha = 0.05\%$)

Table 5. Median lethal time (LT_{50}; in days) of *Tenebrio molitor* larvae after exposure to soil inoculated with mixtures of fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates ESALQ 1426 (*Metarhizium robertsii*; *Mr*), ESALQ 5286 (*M. brunneum*; *Mb*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2; *Ma*). LT_{50} of the three isolates are presented for comparisons (from Table 4).

Treatment		LT_{50} (days)	LCL[1]	UCL[2]
Mr + *Ma*	ESALQ 1426 + ESALQ 1116	7.0	6.7	7.3
Mr + *Mb*	ESALQ 1426 + ESALQ 5286	7.0	6.7	7.4
Mb + *Ma*	ESALQ 5286 + ESALQ 1116	7.1	6.7	7.5
Ma	ESALQ 1116	7.2	6.9	7.5
Mr	ESALQ 1426	7.5	7.2	7.8
Mb	ESALQ 5286	8.8	8.4	9.1

[1]LCL = lower confidence limit (95%)
[2]UCL = upper confidence limit (95%)

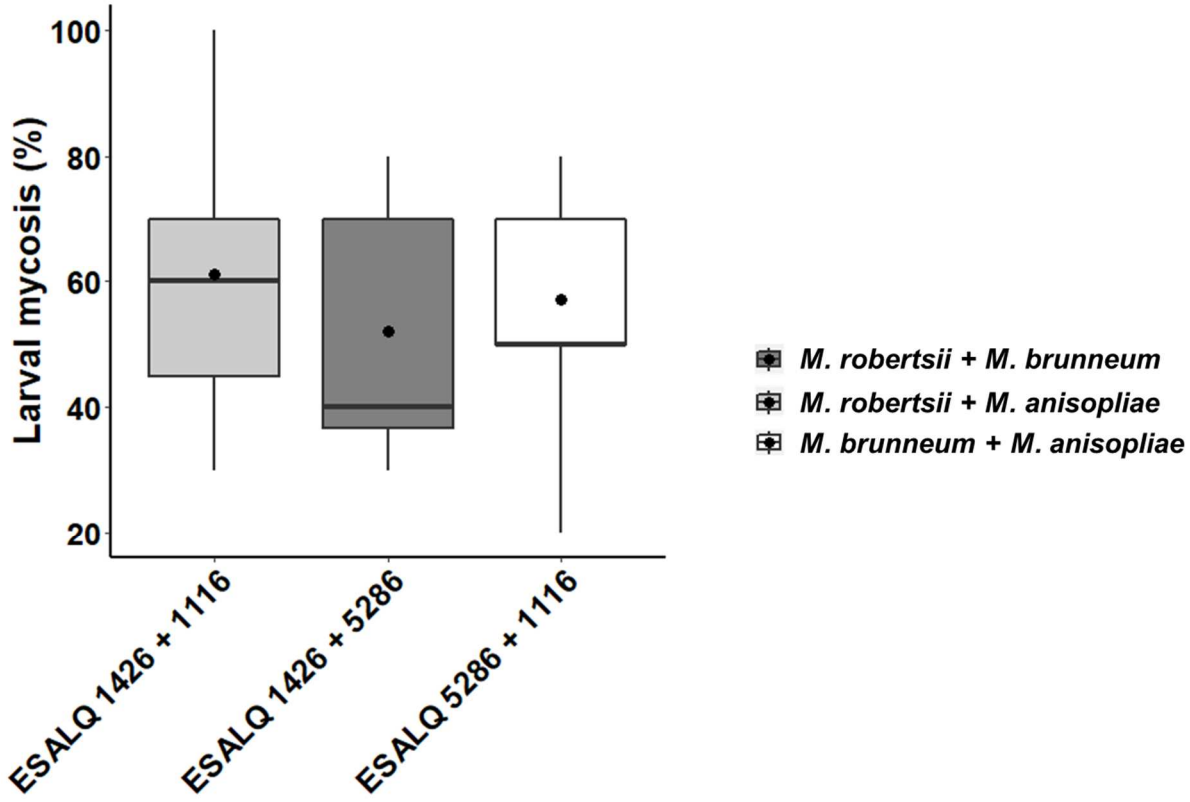

Figure 8. Percentage of sporulation on *Tenebrio molitor* larvae after exposure to soil inoculated with mixtures of fungal suspensions ($2x10^5$ conidia g^{-1} of soil) of isolates ESALQ 1426 (*Metarhizium robertsii*), ESALQ 5286 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae* s.str. Mani 2), adopting a binomial-normal model. According to a Tukey HSD test, means were not significantly different ($\alpha = 0.05\%$). Boxes show the median, 25th and 75th percentiles, and dots inside the boxes represent the mean percentage for each isolate.

4.4. Discussion

In the present study, we used two inoculation methods to expose larvae of *T. molitor* with 12 isolates representing three *Metarhizium* species. This insect species is susceptible to entomopathogenic fungi and has been used as a model insect to recover fungal isolates from soil samples and in tests of virulence, or as a model to evaluate fungal activity against other arthropod species (Bharadwaj and Stafford, 2011; Meyling and Eilenberg, 2007; Oreste et al., 2012; Zimmermann, 2007a).

Our hypothesis was that *M. anisopliae* isolates would be more virulent by the conventional spraying method (representing exposure to infective conidia in the above-ground environment) while isolates of *M. brunneum* and *M. robertsii* would be more virulent by exposure in the soil environment. This hypothesis was not confirmed, since intraspecific differences were as pronounced as interspecific variation. Intraspecies variability in virulence has been reported for *Metarhizium* spp. used against different hosts (e.g. Brunner-Mendoza et al., 2017; Dotaona et al.,

2015; Oreste et al., 2012; Resquín-Romero et al., 2020), and in our study, such variation was also detected when fungal treatments were applied through the spray tower, and for the bait technique. Ondiaka et al. (2008) reported intraspecies variability for *M. anisopliae* and *Beauveria bassiana* tested against adults of the sweet potato weevil *Cylas puncticollis* (concentration: 10^7 conidia mL^{-1}), and stated that strain selection is needed for biological control.

The selection process of an isolate for development as a biopesticide aims to find a suitable isolate that can be mass-produced and control insects under field conditions (Jackson et al., 2010), and should consider aspects such as tolerance to abiotic factors and behavior of the host (Jackson et al., 2010; Lacey et al., 2015). Although a previous study with isolate ESALQ 1604 reported that it is capable of controlling spittlebugs in sugarcane fields for up to sixty days (Iwanicki et al., 2019), the results of the present chapter showed that ESALQ 1604, which is a commercial isolate, caused the lowest larval mortality and lowest percentage of larval sporulation in both methods of exposure against *T. molitor*.

Application of entomopathogenic fungi through spray methods are typically inundative and requires a great amount of inoculum, in high concentration, to induce sufficient mortality in an insect population. In contrast, application in the soil creates an arena containing the conidia, and the infection will develop if the host acquires a sufficient number of propagules during its movement through the soil matrix; in this case, the amount of inoculum depends on factors such as behavior and activity of the host, texture of the soil, moisture content and the microbial community in the soil (Jaronski, 2010). In our study, for the insect bait bioassay, we had a small arena (300 mL plastic cups) in which *T. molitor* larvae were enclosed, constantly moving and coming into contact with fungal conidia, in the absence of light and with relatively high moisture level, conditions which are expected to favor infectivity. Despite the different concentrations used in each inoculation method, there was not a great difference in the total number of conidia applied in both methods, which could explain why they had similar proportion of survival and LT_{50} values.

Sporulation was generally lower using the bait method for isolates of *M. brunneum* and *M. robertsii*, compared to sporulation using the spray tower method. This was evident especially for isolate ESALQ 5168, whose mean sporulation percentage in the spray tower method did not differ from the highest ones, but this isolate was among the ones with lowest mean sporulation in the bait method, not differing from isolate ESALQ 1604. We hypothesized that *M. brunneum* and *M. robertsii* are adapted to living below-ground, where the fungus kills soil-borne insects and can potentially transfer insect-derived nitrogen to plants via mycelial connection to endophytically colonized roots (Behie et al., 2017, 2012; Rezende et al., 2015; Steinwender et al., 2015). Thus, it

is possible that there is a selection pressure for development of mycelia instead of conidia. Similar conclusion was obtained from the data about temperature-tolerance of conidia and mycelia, presented in Manuscript 1. This strategy of survival in the soil would be compatible with the phenotype "creeper", which primarily produces high density of mycelia, increasing the chances of the fungus to encounter a plant or insect host in the soil (Angelone et al., 2018). Regarding *M. anisopliae*, even though sporulation was generally higher using the bait method, isolates of this species (except ESALQ 1604) were among the ones with the highest percentage of sporulation using the spray tower method. It seems that *M. anisopliae* would favor production of conidia, in agreement with its presumed adaptation to explore insect resources above-ground, as conidia would be a way to deal with unfavorable conditions above-ground (Manuscript 1), while mycelia may be conducive for plant root colonization below-ground.

One important factor of the dynamics of an entomopathogen is dispersal, as a mean to encounter new hosts (Hajek and Meyling, 2017). In the phylloplane, dispersion of Hypocrealean fungi is passive and mostly relies on abiotic factors such as wind and rain (Meyling and Eilenberg, 2007). Using the spray tower method, isolate ESALQ 5181 (*M. brunneum*) was among the isolates that caused the highest mortalities, but it had one of lowest sporulation percentages. Even though the results of the present chapter and from Manuscript 1 indicate that *M. brunneum* favors production of mycelia instead of conidia, sporulation of ESALQ 5181 was significantly lower compared to its conspecifics, which may suggest that this isolate has a poor dispersal ability above-ground.

Studies to increase efficiency and resilience of fungal entomopathogens in biological control programs have included combination of isolates of the same or of different species, varying in characteristics such as thermotolerance and virulence (Cruz et al., 2006; Seid et al., 2019; Thomas et al., 2003; Wang et al., 2002). In this chapter, the mortality and the LT_{50} obtained by two mixtures [ESALQ 5168+1116 (*M. robertsii* + *M. anisopliae*) and ESALQ 5022+1116 (*M. brunneum* + *M. anisopliae*)] applied using the spray tower method were at a comparable level to the ones caused by single applications. In contrast, the mixture ESALQ 5168+5022 (*M. robertsii* + *M. brunneum*) had a slightly higher LT_{50} reflected by a slightly lower mortality in relation to single applications. Although the three selected isolates did not differ in larval mortality when applied individually, there was a significant difference in the application of mixtures, in which case ESALQ 5022+1116 resulted in higher mortality than ESALQ 5168+5022. Considering these results, it is possible that isolates ESALQ 5022 and ESALQ 1116 act independently when combined, but isolates ESALQ 5022 and ESALQ 5168 show antagonism, that leads to the lower mortality observed. Using the bait method, the mixtures had LT_{50} comparable to single

infections, and did not differ between each other. Our results are in accordance with report by Thomas, Watson & Valverde-Garcia (2003), in which simultaneous infections of the desert locust *Schistocerca gregaria* with *B. bassiana* and *M. acridum* (reported by the authors as *M. anisopliae* var. *acridum*) had the same effect as *M. acridum* alone. Cruz, Gaitan & Gongora (2006) reported that adults of the coffee berry borer *Hypothenemus hampei* infected with mixtures of *B. bassiana* could exhibit similar, higher or lower mortality than individual applications, depending on the virulence and the genetic profile (based on ITS, β-tubulin and AFLP markers) of the isolates. Likewise, Seid et al. (2019) found that mixtures of *B. bassiana* caused equal or lower mortality than single application of the isolates in *T. molitor* larvae, concluding that the efficiency of combinations depends on isolate selection.

Within-host competition can affect not only virulence of isolates, but fungal sporulation as well. Production of fungal propagules on the host would depend on the interaction of the isolates, either being superinfection (when the most virulent isolate in the mixture outcompetes the others) or coinfection (when isolates share resources) (Hughes et al., 2004). In the present study, the use of mixtures did not increase the percentage of sporulation on cadavers: in both application methods, sporulation caused by combinations of isolates were at a similar or lower level compared to single infections of selected isolates. Our results corroborate those by Hughes et al. (2004), with *M. anisopliae* s.l. against the leaf-cutting ant *Acromyrmex echinatior*, and Seid et al. (2019), with *B. bassiana* against *T. molitor*, and according to these authors, this may indicate a case of superinfection.

As described in Manuscript 2, the number of pathogenicity-related proteins or domains of polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) encoded by each isolate showed little intra- and interspecific variation, and this did not result in similar virulence of isolates of the same species, or consistent virulence across a species. Although we obtained the quantity of proteins encoded, we did not study the mechanism of expression of these molecules, and it is possible that the differences in virulence are related to differential expression of these proteins (Leão et al., 2015) or production of different types of destruxins (Hughes et al., 2004).

In the present chapter, intra- and interspecific variability were found in the virulence and sporulation of *Metarhizium* spp. isolates in two methods of exposure, representing above- and below-ground contamination, reinforcing the need for comprehensive isolate selection for their development as biological control agents.

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5. PERSISTENCE OF *Metarhizium* SPP. IN A NON-STERILE SOIL

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ABSTRACT

Metarhizium comprises a group of entomopathogenic fungi with a worldwide distribution. Propagules of *Metarhizium* can remain in the soil environment forming a reservoir of inoculum. In Brazilian soils, *Metarhizium robertsii* and *M. brunneum* are usually associated with plants in the rhizosphere, while *Metarhizium anisopliae* s.str. subclade Mani 2 causes most of the natural infections of insects in the above-ground environment. For use as biocontrol agents, it is crucial to understand the interaction of entomopathogenic fungi with native microbial communities in soils since both groups can affect the survival of each other. In this study, the persistence over time of isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* applied in a non-sterile, sandy clay soil was evaluated in a greenhouse experiment. The hypothesis was that isolates of *M. brunneum* and *M. robertsii* would exhibit higher density at the end of the experimental time than isolates of *M. anisopliae* Mani 2, since they are expected to be better adapted for the soil environment. The densities of all isolates comparably decreased over time, but we found significant variation between the three experimental replicates. The hypothesis was not confirmed, as we did not find differences between species, which might indicate that the selected isolates are similarly affected by the soil environment under the given conditions.

Keywords: *Metarhizium*; soil microbiota; persistence

5.1. Introduction

Metarhizium Sorokin (Ascomycota: Hypocreales) is an important genus of entomopathogenic fungi, with a worldwide distribution in both natural and agricultural ecosystems (Jaronski, 2007; Lacey et al., 2015; Vega et al., 2012), whose species can infect more than 200 insect species from seven orders (Hesketh et al., 2010; Jaronski, 2007; Zimmermann, 2007). Besides their role as entomopathogens, several species of *Metarhizium* also form

endophytic associations with plants (Behie and Bidochka, 2014; Hu and St. Leger, 2002; Wyrebek et al., 2011), where the fungi can provide nitrogen derived from insects to the plant in exchange for carbohydrates (Behie et al., 2017, 2012; Behie and Bidochka, 2014). In addition, root association by *Metarhizium* can enhance plant growth (Jaber and Enkerli, 2016a, 2016b; Sasan and Bidochka, 2012) and protect against herbivores (Canassa et al., 2020).

The soil is composed of a great diversity of microorganisms, such as fungi and bacteria, which form complex communities of millions of species (Bardgett and van der Putten, 2014). As part of their life cycle, these microorganisms may perform beneficial functions such as nutrient cycling, decomposition of organic residues, promoting plant growth, and controlling insects and plant pathogens (Kennedy, 1999). In the soil, entomopathogenic fungal propagules can remain dormant structures until they contact a suitable host (Hesketh et al., 2010; Vega et al., 2012), forming an inoculum reservoir. Under natural conditions, *Metarhizium* sp. can reach up to 10^6 conidia g^{-1} of soil (Scheepmaker and Butt, 2010).

The persistence of entomopathogenic fungi in the soil is influenced by abiotic and biotic factors (Jaronski, 2007). Among the latter, the interaction with other microorganisms (e.g., bacteria, nematodes, protozoans, and other fungi) can impact propagule survival, either directly or indirectly. Early studies demonstrated that non-sterile soils had a fungistatic effect on fungal entomopathogens, but this effect would wear off in sterilized soils (Pereira et al., 1993; Walstad et al., 1970). A study with *Beauveria bassiana* reported that fungistasis was overcome by supplementing the soil with increasing concentrations of glucose: peptone solutions, indicating that germination inhibition was caused by nutrient deprivation (Groden and Lockwood, 1991). Shreds of evidence also suggest that fungistasis may be a result of an absence of stimuli, e.g., contact with a host or ideal level of soil moisture (Jaronski, 2007), or caused by volatile organic compounds released by competitors (Chuankun et al., 2004; Scheepmaker and Butt, 2010).

Several techniques have been developed to recover hypocrealean fungi from the soil and estimate their abundance. The most widespread method is the cultivation-dependent assessment by a spread-plate method, in which soil samples are collected, homogenized with sterile water or buffer, diluted, and then plated in a selective agar media to allow the growth of fungal colonies. Following a pre-determined amount of time, colony-forming units (CFUs) are counted, and the density is calculated, usually as CFU g^{-1} of soil (Inglis et al., 2012).

The distribution of *Metarhizium* species varies according to the geographic region and is possibly influenced by characteristics such as climate conditions and the availability of hosts (Brunner-Mendoza et al., 2019). *Metarhizium robertsii* Bischoff, Rehner & Humber was recovered as the most abundant species in two open field/forest sites in Ontario, Canada (Wyrebek et al.,

2011), while in agricultural fields in Denmark, its occurrence was low (Steinwender et al., 2015, 2014). In Brazil, a comprehensive study conducted in five biomes reported *M. robertsii* as the most abundant species; despite this, natural infections with this species are rare in the country (Botelho et al., 2019), and association of isolates with plants in the rhizosphere are common (Sasan and Bidochka, 2012; Wyrebek et al., 2011). *Metarhizium brunneum* Petch is another species mainly involved in endophytic associations and possibly more adapted to the rhizosphere (Iwanicki et al., 2019; Wyrebek et al., 2011), being reported as the predominant *Metarhizium* species in an experimental field in Denmark and with a very low natural occurrence in Brazil (Botelho et al., 2019; Iwanicki et al., 2019; Steinwender et al., 2015, 2014). *Metarhizium anisopliae* (Metsch.) Sorokin is not abundant in soil samples across Brazil (Botelho et al., 2019); data obtained from the 5'-TEF region from 96 Brazilian isolates of *Metarhizium* spp. supported the existence of three haplotype-based subclades within *M. anisopliae* s.str., designated Mani 1, Mani 2 and Mani 3 (Rezende et al., 2015), with nearly all isolates recovered from insects in field conditions belonging to Mani 2 (Iwanicki et al., 2019; Rezende et al., 2015).

Studies with fungal entomopathogens for biological control purposes usually focus on their development as inundative agents (Castro et al., 2016; Vega et al., 2009). However, when a fungus is introduced into a biological system, it is crucial to consider its interaction with the native microbial community (Guerrero-Guerra et al., 2013; Hirsch et al., 2013; Meyling and Hajek, 2010). In this context, this chapter evaluated the persistence over time of isolates of *M. anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* after application of conidial suspensions in non-sterile, sandy clay soil, filled into plastic pots in a greenhouse. It was hypothesized that isolates of *M. brunneum* and *M. robertsii* are better adapted to survival in the soil and to compete with the soil microbiota than isolates of *M. anisopliae* Mani 2; thus, the densities of *M. brunneum* and *M. robertsii* are expected to be higher *M. anisopliae* Mani 2 by the end of the experimental period.

5.2. Materials and methods

5.2.1. Fungal isolates

Twelve isolates of *Metarhizium* spp. (six of *M. anisopliae* subclade Mani 2, three of *M. robertsii,* and three of *M. brunneum*) were selected from the Entomopathogen Collection "Prof. Sérgio Batista Alves," of the Laboratory of Pathology and Microbial Control of Insects, at the

Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Piracicaba, State of São Paulo, Brazil. Detailed information about the origin of the isolates is shown in Table 1.

Table 1. List of isolates selected for the study, from the Entomopathogen Collection "Prof. Sérgio Batista Alves", Luiz de Queiroz College of Agriculture (ESALQ), Piracicaba, State of São Paulo, Brazil.

Species	Isolate code	Origin	Collection site (city, state)
Metarhizium anisopliae s.str. subclade Mani 2	ESALQ 43	Hemiptera: Cercopidae	Flexeiras, Alagoas
	ESALQ 1116	Coleoptera: Scarabaeidae	Piracicaba, São Paulo
	ESALQ 1641	Hemiptera: Cercopidae	Boca da Mata, Alagoas
	ESALQ 1076	Meadow soil	Arapongas, Paraná
	ESALQ 1175	Meadow soil	Córrego Rico, São Paulo
	ESALQ 1604	Biotech G, Biotech® Controle Biológico (commercial isolate)	Unknown
Metarhizium brunneum	ESALQ 5022	Sugarcane soil	Iracemápolis, São Paulo
	ESALQ 5286	Sugarcane soil	Iracemápolis, São Paulo
	ESALQ 5181	Sugarcane root	Iracemápolis, São Paulo
Metarhizium robertsii	ESALQ 1426	Soybean soil	Londrina, Paraná
	ESALQ 1635	Native forest soil	Delmiro Gouveia, Alagoas
	ESALQ 5168	Coleoptera: Scarabaeidae	Iracemápolis, São Paulo

5.2.2. Preparation of fungal suspensions

Isolates were cultivated in Petri dishes containing Potato Dextrose Agar (PDA) for 10 days (25 ± 1 °C, 12h photophase), after which suspensions of each isolate were prepared (concentration: 10^8 conidia mL^{-1}) and 5 mL were inoculated in Schott glass bottles (250 mL) containing 50 g of hydrated, sterile parboiled rice; in the case of isolates ESALQ 1635 and ESALQ 5168, which exhibited poor sporulation in the glass bottles, 10 mL of suspensions were inoculated in polypropylene bags containing 300 g of rice. Bottles and bags were shaken and placed in an incubator for ca. ten days (25 ± 1 °C, 12h photophase) for conidial production. Rice was then washed with 100 mL (or 300 mL, in the case of polypropylene bags) of sterile distilled water plus 0.05% Tween 80 and filtered, resulting in conidial suspensions whose concentrations were determined and adjusted to $3x10^8$ conidia mL^{-1}.

5.2.3. Experimental set-up

Twenty milliliters of the $3x10^8$ conidia mL^{-1} conidial suspensions were applied in plastic bags containing 300 g of non-sterile sandy clay soil, collected from the experimental area of the

Department of Entomology and Acarology of ESALQ, resulting in a final concentration of $2x10^7$ conidia g^{-1} of soil. Physical analysis of the soil is presented in Appendix A. The bags were shaken to ensure good soil wetting and put in plastic pots (500 mL), which were placed in a greenhouse in a randomized block design. Control was inoculated with sterile distilled water plus 0.05% Tween 80. Each isolate was considered a treatment, and there were five pots (replicates) per treatment.

The experiment was conducted from January to December 2020 and repeated three times, with each replicate experiment lasting 120 days. Temperatures during this period varied between 5.0 and 41.2 °C (according to climate data records from the Department of Biosystems Engineering of ESALQ).

5.2.4. CFU counting

On the experimental times 1 day, 30 days, 60 days and 120 days, two samples of 1 g of soil were collected from each pot, using a cork borer. The samples were homogenized in 10 mL of sterile distilled water and serially diluted up to 4 times. Aliquots of 100 µL were taken from the dilutions, inoculated into Petri dishes containing PDAY (PDA plus 1 g Yeast extract) plus 0.05 g L^{-1} of antibiotic gentamicin and 1 mL L^{-1} of a 4.5% v/v solution of Derosal 500 SC (carbendazim) (which has fungistatic properties at low concentrations), and spread with a Drigalski spatula. There were 2 plates per dilution; they remained open in laminar flow cabinet until the liquid dried, after which they were incubated (25 ± 1 °C, 12h photophase) for 3 to 7 days, when the densities of the isolates were evaluated through CFU, using plates of the dilution that had a suitable number of colonies to count (between 30 and 300). After this, they were incubated again until fungal sporulation was observed, allowing morphological identification of the isolated fungi.

5.2.5. Statistical analysis

Data analysis was performed using the negative binomial regression model. The regression was estimated using the function "glm.nb" from the package "MASS" (Venables and Ripley, 2002).

For each of the three replicates in time, the number of CFUs was averaged between the plates for each treatment and time point. An analysis was conducted to test if the data from the replicates could be combined. There was variability in the behavior of isolates between replicates in time; thus each replicate was analyzed separately. Means were compared by a Tukey Contrast

analysis using the function "emmeans" from the package "emmeans" (Lenth, 2021), with a significance level $\alpha = 0.05$. Graphics were generated using the package "ggplot2" (Wickham, 2016).

5.3. Results

Metarhizium sp. colonies were not found in any plates from control treatments throughout the experiment. In the first replicate, there was a significant effect of days in the number of CFU g^{-1} of soil ($P = 0.009$). There was no significant difference between the curves of the isolates (Figure 1). The mean concentration varied between $2x10^6$ and $1.5x10^7$ CFU g-1 of soil on the first day of evaluation. After 30 days, all isolates showed a slight decrease in CFU counting, except for *M. anisopliae* ESALQ 1604, which had a slight increase. After 60 and 120 days, densities continued to decrease, and by the end of the experimental time, concentrations were below 10^5 conidia g^{-1} of soil.

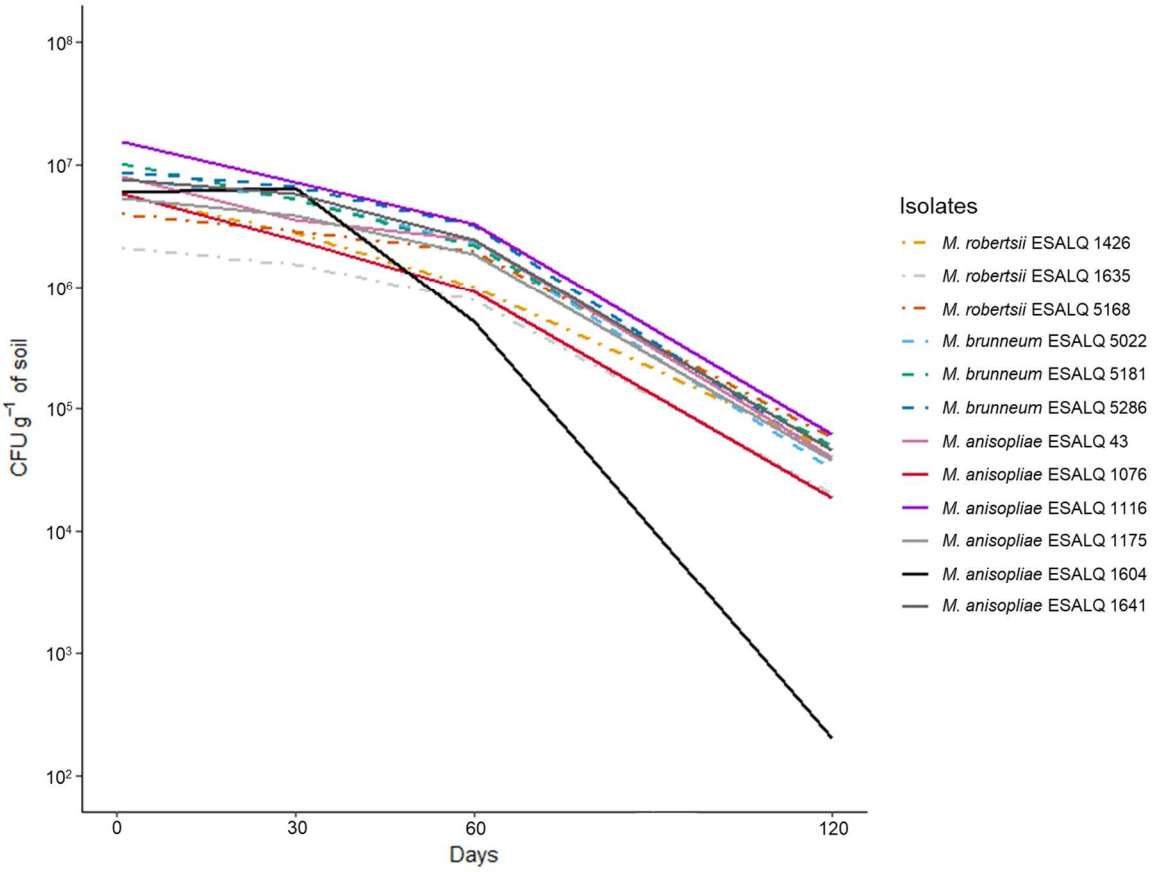

Figure 1. Abundance of isolates (in CFU g^{-1} soil) of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* during 120 days, after inoculation in plastic pots containing non-sterile sandy clay soil (First replicate), adopting a negative binomial model. In this replicate, isolates did not differ according to a Tukey Contrast (α = 0.05%).

In the second replicate, there was also a significant effect of days in the abundance of the isolates (P = 0.004), and there was no difference between the curves of the isolates. The concentration recovered on the first day varied between $2.2x10^6$ and $6.1x10^6$ conidia g^{-1} of soil (Figure 2). Decreases in abundance followed on the 30, 60 and 120-day evaluations, at which point the concentrations varied between $7x10^2$ and $3.2x10^5$ conidia g^{-1} of soil.

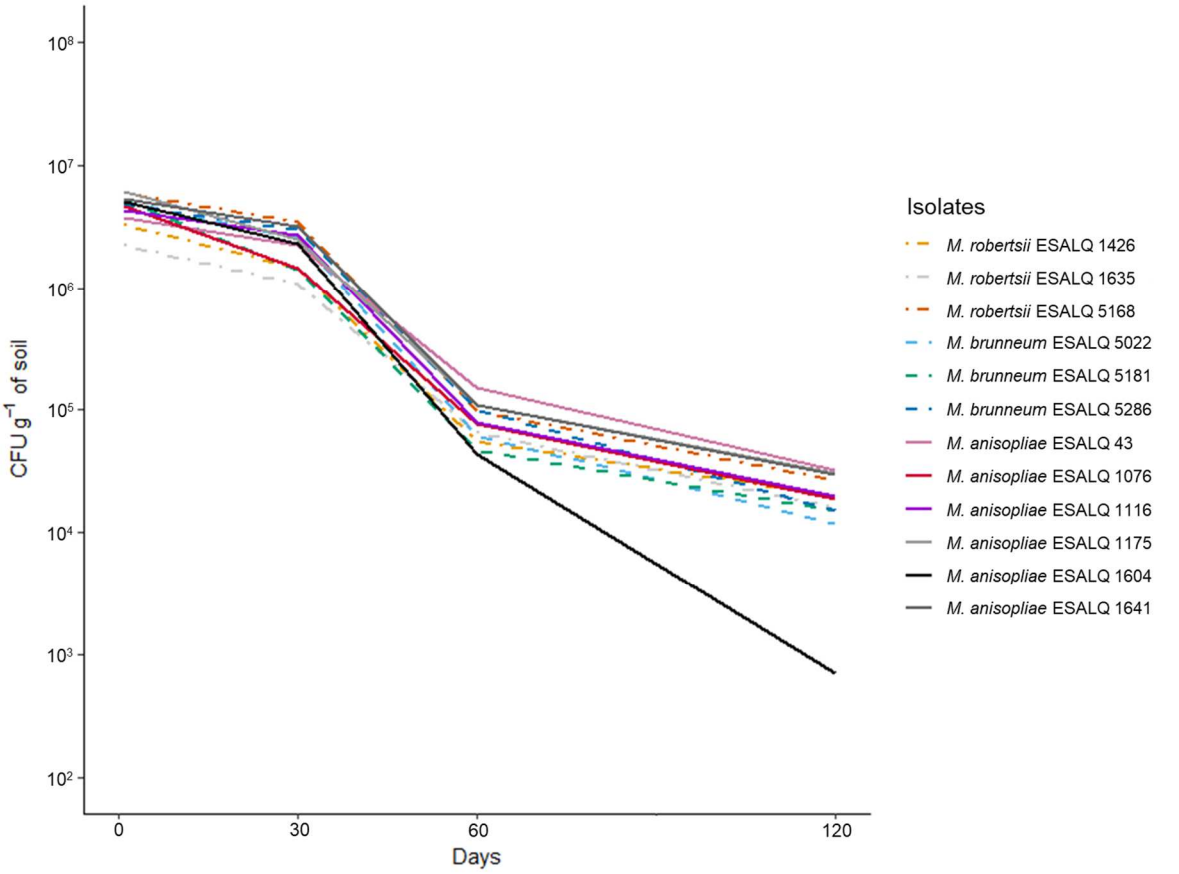

Figure 2. Abundance of isolates (in CFU g^{-1} soil) of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* during 120 days, after inoculation in plastic pots containing non-sterile sandy clay soil (Second replicate), adopting a negative binomial model. In this replicate, isolates did not differ according to a Tukey Contrast (α = 0.05%).

There was a significant effect of days in replicate three (P = 0.001), and the curves of the isolates did not differ. The density of the isolates was similar to replicates 1 and 2 on the first day of evaluation, varying between 10^6 and 10^7 CFU g^{-1} of soil (Figure 3). Isolates ESALQ 5286 (*M. brunneum*) and ESALQ 1116 (*M. anisopliae*) had a slight increase in abundance on day 30, whereas the other isolates showed a decrease; still, densities obtained were above 10^6 CFU g^{-1} of soil. Reductions in CFU were similar for all isolates on day 60, and slightly higher than the other replicates. At the final evaluation after 120 days, densities varied between 10^3 and $1.6x10^4$ conidia g^{-1} of soil.

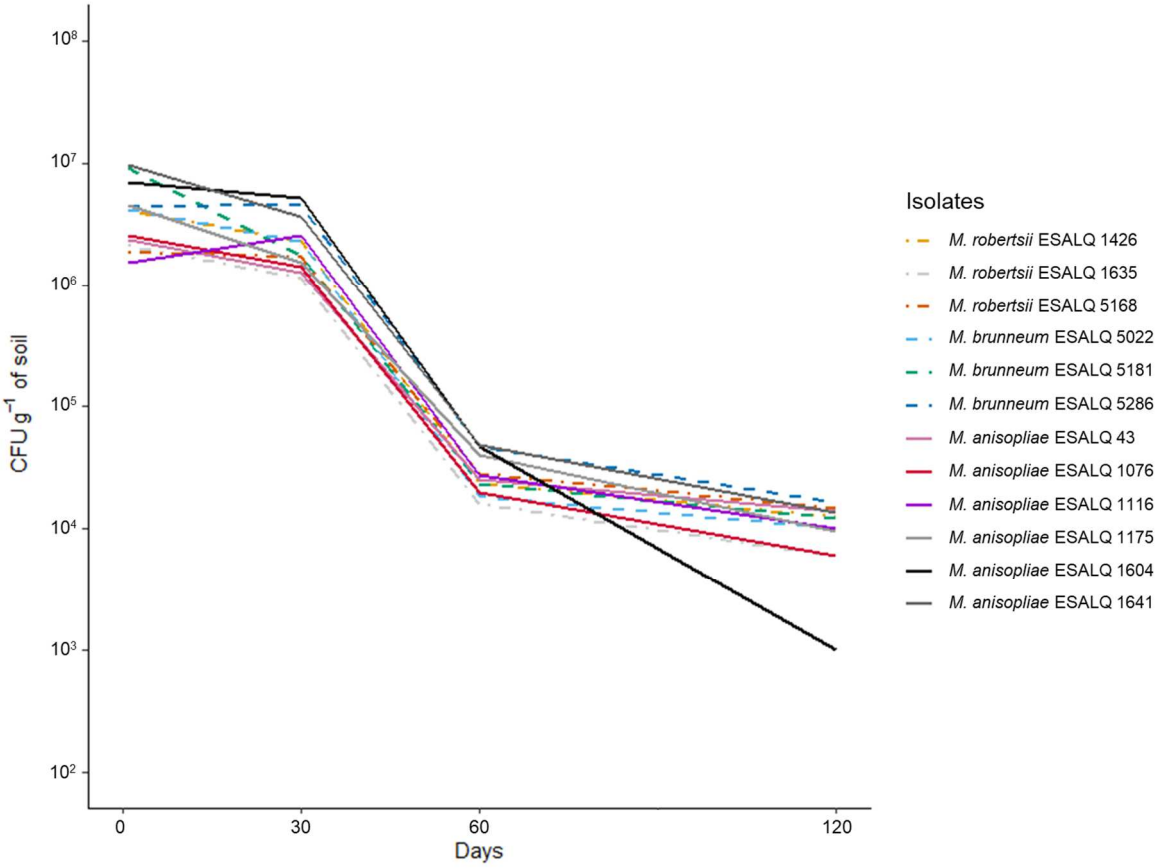

Figure 3. Predicted abundance of isolates (in CFU g^{-1} soil) of *Metarhizium anisopliae* s.str. Mani 2, *M. brunneum* and *M. robertsii* during 120 days, after inoculation in plastic pots containing non-sterile sandy clay soil (Third replicate), adopting a negative binomial model. In this replicate, isolates did not differ according to a Tukey Contrast ($\alpha = 0.05\%$).

5.4. Discussion

We evaluated the persistence of isolates of three species of *Metarhizium* spp. in non-sterile sandy clay soil to test the hypothesis that isolates of *M. brunneum* and *M. robertsii* would show increased persistence compared to *M. anisopliae*. However, the hypothesis was not confirmed; no differences were found between the decline in CFUs abundance among the 12 isolates.

Decreases in the abundance of an entomopathogenic fungal population in the soil are expected over time. In a semi-field experiment, Liu et al. (2016) found that an isolate of *M. anisopliae* s.str. applied at different distances from peanut plant roots had a rapid decrease in density in a period of up to 60 days, regardless of the distance from the plant. In a pot experiment with tobacco soil, Yang et al. (2019) reported a decline in density for *Metarhizium* spp. and *B. bassiana*, with CFUs reaching less than 7% of the initial density after 180 days. In a small scale experiment in a maize field, Pilz et al. (2011) showed that two isolates of *M. anisopliae*,

formulated as fungus colonized barley kernels, increased in density one month after application, followed by a decrease in the subsequent evaluations until it returned to an initial level six months later. A study with two isolates of *M. anisopliae* s.l. in Finnish soils also found a reduction in abundance, with CFUs cm^{-3} up to 46% of the value found two years prior (Vänninen et al., 2000). Consistent with these reports, the curves obtained in the present chapter showed that all isolates decreased in density over time (except for three isolates which had small increases after 30 days), reaching final densities at least 85% lower than the level measured at the first day after 120 days later.

An *in vitro* experiment testing the antagonistic effects of soil fungi (e.g., *Penicillium* sp, *Trichoderma viride*) against *M. anisopliae* s.l. and *Beauveria* spp. demonstrated that these fungal entomopathogens can inhibit and be inhibited by soil fungi (Sharma et al., 1999). In the present study, the decrease in density of the isolates throughout the experimental time indicates some degree of antagonism between the isolates and the native microbiota of the soil. However, this antagonism seems to have affected all isolates similarly since the curves of CFU g^{-1} did not differ in each replicate.

Isolates *M. anisopliae* ESALQ 1604 (replicate 1) and *M. brunneum* ESALQ 5286 and *M. anisopliae* ESALQ 1116 (replicate 3) exhibited an increase in density on the second evaluation (day 30 post-inoculation). This could indicate saprophytic development through conidia germination and mycelial growth or the occurrence of microcycle conidiation, a process in which conidia germinate new conidia, without participation of mycelia, under stress conditions (Jung et al., 2014). Fargues & Robert (1985) incubated conidial samples of four fungal species in an experimental soil and reported that one isolate of *M. anisopliae* s.l. showed a density increase after an initial decline, attributing it to the ability to produce new conidia during microcycles, whereas Vänninen, Tyni-Juslin & Hokkanen (2000) obtained similar results, but stated that the increase could also be due to the presence of a susceptible insect host.

The persistence of fungal entomopathogens in the soil can last several months, or even years, in a system with the presence of insect and/or plant hosts. After a fast reduction in density of an isolate of *M. anisopliae* s.str., Liu et al. (2016) reported that the fungus adapted to the new environment and re-established its population, especially in the rhizosphere, where an association with peanut plant roots could have had a significant role in the process. The application of two isolates of *M. anisopliae* in maize fields to control the western corn rootworm *Diabrotica virgifera virgifera* showed that the fungi persisted for at least 15 months; a positive correlation between the presence of the host and the density of the fungi was found (Pilz et al., 2011). Klingen et al. (2015) evaluated the persistence (in CFU per g of dry soil) of one isolate of *Beauveria pseudobassiana*

and two isolates of *M. brunneum* inoculated in the form of fungal colonized barley kernels in a soil where strawberry plants were planted and observed that the isolates exhibited higher levels of CFU in rhizosphere soil (tightly adhered to plant roots) than in bulk soil (up to 20 cm around the plants), in a period of 94 to 877 days after inoculation.

The **s**oil is a very heterogeneous environment, and variation in its composition (in terms of microbiota, physical and chemical properties) makes it difficult to generalize about the persistence of fungal isolates (Jaronski, 2007). Survival in the soil is subjected to many factors, such as type of soil, moisture content and temperature, and different isolates can have different responses; regarding the latter factor, there is usually an inverse relationship between temperature and persistence (Jaronski, 2007). Studies evaluating the persistence of *B. bassiana* in soil reported that survival of conidia decreased when temperatures increased, with a soil temperature of 30 °C already being detrimental to propagules (Lingg and Donaldson, 1981; Quintela et al., 1992; Studdert and Kaya, 1990). In the present study, in the third replicate, the curves reached a lower point at the end of the experimental time compared to the first two replicates. The ambient temperature was very high between the first and second months (reaching values above 40 °C) due to a heatwave that persisted for several days. Although the soil was contained in plastic pots, this could have been enough to impact the survival of conidia in the top layer, consequently causing the higher reduction observed during the evaluation and lowering the final points of the curves.

Variability in persistence in the soil has been previously reported for isolates of *Metarhizium* spp. (e.g., Vänninen; Tyni-Juslin; Hokkanen, 2000; Yang et al., 2019). We found variation between replicates, possibly related to the influence of abiotic factors, but we did not find significant variation between isolates within replicates. This might indicate that the isolates were similarly affected and have similar reduction rates. A greenhouse experiment with sugarcane plants was being executed to evaluate fungal persistence in the rhizosphere, but high contamination levels forced it to be interrupted. To complement this study, it would be interesting to assess the persistence of the isolates of the present study in field conditions in a natural environment.

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APPENDIX

APPENDIX A - Physical analysis of the soil used for the experiment in this chapter.

Soil separate	**Unit**	**Content**
Sand	g kg^{-1}	529
Silt	g kg^{-1}	119
Clay	g kg^{-1}	352
Textural class	**-**	sandy clay

Methods: Bouyoucos (densimeter) (SSSA Book Series 5. Methods of Soil Analysis, Part 4). Diameter range (mm) according to the USDA.
Five subdivisions of sand: very coarse sand = 2–1; coarse sand = 1–0.5; medium sand = 0.5–0.25; fine sand = 0.25–0.10; very fine = 0.10–0.05;
Sand = 2–0.05; silt = 0.05–0.002; clay < 0.002.