**University of São Paulo**
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# Abundance, genetic diversity and persistence of *Metarhizium* spp. fungi from soil of strawberry crops and their potential as biological control agents against the two-spotted spider mite *Tetranychus urticae*

**Thiago Rodrigues de Castro**

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

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FACULTY OF SCIENCE
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# IMBICONT

## Improved biological control for IPM in fruits and berries

UNIVERSITY OF COPENHAGEN

USP ESALQ

FACULTY OF SCIENCE

Laboratório de Patologia e Controle Microbiano de Insetos

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"Do... or do not. There is no try!".

Master Yoda

**SUMÁRIO**

## RESUMO

### Abundância, diversidade genética e persistência de fungos *Metarhizium* spp. isolados de solos de morangueiro e seu potencial como agentes de controle biológico do ácaro rajado, *Tetranychus urticae*

A crescente demanda por morangos vem impondo desafios, especialmente quanto ao controle das pragas. Muitos agricultores relatam problemas com a redução da eficiência do controle químico, provavelmente devido à seleção de populações resistentes de insetos e ácaros. Uma alternativa é o uso de controle biológico com fungos entomopatogênicos como ferramenta dentro do manejo integrado de pragas. *Metarhizium* spp. (Hypocreales: Clavicipitaceae), são fungos entomopatogênicos generalistas com distribuição cosmopolita e que podem causar doenças em um grande número de hospedeiros. Muitos estudos sobre o desenvolvimento de *Metarhizium* como agente de controle biológico foram realizados, mas este leque de conhecimento está em contraste com a notável falta de investigação sobre a ecologia de *Metarhizium* nos agroecossistemas. Esta tese teve como objetivo avaliar o estabelecimento, persistência e dispersão destes fungos entomopatogênicos em solo de morangueiro em Inconfidentes, Minas Gerais, Brasil; bem como estudar a diversidade e abundância de espécies de *Metarhizium* isolados do solo de cultivos orgânico e convencional de morangueiro, e das margens das plantações no Brasil e Dinamarca. A eficácia de novas espécies de *Metarhizium*, encontradas recentemente no Brasil, foi avaliada contra o ácaro rajado, *Tetranychus urticae*. Os isolados inoculados de *M. anisopliae* (ESALQ1037) e *M. robertsii* (ESALQ1426) foram capazes de persistir por até 12 meses após a aplicação no solo, além de dispersar para outras parcelas e colonizar a rizosfera dos morangueiros. Nas parcelas onde ESALQ1037 e ESALQ1426 foram aplicados, 25% e 87,5% dos isolados recuperados após 12 meses consistiam dos mesmos isolados inoculados. Uma nova linhagem não taxonomicamente identificada, referida neste trabalho como *Metarhizium* sp. Indet. 5, foi encontrada nas margens de morangueiros cultivados. A espécie dominante de *Metarhizium* no Brasil e Dinamarca foi *Metarhizium robertsii* e *M. brunneum,* respectivamente. Além disso, *Metarhizium pemphigi* foi detectado pela primeira vez na Dinamarca neste estudo. Solos de cultivo orgânico de morangueiro em geral apresentaram uma diversidade maior de *Metarhizium* do que solos de cultivos convencionais. Estes estudos revelaram pela primeira vez o potencial de novas espécies de *Metarhizium* como agentes de controle biológico do ácaro rajado, sendo o menor tempo letal mediano ($TL_{50}$= 4 ± 0.17 dias) observado em ácaros tratados com o isolado ESALQ1638 de *Metarhizium* sp. indet. 1. Os melhores isolados foram ESALQPL63 de *B. bassiana*, ESALQ1608 e ESALQ1638 de *Metarhizium* sp. indet. 1 e ESALQ3069 e ESALQ3222 de *M. pingshaense* baseado na curva de sobrevivência, mortalidade total, porcentagem de cadáveres esporulados e $TL_{50}$. O conhecimento da diversidade de *Metarhizium* spp. e persistência em solos de morango, gerados neste estudo, poderão ser úteis no desenvolvimento de estratégias de conservação e maximizar o controle biológico natural de pragas.


Palavras-chave: Controle microbiano; Marcadores microssatélites; Estrutura da comunidade fúngica; Ecologia de populações; “*Insect baiting*”; Virulência.

## ABSTRACT

**Abundance, genetic diversity and persistence of *Metarhizium* spp. fungi from soil of strawberry crops and their potential as biological control agents against the two-spotted spider mite *Tetranychus urticae***

The growing demand for strawberries has imposed challenges, especially regarding the control of pests. Many farmers report problems with reduced chemical control efficiency, probably due to selection of resistant populations of insects and mites. An alternative is the use of biological control using pathogenic fungi as a tool in integrated pest management. *Metarhizium* spp. (Hypocreales: Clavicipitaceae) are generalist entomopathogenic fungi with worldwide distribution and can cause diseases in a large number of hosts. Many studies on the development of *Metarhizium* as a biological control agent were performed, but this bulk of knowledge is in remarkable contrast to the lack of research on the fundamental ecology of *Metarhizium* in agroecosystems. This thesis aimed to evaluate the establishment, persistence and dispersal of these entomopathogenic fungi in strawberry crop soil in Inconfidentes, Minas Gerais, Brazil; and to study the diversity and abundance of species of *Metarhizium* isolated from organic and conventional strawberry crop soils, and the field margins in Brazil and Denmark. The effectiveness of new species of *Metarhizium* recently found in Brazil, was evaluated against two spotted spider mite, *Tetranychus urticae*. Applied isolates of *M. anisopliae* (ESALQ1037) and *M. robertsii* (ESALQ1426) were able to persist for up to 12 months after the application within the soil, and disperse to other plots and colonize the rhizosphere of strawberry plants. In the plots where ESALQ1037 and ESALQ1426 were applied, 25% and 87.5% of the isolates recovered after 12 months consisted of the same isolates inoculated. A new taxonomically unassigned lineage, referred to as *Metarhizium* sp. Indet. 5 in this study, was found in strawberry crop margins. The dominant species of *Metarhizium* in Brazil and Denmark was *Metarhizium robertsii* and *M. brunneum* respectively. Further, *Metarhizium pemphigi* was first detected in Denmark in this study. Soil in organically grown strawberries harbored a more diverse population of *Metarhizium* spp. compared with conventionally grown strawberries. These studies showed for the first time the potential of new species of *Metarhizium* as spider mite biological control agents, the lowest median lethal time ($LT_{50}$ = 4 ± 0.17 days) was observed in mites treated with the isolate ESALQ1638 of *Metarhizium* sp. indet. 1. The best isolates were ESALQPL63 of *B. bassiana*, ESALQ1608 and ESALQ1638 of *Metarhizium* sp. indet. 1 and ESALQ3069 and ESALQ3222 of *M. pingshaense* based on the survival curve, total mortality, percentage of sporulated cadavers and $LT_{50}$. Knowledge of the diversity of *Metarhizium* spp. and persistence in strawberry soil generated in this study may be useful in developing conservation strategies and maximize the natural biological pest control.

Keywords: Microbial control; Microsatellite markers; Fungal community structure; Population ecology; Insect baiting; Colony-forming unit; Virulence; Pathogenicity

# 1 INTRODUCTION

## 1.1 Strawberry

The wild and cultivated species of strawberry had historical ornamental and medicinal purpose, and was widely used in Europe (ASSIS, 2006). The strawberry genotype that is currently cultivated, *Fragaria* × *ananassa* (Rosales: Rosacea) had its origins from the inbreed of *Fragaria virginiana* Mill (originated from North America) and *Fragaria chiloensis* (Linnaeus) Duchesne, 1766 (originated from Chile). This occurred through commercial plants natural hybridization (REBELO; BALARDIN, 1989; RESENDE et al., 1999; MALAGODI-BRAGA, 2002).

The world´s 2013 strawberry production was approximately 7.8 million tons, the biggest producing country was China with a production of 2.9 million tons. Brazil produced more than three thousand tons, producing 8.500 kg/ha and Denmark more than six thousand tons producing 6.200 kg/ha in 2013 (FAO, 2016). Strawberries for direct consumption is a growing and profitable promising market and normally represents the main crop in the regions that is developed (CRUZ, 1999). However, damage due to pests and diseases cause losses to farmers (WILSON; TISDELL, 2001).

## 1.2 Strawberry pests

Strawberry plants at different growth stages are susceptibility to various pests. The main groups of insects that causes damage are mites, beetles, aphids, leaf cutting ants and caterpillars (FADINI; ALVARENGA, 1999).

Mites are the most harmful group of strawberry crop pests in Brazil (FADINI; ALVARENGA, 1999). Among the mites found in strawberries, the main pests are the red mites *Tetranychus cinnabarinus* (Boisduval) Boudreaux, 1956, *Tetranychus desertorum* Banks, 1900 and *Tetranychus ludeni* Zacher, 1913); and also the Two-Spotted Spider Mite, *Tetranychus urticae* Koch, 1836 considered a main pest of strawberry (FADINI; ALVARENGA, 1999; FADINI; PALLINI, 2004; DE MORAES; FLECHTMANN, 2008). Freitas et al. (2011) reported spider mite to be the main problem of strawberry crops in south Minas Gerais state in Brazil. Mites from family Tarsonemidae may also occur in strawberries, for example *Phytonemus pallidus* (Banks, 1899) and broad mite, *Polyphagotarsonemus latus* (Banks, 1904) which attack mainly the tip of the plants leaving them wrinkled (FADINI; ALVARENGA, 1999; DE

MORAES; FLECHTMANN, 2008). *Tetranychus urticae* is an important pest of more than 150 host plants of economic importance worldwide (JEPPSON et al., 1975). At room temperature, the development from egg to adult is about 10 days and each female may lay up to 100 eggs, allowing the rapid increase in population (MITCHELL, 1973). For these reasons, it is considered one of the most important species of mite pests worldwide (DE MORAES; FLECHTMANN, 2008). According to Chiavegato e Mischan (1981) the potential productivity reduction in strawberry plants can reach up to 80% in case of high population density of this pest.

### 1.3 Control tactics

Chemical control (also known as conventional control) is the main method of pest control used by most producers that adopt the conventional cropping systems (VAN LEEUWEN et al., 2015). Such systems include the use of chemical pesticides, especially Abamectin, Milbemectin, and Fenpropathrin. However, many producers have had problems with the efficiency of these products, most likely due to selection of resistant populations in strawberry (SATO et al., 2005). Insects and mites can quickly develop resistance to chemical pesticides when they are frequently used and under strong selection pressure that leads to the increasing use of new pesticides and intensive mixing products for satisfactory control.

An alternative to these conventional systems is the use of biological control, which is broadly used in organic farm systems. Biological control can be defined as “the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging then it would otherwise be” (EILENBERG et al., 2001). Insect pathogenic fungi from Ascomycota (genera *Metarhizium* and *Beauveria*) were the first pathogens to be used in biological control of arthropods (DAVIDSON, 2012). While research on such fungi aiming to develop products has been intense, less attention has been given to research on the ecology of the fungi. Inconsistent results from biological control experiments in the field might be due to a lack of understanding of the ecology and biology of these fungi (VEGA et al., 2008).

Entomopathogenic fungi are able to penetrate directly into the host cuticle, so there is no need for ingestion by the host as happens with most of other insect pathogens like bacteria and virus (HALL; PAPIEROK, 1982). The infection process of a fungus (exemplified with *Metarhizium* spp.) to a host can be briefly explained by: 1. Attachment: the fungal conidia adhere to the host cuticle using a combination of

hydrophobic interaction and specialized adhesion proteins; 2. Germination and appressoria formation; 3. Penetration through the cuticle, which is mechanical but aided by the production of enzymes including proteases, chitinases and lipases; 4. Overcoming host defenses: production of destruxins; 5. Proliferation within the host: mainly through the production of blastospores or hyphae; and 6. Outgrowth and production of new infective fungal conidia. Mostly, the fungi require high relative humidity to infect, and this condition is actually found in protected cultivations in greenhouses, or crops that generate a suitable microclimate, as is the case of strawberry (ZIMMERMANN, 2007).

Entomopathogenic fungi are good candidates for implementation in Integrated Pest Management (IPM), a practice increasingly adopted in different agroecosystems, which includes the maintenance of natural enemies within crops. This can be combined with inundative releases of natural enemies. If the use of pesticides is necessary, the decision is always to use efficient products against the pests, but selective, so they do not harm to the natural enemies. Such practices should be adopted with the aim of increasing the maximum production with minimum possible impact on the environment (KOGAN, 1998).

The need to develop alternative control strategies is critical considering the cost, reduced efficiency and environmental impact of pesticides. So it is essential that multiple strategies of biological control are developed that can be used by farmers and incorporated into IPM programs. One option is the use of entomopathogenic fungi, since most of these have a high specificity, proportionating a restricted host range, which results in a low risk to natural enemies such as predators and parasitoids. Even in the case of pathogens with a wider range of hosts, they present a lower risk than pesticides (JACOBSON et al., 2001). Most of the commercially produced fungi are species of *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria*, which are all relatively easy to mass produce (FARIA; WRAIGHT, 2007). Although recent discoveries assign new ecological functions to this group of fungi, it has been broadly studied for its insect control.

### 1.4 The genus *Metarhizium*

*Metarhizium* spp. (Hypocreales: Clavicipitaceae), are generalist entomopathogenic fungi with a cosmopolitan distribution occurring in the tropics, temperate, sub-Arctic and sub-Antarctic regions (JARONSKI, 2007; ZIMMERMANN,

2007). They can cause diseases in a large number of hosts (ROBERTS; HAJEK, 1992), act as plant endophytes by rhizosphere colonization (BEHIE et al., 2012) or in the soil as saprophytic (MEYLING; EILENBERG, 2007). Much effort has been put into research on the development of *Metarhizium* as biological control agent (for inundation biological control) to be applied in agriculture and forestry. However, this bulk of knowledge is in striking contrast to the lack of research into the fundamental ecology of *Metarhizium* in agroecosystems.

*Metarhizium* spp can infect more than 200 species of insects and other arthropods (ROBERTS; HAJEK, 1992). According to Zimmermann (1993), beetles, grasshoppers, leafhoppers and termites represent the most susceptible groups to these fungi. For these reasons, *Metarhizium* is one of the most studied group of insect pathogens for use in biological pest control.

Temperature, humidity, and UV-radiation are the most important abiotic factors that affect entomopathogenic fungal performance. Temperature can affect *Metarhizium* spp. germination, hyphal growth and infection rates (KEYSER et al., 2014). Temperature fluctuations have also been shown as one of the primary limiting factors in field release success (NELSON FOSTER et al., 2010; FOSTER et al., 2011). Similarly, UV radiation, especially UV-B radiation can be highly detrimental to conidia survival (BRAGA et al., 2001; RANGEL et al., 2005). Relative humidity is an important factor in determining sporulation, infection, growth and conidial longevity (DAOUST; ROBERTS, 1983; MILNER et al., 1997; ARTHURS; THOMAS, 2001; VEGA et al., 2012). These factors are also relevant in understanding the natural distribution and abundance in the field. It is possible to verify that the climate zones have different abiotic factors and this will reflect and have strong influence on abundance and distribution of these fungi. In Fernandes et al. (2008) *Metarhizium* spp. showed to be less cold tolerant than *Beauveria* spp., which may explain for example, why *Beauveria* was isolated all over Finland while *Metarhizium* spp were mostly recovered from the southern Finland areas (VÄNNINEN, 1996). No *Metarhizium* isolates were found in one area of southern Alberta, Canada, this is possibly due to the semi-arid and cold area sampled (INGLIS et al., 2008). Some differences in the response to abiotic factors can have direct association with the isolate origin, since isolates sampled from forested areas were more likely to grow at low temperatures while those found in open fields showed a propensity for growth at higher temperatures and were more UV-B tolerant (BIDOCHKA et al., 2001).

Soil is the most appropriate environment for recovery of these entomopathogenic fungi since it serves as a natural reservoir enabling the survival of fungal propagules when they find a host (MEDO; CAGÁŇ, 2011; VEGA et al., 2012). The entomopathogenic fungi species composition and its occurrence in the soil can be significantly influenced by factors related to soil characteristics, climatic conditions, agricultural inputs and practices (JARONSKI, 2007; MEYLING; EILENBERG, 2007; VEGA et al., 2012). In addition to these factors, the various methods used for isolation of these fungi, (e.g., sampling and isolation method) makes direct comparison between the studies difficult, since until now, these works have shown diverse occurrence patterns.

### 1.4.1 Metarhizium anisopliae

The successful use of this fungus in Brazil started in the 70's as the biological control program of leafhopper in northeast region of Brazil on sugarcane crops (ALVES et al., 2008). Brazil has one of the most successful program using commercial products and non-commercial based in *M. anisopliae* complex that controls 16 leafhoppers (Hemiptera: Cercopidae) including *Mahanarva fimbriolata* (Stal, 1854) and *M. posticata* (Stal, 1855) in sugarcane and *M. fimbriolata*, *Deois flavopicta* (Stal, 1854) and *Notozulia entreriana* (Berg, 1879) on pasture (ALVES et al., 2008). This fungus has provided efficient levels of leafhoppers control (LI et al., 2010) and the estimation is that the annual application of this fungi exceeds two million hectares of sugar cane every year (PARRA, 2014).

Due to this success, *M. anisopliae* is the fungus species with the highest number of bio-product registered (n=16) (AGROFIT) in the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) beside the fact of being the most commercialized entomopathogen by companies it has a history of being produced on a large scale by sugar cane producers, as non-commercial product (LI et al., 2010).

These products are used mainly on sugarcane crops and pastures. Although the records are restricted to leafhoppers, the application of *M. anisopliae* products had shown good results in the control of other pests like termites, ticks and ants (GARCIA, 2008; CASTILHO et al., 2010; HUSSAIN et al., 2011; QUINELATO et al., 2012). Studies have shown an efficiency of 60 to 100% in control of leafhoppers pest populations in the sugarcane fields of São Paulo state (DINARDO-MIRANDA et al., 2001; LOUREIRO et al., 2012). Despite the widespread use of *M. anisopliae* to control

these pests, little is known about the diversity, distribution and ecology of currently recognized species (BISCHOFF et al., 2009) in natural and agricultural environments in Brazil (REZENDE et al., 2015).

The virulence of different species can vary greatly between different host species (BUTT et al., 1992) because of it, virulence tests towards each insect species of interest become necessary. Additionally, virulence varies among isolates even within the same species of fungus, which complicates even more the appropriate isolate selection for applied use. The variation in virulence is also important when considering pest control strategies, since a very small percentage of all insect species are important pests and some beneficial and natural enemies like pollinators, parasitoids and predators are important to a balanced insect community, they should not be harmed by the applied entomopathogenic fungi, so, it is possible to use this virulence variation as an advantage and a tool in IPM. Although is it clear that the use of entomopathogenic fungi is advantageous for farmers, consumers, and the environment, it is also clear that much research is needed to reach the full potential of these fungi.

*Metarhizium* spp. can be more abundant than other entomopathogenic fungi in crop fields and open meadows (VÄNNINEN, 1996; BIDOCHKA et al., 1998; QUESADA-MORAGA et al., 2007; SUN et al., 2008). The studies until now reported that *Metarhizium* spp. have a tendency to be less commonly found in colder regions than in temperate and tropical regions (KLINGEN et al., 2002; INGLIS et al., 2008; SUN; LIU, 2008), this is also supported by laboratory studies which has demonstrated that most species of *Metarhizium*, with the exception of *M. frigidum*, do not grow at cold temperatures (FERNANDES et al., 2010a, 2008). It seems *Metarhizium* spp. distribution is more associated with habitats than with host insects as firstly hypothesized (BIDOCHKA et al., 2001; FISHER et al., 2011; WYREBEK et al., 2011; STEINWENDER et al., 2015), which shows the importance to study ecological aspects of this fungi group in each crop system.

### 1.5 Fungal identification

The morphological identification can be done by the visualization of hyphae, phialides, conidiophore and conidia, through microscope observation. The morphological characteristics can be assessed using identification keys (HUMBER, 2012)

Recently, in a multilocus phylogenetic study, the fungus previous known as *M. anisopliae* actually comprises a nine species complex: *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acridum*, *M. majus*, *M. lepidiotae*, *M. brunneum*, *M. globosum* and *M. robertsii (BISCHOFF* et al., *2009)*, and *M. flavoviridae* already known as a different species by its different morphology. In this study, was also highlighted that it is difficult to separate some species of the *M. anisopliae* complex based only on morphological characters, for example, *M. anisopliae* has identical morphology of *M. robertsii*. They suggest that the most reliable way to distinguish between species within this complex is the use of molecular tools and analyzes. Based on their results, was determined that the 5' region of the gene for the translational elongation factor (TEF-1α) is the most informative region for routine use in species identification within the genus. This region requires only two primers and is easily amplified. Recently Kepler e Rehner (2013) sought to identify new nuclear regions that are more informative for *Metarhizium* complex. Primers developed for seven new regions were evaluated (MzFG543igs, MzFG546igs, MzBTigs, MzIGS2, MzIGS3, MzIGS5 and MzIGS7) and observed that the combined phylogenetic analysis of these segments provided a satisfactory genealogy for the group.

**1.6 Diversity**

In Brazil, until now 12 species have been reported in different habitats (*M. anisopliae*, *M. acridum*, *M. majus*, *M. flavoviride*, *M. pingshaense*, *M. robertsii*, *M. brunneum*, *M. lepidiotae* and four undetermined species: *Metarhizium* sp indet.1, *Metarhizium* sp indet.2 and *Metarhizium* sp indet.3, *Metarhizium* sp indet.4 (ROCHA et al., 2009, 2013; LOPES; MESQUITA, 2013; LOPES; SOUZA, 2013; LOPES et al., 2014; REZENDE, 2014; REZENDE et al., 2015; ZANARDO, 2015; IWANICKI, 2016), but few studies were conducted to understand the diversity of these fungi. The knowledge until now allows inferring that the diversity of *Metarhizium* species recovered from soil is high. *M. robertsii* and *M. anisopliae* are the two most common species in Brazil, being *M. robertsii* commonly recovered in five Brazilian biomes soils under different vegetation covers (ROCHA et al., 2013; REZENDE, 2014; ZANARDO, 2015). *Metarhizium anisopliae* is the species most commonly found infected insects (ALVES et al., 2004; LOPES; MESQUITA, 2013; REZENDE et al., 2015; IWANICKI, 2016). Unlike the high species diversity in soil, it was only observed one *M. anisopliae* clade naturally infecting root-leafhopper (REZENDE et al., 2015).

The Danish diversity of *Metarhizium* is lower than the Brazilian and six species are reported until now in different agroecosystems: *M. brunneum, M. robertsii, M. majus,* M. *guizhouense* and *M. flavoviride*. Different diversity was found between the studies, predominance of *M. brunneum* was found in most of the works performed in the country (MEYLING et al., 2011; STEINWENDER et al., 2014, 2015) and Keyser et al. (2015) found majority of *M. flavoviridae*. These differences in the composition of the populations could be due to that plants can have some sort of interaction with the surrounding soil environment and recruit fungal associates or even determine the composition of *Metarhizium* populations depending on the established crop (STEINWENDER et al., 2015).

**1.7 Molecular tools**

With advances in molecular biology techniques, a new research niche has been gaining momentum in recent years for entomopathogenic fungi. Molecular markers are precise tools, able to detect DNA polymorphisms in different populations of a species, therefore, considered an ideal technique to monitor and study the ecology of naturally occurring entomopathogenic fungi and applied isolates in the agricultural fields. Molecular techniques have been applied in ecological studies and virulence of entomopathogenic fungi, especially *Metarhizium* spp., which is the most studied genera in molecular and biochemical level (ZIMMERMANN, 2007). Genetic diversity and population structure of this fungus has been recently studied to understand the relationship of different genotypes with the host and / or habitat on a regional or global scale, and allows you to monitor your persistence and behavior in the environment that was introduced. Several methods have been used for gene frequency analysis for genotyping studies such as RFLP (restriction fragment length polymorphism), AFLP (polymorphic amplified fragment length), RAPD (Random Amplified Polymorphic DNA) and microsatellite (SSR - Simple sequence repeat) (ENKERLI; WIDMER, 2009).

A molecular marker is a polymorphic DNA region or "locus" that characterizes the genotype of the individual who owns it. Therefore, molecular markers may be derived from any given molecular data providing a detectable polymorphism among the organisms being compared (SALLES et al., 2003). The uses of microsatellites (SSR) have been gaining prominence in phylogenetic studies of population structure and genetic diversity of fungi. These markers consist of tandem repeated sequences of one to six nucleotides found scattered throughout the genome (JARNE; LAGODA,

1996). The polymorphism is represented by the variation in the number of repeating elements that constitute the microsatellite, that produce fragments of different sizes (CARRER et al., 2010). SSR markers are characterized for being codominant, abundant, dependent on small amount of individual's DNA and once obtained the primers (markers) informative to one species or isolate, the costs and the demand for hand labor are reduced dramatically (SALLES et al., 2003). In studies of Enkerli et al. (2005) and Oulevey et al. (2009) 41 microsatellites markers were described to recognized *M. anisopliae*, *M. brunneum* and *M. robertsii*, however, more recently, Mayerhofer et al. (2015) also validated between 15-34 microsatellite regions from those 41 previously described to the *M. guizhouense*, *M. lepidiotae*, *M. majus* and *M. pingshaense*.

Studies using microsatellites markers for *Metarhizium* spp. isolates differentiation showed the efficiency of this technique and can therefore be considered as an effective monitoring tool to studies of fungi application in the field (BECERRA VELÁSQUEZ et al., 2007; STEINWENDER et al., 2014; KEPLER et al., 2015). However, few studies were done in Brazil using DNA sequences obtained from a gene for haplotype and nucleotide diversity studies until now.

### 1.8 Highlights and future perspectives

The use of microorganisms to control pest insects is an important part of current and future crop protection. Understanding the fundamental ecology of these organisms is vital to their successful use. For example, research regarding their natural occurrence, persistence and pathogenicity of new species greatly enhances the potential for more efficient utilization in pest regulation. This thesis advances the current scientific knowledge regarding the ecology and biological control use of *Metarhizium* spp. fungi in several areas:

- Persistence and dispersal was confirmed up to one year after application of two isolates of *Metarhizium* spp. in a strawberry crop in Brazil and rhizosphere colonization was detected.
- A new taxonomically unassigned lineage, referred to as *Metarhizium* sp. Indet.5 was found in the margins of conventional strawberry management in Brazil.

- *Metarhizium robertsii* is the dominant species of the *Metarhizium* community found in strawberry crop soil in Brazil which were different form the Danish diversity where *M. brunneum* was the dominant species.
- *Metarhizium pemphigi* was detected for the first time in Denmark.
- Organically management system seems to improve the diversity and the crop margins soil seems to contribute (by exchange of species) with the regulation of the fungi community inside the crop.
- The new species of *Metarhizium* showed good potential as biological control agents

Probably, one of the most important components of any research is not the conclusion generated but rather the perspectives and new hypothesis that the conclusions leads to. Based on the observations of this thesis there are several research questions that should be addressed in the future, including:

- Worldwide survey studies that attempt to clarify the distribution and occurrence of *Metarhizium* spp. in agriculture. These studies should highlight habitat associations as well seeking to understand what characteristics promote higher density or diversity in particular areas. Essential to these studies is to continue to develop the molecular based ecological tools, like SSR markers (microsatellites) to discriminate genotypic diversity within all *Metarhizium* species.
- Field studies to evaluate the effectiveness of the new *Metarhizium* species, found in recent bioprospection studies against pest insects. These studies should not only evaluate infectivity, but also the fungal persistence in the field, and effects of crop type.
- Future studies to better understand if the rhizosphere colonization provides benefits such as plant growth promotion and antagonism towards pests and diseases as has been seen in laboratory studies for other crops.

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Manuscript 1

TITLE: Persistence of Brazilian isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *M. robertsii* in strawberry crop soil after soil drench application

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# 2 PERSISTENCE OF BRAZILIAN ISOLATES OF THE ENTOMOPATHOGENIC FUNGI *Metarhizium anisopliae* AND *M. robertsii* IN STRAWBERRY CROP SOIL AFTER SOIL DRENCH APPLICATION


## Abstract

Establishment, persistence and dispersal of the entomopathogenic fungi *Metarhizium* anisopliae (ESALQ1037) and *M. robertsii* (ESALQ1426) (Ascomycota: Hypocreales) were investigated in the soil and rhizosphere following soil drench application in strawberries between 2012 and 2013 at a single location in Inconfidentes, Minas Gerais, Brazil. *Metarhizium* spp. isolates (n= 108) were collected using selective agar media and insect bait methods, and characterized by sequence analysis of 5' -TEF (elongation factor 1-α) and MzFG543igs intergenic region and by multilocus simple sequence repeat (SSR) analysis. Both applied fungal isolates were frequently recovered, suggesting that they were able to establish and disperse within the soil, showing rhizosphere competence. Persistence within the soil and rhizosphere for both fungi was confirmed up to 12 months after application. In the plots where ESALQ1037 and ESALQ1426 were applied, 25% and 87.5% of the respective isolates recovered after 12 months consisted of the same isolates inoculated. *Metarhizium robertsii* was the most abundant species in the agroecosystem studied representing 77.8% of the isolates recovered across all sample dates.


Keywords: Microbial control; Microsatellite markers; Fungal community structure

## 2.1 Introduction

The world´s strawberry production was approximately 7.8 million tons in 2013 and Brazil produced more than three thousand tons in 2013, with a yield of 8,500 Kg/ha (FAO, 2016). Strawberries (*Fragaria x ananassa* Duch.; Rosales: Rosacea) produced for direct consumption, are a growing, and promising market in Brazil, with profit margins at the point of sale around 20% with a current price of the final product of approx. USD 4.4/Kg (AGRA-FNP-PESQUISAS, 2015). However, damage from pests and diseases cause significant losses for farmers (WILSON; TISDELL, 2001).

The high load of chemical pesticides used in the Brazilian conventional strawberry production system is of concern, especially regarding negative impacts on the environment and natural enemies of agricultural pests (SATO et al., 2007). Several studies have demonstrated that fungicides, and in some instances herbicides, can significantly reduce the germination and growth of entomopathogenic fungi (SAMSON et al., 2005; YÁÑEZ; FRANCE, 2010; D'ALESSANDRO et al., 2011). In recent years, it has been observed that strawberry producers have experienced problems with the efficacy of chemical products used in pest control, likely due to the selection of resistant

populations after prolonged cyclical application (SATO et al., 2005). In addition, the use of chemical control increases the risk of pesticide residues in the fruits, as well as causing health problems for farm workers and the contamination of the environment (MAREDIA, 2003).

A viable alternative to the use of chemical pesticides in strawberry production is biological control, such as the use of entomopathogenic fungi- in particular species of the Ascomycota, which often have a broad host range. The genus *Metarhizium* (Hypocreales: Clavicipitaceae) contains species which occur naturally within the soil environment (MEYLING; EILENBERG, 2007; VEGA et al., 2009; RUDEEN et al., 2013; STEINWENDER et al., 2014), from which they can be recovered. It has been suggested that *Metarhizium* spp. use the soil as a reservoir, ensuring long-term persistence, even when crops and insects are not present in the field (KLINGEN; HAUKELAND, 2006).

Much effort has been put into the research and development of *Metarhizium* spp. as biological control agents (for inundation biological control) to be applied in agriculture and forestry (VEGA et al., 2012). Brazil has a long history of using *M. anisopliae* as a biocontrol agent especially against spittlebugs (Hemiptera: Cercopoidea) in sugarcane and it is estimated that around two million hectares are treated annually (PARRA, 2014). However, the impact of the aerial application of *Metarhizium* spp. on the indigenous community of the fungus in the soil still need to be investigated. More recently, an increasing number of studies have demonstrated that *Metarhizium* spp. benefit plant growth (BEHIE et al., 2012; KHAN et al., 2012; SASAN; BIDOCHKA, 2012; BEHIE; BIDOCHKA, 2014) and are antagonistic towards pests and diseases (SASAN; BIDOCHKA, 2013). Understanding the persistence of *Metarhizium* in the soil and rhizosphere is important to predict the possible duration of the beneficial effects of soil inoculation.

Molecular markers with high discriminatory power are required when studying behavior and fate of a specific applied fungal isolate in the field. Simple sequence repeats (SSR or microsatellite markers) have proven to be very useful for such purposes and allow for consistent and explicit assessment of isolate identity and persistence (PILZ et al., 2011; STEINWENDER et al., 2014, 2015; KEPLER et al., 2015).

The aim of this study was to evaluate the diversity of *Metarhizium* spp. and persistence over one year of two Brazilian isolates of *Metarhizium* (*M. anisopliae* and

*M. robertsii*) applied in a strawberry crop in Minas Gerais state in Brazil. We used SSR markers and phylogenetic analyses of DNA sequence data for characterization of *Metarhizium* spp. diversity and identification of the two applied fungal isolates among the recovered isolates during the study period.

## 2.2 Material and Methods

### 2.2.1 Experimental field description

The experiment was performed in a strawberry crop in Inconfidentes city in the state of Minas Gerais (MG) Brazil (22°19′2″S; 46°19′42″W; 904 m altitude). The strawberry field was not subjected to any treatments with chemical pesticides during the experimental period (May 2012 to August 2013). Approximately 1,520 strawberry seedlings (San Andreas variety) were planted in 12 beds, each 15 m long, with three rows per bed, with 35 cm between each plant in May 2012.

### 2.2.2 Treatment application

Two *Metarhizium* isolates were used:1) *M. anisopliae* ESALQ1037 – isolated in March 1992 in Porto Alegre – RS from *Solenopsis* sp. (Hymenoptera: Formicidae) and 2) *M. robertisii* ESALQ1426 – Isolated from soybean crop soil (selective agar medium) in December 2007 in Londrina-PR. Both isolates were deposited at the Collection of Entomopathogenic Microorganisms of the Laboratory of Pathology and Microbial Control of Insects (LPCMI) of the Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ-USP).

Aerial conidia were produced using parboiled rice as the substrate by the plastic bag method (JARONSKI; JACKSON, 2012), and was then mechanically harvested from a fungus-rice mixture using an electrically vibrating sieve containing a set of three 20 cm round sieves of 32 mesh (pore size 500 µm) (Bertel Indústria Metalúrgica Ltd., Brazil). Afterward, different batches of dried conidial powder (< 13% w/w final moisture) were vacuum sealed and stored at –20 °C until use. The conidia viability was determined using the direct count method (ESALQ method), on 4-mL of PDA amended with 0.001% (v/v) Derosal® 500 SC (Carbendazim, Bayer CropScience, SP, Brazil) on Rodac® plates (OLIVEIRA et al., 2015).

Immediately after the initial soil sampling in September 2012 for baseline characterization, the two *Metarhizium* isolates were applied to the experimental

strawberry field as a randomized block design, containing four blocks with the three treatments (1. *M. anisopliae* ESALQ1037; 2. *M. robertisii* ESALQ1426 and 3. Control, water), yielding 12 plots (beds) in total. The fungal suspension (unformulated) was applied by drenching 100 mL of $1x10^8$ viable conidia/mL on the soil surface around each of the 43 strawberry plant in the middle row of the beds after fungal suspension intense homogenization. The control treatment was carried out by applying only water in a similar manner. The application was done between 6 and 7 pm, to minimize UV effects on conidial viability.

### 2.2.3 Sampling dates

Soil samples were taken across four sampling time points: 1) September 4th, 2012, prior to fungal application in order to characterize the indigenous *Metarhizium* spp. community in the soil, 2) January 9th, 2013; 3) April 16th, 2013, and 4) August 21th, 2013; to evaluate the persistence of the inoculated isolates at the experimental field site.

### 2.2.4 Soil sampling

Each soil sample was taken to a depth of 10 cm using a cylindrical sampler (Sondaterra, Brazil) with defined volume (70 $cm^3$). The samples were stored in sterile plastic bags and kept in Styrofoam boxes at approximately 5°C during transport to the laboratory following the methodologies pointed out by Inglis et al. (2012).

Each sample consisted of five sub-samples of soil collected in four middle row beds of each treatment per sampling occasion, at 2 m intervals and 10 cm away from the strawberry roots. These five combined sub-samples were stored in a sterile plastic bag and manually homogenized, forming one composite sample per bed on each occasion, yielding 48 composite samples per treatment for the total experimental period. In addition, for each sampling occasion, four composite samples were taken from the soil 10 cm away from the roots of five plants of *Bidens pilosa* L. (Asterales: Asteraceae) - the most abundant species of spontaneous herb found close to the crop field (maximum 3 m distance from strawberry plants). This sampling thus generated in total 20 composite samples from the crop margins.

On the last sampling date, we also conducted a root sampling of the strawberry crop by taking five randomly selected whole plants from the middle row of each bed using a garden shovel to pull out the entire root system and adjoining

rhizospheric soil. These samples were stored as mentioned for the soil samples above. This was carried out to evaluate the rhizosphere colonization of the inoculated *Metarhizium* species.

### 2.2.5 Isolation of entomopathogenic fungi

The isolations of entomopathogenic fungi from the soil were carried out using selective agar medium and insect baiting.

#### 2.2.5.1 Selective agar medium

The isolation of entomopathogenic fungi using selective agar medium was done using Potato Dextrose Agar (PDA) with 0.002% Dodine (Dodex 450 SC - Sipcam Isagro, Brazil) and 0.05 g / L Gentamicin (Amresco Inc., USA) to reduce the growth of contaminant fungi and bacteria that normally occur in the soil, as described by Fernandes et al. (2010). Each soil sample was homogenized by hand and 10 g of soil from each composite sample was added to a Scotch tube (250 ml) containing 90 mL of sterile distilled water and 0.01% of Tween 80 (Oxiteno, Brazil). The solutions were homogenized on a Vortex-type stirrer and serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$). Each dilution (0.1 ml) were then inoculated in duplicates into Petri dishes (90 x 15 mm) containing selective medium and incubated in a climatic chamber at a temperature of 25 ± 1 °C, relative humidity of 70 ± 10% and photoperiod of 12 hours for seven days. After this period, Petri dishes were assessed daily for the presence of *Metarhizium* colonies, of which, identities were confirmed through the analysis of morphological characteristics. Colonies were counted and the number of Colony Forming Unities (CFU) per gram of humid soil were calculated. Pure *Metarhizium* colonies were obtained by streaking small quantities of conidia using platinum sterile loops in PDA medium plates (INGLIS et al., 2012).

#### 2.2.5.2 Insect baiting

The insect baiting method used was described by Inglis et al. (2012). Soil samples were homogenized by hand using a metal sieve with pore size of 4 mm. One hundred grams of the sieved soil were transferred to transparent plastic pots with 200 mL capacity with perforated lids. Ten $3^{th}$ or $4^{th}$ instar larvae of *Tenebrio molitor* L. (Coleoptera: Tenerionidae) from a stock rearing in the laboratory (LPCMI – ESALQ/USP) were transferred to the surface of the soil in plastic pots. Soil samples

were moistened with sterile distilled water using a manual spray whenever deemed necessary.

The pots were stored in a climatic chamber at 25 ± 1 °C, 70 ± 10% in darkness and each container was turned up-side down every day during the first week to induce the movement of the larvae through the soil. From the 5$^{th}$ day onwards, inspections of dead larvae were made every three days for a period of three weeks. Dead larvae were surface sterilized by immersion in sodium hypochlorite 2% for 30 s to prevent the growth of external contaminant saprophytic fungi, and washed three times in sterile distilled water and then individually placed in 24 cell well culture plates with a lid on. Moistened cotton with sterile distilled water were added to ensure conditions of high relative humidity. After observation of external fungal growth on the insect cadavers, pure *Metarhizium* colonies were obtained by streaking small quantities of conidia using platinum sterile loops in PDA medium plates (INGLIS et al., 2012).

#### 2.2.5.3 Isolation from strawberry rhizospheres

For the isolation of the fungi from the rhizosphere, the roots were first shaken manually to remove loose soil. Roots were then washed with sterile distilled water and manually cut using laboratory scissors into small pieces (≈ 0.5 cm). Ten grams of root cuttings were placed together in 20 mL of distilled water plus 0.05% Tween 80 in a 40 mL flat bottom glass tube. The tubes were vortexed for 10 min and 100 µL of this solution was serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$) and 0.1 mL of each dilution were plated in duplicate plates on selective media as described above (WYREBEK et al., 2011). After seven days, *Metarhizium* colonies were identified by the analysis of morphological characteristics and the number of CFU's per gram of humid roots was calculated. *Metarhizium* isolates with different colony morphology from each sample and each isolation method were cryopreserved in the Collection of Entomopathogenic Microorganisms of LPCMI-ESALQ-USP and included in the studies described below. The identities of *Metarhizium* colonies were confirmed based on colony morphology and characteristics of hyphae, phialides, conidiophores and conidia using a microscope (x400) and an identification key (HUMBER, 2012).

### 2.3 Statistical analyses

CFU counts of *Metarhizium* spp. in each plot were analyzed by fitting a linear mixed model to the proportion of *Metarhizium* CFU data with a full interaction

between sample dates and plot treatment with a linear predictor, including random intercepts and slopes for each plot. Sub-models were tested using likelihood-ratio (LR) tests (MCCULLAGH; NELDER, 1989).

The proportions of *T. molitor* larvae killed by *Metarhizium* in soil samples were analyzed by fitting a binomial generalized linear mixed model with a full interaction between sampling time points and treatment linear predictor and including random intercepts and slopes for each plot, as well as an observation-level random effect to model overdispersion. Submodels were tested using Likelihood-Ratio (LR) tests (DEMÉTRIO et al., 2014). All analyses were performed using the R statistical software environment (R DEVELOPMENT CORE TEAM, 2015)

## 2.4 Molecular identification

### 2.4.1 DNA extraction

DNA was extracted using ABI PrepMan Ultra protocol as described by Kepler et al. (2014). DNA was extracted from vegetative hyphae and conidia grown 5–10 days on sterile filter paper strips overlaid on PDA plates. Mycelium and conidia were transferred to a 2 mL sterile tube containing 300 mL Prepman extraction buffer and zirconia-silica beads. Cells were disrupted in a FastPrep-2 5G Instrument (MP Biomedicals) with two 10 s cycles at a speed setting of 5.5. The tubes were incubated in boiling water for 10 min and subsequently centrifuged for 10 min at 14,000 g. One hundred and seventy-five µL of the supernatant were transferred to a fresh tube and stored at -20 °C.

### 2.4.2 Gene sequencing and SSR marker analyses

SSR marker analyses were performed as described by Mayerhofer et al. (2015) using 11 markers: Ma307, Ma145, Ma165, Ma416, Ma2097, Ma164, Ma2098, Ma2065, Ma2089, Ma2063, Ma2054, (ENKERLI et al., 2005; OULEVEY et al., 2009). SSR loci were PCR amplified as described by Mayerhofer et al. (2015) and products analyzed on an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Fragment sizes (allele sizes) were determined using the GenMarker v1.51 (SoftGenetics LLC, State College, PA) software and GeneScan ROX400 (Applied Biosystems) as internal size standard. SSR data were analyzed using GenAlEx 6.5 (PEAKALL, 2006, 2012).

Individual multilocus SSR haplotypes were assigned to species by sequencing the nuclear encoded translation elongation factor 1-alpha (5'-TEF1-α) (BISCHOFF et al., 2009) and a nuclear intergenic marker MzFG543igs (KEPLER; REHNER, 2013). PCR amplifications were performed for one representative isolate of each SSR haplotype with primers EF2F (5´-GGAGGACAAGACTCACATCAACG- 3´) and EFjR (5´-TGYTCNCGRGTYTGNCCRTCYTT-3´) and MzFG543igs_1F (5'-ATTCATTCAGAACGCCTCCAA-3') and MzFG543igs_4R (5'-GGTTGCGACTGAGAATCCATG-3'). PCR products were purified using the geneMAG-PCR Kit (Chemicell, Germany) and sequenced. Sequencing was performed by Beckman Coulter Genomics (United Kingdom).

Sequences were edited and aligned with 17 reference sequences obtained from GenBank, representing ex-type cultures or taxonomically confirmed isolates of *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. pingshaense* and *M. robertisii*, (BISCHOFF et al., 2009; STEINWENDER et al., 2014; REZENDE et al., 2015) using MAFFT with the FFT-NS-i alignment option in Geneious 7.1.8. software (KEARSE et al., 2012). The sequence of *M. lepidiotae* (ARSEF7412) was included as outgroup and jModelTest 2.1.7 (DARRIBA et al., 2012) was used to calculate the evolution model for the phylogenetic tree (best-fit models of nucleotide substitution). The most parsimonious tree was calculated based on Bayesian (GTR-GAMMA) and Maximum Likelihood (GTR-GAMMA) model parameters using 1000 bootstrap replicates with the software Geneious 7.1.8 (KEARSE et al., 2012) of the combined single gene alignments (5'-TEF1- α and MzFG543igs) made in the software Mesquite 3.04 (MADDISON; MADDISON., 2015).

## 2.5 Results

### 2.5.1 Isolation of entomopathogenic fungi

#### 2.5.1.1 Selective agar media

No significant differences were found between the densities of *Metarhizium* spp. in soil of the different treatments (soil from strawberry plots inoculated with *M. anisopliae* or *M. robertsii*, uninoculated control plots and margin crop area) at each sampling time point, except for September 2012 ($3.5\pm1.0 \times 10^3$ CFU/g) where the concentration in the *M. robertsii* treatment was lower than the concentration in the other

treatments (LR=18.35, d.f.=9, p=0.0313) (Figure 2.1). The abundance of *Metarhizium* spp. in soil, within each treatment over time, differed only in plots where *M. robertsii* and *M. anisopliae* were applied. The highest concentrations in these treatments were observed in August 2013 (1.4±0.6 x $10^4$ CFU/g) and September 2012 (7.5±1.2 x $10^3$ CFU/g), respectively, indicating that the application of *M. robertsii* and *M. anisopliae* did not cause an overall increase of *Metarhizium* density in the soil.

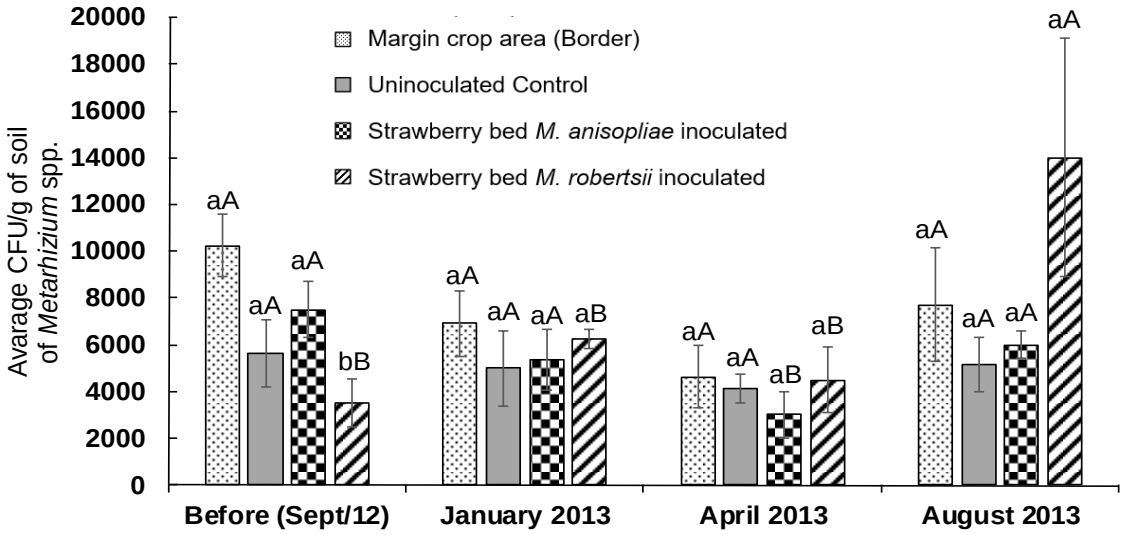


Figure 2.1 - Density [Colony forming units (CFU) per gram of humid soil] of *Metarhizium* spp. in strawberry crop soil. Different lower-case letters indicate differences among treatments at the same date; different upper-case letters indicate treatment differences at different dates both using linear mixed models and with a full interaction between sampling time point and treatment as the linear predictor (P ≤ 0.05)

### 2.5.1.2 Insect bait mortality

The percentages of *T. molitor* larvae killed by *Metarhizium* spp. exposed to different soil samples were not significantly different among treatments, except in August 2013, when the values were higher in *M. anisopliae* treated plots (77.5 ± 6.3%) than all other treatments (LR=1.50, d.f.=3, p = 0.6813). The effect of sampling time points was significant (LR=28.70, d.f.=1, p < 0.0001).

The larval mortality proportion in the soil samples increased in those strawberry plots inoculated with *M. anisopliae* or *M. robertsii*, as well as uninoculated control plots after August 2013, January 2013 and April 2013 compared to the previous sample dates, respectively. At the last sampling, the percentage of larvae killed by *Metarhizium*

were 50.0 ± 15.8%, 45.0 ± 6.5% and 36.0 ± 12.1% in treatment plots inoculated with *M. robertsii*, uninoculated control and border plants, respectively (Figure 2.2).

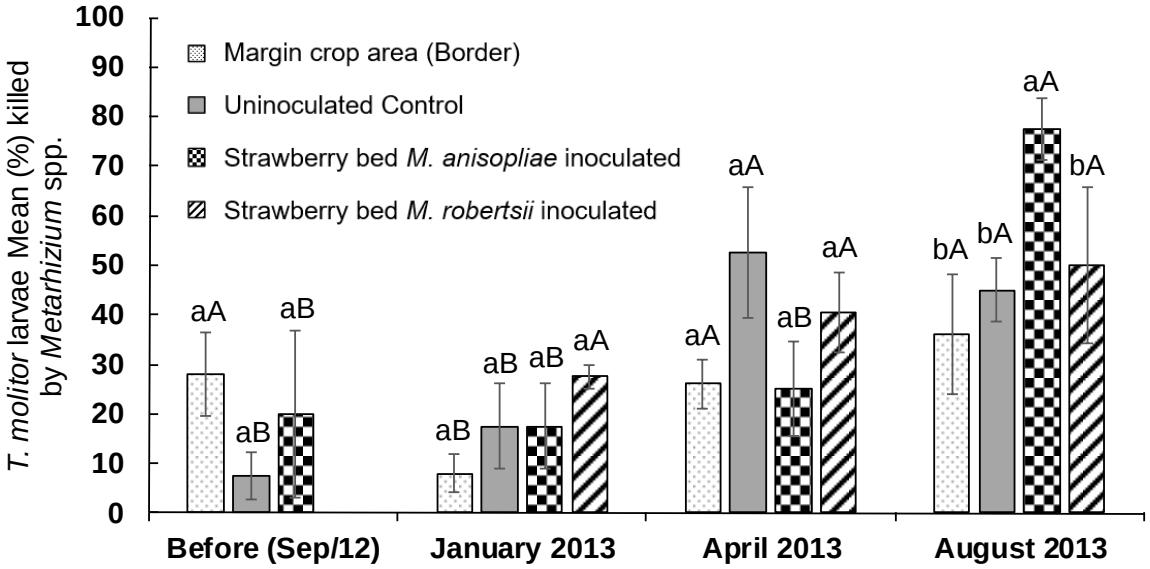


Figure 2.2 - Mortality (Mean ± SE) of *Tenebrio molitor* larvae used in the isolation of *Metarhizium* spp. by the insect bait method. Different lower-case letters indicate differences among treatments at the same date; different upper-case letters indicate treatment differences at different dates both using binomial generalized linear mixed models and with a full interaction between sampling time point and treatment as the linear predictor ($P \leq 0.001$)

### 2.5.2 Gene sequencing and SSR marker analyses

Of the 108 isolates recovered from all the plots during the period of the experiment and used in the SSR marker analyses: 27.8% were found in the margins of the crop associated with the border herb plants, 25.0% were found in the uninoculated control plots, 20.4% were in *M. anisopliae* treated plots, 16.7% were from the *M. robertsii* treated plots, 5.5% were recovered from rhizosphere in *M. robertsii* treated plots and 4.6% were from rhizosphere in *M. anisopliae* treated plots (Table 2.1). These 108 isolates were used in the SSR analyses. All loci displayed polymorphism. The analysis resolved 11 haplotypes among them (Table 2.2).

Table 2.1 - Occurrence of each *Metarhizium* haplotype in strawberry crop soil before the application and at three subsequent sampling time points. (*Ma = Metarhizium anisopliae, Mr = M. robertsii, Mb = M. brunneum* and *Mp = M. pingshaense*)

| | | Strawberry Plots | | | | | | | | | | | | Margins | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***M. anisopliae*** | | | | ***M. robertsii*** | | | | **Control** | | | | **Border plants** | | | | **Roots** | Total |
| | application | Before | After | | | Before | After | | | Before | After | | | Before | After | | | After | |
| Species | **Haplotype** | **Sep12** | **Jan13** | **Apr13** | **Aug13** | **Sep12** | **Jan/3** | **Apr13** | **Aug13** | **Sep12** | **Jan13** | **Apr13** | **Aug13** | **Sep12** | **Jan13** | **Apr13** | **Aug13** | **Aug13** | |
| *Ma* | **A*** | 0 | 6 | 9 | 1 | 0 | 0 | 0 | 1 | 0 | 3 | 3 | 0 | 0 | 1 | 0 | 0 | 5 | **29** |
| | **B** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** |
| | **C** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | **1** |
| *Mr* | **D** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | **1** |
| | **E** | 1 | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 1 | 4 | 7 | 3 | 2 | 4 | 5 | 2 | 3 | **36** |
| | **F** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 2 | 0 | 0 | **7** |
| | **G** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | **1** |
| | **H** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | **1** |
| | **I*** | 0 | 0 | 0 | 0 | 0 | 2 | 7 | 7 | 0 | 0 | 0 | 2 | 0 | 1 | 4 | 3 | 3 | **29** |
| *Mb* | **J** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** |
| *Mp* | **K** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** |
| **Total n isolates** | | **2** | **6** | **10** | **4** | **0** | **2** | **8** | **8** | **1** | **8** | **11** | **7** | **3** | **9** | **12** | **6** | **11** | **108** |

* haplotype of applied isolates (*M. anisopliae* - ESALQ1037 and *M. robertsii* – ESALQ1426)

Table 2.2 - Number of alleles, allele size range [base pairs] for 11 SSR loci for each of the eleven haplotypes identified with DNA sequence based analyses

| Locus | *M. brunneum* n=1 and *M. pingshaense* n=1 | *M. anisopliae* n=22 | *M. robertsii* n=84 |
|---|---|---|---|
| **Ma145** | 1 [106] | 1 [106] | 2 [107; 108] |
| **Ma164** | 2 [116;121] | 1 [116] | 2 [116; 117] |
| **Ma165** | 2 [140;141] | 2 [140;141] | 1 [143] |
| **Ma307** | 2 [112;161] | 2 [161;164] | 3 [149-161] |
| **Ma416** | 2 [115;116] | 1 [115] | 1 [126] |
| **Ma2054** | 2 [218;238] | 1 [218] | 2 [217; 220] |
| **Ma2063** | 2 [144;143] | 2 [144;146] | 1 [137] |
| **Ma2065** | 2 [131;146] | 1 [131] | 2 [129; 131] |
| **Ma2089** | 2 [196;199] | 2 [196;198] | 1 [194] |
| **Ma2097** | 2 [189;191] | 2 [191;195] | 3 [181-185] |
| **Ma2098** | 2 [171;179] | 1 [171] | 3 [171-197] |
| **Number of haplotypes** | 2 (J, K) | 3 (A* - C) | 6 (D – I*) |

* haplotype of applied isolates (*M. anisopliae* - ESALQ1037 and *M. robertsii* – ESALQ1426)

A species assignment based on 5'TEF1-α and MzFG543igs sequence analyses was performed with all different haplotypes. Sequence alignments and subsequent phylogenetic analyses revealed the presence of four *Metarhizium* species clustering with *M. anisopliae*, *M. robertsii*, *M. brunneum* and *M. pingshaense* reference isolates (Figure 2.3). Sequence alignments consisted of 625 positions for 5'TEF1-α and 911 position for MzFG543igs and after combining them, sequence differences were compared in an alignment of 1,210 positions of 5'TEF1-α and MzFG543igs concatenated dataset (Table 2.3). Here, *M. robertsii* intraspecific differences ranged between one up to 53 positions while lower intraspecific variation was observed within *M. anisopliae*, maximum of 6 base pair differences. Base pair differences between *M. robertsii* and *M. brunneum* haplotypes ranged from 158 to 193 positions, representing the largest differences found among the haplotypes studied. The forth species recovered was *M. pingshaense*, with a difference ranging between 41 positions (*M. robertsii)* to 161 positions (*M. brunneum*).

Table 2.3 - Base pair difference among the 11 *Metarhizium* haplotypes in sequences of the 5' end of Elongation Factor 1-α and MzFG543igs concatenated dataset (total positions 1,210 bp). (*Ma* = *Metarhzium anisopliae, Mr* = *M. robertsii, Mb* = *M. brunneum* and *Mp* = *M. pingshaense*)

| **Species** | | *Ma* | *Ma* | *Ma* | *Mp* | *Mr* | *Mr* | *Mr* | *Mr* | *Mr* | *Mr* | *Mb* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Haplotype** | **A*** | **C** | **B** | **K** | **D** | **E** | **I*** | **F** | **G** | **H** | **J** |
| *Ma* | **A*** | | | | | | | | | | | |
| *Ma* | **C** | 6 | | | | | | | | | | |
| *Ma* | **B** | 5 | 1 | | | | | | | | | |
| *Mp* | **K** | 46 | 40 | 41 | | | | | | | | |
| *Mr* | **D** | 66 | 60 | 61 | 53 | | | | | | | |
| *Mr* | **E** | 89 | 89 | 90 | 82 | 49 | | | | | | |
| *Mr* | **I*** | 56 | 50 | 51 | 43 | 10 | 39 | | | | | |
| *Mr* | **F** | 90 | 90 | 91 | 83 | 50 | 1 | 40 | | | | |
| *Mr* | **G** | 63 | 57 | 58 | 50 | 5 | 52 | 13 | 53 | | | |
| *Mr* | **H** | 54 | 48 | 49 | 41 | 14 | 43 | 4 | 44 | 13 | | |
| *Mb* | **J** | 159 | 163 | 164 | 161 | 170 | 192 | 160 | 193 | 167 | 158 | |

Three haplotypes were found among the 22 *M. anisopliae* isolates, and were referred to as haplotypes A (including applied ESALQ1037), B and C; six haplotypes among 84 *M. robertsii* isolates: D, E, F, G, H and I (including applied ESALQ1426); and the single isolates of *M. brunneum* and *M. pingshaense* were referred to haplotypes J and K, respectively (Figure 2.3).

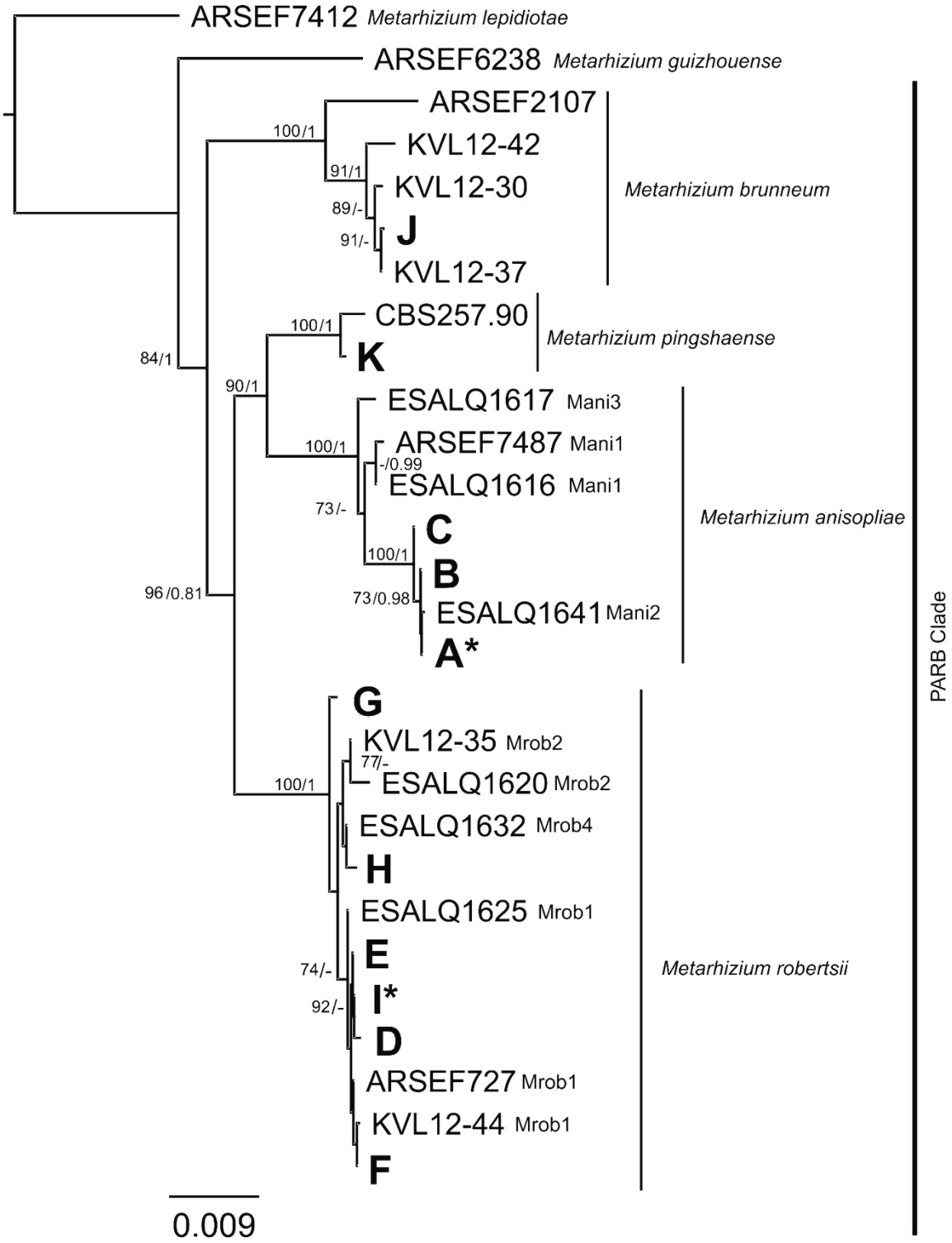


Figure 2.3 - (ML) Maximum likelihood (GTR-gamma) / (B) Bayesian (GTR-gamma) phylogeny of the combined data set of 5` TEF and MzFG543igs sequences of 11 haplotypes identified in the experiment crop, including 17 taxonomically validated reference strains accessioned in ARSEF (ARS Entomopathogenic Fungal Culture Collection, CBS (Fungal Biodiversity Centre) and ESALQ (Collection of Entomopathogenic Microorganisms of LPCMI-ESALQ-USP). Bootstrap support values ≥ 70% are listed near nodes of each type of analyses (ML/B)

### 2.5.3 Species and haplotype frequencies

Three of the haplotypes, two assigned to *M. robertsii* (E, I) and one assigned to *M. anisopliae* (A, the applied isolate) were most frequently isolated. *M. robertsii* was the most frequent species comprising 77.8% of all isolates, followed by 20.4% of *M. anisopliae*, and one isolate each of *M. brunneum* and *M. pingshaense*. Among the *M. robertsii* haplotypes two were most frequent; haplotype E (33.3% of the *Metarhizium* spp. isolates) an indigenous haplotype and I (26.8%) the haplotype of the applied *M. robertsii* isolate. Among the *M. anisopliae*, haplotype A, representing the applied isolate, was most frequent (26.8%). Except for the *M. robertsii* haplotype F (6.5%) all remaining haplotypes were represented by single isolates only (Table 2.4). The most frequent haplotype E (*M. robertsii*) was detected in all the treatments. The haplotypes (A and I) of the applied isolates were not detected in any of the treatments before application (Table 2.1).

Of the three most frequently isolated haplotypes, two were assigned to *M. robertsii* (E, I) and one assigned to *M. anisopliae* (A, the applied isolate). *M. robertsii* was the most frequent species comprising 77.8% of all isolates, followed by 20.4% for *M. anisopliae*, and a single isolate for both *M. brunneum* and *M. pingshaense*. Among the *M. robertsii* haplotypes; haplotype E (33.3% of the *Metarhizium* spp. isolates) - an indigenous haplotype and I (26.8%) - the haplotype of the applied *M. robertsii* isolate, were the most frequently isolated. Among the *M. anisopliae*, haplotype A, representing the applied isolate, was most frequent (26.8%). Except for the *M. robertsii* haplotype F (6.5%), all remaining haplotypes were represented by single isolates only (Table 2.4). The most frequent haplotype E (*M. robertsii*) was detected in all the treatments. The haplotypes (A and I) of the applied isolates were not detected in any of the treatments before application (Table 2.1).

The two applied *Metarhizium* isolates (haplotype A and I) were recovered in several samples in the plots from which they were inoculated, as well as a number of plots from which they were not. The inoculated *M. anisopliae* haplotype A was found after four months of inoculation in all treatments except in *M. robertsii* treated plots; in this latter treatment the applied haplotype A was found after one year. The inoculated *M. robertsii* haplotype I was detected after four months in the plots where it was applied and in the soil around border plants. After one year (August, 2013) this haplotype was also detected in the uninoculated control plots while it was never recovered in the *M.*

*anisopliae* treated plots. The number of haplotypes detected after the inoculation (not including haplotypes of the inoculated isolates) was lower in the *M. robertsii* (n=1) and *M. anisopliae* (n=2) treated plots than in the uninoculated control plots (n=5) and around border plants (n= 4). More *M. robertsii* isolates (n=22) where obtained at the last sample occasion than *M. anisopliae* isolates (n=3).

Table 2.4 - Allele of sizes of 11 SSR markers of all haplotype found at the strawberry field studied

| | **SSR Markers** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HAPLO TYPE | **Ma 307** | **Ma 145** | **Ma 165** | **Ma 416** | **Ma 2097** | **Ma 164** | **Ma 2098** | **Ma 2065** | **Ma 2089** | **Ma 2063** | **Ma 2054** | Number |
| A | 164 | 106 | 140 | 115 | 191 | 116 | 171 | 131 | 198 | 146 | 218 | 29 |
| B | 164 | 106 | 140 | 115 | 195 | 116 | 171 | 131 | 198 | 144 | 218 | 1 |
| C | 161 | 106 | 141 | 115 | 191 | 116 | 171 | 131 | 196 | 144 | 218 | 1 |
| D | 161 | 108 | 143 | 126 | 183 | 117 | 197 | 131 | 196 | 137 | 217 | 1 |
| E | 161 | 108 | 143 | 126 | 183 | 117 | 197 | 131 | 194 | 137 | 217 | 36 |
| F | 161 | 108 | 143 | 126 | 183 | 117 | 171 | 131 | 194 | 137 | 217 | 7 |
| G | 161 | 108 | 143 | 126 | 183 | 117 | 171 | 131 | 194 | 137 | 253 | 1 |
| H | 149 | 107 | 143 | 126 | 181 | 117 | 183 | 129 | 194 | 137 | 220 | 1 |
| I | 152 | 108 | 143 | 126 | 185 | 117 | 195 | 129 | 194 | 137 | 217 | 29 |
| J | 112 | 0 | 140 | 116 | 189 | 121 | 179 | 146 | 199 | 143 | 238 | 1 |
| K | 148 | 103 | 139 | 126 | 183 | 122 | 174 | 127 | 194 | 154 | 226 | 1 |

Three haplotypes of *M. anisopliae* and *M. robertsii* were recovered from the rhizosphere samples. Besides the inoculated isolates (haplotype A and I), only the most common indigenous isolate in the soil (*M. robertsii* haplotype E) was found from the rhizosphere (Table 2.1).

The haplotype frequency of the applied *M. robertsii* isolate (I) increased from zero- before inoculation- to 0.12, 0.29 and 0.40, after 4, 8 and 12 months, respectively. The haplotype frequency of the applied *M. anisopliae* isolate (A) increased 4 months after application to 0.40 but decreased after 8 and 12 months to 0.29 and 0.20, respectively. The haplotype frequency of most common indigenous isolate (haplotype E) - before the inoculation of the treatments- was 0.67 and decrease to 0.32 after 4

months. The frequency of this haplotype was stable after this period (0.31 and 0.31 after 8 and 12 months).

## 2.6 Discussion

In this study, we demonstrate that two *Metarhizium* isolates applied in the strawberry field showed persistence and dispersion in the soil and colonized the rhizosphere. The inclusion of SSR markers allowed for a reliable and transparent identification of the applied fungal isolates among the indigenous *Metarhizium* haplotypes. The applied isolates were recovered most frequently in their specific treatment for up to one year after application, but they were also found in uninoculated strawberry control plots and in soil of field margins. A high frequency of haplotypes of *M. robertsii* was recovered across all plots compared to other *Metarhizium* species. In addition, the occurrence of the *M. robertsii* in the uninoculated plots (control and border plants) was higher than of other *Metarhizium* spp. indicating that this species is the most naturally abundant at the field site and may be better adapted to the prevailing conditions. Regarding the final sampling occasion, the inoculated *M. anisopliae* isolate comprised only 25% of the haplotypes found in the plots in which it was applied, and the *M. robertsii* isolate comprised 87.5% of the haplotypes found in the plots in which it was applied. Our results support the hypothesis raised by Rezende et al. (2015) that Brazilian *M. robertsii* haplotypes may be better adapted to persistence in the soil environment as opposed to being an entomopathogen- above ground. The inoculated *M. robertsii* isolate ESALQ1426 was originally obtained from soil and it seems to persist better than the *M. anisopliae* isolate ESALQ1037 which was isolated from a fire ant. So far, most characterized isolates originating from insect hosts in Brazil belong to the *M. anisopliae* Mani2 clade (REZENDE et al., 2015), including the inoculated isolate ESALQ1037 of the present study. The seemingly primarily soil-based ecology of *M. robertsii* including the indication of an association with plant roots reported here is consistent with other studies (BEHIE et al., 2012; STEINWENDER et al., 2015). We suggest that additional studies using microsatellites should be initiated to understand the primarily above-ground ecology of *M. anisopliae* in Brazil.

The *M. anisopliae* isolate applied in the soil was recovered from the rhizosphere of the strawberry plants at the final sampling occasion one year after application, indicating that a member of Mani2 clade can be associated with roots. Rhizosphere

soils are a potential reservoir for *Metarhizium* spp.- being the soil/root interphase where plants, insects, and microbes interact (HU; ST. LEGER, 2002). Persistence of *M. brunneum* in the strawberry rhizospheres has been shown to depend on local adaptations to the prevailing abiotic conditions at the field site (KLINGEN et al., 2015), and rhizosphere compatibility of *M. anisopliae* in Brazil could be an important trait of a biocontrol agent for long term persistence and prolonged biocontrol efficacy.

Our study is the first in Brazil to implement SSR markers, and therefore we got much more accurate data, detailing the natural occurrence of individual isolates, focusing on within species diversity and persistence over time of each specific applied isolate in different crops. Comparison with earlier studies (from Brazil and elsewhere) should take this into account. Vieira Tiago et al. (2012) studied the persistence of *M. anisopliae* in sugarcane soil, but in laboratory conditions (soil brought from field and used in pots) and detected the fungus for up to 60 days. The extrapolation of these laboratory data into a field situation is, however, difficult. A high persistence of *M. bruneum* isolate Bipesco 5 and *M. anisopliae s.l.* isolate 2277 in corn field soil in Hungary, reaching up to 15 months, was demonstrated by Pilz et al. (2011) using some of the same SSR markers as in the present study. These data corroborate our findings regarding the long-term persistence of inoculated *Metarhizium* isolates.

Although the treatment plots had a greater abundance of the specific isolates which were applied, and there was a number of cases in which the treatment isolates dispersed into neighboring control plots, there was high degree of diversity of indigenous haplotypes were still found in all plots. It therefore appears that the application of biocontrol fungal isolates did not significantly alter the *Metarhizium* community composition within the one-year time frame of this study. Earlier studies have also reported high diversity of *Metarhizium* species in Brazil. Lopes, Mesquita et al. (2013) found in small agricultural habitats (commercial banana fields) *M. anisopliae*, *M. pingshaense* and what is likely to be an undescribed *Metarhizium* species. Lopes, Souza et al. (2013) further found *M. lepidiotae* infecting a Melonthid species in maize crop, and *M. brunneum* infecting a hemipteran species in south Brazil (LOPES et al., 2014). Rocha et al. (2013), studying the *Metarhizium* diversity of soils in central Brazil found a large number of isolates of *M. anisopliae*, *M. robertsii* and *M. pemphigi*. Rezende et al. (2015) focused on the *Metarhizium* diversity associated with sugarcane agriculture and identified two new taxonomically unassigned linages besides the ones found in the studies above. In the present study we recovered the most commonly

reported *Metarhizium* species of Brazil, *M. robertsii* and *M. anisopliae*, also to occur in strawberry field soil as well as the seemingly less abundant species of Brazil: *M. brunneum* and *M. pingshaense*.

Besides the two applied *Metarhizium* isolates, we identified five indigenous haplotypes in the strawberry field margins and the control plots, indicating that the strawberry cultivation regimes did not negatively affect fungal diversity directly. High *Metarhizium* densities were found in soil samples collected in agriculture sites in Switzerland from low-input permanent grassland and improved field margins compared to arable, intensively cultivated fields, indicating that both semi-natural habitat types may provide potential refuges for *Metarhizium* species (SCHNEIDER et al., 2012). Clifton et al. (2015) proposed that the abundance of *M. anisopliae s.l.* in field margins was negatively affected by proximity to conventional fields, suggesting that cropping practices within a field could affect soil-borne microorganisms outside of a field. In our study, the margin and border plants presented comparable abundance and diversity of *Metarhizium* spp. to uninoculated control plots. The lack of chemical control measures in the experimental strawberry fields could have been an important contributor to this observation, but more studies are needed to evaluate potential short term and long term negative effects of agro-chemicals on the *Metarhizium* community.

The persistence and dispersal of *M. anisopliae* and *M. robertsii* applied through soil drenching in strawberry soils demonstrated in this study provides valuable information regarding predictions and efficacy of biocontrol agents in the field. Further studies are needed to evaluate if rhizosphere colonization indicated in the present study, are beneficial to plant growth promotion (SASAN; BIDOCHKA, 2012) whilst being antagonistic towards pests and diseases (SASAN; BIDOCHKA, 2013) as has been seen in laboratory settings for other crops.

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Manuscript 2

TITLE: Diversity and abundance of Brazilian and Danish *Metarhizium* spp. in organic and conventional strawberry crop soil.

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# 3 DIVERSITY AND ABUNDANCE OF BRAZILIAN AND DANISH *Metarhizium* spp. IN ORGANIC AND CONVENTIONAL STRAWBERRY CROP SOIL


## Abstract

Studies on the community composition and population structure of entomopathogenic fungi are imperative to link to ecosystem functions such as the contribution to conservation biological control. We studied the diversity and abundance of *Metarhizium* species isolated from soil from conventionally and organically grown strawberry crops and the field margins in two different climatic zones: Brazil (tropical) and Denmark (temperate). In Brazilian strawberry soil, *M. robertsii* (n=129 isolates) was the most commonly species, followed by an undescribed species, *Metarhizium* sp. Indet. 1 (n=16); *M. anisopliae* (n=6); a new taxonomically unassigned lineage, *Metarhizium* sp. Indet. 5 (n=4) reported here for the first time; *M. pingshaense* (n=1) and *M. brunneum* (n=1). In Denmark, species composition was very different from Brazil, with *M. brunneum* (n=31) being the most common species, followed by *M. flavoviridae* (n=6) and *M. pemphigi* (n=5), detected for the first time in Denmark. No overall difference between the two climatic regimes was detected about the density of *Metarhizium* spp. In soil in strawberries, we found indication that soil in organically grown strawberries harbored a more diverse population of *Metarhizium* species compared with conventionally grown strawberries.


Keywords: Microbial control; Population ecology; Fungal community structure; Insect baiting; Colony-forming unit

## 3.1 Introduction

Brazilian climate is mostly tropical with 12 months with mean temperatures ≥ 18 °C and day length relatively constant throughout the year, while seasonal variations are mostly dominated by variation in precipitation (ALVARES et al., 2013). Denmark is characterized by a temperate climate with 12 months with mean temperatures ≥ 10 °C and variable day length throughout the year and seasonal variation consists of four well distinguished seasons (AGERSKOV et al., 2015). Strawberry *Fragaria* × *ananassa* (Rosales: Rosacea) is a growing and profitable promising market in both countries. Brazil produced more than three thousand tons, producing 8,500 kg/ha while Denmark produced more than six thousand tons producing 6,200 kg/ha in 2013 (FAO, 2016).

Although Brazil and Denmark have different climates, they share several pests in strawberries, such as the two-spotted spider mite (*Tetranychus urticae* Koch). The main strategy to fight against these mites and other pests in strawberries is mostly by using chemical control (WILSON; TISDELL, 2001), despite the high load of pesticides which may interfere with the action of naturally occurring pest enemies, such as

entomopathogenic fungi (SATO et al., 2007). Further, producers have experienced problems with decreased efficacy of products for chemical control, most likely due to the selection of resistant populations in strawberry (SATO et al., 2005). This conventional management increases the risk of pesticides residues in fruits, as well as the contamination of the environment and the poisoning of farmers (MAREDIA, 2003). Therefore, it is essential that novel possibilities to control mites in strawberries are explored.

Organic crop system uses several non-chemical methods for pest management (SUNDRUM, 2001; SEUFERT et al., 2012), which also can be used in conventional production. Biological control is one option, for example the use of insect pathogenic fungi. The genus *Metarhizium* (Hypocreales: Clavicipitaceae) contains species that occur naturally in soil (KLINGEN; HAUKELAND, 2006; MEYLING; HAJEK, 2009; MEYLING et al., 2011) and infect insects above- and belowground. Over 170 products have been developed based on at least 12 species of fungi (FARIA; WRAIGHT, 2007). Much effort has been put into the development of biological control products based on *Metarhizium* spp. to be applied in agriculture and forestry throughout the world, mainly for inundative biological control (ZIMMERMANN, 2007). For example, *M. anisopliae* is a biocontrol agent against spittlebugs (Hemiptera) in sugarcane in Brazil since many years and it is estimated that around two million hectares are sprayed every year (PARRA, 2014).

Although *Metarhizium* have been intensely used for pest control, limited focus has been given to the assessment of natural abundance and distribution of this widespread fungal genus (KEYSER et al., 2015). Knowledge of the community composition and population structure of this fungal genus is important to understand their ecological function and contribution to host regulation and potential for conservation biological control (MEYLING; EILENBERG, 2007). Maniania et al. (2008) also highlighted the importance of bioprospection for obtaining more isolates and species of entomopathogenic fungi with varied virulence from diverse geographic regions and hosts allowing to increase biological control efficacy in different agroecosystems.

The aim of the current study was to compare the natural diversity and abundance of *Metarhizium* spp. obtained from soil of conventional and organic strawberry crops and its margins in Brazil (tropical) and Denmark (temperate) using novel molecular tools to allow haplotype detection.

## 3.2 Material and Methods

### 3.2.1 Soil Sampling

Brazil

Soil samples were taken at four sampling time points: 1) September 4th, 2012; 2) January 9th, 2013; 3) April 16th, 2013, and 4) August 21th, 2013; in three locations in South of Minas Gerais state in Brazil, in organic and conventional management production system in each location: Estiva (conventional: 46° 01' 39"W, 22º 26' 07"S 953 m altitude; organic: 46° 02' 25"W, 22° 27' 23"S 933 m altitude); Cambuí (conventional: 46° 03' 07"W, 22° 37' 20"S 912 m altitude; organic: 46° 03' 04"W, 22° 34' 34"S 860 m altitude) and Senador Amaral (conventional: 46° 12' 06"W, 22° 36' 21"S 1500 m altitude; organic: 46° 11' 05" W, 22° 34' 41"S 1543 m altitude).

Denmark

Two sampling time points, June 11th, 2014 and October 22th, 2014, in organic and conventional production system were performed in Denmark; in two locations: Klippinge (conventional: 12° 18' 47"E, 55° 21' 29"N 2569 m altitude; organic: 12° 18' 51"E, 55° 21' 52"N 2570 m altitude) and Skælskør (conventional: 11° 20' 04"E, 55° 17' 02"N 2560 m altitude; organic: 11° 20' 08"E, 55° 16' 46"N 2549 m altitude).

### 3.2.2 Sampling method

The soil was taken from five plots (beds) of the strawberry crops at each farm from a depth of 0-10 cm, using a cylindrical sampler with defined volume (70 $cm^3$) (INGLIS et al. 2012). The samples were stored in sterile plastic bags and kept within Styrofoam box with ice during transport to the laboratory. Five sub-samples of soil were taken per plot with 2 m distance apart, forming one composite sample per plot. In addition, four sub-samples were taken from the soil 10 cm from the roots of the most abundant specie of spontaneous herb in the margin of the crop field (maximum 3m distance). A total of five samples were taken on each farm, totalizing 25 sub-samples per farm of strawberry and 20 sub-samples per farm of soil around spontaneous herb of the crop margins in each date and location, summing up 300 sub-samples in Brazil and 100 in Denmark inside the strawberry crop and 240 sub-samples in Brazil and 80 in Denmark for margins crop soil.

### 3.2.3 Isolation of entomopathogenic fungi

The isolation of entomopathogenic fungi from the soil was carried out using selective agar medium and insect baiting as described in chapter two (Item 2.2.5).

### 3.2.4 Molecular identification of entomopathogenic Hypocreales fungi

#### 3.2.4.1DNA extraction

DNA was extracted from the 203 isolates using the same procedure as described in chapter two (Item 2.4.1).

#### 3.2.4.2 PCR amplification and sequencing

The methods used for amplification and sequencing followed the steps of Steinwender et al. (2014). The diversity of *Metarhizium* spp. was estimated by sequences of the nuclear intergenic (IGS) marker MzFG543igs (KEPLER; REHNER, 2013). The nuclear encoded translation elongation factor 1-alpha (5'-TEF1-α) of each haplotype was sequenced for assignment to species level (BISCHOFF et al., 2009). This was carried out by PCR amplifications of one representative isolate of each haplotype with markers EF2F (5´-GGAGGACAAGACTCACATCAACG- 3´) and EFjR (5´-TGYTCNCGRGTYTGNCCRTCYTT-3´) and for all isolates with markers MzFG543igs_1F (5'-ATTCATTCAGAACGCCTCCAA-3') and MzFG543igs_4R (5'-GGTTGCGACTGAGAATCCATG-3'). PCR products were purified using the geneMAG-PCR Kit (Chemicell, Germany) and sequenced. Sequencing was performed by Beckman Coulter Genomics (United Kingdom).

Sequences were edited and aligned with 52 reference sequences obtained from GenBank, representing ex-type cultures or taxonomically confirmed isolates of *M. acridum, M. anisopliae, M. brunneum, M. flavoviridae, M. guizhouense, M. lepidiotae, M. majus, M. minus, M. pemphigi, M. pingshaense and M. robertisii*, (BISCHOFF et al., 2009; ROCHA et al., 2009, 2013; LOPES; MESQUITA, 2013; LOPES; SOUZA, 2013; LOPES et al., 2014; STEINWENDER et al., 2014) three indeterminate species of *Metarhizium* (*M*. sp. Indet 1, 2 and 3) and two species not identified (REZENDE, 2014; REZENDE et al., 2015; ZANARDO, 2015; IWANICKI, 2016) using MAFFT with the FFT-NS-i alignment option in Geneious 7.1.8. software (KEARSE et al., 2012). The

sequence of *M. minus* (ARSEF1099) was included as outgroup and jModelTest 2.1.7 (DARRIBA et al., 2012) was used to calculate the evolution model for the phylogenetic tree (best-fit models of nucleotide substitution). The most parsimonious tree was calculated based on Bayesian (GTR-GAMMA) and Maximum Likelihood (GTR-GAMMA) model parameters using 1000 bootstrap replicates with the software Geneious 7.1.8 (KEARSE et al., 2012) of the gene alignments of 5'-TEF1- α. The Haplotype network was done using the intergenic spacer region IGS (MzFG543igs) and 203 sequences were obtained from the fungi sampled in Brazilian and Danish strawberry crop soil and in field margins generating 17 haplotypes. Gathering this sequences with 14 sequences from ex-type cultures or taxonomically authenticated reference (ARSEF, ESALQ, CBS and KVL) totalizing 225 sequences, in which 31 haplotypes were set and run with 100 fixed steps connection limit and gaps equal missing data using the software TCS: Phylogenetic network estimation using statistical parsimony 1.21 (CLEMENT et al., 2000) and Network 4.6.1.3. (BANDELT et al., 1999).

### 3.2.5 Statistical Analyses

Analysis of variance models were fitted to the logarithm of *Metarhizium* CFU (selective agar medium method) and the average proportion of infected insects (insect baiting method) data including the effects of location (Brazil: Estiva; Cambuí; Senador Amaral; Denmark: Klippinge or Skælskør) where the soil was sampled as random and the effects of sampling date (Sep/12; Jan/13; Apr/13, Aug/13, Jul/14 or Oct/14), management (conventional or organic), and place of sampling (inside the crop or in field margins) and their interactions as fixed for each Country.

### 3.2.6 Diversity analyses

The Shannon-Wiener diversity index (SHANNON, 1948) was calculated by using the haplotypes as operational taxonomic unit (OTU) and using the software DivEs 3.0.7.1385 (RODRIGUES, 2015). The haplotype and nucleotide diversity index were calculated using the DnaSP 5 (LIBRADO; ROZAS, 2009) and the Analyses of Molecular Variance (AMOVA) was done using the software Arlequin 3.5.2.2 (EXCOFFIER, 2010).

## 3.3 Results

### 3.3.1 Abundance of *Metarhizium* spp. in strawberry crops

There was no significant effect of country on abundance of *Metarhizium* spp. (CFU/g of soil) (Figure 3.1a) using the selective agar medium method and hence analyses were split by Country ($F_{1,34}$=0.01, p=0.93). For Brazil, the only significant difference was found between the dates of sampling (Table 3.1) where density of *Metarhizium* sp. was lower in August 2013 (4.17 ± 0.34x$10^3$) then in January 2013 (2.85 ± 0.62x$10^4$) ($F_{3,28}$=3.80; p ≤ 0.02). The highest mean of *Metarhizium* spp. CFU/g of soil (8.00 ± 1.78x$10^2$) was found in Senador Amaral in April 2013. For Denmark, the only difference was found in the interaction between management and dates where density of *Metarhizium* sp. was lower in conventional management system in October 2014 (3.00 ± 1.08x$10^4$) then in organic management system in the same date (9.00 ± 2.61x$10^4$), and was lower in June 2014 (2.75 ± 1.03x$10^4$) then October 2014 (9.00 ± 2.61x$10^4$) for organic management system ($F_{1,6}$=9.44; p ≤ 0.02). The highest total mean of *Metarhizium* spp. CFU/g of soil (3.85 ± 0.86x$10^3$) was recorded in Skælskør in June 2014

There was no significant effect of country on the proportion of infected *T. molitor* larvae (Figure 3.1b) using the insect bait method and hence analyses were split by country ($F_{1,34}$=0.35, p=0.56). In Brazil, the only difference was found between the dates of sampling where the proportion of infected insects was higher in August 2013 (40.5 ± 9.52%) than September 2012 (11.5 ± 4.2%), January 2013 (13.3 ± 3.8%) and April 2013 (14.7 ± 5.1%) ($F_{3,28}$=4.91; p < 0.01). The highest proportion of *T. molitor* larvae killed by *Metarhizium* spp. (74 ± 4.5%) was recorded in organic management system soil of Cambuí in August 2013. In Denmark, no difference was found on proportion of infected insects for any of the parameters evaluated (location, date, management). The highest proportion of *T. molitor* larvae killed by *Metarhizium* spp. (38 ± 7.7%) was recorded in organic management system soil of Klippinge in June 2014.

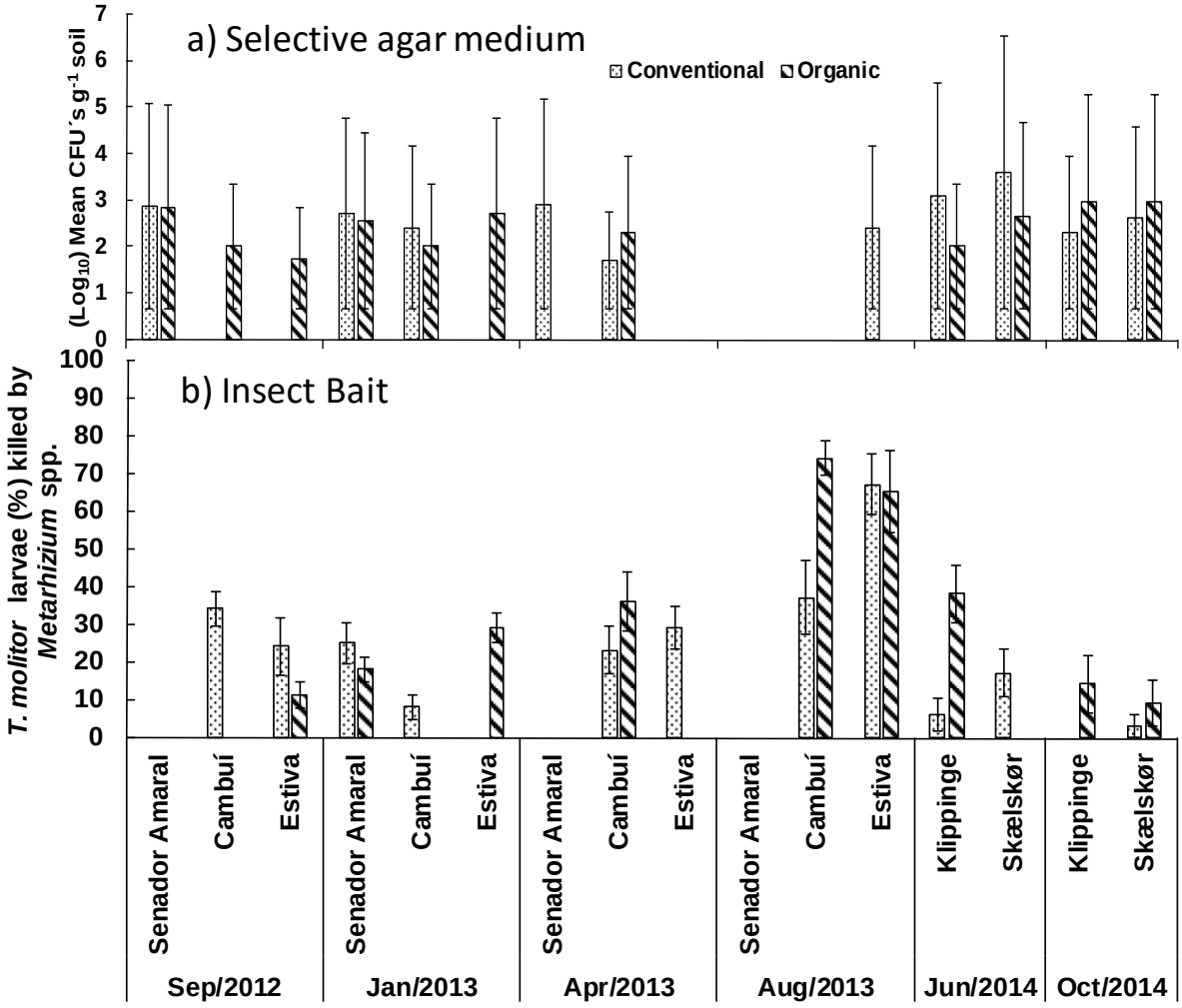


Figure 3.1 - a) Density [Colony forming units (CFU ± SE) per gram of humid soil] of *Metarhizium* spp. in strawberry crop soil in Brazil. b) Mortality (Mean ± SE) of *Tenebrio molitor* larvae used in the isolation of *Metarhizium* spp. by the insect bait method

Table 3.1 - Analysis of variance output of the logarithm of *Metarhizium* CFU, and average proportion of infected insects (insect baiting method) including the effects of place of sampling and Farm as random and the effects of Date, Management and place of sampling and their interactions as fixed for each Country

| Density (CFU) analyses | Brazil | | Denmark | |
|---|---|---|---|---|
| Effect | Test statistic | p-value | Test-statistic | p-value |
| Place of sampling | $F_{1,28}$=0.01 | 0.93 | $F_{1,6}$=1.03 | 0.35 |
| Date | $F_{3,28}$=3.80 | **0.02** | $F_{1,6}$=0.18 | 0.69 |
| Management | $F_{1,2}$=0.02 | 0.89 | $F_{1,1}$=4.17 | 0.29 |
| Place of sampling x Management | $F_{1,28}$=0.38 | 0.55 | $F_{1,6}$=0.56 | 0.48 |
| Place of sampling x Date | $F_{3,28}$=0.19 | 0.90 | $F_{1,6}$=0.74 | 0.42 |
| Management x Date | $F_{3,28}$=0.96 | 0.43 | $F_{1,6}$=9.44 | **0.02** |
| Place of sampling x Management x Date | $F_{3,28}$=0.08 | 0.97 | $F_{1,6}$=0.81 | 0.40 |
| Insect Bait method analyses | | | | |
| Location | $F_{1,28}$<0.01 | 0.96 | $F_{1,6}$=1.38 | 0.28 |
| Date | $F_{3,28}$=4.91 | **<0.01** | $F_{1,6}$=2.27 | 0.18 |
| Management | $F_{1,2}$=0.48 | 0.56 | $F_{1,1}$=0.38 | 0.65 |
| Location x Management | $F_{1,28}$=0.02 | 0.89 | $F_{1,6}$=1.30 | 0.30 |
| Location x Date | $F_{3,28}$=0.17 | 0.92 | $F_{1,6}$=2.06 | 0.20 |
| Management x Date | $F_{3,28}$=0.93 | 0.44 | $F_{1,6}$=0.14 | 0.72 |
| Location x Management x Date | $F_{3,28}$=0.04 | 0.99 | $F_{1,6}$=0.06 | 0.81 |

Location = place of sampling: inside the crop or in field margins
Date = Sep/12; Jan/13; Apr/13, Aug/13, Jul/14 or Oct/14
Management = conventional or organic

### 3.3.2 Diversity of *Metharizium* spp. in strawberry crops

The 203 isolates were grouped in 17 haplotypes based on the MzFG543igs (Figure 3.2) and a haplotype network was performed (Figure 3.3).

#### 3.3.2.1 Brazil

Based on 5'TEF1-α gene analysis, the Brazilian isolates had representatives from six *Metarhizium* species. The single haplotype of *M. anisopliae* (n=6 isolates) clustered with Mani2 clade (Figure 3.2). *M. robertsii* (n=131), was the most abundant but only 3 haplotypes were found, all isolates clustered with Mrob4 clade except one

isolate that clustered with Mrob1 clade. Two haplotypes of *Metarhizium* sp. Indeterminate 1 (n=16), one haplotypes each of *M. pingshaense* (n=1) and *M. brunneum* (n=1) and two haplotypes of a taxonomically unassigned lineage between *M. acridum* and *M. lepidiotae* with great support in both Bayesian and maximum likelihood analyses. This new lineage is referred to here as *Metarhizium* sp. Indet. 5 (n=4). All these isolates formed well-supported monophyletic groups with the reference sequences.

#### 3.3.2.2 Denmark

The most representative species of the Danish isolates is *M. brunneum* (n=33) and consisted of one haplotype clustering with the Danish taxonomically authenticated reference (KVL12-30) and the second haplotype with the Brazilian reference (CG1126) isolated from Hemiptera in south of Brazil. One haplotype each of *M. flavoviridae* (n=6) and *M. pemphigi* (n=5) detected for the first time in Denmark.

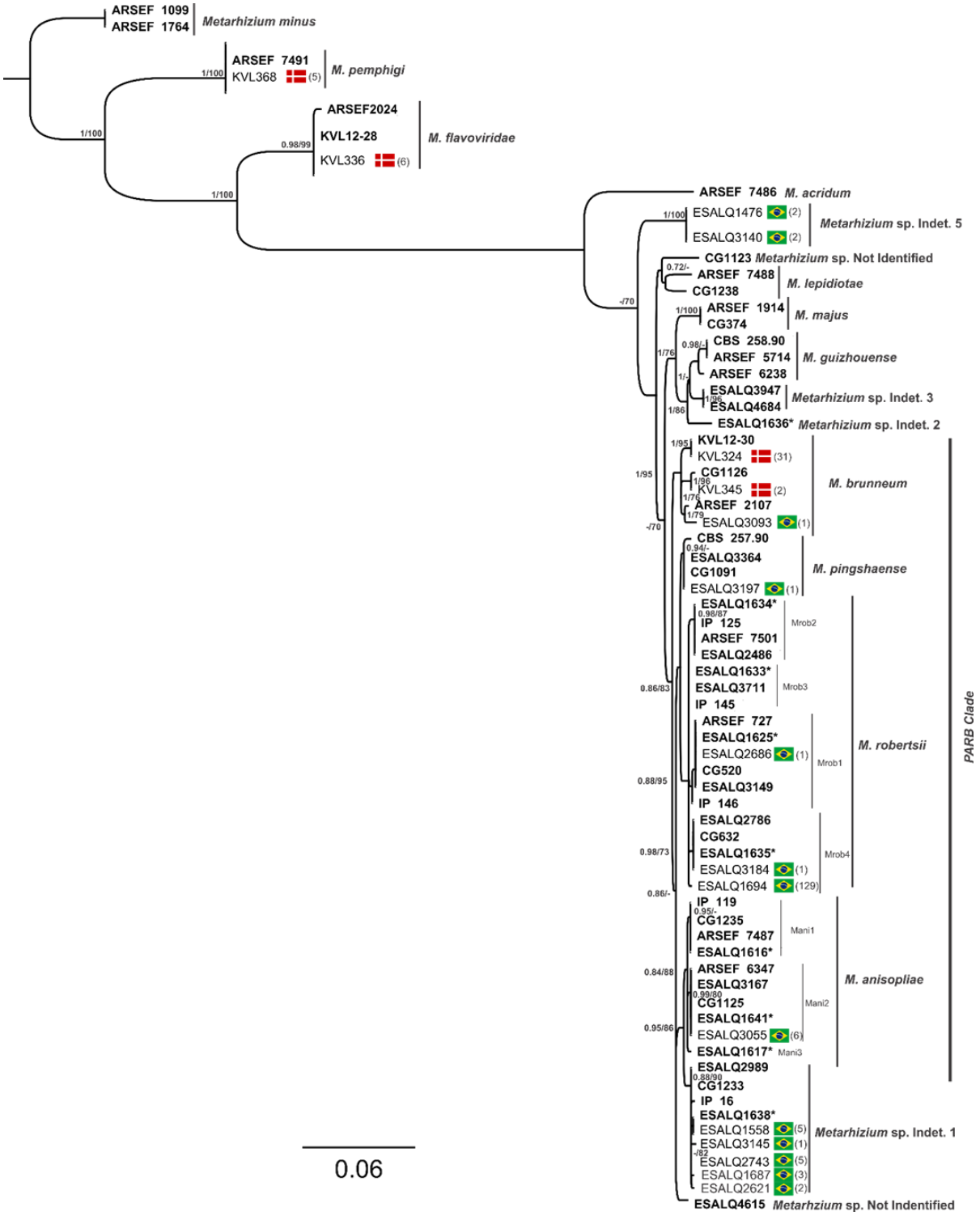


Figure 3.2 - Bayesian (B) [GTR-gamma] / Maximum likelihood (ML) [GTR-gamma] phylogeny of the data set of 5` TEF sequences of 17 haplotypes found in the experiment crops, including 52 taxonomically validated reference strains (in bold) accessioned in ARSEF (ARS Entomopathogenic Fungal Culture Collection, CBS (Fungal Biodiversity Centre), ESALQ (Collection of Entomopathogenic Microorganisms of LPCMI-ESALQ-USP) CG (EMBRAPA – Cenargen) and IP (Rocha et al. 2013). Bootstrap support values ≥ 70% are listed near nodes (B/ML). Numbers in the bracket in front of the flags refer to number of isolates collected of each haplotype

### 3.3.3 Distances

Sequence differences were compared in an alignment of 515 positions of 5'TEF1-α (Table 3.2). *Metarhizium robertsii* intraspecific differences were detected at up to four positions while intraspecific variation within *M. brunneum* were found up to 8 positions, *Metarhizium* sp. Indet. 1 intraspecific differed up to 3 base pairs, while *Metarhizium* sp. Indet. 5 intraspecific had 1 position difference between the two haplotypes. The longest interspecific distance was found between *M. flavoviridae* and the new unsigned species *Metarhizium* sp. Indet. 5 with 111 base pairs. The distances between *M. flavoviridae* and the other species were: 103 and 104 differences from *Metarhizium* sp. Indet. 1, 103 from *M. anisopliae*, 105 from *M. pingshaense*, 103 to 105 from *M. robertsii* and 80 to 105 from *M. brunneum*. The differences between, *Metarhizium* sp. Indet. 1 and *Metarhizium* sp. Indet. 5 ranged from 44 to 45 base pairs difference.

Table 3.2 - Base pair difference among the 17 *Metarhizium* haplotypes in sequences of the 5' end of Elongation Factor 1-α (total positions 512 bp). (*Ma = Metarhzium anisopliae, Mr = M. robertsii, Mb = M. brunneum, Mf= M. flavoviridae*, *Mping = M. pingshaense*, M.sp. I. 1 = *Metarhizium* sp. Indet. 1, M.sp. I. 5 = *Metarhizium* sp. Indet. 5 and *Mp = M. pemphigi*)

| **Isolates** | | **ESALQ 1476** | **ESALQ 3140** | **ESALQ 1558** | **ESALQ 2743** | **ESALQ 2621** | **ESALQ 1687** | **ESALQ 3145** | **ESALQ 3055** | **ESALQ 3197** | **ESALQ 1694** | **ESALQ 2686** | **ESALQ 3184** | **ESALQ 3093** | **KVL 324** | **KVL 345** | **KVL 336** | **KVL 368** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Species | *M.*sp. I. 5 | *M.*sp. I. 5 | *M.*sp. I. 1 | *M.*sp. I. 1 | *M.*sp. I. 1 | *M.*sp. I. 1 | *M.*sp. I. 1 | *Ma* | *M.ping* | *Mr* | *Mr* | *Mr* | *Mb* | *Mb* | *Mb* | *Mf* | *Mp* |
| **ESALQ 1476** | *M.*sp. I. 5 | | | | | | | | | | | | | | | | | |
| **ESALQ 3140** | *M.*sp. I. 5 | 1 | | | | | | | | | | | | | | | | |
| **ESALQ 1558** | *M.*sp. I. 1 | 44 | 44 | | | | | | | | | | | | | | | |
| **ESALQ 2743** | *M.*sp. I. 1 | 44 | 44 | 2 | | | | | | | | | | | | | | |
| **ESALQ 2621** | *M.*sp. I. 1 | 45 | 45 | 3 | 3 | | | | | | | | | | | | | |
| **ESALQ 1687** | *M.*sp. I. 1 | 44 | 45 | 3 | 3 | 2 | | | | | | | | | | | | |
| **ESALQ 3145** | *M.*sp. I. 1 | 44 | 45 | 2 | 3 | 3 | 2 | | | | | | | | | | | |
| **ESALQ 3055** | *Ma* | 43 | 43 | 7 | 7 | 7 | 7 | 8 | | | | | | | | | | |
| **ESALQ 3197** | *M.ping* | 40 | 40 | 12 | 12 | 12 | 13 | 13 | 11 | | | | | | | | | |
| **ESALQ 1694** | *Mr* | 44 | 44 | 16 | 16 | 16 | 17 | 17 | 15 | 10 | | | | | | | | |
| **ESALQ 2686** | *Mr* | 46 | 46 | 18 | 18 | 18 | 19 | 19 | 17 | 12 | 4 | | | | | | | |
| **ESALQ 3184** | *Mr* | 45 | 45 | 17 | 17 | 17 | 18 | 18 | 16 | 11 | 3 | 3 | | | | | | |
| **ESALQ 3093** | *Mb* | 44 | 44 | 20 | 20 | 20 | 21 | 21 | 19 | 18 | 20 | 22 | 21 | | | | | |
| **KVL 324** | *Mb* | 33 | 33 | 11 | 12 | 12 | 13 | 13 | 12 | 12 | 14 | 15 | 15 | 8 | | | | |
| **KVL 345** | *Mb* | 31 | 31 | 11 | 12 | 12 | 13 | 13 | 12 | 13 | 15 | 15 | 15 | 7 | 6 | | | |
| **KVL 336** | *Mf* | 111 | 111 | 103 | 104 | 104 | 104 | 104 | 103 | 105 | 103 | 105 | 103 | 105 | 80 | 85 | | |
| **KVL 368** | *Mp* | 105 | 105 | 94 | 95 | 95 | 95 | 95 | 98 | 97 | 97 | 98 | 97 | 98 | 79 | 84 | 61 | |

### 3.3.4 Ecological aspects

#### 3.3.4.1 Analyses of Molecular Variance

The Molecular Variance between Brazil and Denmark (49.2%) was similar to the variance within each country (50.8%) ($p < 0.001$) (Table 3.3). The variance between organic and conventional was very small and the biggest variation was found within each management type, 99.45% of the variance in Brazil ($p < 0.01$) and 82.41% in Denmark ($p < 0.001$).

Table 3.3 - Analyses of molecular variance (AMOVA) of *Metarhizium* spp. found in strawberry crops and field margins in Brazil and in Denmark

| Group | SS | Variance component | Variance (%) | Fixation Index ($\phi$st) | P-value |
|---|---|---|---|---|---|
| Country | | | | | |
| Among countries | 1033.5 | 14.77399 | 49.22 | 0.49217 | **< 0.001** |
| Within countries | 3064.1 | 15.24422 | 50.78 | | |
| Brazil | | | | | |
| Among managements | 6508 | 0.02582 | 0.55 | 0.00547 | **0.0136** |
| Within managements | 7043.4 | 4.69560 | 99.45 | | |
| Denmark | | | | | |
| Among managements | 261.7 | 10.79972 | 17.59 | 0.17590 | **< 0.001** |
| Within managements | 1922.6 | 50.59591 | 82.41 | | |

Country= Brazil and Denmark; Management= Organic and Conventional

#### 3.3.4.2 Diversity

The haplotype and nucleotide diversity of *Metarhizium* spp in Denmark (Hd= 0.481, $\pi$= 0.0780) was higher than Brazil (Hd= 0.339, $\pi$= 0.0102) but the Shannon-Wiener index was similar (0.3953 and 0.3935 for Brazil and Denmark) (Table 3.4). The *Metarhizium* spp. diversity found in organic crop systems in Denmark was higher than in conventional system for all indexes. In Brazil, the haplotype and nucleotide diversity were similar but the Shannon-Wiener index was a bit higher in the organic system. The haplotype diversity (Hd) in the Danish organic system (Hd = 0.627) was more than double of the diversity found in Brazil (Hd = 0.300) in the same system. Higher

polymorphism in the organic management was observed in Denmark with $\pi$ = 0.1123 almost 15 fold of the nucleotide diversity ($\pi$ = 0.0075) in Brazil.

Table 3.4 - Diversity of *Metarhizium* spp. found in strawberry crops and field margins in Brazil and in Denmark

| Group | N | H | Hd | $\pi$ | V | Pi | S | Shannon-Wiener |
|---|---|---|---|---|---|---|---|---|
| Brazil | 159 | 13 | 0.339 | 0.0102 | 106 | 87 | 19 | 0.3953 |
| Conventional | 100 | 9 | 0.299 | 0.0108 | 98 | 84 | 14 | 0.3338 |
| Organic | 59 | 7 | 0.300 | 0.0075 | 45 | 41 | 4 | 0.4227 |
| Denmark | 44 | 4 | 0.481 | 0.0780 | 200 | 200 | 0 | 0.3935 |
| Conventional | 27 | 3 | 0.236 | 0.0440 | 198 | 150 | 48 | 0.2042 |
| Organic | 17 | 3 | 0.627 | 0.1123 | 198 | 198 | 0 | 0.5106 |

### 3.3.5 Haplotype network

The *M. robertsii* haplotype 11 (H11) was the most commonly found (n= 129 isolates) and was consistently distributed in the sampled area, 31% was observed in soil of the conventional management system, 31% in the margins of the conventional field, 24% in the organic system and 14% in margins of the organic system (Figure 3.3). The *M. brunneum* haplotype 1 (H1) was the most commonly found (n= 30 isolates) in Denmark with 44% of isolates found in margins of conventional system, 28% inside the conventional system, 22% inside the margins of organic system and 6% in the margins of the organic fields.

*Metarhizium* sp. Indet. 1 haplotypes (H9 and H10) was the second most common species in Brazil and was found in most of the sampled areas, and it was more abundant than the only haplotype (H6) of *M. anisopliae* which was found only in the organic system and in margins of the conventional system. The second most common species in Denmark, *M. flavoviridae* (H24) was found only in margins of the organic and conventional systems (67% and 33%, respectively).

The two haplotypes (H21 and H22) of the *Metarhizium* sp. Indet. 5 were found only in margins of the conventional system in Brazil. *M. pemphigi* was found only in Denmark (H25), 40% in margins of the conventional system, 40% in the organic crops and 20% in conventional crops.

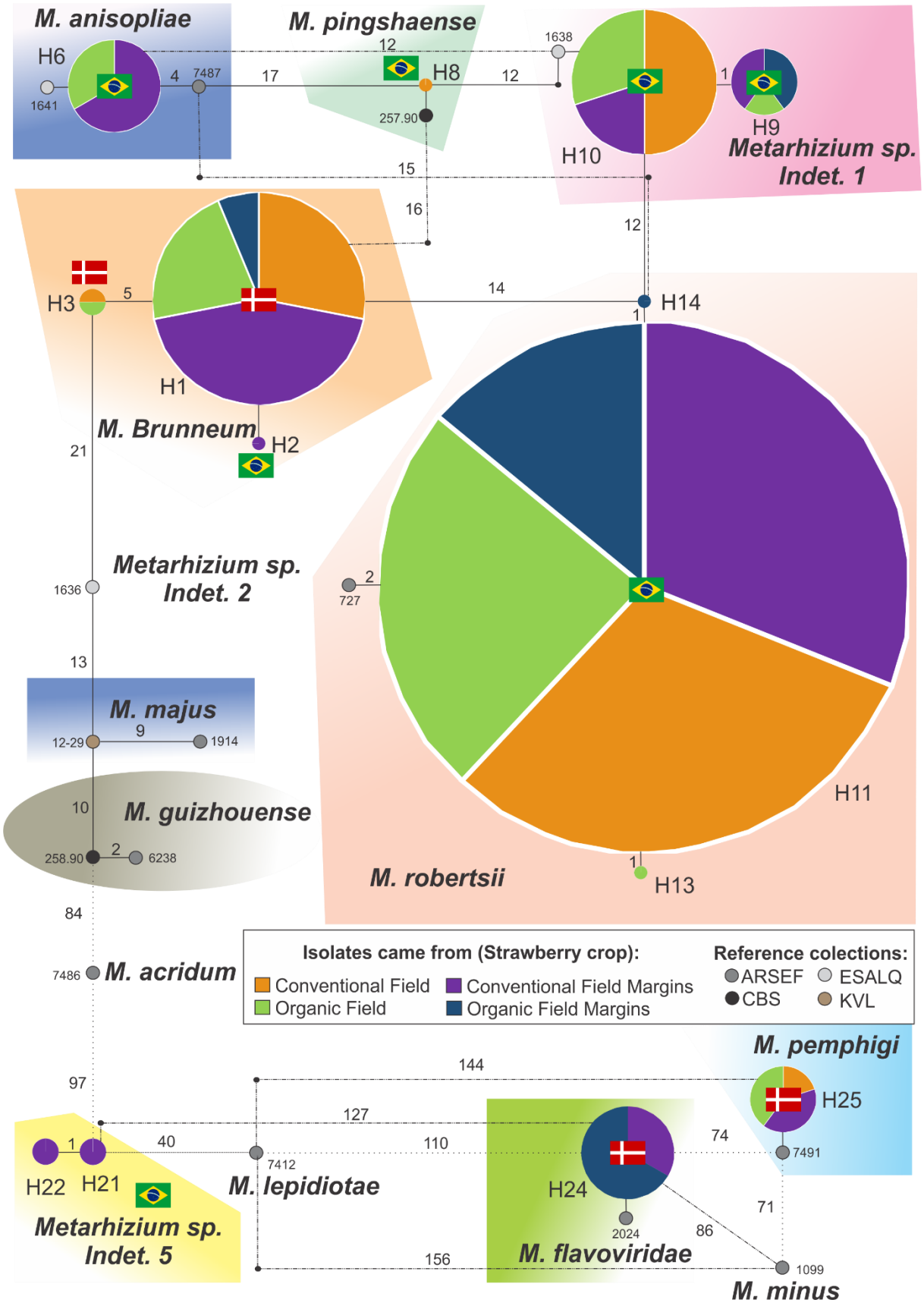


Figure 3.3 - Haplotype network of *Metarhizium* spp. found in strawberry crops and field margins in Brazil and in Denmark

### 3.4 Discussion

This is the first study comparing directly the diversity and abundance of *Metarhizium* species in the same crop (strawberry) grown under two very different climatic regimes. Sun and Liu (2008) compared the occurrence and the species diversity of fungi in soil of different climatic regions of China, and they found in overall that 10.6% of the entomopathogenic fungi isolated belonged to those *Metarhizium anisopliae sensu lato,* but with some geographical difference related to the four different climatic zones of sampling. Our comparison is, however, different, since we restricted our studies to solely two climatic regimes, one crop, but then actually both hemispheres and we used the new multilocus phylogenic classification focusing only in *Metarhizium* spp. Also, our haplotype assessment gave a much higher resolution of the diversity found than in previous *Metarhizium* studies.

*Metarhizium* community in temperate North America appeared to be dominated by *M. brunneum, M. robertsii* and *M. guizhouense* (FISHER et al., 2011; WYREBEK et al., 2011; KEPLER et al., 2015), with *M. anisopliae* a minor component or absent altogether. Our results corroborate other studies regarding the most frequent *Metarhizium* species found in South America and Europe. In South America, the predominance of *M. anisopliae* and *M. robertsii* has been observed (ROCHA et al., 2009, 2013; LOPES, MESQUITA, 2013; LOPES; SOUZA, 2013; LOPES et al., 2014; REZENDE et al., 2015). In contrast, *Metarhizium* community in Europe appeared to be dominated by *M. brunneum, M. flavoviride* and *M. robertsii* (MEYLING et al., 2011; STEINWENDER et al., 2014, 2015; KEYSER et al., 2015).

Rezende et al. (2015) found two new taxonomically unassigned linages in Brazil, being phylogenetically close to *M. anisopliae* (*Metarhizium* sp. indet. 1) and the second more basal in the phylogenetic tree (*Metarhizium* sp. indet. 2). We reported here that *Metarhizium* sp. indet. 1 is actually more abundant in strawberry than *M. anisopliae* a common species in Brazil. In our study, another new taxonomically unassigned lineage was found (*Metarhzium* sp. Indet. 5) that is even more basal than the ones found in the previous study (REZENDE et al., 2015). There is only a single report of *M. brunneum* (LOPES et al., 2014) from Brazil and here we report a second isolate collected in the country.

The results we observed from strawberry soils corroborate other studies demonstrating that the most frequent species found in Denmark was *M. brunneum* (STEINWENDER et al., 2014, 2015) but differed from that observed by Keyser et al.

(2015) where *M. flavoviride* was the dominant species, and *M. pemphigi* was detected for the first time in Denmark in our study.

We found a higher diversity of *Metarhizium* in organic strawberry farms in Denmark compared with conventional strawberry, which supports earlier findings in other crops. Klingen et al. (2002) found significantly higher occurrence of insect pathogenic fungi in soils from arable fields of organically managed farms in Norway. Clifton et al. (2015) compared soil-borne entomopathogenic fungi in organic and conventional fields in Midwestern USA in corn and soybean fields and found a similar result where occurrence of entomopathogenic fungi was significantly greater in the soil of organic agroecosystems than conventional. The differences between *Metarhizium* occurrence in conventional and organic soils were bigger in Denmark compared to Brazil. One explanation for that could be due to the definition of 'organic crop' differs between countries. In Denmark, there are stricter rules and more surveillance for organic fields than in Brazil which may lead to the suggestion that the organically grown areas in Denmark have had more time to be settled as being 'organic'. We hypothesize that if organically grown strawberries in Brazil in the future will be subjected to better surveillance it will be possible to detect differences in *Metarhizium* spp. composition, more comparable with the results from Denmark.

The importance of the field margins to the diversity of the fields can be seen when evaluating the haplotype network (Figure3.3). The margins seem important as a sort of biodiversity pool once the abundance of *M. anisopliae* was negatively affected by proximity to conventional fields, in accordance with Clifton et al. (2015), who suggested that cropping practices within a field can affect soil-borne microorganisms even outside the field. The management of crop margins is important to ensure the natural diversity of *Metarhizium* species. Overall, insufficient knowledge about main drivers in *Metarhizium* spp. diversity does not yet allow for general conclusions, if crop, climatic zone, or cropping system is of similar importance. Bidochka et al. (2001) reported that *M. anisopliae* population structure may be driven by habitat selection and not insect host selection. Our studies seem to support this hypothesis, although in our case the scale (climatic regimes) is larger than habitat.

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Manuscript 3

# Exploring the diversity of *Metarhizium* spp. from Brazil for the control of the Two-spotted spider mite

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# 4 EXPLORING THE DIVERSITY OF *Metarhizium* spp. FROM BRAZIL FOR THE CONTROL OF THE TWO-SPOTTED SPIDER MITE


## Abstract

The two-spotted spider mite, *Tetranychus urticae* is the most important pests of strawberry crops in Brazil and many other countries. Although control of this pest is mostly by chemical pesticides, the use of biological control with entomopathogenic fungi, specially *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium* spp. (Hypocreales: Clavicipitaceae), is increasing. With the latest changes in the classification of *Metarhizium,* new species has been described, but most studies are still concentrated in only three species, *M. anisopliae, M. brunneum* and *M. robertsii*. Recently, new undescribed *Metarhizium* species have been found in Brazil and these species may add a variety of new options in the pest control. The local species may be more adapted to specific agroecosystems and more virulent towards local pests. To test these hypotheses, the mortality of adult females of *T. urticae* by six isolates of four undescribed *Metarhizium* species from different regions and crops were compared to two isolates of each species of *M. anisopliae, M. brunneum, M. pingshaense* and *M. robertsii*, isolated from strawberry crops in Brazil. *Beauveria bassiana* ESALQPL63, commercially available in Brazil against this pest was used as control. Fungal suspensions were sprayed in laboratory at $1x10^7$ conidia/mL with 0.05% tween 80. The best isolates were ESALQPL63 of *B. bassiana*, ESALQ1608 and ESALQ1638 of *Metarhizium* sp. indet. 1 and ESALQ3069 and ESALQ3222 of *M. pingshaense* based on survival curve, total mortality, percentage of sporulated cadavers and median lethal time ($LT_{50}$). The lowest $LT_{50}$, 4 (±0.17) was observed in mites treated with ESALQ1638 of *Metarhizium* sp. indet. 1. This study revealed for the first time the potential of unassigned new lineages and less studied species of *Metarhizium* as a biocontrol agent of *T. urticae*. These less known species should be considered in the development of new biopesticides and in further studies to assess their potential as biological control agents of other pest in the future.


Keywords: *Tetranychus urticae*; Microbial control; Strawberry; *Beauveria bassiana*; Virulence; Pathogenicity

## 4.1 Introduction

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae) is considered one of the main pests of horticultural crops worldwide (ATTIA et al., 2013) and is one of the main pests on strawberry crops in Brazil (IWASSAKI et al., 2015), affecting crops by feeding on the undersurface of leaves covered with fine webbing, causing curling and discoloration, reducing photosynthetic activity and causing leaf abscission in severe infestations (DE MORAES ; FLECHTMANN, 2008; ATTIA et al., 2013). *Tetranychus urticae* has developed resistance against many chemical pesticides (STUMPF ; NAUEN, 2001; NICASTRO et al., 2010).

One alternative to chemical pesticides is the biological control with entomopathogenic fungi, in particular species from the Ascomycota. Species from the genus *Metarhizium* (Hypocreales: Clavicipitaceae) are easy to mass produce and formulate, so several mycopesticides based on these fungi are available (FARIA ; WRAIGHT, 2007; VEGA et al., 2012)

Recently, after Bischoff et al. (2009) discoveries in a multilocus phylogenetic study, the fungus previous known as *Metarhizium anisopliae* actually comprises a nine species complex: *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acridum*, *M. majus*, *M. lepidiotae*, *M. brunneum*, *M. globosum* and *M. robertsii* and also *M. flavoviridae* already known as a different species by its different morphology. They suggest that the most reliable way to distinguish between species within this complex is the use of molecular tools and analyzes. Based on their results, was determined that the 5' region of the gene for the translational elongation factor (TEF) is the most informative region for routine use in species identification within the genus. Due to these new findings, all work done using *Metarhizium* spp. identified only by morphological means, makes impossible to determine the true identity of the species and because of that all studies performed without molecular identification of these fungi needs to be cited as *M. anisopliae sensu latu* (*s.l.*).

Besides of that, other important fact that needs to be highlighted is that most of the studies using fungi from the genus *Metarhizium* were performed using different isolates of the same species. Some studies show that *M. anisopliae s.l.* is pathogenic to other mite species (WEKESA et al., 2005; BUGEME, et al., 2008, 2015; SHI et al., 2008; TAVASSOLI et al., 2011; QUINELATO et al., 2012) and by that, it is possible to infer that fungi from this genus may offer a good opportunity for biological control of *T. urticae*. Testing different species of *Metarhizium*, identified by molecular means, may provide a more versatile background for selection of species (and not just strains or isolates) for further work.

Pathogenicity and virulence studies of *Metarhizium anisopliae s.l.* have been performed in *T. urticae* (TAMAI et al., 2002; CHANDLER et al., 2005; BUGEME et al., 2008; AMJAD et al., 2012). These kinds of laboratory bioassays are essential to select isolates for subsequent more deep and detailed studies. Such studies also work as a first step and if the results merit it, one or more isolates might then be worthy for field

trials and, in the best case, later registration and commercialization (VEGA et al., 2012).

The following *Metarhizium* species are known to occur naturally in soils or insects in Brazil: *M. anisopliae*, *M. acridum*, *M. majus*, *M. flavoviride*, *M. pingshaense*, *M. robertsii*, *M. brunneum*, *M. lepidiotae* (LOPES; MESQUITA, 2013; LOPES; SOUZA, 2013; LOPES et al., 2014; REZENDE et al., 2015). Scientifically undescribed *Metarhizium* lineages have also been detected in Brazil: refer to as *Metarhizium* sp. Indet. 1, *Metarhizium sp.* Indet.2, *Metarhizium sp.* Indet.3, *Metarhizium sp.* Indet.4 (REZENDE, 2014; REZENDE et al., 2015; ZANARDO, 2015; IWANICKI, 2016) and *Metarhizium sp.* Indet.5 (T. CASTRO, unpublished). Even though these species have not been formally described yet, it will be highly relevant to evaluate their potential for pest control.

Maniania et al. (2008) highlighted the importance of bioprospection for obtaining more isolates and species of entomopathogenic fungi with varied virulence from diverse geographic regions and hosts allowing to increase biological control efficacy in different agroecosystems. A number of isolates from different species of *Metarhizium* have recently been obtained from soil of strawberry crops in Brazil. In this study, we aimed to compare the pathogenicity and virulence of six isolates of four undescribed *Metarhizium* species (from strawberry, sugarcane crops and native forest from different regions) with two isolates of each species of *M. anisopliae, M. brunneum, M. pingshaense* and *M. robertsii*, isolated from strawberry crops, all from Brazil. Also, we obtained information about host adaptation by qualitatively evaluation of sporulation from mite cadavers.

## 4.2 Material and Methods

### 4.2.1 Fungal origin

Two isolates each of M*. robertsii*, *M. anisopliae*, *M. brunneum*, *M. pingshaense*, and one isolate each of *Metarhizium* indet. sp. 2 and *Metarhizium* indet. sp. 5 obtained from strawberry crop in south of Minas Gerais State in Brazil between 2012 and 2013, as described in chapter two and three (Item 2.2.1 and 3.2.1, respectively), were selected (Table 4.1). These were compared to two isolates each of *Metarhizium* sp.

indet. 1 and *Metarhizium* indet. sp. 4. (REZENDE, 2014; ZANARDO, 2015) from the Collection of Entomopathogenic Microorganisms of ESALQ-USP. *Beauveria bassiana* (ESALQPL63), commercially available in Brazil against this pest, was used as control. All fungi used were molecularly identified using 5`TEF-1α marker as suggested by Bischoff et al. (2009).

### 4.2.2 *Tetranychus urticae* stock culture

*Tetranychus urticae* was reared on Jack bean plants *Canavalia ensiformis* (Fabales: Fabaceae) in an acclimatized room at 25 °C, 60% RH, and 12h photoperiod at the University of São Paulo (ESALQ-USP) campus in Piracicaba – SP Brazil since 2014. The plants were watered three times per week. Old and highly infested plants were replaced by new ones as required.

### 4.2.2 Fungal production

All selected isolates were grown in Petri dishes (90 x 15 mm) at 25 ± 1°C on PDA media (Potato Dextrose Agar, Difco™, USA). Conidia were harvested by scraping the surface of 2-week-old sporulating cultures and then were suspended in 5 ml sterile distilled water containing 0.05% Tween 80 (Oxiteno, Brazil) in a 40 mL flat bottom glass tube (stock suspension). The conidial suspension was vortexed for 1 min to produce a homogenous conidial suspension and counted on Neubauer chamber (K5-0111 model, KASVI, Brazil) to obtain a $1x10^7$conidia/mL suspension of each isolate.

### 4.2.3 Bioassay

Petri Dishes with 3.5 cm of diameter and 1.5 cm of height were filled with 3 mL of 0.5% of water agar and a Jack Bean disc leaf was placed on top of the agar with the abaxial side of the leaf facing up. Thereafter, 12 *T. urticae* females were transferred by a fine paintbrush in the center of the leaf disc. Each Petri dish was sprayed with 1 mL of a $1 \times 10^7$ conidia/mL suspensions of one isolate with 0.05% Tween 80 using a Potter Spray Tower (Burkard Manufacturing Co. Ltd., Rickmansworth, Herts, England). The device was adjusted to a pressure of 0.7 psi, with an average of 1.5 mg/cm$^2$. To avoid contamination between treatments, the sprayer was cleaned with alcohol and washed three times with distilled water, and the first spray of each treatment was discarded. A control with distilled water plus 0.05% Tween 80 was included. Five replicates (Petri dishes) were sprayed separately. The entire experiment was repeated three times

yielding 180 mites per treatment, totalizing 2880 mites used in the entire experiment. The data from the three experiments were analyzed together.

Mortality was recorded daily for 10 days. Dead mites were individually placed in 24 cell well culture plates with a lid on, containing moistened cotton with sterile distilled water to ensure conditions of high relative humidity to allow the growth of fungus on the surface of the cadaver. Mortality caused by fungus was confirmed by microscopic examination.

### 4.2.4 Statistical analyses

The survival parameters of infected mites were analyzed using the Kaplan–Meier survival analysis using Log Rank (Mantel-Cox) test in SPSS [v. 22.0.0,(2013)]. Quasi-binomial models were fitted to the total mortality and sporulated cadaver's data separately; F-tests were performed to assess significance of effects using a GLM procedure (multcomp R package) through ANOVA. Treatment differences were tested using 95 % confidence intervals using Tukey HSD contrasts. Analyses were performed using the R statistical software environment (R DEVELOPMENT CORE TEAM, 2015).

## 4.3 Results

All *Metarhizium* isolates were pathogenic to *T. urticae* and differed from untreated control in the survival analyses (Figure 4.1) and Tukey HSD comparison (Figure 4.2). The survival curve of the mites treated with our control isolate, *B. bassiana* ESALQPL63, was similar to *Metarhizium* sp. indet. 1 (ESALQ1608 and ESALQ1638), and *M. pingshaense* (ESALQ3069 and ESALQ3222) but differed from all other isolates [Log Rank (Mantel-Cox) $p<0.05$], confirming the high efficacy of this isolate. The mites treated with *Metarhizium* sp. indet. 4 (ESALQ1660 and ESALQ1684), *M. brunneum* (ESALQ2623) and *Metarhizium* sp. indet. 5 (ESALQ3140) presented higher survival and differed from all other isolates.

The lowest $LT_{50}$ (4 ± 0.166 days, CI= 3.68-4.32) were observed in treatment with *Metarhizium* sp. indet. 1 ESALQ1638 (Table 4.1). All other treatments did not differ based on comparison of the confidence interval of $LT_{50}$. The mites treated with *Metarhizium* sp. indet. 4 ESALQ1660 presented a $LT_{50}$ of 8 ±1.487 (CI= 5.08-10.91).

The total mortality of mites (Figure 4.2) treated with the control isolate, *B. bassiana* ESALQPL63, 93 ± 1.0% after 10 days of evaluation, was similar to the mortality of eight isolates. *Beauveria bassiana* ESALQPL63 and *Metarhizium* sp. indet. 1 (ESALQ1608 and ESALQ1638), differed ($F = 29.92$; $df = 15$; $p < 0.001$) from *M. anisopliae* (ESALQ2651 and ESALQ3054), *M. brunneum* (ESALQ2623), *Metarhizium* sp. indet. 4 (ESALQ1660 and ESALQ1684) [56 ± 9.7% and 59 ± 2.8%] and *Metarhizium* sp. indet. 5 (ESALQ3140), the isolates with lowest mortalities.

The total mortality pattern, in general, followed the pattern of the confirmed mortality. Sporulation was observed in approximately 80% of spider mite cadavers in all treatments. Mites treated with *B. bassiana* (ESALQPL63) presented 75 ± 1.0% of the cadavers sporulated, and did not differed from eight other isolates. *Beauveria bassiana* (ESALQPL63) and *Metarhizium* sp. indet. 1 (ESALQ1608 and ESALQ1638), *Metarhizium pingshaense* (ESALQ3222), presented higher percentage of sporulation than ($F = 67.45$; $df = 15$; $p < 0.001$) *M. anisopliae* (ESALQ2651 and ESALQ3054), *M. brunneum* (ESALQ2623), *Metarhizium* sp. indet. 4 [ESALQ1660 (37 ± 1.0%) and ESALQ1684] and *Metarhizium* sp. indet. 5 (ESALQ3140).

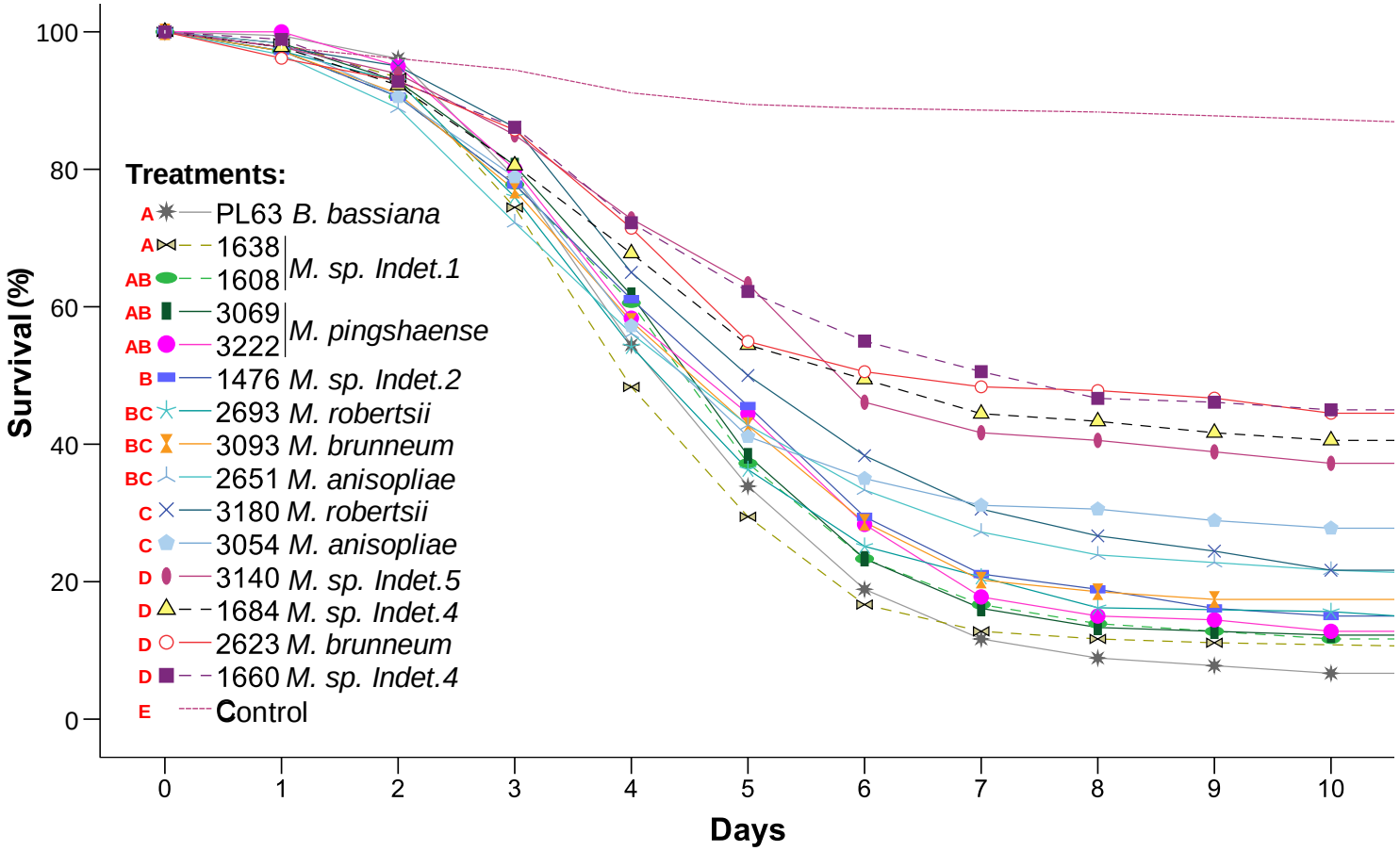


Figure 4.1 - Survival curves of *Tetranychus urticae* (n = 180 mites per treatment) inoculated with *Metarhizium* spp. The survival curve refers to the percentage of mites surviving up to 10 days post-treatment. Different letters indicate differences among treatments [Log Rank (Mantel-Cox) $p<0.05$]

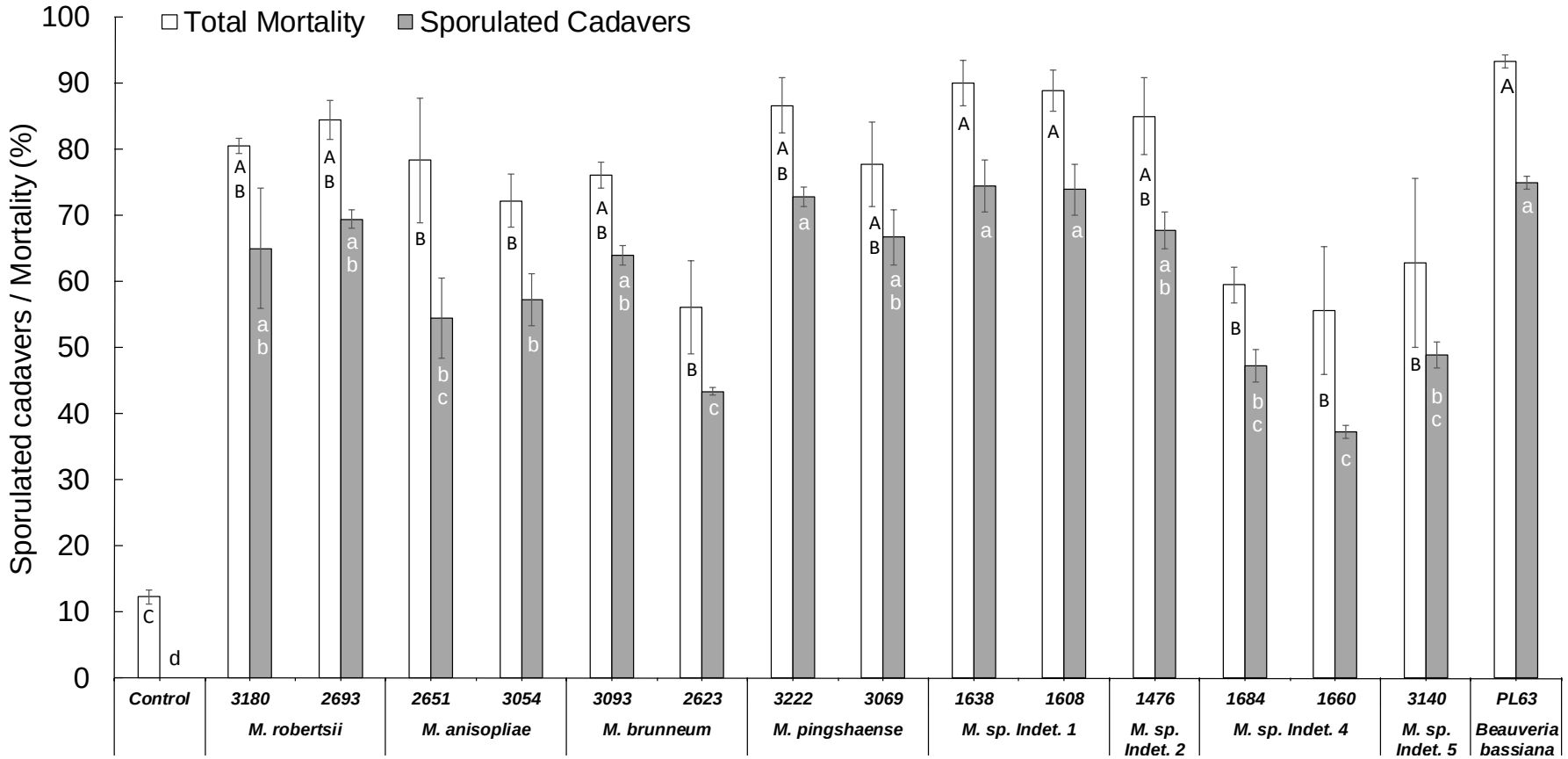


Figure 4.2 - Mortality and sporulated cadavers of *Tetranychus urticae* females at 25 °C and 12:12 L:D 10 days after being sprayed with 14 isolates of *Metarhizium* spp. or *Beauveria bassiana* (isolate ESALQPL63) with 0.05% Tween-80. Different lower-case letters indicate treatment differences in sporulated cadavers; different upper-case letters indicate treatment differences in total mortality by quasi-binomial models and F-tests through ANOVA and Tukey HSD comparison ($p < 0.05$)

Table 4.1 - Origin of *Metarhizium* spp. and *Beauveria bassiana* isolates and Median Lethal Time ($LT_{50} \pm SE$) of *Tetranychus urticae* mites sprayed with each isolate

| Collection Number (ESALQ) | Species | Type of isolation: Origen[1] | City | Environment | $LT_{50}$ (±SE) | CI[2] |
|---|---|---|---|---|---|---|
| 3180 | *M. robertsii* | IB: Soil | Estiva / MG | Strawberry | 5 (±0.28) | (4.45-5.55) |
| 2693 | *M. robertsii* | SM: Rhizosphere | Inconfidentes / MG | Strawberry | 5 (±0.18) | (4.65-5.35) |
| 2651 | *M. anisopliae* | Insect Bait: Soil | Cambuí / MG | Strawberry | 5 (±0.28) | (4.46-5.54) |
| 3054 | *M. anisopliae* | SM: Rhizosphere | Inconfidentes / MG | Strawberry | 5 (±0.23) | (4.55-5.45) |
| 3093 | *M. brunneum* | IB: Soil | Senador Amaral / MG | Strawberry | 5 (±0.24) | (4.52-5.48) |
| 2623 | *M. brunneum* | IB: Soil | Inconfidentes / MG | Strawberry | 7 (±0.83) | (5.38-8.62) |
| 3222 | *M. pingshaense* | IB: Soil | Estiva / MG | Strawberry | 5 (±0.27) | (4.48-5.52) |
| 3069 | *M. pingshaense* | IB: Soil | Cambuí / MG | Strawberry | 5 (±0.15) | (4.70-5.30) |
| 1638 | *Metarhizium sp.* Indet.1 * | IB: Soil | Rio Verde / GO | Cerrado | 4 (±0.17) | (3.68-4.32) |
| 1608 | *Metarhizium sp.* Indet.1 * | IB: Soil | Rio Verde / GO | Cerrado | 5 (±0.15) | (4.70-5.30) |
| 1476 | *Metarhizium sp.* Indet.2 * | IB: Soil | Cambuí / MG | Strawberry | 5 (±0.23) | (4.54-5.46) |
| 1684 | *Metarhizium sp.* Indet.4 * | SM: Rhizosphere | Iracemapolis / SP | Sugar Cane | 6 (±0.48) | (5.06-6.94) |
| 1660 | *Metarhizium sp.* Indet.4 * | IB: Soil | Piracicaba / SP | Sugar Cane | 8 (±1.49) | (5.08-10.91) |
| 3140 | *Metarhizium sp.* Indet.5 * | IB: Soil | Cambuí / MG | Strawberry | 6 (±0.34) | (5.33-6.67) |
| PL63 | *Beauveria bassiana* | Insect | Piracicaba / SP | *Atta* sp. | 5 (±0.16) | (4.69-5.31) |

* New M*etarhizium* spp. not yet described. [1] IB= Insect Baiting SM= Selective Medium [2] CI= confidence interval of $LT_{50}$

## 4.4 Discussion

Although all the 14 fungal isolates tested were able to infect adult female of *T. urticae* mite in laboratory, there were significant variations amongst the isolates. These results revealed the potential of the unassigned new lineages of *Metarhizium* as biocontrol agents. *Metarhizium* sp. Indet. 1 ESALQ1638, stands out by presenting the lowest $LT_{50}$ and it was similar to the best isolates, including *B. bassiana* (ESALQPL63) registered against this pest, when considering the other parameters (survival curve, total mortality and sporulated cadavers). *Metarhizium* sp. Indet. 1 (ESALQ1608) and *M. pingshaense* (ESALQ3069 and ESALQ3222) were as good as the isolates of the more studied and commercial available *Metarhizium* species: *M. anisopliae, M. brunneum* and *M. robertsii*.

Even though *M. anisopliae* is most frequently species found naturally infecting insects in Brazil (REZENDE, 2014; IWANICKI, 2016) and it is the only species used in all commercial products in the country, all other species tested here, except *Metarhizium* sp. Indet. 4 and *Metarhizium* sp. Indet. 5, presented at least one isolate among the best against *T. urticae* in all parameters. Variations between isolates in the virulence of *M. anisopliae* have been reported in *T. urticae* (TAMAI et al., 2002; CHANDLER et al., 2005; BUGEME et al., 2008). Based on the new classification of *Metarhizium* (BISCHOFF et al., 2009) it is likely that Chandler et al. (2005) and Bugeme et al. (2008), had compared isolates from diferent species of *Metarhizium.* However, we now know that the isolates used by Tamai et al. (2002) is probably *M. anisopliae* based on later studies on molecular identification of ESALQ collection of *Metarhizium* (REZENDE, 2014).

The control levels obtained in our study was similar to other laboratory screenhouse and field studies elsewhere. Chandler et al. (2005) showed reductions of all developmental stages (eggs, nymphs, and adults) of *T. urticae* when *M. anisopliae* was applied; they also studied the efficacy of Naturalis-L (*B. bassiana*-based mycopesticide - Troy Biosciences, Phoenix, TX, USA) which reduced *T. urticae* populations by 97%. Up to 93% and 96% reductions in *T. urticae* population density on cucumber and tomato, respectively, was observed following application of Naturalis-L (MARCIC et al., 2012). Bugeme et al. (2015) recently reported that *M. anisopliae* formulations were as effective as abamectin (conventional chemical control) in reducing *T. urticae* densities on common bean in both screenhouse and field experiments. An emulsifiable formulation of *M. anisopliae* was tested in the control of five Tetranychidae mites

including *T. urticae* in irrigated cotton fields in a desert area on the Tarim Basin of northwestern China and a high potential for practical use in the management of the spider mites using this technique was found (SHI et al., 2008).

The potential of *M. pingshaense* for pest control was largely unexplored. The pathogenicity of *M. pingshaense* isolate MGC02 caused more than 80% larval mortality of *Anomala cincta* (Coleoptera: Scarabeidae) in laboratory assays (GUZMÁN-FRANCO et al., 2011). Pena-Pena et al. (2015) reported that the same isolate has a potential in controlling root-feeding pests by seed inoculation, confirming its ability to endophytic colonize the maize roots.

This study revealed for the first time the potential of new and less studied species of *Metarhizium* as biocontrol agent of *T. urticae*. These less known species should be considered in further studies to assess their potential as biological control agents of other pest in the future.

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