CAMILA COSTA MOREIRA

THE DOUBLE LIFE OF AN INSECT PATHOGEN: *Metarhizium* AS A PLANT SYMBIONT AND ITS GENETIC DIVERSITY IN COFFEE AGROECOSYSTEMS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2016

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
M838d
2016

Moreira, Camila Costa, 1985-
The double life of an insect pathogen : *Metarhizium* as a plant symbiont and its genetic diversity in coffee agroecosystems / Camila Costa Moreira. – Viçosa, MG, 2016.
xii, 118f. : il. (algumas color.) ; 29 cm.

Orientador: Simon Luke Elliot.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Fungos entomopatogênicos. 2. *Metarhizium anisopliae*. 3. Biologia molecular. 4. Simbiose. 5. Genética de populações. 6. Café - Raízes . I. Universidade Federal de Viçosa. Departamento de Entomologia. Doutorado em Entomologia. II. Título.

CDD 22. ed. 579.5677

CAMILA COSTA MOREIRA

THE DOUBLE LIFE OF AN INSECT PATHOGEN: *Metarhizium* AS A PLANT SYMBIONT AND ITS GENETIC DIVERSITY IN COFFEE AGROECOSYSTEMS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

APROVADA: 26 de fevereiro de 2016.

Irene Maria Cardoso

Raul Narciso Carvalho Guedes

Braz Tavares da Hora Júnior

Eduardo Seiti Gomide Mizubuti
(Coorientador)

Simon Luke Elliot
(Orientador)

“A gente quer passar um rio a nado, e passa; mas vai dar na outra banda é num ponto muito mais embaixo, bem diverso do em que primeiro se pensou. Viver nem não é muito perigoso?”

João Guimarães Rosa

Aos agricultores familiares de Araponga e a todos que acreditam que é possível produzir alimentos de forma sustentável. Dedico.

AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Programa de Pós-Graduação em Entomologia pela oportunidade e estrutura concedida para a realização deste trabalho. Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pela bolsa de doutorado e ao Programa de Doutorado Sanduíche no Exterior da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PDSE-CAPES) pela bolsa sanduíche. À FAPEMIG pelo financiamento de parte desta pesquisa.

Ao meu orientador Simon L. Elliot, por todos os ensinamentos, orientação e amizade ao longo desses seis anos. Sua confiança em mim e no meu trabalho foram essenciais para minha formação pessoal e científica. Você sempre será uma fonte de inspiração. Muito obrigada!

Ao meu co-orientador Eduardo S. G. Mizubuti, por toda ajuda e por ter aberto as portas do BIOPOP durante os últimos seis anos. Obrigada também pelas discussões, conversas e confiança no meu trabalho. Aprendi muito com seu exemplo.

À professora Irene Cardoso, por toda ajuda nos últimos seis anos e por ter ajudado no contato com os agricultores. Obrigada pela oportunidade de participar dos Intercâmbios, Troca de Saberes e escrever o Nossa Pesquisa na Roça. Essas experiências foram muito enriquecedoras e me ensinaram muito. Muito obrigada também pelo exemplo de dedicação ao seu trabalho e às suas nobres causas!

Ao professor Michael J. Bidochka por todos os ensinamentos sobre microbiologia e *Metarhizium*, por ter me recebido em seu laboratório e pelo suporte durante a minha estadia em Saint Catharines.

Ao Braz H. Junior, pela participação na defesa de doutorado e por todos os ensinamentos no BIOPOP. Esse trabalho não teria se concretizado sem a sua ajuda.

Ao professor Raul pela participação na defesa de doutorado e pela convivência na Comissão Coordenadora do PPG Entomologia.

Aos membros da banca de qualificação: Carla C. M. Arce, Davi Mesquita, Eduardo S. G. Mizubuti e Olinto Liparini pelas discussões e sugestões sobre o tema dessa tese.

Aos agricultores que gentilmente cederam suas áreas para a realização das coletas de solo e raízes: Jesus e Alcione, Clauvinei e Raquel, João dos Santos Souza e Santinha e Manoel e Graciela.

Aos amigos do Laboratório de Interações Inseto-Microrganismos: Aline, Ângela, Dani, Daniel, Tiago, Débora, Fábio, Farley, Fernanda, Marcela, Marcos, Mayara, Silma, Talitta e Thairine pela ótima convivência. À técnica Verônica por toda ajuda e por tornar vida mais fácil no laboratório. Em especial, agradeço as estagiárias e amigas Fernanda, Mayara e Thairine, sem vocês esse trabalho teria sido muito mais difícil. Muito obrigada pelo suporte, disposição e amizade. Amo vocês!

Aos amigos do Bidochka's Lab, Emily Freeman, Irina Sementchoukova, Larissa Barelli, Scott Behie, Soumya Moonjely e Tara Koch com quem compartilhei ótimos momentos. Muito obrigada por proporcionarem um excelente ambiente de trabalho repleto de aprendizados e amizade. Em especial agradeço ao Scott Behie por toda a ajuda e amizade durante o meu período na Brock University, aprendi mais com você do que eu mesma imaginava. Obrigada também pelas nossas Downton Abbey's Mondays! Agradeço também à amiga Kristin Schaven-Behie, pela amizade e pela companhia nos almoços! Miss you guys!

Aos amigos do Laboratório de Biologia de Populações de Fitopatógenos, pela ótima convivência e por terem me recebido tão bem. Em especial agradeço ao Miller pelos ensinamentos e ajuda com os microssatélites.

Aos funcionários do Núcleo de Microscopia da UFV pela ajuda com o confocal, principalmente a Carlota e a Karla.

À Jamile Camargos e à Natália Penido do Laboratório de Genômica pela genotipagem dos microssatélites.

À amiga Raquel, que mesmo longe esteve sempre presente me encorajando durante os últimos 6 anos.

Aos Subordinados de Gracinha por terem me ajudado a gostar de Viçosa e por todos os momentos maravilhosos que compartilhamos. Em especial agradeço aos amigos Paulo Cristaldo e Carla C. M. Arce: obrigada pelo exemplo, amizade e apoio em todas as horas. Amo muito vocês.

Aos meus amigos de Cana Verde, especialmente as minhas Bests: Jaqueline, Marinês, Michelle e Priscila, obrigada pela nossa amizade de vida toda e por todo apoio no nosso grupo durante a minha estádia no Canadá. Amo vocês!

Às minhas amigas-irmãs mais distantes e ao mesmo tempo mais próximas: Mariana Toledo e Mônica Carvalho. Obrigada pela nossa amizade e por estarem presentes nos meus pensamentos mais felizes!

Às minhas amigas de morada em Viçosa, Dalila e Priscila pelos ótimos momentos que passamos juntas e conversas acolhedoras. À Dani pela amizade, carinho e por estar presente nos momentos mais decisivos dessa jornada!

Às minhas roommates, Bruna e Carol, por terem sido minha família no Canadá, pela amizade e por terem compartilhado momentos que vão ficar pra sempre no meu coração. À Elza, por sua presença acolhedora e pelo amor durante nosso tempo no Canadá.

À todos os amigos do CSF na Brock University em 2014, obrigada por terem feito a vida mais leve. Aos amigos da República Delícia, obrigada pelas comemorações nas datas mais significativas e pela hospedagem nos últimos dias em Saint Catharines.

Ao meu namorado Ivan, por ter sido meu companheiro, meu amigo, meu amor e minha duplinha durante esse doutorado. Obrigada por ter "entrado" nessa comigo e me apoiado em todos os momentos. Não foi fácil chegar até aqui, mas com certeza sua presença fez tudo mais tranquilo e mais leve. Esse título também é seu! Amo-te!

Aos meus sogros, Lucília e José Maria, e à família Santana, pelo apoio, encorajamento e por terem me recebido de forma tão acolhedora.

À minha família, pela confiança e amor devotados a mim. À minha mãe, Sonia, por ser meu porto seguro, minha confidente e minha melhor amiga. Nos momentos mais difíceis, seu exemplo e seu amor me fazem seguir em frente. A cada dia que percebo algum traço seu em mim me sinto uma pessoa melhor. Obrigada Mamãe!

Aos meus irmãos, Juliana e Saulo, por todo amor e força. Obrigada também pelo exemplo e por partilharem dos mesmos sonhos. Com certeza nossas jornadas não foram fáceis, mas sempre podemos nos apoiar uns nos outros!

Ao vovô Adonias e a vovó Elza pelo carinho, exemplo e pelos deliciosos domingos.

À Deus pela força de todas as horas.

Enfim, agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

SUMÁRIO

RESUMO

MOREIRA, Camila Costa, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **A vida dupla de um patógeno de insetos: *Metarhizium* como simbionte de plantas e sua diversidade genética em agroecossistemas de café**. Orientador: Simon Luke Elliot. Coorientador: Eduardo Seiti Gomide Mizubuti.

O gênero *Metarhizium* é amplamente conhecido por sua capacidade entomopatogênica. No entanto, recentemente também foi reconhecido como simbionte de planta, capaz de transferir nitrogênio de cadáveres de insetos para plantas, atuar como antagonista de fitopatógenos e promover o crescimento vegetal. Essas funções podem ser consideradas como serviços de ecossistema que podem ser fornecidos por esses fungos para plantas em sistemas de cultivos sustentáveis. Todavia, esses fungos são pouco considerados no contexto ecológico e dados sobre sua diversidade em solos agrícolas e na rizosfera são muito escassos, especialmente em ecossistemas tropicais. Além disso, nenhum método molecular para detectar e quantificar especificamente *Metarhizium* em associação com sistema radicular está disponível. Dessa forma, nesta tese nós focamos no estabelecimento de um método para detectar e quantificar *Metarhizium* em raízes de plantas e na investigação de sua diversidade em cultivos de café agroflorestal e pleno sol. Nós estabelecemos um método baseado na reação em cadeia da polimerase em tempo real (qPCR) confiável e reprodutível para detectar e quantificar *Metarhizium* em raízes de plantas. Tal método foi verificado por meio de detecção via método dependente de cultivo e microscopia confocal. Considerando a diversidade de *Metarhizium* em solos agroflorestais e pleno sol, nós encontramos três espécies, sendo que *M. robertsii* foi a espécie mais frequente em ambos sistemas. Ao comparar a diversidade entre os sistemas agroflorestais e pleno sol, duas das três agroflorestas amostradas apresentaram maior diversidade de *Metarhizium* e a diversidade total, considerando as seis áreas, também foi maior nas agroflorestas. A população de *M. robertsii* foi dividida em três diferentes clados, porém nenhum padrão de distribuição ou recombinação foi observado em relação aos mesmos. Com a relação à diversidade de *Metarhizium* na rizosfera, *M. robertsii* também foi a espécie mais abundante, sendo encontrada em todos os grupos de plantas amostrados. *Metarhizium pemphigi* foi a mais frequente na rizosfera de plantas de café, indicando a possibilidade de sua especialização ecológica em relação às raízes de café. Nós fornecemos resultados importantes sobre a associação de *Metarhizium* no solo e em associação com plantas, incluindo: (*i*) um método de laboratório pra estudar associação *Metarhizium*-raiz, (*ii*) a diversidade de *Metarhizium* no solo em dois sistemas de cultivo e

(*iii*) a diversidade de *Metarhizium* na rizosfera de plantas presentes em sistema de cultivo diversificado.

ABSTRACT

MOREIRA, Camila Costa, D.Sc., Universidade Federal de Viçosa, February, 2016. **The double life of an insect pathogen: *Metarhizium* as a plant symbiont and its genetic diversity in coffee agroecosystems.** Adviser: Simon Luke Elliot. Co-adviser: Eduardo Seiti Gomide Mizubuti.

The *Metarhizium* genus is widely recognized for its entomopathogenic capacity, but more recently was recognized as a plant symbiont, being able to transfer nitrogen from insect cadavers to plants, act as plant pathogen antagonist and plant growth promoter. All those functions could be valuable as ecosystem services provided by these fungi to plants in sustainable agricultural schemes. However, these fungi are poorly considered in an ecological context and data about their diversity and abundance in agricultural soils and in association with plant rhizosphere are very sparse, especially in tropical ecosystems. Also, considering the ability to form mutualistic association with plants, no *Metarhizium's* specific molecular method is available to detect and quantify association in plant root systems. Given this, in this thesis we focused in the establishment of a method to detect and quantify *Metarhizium* in plant roots and investigate its diversity in coffee based agroforestry and full sun systems. In doing so, we aimed to provide a better understanding of *Metarhizium´*s association with plants, to get a better insight of how its inter- and intraspecific diversity is distributed in soils of coffee agroforestry and full sun soils and to understand how *Metarhizium* species are distributed in the rhizosphere of coffee plants and non-crop plants in a coffee agroforestry system. We established a reliable and reproducible real-time polymerase chain reaction (qPCR) method to quantify and detect *Metarhizium* in plant roots and also detect the association through cultivation methods and confocal microscopy. In the surveys from the diversity of *Metarhizium* in soils from agroforestry and full sun coffee systems, we found three *Metarhizium* species, *M. robertsii* being the most prevalent of these. Comparing the diversity between agroforestry and full-sun systems we found higher diversity in agroforestry systems in two of the three sampled fields, and overall diversity was also higher in agroforestry. The *M. robertsii* population exhibited presented three clades and no specific distribution pattern and recombination was observed in the clades. Regarding *Metarhizium* diversity in the rhizosphere, *M. robertsii* was also the most abundant species and was present in all groups of surveyed plants, *M. pemphigi* presented the highest levels in the coffee rhizosphere indicating a possible ecological specialization of this species to coffee roots. We provided important findings regarding the association of the insect pathogen *Metarhizium* when in association with plants, including: (i) a laboratory method to study the

Metarhizium-plant association, (ii) the diversity of *Metarhizium* in soil and its comparison between agricultural systems and (iii) the *Metarhizium* diversity in the rhizosphere of crop and non-crop plants in a biodiverse agroecosystem.

GENERAL INTRODUCTION

This thesis concerns an insect-pathogenic fungus, *Metarhizium* Sorokin, that is well known as an entomopathogen but is also endophytically associated with plant roots and is rhizosphere competent. More specifically, the thesis focuses on means to evaluate *Metarhizium* ability to colonize plant roots and on its genetic diversity in agroecosystems.

This general introduction is divided into 6 parts. First I briefly introduce the fungus *Metarhizium*. Secondly, I discuss the importance of the new discoveries regarding *Metarhizium*-plant associations. Third, I discuss *Metarhizium* diversity in agroecosytems, means to detect diversity and the importance of the characterization of *Metarhizium* communities and populations for biological control and promotion of plant health. Finally, I offer a conclusion of the thesis.

THE FUNGUS *METARHIZIUM*

Metarhizium is an Ascomycete fungus belonging to the order Hypocreales and the family Clavicipitaceae. The Hypocreales comprises fungi with diverse nutritional modes (insect-, fungus-, and plant-parasites, woody plant saprobes and yeast-like symbionts) (Vega *et al.* 2009) and in the family Clavicipitaceae the majority of the species are pathogenic at some point in their life cycle (Kepler *et al.* 2014). The genus *Metarhizium* has hyaline, brown or green asexual conidia and in its most recent taxonomic review includes 30 species (Kepler *et al.* 2014). The teleomorphs of *Metarhizium* species are members of the *Metacordyceps* genus (Kepler *et al.* 2012), however there are few records of teleomorphs (Sung *et al.* 2007; Li *et al.* 2010; Kepler *et al.* 2012) and the most frequently found species, *M. robertsii, M. brunneum* and *M. anisopliae*, have not been linked to a sexual stage (Kepler *et al.* 2014). The importance of the genus is related to the ability of most species to infect insects, however in the new classification the genus includes species pathogenic to chameleons (Reptilia) (Kepler *et al.* 2014). *Metarhizium* species have also been reported in symbiotic association with plant roots (Hu & St Leger 2002; Behie & Bidochka 2014), revealing another important aspect of its life style.

The species included in the *Metarhizium anisopliae* species complex (Bischoff *et al.* 2009) are the most common species of the *Metarhizium* genus in soils worldwide and have been used for biological control against various insect pests (Shah & Pell 2003). The former *M. anisopliae* is now recognized as ten distinct phylogenetic species (Bischoff *et al.* 2009; Kepler *et al.* 2014). This species were shown to be effective for controling malaria vectors (Scholte *et al.* 2005; Kanzok & Jacobs-Lorena 2006), spittle bugs (Roberts & St. Leger 2004; Tiago *et al.* 2011) and locusts (Lomer *et al.* 2001). However, in other cases *Metarhizium* biological control potential has been inconsistent (St. Leger & Screen 2001). In part, the failure to explore *Metarhizium*'s entomopathogenic ability for biological control purposes came from the expectation that it will have equivalent performance to synthetic pesticides (Roy *et al.* 2010). Yet, the most the important cause of *Metarhizium´*s poor performance can be attributed to a lack of understanding of its response to ecological variables and how naturally occurring species impact ecosystem functioning through its ability to associate with plants and kill insects (Meyling & Eilenberg 2007). Expanding the knowledge of the ecological aspects is necessary to promote feasible and consistent management of *Metarhizium* as a biocontrol agent and plant health promoter.

METARHIZIUM AND ITS VERSATILE LIFE STYLE

A critical question in understanding an insect pathogen's life cycle is to understand how they survive in the environment in the absence of insect hosts. *Metarhizium* is considered primarily an insect parasite, however much recent evidence suggests that the association with plants roots is key to its survival and sustaining highly abundant populations in soil environments (St. Leger 2008). Entomopathogenic fungi exhibit a diverse array of adaptations to insect parasitism, that includes the general ability to overcome insect immune defenses and obtain nutrition from insects (Roy *et al.* 2006). On the other hand recent studies report *Metarhizium* adaptations in associating with plant roots and obtaining nutrition (Wang & St Leger 2007; Fang & St. Leger 2010; Behie *et al.* 2012; Wyrebek & Bidochka 2013; Behie & Bidochka 2014; Behie *et al.* submitted).

One of the mentioned adaptations is the expression of *Metarhizium* adhesion 2 (*Mad2*) on plants surfaces. Adhesin-like protein 1 (*Mad*1) and adhesin-like protein 2 (*Mad*2) enable attachment to insect cuticle and plant root surfaces, respectively (Wang & St Leger 2007). Adherence assays demonstrated that disruption of *Mad*1 gene eliminated the ability to adhere to insect cuticle. Wang and St Leger (2007) also showed that yeast cells expressing *Mad*1, but not *Mad*2, were able to adhere to insect cuticle. Furthermore, a *Mad*2-disrupted mutant showed the inability to adhere to plant epidermis, while yeast cells expressing only *Mad*2 were able to adhere (Wang & St Leger 2007). These adhesins give *Metarhizium* the ability to adhere to insect and plant surfaces, enabling it to effectively colonize and persist in these different phases of its life cycle. Also, *Mad*2-disrupted mutants presented poor survival in cabbage fields, indicating that *Metarhizium* persistence in the soil is directly linked to its ability to associate with plant roots (Wang *et al.* 2011; Liao *et al.* 2014)

Another adaptation associated with the acquisition of plant-derived sugars in *Metarhizium* is the presence of Metarhizium *raffinose transporter* gene (*Mrt*), an oligosaccharide transporter that is necessary for root colonization (Fang and St. Leger, 2010). An *Mrt*-disrupted mutant grows poorly in root exudate and its rhizosphere competence was greatly reduced. Notably, disruptions in *Mrt* did not have an effect on virulence to insects, demonstrating that this gene is exclusively used in *Metarhizium*'s interactions with plants. *Mrt* disrupted mutants also presented poor persistence in the field (Liao *et al.* 2014).

Metarhizium was also able to increase plant growth, mitigate salt stress and act as a plant pathogen antagonist (Khan *et al.* 2012; Sasan & Bidochka 2012, 2013). The close association of *Metarhizium* species and plant hosts is also evidenced by its ability to transfer nitrogen from insect cadavers to plant hosts (Behie *et al.* 2012; Behie & Bidochka 2014). On the other hand, compounds derived from plant photosynthate acquisition were detected in *M. robertsii* growing endophytically (Behie *et al.* submitted).

All this evidence for adaptation to plant association highlights the prospective potential of *Metarhizium* as a plant health promoter and means of insect control. However, the several mechanisms that should be involved in this association and the specific association of *Metarhizium* with plants must to be elucidated in order to work towards better results to manage naturally occurring populations, and if necessary, in *Metarhizium* application for biological control.

METARHIZIUM DIVERSITY

Metarhizium anisopliae species taxonomy

The genus *Metarhizium* was described by Sorokin (1879) (Sorokin 1883). The taxonomic revision of Bischoff and colleagues (2009) has significantly revised and resolved *Metarhizium* species diversity and systematics, in particular for *M. anisopliae* species complex. They used a multilocus approach, with sequences from the nuclear encoded genes *translation elongation factor (TEF), RNA polymerase II largest subunit (RPBl), RNA polymerase second largest subunit (RPB2) and B-tubulin (Bt)*. Based on the phylogenetic evidence, nine terminal taxa were proposed in the *M. anisopliae* complex to be recognized as species, including: *M. anisopliae, M. robertsii, M. pingshaense, M. brunneum, M. majus, M. guizhouense, M. lepididotie* and *M. acridum (Bischoff et al. 2009),* most of these being cryptic species. *Metarhizium indigotica* was recently added to the *M. anisopliae* species complex (Kepler *et al.* 2014). One of the greatest achievement of Bischoff *et al.* (2009) was the establishment of the intron rich portion of the translation enlogation factor (5'TEF) as the most informative region to identify *Metarhizium* species that can be easily applied as a barcode for routine species identification. This method has been applied with success in *Metarhizium* species recognition for ecological purposes (Fisher *et al.* 2011; Wyrebek *et al.* 2011; Lopes *et al.* 2013; Rocha *et al.* 2013; Steinwender *et al.* 2014; Kepler *et al.* 2015; Rezende *et al.* 2015).

Genetic Variability of *Metarhizium*

The assumption that host insect taxa are the predominant influence in population genetics of insect pathogenic fungi have propelled many studies (Riba *et al.* 1986; St. Leger *et al.* 1992; Fegan *et al.* 1993; Leal *et al.* 1994; Tigano-Milani *et al.* 1995), however no clear population structure related to the insect host has been detected. Bidochka *et al*. (2001) studied the genetic diversity of *Metarhizium* isolates from forest and agricultural habitats in temperate region. Based on multilocus analyses, two genetically distinct groups were identified, OG1 and OG2, that were associated with agricultural and forest habitats respectively. Pathogenicity of OG1 and OG2 isolates to several insects related to both surveyed ecosystems did not show any specificity. Subsequently, the isolates belonging to those groups were identified as *M. robertsii* (OG1) and *M. brunneum* (OG2) (Wyrebek *et al.* 2011). The further discovery of *Metarhizium* associated with plant roots explain better the observed pattern and posterior studies suggests that plant hosts are the main influence on the species and genotype distributions (Fisher *et al.* 2011; Wyrebek *et al.* 2011; Wyrebek & Bidochka 2013; Kepler *et al.* 2015). Evidence from plant taxa governing *Metarhizium* species distribution also came from comparisons using 5'TEF phylogeny, which is used for species identification, and phylogenies reconstructed with *Mad*1 and *Mad*2 genes (Wyrebek & Bidochka 2013). *Mad*2 phylogeny was more congruent with 5′ TEF than *Mad1*, indicating its divergence among *Metarhizium* lineages, contributing to clade- and species-specific variation, while it appears that *Mad1* has been largely conserved (Wyrebek & Bidochka 2013). The results suggest that plant relationships, rather than insect hosts, have been a major driving factor in the divergence of the genus *Metarhizium* (Wyrebek & Bidochka 2013). *Metarhizium* species associations with specific plants have already reported (Fisher *et al.* 2011; Wyrebek *et al.* 2011), however few studies of intraspecific structure have been performed since the group´s taxonomy was revised.

Highly sensitive single sequence repeat (SSR) markers for *Metarhizium* population studies were developed by Enkerli *et al*. (2005) and Oulevey *et al.* (2009). Those markers have made possible the determination of population structures in closely related isolates (Oulevey *et al.* 2009), such as isolates originating from different soil samples or plant hosts

in a single agricultural field. Detecting intraspecific differences can provide an appropriate knowledge of how *Metarhizium* species are affected by evolutionary mechanisms and what factors are responsible to lead community and the genetic structure of the population, habitat association or host insect or plant association. Furthermore, better understanding of *Metarhizium* genetic structure can be helpful to manage natural communities to reach pest control and promote plant health or for optimizing other pest control strategies (Kepler *et al.* 2015).

Population structure of fungal species is deeply affected by the presence or absence of recombination (Kepler *et al.* 2015). The assessment of the genetic structure through SSR markers enables to infer about the reproductive mode. However, surveys for the presence and distribution of mating types can directly provide data about the fungal reproductive mode, increasing the comprehension of the genetic mechanisms following sexual or asexual reproduction and potential pathways of genetic exchange (Pattemore *et al.* 2014). These data can help explain the strong bias found in clonal mode of reproduction. Reproductive stages of *Metarhizium* have been reported by Sung *et al.* (2007), Li *et al.* (2010), Kepler *et al.* (2015), however the occurrence of sex shaping genetic structure of *Metarhizium* species remains obscure. Kepler (2015) developed a *Metarhizium* specific PCR-based assay to characterize mating type idiomorphs (MAT) based on genomic information (Gao *et al.* 2011) and have successfully determined the MAT idiomorphs present in a *Metarhizium* community. Evidence of sex was reported in *M. robertsii* Clade 4 by multilocus analyses; however, the presence of an alternative mating type was not detected (Kepler *et al.* 2015). A survey for the presence of MATs in the population together with its genetic structure can be useful to support decisions related to the release of commercial biocontrol strains. Furthermore, the complete understanding of *Metarhizium* genetic structure can be helpful for optimizing pest control strategies or to manage the natural community to reach pest control and promote overall plant health (Kepler *et al.* 2015).

METARHIZIUM AND COFFEE CROPS

The increased persistence of *Metarhizium* in soils associated with plant roots and its abundant natural occurrence (Milner 1992), emphasizes the opportunity of managing *Metarhizium* natural populations to achieve biological control and plant health goal. This is particularly relevant to an agroecological farms where soil conservation and plant diversity are the main factors responsible for achieving sustainable agriculture. The increase of plant diversity in agricultural field is a key factor for building up a beneficial population of rhizosphere microorganisms, without the need for direct inoculation of specific microorganisms (Ratnadass *et al.* 2012). In addition, different plant species have specific rhizosphere exudates that can harbor different microbial communities, thus a variety of plants can provide different ecological niches encouraging microbial diversity (Ratnadass *et al.* 2012). The main cultivated crop and the additional co-occurring plants may influence abundance and diversity of *Metarhizium* in soils and must be taken into account in surveys.

Agroforestry systems in general and agroforestry coffee systems in particular are important management type and have been adopted for family farmers (Cardoso *et al.* 2001; Haggar *et al.* 2011). Recent efforts have focused on improving coffee yield coupled with more sustainable production (Jha *et al.* 2014) and, furthermore, there is a growing open market for increased quality products obtained from agroforestry coffee systems (Loureiro & Lotade 2005). These systems have important positive consequences for agricultural fields. Shade trees in agroforestry enhance functional biodiversity, carbon sequestration, soil fertility, drought resistance as well as weed and biological pest control (Tscharntke *et al.* 2011) . Yet, there are few reports of the occurrence of entomopathogenic fungi in coffee and agroforestry soils. Comparing agroforestry and full sun soils we observed higher abundance and activity of insect-pathogenic fungi in the agroforestry system (Moreira *et al.* submitted) (Fig. 1, extracted from Moreira and Elliot, (in prep.)). Bait insects in contact with agroforestry soils died from insect pathogenic fungi infection sooner than those in contact with samples from full sun systems. The differences observed between the systems can be explained by differences in insect-pathogenic fungi community composition (Fig. 1). Since

Metarhizium was the most frequently found genus we investigated its community composition and genetic variability in each production system. Understanding species distribution and pattern of plant association is the first step in the attempt to manage *Metarhizium* in a conservative biological control approach.

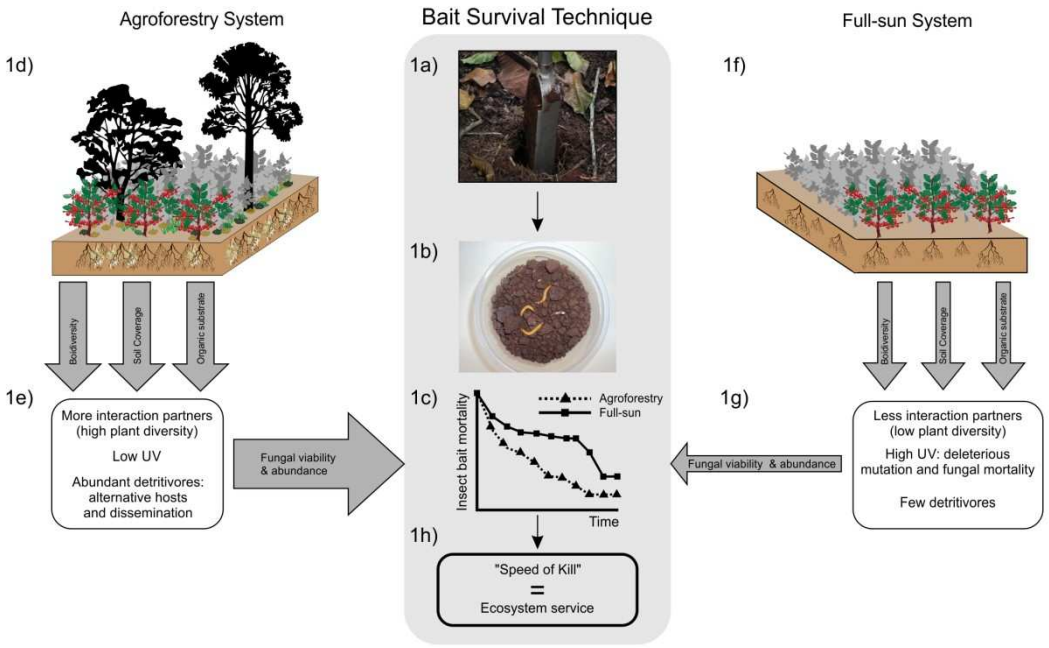

Figure 1. Comparison of the entomopathogenic fungi activity in soils under two coffee cultivation regimes: agroforestry and full-sun. The differential "speed of kill" of the bait insects is taken as a measure of "ecosystem services provided" by the entomopathogenic fungi, since the speed at which a fungus kill a bait is directly correlated with the its virulence and potential of survive in that soil environment, and consequently it is directly correlated with the soil characteristics favorable to fungal performance.

OVERVIEW

This thesis focuses on a means to establish a method to quantify *Metarhizium* colonization in plant roots, assess its genetic diversity in agroecosystems and also evaluate *Metarhizium* species diversity in the rhizosphere of coffee plants and non-crop plants present in the system.

In Chapter 1, we described a qPCR method to detect and quantify *M. robertsii* association in plant roots. The method showed specificity and reliable results through the utilization of SYBR green detection system. The method was validated through the quantification of a time course association of *M. robertsii* in bean plants from 7 to 35 days of association. Quantification through a cultivable method (CFU count) was used to compare the results. Laser scanning confocal microscopy was used to describe the association along the time course.

The three methods applied to quantify association displayed similar patterns. Association was higher in the first days of association followed by a decrease in the following days. The observed pattern could be associated with the massive growth of hyphae on the root surface following experimental inoculation. The method proposed is a valuable tool for investigation of *M. robertsii* colonization in experimental settings and detection in field plants.

In Chapter 2, *Metarhizium* community and population diversity in coffee based agroforestry and full sun management were investigated. We hypothesized that the most diverse agroforestry system would harbor a more diverse *Metarhizium* community. A set of 118 isolates obtained from the both fields were characterized through molecular methods. Three species *M. anisopliae*, *M. guizhouense* and *M. robertsii* were recorded, and the last was the most abundant. Comparison of diversity indices between the fields in the area pairs revealed higher diversity in agroforestry system for two of the sampled areas and the overall diversity was also higher in agroforestry. *M. robertsii* population structure did not show

structuration according to the management systems. Three intraspecific clonal clades were detected in the *M. robertsii*. We suggested that the prevalence of *M. robertsii* could be due its preferential association with coffee plants.

Based on the results of Chapter 2, we asked if a more directional sample of *Metarhizium* isolates, in plant roots, could reveal a specific-plant rhizosphere association. In Chapter 3, we aimed to characterize through molecular methods the community diversity of *Metarhizium* isolates associated with different plant in the agroforestry system. Isolates from coffee plants, trees, banana and non-crop herbaceous plants were sampled and characterized. We found that spontaneous monocotyledonous plants harbored the higher amount of isolates, followed by coffee plants. *Metarhizium robertsii* was the most prevalent species, found in all groups of sampled plants and soil. Contrary to what we expected *M. pemphigi* was the prevalent species in coffee rhizosphere. We suggest that this species may present ecological specialization to coffee roots.

REFERENCES

Behie S.W. & Bidochka M.J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and Environmental Microbiology*, 80, 1553-1560.

Behie S.W., Moreira C.M., Sementchoukova I., Barelli L., Zelisko P.M. & Bidochka M.J. (submitted). Trading insect nitrogen for photosynthate: Carbon translocation from a plant to an insect pathogenic, endophytic fungus. *Science*.

Behie S.W., Zelisko P.M. & Bidochka M.J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science*, 336, 1576-1577.

Bidochka M.J., Kamp A.M., Lavender T.M., Dekoning J. & De Croos J.N.A. (2001). Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: Uncovering cryptic species? *Applied and Environmental Microbiology*, 67, 1335-1342.

Bischoff J.F., Rehner S.A. & Humber R.A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 101, 512-530.

Cardoso I.M., Guijt I., Franco F.S., Carvalho A.F. & Neto P.S.F. (2001). Continual learning for agroforestry system design: university, NGO and farmer partnership in Minas Gerais, Brazil. *Agricultural Systems*, 69, 235-257.

Enkerli J., Kölliker R., Keller S. & Widmer F. (2005). Isolation and characterization of microsatellite markers from the entomopathogenic fungus *Metarhizium anisopliae*. *Molecular Ecology Notes*, 5, 384-386.

Fang W. & St. Leger R.J. (2010). *Mrt*, a gene unique to fungi, encodes an oligosaccharide transporter and facilitates rhizosphere competency in *Metarhizium robertsii*. *Plant Physiology*, 154, 1549-1557.

Fegan M., Manners J.M., Maclean D.J., Irwin J.A.G., Samuels K.D.Z., Holdom D.G. & Li D.P. (1993). Random amplified polymorphic DNA markers reveal a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*. *Microbiology*, 139, 2075-2081.

Fisher J.J., Rehner S.A. & Bruck D.J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology*, 106, 289-295.

Gao Q., Jin K., Ying S.-H., Zhang Y., Xiao G., Shang Y., Duan Z., Hu X., Xie X.-Q., Zhou G., Peng G., Luo Z., Huang W., Wang B., Fang W., Wang S., Zhong Y., Ma L.-J., St. Leger R.J., Zhao G.-P., Pei Y., Feng M.-G., Xia Y. & Wang C. (2011). Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet*, 7, e1001264.

Haggar J., Barrios M., Bolaños M., Merlo M., Moraga P., Munguia R., Ponce A., Romero S., Soto G., Staver C. & de M. F. Virginio E. (2011). Coffee agroecosystem performance under full sun, shade, conventional and organic management regimes in Central America. *Agroforest Syst*, 82, 285-301.

Hu G. & St Leger J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology*, 68, 6383-6387.

Jha S., Bacon C.M., Philpott S.M., Ernesto Méndez V., Läderach P. & Rice R.A. (2014). Shade coffee: Update on a disappearing refuge for biodiversity. *BioScience*, 64, 416-428.

Kanzok S.M. & Jacobs-Lorena M. (2006). Entomopathogenic fungi as biological insecticides to control malaria. *Trends in Parasitology*, 22, 49-51.

Kepler R.M., Humber R.A., Bischoff J.F. & Rehner S.A. (2014). Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia*, 106, 811-829.

Kepler R.M., Sung G.-H., Ban S., Nakagiri A., Chen M.-J., Huang B., Li Z. & Spatafora J.W. (2012). New teleomorph combinations in the entomopathogenic genus Metacordyceps. *Mycologia*, 104, 182-197.

Kepler R.M., Ugine T.A., Maul J.E., Cavigelli M.A. & Rehner S.A. (2015). Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology*, 17, 2791–2804.

Khan A., Hamayun M., Khan S., Kang S.-M., Shinwari Z., Kamran M., ur Rehman S., Kim J.-G. & Lee I.-J. (2012). Pure culture of *Metarhizium anisopliae* LHL07 reprograms soybean to higher growth and mitigates salt stress. *World J Microbiol Biotechnol*, 28, 1483-1494.

Leal S.C.M., Bertioli D.J., Butt T.M. & Peberdy J.F. (1994). Characterization of isolates of the entomopathogenic fungus *Metarhizium anisopliae* by RAPD-PCR. *Mycological Research*, 98, 1077-1081.

Li C., Huang B., Fan M., Lin Y. & Li Z. (2010). *Metacordyceps guniujiangensis* and its *Metarhizium* anamorph: a new pathogen on cicada nymphs. *Mycotaxon*, 111, 221-231.

Liao X., O'Brien T., Fang W. & St. Leger R. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Appl Microbiol Biotechnol*, 98, 7089-7096.

Lomer C.J., Bateman R.P., Johnson D.L., Langewald J. & Thomas M. (2001). Biological control of locusts and grasshoppers. *Annual Review of Entomology*, 46, 667-702.

Lopes R.B., Souza D.A., Oliveira C.M. & Faria M. (2013). Genetic diversity and pathogenicity of *Metarhizium* spp. associated with the white grub *Phyllophaga capillata* (Blanchard) (Coleoptera: Melolonthidae) in a soybean field. *Neotropical Entomology*, 42, 436-438.

Loureiro M.L. & Lotade J. (2005). Do fair trade and eco-labels in coffee wake up the consumer conscience? *Ecological Economics*, 53, 129-138.

Meyling N.V. & Eilenberg J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control*, 43, 145-155.

Milner R.J. (1992). Selection and characterization of strains of *Metarhizium anisopliae* for control of soil insects in Australia. . In: *Biological Control of Locusts and Grasshoppers.* (eds. Lomer CJ & Prior C). CAB International Wallinford, pp. 200-207.

Moreira C.C., Celestino D., Guerra T.G., Cardoso i.M. & Elliot S.L. (submitted). Ecosystem services offered by entomopathogenic fungi: Agroforestry coffee soils are more insect-suppressive than Full-sun soils. *Submitted to Journal of Invertebrate Pathology*.

Moreira C.C. & Elliot S.L. (in prep.). Bait survival technique to estimate the potential for soil-borne insect-pathogenic fungi as cryptic providers of ecosystem services. *In preparation*.

Oulevey C., Widmer F., Kölliker R. & Enkerli J. (2009). An optimized microsatellite marker set for detection of *Metarhizium anisopliae* genotype diversity on field and regional scales. *Mycological Research*, 113, 1016-1024.

Pattemore J., Hane J., Williams A., Wilson B., Stodart B. & Ash G. (2014). The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. *BMC Genomics*, 15, 660.

Ratnadass A., Fernandes P., Avelino J. & Habib R. (2012). Plant species diversity for sustainable management of crop pests and diseases in agroecosystems: a review. *Agron. Sustain. Dev.*, 32, 273-303.

Rezende J.M., Zanardo A.B.R., Lopes M.D., Delalibera I. & Rehner S.A. (2015). Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. *Biocontrol*, 60, 495-505.

Riba G., Bouvier-Fourcade I. & Caudal A. (1986). Isoenzymes polymorphism in *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) entomogenous fungi. *Mycopathologia*, 96, 161-169.

Roberts D.W. & St. Leger R.J. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological Aspects. In: *Advances in Applied Microbiology*. Academic Press, pp. 1-70.

Rocha L.F.N., Inglis P.W., Humber R.A., Kipnis A. & Luz C. (2013). Occurrence of *Metarhizium* spp. in Central Brazilian soils. *Journal of Basic Microbiology*, 53, 251-259.

Roy H.E., Brodie E.L., Chandler D., Goettel M.S., Pell J.K., Wajnberg E. & Vega F.E. (2010). Deep space and hidden depths: understanding the evolution and ecology of fungal entomopathogens. In: *The Ecology of Fungal Entomopathogens* (eds. Roy H, Vega F, Chandler D, Goettel M, Pell J & Wajnberg E). Springer Netherlands, pp. 1-6.

Roy H.E., Steinkraus D.C., Eilenberg J., Hajek A.E. & Pell J.K. (2006). Bizarre interactions and end games: Entomopathogenic fungi and their arthropod hosts. *Annual Review of Entomology*, 51, 331-357.

Sasan R.K. & Bidochka M.J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany*, 99, 101-107.

Sasan R.K. & Bidochka M.J. (2013). Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*, 35, 288-293.

Scholte E.-J., Ng'habi K., Kihonda J., Takken W., Paaijmans K., Abdulla S., Killeen G.F. & Knols B.G.J. (2005). An entomopathogenic fungus for control of adult african Malaria mosquitoes. *Science*, 308, 1641-1642.

Shah P.A. & Pell J.K. (2003). Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol*, 61, 413-423.

Sorokin N. (1883). Plant parasites of man and animals as causes of infectious diseases. In, pp. 268-291.

St. Leger R.J. (2008). Studies on adaptations of *Metarhizium anisopliae* to life in the soil. *Journal of Invertebrate Pathology*, 98, 271-276.

St. Leger R.J., May B., Allee L.L., Frank D.C., Staples R.C. & Roberts D.W. (1992). Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, 60, 89-101.

St. Leger R.J. & Screen S. (2001). Prospects for strain improvement of fungal pathogens of insects and weeds. In: *Fungal biocontrol agents: progress, problems and potential* (eds. Butt TM, Jackson C & Morgan N). CAB International Wallingford, UK, pp. 219-238.

Steinwender B.M., Enkerli J., Widmer F., Eilenberg J., Thorup-Kristensen K. & Meyling N.V. (2014). Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. *Journal of Invertebrate Pathology*, 123, 6-12.

Sung G.-H., Hywel-Jones N.L., Sung J.-M., Luangsa-ard J.J., Shrestha B. & Spatafora J.W. (2007). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Studies in Mycology*, 57, 5-59.

Tiago P.V., Souza H.M.d.L., Moysés J.B., Oliveira N.T.d. & Lima E.Á.d.L.A. (2011). Differential pathogenicity of *Metarhizium anisopliae* and the control of the sugarcane root spittlebug *Mahanarva fimbriolata*. *Brazilian Archives of Biology and Technology*, 54, 435-440.

Tigano-Milani M.S., Gomes A.C.M.M. & Sobral B.W.S. (1995). Genetic Variability among Brazilian Isolates of the Entomopathogenic Fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, 65, 206-210.

Tscharntke T., Clough Y., Bhagwat S.A., Buchori D., Faust H., Hertel D., Hölscher D., Juhrbandt J., Kessler M., Perfecto I., Scherber C., Schroth G., Veldkamp E. & Wanger T.C. (2011). Multifunctional shade-tree management in tropical agroforestry landscapes – a review. *Journal of Applied Ecology*, 48, 619-629.

Vega F.E., Goettel M.S., Blackwell M., Chandler D., Jackson M.A., Keller S., Koike M., Maniania N.K., Monzon A., Ownley B.H., Pell J.K., Rangel D.E.N. & Roy H.E. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology*, 2, 149-159.

Wang C. & St Leger R.J. (2007). The *MAD*1 Adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the *MAD*2 adhesin enables attachment to plants. *Eukaryotic Cell*, 6, 808-816.

Wang S., O'Brien T.R., Pava-Ripoll M. & St. Leger R.J. (2011). Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations. *Proceedings of the National Academy of Sciences*, 108, 20449-20454.

Wyrebek M. & Bidochka M.J. (2013). Variability in the insect and plant adhesins, *Mad1*and *Mad2*, within the fungal genus *Metarhizium* suggest plant adaptation as an evolutionary force. *PLoS ONE*, 8, e59357.

Wyrebek M., Huber C., Sasan R.K. & Bidochka M.J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology*, 157, 2904-2911.

CHAPTER 1

REAL TIME PCR QUANTIFICATION OF ENDOPHYTIC COLONIZATION OF *METARHIZIUM ROBERTSII* AND THE DESCRIPTION OF ITS ASSOCIATION DEVELOPMENT IN PLANT ROOTS

Abstract

A quantitative PCR (qPCR) assay was developed to estimate endophytic colonization of the fungal insect pathogen and root symbiont *Metarhizium robertsii* in bean roots (*Phaseolus vulgaris*). The qPCR method was validated through the quantification of the time course development of the association in bean roots from 7 to 35 days. The method was compared with CFU recovery from root homogenate suspensions and the spatial and temporal colonization development was assessed through laser scanning confocal microscopy using a *GFP*-expressing isolate. The primer pair *MtNit* was chosen through specificity tests performed by conventional and quantitative PCR. A linear relationship between the Ct value (qPCR amplification) and DNA concentration was achieved for pure target DNA (*M. robertsii*) and target DNA contaminated with plant DNA in the spike test with *MtNit*. Quantification of fungal biomass in the time course assay showed high fungal biomass at 7 days and a decrease over the following days, stabilizing at 21, 28 and 35 days. Quantification by CFU counting also showed a related pattern. Spatial description through microscopy explained the pattern achieved by quantification methods, presenting an extensive mycelial network growing over the root surface in the first days and a following decrease of external colonization in the subsequent days. Both quantification methods were suitable for estimation of endophytic colonization. Nevertheless, qPCR is preferable because it is highly specific and less time-consuming than quantification by culturing methods and observation through microscopy.

Introduction

The insect-pathogenic fungus *Metarhizium* (Hypocreales: Clavicipitaceae) has been used extensively as a biological control agent (Shah & Pell 2003) and many of the biochemical and molecular factors involved in insect pathogenesis have been elucidated (Gao *et al.* 2011). However, there are many instances where *Metarhizium* has not performed optimally as a biological control agent in the field (Meyling & Eilenberg 2007). One of the main reasons for this may be that the ecological role of this fungus has generally been neglected (Vega *et al.* 2009). Regardless of the potential utility of the fungus as a biocontrol agent one cannot ignore its phylogenetic history as a relative of plant endophytes (Behie *et al.* 2013) and this must be considered in any biocontrol effort. The discovery of *Metarhizium* as a root endophyte (Sasan & Bidochka 2012) and its nutrient transfer to plants from insect cadavers (Behie *et al.* 2012) has highlighted its importance in the ecosystem. The endophytic capability of *Metarhizium* is now being evaluated alongside its use in biological control, and is potentially critical in developing novel and effective biological control strategies.

Endophytes play key roles in the ecosystem and influence health, evolution, and ecology of the host plant (Brundrett 2006). For example, *Metarhizium* was reported as plant growth promoter (Khan *et al.* 2012; Sasan & Bidochka 2012; Liao *et al.* 2014), plant pathogen antagonist, nitrogen transfer to a broad plant host range (Behie & Bidochka 2014b) and photosynthate compounds receiver from plant host (Behie *et al.* submitted). However, the mechanisms involved in endophytic associations have not been fully elucidated.

In order to accurately assess the ability of *Metarhizium* to colonize plant roots, it is critical to quantify endophytic association which may be dependent on environmental factors (Lovett & St. Leger 2014), plant species, plant localization (Behie *et al.* 2015) as well as fungal strain variation. Typically, plant colonization may be quantified through cultivation dependent techniques such as the percentage of plant tissue fragments colonized or amount of colony forming units (CFU) from crushed and homogenized tissues. Cultivation techniques have serious limitations, however, especially when trying to quantify endophytic association under

non-axenic conditions which could potentially lead to an inaccurate estimation of plant colonization (Porras-Alfaro & Bayman 2011). The use of green fluorescence protein (*GFP*)-transformed fungi and microscopic evaluation of plant tissues has proven useful in determining the presence of fungus in plant tissues (Maciá-Vicente *et al.* 2009), but it is not quantitatively relevant. Nevertheless, *GFP*-transformed fungus has the additional advantage of the description of the association spatially and the identification of fungal and plant special structures (Maciá-Vicente *et al.* 2009).

Quantitative real-time polymerase chain reaction (qPCR) is an accurate method for quantifying fungal colonization in plant tissues, and is reported to be more reliable than culture- and microscopy-based (Tellenbach *et al.* 2010) .Here we show a method for the detection and quantification of *Metarhizium robertsii* on and in plant roots based on qPCR utilizing SYBR Green fluorescence. The method was designed and tested according to its sensitivity, consistency and specificity in target (*M. robertsii*), and non-target (plant) genomic DNA. The method was verified by quantifying the time course of colonization of bean roots (*Phaseolus vulgaris*) by *M. robertsii*. The qPCR values were correlated with traditional CFU quantification methods and a spatially description of the association was performed using a *M. robertsii* isolate expressing *GFP* through laser confocal scanning microscopy.

Material and Methods

Fungal and plant material

A *Metarhizium robertsii* (ARSEF 2575) transformant expressing green fluorescence protein (-*GFP*) was used for real time PCR calibration and quantification experiments, colony counting and for laser confocal microscopy. The construction of the *GFP*-expressing plasmids, transformation, and transgenic fungal lines have been previously described by Fang *et al.* (2006). The fungal isolate was maintained on PDA (39g l^{-1} of potato dextrose agar). For fungal DNA extraction, 100 µl of fungal conidia (10^7 per ml) were inoculated into 50 ml 0.2% (w/v) yeast extract, 1% peptone, 2% glucose broth (YPD) in flasks shaken at 280 rpm at 27°C for 5 days. *Phaseolus vulgaris* seeds (haricot bean, cultivar 'Soldier') were used as the host plant and seeds were obtained from OSC Seeds, Ontario, Canada.

Seed sterilization and fungal inoculation

Bean seeds were surface sterilized using chlorine gas method (Gamborg & Phillips 1995). Chlorine gas was produced by the combination of 100 ml of 5.25% hypochlorite (bleach) and 4 ml of 37% hydrochloric acid in a small beaker placed in glass dissector under the fume hood for 18 hr. Surface sterilized seeds were placed on water-agar (1.5 % agar) and kept at 25°C for a photoperiod of 16 h a day for 5 days in order to obtain seedlings. The seedling were inoculated with 100µl of ARSEF 2575-*GFP* conidial suspension (10^7 conidia/ml) in Triton X-100 (0.001%) and kept for a further 3 days in water agar. Control seedlings were inoculated with 100 µl of 0.01% sterile distilled solution of Triton X-100.

Planting, harvesting, CFU count and plant material processing

Seedlings were placed in sterile soil and harvested at 1, 7, 14, 21, 28 and 35 days after fungus inoculation. Five plants were used for each time period for a total of 70 plants including controls. At each time period 5 fungal inoculated plants and 5 control plants were harvested. Roots were removed from soil and the roots were washed in distilled water. The roots were cut

into six sections of 0.5 cm lengths. Six randomly chosen 0.5 cm pieces were placed into 5 ml distilled water and homogenized using a rotary homogenizer (Greiner Scientific). Samples (100 µl) of homogenate were spread, in duplicate, on to selective media, containing: PDA, 0.5 g cycloheximide, 0.2 g chloramphenicol, 0.5 g 65% dodine and 0.01 g crystal violet. The plates were kept at 27°C for 20 days and *M. robertsii* CFU's were counted in five plant replicates.

The remaining root pieces were frozen at -80 °C for 12 hours and then freeze-dried for 24h. An aliquot of 2 mg of the lyophilized material was ground using a ball mill (Retsch, MM 300) and DNA was extracted from the powdered material.

DNA extractions

Genomic DNA was extracted from fungal isolate grown in YPD using the Wizard® Genomic DNA Purification Kit (Promega). The obtained DNA was used to evaluate primer sets for qPCR and also to construct standard calibration curves (DNA concentration versus qPCR CT values). DNA from uninoculated plants was also extracted using Plant DNeasy Mini Kit (Qiagen) as the negative control. DNA from lyophilized plant-ARSEF 2575-*GFP* association samples were extracted using a Soil Isolation DNA Kit (Norgen). This kit was finally selected after several attempts using various DNA extraction kits to produce DNA free of potential PCR inhibitors present in soil residues associated with plant roots. DNA quality verification and quantification were checked spectrophotometrically at 260 nm/280 nm (Implen Nanophotometer^{TM} Pearl). DNA from fungal, plant only and inoculated plants were stored until use at -20°C.

Design of PCR primers and specificity

The primers for detection of *M. robertsii* ARSEF 2575-*GFP* by real-time PCR (Table 1) were developed using Primer 3 Software (S. Rozen and HJ Skaletsky; available at http://www-genome.wi.mit.edu/ genome_ software/other/primer3.html) based on genome data for *M. robertsii* (Gao *et al.* 2011). Primer specificity was checked by amplifying template (ARSEF 2575-*GFP*), plant DNA and blanks without DNA by conventional PCR. The reactions were performed in a total volume of 25 µl containing 2 µl of template ARSEF 2575-*GFP* DNA (25

ng/µl), 12.5 µl Jump Start Taq Ready Mix (Sigma), 0.5 µM of forward and reverse primers and 8.5 µl PCR-grade water. The PCR conditions were; 2 min 98 °C, 40 cycles of 98 °C for 30 sec, 58.7 °C for 30 sec and 72 °C for 30 sec. The amplicons were visualized by ultraviolet fluorescence on 3% agarose gel stained with ethidium bromide. The *MtNit* primer pair provided the most consistent results in conventional PCR and was selected for the subsequent tests performed by qPCR.

Quantitative real time-PCR

The qPCR reactions contained SSO Fast Eva Green Ready mix (2x) (Bio-Rad), 0.3 µM of *MtNit* forward and reverse primers, 3 µl of template DNA and PCR-grade water to a final volume of 20 µl. Each reaction was performed in triplicate and controls were included in each reaction set. The qPCR reactions were performed using Real Time Detection Sytem MyIQ (BioRad Laboratories Ltda., Canada, Mississauga, Ont.) at: 2 min 98 °C, 40 cycles of 98 °C for 5 sec, 58.7 °C for 10 sec and 72 °C for 20s. The Ct cycle was calculated using the Optical System software, version 2.0 for MyIQ (BioRad). *MtNit* specificity was also checked by qPCR using template (ARSEF 2575-*GFP*), bean DNA and blanks without DNA to construct a dissociation curve. The qPCR products were also visualized by ultraviolet fluorescence on 3% agarose gel stained with ethidium bromide.

Standard curve using genomic DNA

A standard curve for qPCR using different concentrations of fungal genomic DNA (ARSEF 2575-*GFP*) was prepared by using spectrophotometrically quantified DNA. The DNA concentration gradients ranged from 10 ng to 1pg. qPCR was done in triplicate for each DNA concentration and the assay was repeated twice. The template concentration was plotted against the threshold cycle (Ct), using Optical System software, version 2.0 for MyIQ (BioRad). In order to generate a standard quantification curve the Ct values were plotted versus the logarithmic DNA concentration. The correlation coefficient (R^2) and PCR efficiency was calculated based on the slope of the standard curve [$E= 10^{-1/slope}$; % E= (E – 1)x100%].

Spike test

A spike test was conducted in order to verify the ability of the *MtNit* primers to recognize target DNA with an overwhelming concentration of non-target plant DNA. Fungal DNA concentrations ranging from 10 to 0.01ng were spiked with 20 ng of plant DNA and subjected to qPCR. Ct values were plotted versus the logarithmic value for each fungal DNA concentration.

Real time quantification of time course of colonization in roots

Plants from a time-course development of ARSEF 2575-*GFP* association and control plant (uninoculated) were harvested and processed as described above. Plant-ARSEF 2575-*GFP* association DNA was extracted also as described above, and used as templates for qPCR. The DNA from five plant replicates was used and three qPCR reactions were performed in each plant replicate. The result of the amount of fungal DNA in the root experimental sample was obtained as a Ct value. In order to obtain the amount of fungal DNA (ng) in the samples, the Ct values obtained in the given calibration curve were correlated with the amount of DNA used to construct the curve. Subsequently, the Ct value from experimental samples was interpolated in the calibration curve to estimate amounts of DNA (ng) in every experimental sample. To compare the mean amount of fungal colonization in the five plant replicates among the time points (7, 14, 21, 28, 35 days) a generalized linear model was constructed and an ANOVA with *F* test was performed using R (R Development Core 2008). Sampling was destructive, so separate plants were evaluated at each time point. For this reason, we did not use repeated measures in the analysis. The differences among the mean colonization (ng) at each time point were obtained through a simplification of the original model with amalgamation of time points, the models were then contrasted with the original model and the significance differences were obtained (Crawley 2007).

Laser Confocal Scanning Microscopy of plant-ARSEF 2575-*GFP*

Bean seedlings were inoculated with ARSEF 2575-*GFP* and planted as described above. Three plants from 3, 7, 14, 21 and 28 days post-inoculation were harvested and processed for laser scanning confocal microscopy. Root pieces were examined under the Zeiss LSM 510 confocal microscope at the Microscopy and Microanalysis Unit of the Federal University of Viçosa (Núcleo de Microscopia e Microanálise da Universidade Federal de Viçosa — NMM-UFV) to analyze the dynamics of root colonization by ARSEF 2575-*GFP*. The GFP was excited with a 488-nm laser and emission was detected at 505-530 nm. Root cell wall autofluorescence was detected at 580-620 nm. Images were processed using Zeiss LSM Image Browser.

Results

Primer specificity

The fidelity of all the primers designed (Table 1) was tested by conventional PCR with *M. robertsii* ARSEF 2575-*GFP* (target) and bean (non-target) DNA templates (Fig. 1). Any non-specific homology was checked by Blast alignment of the primer target sequences from the Genbank. The primers were also tested using qPCR and the *MtNit* primer set was selected for its consistent performance based on Ct value and specificity visualized by the dissociation curve of the qPCR (Fig. 2A). The dissociation profile showed amplification in the presence of the ARSEF 2575-*GFP* template, but no amplification was observed using bean DNA or water (negative control); these results were also confirmed by agarose gel electrophoresis of the reaction product (Fig. 2B).

Table 1. Quantitative real-time polymerase chain reaction primers designed for the present study

Primer name	Target Sequence	GenBank accession n°	Primer sequence and position (5′ to 3′)	Length, T_m
MtNit	Pathway-specific nitrogen regulator	NW_006916915.1	ACCCAAGGAGTTTGAGCCAG (F) GCAGGCACAGCAAGAAAGAC (R) (4465636-4465736)	101 bp, 58.7 °C
MtChi1	Chitin synthase 1	NW_006916917.1	GAGACGGTCGTGGTCATAGC (F) TGCCTGAATAGGGCTAACCG (R) (1610817-1610939)	123 bp, 58.7 °C
MtPep	D-alanyl-D-alanine carboxypeptidase	NW_006916956.1	TGACAATTTTGCCGGTGCTG (F) ATCCAATCCTGGGTGCAAGG (R) (30855-30972)	118 bp, 58.7 °C
MtCarb	Carbon response regulator	NW_006916923.1	CAGTTGTCGTTCTCCCTCCC (F) GACCTTCTGGCTGTTCTGGG (R) (1228426-1228500)	75 bp, 58.7 °C
MtITS	Internal transcribed spacer		GTGGACTTGGTGTTGGGGAT (F) GCTCCTGTTGCGAGTGTTTT (R) (321-438)	118 bp, 58.7 °C

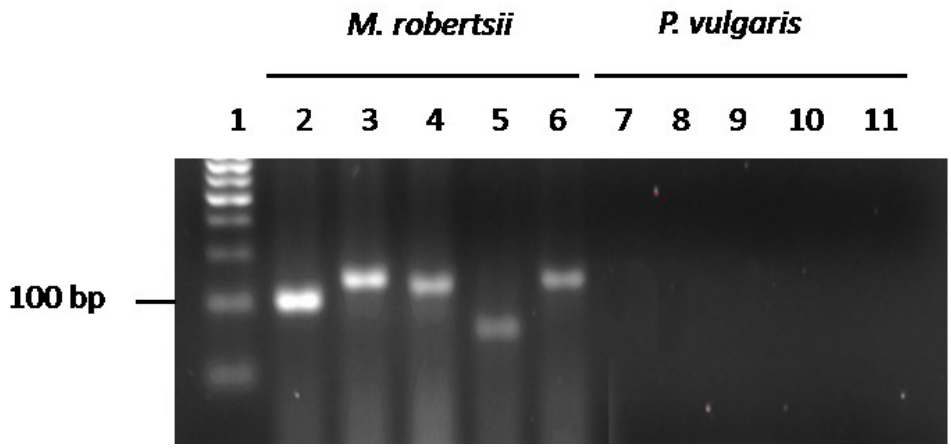

Figure 1. Specificity test of the five *Metarhizium robertsii* specific primer pairs by conventional polymerase chain reaction amplification. Test for *M. robertsii* genomic DNA amplification from lanes 2-6. Test for plant (*Phaseolus vulgaris*) genomic DNA from lanes 7-11. Lane 1 is a 100-bp ladder (Norgen). Lanes 2 and 7: *MtNit*; lanes 3 and 8: *MtChi1*, lanes 4 and 9: *MtPep*, lanes 5 and 10: *MtCarb*, lanes 6 and 11: *MtITS*.

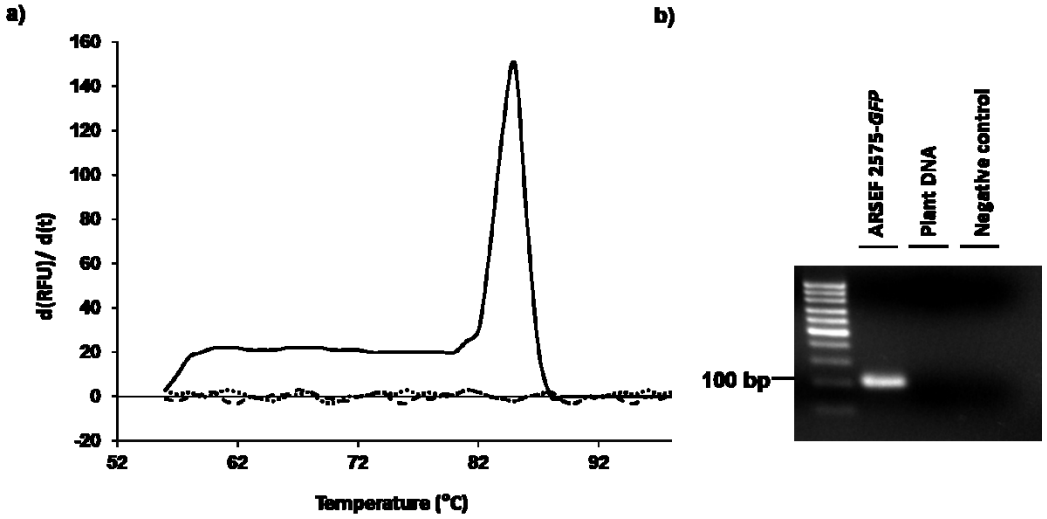

Figure 2. Dissociation curve and electrophoresis gel of quantitative real-time polymerase chain reaction amplification using the MtNit primer. a) Specific amplification is seen by the amplification of the reaction containing M. robertsii genomic DNA as the template (-) . No amplification is seen with plant DNA (---) and negative control (…). b) Specific amplification is also visualized in the electrophoresis. Lane 1 is a 100-bp ladder (Norgen).

Standard curve and Spike test

Amplification of a dilution series of *Metarhizium* DNA (10, 1, 0.1, 0.01, 0.001 ng) showed a linear relationship between the Ct value (qPCR amplification) and DNA concentration (Fig.

3A). The 101-bp *M. robertsii* ARSEF 2575- *GFP* fragment was amplified over a log concentration range showing a correlation coefficient of 0.99 and PCR efficiency of 102.65%.

In the spike test the concentration of plant genomic DNA used (20 ng), was 2000 times greater than the smallest amount (0.01ng) of fungal DNA. Figure 3B showns that the 101-bp *MtNit* target amplified was selectively amplified in the presence of contaminating plant DNA with a high correlation coefficient.

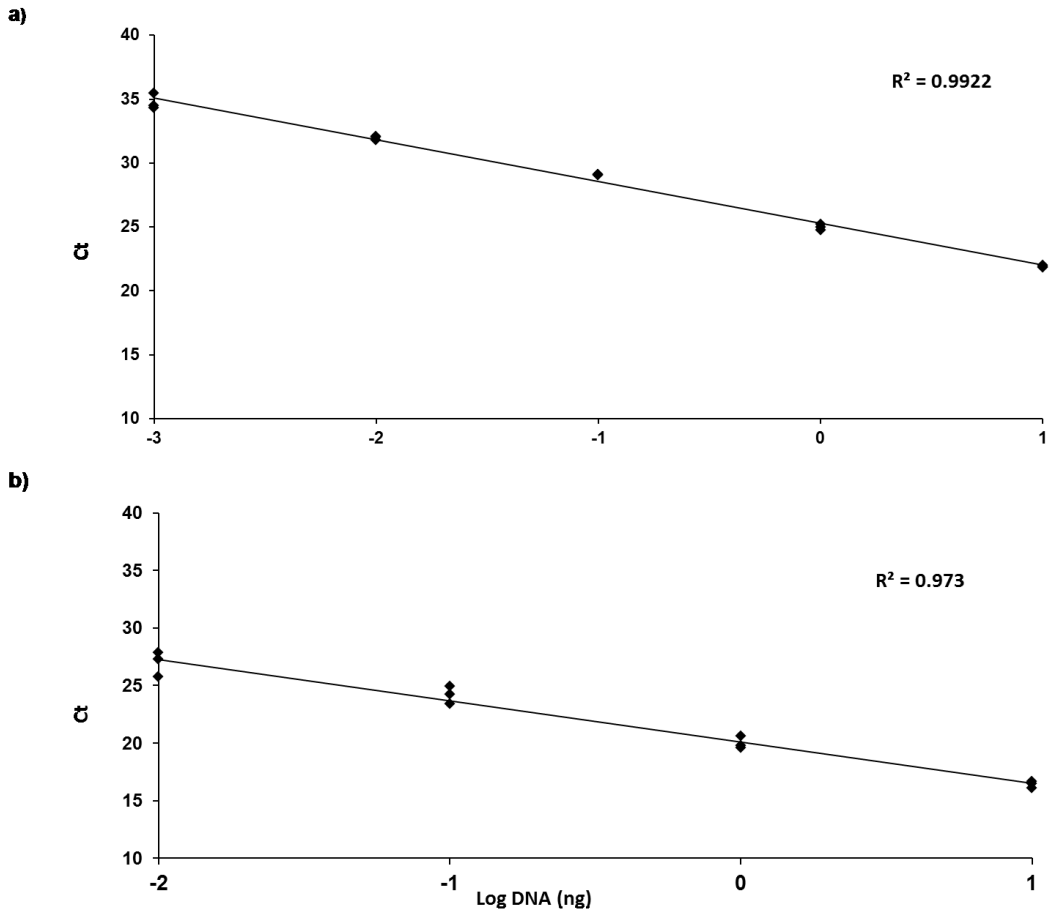

Figure 3. Standard amplification curves of Metarhizium robertsii ARSEF 2575-GFP DNA with MtNit primer. (a) Standard curve amplification of dilutions of M. robertsii ARSEF 2575-GF pure genomic DNA (0.001, 0.01, 0.1, 1 and 10 ng) represented by its correspondent log value. Threshold cycle (Ct) is plotted against genomic DNA concentration (y= - 3.268x + 25.269, R2= 0.99, PCR Efficiency=102.65%). (b) Spike test curve of varying amounts of M. robertsii ARSEF 2575-GFP DNA (0.0, 0., 1.0 and 10 ng) mixed with 20ng of Phaseolus vulgaris DNA. Threshold cycle (Ct) is plotted agaist genomic DNA concentration (y= - 3.5843x + 20.105, R2= 0.973, PCR Efficiency=90.11%).

Real time quantification of time course of *M. robertsii* colonization in roots and CFU count

Real time PCR with the primer *MtNit* template DNA from root experimental samples shows production of templates in inoculated plants (Fig. 4A). Uninoculated plants did not yield substantial production of templates. Colonization at seven days was much higher than at all the other time points (4.30 ng± 0.30 S.E.M.; $P<0.001$). At fourteen days, reduced colonization was detected (1.33±0.38; $P<0.001$). At the other time points, colonization was detected only at low levels and no significant differences were detected.

The colonization of bean roots using culturing techniques (CFU counts) showed growth in all plated root homogenates (Fig 4B). Colonization at seven days was greater than at the other time points (139 CFU/100µl±12.44; $P<0.001$). At fourteen days a decrease in colonization rate was observed (74.6 CFU/100µl±5.54; $P<0.001$). Colonization continued decreasing until it reached 11.6 CFU/100µl (±1.80; $P<0.001$) and 11 CFU/100µl (±1.37; $P<0.001$), at 21 and 35 days respectively. A small increase was observed at 28 days to 20.2 CFU/100µl (±3.12; $P<0.001$).

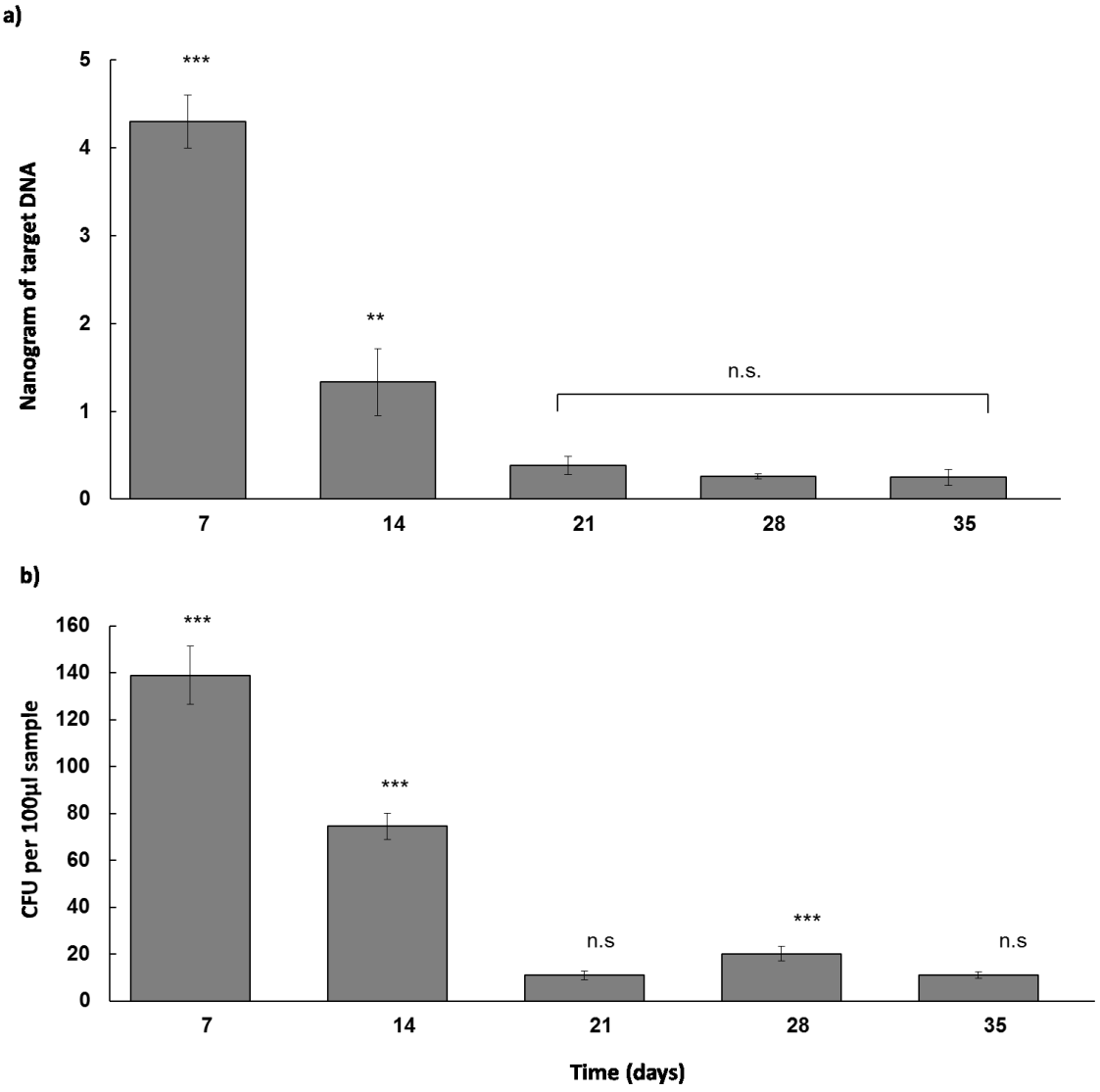

Figure 4. Time course quantification (7, 14, 21, 28 and 35 days) of endophytic colonization of Metarhizium robertsii ARSEF 2575-GFP in bean roots. (a) Time course quantification obtained by qPCR. (b) Time course quantification obtained from CFU counts. *** P <0.001, ** P <0.01, n.s. – not significant.

Laser Confocal Scanning Microscopy of the plant- ARSEF 2575-*GFP* association

Discrimination between *GFP* fluorescence and root autofluorescense was possible due to the differential detection of fluorescence wavelengths. *Metarhizium robertii* ARSEF 2575-*GFP* rapidly colonized bean root surfaces. At three days post-inoculation, appressorium-like structures appeared to be initiating penetration of the root epidermis (Fig 5A), conidia adhered to the plant surfaces was observed (Fig. 5B) and hyphae were growing from germinating conidia (Fig. 5C). On the seventh day an extensive hyphal network colonizing the root surface

was observed on the upper part of the roots (Fig. 5D) and hyphae were observed penetrating the epidemic cell layer and some hyphae were observed internally in the root (Fig. 5E). Also on the seventh day, a mycelial network was oberserved on the root meristematic region (Fig. 5F). More hyphae growing between cortical cells were observed at day fourteen (Fig. 6A) and the density of the external mycelial network decreased and a sparser external mycelial network was to be seen (Fig. 6B). At twenty-one days, conidia germinating at the cell surface were also to be seen (Fig. 6C) and roots were hosting internal colonization (Fig. 6D). At twenty-eight days, colonization spots were less common, but colonization at epidermal and cortical cell layers was detected (Fig. 6E). At the same time point (twenty-eight days), colonization of the root hairs was to be seen (Fig. 6F). At all the time points, hyphal regions with low fluorescence were frequently observed. Also, penetration of the vessels region or hyphal special structures was not observed in all the time points.

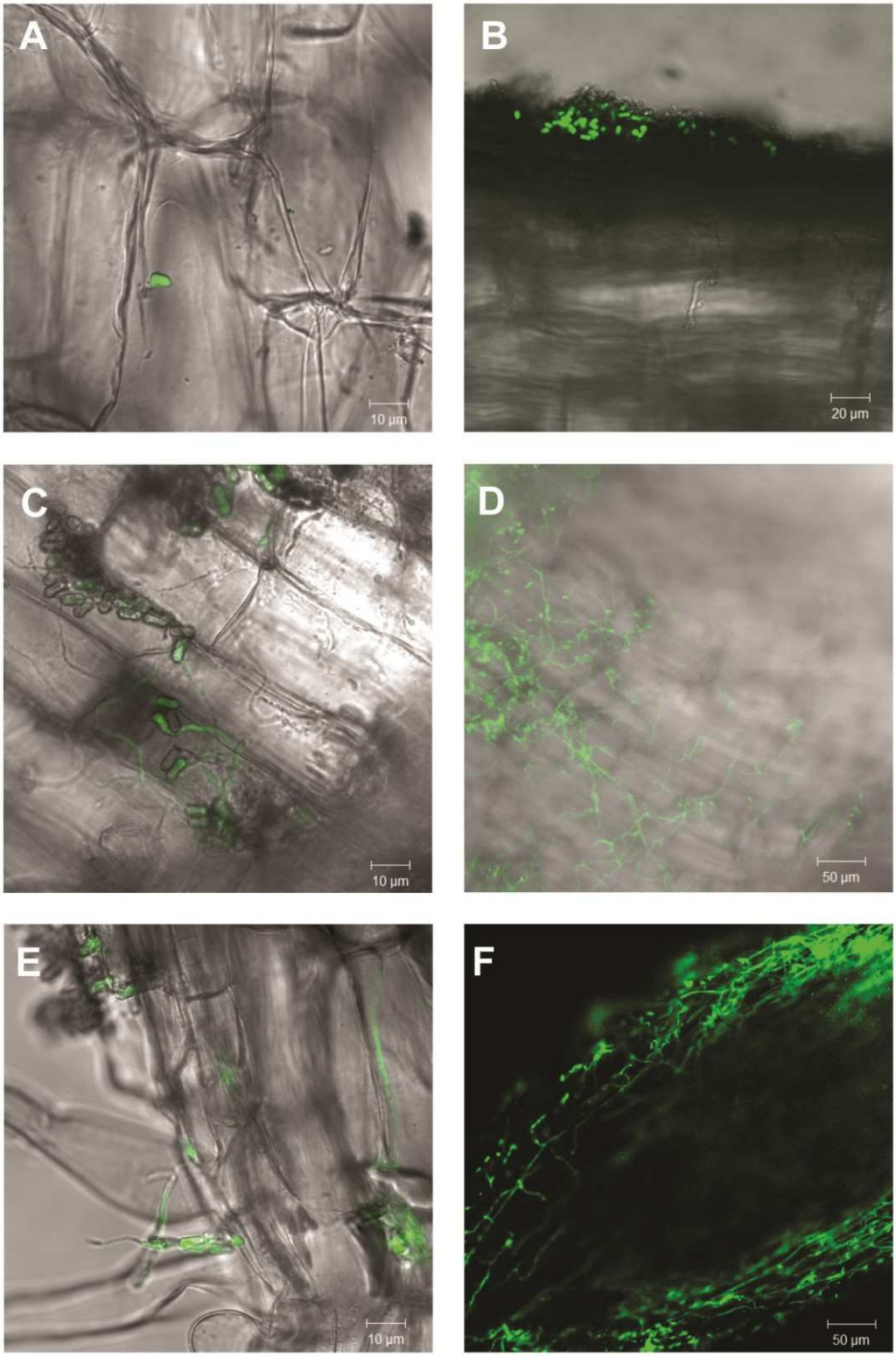

Figure 5. Merged laser scanning confocal microscopy and brightfield microscopy of bean (*Phaseolus vulgaris*) root colonization by *Metarhizium robertsii* ARSEF 2575-GFP. (a-c) 3 days post inoculation – (a) Germinating conidium showing an appressorium-like structure in root cell epidermis; (b) Conidia adhered to the root epidermis; (c) Germinating conidia with penetration of the root cell epidermis. (d-f) 7 days post inoculation – (d) Massive colonization of the root surface with formation of a hyphal network; (e) Penetration of root epidermal cell and internal colonization of epidermal root layer; (f) Massive internal and external colonization of root meristematic region with formation of hyphal network.

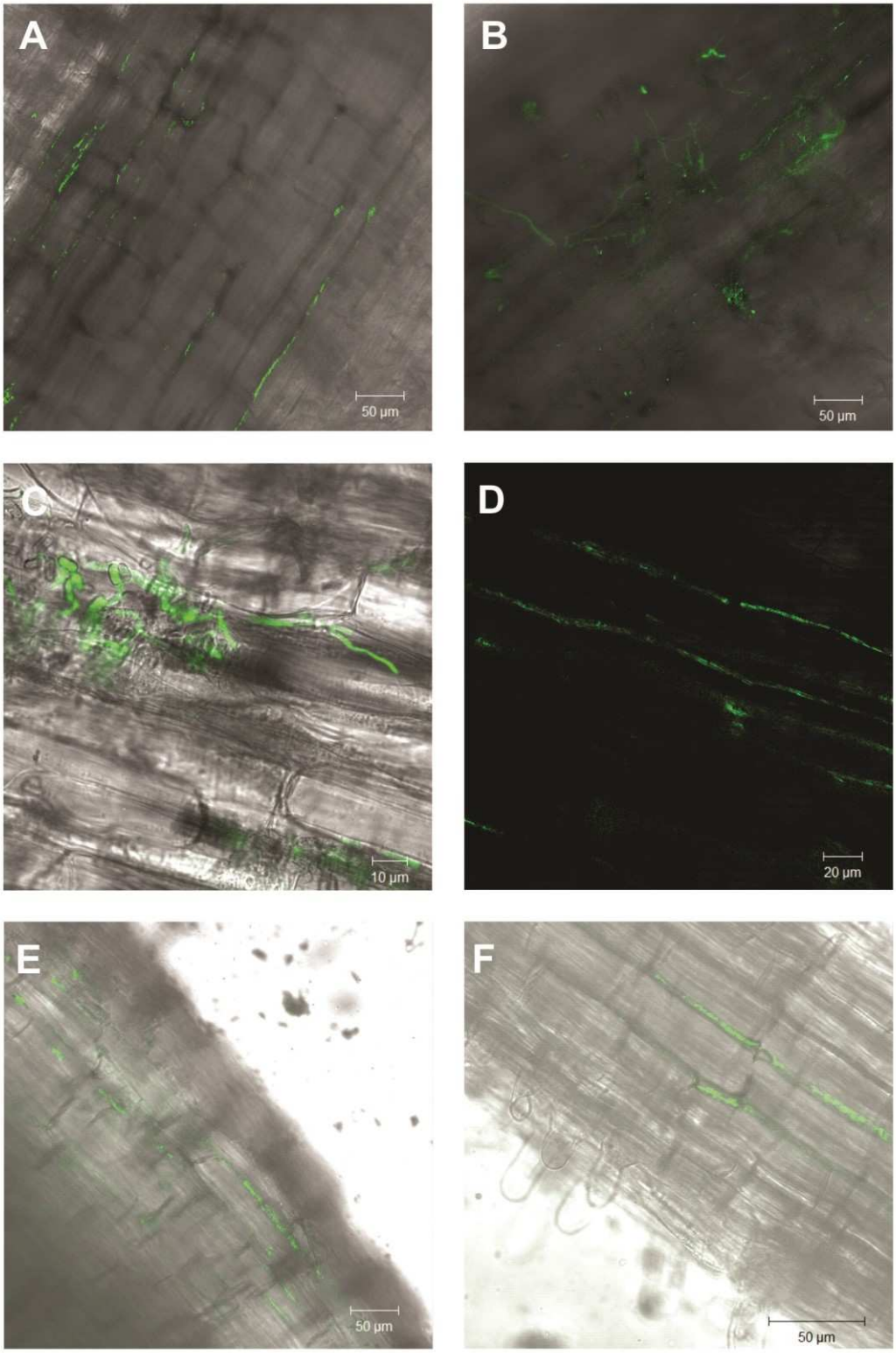

Figure 6. Merged laser scanning confocal microscopy and brightfield of bean (Phaseolus vulgaris) root colonization by Metarhizium robertsii ARSEF 2575-GFP. (a-b) 14 days post inoculation – (a) Internal colonization of cortical cells; (b) External and internal hyphal growth. (c-d) 21 days post inoculation – (c) Germinating conidia and hyphal growth at root surface; (d) Internal growth in the intercellular space at the cortical cell layer. (e-f) 28 days – (e) Colonization of epidermal cell layer; (f) Internal colonization of a root hair.

Discussion

The recent discovery that *Metarhizium* can form intimate and apparently mutualistic associations with plants could have great agricultural potential to plant protection from insect pests, pathogens and plant nutrition (Fang & St. Leger 2010; Behie *et al.* 2012; Sasan & Bidochka 2012, 2013; Behie & Bidochka 2014b; Behie *et al.* submitted). At present, though, studies of the association between roots and insect pathogenic fungi are at a very initial stage; much still needs to be explored regarding molecular and biochemical mechanisms, interaction dynamics and consequences of the association for both partners. One of the first steps for this is to establish methods to assess, quantify and describe the development of the association. Here we established a qPCR based method to detect and quantify *M. robertsii* in plant root tissues. We also described the time course colonization of bean roots using qPCR, a culturing based method and *GFP* detection with laser scanning confocal microscopy. Although it could be considered that this is a very specific tool, it is becoming ever more apparent that plant root associations with *M robertsii* and congeneric fungi are the norm rather than isolated instances (Behie & Bidochka 2014b; Behie *et al.* 2015), with potentially important implications for plant biology and ecology in general.

The method developed here allows the detection and quantification of root colonization by *M. robertsii* with high sensitivity, specificity, consistency, reduced amplicon size, and with the possibility to assess colonization in a large number of samples. The utilization of SYBR Green fluorescence detection provides a reliable method with low cost, and has proven effective in previous studies to detect other fungal endophytes (Landa *et al.* 2013), plant pathogens (Pasche *et al.* 2012) and arbuscular mycorrizal fungi (Alkan *et al.* 2004). Quantification and detection of *Metarhizium* in soils (Schneider *et al.* 2011) and insect tissues (Bell *et al.* 2009) has been performed in previous studies using qPCR, however this is the first time that this technology is applied to quantify *M. robertsii* in association with host plant tissue, coupled with culturing method and imaging through laser scanning confocal microscopy.

The *MtNit* primer was sufficiently sensitive to detect up to 1 pg of *M. robertsii* purified DNA. The linear range of amplification obtained in the standard curve provided a reliable degree of confidence in quantification of experimental samples. Also, the degree of specificity seen under spike test conditions in a fixed large amount of non-specific DNA is a strong indication of the consistency and sensitivity of the qPCR diagnostic. The methodology provided here can be extremely useful for further investigation of the *M. robertsii*-plant association. Association detection and quantification can be included as a response variable in experimental set-ups investigating the effects of *M. robertsii* in plants such as: protection against biotic and abiotic stress, production of toxic compounds, activation of host defense mechanisms and others, unveiling molecular and biochemical paths of the interaction and others.

The use of isolates with knockouts of specific genes is one of the most informative approaches to unravelling the roles of those genes (Fang *et al.* 2006); such mutants have proven to be useful to investigate the role of specific genes in plant-fungus associations (Fang & St. Leger 2010; Wang *et al.* 2011; Liao *et al.* 2014). The comparison of the amount of association of the wild type and mutants can be a relevant response and explain much of the role of the surveyed genes; the method presented here can be a very valuable tool to understand the role of specific genes involved in nutrient transfer between *Metarhizium* and plants.

The qPCR based approach can be relatively simple and fast compared to other methods to detect and quantify endophytic associations; however, several technical problems must be overcome to achieve consistent and reliable results. One of the critical factors is the isolation of DNA from experimental or environmental plant-fungus samples (Malvick & Grunden 2005). Successful detection of fungal DNA in experimental root samples, contaminated with non-target DNA and residual soil particles, depends on isolation of DNA free of compounds that inhibit amplification. In the present study amplification was obtained in the experimental samples when soil PCR inhibitors were removed using soil DNA isolation kit. An additional technical problem that must be overcome is the recovery of fungal DNA in a sample with an overwhelming amount of plant DNA and high water content. A certain amount of plant material must to be

used in order to reach a detectable amount of the fungal DNA, high water content in roots can also reduce the amount of plant material processed for DNA extraction and consequently the amount of fungal DNA in the sample. The utilization the fine-ground freeze dried material for DNA extraction optimized the recovery of fungal DNA due the removal of water from sample and increasing the contact surface of the DNA isolation substances present in the kit. The small particles of the plant-fungus material also increased the recovery of fungal DNA in the sample.

Besides the differences in the scale, qPCR detection and CFU counting seems to follow the same pattern along the time course development of the association. Usually cultivation methods relying on CFU counts tend to be challenging under non-axenic conditions (Porras-Alfaro & Bayman 2011) but here this problem was solved through the utilization of selective media. Cultivating methods can also over- or underestimate colonization when accounting the amount of colonization through the percent of fragments colonized. In this case, a root fragment completely colonized by the endophyte and a fragment containing only a small propagule are scored as positive (Maciá-Vicente *et al.* 2009). Unless a great number of fragments are used, the method is frequently saturated. Here the homogenate method resulted in a more uniform sampling and consequently in a more accurate translation of the colonization pattern.

In the present study the influence of the *M. robertsii* external growth was not excluded from the quantification through surface sterilization because it can compromise both methods of quantification used here. Surface sterilization methods commonly use a disinfectant agent as sodium hypochlorite to kill external fungal propagules. However, to achieve a complete clean root surface the endophytic fungi can also be compromised, since sodium hypochlorite can penetrate root tissues consequently killing endophytic fungi (Hallmann *et al.* 2006). Additionally, the DNA denaturing properties of sodium hypochlorite wers also already reported (Thornbury & Farman 2000; Phe *et al.* 2004). Besides the importance of the endophytic association, the role of the externally growing *M. robertsii* in root surface is not fully understood. Fungi and bacteria growing externally on the root surface have great impact on rhizosphere functioning and consequently in the plant health (Badri *et al.* 2009; Fang & St.

Leger 2010), while externally growing fungal mycelium is essential for increasing nutrient scavenging (Behie & Bidochka 2014a) communication between plants (Badri *et al.* 2009), nutrient recycle and exchange (Morgan *et al.* 2005) and influence the controlo f soil pests (Keyser *et al.* 2014). Based on that, external mycelial growth was kept in the analysis; however, if the interest is solely in the fungus growing inside the roots alternative methods for surface sterilization without sodium hypochlorite can be tested.

The great amount of colonization at the seventh day achieved by the both quantification methods is clearly influenced by the inoculum applied to the roots initially. The observation of the colonization pattern obtained by the *GFP*-tagged *M. robertsii* supported this observation. A great amount of germinating spores and the formation of an extensive external mycelial network is observed in the first days. Penetrating hyphae are also observed and at 7 days post inoculation and it is already possible to see growth in the intercellular spaces in the epidermal layer. Penetration seems to occur through the formation of appressorium-like structures. Regions with degraded hyphae showing loss of fluorescence were frequently observed, which may indicate hyphal death by the action of the host defense mechanisms. Further studies can elucidate what plant defense mechanisms are triggered by *M. robertsii* colonization and what factors maintain the long term association.

Endophytic fungal hyphae can establish an intimate interaction with the host cells, the internal hyphal net can be more extensive and limited to epidermis or include cortex layer (Zuccaro *et al.* 2014). Very well adapted interfaces for nutrient transfer are absent in endophytes, nevertheless recent reports show evidence of biotrophic interfaces with plant cells and fungal hyphae wrapped by host membranes (Maciá-Vicente *et al.* 2009; Jacobs *et al.* 2011; Lahrmann *et al.* 2013). Here we did not observe any special structures regarding plant or the symbiont fungus but further observations will be required, increasing the contrast of the plant cells through application of dyes or other microscopic approaches to confirm this observation.

A great number of techniques is used to quantify the amount of association of endophytic organisms (Schulz & Boyle 2005; Porras-Alfaro & Bayman 2011). Methods based on qPCR quantification are frequently used and are more reliable (Tellenbach *et al.* 2010). When correlated with images, this new tool allows more in-depth study of root colonization. Further studies applying the new tool will be required to understand dynamics of the *M. robertsii*–plant association and consequently to develop the utilization of this symbiosis to achieve agricultural benefits to plants.

References

Alkan N., Gadkar V., Coburn J., Yarden O. & Kapulnik Y. (2004). Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. *New Phytologist*, 161, 877-885.

Badri D.V., Weir T.L., van der Lelie D. & Vivanco J.M. (2009). Rhizosphere chemical dialogues: plant–microbe interactions. *Current Opinion in Biotechnology*, 20, 642-650.

Behie S.W. & Bidochka M.J. (2014a). Nutrient transfer in plant–fungal symbioses. *Trends in Plant Science*, 19, 734-740.

Behie S.W. & Bidochka M.J. (2014b). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and Environmental Microbiology*, 80, 1553-1560.

Behie S.W., Jones S.J. & Bidochka M.J. (2015). Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. *Fungal Ecology*, 13, 112-119.

Behie S.W., Moreira C.M., Sementchoukova I., Barelli L., Zelisko P.M. & Bidochka M.J. (submitted). Trading insect nitrogen for photosynthate: Carbon translocation from a plant to an insect pathogenic, endophytic fungus. *Science*.

Behie S.W., Padilla-Guerrero I.E. & Bidochka M.J. (2013). Nutrient transfer to plants by phylogenetically diverse fungi suggests convergent evolutionary strategies in rhizospheric symbionts. *Communicative & Integrative Biology*, 6, e22321.

Behie S.W., Zelisko P.M. & Bidochka M.J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science*, 336, 1576-1577.

Bell A.S., Blanford S., Jenkins N., Thomas M.B. & Read A.F. (2009). Real-time quantitative PCR for analysis of candidate fungal biopesticides against malaria: Technique validation and first applications. *Journal of Invertebrate Pathology*, 100, 160-168.

Brundrett M. (2006). Understanding the Roles of Multifunctional Mycorrhizal and Endophytic Fungi. In: *Microbial Root Endophytes* (eds. Schulz BE, Boyle CC & Sieber T). Springer Berlin Heidelberg, pp. 281-298.

Crawley M.J. (2007). *The R Book*. Wiley Publishing.

Fang W., Pei Y. & Bidochka M.J. (2006). Transformation of *Metarhizium anisopliae* mediated by *Agrobacterium tumefaciens*. *Canadian Journal of Microbiology*, 52, 623-626.

Fang W. & St. Leger R.J. (2010). *Mrt*, a gene unique to fungi, encodes an oligosaccharide transporter and facilitates rhizosphere competency in *Metarhizium robertsii*. *Plant Physiology*, 154, 1549-1557.

Gamborg O. & Phillips G. (1995). Sterile Techniques. In: *Plant Cell, Tissue and Organ Culture* (eds. Gamborg O & Phillips G). Springer Berlin Heidelberg, pp. 35-42.

Gao Q., Jin K., Ying S.-H., Zhang Y., Xiao G., Shang Y., Duan Z., Hu X., Xie X.-Q., Zhou G., Peng G., Luo Z., Huang W., Wang B., Fang W., Wang S., Zhong Y., Ma L.-J., St. Leger R.J., Zhao G.-P., Pei Y., Feng M.-G., Xia Y. & Wang C. (2011). Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet*, 7, e1001264.

Hallmann J., Berg G. & Schulz B. (2006). Isolation Procedures for Endophytic Microorganisms. In: *Microbial Root Endophytes* (eds. Schulz BE, Boyle CC & Sieber T). Springer Berlin Heidelberg, pp. 299-319.

Jacobs S., Zechmann B., Molitor A., Trujillo M., Petutschnig E., Lipka V., Kogel K.-H. & Schäfer P. (2011). Broad-spectrum suppression of innate immunity is required for colonization of *Arabidopsis* Roots by the fungus *Piriformospora indica*. *Plant Physiology*, 156, 726-740.

Keyser C.A., Thorup-Kristensen K. & Meyling N.V. (2014). Metarhizium seed treatment mediates fungal dispersal via roots and induces infections in insects. *Fungal Ecology*, 11, 122-131.

Khan A., Hamayun M., Khan S., Kang S.-M., Shinwari Z., Kamran M., ur Rehman S., Kim J.-G. & Lee I.-J. (2012). Pure culture of *Metarhizium anisopliae* LHL07 reprograms soybean to higher growth and mitigates salt stress. *World J Microbiol Biotechnol*, 28, 1483-1494.

Lahrmann U., Ding Y., Banhara A., Rath M., Hajirezaei M.R., Döhlemann S., von Wirén N., Parniske M. & Zuccaro A. (2013). Host-related metabolic cues affect colonization strategies of a root endophyte. *Proceedings of the National Academy of Sciences*, 110, 13965-13970.

Landa B.B., López-Díaz C., Jiménez-Fernández D., Montes-Borrego M., Muñoz-Ledesma F.J., Ortiz-Urquiza A. & Quesada-Moraga E. (2013). In-planta detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed nested and quantitative PCR-based assay and confocal laser scanning microscopy. *Journal of Invertebrate Pathology*, 114, 128-138.

Liao X., O'Brien T., Fang W. & St. Leger R. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Appl Microbiol Biotechnol*, 98, 7089-7096.

Lovett B. & St. Leger R.J. (2014). Stress is the rule rather than the exception for *Metarhizium*. *Curr Genet*, 1-9.

Maciá-Vicente J.G., Jansson H.-B., Talbot N.J. & Lopez-Llorca L.V. (2009). Real-time PCR quantification and live-cell imaging of endophytic colonization of barley (*Hordeum vulgare*) roots by *Fusarium equiseti* and *Pochonia chlamydosporia*. *New Phytologist*, 182, 213-228.

Malvick D.K. & Grunden E. (2005). Isolation of fungal DNA from plant tissues and removal of DNA amplification inhibitors. *Molecular Ecology Notes*, 5, 958-960.

Meyling N.V. & Eilenberg J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control*, 43, 145-155.

Morgan J.A.W., Bending G.D. & White P.J. (2005). Biological costs and benefits to plant–microbe interactions in the rhizosphere. *Journal of Experimental Botany*, 56, 1729-1739.

Pasche J.S., Mallik I., Anderson N.R. & Gudmestad N.C. (2012). Development and Validation of a Real-Time PCR Assay for the Quantification of *Verticillium dahliae* in Potato. *Plant Disease*, 97, 608-618.

Phe M.H., Dossot M. & Block J.C. (2004). Chlorination effect on the fluorescence of nucleic acid staining dyes. *Water Research*, 38, 3729-3737.

Porras-Alfaro A. & Bayman P. (2011). Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. *Annu. Rev. Phytopathol.*, 49, 291-315.

R Development Core T. (2008). R: A Language and Environment for Statistical Computing. In. R Foundation for Statistical Computing Vienna, Austria.

Sasan R.K. & Bidochka M.J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany*, 99, 101-107.

Sasan R.K. & Bidochka M.J. (2013). Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*, 35, 288-293.

Schneider S., Rehner S.A., Widmer F. & Enkerli J. (2011). A PCR-based tool for cultivation-independent detection and quantification of *Metarhizium* clade 1. *Journal of Invertebrate Pathology*, 108, 106-114.

Schulz B. & Boyle C. (2005). The endophytic continuum. *Mycological Research*, 109, 661-686.

Shah P.A. & Pell J.K. (2003). Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol*, 61, 413-423.

Tellenbach C., Grünig C.R. & Sieber T.N. (2010). Suitability of Quantitative Real-Time PCR To Estimate the Biomass of Fungal Root Endophytes. *Applied and Environmental Microbiology*, 76, 5764-5772.

Thornbury D. & Farman M. (2000). Reusing nylon-membrane for radioactive hybridizations. *Bio Techniques*, 29, 1250-1254.

Vega F.E., Goettel M.S., Blackwell M., Chandler D., Jackson M.A., Keller S., Koike M., Maniania N.K., Monzon A., Ownley B.H., Pell J.K., Rangel D.E.N. & Roy H.E. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology*, 2, 149-159.

Wang S., O'Brien T.R., Pava-Ripoll M. & St. Leger R.J. (2011). Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations. *Proceedings of the National Academy of Sciences*, 108, 20449-20454.

Zuccaro A., Lahrmann U. & Langen G. (2014). Broad compatibility in fungal root symbioses. *Current Opinion in Plant Biology*, 20, 135-145.

CHAPTER 2

METARHIZIUM ASSOCIATED WITH AGROFORESTRY AND FULL SUN COFFEE SYSTEMS: COMMUNITY CHARACTERIZATION AND POPULATION GENETICS

Abstract

Metarhizium species are insect pathogens and plant symbionts very abundant in soils worldwide. They also have the potential to provide important ecosystem services in agricultural settings as reduction of insect populations, improved plant nutrient uptake and protection from plant pathogens. The use of more diverse and sustainable land use practices such as tree-based intercropping can have the potential to reduce the negative impact of agricultural practices on beneficial microorganisms communities, however the effects of those practices in *Metarhizium* species diversity and distribution is unknown. In this paper we compare the effects of agroforestry and full sun coffee cultivation systems on *Metarhizium* community diversity and population structure. One hundred and eighteen isolates from three areas, containing each an agroforestry and full sun field were included in this study. The isolates were characterized through sequencing of the final portion of the translation enlongation factor 5'TEF and SSR markers. Phylogeny of 5'TEF region reveled three species, with *M. robertsii* predominating. Comparison of diversity indices between the fields in the area pairs revealed higher diversity in agroforestry system for two of the sampled areas and the overall diversity was also higher in agroforestry. *Metarhizium robertsii* was found to contain high SSR multilocus haplotypes diversity, belonging to three major clades, however no evidences for recombination were observed in clades. Our findings record *Metarhizium* diversity, for community and population levels, on soils of coffee based agroecosystem and can be very valuable to establish strategies for management of indigenous community to promote insect control and improve plant health in sustainable agricultural settings.

Introduction

The substantial and irreversible loss of biodiversity and the consequent deterioration of ecosystem services are consequences of the growing amount of land employed to agriculture and agricultural intensification (Matson *et al.* 1997; Tilman *et al.* 2002). Paradoxically, the biodiversity loss can affect ecosystem functioning and the delivery of services and consequently decreasing soil fertility and agricultural production (Tilman *et al.* 2002; Foley *et al.* 2005). The role of biodiversity in the provision of ecosystem services is widely recognized nowadays (Cardinale *et al.* 2012). However the importance of organisms that provide support (e.g., soil formation and fertility) and regulation (e.g., pest control and crop pollination) services are generally neglected (Bommarco *et al.* 2013).

The specific roles of many groups of organisms remain undervalued in natural and agricultural context, mostly microorganisms, such as fungi in the genus *Metarhizium*. *Metarhizium,* one of the most studied groups in the order Hypocrelaes, are capable of colonizing and establishing mutualistic association with plants (Behie *et al.* 2012; Sasan & Bidochka 2012) and they are also very valued as insect pathogens and used as biological insecticides (Shah & Pell 2003). However, their roles in an ecological context have received little attention and, despite how well documented is *Metarhizium´*s insect pathogenic capacity, their biological control potential is frequently inconsistent (St. Leger & Screen 2001). Thus, the recognition of *Metarhizium* as a ubiquitous component of the soil environment, and accounting for its responses to ecological variables, distribution, diversity and ecological interactions have been emphasized by the need for better performance as biocontrol agents (Hu & St Leger 2002; Meyling & Eilenberg 2007; Bischoff *et al.* 2009; Wyrebek *et al.* 2011; Rocha *et al.* 2013; Steinwender *et al.* 2014; Rezende *et al.* 2015; Steinwender *et al.* 2015). This new perspective led to serendipitous findings in the plant-insect pathogen partnership (Fang & St. Leger 2010; Behie *et al.* 2012; Wyrebek & Bidochka 2013) and emphasized how important is to consider

Metarhizium as a provider of ecosystem services. One of the breakthrough findings in *Metarhizium* ecology is its ability to transfer nutrients from insect cadavers to plants (Behie *et al.* 2012), representing an additional branch in the nitrogen cycle. This ability seems to be widespread within the genus and across plant taxa (Behie & Bidochka 2014). Also, *Metarhizium* species had already been reported as plant growth promoters and plant pathogen antagonists (Sasan & Bidochka 2012). Thereby, these fungi provide supporting ecosystem services, providing nutrition to plants, and regulation ecosystem servives, controlling insect pest and plant pathogens.

Faced with the above findings, we predict that in environments with greater plant abundance and variety, and consequently more complex root systems, there could be more opportunities to enter in association with plants, due to the increased availability of plant hosts. Once in association with the plant root system, the fungi will be more widely distributed within the soil systems (Keyser *et al.* 2014), increasing the possibility of encountering insect hosts and ultimately facilitating the provision of ecosystem services by these organisms. Also, the distribution of *Metarhizium* species can be correlated with specific plant taxa (Fisher *et al.* 2011; Wyrebek *et al.* 2011). These characteristics highlight the need to consider specific crops of interest, plant diversity and management systems in diversity surveys.

Biodiversity-friendly means of agriculture, such as agroforestry systems, can harbor increased biodiversity in agricultural systems and consequently provide better soil conditions to encourage microbial diversity (Bommarco *et al.* 2013). Coffee-based agroforestry systems can promote complex agroecosystems containing high diversity of tree species which can enhance functional biodiversity, carbon sequestration, soil fertility, drought resistance as well as weed and biological pest control (Tscharntke *et al.* 2011). Although full sun systems are believed to have increased yield, their adoption frequently imply increases in the use of pesticides, and fertilizers (Jha *et al.* 2014). In our previous work (Moreira *et al.* submitted) a survey was conducted comparing the occurrence of

entomopathogenic fungi using insect-bait method in agroforestry and full-sun cultivation systems. The abundance of entomopathogenic fungi was higher in agroforestry than in full sun systems; however our major finding was the increased activity of those fungi in agroforestry system (Moreira *et al.* submitted). Assessing the survival of the bait insects in soil samples from both management systems, it was possible to detect that bait in contact with agroforestry soil samples died from insect pathogenic fungal infection sooner than those in contact with samples from full sun systems. *Metarhizium* spp. were the most abundant group of fungi pathogenic to insects sampled in both management systems (Moreira *et al.* submitted). Thus, the characterization of the *Metarhizium* community and its genetic variability in each production system can be useful to understand the factors that affect abundance and activity of *Metarhizium* in agroforestry and full-sun systems. This is especially important in tropical areas where coffee crops are abundant, but the entomopathogenic fungi community is poorly characterized. This knowledge could be crucial to the valuation of a neglected portion of the biodiversity in its agroecosystem. Also the comparison of the diversity in systems under agroforestry and full-sun could reveal the effects of those practices on *Metarhizium* community and therefore encourage the adoption of practices to promote insect control, coffee plant health and nutrition.

The purpose of this study was to investigate the community diversity and population structure of *Metarhizium* in the soil of coffee crop systems using the isolates obtained previously by Moreira *et al.* (submitted). First the collection of 118 *Metarhizium* isolates was identified to the species level using 5'TEF region as barcode followed by the reconstruction of the DNA phylogeny. Secondly, we analyzed the genotypic diversity by using multilocus SSR markers and the genetic diversity and structure of the population of *M. robertsii* comparing agroforestry and full-sun management systems. *Metarhizium´*s reproductive mode was also assessed by polymerase chain reaction (PCR) assay for diagnosis of polymorphism at the mating type (MAT) locus. To date, this is the first study

to address the diversity of entomopathogenic at the population level in small scale agricultural fields in Brazil.

Material and Methods

Sampling and fungal isolation

Metarhizium isolates were obtained from soil samples collected from smallholdings under organic coffee cultivation in the municipality of Araponga, Minas Gerais, southeastern Brazil (20° 48´ S and 42° 32´ W) between June and August, 2010 (Moreira *et al.* submitted). The sampling process was designed to compare organic agroforestry and full-sun coffee production systems. In this scenario, agroforestry consists of shade trees planted between coffee rows. Fertilization was provided by green manure and organic fertilizers with no pesticide use. The full sun system consists of unshaded open cultivation. Inorganic fertilizers were used, but no pesticides were applied. Three areas (A1, A2, A3) were chosen, each of which had an organic agroforestry (AG) and a full-sun (FS) coffee plantation. In total six fields (three pairs) were sampled. The distances between each field of a pair varied between 10 and 100 m. Samples were collected from points in a rough grid of nine or ten planted adjacent rows (spaced ca. 3m from each other) by eight to ten samples per row (every third bush, so a spacing of ca. 4m). Soil was collected using a core sampler to 20cm depth, from the base of the bushes, beneath the canopy but ca. 75cm from the plants' trunks. In total 490 soil samples were taken from three areas, divided as follow: A1: 97 (AG) and 97 (FS); A2: 78 (AG) and 76 (FS); A3: 70 (AG) and (72) (FS).

Entomopathogenic fungi were isolated from the soil samples using the insect bait method (Zimmermann 1986). Soil samples were placed in 200 ml plastic cups, moistened with sterile distilled water and four larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) were transferred to each cup. The samples were kept in the dark, inverted daily and inspected for presence of dead larvae at every three days. Insect cadavers were incubated in moist chambers and emerging fungi were isolated in PDA (Potato 20 %, Dextrose 2% and Agar 1.5%) plates.

DNA extraction

A total of 118 *Metarhizium* isolates were recovered. To obtain single-spore cultures, a small amount of conidia was suspended in 1ml of 0.01% water solution of Tween 80. An aliquot of 100 µl of the suspension was spread onto PDA plates and incubated for 12h at 25°C. A single germinating conidium was transferred into a new PDA plate. Conidia from each single-spore culture isolate were inoculated into 50 ml of liquid medium (0.25% peptone, 0.25% yeast extract and 1% dextrose) and left to grow at room temperature on a rotary shaker at 140 rpm for 7 days. Fungal mycelium was washed with sterile distilled water then transferred to a filter paper to dry and macerated with mortar and pestle in liquid nitrogen.

Genomic DNA was extracted from ground mycelium using Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). DNA integrity was visualized by ultraviolet fluorescence on 1% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in a 1× TBE in a Agarose gel 1%. DNA concentration was adjusted to 25 ng/µL in a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Metarhizium species phylogeny

The final portion of 5'TEF (Rehner and Buckley, 2005; Bischoff *et al.*, 2009) was used to reconstruct the DNA phylogeny of the 118 *Metarhizium* isolates. The nuclear 5'TEF was amplified with the primers EF1T (5'ATGGGTAAGGARGACAAGAC) and EF2T (5'GGAAGTACCAGTGATCATGTT). PCR amplifications were performed in total volume of 25µL consisting of 12.5 µl Dream Taq^{TM} PCR Master Mix 2X (Thermo Fisher Scientific), 1 µl each of 10 µM forward and reverse primers (Sigma- Aldrich), 1 µl dimethyl sulphoxide (DMSO, Sigma-Aldrich), 2.5 µl 100× (10 mg/mL) Bovine Serum Albumin (BSA, Sigma-Aldrich), 2 µl genomic DNA, and 5 µL of nuclease-free water to complete the total volume. The PCR reactions were performed at: 2 min 94°C, 40 cycles of 30s of denaturation at 94°C, a 30s annealing step at 56°C, and a 45s extension step at 72°C, concluding with 10 min of incubation at 72°C. PCR products were visualized by

ultraviolet fluorescence on 1% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in a 1XTBE and check for amplification size. PCR products were purified by EXO-IT® (Affymetrix) and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). Sequences were edited using DNA Dragon software (Hepperle 2010). To help clarify the phylogenetic relationship of the collected *Metarhizium* isolates, sequences from the *Metarhizium* species ex-type isolates (Bischoff *et al.* 2009) and additional representative sequences from recent Brazilian surveys conducted by Rezende *et al.* (2015) and Rocha *et al.* (2013) were also included. These sequences were obtained from GenBank (Table S1). *Metarhizium flavoviridae* (ARSEF 2133) was used as the outgroup. All sequences were aligned with Muscle v.3.6. implemented in Mega 6.0 (Tamura *et al.* 2013).

The 5'TEF sequence data were analyzed by Bayesian Inference conducted with MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) and Maximum Likelihood (ML) conducted with Paup v. 40b10 (Swofford 2003). MrModeltest v.2.2 (Nylander 2004) was used to select the best fit nucleotide substitution model. For Bayesian analysis the number of generations was 10,000,000 and the trees were saved every 1,000 generations, resulting in 10,000 saved trees. The first 2,500 trees were discarded as the burn-in phase of each analysis. For ML analysis Heuristic ML bootstrap analysis consisted of 1000 pseudoreplicates (TBR branch swapping). The phylogenetic tree was visualized using FigTree v. 1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Mating type determination

The mating type of all *Metarhizium* isolates was determined using the primers developed by Kepler *et al.* (2015) for idiomorphs MAT1-1 and MAT1-2 based on *M. robertsii* and *M. acridum* genome data (Gao *et al.* 2011). The primer pairs MAT111-1F/ MAT111-3R (991 bp) and MAT121-3F/MAT121-4R (436 bp), were used to amplify the genomic fragments corresponding to MAT1-1 and MAT1-2, respectively. Amplifications for each

locus were carried out in separate reactions and the PCR conditions were used as described by Kepler *et al.* (2015). The isolate L47A, identified as *M. anisopliae*, was used to adjust PCR conditions because it has both mating type idiomorphs as described by Pattemore *et al.* (2014). PCR products were visualized by ultraviolet fluorescence on 1% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in 1XTBE and checked for amplification size.

Microsatellite genotyping

Sixteen microsatellite markers were used to amplify microsatellite loci in *Metarhizium* (Enkerli *et al.* 2005; Oulevey *et al.* 2009) (Table 2). All loci were used to assess the multilocus genotypes for all isolates. The PCR fluorescent products were generated using a forward primer labelled with fluorescent dye (D dye set:6-FAM, NED, HEX; Applied Biosystems). Multiplex PCR reactions were performed with Type-it Microsatellite PCR kit (QIAGEN).

The molecular size standards GeneScan™ 500 ROX™ dye (Applied Biosystems) and PCR products were separated on ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Allele sizes were estimated using GeneMaker v. 1.191 (Sounits Genetics). All alleles that showed frequencies of <10% were repeated. Alleles were scored according to the number of repeat units.

Microsatellite analysis

The total number of alleles and the private alleles were estimated for every locus in each *Metarhizium* lineage. Using the R package POPPR (Kamvar *et al.* 2014) any invariant loci were determined and removed from the analysis. The isolates were assigned to haplotypes and the number of expected haplotypes at the smallest sample size was determined also using POPPR.

***Metarhizium* diversity in agroforestry and full sun systems**

Shannon (H_{Sh}) and Simpson (H_{Si}) information indeces were calculated to compare diversity between the both coffee management systems (AG and FS) using Vegan R Package. Haplotype diversity was compared between each area pair (A1, A2, A3) as well as between management system (AG and FS). Diversity indices were compared using a permutation test suggested by Pallmann *et al.* (2012). Tests were performed comparing each area pair to overall diversity, using the mcpHill function implemented in the SimBoot R package. *P-values* were adjusted for multiple comparisons with groups and with diversity indices (H_{Sh} and H_{Si}) (Pallmann *et al.* 2012). The indices were calculated by the total number of haplotypes recorded without distinction of species (including *M. robertsii*, *M. anisopliae* and *M. guizhouense*) and by the *M. robertsii* haplotypes.

Genetic variability of *M. robertsii*

For the subsequent analysis only the microsatellite data set from *M. robertsii* lineage (108 isolates) was used. Population genetic parameters were estimated in two scenarios: first, the isolates were assigned *a priori* to populations in accordance with the management systems (AG and FS); secondly, the clades resolved by 5'TEF phylogeny were used. Clonal fraction was calculated as proposed by Zhan *et al.* (2003),1─[(no. of different genotypes)/(total no. of isolates)]. Genotypic evenness, the measure of the distribution of genotype abundances (Grunwald *et al.* 2003), was also estimated using POPPR. Allelic richness was determined using HP-Rare, after rarefaction for the smallest sample size (Kalinowski 2005).

The clones in the data set were removed, i.e. clone corrected, for estimation of Gene diversity and linkage disequilibrium. Gene diversity (Nei 1973), was calculated in GENEPOP (Rousset 2008). The index of association (I_A) (Brown *et al.* 1980) and r_d (Agapow & Burt 2001) was assessed as a measure of linkage disequilibrium; both were calculated in POPPR at 1,000 randomizations. All these analyses, when possible, were calculated for the populations from different management systems, agroforestry (AG) and

full sun (FS). For the *M. robertsii* clades, I_A was calculated only for Clade 1 due the limitations of the small population size.

Locus-based parameters were also determined. Allelic richness was estimated in HP-Rare and Gene diversity in GENEPOP for all loci.

Population structure

Bruvo's genetic distance (Bruvo *et al.* 2004), based on stepwise mutation model, was used to estimate the relationship between haplotypes and a minimum spanning network was built in POPPR.

The R_{ST} (Goodman 1997), was used to measure differentiation between populations using Arlequin (Excoffier & Lischer 2010). Also, to detect population differentiation in the hierarchical levels of the populations, an Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) was used. Estimation of variance components was based on the sum of squares of the differences among two haplotypes. The levels of variation in AMOVA were: between management systems, among sampled fields within management systems, among clades within fields and within fields. Discriminant analyses of principal components (DAPC) (Jombart *et al.* 2010) were used to infer the number of clusters of genetically related individuals in the *M. robertsii* data set without prior knowledge. DAPC was performed in the ADEGENET package for R (Jombart & Ahmed 2011). The number of clusters (K) varied from one to 40 and the optimal K was determined based on Bayesian Information criterion (BIC).

Results

Metarhizium distribution

All sampled fields yielded bait insects infected by *Metarhizium* spp. A total of 118 isolates were recovered from all fields. Table 1 contains the number of isolates recovered in each field.

Table 1. *Metarhizium* isolates recovered from agroforestry and full-sun coffee based systems. Shown are the total number of isolates and species in each sampled field.

	Agroforestry			Full-sun			
Sampled fields	A1	A2	A3	A1	A2	A3	Total
Number of isolates	41	27	7	23	15	5	118
M. anisopliae	2	1	1	-	2	3	9
M. guizhoeunse	1	-	-	-	-	-	1
M. robertsii	38	26	6	23	13	2	108

Metarhizium species phylogeny

The alignment of 5'TEF sequences (697 bp) from the 118 *Metarhizium* isolates revealed eight haplotypes. The phylogeny based on Bayesian Inference is shown in Figure 1. The *Metarhizium* isolates grouped to three already described species: *M. robertsii*, *M. anisopliae* and *M. guizhouense* (Fig. 1). The most frequently found species *M. robertsii* was subdivided into three well supported clades and all isolates of a clade had the same 5'TEF haplotype. Clade identification was based on previous publications where the same haplotypes were reported. Clade 1 was comprised of the most common haplotype which was assigned to 101 sampled isolates. The *M. robertsii* reference isolate ARSEF 727 and the Brazilian reference isolates ESALQ 1625 and IP146 also grouped in clade 1. Clade 2, with 13 isolates, included *M. robertsii* reference ARSEF 7501, and the Brazilian isolates ESALQ 1630 and IP125. Clade 4 was the smallest *M. robertsii* group, with 4 isolates, and included the ARSEF 4739 and ESALQ 1632. One isolate, L47A, was placed at the core group of the *M. anisopliae* lineage. Isolate L47A grouped with the

ex-type isolate for this species, ARSEF 7487 and IP119 and ESALQ 1607. Isolates L47A and ESALQ 1607 had the same haplotype. The group comprised of isolates L76B, R64D, N3A, N5A, N18A, J33A, J83B and S62B included the *M. anisopliae* lineage. This group of isolates was placed out of the *M. anisopliae stricto sensu* lineage described by Bischoff *et al.* (2009), however it shares similarities with the Brazilian isolates ESALQ 1639 and IP46. The isolate J33B clustered in the *M. guizhouense* branch, although they did not share the same 5'TEF haplotype. Isolates of *M. robertsii* were sampled in all surveyed areas and were included in the most representative clade; whereas haplotypes of other species were confined to a given area or field (Fig. 1).

Mating type determination

All isolates in the *M. robertsii* clades had the MAT1-1 idiomorph (Fig. 1). All isolates of the *M. anisopliae* clade had the MAT1-1 idiomorph, but isolate L47 in which idiomorphs MAT1-1 and MAT1-2 were detected. Isolate J33B, of the *M. guizhouense* clade also had only MAT1-1.

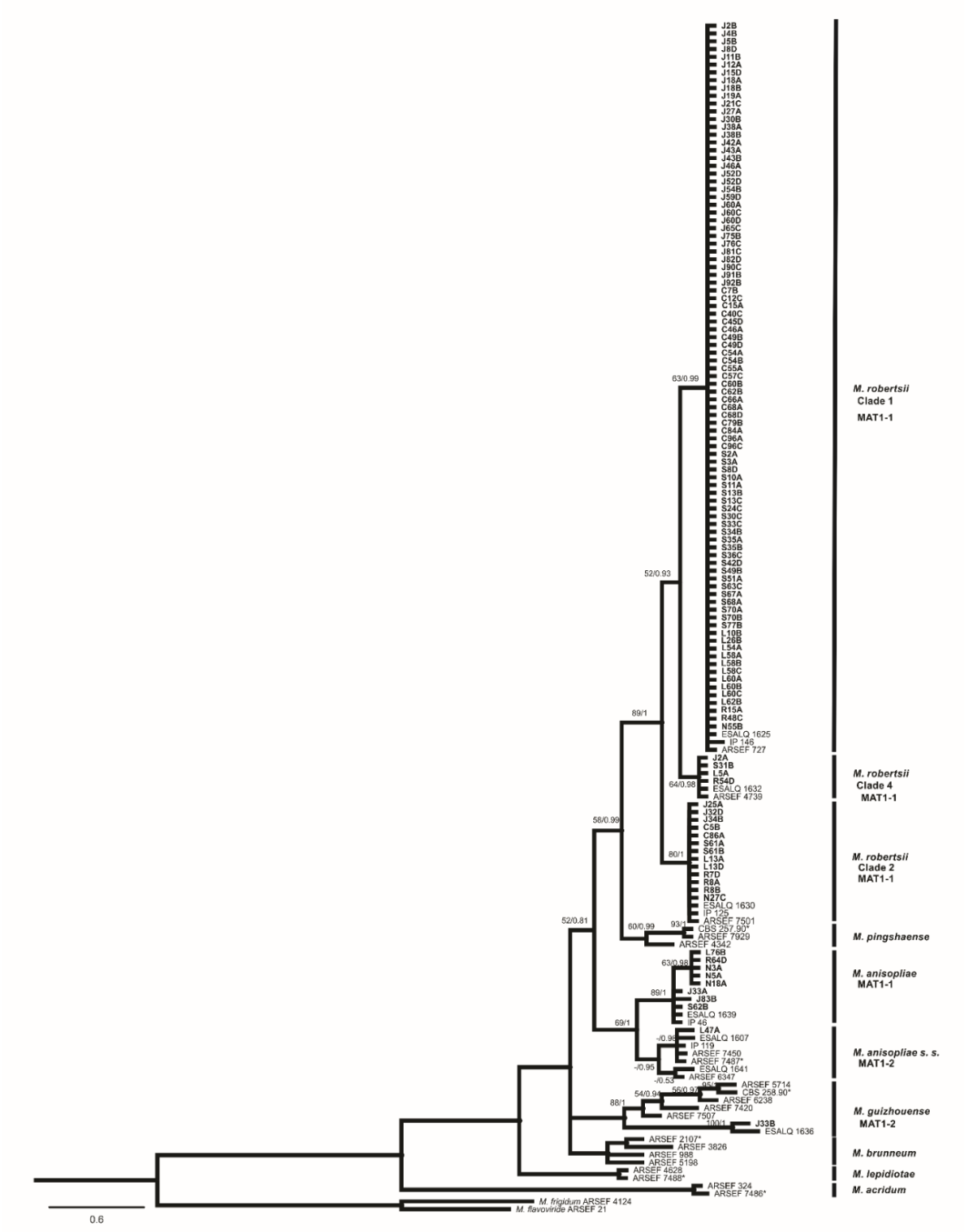

Figure 1. Bayesian analysis showing the phylogenetic relationships of *Metarhizium* species based on the 5'TEF sequence alignment. Bayesian posterior probabilities and Maximum Likelihood boostrap values are given at the nodes. The black line scale bar shows 0.6 expected changes per site. The tree was rooted with *Metarhizium flavoviridae*.

Microsatellites analysis

Isolates from all clades determined using 5'TEF were characterized using 16 microsatellites markers. The loci Ma2060, Ma2070, Ma325 and Ma2077 were excluded from analyses due the high number of null alleles for most isolates. A total of 67 haplotypes out of 118 isolates were identified and no multilocus haplotypes were shared between the clades resolved by 5'TEF. Private alleles (Table 2) were present in each *Metarhizium* lineage.

Table 2. Number of alleles and private alleles in each microsatellite locus in *M. anisopliae*, *M. guizhouense* and *M. robertsii*

Locus	*M. anisopliae*	*M. guizhoeunse*	*M. robertsii*
n[a]	9	1	108
Ma307	2/-	1/-	6/1
Ma375	2/1	1/-	1/-
Ma2283	2/1	1/-	2/1
Ma2049	2/1	1/1	5/4
Ma2069	3/1	1/1	5/2
Ma327	3/1	1/-	4/1
Ma2063	3/1	1/-	2/-
Ma2056	6/2	1/-	5/1
Ma097	2/-	1/-	5/2
Ma2089	2/1	1/1	1/-
Ma2054	4/1	1/1	6/3
Ma2296	4/2	1/-	5/3

[a]Sample size

***Metarhizium* diversity in agroforestry and full sun systems**

Differences in diversity between the management systems in each area pair were observed (Table 3). For areas 1 and 3 diversity was higher in agroforestry systems (A1: P <0.01; A3: P<0.001). However, in area 2 diversity was higher in the full sun system regardless of the index (H_{Sh} and H_{Si}) used for comparison (A2: P=0.016). When systems

were averaged across areas, diversity was higher in agroforestry than in full sun ($P<$ 0.01). The same pattern is observed for *M. robertsii* haplotypes (Table 3). Statistical differences were detected in all comparisons (Table 3).

Table 3. Management systems (AG and FS) diversity analyses using Shannon (H_{Sh}) and Simpson (H_{Si}) indices. Calculations by the total number of multilocus haplotypes from all *Metarhizium* species and by the *M. robertisii* multilocus haplotypes. P-values were adjusted P for multiple comparisons across groups.

Metarhizium spp. haplotypes		A1	A2	A3	Total
H_{Sh}	AG	2.82	2.16	1.94	3.21
	FS	2.28	2.49	1.61	3.03
	P	>0.001	>0.001	0.016	>0.001
H_{Si}	AG	0.91	0.80	0.86	0.92
	FS	0.82	0.91	0.8	0.90
	P	>0.001	>0.001	0.016	>0.001
M. robertsii haplotypes		A1	A2	A3	Total
H_{Sh}	AG	2.67	2.10	1.80	3.03
	FS	2.19	2.31	0.83	2.82
	P	0.001	0.0092	0.072	>0.001
H_{Si}	AG	0.90	0.79	0.83	0.902
	FS	0.81	0.89	0.5	0.88
	P	0.001	0.0092	0.072	>0.001

M. robertsii population parameters

All 108 isolates of *M. robertsii* were used for population analyses. Locus Ma327 displayed low gene diversity (Table 4) and considered as uninformative and excluded from the analyses; all other loci were polymorphic. Population diversity did not differ after removal of locus Ma327 (data not shown). The number of alleles at each locus varied from two to seven (Table 4). Gene diversity varied from 0.019 (Ma327) to 0.498 (Ma307). Allelic richness was also smallest for Ma327 and greatest for Ma2296. In total 57 haplotypes were identified among the 108 isolates. Haplotype 6 (Table S2.) was the most frequently detected. Thirty-one isolates were assigned to haplotype 6 which was present in both management systems. No haplotypes were shared between the *M. robertsii* clades.

Estimators of population genetic parameters vary according to management systems (Table 5). The clonal fraction was higher for AG populations. The I_A values estimated for AG (0.857) and FS (0.889) systems were similar, and revealed significant linkage disequilibrium in both subpopulations ($P<0.01$). Higher variability was detected within Clade 1 (Table 5). Isolates of clade 1 probably had the largest influence on the pattern of diversity observed according to management. Clade 1 was the most frequently sampled *M. robertsii* clade (70) occurring in both management systems. Clades 2 and 4 have small sample sizes and did not show any multilocus haplotype clones. The I_A for Clade 1 also revealed significant linkage disequilibrium.

Table 4. Information and statistics for each microsatellite locus used to characterize *Metarhizium robertsii* isolates from coffee based agroforestry (AG) and full-sun sytems (FS).

Locus[a]	Repetive sequence	No. of Alleles	Size range (bp)	Allelic Richness	Gene Diversity
Ma307	(AGG)3CTG(AAG)2GAG(AAG)4 (AGG)3	6	143 – 161	5.675	0.4898
Ma375	(AAG)2(GAAAAGAAG)2	3	140 – 158	2.5023	0.0369
Ma2283	(CT)13/(C)4G(C)4/(CTT)3	3	264 – 268	2.7504	0.1068
Ma2049	(GT)12	5	111 – 141	4.7366	0.3273
Ma2069	(AG)2(G)6(AG)2GG(AG)5/(GT)11	5	204 – 234	4.7511	0.4901
Ma327	(ACACAT)2(AC)4/(A)7G(GAA)3AAGA(AG)5/(AAG)3AG(AAG)2	4	194 - 206	3.6904	0.2480
Ma2063	(GT)11	5	134 – 150	4.0047	0.0730
Ma2056	(GT)12/(TA)4	6	134 – 170	5.4416	0.4784
Ma097	(CA)3A(AC)4(A)8	5	144 – 170	4.6289	0.1753
Ma2089	(GT)13	2	188 – 194	1.7512	0.0185
Ma2054	(GT)14/(ATAC)4/(TATG)6	7	218 – 244	6.6150	0.3728
Ma2296	(CT)8CCAT(CT)7	7	132 – 152	6.6117	0.3956

[a] Microssatellite loci published by Enkeli *et al.* (2005) and Oulevey *et al.* (2011)
[f]Allelic richness (Kalinowski, 2005)
[c]Gene diversity (Nei, 1973) among individuals within populations, averaged over all loci

Table 5. Basic population parameters, genetic diversity and multilocus linkage disequilibrium of *Metarhizium robertsii* populations from coffee based agroforestry (AG) and full-sun (FS) systems. The same set of *M. robertsii* individuals were assigned a priori to populations by management systems, AG and FS, and by the clades resolved by 5'TEF phylogeny, Clade 1, Clade 2 and Clade 4.

							Clone corrected dataset		
Population	n[a]	H[b]	$E(g_n)$[c]	Clonal Fraction[d]	E_5[e]	Allelic richness[f]	H_e[g]	I_A[h]	rd[i]
AG	70	36	22.4	0.48	0.470	3.30	0.3983	0.857**	0.088**
FS	38	26	26.0	0.31	0.471	3.60	0.4358	0.889**	0.101**
Total	108	57	24.0	0.47	0.346	4.67	0.4278	0.860**	0.089**
Clade 1	91	40	7.13	0.56	0.372	1.46	0.378	0.112**	0.111**
Clade 2	13	13	10.00	0.00	1.00	1.93	0.441	nc	nc
Clade 4	4	4	4.00	0.00	1.00	1.83	0.454	nc	nc
Total	108	57	7.78	0.47	0.346	4.67	0.428	nc	nc

[a]Population sample size.
[b] Number of haplotypes.
[c]Number of haplotypes expected for a sample of 38 and four isolates, for management and clades scenarios respectively.
[d]Clonal fraction (Zhan *et al.*, 2003).
[e]Evenness (Grünwald *et al.*, 2003)
[f]Allelic richness (Kalinowski, 2005)
[g]Gene diversity (Nei, 1973) averaged over all loci.
[h]Index of association, measure of linkage disequilibrium (Agapow & Burt, 2001)
**Significant at $P<0.001$
nc, not calculated

Population structure

The minimum spanning network (Fig 2.) did not reveal any structure related to management systems. On the other hand, the network topology was influenced by the 5'TEF Clades. Clade 1, the most commonly sampled clade, dominated the core of the diagram and Clade 2 and 4 were divided into 3 or 2 subgroups, respectively. AMOVA was carried only for the isolates of *M. robertsii*. Comparisons according to management types and areas resulted in R_{ST} values close to 0 and AMOVA showed negative variance, suggesting no population structure (Meirmans 2006).

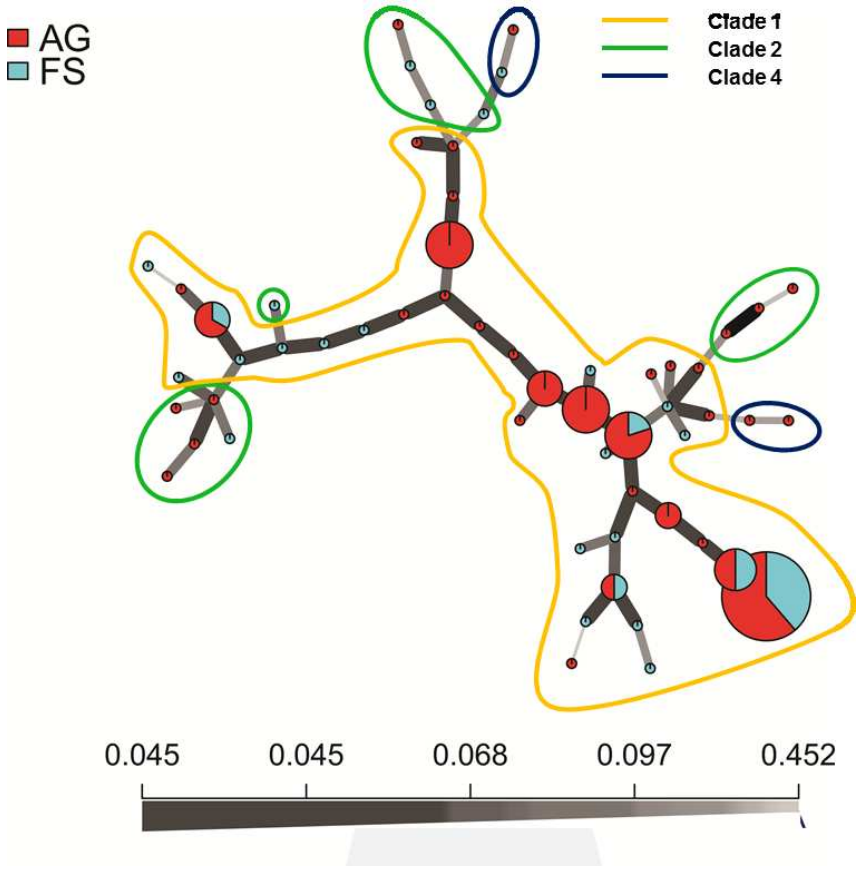

Figure 2. Minimum spanning network showing the relationship among microsatellite haplotypes of *Metarhizium robertsii* from agroforestry and full-sun coffee based systems. Each node represents a given haplotype. Node sizes and colors correspond to the number of isolates of the same haplotype and management system membership (AG &FS), respectively. Thickness and grey scale of the lines are proportional to the Bruvo's distance, thicker and darker lines correspond to smaller distances. The colors of the lines represent 5 'TEF Clade of each haplotype.

The R_{ST} values were 0.2 between Clades 1 and 2 and 0.7 between Clades 1 and 3 (Table 6). Based on the AMOVA, 48.7% of the variance was assigned to genetic differences within clades; thus, 51.3% was assigned to variance between clades (Table 7).

Table 6. Pairwise genetic differention (R_{ST}) between *Metarhizium robertsii* clades

Population	Clade 1	Clade 2	Clade 4
Clade 1			
Clade 2	0.27 **		
Clade 4	0.79 **	0.31	

*$R_{ST,}$ distance based on sum of square size difference, calculated in Arlequin.
**Significant at P<0.01

Table 7. . Analysis of molecular variance (AMOVA) for *Metarhizium robertsii* isolates from Agroforestry and Full-sun coffee based systems, variance in divided by Clade resolved by 5' TEF phylogeny

Source of variation	d.f.	Sum of squares	Variation (%)	Fixation Index	*P*
Among clades	2	89692.78	51.35		
Within clades	105	283178.64	48.65	F_{ST}= 0.51	0.03
Total	107	372871.43			

DAPC main axes explained 97.4 % of the total variability among groups, and the most likely number of clusters was K=5 (Fig. 3). The dark blue group was composed mostly by isolates from Clade 2. One Clade 1 isolates were assigned to the dark blue group. All isolates of the green group belong to Clade 1, with one exception, isolate S61A that is from Clade 2. The yellow group clustered isolates just from Clade 1. The light blue group clustered the isolates belonging to the three clades, but most of these were from Clade 4. The pink group was mostly composed of isolates of Clade 1, except for isolate R54D that is from Clade 4.

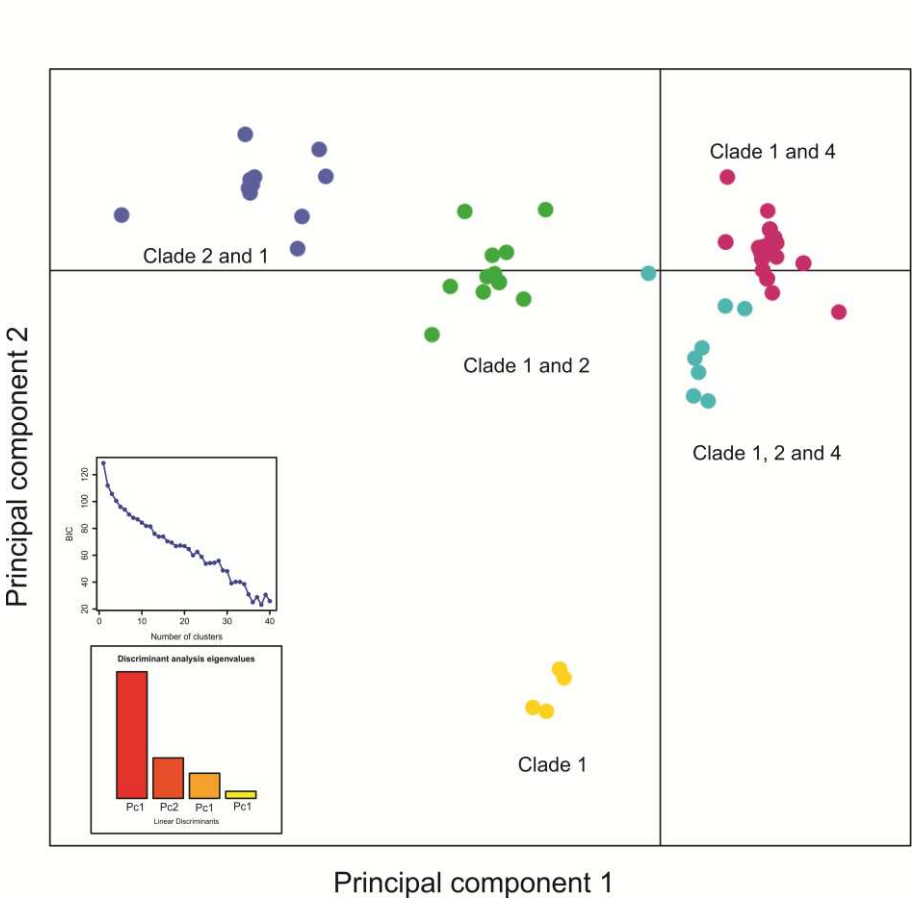

Figure 3. Scatterplot representing the discriminant analysis of principal components (DAPC) of *Metarhizium robertsii* isolates from agroforestry and full-sun coffee based systems. Each cluster is indicating by a different color. In the bottom left are the Bayesian Information criterion (BIC) and the first four principal components.

Discussion

Soil-borne entomopathogenic fungi are important components of ecosystems, but in order to properly explore the potential benefits of these microorganisms it is necessary to understand how these fungi respond to the ecological context in which they are placed and how and to what extent their multiples roles are valuable as ecosystem services. Here we combined different analytical tools, phylogenetic, SSR genotyping and mating type data analysis to characterize the composition of communities in regards to different species of *Metarhizium* and we conducted a population genetics study of the population of *M. robertsii*. Both analyses were designed to assess the effects coffee plants grown under agroforestry and full sun systems. This is the first study of *Metarhizium* community conducted in a fine-grained scale with the application of high resolution SSR markers in a tropical area.

Metarhizium community composition

Our study reveals three *Metarhizium* species: *M. robertsii* was the most prevalent species and *M. anisopliae* and *M. guizhouense* occurred at low levels. Based on other phylogenies built with Brazilian isolates collected from a broad geographical range (Rocha *et al.* 2013; Rezende *et al.* 2015) the number of clades resolved in the 5'TEF phylogeny were representative. Our findings confirm the predominant occurrence of *M. robertsii* and *M. anisopliae* in tropical areas (Carrillo-Benitez *et al.* 2013; Lopes *et al.* 2013; Rocha *et al.* 2013; Rezende *et al.* 2015) and the absence of *Metarhizium brunneum*. In surveys conducted in North hemisphere *M. brunneum* is frequently detected (Bidochka & De Koning 2001; Wyrebek *et al.* 2011; Steinwender *et al.* 2014; Kepler *et al.* 2015), however it was never recorded in southern areas (Rocha *et al.* 2013) (Carrillo-Benitez *et al.* 2013; Pérez-González *et al.* 2014; Rezende *et al.* 2015). *Metarhizium robertsii* seems to be widespread in both hemispheres and most prone to be associated to soil environment and different plant rhizosphere including engaging in endophytic association

in root (Sasan & Bidochka 2012). This species was reported in remarkably close association to plant partners is also known for its ability to transfer nitrogen from insect cadavers to plant hosts (Behie *et al.* 2012) and receiving plant photosynthetic compounds in reward (Behie *et al.* submitted).

Metarhizium species are reporteded to grow preferentially in the rhizosphere of a considerable number of plant species and some degree of "host-specificity" is observed (Fisher *et al.* 2011; Wyrebek *et al.* 2011; Liao *et al.* 2014). Here, *M. robertsii* was isolated across both agroforestry and full sun areas and no differences of its occurrence between coffee management regimes was detected. This suggests that *M. robertsii* is probably associated to coffee rhizosphere. Kepler *et al.* (2015) suggest that plant species-specific effects may influence the abundance and diversity of *Metarhizium* species both in rhizospheric and non-rhizospheric soil but under the influence of plant roots. In coffee monocultures, as the full-sun management system, coffee rhizosphere is likely to exert large influence on the soil environment. Considering that in agroforestry systems, coffee is still the most abundant plant species its rhizosphere is also likely to influence soil microbiota. Based on that, we suggest that *M. robertsii* is the main species associated to coffee plant roots. This entomopathogenic fungus was reported to be able to grow in high temperatures and is more resistant to UV exposure than other *Metarhizium* spp. (Bidochka *et al.* 2001). These aspects help explain its widespread occurrence even in full sun management systems.

In addition of being the most abundant *Metarhizium* species found, *M. robertsii* apparently has relatively high intraspecific variation. Three 5'TEF haplotypes of *M. robertsii* were found associated to three clades in the phylogeny. The diversity of *M. robertsii* lineage assessed through 5'TEF was similar to previous studies conducted in Brazil (Rocha *et al.* 2013; Rezende *et al.* 2015), Denmark (Steinwender *et al.* 2014) and United States (Kepler *et al.* 2015).

In previous works characterizing *Metarhizium* isolates in Brazil the most frequently found species was *M. anisopliae* (Rocha *et al.* 2013; Rezende *et al.* 2015), but in the current study only 9 isolates of *M. anisopliae* were found. A direct comparison between our study and the previous ones cannot be established because of major differences regarding geographical scale, isolation methods and habitat surveyed. However, general differences can at least be mentioned. Rocha *et al.* (2013) recovered isolates using *Triatoma infestans* as bait in native soil from Brazilian Cerrado habitats. On the other hand, Rezende *et al.* (2015) characterized isolates from a broad geographical range, isolated from plant pest insects infected by the fungus in sugarcane fields and soils cultivated with other crops, native ecosystems soil and commercially registered isolates. The great majority of the *M. anisopliae* described in Rezende *et al.* (2015) were isolated from infected insects, particularly grass feeding spittle bugs (Hemiptera: Cercopidae). Our results cannot be compared to those obtained by Rocha *et al.* (2013) because these authors used larvae of a distinct hemipteran insect as bait; this insect order could be more susceptible to *M. anisopliae* infections. Other studies characterizing a reduced number of isolates also recovered *M. anisopliae* isolates from live baits and infected insects other than *T. molitor* (Lopes *et al.* 2013).

The only *M. guizhouense* isolate came from an agroforestry plot. Unfortunately, only a single isolate was obtained, but we speculate that this species is more likely to be associated with soils in undisturbed ecosystems and to tree roots rather than in intensively cultivated soil. This has been demonstrated in previous works (Fisher *et al.* 2011; Wyrebek *et al.* 2011; Rezende *et al.* 2015).

Complementary to boundaries of *Metarhizium* species revealed by the phylogenetic analysis, the delimitation in all three *Metarhizium* species was highlighted by a high proportion of private SSR alleles and distinct multilocus haplotypes. The absence of any shared haplotypes between the three *M. robertsii* clades also demonstrate delimitation of the genetic entities in this group and the concordance between 5'TEF

region and multilocus haplotypes data, although SSR markers allowed a much higher resolution.

Metarhizium diversity in agroforestry and full sun systems

Greater *Metarhizium* haplotype diversity in agroforestry fields was observed in two of the three sampled areas and overall, the diversity was higher in agroforestry. This difference can be explained by factors intrinsically related to each sampled area. In the sampled areas vegetation within agroforestry systems is managed based on compatibility with coffee, biomass production, labour intensity and production diversity, however each agroforestry had a unique plant species composition (Souza *et al.* 2010). The differences in vegetation composition can also be a determinant factor in the differences observed between the agroforestry fields in each area. Also, the composition of root exudates and the microbiota associated to roots contribute to specific microorganism communities (Ratnadass *et al.* 2012), which in turn influence the overall soil microbial community. The differences in non-crop plant composition within each agroforestry fields in each area can be related to differences in *Metarhizium* abundance and diversity. Yet, the time of conversion from monoculture crop to agroforestry also has influenced the system stability, which progresses towards that of natural systems as conversion time advances (Isaac *et al.* 2005; Jose 2009). In the present study A1 agroforestry has the longest conversion time, ca. 22 years. Agroforestry systems in areas A2 and A3 are more recent, ca. 15 years.

The greatest difference in diversity between AG to FS was recorded in A1 area. The largest number of isolates were sampled in A1. Thus, as demonstrated in other studies, diversity was sample-size dependent. Expanded samplings of *Metarhizium* using different isolation methods are needed to determine whether agroforestry systems increase *Metarhizium* diversity.

Population structure of *M. robertsii*

The clonal population structure of *M. robertsii* population is evidenced by high linkage disequilibrium and the presence of a single mating type idiomorph in the population. An additional combination of factors also highlight the strong evidence of clonal structure: 84% of the isolates were attributed to only one Clade; allele diversity was mostly due to small changes in allele size and haplotypes differed from one another by one to three loci; genetic distances between individuals was small.

Clade 1 includes 84% of all isolates sampled in the study. Isolates of clade 1 were widespread along all sampled fields. One haplotype in Clade 1 was highly prevalent (35%) and others least frequent haplotypes co-occurred. Previous studies also report the prevalence of single dominant *Metarhizium* haplotype in agricultural surveyed areas (Inglis *et al.* 2008; Steinwender *et al.* 2014; Kepler *et al.* 2015).

Many haplotypes differed from each other in allele size at one or two SSR loci. Also most differences in allele size were due to small changes, ie. di- or tri-nucleotides. These differences can be attributed to the high resolution of SSR markers and the high propensity of deletions or additions in loci harboring dinucleotides repeats (Chakraborty *et al.* 1997; Lehner *et al.* 2015). These changes create new genotypes, but they are still closely related. The low distances between *M. robertsii* haplotypes can also be confirmed by the minimum spanning network topology that highlights the close relationship between then.

Clonal population structure can cause association between independent markers, generating linkage disequilibrium. *Metarhizium robertsii* population has significant linkage disequilibrium and the clonal structure was also confirmed by absence of alternative MAT idiomorphs in the three *M. robertsii* clades, preventing the occurrence of recombination. Genomic analyses of *M. robertsii* also provide evidences for its probable exclusively clonal reproduction (Gao *et al.* 2011) . There is particularly strong evidence for clonal population structure in Clade 1, a clade equivalent to Clade A reported by

Kepler *et al.* (2015), that was also fixed for the MAT1-1 idiomorph. Clade 4 in the present study is equivalent to the Clade 1 in Kepler *et al.* (2015) this clade is fixed for MAT1-1 and for MAT1-2 idiomorphs in the respectivly studies. Taking the reduced sample size of Clade 4 into account, the absence of the alternative idiomorph MAT1-2 could be a sampling bias and a bigger sample size of this clade could reveal the presence of the alternative mating type. The Clade 2 in both studies was fixed for MAT1-1. The clonal population structure resembles the experimental findings of Wang *et al.* (2011), suggesting that beneficial mutations are the explanation of the increased fitness of *Metarhizium* mutants released in the field.

The negative variance observed in AMOVA suggests no population structure (Meirmans 2006), relative to management systems. The biological interpretation for this is that, overall, alleles are more related to one another between populations than within populations. This is expected here as the three *M. robertsii* clades are present in all areas and consequently in both management systems. These results indicate a lack of genetic structure according to management types and area.

The only structure encountered in the *M. robertsii* population was due to differences between the clades. R_{ST} distances suggest differentiation between Clade 4 isolates in relation to those of Clades 1 and 2. Clusters assigned in the DAPC analyses seems to reflect 5'TEF clades, except for 1 or 2 isolates from different clades belonging to different clusters. The independent evolution of identical SSR allele sizes can explain this pattern or the SSR multilocus haplotypes of those isolates can be truly related to the groups to which they were assigned. If so the data in 5'TEF phylogeny and for SSR markers have different evolutionary histories in those isolates. The occurrence of the three *M. robertsii* clades in the absence of genetic recombination could be due to multiple migration events what is also demonstrated by the occurrence of the same 5'TEF clades in surveys conducted in north hemisphere (Steinwender *et al.* 2014; Kepler *et al.* 2015).

Limited sexual reproduction seems to be a common virulence strategy in many pathogens. Clonality enables the generation of populations well adapted to host and the environmental niches, however retaining the ability to engage in sexual or parasexual reproduction can be a key to respond to selective pressures (Heitman 2006). Reproduction through the mitotic production of haploid offspring and the exposure of a high number of spores to environmental conditions increase the efficiency of purification from deleterious alleles (Henk *et al.* 2012). The clonal propagation of *M. robertsii* through massive spore production in insect hosts and through plant root systems maximizes its occupation of the soil environment enabling the widespread distribution and the absence of recombination could be the key for its intimate mutualistic association to plant hosts.

In practical terms, the clonal population and barriers to gene flow, are compatible with release strategies of natural enemies in large-scale. The introduction of new *Metarhizium* genotype (or clone) in an already subdivided population appears to have low possibilities of hybridization between the introduced biological strain and native strains. The introduced haplotypes can have higher fitness than the indigenous population causing a reduction in those populations. The possibility of the released isolate to associate to plant roots represents an increase of its potential to persist in the environment. In one way it can provide long term biological control, but in another this can represent difficulties in eliminating the introduced agent (Wang *et al.* 2011). Considering those facts and the widespread occurrence of *Metathizium* isolates the best strategy would be the management of natural populations to achieve pest control, plant health and nutrient acquisition. Understanding the evolutionary forces that govern their distribution, diversity and their ecological interactions in natural and cultivated systems is a key to achieve those goals. Our hypothesis of increased genetic diversity in agroforestry systems was not conclusive, however this management system can still play a role in *Metarhizium* provision of ecosystem services in agricultural scenarios. A sampling strategy directed to recognizing the species-specific relationship between *Metarhizium*

genotypes and plant species that integrate agroflorestry or others diversified agricultural systems could reveal interesting results about *Metarhizium* community composition. Furthermore, this can help to determine if it is possible to achieve conservative biological control goals.

The results presented here are a step forward in understanding *Metarhizium* ecology an in agricultural scenario, especially in tropical environments and in worldwide important coffee crops. They are particularly important due to the context where the sampled areas are inserted. The agroforestry systems has been a long-term experimentation carried out by a non-governmental organization (Centre of Alternative Technologies of Zona da Mata) and local farmers since 1993 and the region is enrolled in research in various fields (Cardoso *et al.* 2001; Mendonça & Stott 2003; Souza *et al.* 2010; Souza *et al.* 2012) including the effect trees in insects natural enemies diversity (Rezende *et al.* 2014). Our results contribute to the knowledge of biodiversity in the region and reinforce the importance of the adoption of sustainable agricultural practices.

Supplementary table 1.

Species	Voucher	Genbank Number 5'TEF	Reference
M. acridium	ARSEF 324	EU248844	Bischoff *et al.*, 2009
M. acridium	ARSEF 7486*	EU248845	Bischoff *et al.*, 2009
M. anisopliae	ARSEF 6347	EU248881	Bischoff *et al.*, 2009
M. anisopliae	ARSEF 7450	EU248852	Bischoff *et al.*, 2009
M. anisopliae	ARSEF 7487*	DQ463996	Bischoff *et al.*, 2009
M. anisopliae	ESALQ 1607	KP0279611	Rezende *et al.*, 2014
M. anisopliae	ESALQ 1641	KP0279641	Rezende *et al.*, 2014
M. anisopliae	IP 119	JQ061234	Rocha *et al.*, 2013
M. brunneum	ARSEF 2107*	EU248855	Bischoff *et al.*, 2009
M. brunneum	ARSEF 3826	EU248874	Bischoff *et al.*, 2009
M. brunneum	ARSEF 5198	EU248876	Bischoff *et al.*, 2009
M. brunneum	ARSEF 988	EU248890	Bischoff *et al.*, 2009
M. flavoviride	ARSEF 2133*	DQ463988	Bischoff *et al.*, 2009
M. frigidum	ARSEF 4124*	DQ463978	Bischoff *et al.*, 2009
M. guizhouense	ARSEF 5714	EU248856	Bischoff *et al.*, 2009
M. guizhouense	ARSEF 6238	EU248857	Bischoff *et al.*, 2009
M. guizhouense	ARSEF 7420	EU248892	Bischoff *et al.*, 2009
M. guizhouense	ARSEF 7507	EU248858	Bischoff *et al.*, 2009
M. guizhouense	CBS 258.90*	EU248862	Bischoff *et al.*, 2009
M. guizhouense	ESALQ 1636	KP0279821	Bischoff *et al.*, 2009
M. lepidiotae	ARSEF 4628	EU248863	Bischoff *et al.*, 2009
M. lepidiotae	ARSEF 7488	EU248865	Bischoff *et al.*, 2009
M. pingshaense	ARSEF 4342	EU248851	Bischoff *et al.*, 2009
M. pingshaense	ARSEF 7929	EU248847	Bischoff *et al.*, 2009
M. pingshaense	CBS 257.90*	EU248850	Bischoff *et al.*, 2009
M. pingshaense	ESALQ 1639	KP0279561	Rezende *et al.*, 2014
M. pingshaense	IP 46	JQ061205	Rocha *et al.*, 2013
M. robertsii	ARSEF 4739	EU248848	Bischoff *et al.*, 2009
M. robertsii	ARSEF 727	DQ463994	Bischoff *et al.*, 2009
M. robertsii	ARSEF 7501*	EU248849	Bischoff *et al.*, 2009
M. robertsii	ESALQ 1625	KP0279741	Rezende *et al.*, 2014
M. robertsii	ESALQ 1630	KP0279721	Rezende *et al.*, 2014
M. robertsii	ESALQ 1632	KP0279761	Rezende *et al.*, 2014
M. robertsii	IP 125	JQ061241	Rocha *et al.*, 2013
M. robertsii	IP 146	JQ061236	Rocha *et al.*, 2013

Suplementary Table 2.

			Loci											
Voucher	Lineage	Haplotype	Ma307	Ma375	Ma2283	Ma2049	Ma2069	Ma327	Ma2063	Ma2056	Ma097	Ma2089	Ma2054	Ma2296
J2B	*M. robertsii* Clade 1	1	158	149	266	123	218	206	134	138	168	194	218	140
J4B	*M. robertsii* Clade 1	2	158	149	266	125	218	206	134	138	168	194	218	138
J5B	*M. robertsii* Clade 1	3	149	149	266	123	218	206	134	138	168	194	218	142
J8D	*M. robertsii* Clade 1	4	158	149	266	125	218	204	134	138	168	194	218	142
J11B	*M. robertsii* Clade 1	5	158	149	266	125	218	206	134	138	168	194	218	142
J12A	*M. robertsii* Clade 1	6	158	149	266	125	218	206	134	138	168	194	218	140
J15D	*M. robertsii* Clade 1	7	161	149	266	125	218	206	134	138	168	194	218	140
J19A	*M. robertsii* Clade 1	8	158	149	266	125	218	206	134	138	168	194	218	144
J21C	*M. robertsii* Clade 1	9	158	158	266	125	220	206	134	138	168	194	218	140
J27A	*M. robertsii* Clade 1	10	161	149	266	125	218	206	134	138	168	194	218	142
J38A	*M. robertsii* Clade 1	11	158	149	266	125	218	206	134	140	168	194	218	140
J38B	*M. robertsii* Clade 1	12	158	149	266	125	218	206	134	138	162	194	218	140
J43A	*M. robertsii* Clade 1	13	149	149	266	125	218	206	134	138	168	194	218	140
J43B	*M. robertsii* Clade 1	14	158	149	266	125	220	206	134	138	168	194	218	140
J54B	*M. robertsii* Clade 1	15	146	149	266	125	218	206	134	138	168	194	218	140
J65C	*M. robertsii* Clade 1	16	149	149	264	123	218	206	134	138	168	194	218	140
C7B	*M. robertsii* Clade 1	17	161	149	264	127	220	206	134	140	168	194	220	142
C12C	*M. robertsii* Clade 1	18	152	149	264	127	220	206	134	140	170	194	220	142
C46A	*M. robertsii* Clade 1	19	161	149	266	127	220	206	134	140	168	194	220	142
C54A	*M. robertsii* Clade 1	20	158	149	266	127	220	206	134	140	168	194	220	142
C55A	*M. robertsii* Clade 1	21	158	149	266	125	218	206	134	138	168	194	218	136
C57C	*M. robertsii* Clade 1	22	143	149	268	125	218	196	144	142	162	194	222	140
C66A	*M. robertsii* Clade 1	23	158	149	266	125	218	204	134	140	168	194	218	140

Suplementary Table 2. Continue.

Voucher	Lineage	Haplotype	Ma307	Ma375	Ma2283	Ma2049	Ma2069	Ma327	Ma2063	Ma2056	Ma097	Ma2089	Ma2054	Ma2296
C79B	*M. robertsii* Clade 1	24	158	149	266	125	218	206	134	140	168	198	224	132
S24C	*M. robertsii* Clade 1	25	161	149	266	125	218	206	134	140	168	194	218	140
S30C	*M. robertsii* Clade 1	26	143	149	266	127	204	206	134	134	168	194	218	140
S35A	*M. robertsii* Clade 1	27	158	149	266	125	218	206	134	138	168	194	218	132
S36C	*M. robertsii* Clade 1	28	158	149	266	125	204	206	134	140	144	194	218	140
S49B	*M. robertsii* Clade 1	29	158	149	266	125	218	206	134	140	168	194	218	132
S68A	*M. robertsii* Clade 1	30	158	140	266	129	220	196	142	146	168	194	230	150
L10B	*M. robertsii* Clade 1	31	158	149	266	125	218	204	134	138	168	194	218	140
L26B	*M. robertsii* Clade 1	32	158	149	266	125	218	204	134	138	168	194	218	136
L60B	*M. robertsii* Clade 1	33	158	149	266	129	218	206	134	138	168	194	218	140
L60C	*M. robertsii* Clade 1	34	158	149	266	125	216	202	134	138	168	194	218	140
L62B	*M. robertsii* Clade 1	35	158	149	266	125	204	206	134	140	168	194	218	140
R8A	*M. robertsii* Clade 1	36	158	149	266	125	204	204	134	138	170	194	230	140
R15A	*M. robertsii* Clade 1	37	158	149	266	125	204	206	134	138	168	194	218	140
R48C	*M. robertsii* Clade 1	38	149	149	264	125	204	206	138	140	168	194	218	140
N55B	*M. robertsii* Clade 1	39	158	149	266	125	204	206	134	140	144	194	220	140
C45D	*M. robertsii* Clade 1	40	161	149	266	125	220	206	134	140	168	194	218	140
C84A	*M. robertsii* Clade 1	41	161	149	264	125	220	206	134	138	168	194	218	140
J2A	*M. robertsii* Clade 4	42	143	149	266	129	208	206	150	134	168	194	218	138
S31B	*M. robertsii* Clade 4	43	149	149	266	127	216	206	134	138	168	194	230	140
L5A	*M. robertsii* Clade 4	44	149	149	266	127	218	206	134	138	168	194	226	140
R54D	*M. robertsii* Clade 4	45	158	149	266	125	204	206	134	138	168	194	218	140
J25A	*M. robertsii* Clade 2	46	161	149	266	133	204	204	134	134	170	194	216	140
J32D	*M. robertsii* Clade 2	47	161	149	266	125	216	206	134	134	168	194	222	140

Suplementary Table 2. Continue

Voucher	Lineage	Haplotype	Ma307	Ma375	Ma2283	Ma2049	Ma2069	Ma327	Ma2063	Ma2056	Ma097	Ma2089	Ma2054	Ma2296
J34B	*M. robertsii* Clade 2	48	146	149	266	125	216	204	134	134	168	194	222	140
C5B	*M. robertsii* Clade 2	49	149	149	266	123	218	206	134	136	168	194	224	138
C86C	*M. robertsii* Clade 2	50	158	149	266	125	216	204	134	134	168	194	226	140
S61A	*M. robertsii* Clade 2	51	158	149	266	125	216	206	134	134	168	194	218	140
S61B	*M. robertsii* Clade 2	52	158	149	266	125	216	204	134	134	168	194	220	144
L13A	*M. robertsii* Clade 2	53	149	149	266	125	220	206	134	138	168	194	230	138
L13D	*M. robertsii* Clade 2	54	149	149	266	125	220	204	134	140	168	194	230	140
R7D	*M. robertsii* Clade 2	55	158	149	266	125	220	204	134	140	170	194	230	140
R8B	*M. robertsii* Clade 2	56	158	149	266	125	204	206	134	138	170	194	230	140
N27C	*M. robertsii* Clade 2	57	158	149	266	125	218	204	134	142	148	194	224	140
J33A	*M. anisopliae*	58	158	149	266	141	204	196	142	170	170	198	238	148
J83B	*M. anisopliae*	59	161	149	266	141	208	206	142	134	170	198	226	148
S62B	*M. anisopliae*	60	161	158	266	141	212	196	142	158	170	198	238	140
L47A	*M. anisopliae*	61	161	149	268	125	220	196	144	142	162	194	222	140
L76B	*M. anisopliae*	62	161	149	266	141	208	206	134	138	162	198	222	140
R64D	*M. anisopliae*	63	161	149	266	141	204	194	144	134	162	198	216	138
N3A	*M. anisopliae*	64	161	149	266	141	204	194	134	134	162	198	226	152
N5A	*M. anisopliae*	65	161	149	266	141	208	194	150	134	162	198	216	140
N18A	*M. anisopliae*	66	161	149	266	141	204	194	134	134	162	198	222	140
J33B	*M. guizhouense*	67	143	149	266	111	234	202	150	136	168	186	244	138

References

Agapow P.M. & Burt A. (2001). Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, 1, 101-102.

Behie S.W. & Bidochka M.J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and Environmental Microbiology*, 80, 1553-1560.

Behie S.W., Moreira C.M., Sementchoukova I., Barelli L., Zelisko P.M. & Bidochka M.J. (submitted). Trading insect nitrogen for photosynthate: Carbon translocation from a plant to an insect pathogenic, endophytic fungus. *Science*.

Behie S.W., Zelisko P.M. & Bidochka M.J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science*, 336, 1576-1577.

Bidochka M.J. & De Koning J. (2001). Are teleomorphs really necessary?: modelling the potential effects of Muller's Ratchet on deuteromycetous entomopathogenic fungi. *Mycological Research*, 105, 1014-1019.

Bidochka M.J., Kamp A.M., Lavender T.M., Dekoning J. & De Croos J.N.A. (2001). Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: Uncovering cryptic species? *Applied and Environmental Microbiology*, 67, 1335-1342.

Bischoff J.F., Rehner S.A. & Humber R.A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 101, 512-530.

Bommarco R., Kleijn D. & Potts S.G. (2013). Ecological intensification: harnessing ecosystem services for food security. *Trends in Ecology & Evolution*, 28, 230-238.

Brown A.H.D., Feldman M.W. & Nevo E. (1980). Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics*, 96, 523-536.

Bruvo R., Michiels N.K., D'Souza T.G. & Schulenburg H. (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, 13, 2101-2106.

Cardinale B.J., Duffy J.E., Gonzalez A., Hooper D.U., Perrings C., Venail P., Narwani A., Mace G.M., Tilman D., Wardle D.A., Kinzig A.P., Daily G.C., Loreau M., Grace J.B., Larigauderie A., Srivastava D.S. & Naeem S. (2012). Biodiversity loss and its impact on humanity. *Nature*, 486, 59-67.

Cardoso I.M., Guijt I., Franco F.S., Carvalho A.F. & Neto P.S.F. (2001). Continual learning for agroforestry system design: university, NGO and farmer partnership in Minas Gerais, Brazil. *Agricultural Systems*, 69, 235-257.

Carrillo-Benitez M.G., Guzman-Franco A.W., Alatorre-Rosas R. & Enriquez-Vara J.N. (2013). Diversity and genetic population structure of fungal pathogens infecting white grub larvae in agricultural soils. *Microb Ecol*, 65, 437-49.

Chakraborty R., Kimmel M., Stivers D.N., Davison L.J. & Deka R. (1997). Relative mutation rates at di-, tri-, and tetranucleotide microsatellite loci. *Proceedings of the National Academy of Sciences*, 94, 1041-1046.

Enkerli J., Kolliker R., Keller S. & Widmer F. (2005). Isolation and characterization of microsatellite markers from the entomopathogenic fungus *Metarhizium anisopliae*. *Molecular Ecology Notes*, 5, 384-386.

Excoffier L. & Lischer H.E.L. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10, 564-567.

Excoffier L., Smouse P.E. & Quattro J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131, 479-91.

Fang W. & St. Leger R.J. (2010). *Mrt*, a gene unique to fungi, encodes an oligosaccharide transporter and facilitates rhizosphere competency in *Metarhizium robertsii*. *Plant Physiology*, 154, 1549-1557.

Fisher J.J., Rehner S.A. & Bruck D.J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology*, 106, 289-295.

Foley J.A., DeFries R., Asner G.P., Barford C., Bonan G., Carpenter S.R., Chapin F.S., Coe M.T., Daily G.C., Gibbs H.K., Helkowski J.H., Holloway T., Howard E.A., Kucharik C.J., Monfreda C., Patz J.A., Prentice I.C., Ramankutty N. & Snyder P.K. (2005). Global consequences of land use. *Science*, 309, 570-574.

Gao Q., Jin K., Ying S.-H., Zhang Y., Xiao G., Shang Y., Duan Z., Hu X., Xie X.-Q., Zhou G., Peng G., Luo Z., Huang W., Wang B., Fang W., Wang S., Zhong Y., Ma L.-J., St. Leger R.J., Zhao G.-P., Pei Y., Feng M.-G., Xia Y. & Wang C. (2011). Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet*, 7, e1001264.

Goodman S.J. (1997). R_{ST} Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Molecular Ecology*, 6, 881-885.

Grunwald N.J., Goodwin S.B., Milgroom M.G. & Fry W.E. (2003). Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology*, 93, 738-746.

Heitman J. (2006). Sexual reproduction and the evolution of microbial pathogens. *Current Biology*, 16, R711-R725.

Henk D.A., Shahar-Golan R., Devi K.R., Boyce K.J., Zhan N., Fedorova N.D., Nierman W.C., Hsueh P.-R., Yuen K.-Y., Sieu T.P.M., Kinh N.V., Wertheim H., Baker S.G., Day J.N., Vanittanakom N., Bignell E.M., Andrianopoulos A. & Fisher M.C. (2012). Clonality despite sex: The evolution of host-associated sexual neighborhoods in the pathogenic fungus *Penicillium marneffei. PLoS Pathog*, 8, e1002851.

Hepperle D. (2010). DNA Dragon 1.4. 1–DNA Sequence Contig Assembler Software. URL Available at: www. dna-dragon. com.

Hu G. & St Leger J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology*, 68, 6383-6387.

Inglis G.D., Duke G.M., Goettel M.S. & Kabaluk J.T. (2008). Genetic diversity of *Metarhizium anisopliae* var. anisopliae in southwestern British Columbia. *Journal of Invertebrate Pathology*, 98, 101-113.

Isaac M.E., Gordon A.M., Thevathasan N., Oppong S.K. & Quashie-Sam J. (2005). Temporal changes in soil carbon and nitrogen in west African multistrata agroforestry systems: a chronosequence of pools and fluxes. *Agroforest Syst*, 65, 23-31.

Jha S., Bacon C.M., Philpott S.M., Ernesto Méndez V., Läderach P. & Rice R.A. (2014). Shade coffee: Update on a disappearing refuge for biodiversity. *BioScience*, 64, 416-428.

Jombart T. & Ahmed I. (2011). ADEGENET 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics*, 27, 3070-3071.

Jombart T., Devillard S. & Balloux F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11, 94.

Jose S. (2009). Agroforestry for ecosystem services and environmental benefits: an overview. *Agroforest Syst*, 76, 1-10.

Kalinowski S.T. (2005). HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, 5, 187-189.

Kamvar Z.N., Tabima J.F. & Grünwald N.J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ*, 2, e281.

Kepler R.M., Ugine T.A., Maul J.E., Cavigelli M.A. & Rehner S.A. (2015). Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology*, 17, 2791–2804.

Keyser C.A., Thorup-Kristensen K. & Meyling N.V. (2014). Metarhizium seed treatment mediates fungal dispersal via roots and induces infections in insects. *Fungal Ecology*, 11, 122-131.

Lehner M.S., Paula Júnior T.J., Hora Júnior B.T., Teixeira H., Vieira R.F., Carneiro J.E.S. & Mizubuti E.S.G. (2015). Low genettic variability in *Sclerotinia sclerotiorum* populations from commom bean fields in Minas Gerais State, Brazil, at regional, local and micro-scales. *Plant Pathology*, 64, 921-931.

Liao X., O'Brien T., Fang W. & St. Leger R. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Appl Microbiol Biotechnol*, 98, 7089-7096.

Lopes R.B., Souza D.A., Oliveira C.M. & Faria M. (2013). Genetic diversity and pathogenicity of *Metarhizium* spp. associated with the white grub *Phyllophaga capillata* (Blanchard) (Coleoptera: Melolonthidae) in a soybean field. *Neotropical Entomology*, 42, 436-438.

Matson P.A., Parton W.J., Power A.G. & Swift M.J. (1997). Agricultural intensification and ecosystem properties. *Science*, 277, 504-509.

Meirmans P.G. (2006). Using the AMOVA framework to estimante a standardized genetic differentiation measure. *Evolution*, 60, 2399-2402.

Mendonça E.S. & Stott D.E. (2003). Characteristics and decomposition rates of pruning residues from a shaded coffee system in Southeastern Brazil. *Agroforest Syst*, 57, 117-125.

Meyling N.V. & Eilenberg J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control*, 43, 145-155.

Moreira C.C., Celestino D., Guerra T.G., Cardoso i.M. & Elliot S.L. (submitted). Ecosystem services offered by entomopathogenic fungi: Agroforestry coffee soils are more insect-suppressive than Full-sun soils. *Submitted to Journal of Invertebrate Pathology*.

Nei M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, 70, 3321-3323.

Nylander J.A.A. (2004). MrModeltest v2. Program distributed by the author. In. Uppsala University 2 Evolutionary Biology Centre,.

Oulevey C., Widmer F., Kolliker R. & Enkerli J. (2009). An optimized microsatellite marker set for detection of *Metarhizium anisopliae* genotype diversity on field and regional scales. *Mycological Research*, 113, 1016-1024.

Pallmann P., Schaarschmidt F., Hothorn L.A., Fischer C., Nacke H., Priesnitz K.U. & Schork N.J. (2012). Assessing group differences in biodiversity by simultaneously testing a user-defined selection of diversity indices. *Molecular Ecology Resources*, 12, 1068-1078.

Pattemore J., Hane J., Williams A., Wilson B., Stodart B. & Ash G. (2014). The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. *BMC Genomics*, 15, 660.

Pérez-González V.H., Guzmán-Franco A.W., Alatorre-Rosas R., Hernández-López J., Hernández-López A., Carrillo-Benítez M.G. & Baverstock J. (2014). Specific diversity of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. *Journal of Invertebrate Pathology*, 119, 54-61.

Ratnadass A., Fernandes P., Avelino J. & Habib R. (2012). Plant species diversity for sustainable management of crop pests and diseases in agroecosystems: a review. *Agron. Sustain. Dev.*, 32, 273-303.

Rezende J.M., Zanardo A.B.R., Lopes M.D., Delalibera I. & Rehner S.A. (2015). Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. *Biocontrol*, 60, 495-505.

Rezende M.Q., Venzon M., Perez A.L., Cardoso I.M. & Janssen A. (2014). Extrafloral nectaries of associated trees can enhance natural pest control. *Agriculture, Ecosystems & Environment*, 188, 198-203.

Rocha L.F.N., Inglis P.W., Humber R.A., Kipnis A. & Luz C. (2013). Occurrence of *Metarhizium* spp. in Central Brazilian soils. *Journal of Basic Microbiology*, 53, 251-259.

Ronquist F. & Huelsenbeck J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572-1574.

Rousset F. (2008). GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*, 8, 103-106.

Sasan R.K. & Bidochka M.J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany*, 99, 101-107.

Shah P.A. & Pell J.K. (2003). Entomopathogenic fungi as biological control agents. *Appl Microbiol Biotechnol*, 61, 413-423.

Souza H., Cardoso I., Fernandes J., Garcia F.P., Bonfim V., Santos A., Carvalho A. & Mendonça E. (2010). Selection of native trees for intercropping with coffee in the Atlantic Rainforest biome. *Agroforest Syst*, 80, 1-16.

Souza H.N.d., de Goede R.G.M., Brussaard L., Cardoso I.M., Duarte E.M.G., Fernandes R.B.A., Gomes L.C. & Pulleman M.M. (2012). Protective shade, tree diversity and soil properties in coffee agroforestry systems in the Atlantic Rainforest biome. *Agriculture, Ecosystems & Environment*, 146, 179-196.

St. Leger R.J. & Screen S. (2001). Prospects for strain improvement of fungal pathogens of insects and weeds. In: *Fungal biocontrol agents: progress, problems and potential* (eds. Butt TM, Jackson C & Morgan N). CAB International Wallingford, UK, pp. 219-238.

Steinwender B.M., Enkerli J., Widmer F., Eilenberg J., Kristensen H.L., Bidochka M.J. & Meyling N.V. (2015). Root isolations of *Metarhizium* spp. from crops reflect diversity in the soil and indicate no plant specificity. *Journal of Invertebrate Pathology*, 132, 142-148.

Steinwender B.M., Enkerli J., Widmer F., Eilenberg J., Thorup-Kristensen K. & Meyling N.V. (2014). Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. *Journal of Invertebrate Pathology*, 123, 6-12.

Swofford D.L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*.

Tilman D., Cassman K.G., Matson P.A., Naylor R. & Polasky S. (2002). Agricultural sustainability and intensive production practices. *Nature*, 418, 671-677.

Tscharntke T., Clough Y., Bhagwat S.A., Buchori D., Faust H., Hertel D., Hölscher D., Juhrbandt J., Kessler M., Perfecto I., Scherber C., Schroth G., Veldkamp E. & Wanger T.C. (2011). Multifunctional shade-tree management in tropical agroforestry landscapes – a review. *Journal of Applied Ecology*, 48, 619-629.

Wang S., O'Brien T.R., Pava-Ripoll M. & St. Leger R.J. (2011). Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations. *Proceedings of the National Academy of Sciences*, 108, 20449-20454.

Wyrebek M. & Bidochka M.J. (2013). Variability in the insect and plant adhesins, *Mad1*and *Mad2*, within the fungal genus *Metarhizium* suggest plant adaptation as an evolutionary force. *PLoS ONE*, 8, e59357.

Wyrebek M., Huber C., Sasan R.K. & Bidochka M.J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology*, 157, 2904-2911.

Zhan J., Pettway R. & McDonald B. (2003). The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics ans Biology*, 38, 286-97.

Zimmermann G. (1986). The '*Galleria* bait method' for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology*, 102, 213-215.

CHAPTER 3

DIVERSITY OF *METARHIZIUM* SPECIES IN THE RHIZOSPHERE OF COFFEE AND NON-CROP PLANTS IN AN AGROFORESTRY SYSTEM

Abstract

Understanding the diversity of the insect-pathogenic and plant symbiont fungus of the genus *Metarhizium* in plant-diverse agroecosystems could give important insights to exploit its ecological functions and to establish management strategies. In this study we aimed to determine *Metarhizium* species diversity and the prevalence in soil and the rhizosphere of coffee and non-crop plants in coffee cultivation under a diversified agroforestry system. *Metarhizium* was isolated using selective media from five groups of plants, including: coffee, trees, banana, spontaneous monocotyledons and dicotyledons plants. Coffee, trees and spontaneous monocotyledons harbored the greatest amount of total and *Metarhizium* colony forming units. *Metarhizium* diversity revealed by 5'TEF showed four species: *M. robertsii*, *M. anisopliae*, *M. brunneum* and *M. pemphigi*. *Metarhizium robertsii* was the most frequent species, isolated in all the groups of samples. *Metarhizium pemphigi* was the second most abundant species and was isolated mostly from coffee roots, indicating probable ecological specialization of this fungus species to coffee plants. The plant-rhizosphere association can increase *Metarhizium* persistence and population levels in the soil. Here we presented great abundance and diversity of *Metarhizium* in plant roots in a diversified agroecological system where these fungi may play important roles. Also, *M. pemphigi* could be explored as a preferential coffee symbiont to estabilish management strategies and the development of seed treatments.

Introduction

The biggest challenge faced by agriculture in this century is to increase agricultural yield while limiting impact to the environment or even encouraging natural resource preservation (Tilman *et al.* 2002). This challenge is in accordance with the ecological intensification perspective; this can be defined in a broad sense as the understanding of natural functions so as to exploit resources without destroying then, increasing yield, and breaking with practices based on intensive use of pesticides, chemical fertilizers, water and fossil fuels (Bommarco *et al.* 2013). In the current world scenario this approach can be ultimately the solution for food production for present and especially for future generations (Gaba *et al.* 2014). However, to apply the principles of ecological intensification, it is necessary to have a deep understanding of the interactions between crops, pests, diseases and, in particular, to understand how biodiverse environments can encourage mutualistic interactions that can be converted into crucial ecological services (Bommarco *et al.* 2013).

The maintenance of plant diversity is one of the keys to support multiple ecosystem functions and consequently ecosystem services (Isbell *et al.* 2011). Besides the services provided by each plant species by itself, plant diversity can also favor the abundance and diversity of natural enemies (Tscharntke *et al.* 2005; Tscharntke *et al.* 2007) and the recruitment of diversified rhizospheric microbiota (Berendsen *et al.* 2012). Agroforestries are plant diversified agricultural production systems in which trees are managed together with the crops. The incorporation of trees diversifies and sustains production in order to increase social, economic and environmental benefits for land users at all scales (Leakey 2014). Coffee agroforestry has important consequences for biodiversity conservation in agricultural settings (Perfecto *et al.* 2005; Williams-Guillén *et al.* 2006; Tscharntke *et al.* 2015) besides the more recent open market for increased quality products obtained from shade-grown coffee (Loureiro & Lotade 2005).

Many studies have found that agroforestries maintain a level of biodiversity that is considerably higher than other agricultural systems but generally a little lower than that of natural forests (Leakey 2014). Most of those studies consider the diversity of plant, natural enemies and wildlife species (Bali *et al.* 2007; Lozada *et al.* 2007; Caudill *et al.* 2013; De la Mora *et al.* 2015; Smith *et al.* 2015). However, the diversity of the belowground microbiota is still neglected, despite its complexity and crucial importance to proper functioning of the agroecosystem (Leakey 2014).

Beneficial rhizosphere microorganisms play a crucial role in natural and cultivated ecosystem functioning by influencing plant physiology and development (Berendsen *et al.* 2012). These microorganisms can have an important role in plant nutrient acquisition (Behie & Bidochka 2014a; Hacquard *et al.* 2015) and defense against abiotic (Coleman-Derr & Tringe 2014) and biotic challenges (Mendes *et al.* 2011; Berendsen *et al.* 2012). The potential of key rhizosphere microorganisms such as arbuscular mycorrhiza (Pellegrino & Bedini 2014; Yang *et al.* 2014) and plant growth promoting rhizobacteria (Sayyed *et al.* 2012; Nehra & Choudhary 2015) are already considered and valued as important providers of ecosystem services to agricultural systems, especially in low-input cropping schemes. The increase of plant diversity in agricultural contexts is also considered a key factor for building up a beneficial population of rhizosphere microorganism without the need for direct inoculation of specific microorganisms (Ratnadass *et al.* 2012). In addition, different plant species have specific rhizosphere exudates that can harbor different microbial communities. In this way a variety of plant can provide different ecological niches encouraging microbial diversity (Ratnadass *et al.* 2012). Nevertheless, many groups of microorganisms are still not considered or their diversity and potential to deliver ecosystems services in agricultural settings are not well-known. Few studies have addressed the microbial diversity in coffee agroforestry systems to date, especially fungal diversity. Entomopathogenic fungi from coffee agroforestry soils have been reported to be more

infective to soil insects when compared to coffee monoculture full-sun soils (Moreira *et al.* submitted). Also, three species of *Metarhizium* and a variety of *Metarhizium robertsii* genotypes were also reported in agroforestry coffee soils (Moreira *et al.* in prep.).

Entomopathogenic fungi of the genus *Metarhizium* have long being recognized as insect pathogens (Roberts & St. Leger 2004) . However, recent studies found that the insect pathogenic ability is not the only role played by these fungi (Behie *et al.* 2012; Sasan & Bidochka 2012, 2013). *Metarhizium* species are also able to associate symbiotically with plant roots, as rhizosphere competent (Hu & St Leger 2002) or endophytic simbionts (Sasan & Bidochka 2012), and transfer nutrients from insect cadavers (Behie *et al.* 2012; Behie & Bidochka 2014b). It has also been hypothesized that they can act as plant bodyguards against insect pests (Elliot *et al.* 2000).

The genus *Metarhizium* is distributed in natural and agricultural soils worldwide (Roberts & St. Leger 2004) and its association with various plant types and taxa has already been observed (Fisher *et al.* 2011; Wyrebek *et al.* 2011; Khan *et al.* 2012; Behie & Bidochka 2014b; Steinwender *et al.* 2015). Research indicates that the abilities to associate and transfer nitrogen to plant hosts are widespread in the genus (Behie & Bidochka 2014b). Some studies indicate that *Metahizium* species may present plant-rhizosphere-specificity (Fisher *et al.* 2011; Wyrebek *et al.* 2011), however others indicate no specificity (Steinwender *et al.* 2015). Plant-rhizosphere specificity of *Metarhizium* species could be an important trait driving enhanced persistence and growth in the rhizosphere of preferential hosts leading to enhanced pest control, nutrient transfer and control of insect pests. Plant-host specificity could also drive increased diversity of *Metarhizium* species in plant diversified agricultural systems like coffee agroforestry due to the opportunity for different fungal species to associate with a variety of plant hosts. This knowledge is crucial to understand the relationships of these fungi in agroforestry coffee ecosystem and may lead to strategies of ecological intensification that maintain or improve the services delivered by them.

Here we aimed to investigate if the diversity of plant species in the coffee crop under a diversified agroforestry system can influence *Metarhizium* species diversity, i.e. if different plant species recruit specific *Metarhizium* species and influence in the abundance of these species. For this we sampled entomopatogenic fungi from different categories of plants, comprising coffee bushes, banana plants, woody trees, and spontaneous plants, and soil from a single field of coffee cultivation. We accounted for the abundance of *Metarhizium* colonies in the plant root system and for the identity of the fungi in the plant roots.

Material and Methods

Root and soil sampling

Roots were sampled from a single coffee field that has been under agroforestry, organic management since 1994 (Cardoso *et al.* 2001), within the Atlantic Rainforest Biome in the municipality of Araponga, Minas Gerais, southeastern Brazil (20° 48´ S and 42° 32´ W). The location was chosen because it has a great variety of trees and spontaneous herbs between coffee rows. The field also presented a great variety *of Metarhizium robertsii* haplotypes in soil samples in our previous study (Moreira *et al.* in prep.). One hundred and fifty plant roots were collect randomly in a rough grid of 12 planted rows of approximately 30m. Thirty root samples were sampled from each plant type, including: coffee bushes, woody trees, banana plants, spontaneous monocotyledons and spontaneous dicotyledons plants. The plant types were classified by plant habit in trees, spontaneous monocotyledons and spontaneous dicotyledons plants; plant species are presented in Table 1. Thirty soil samples were also taken in the same sampling scheme with the help of a core soil sampler to 20cm depth.

Roots were pulled out from soil with a shovel; soil excess was removed and they were placed in pre-identified plastic bags. For spontaneous monocotyledonous and dicotyledonous spontaneous plants the entire root system was taken, for coffee bushes, banana and trees, lateral roots coming from large roots were taken. For each plant, pictures were taken and a foliage sample was collected for plant identification. In the laboratory roots were refrigerated (5°C) until the following day when they were processed. All the samples were processed within one week.

Table 1. Plant taxa sampled in each plant group and the number of samples collected from each taxa. The number of positive sample for and Metarhizium species are indicated.

Category	Plant taxa	Number of samples	N° of *Metarhizium* positive samples	*Metarhizium* specie
Coffee	*Coffea* sp.	30	22	*M. robertsii clade* 1, *M. robertsii* clade 2, *M. pemphigi*

Tree	*Aegephila sellowiana*	2	1	*M. robertsii* clade 1
	Apidosperma pollineurum	1	0	
	Carica papaya	1	0	
	Cariniana sp.	1	0	
	Ceiba speciosa	2	2	*M. robertsii* clade1
	Inga sp.	8	7	*M. robertsii* clade1, *M. brunneum*
	Joannesia princeps	1	1	*M. robertsii* clade1
	Mangifera indica	4	1	*M. pemphigi*
	Persea sp.	7	1	*M. robertsii* clade1
	Rollinia dolabripetala	1	1	*M. robertsii* clade1
	Solanum mauritianum	2	2	*M. robertsii* clade 1, *M. pemphigi*
Banana	*Musa* sp.	30	7	*M. robertsii* clade1, *M. anisopliae*
Spontaneous monocots	*Commelina benghalensis*	27	20	*M. robertsii* clade 1, *M. robertsii* clade 4, *M. anisopliae*, *M. pemphigi*
	Eleusine indica	3	2	*M. robertsii* clade 1, *M. pemphigi*
Spontaneous dicots	*Ageratum conyzoides*	3	0	
	Amaranthus deflexus	2	1	*M. robertsii* clade 1
	Arachis pintoi	3	2	*M. robertsii* clade 1
	Cela asiatica	1	0	
	Conyzia canadensis	3	1	*M. robertsii* clade 1
	Emila fosbergii	4	0	
	Galinsoga parviflora	3	1	*M. robertsii* clade 1
	Galinsoga quadrilata	2	0	
	Oxalis corniculata	2	0	
	Spermacoceae latifolia	1	1	*M. robertsii* clade 1
	Sonchus oleraceus	4	0	
	Urena lobata	2	1	*M. robertsii* clade 2

Fungal isolation

Roots were shaken by hand to remove most of the soil particles. They were not washed, in order to keep the soil directly adhered to the root as representative of the rhizosphere. Five grams of roots were ground with mortar and pestle and then diluted in 15 ml of sterile distilled water solution of Tween 0.01% in a Falcon® tube. Tubes were rotated for one hour in a rotary shaker at 150 rpm. For soil samples, 5g of the sample were diluted in 15ml of sterile distilled water solution of Tween 0.01% and the same procedure used for root samples was used. Entomopathogenic fungi from the order Hypocreales were isolated from the root suspensions in selective medium with two concentrations of the active ingredient CTAB (cetyl trimethylammonium bromide), 0.35g l^{-1} and 0.175g l^{-1}, setting up half strength and quarter strength CTAB media, respectively (Posadas *et al.* 2012; Kepler *et al.* 2015). Soil samples were only plated in half strength CTAB media. The basic composition for the two media was: 10g peptone L^{-1}, 20g glucose L^{-1}, 0.6g streptomycin L^{-1}, 0.05g cycloheximide L^{-1}, 0.05 tetracycline L^{-1}. From the suspension, 200 µl were plated in two Petri dishes (9 cm diameter) for each medium type. Plates were kept at 26 °C for 20 days in the dark. Colony forming units (CFU) were counted from each plate and the results were recorded according to the plant group. *Metarhizium* colonies were isolated in PDA (39 g of potato dextrose agar L^{-1}) to obtain pure cultures. In plates in which different colony morphologies were present, a representative of the morphologies was selected and isolated in PDA in different Petri dishes.

Statistical analyses of CFU counting

All statistical analyses were performed with R software version 3.2.0 (R Development Core Team 2015). The total and *Metarhizium* number of CFU recovered were analyzed using generalized linear models (GLM). Numbers of CFUs recovered from plant samples on each media type were analyzed separately. All dependent variables were accorded quasipoisson distributions. Prior to all analyses, we checked for data overdispersion and

residual analyses were carried out to determine model acceptability and error distribution. Analyses were all ANOVAs with χ^2 tests (Crawley 2007). The mean number of colonies from each plant host was compared through multiple contrasts of means using the function 'glht' implemented in the R package MULTCOMP.

The number of positive samples for *Metarhizium* per plant host type was also analyzed through GLM. The number of positive samples for *Metarhizium* constituted the response variable and the plant host the explanatory variable. Data overdispersion and residual analyses were also carried out to determine model acceptability and error distribution. The response variable was accorded a binomial distribution and analyzed by ANOVAs with χ^2 tests. The percentage of samples positive for *Metarhizium* from each plant host was also compared through multiple contrasts of means using the function 'glht' implemented in the R package MULTCOMP. CFU counting and frequency of positive samples from soil samples were excluded from the analysis because there was only isolate in the half strength media.

DNA extraction

From each pure culture, single-spore cultures were obtained. A small amount of conidia were suspended in 1 ml of 0.01% water solution of Tween 80. An aliquot of 100 µl of the suspension was spread in PDA plates and incubated for 12h at 25°C. A single germinating conidium was transferred into a new PDA plate. Conidia from each single-spore culture were inoculated into 20 ml of liquid medium (2.5g peptone l^{-1}, 2.5g yeast extract l^{-1} and 10g dextrose l^{-1}) in a 3.5 cm diameter Petri dish and left to grow at 26 °C for 5 days. Fungal mycelium was washed with distilled sterile water then transferred to a filter paper to dry. Dry mycelium was ground in 2 ml tube with two 5 mm steel balls using a mechanical Tissueliser (QIAGEN Ltd.) at 30 Hz for 2 minutes. From the ground mycelium genomic DNA were extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). DNA integrity was visualized by ultraviolet

fluorescence on 1% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in a 1× TBE in an 1% agarose gel. DNA concentrations were adjusted to 25ng/µL in a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

PCR amplification, sequencing and phylogenetic analyses

Metarhizium isolates were assigned to species by amplification of 5'TEF using the primers EF1T (5'ATGGGTAAGGARGACAAGAC) and EF2T (5'GGAAGTACCAGTGATCATGTT), according to previously described conditions (Rehner & Buckley 2005; Bischoff *et al.* 2006). PCR products were visualized by ultraviolet fluorescence on 1% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in a 1XTBE and checked for amplification size. PCR products were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). Sequences were edited using DNA Dragon software (Hepperle 2010). Genbank sequences of 5'TEF from *Metarhizium* ex-type isolates (Bischoff *et al.* 2009; Kepler *et al.* 2014) and from previous Brazilian studies (Rocha *et al.* 2013; Moreira *et al.* in prep.) were include in the analyses to elucidate phylogenetic relationship among species (Table S1.). Sequences were aligned by Muscle v.3.6. in the software Mega 6.0 (Tamura *et al.* 2013).

Phylogenetics analysis

The 5'TEF sequence data were analyzed by Maximum Likelihood conducted with Paup v. 40b10 (Swofford 2003) and Bayesian Inference conducted with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). For both analyses the DNA sequence evolution model was established based on the Akaike information criterion (AIC) and likelihood ratio of MrModelTest (Nylander 2004). The HKY+I model (Hasegawa-Kishino-Yano with a proportion of invariable sites) was used for the 5'TEF ML and Bayesian analysis.

Heuristic ML bootstrap analysis consisted of 100 pseudoreplicates (TBR branch swapping). For Bayesian Inference a parallel run, consisting of four chains, was subjected to Markov Chain Monte Carlo (MCMC) analysis until the runs converged with a split frequency of <0.01. The MCMC analysis started with a heating parameter 0.1 from a random tree topology and lasted 10,000,000 generations. Trees were saved every 1,000 generations, resulting in 10,000 saved trees. The first 2,500 trees were discarded as the burn-in phase. The phylogenetic tree was visualized using FigTree v. 1.4 23 (http://tree.bio.ed.ac.uk/software/figtree). The species *M. flavoviridae* was used to root the tree. *Metarhizium* 5'TEF genotypes were linked specifically to the host plant species or to the plant type.

Results

Total colony-forming units

A negative effect of the increasing CTAB concentration was observed on the total number of colonies formed after 20 days of growth ($\chi^2_{[298]}$= 9858.5, P<0.001). Fewer colonies were formed (40% fewer) in half strength CTAB media (17.96± 1.92 cfu/200µl; mean±SE), than quarter strength media (30.26± 3.24 cfu/200µl). Within half strength CTAB medium, spontaneous monocotyledons (MN) harbored the greatest amount of colonies (37.86± 5.08 cfu/200µl; P<0.001) and was significantly different from the amount of colonies harbored for all the other hosts (Fig. 1A). Within the quarter strength CTAB medium, coffee (CF) and MN plants harbored the highest numbers of colonies, (57.83± 8.06 cfu/200µl and 40.03±7.48 cfu/200µl, respectively), and did not differ significantly (P=0.29) from each other (Fig. 1A). Banana (BN) and trees (TR) also presented a greater amount of colonies (21.13± 3.74 cfu/200µl and 30.13± 8.61 cfu/200µl, respectively) in the quarter strength medium but it did not differ from spontaneous dicotyledons (DC) samples that harbored the smallest number of colonies (2.16± 1.18 cfu/200µl; P= 0.23) (Fig.1A). Colonies of *Metarhizium* spp., *Beauveria* spp., *Isaria* spp. and *Pochonia chlamidospora* were isolated.

Metarhizium colony-forming units

A negative effect of the increasing CTAB concentration on the amount of *Metarhizium* colonies was observed after 20 days of growth ($\chi^2_{[298]}$= 2914.4, P=0.007). Quarter strength CTAB medium harbored more *Metarhizium* colonies then half strength CTAB medium (3.92± 1.21 cfu/200µl and 1.24± 0.19 cfu/200µl, respectively). Within half strength CTAB medium, the mean number of *Metarhizium* colonies recovered from the different plant roots ranged from 2.8 to 0.3. MN samples harbored the greatest amount of *Metarhizium* colonies (2.8±0.56 cfu/200µl) and it was not significantly different (P=0.26) from the number of colonies recovered from TR samples (1.63± 0.44

cfu/200µL). BN and DC samples yielded the smallest amount of *Metarhizium* colonies in half strength CTAB medium (0.3±0.13 cfu/200µl and 0.43± 0.24 cfu/200µl; P=0.99, respectively). Within the quarter strength CTAB medium the greatest amount of *Metarhizium* colonies was recovered from CF (11.73± 5.38 cfu/200µl), besides of only differing statistically from the number of colonies recovered from BN (0.30± 0.15 cfu/200µl; P<0.01) and DC (0.20 ± 0.14 cfu/200µl, P<0.01). TR and MN samples harbored 3.8± 2.14 cfu/200µl (P=0.22) and 3.5± 1.18 cfu/200µl (P=0.18) *Metarhizium* colonies respectively, and did not differ from the number of colonies yielded by CF samples.

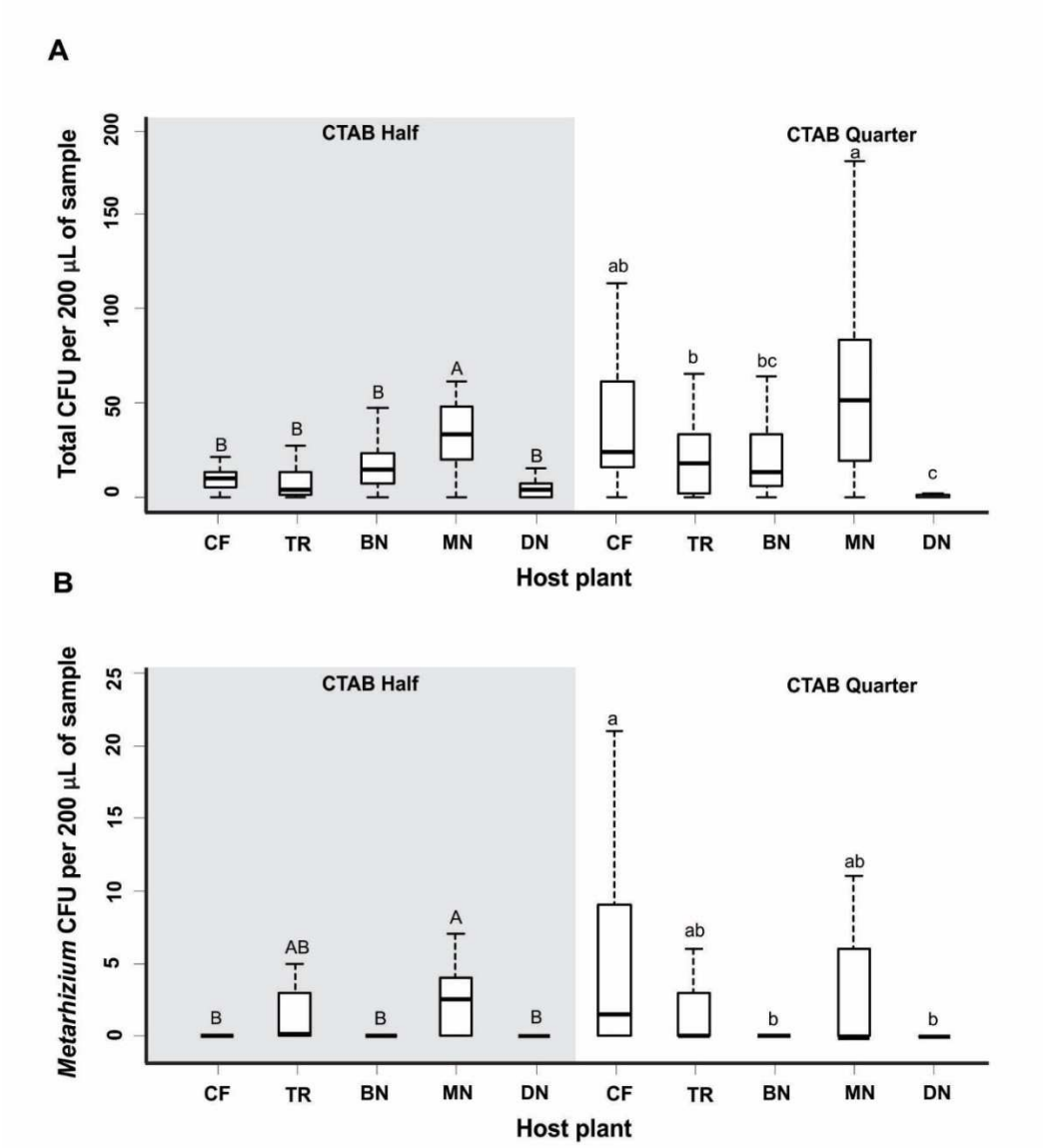

Figure 1. Box plots showing the number of (A) total colony-forming units and (B) *Metarhizium* spp. colony forming units (cfu) from the different groups of samples plants(CF=Coffee, TR=Trees, BN=Banana, MN=Spontaneous monocotyledons, DN=Spontaneous dicotyledons) recovered on CTAB selective media at two concentrations, half (0.35 mg l-1; gray area) and quarter (0.0175mg l-1, white area) strengh. Lower and upper boxes represent the first and third quartile, respectively, their intersection is the median, and the whiskers represent the minimum and maximum cfu values. Significance levels are indicated as letters; uppercase letters represent the differences between values in half strength media; lowercase indicated the differences between values in quarter strength media as $P<0.05$.

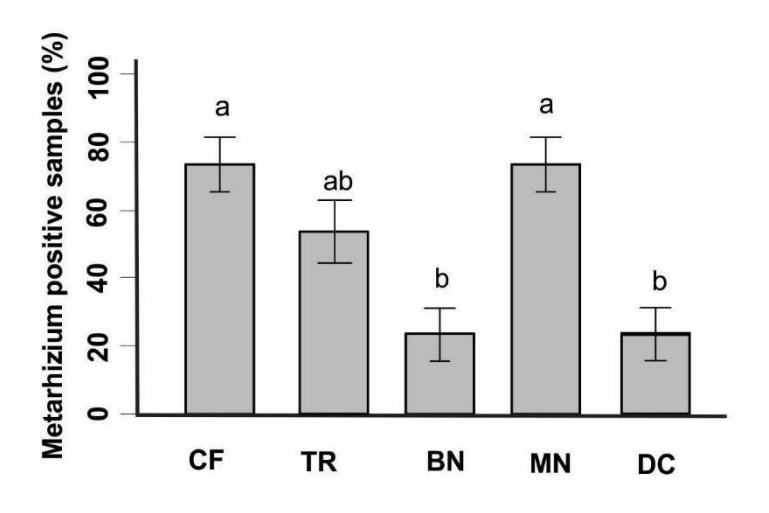

Figure 2. Percentage (Mean±SE) of root samples positive for *Metarhizium* from the different groups of sampled plant roots (CF=Coffee, TR=Trees, BN=Banana, MN=Spontaneous monocotyledons, DN=Spontaneous dicotyledons) recovered on CTAB selective media. Significance levels are indicated as letters; uppercase letters represent the differences between values in half strength media; lowercase indicate the differences between values in quarter strength media as $P<0.05$.

Molecular taxonomic assignment of *Metarhizium* isolates

A total of 134 *Metarhizium* isolates were recovered from plant hosts roots and soil. Seven haplotypes were revealed by the alignment of *Metarhizium* 5'TEF sequences (726 bp). Phylogenetic analyses using Maximum Likelihood (ML) and Bayesian methods on 5'TEF dataset resulted in reconstructed trees with similar topologies (Fig. 3). Four *Metarhizium* species were recovered: *M. robertsii*, *M. anisopliae*, *M. brunneum* and *M. pemphigi*. The most common species was *M. robertsii*, representing 67% of the isolates (90 isolates). *Metarhizium robertsii* branch harbored three different 5'TEF haplotypes. These different haplotypes paired with previous described 5'TEF *M. robertsii* clades: Clade 1, Clade 2, Clade 4 (Kepler *et al.* 2015) comprising 86, 2 and 2 isolates respectively . The second most abundant species was *M. pemphigi*, 40 isolates, representing 29.8% of the total. *Metarhizium pemphigi* haplotype paired closely with the Brazilian isolate IP143 (Rocha *et al.* 2013), and with the reference isolate ARSEF 6569 (Kepler *et al.* 2014). Three *M. anisopliae* isolates were recovered, two of these paired with the reference isolate ARSEF 7487 and the isolate BN_43 was placed outside the *M.*

anisopliae core group. Only one isolate of *M. brunneum* was recovered and it paired with the reference isolate ARSEF 988.

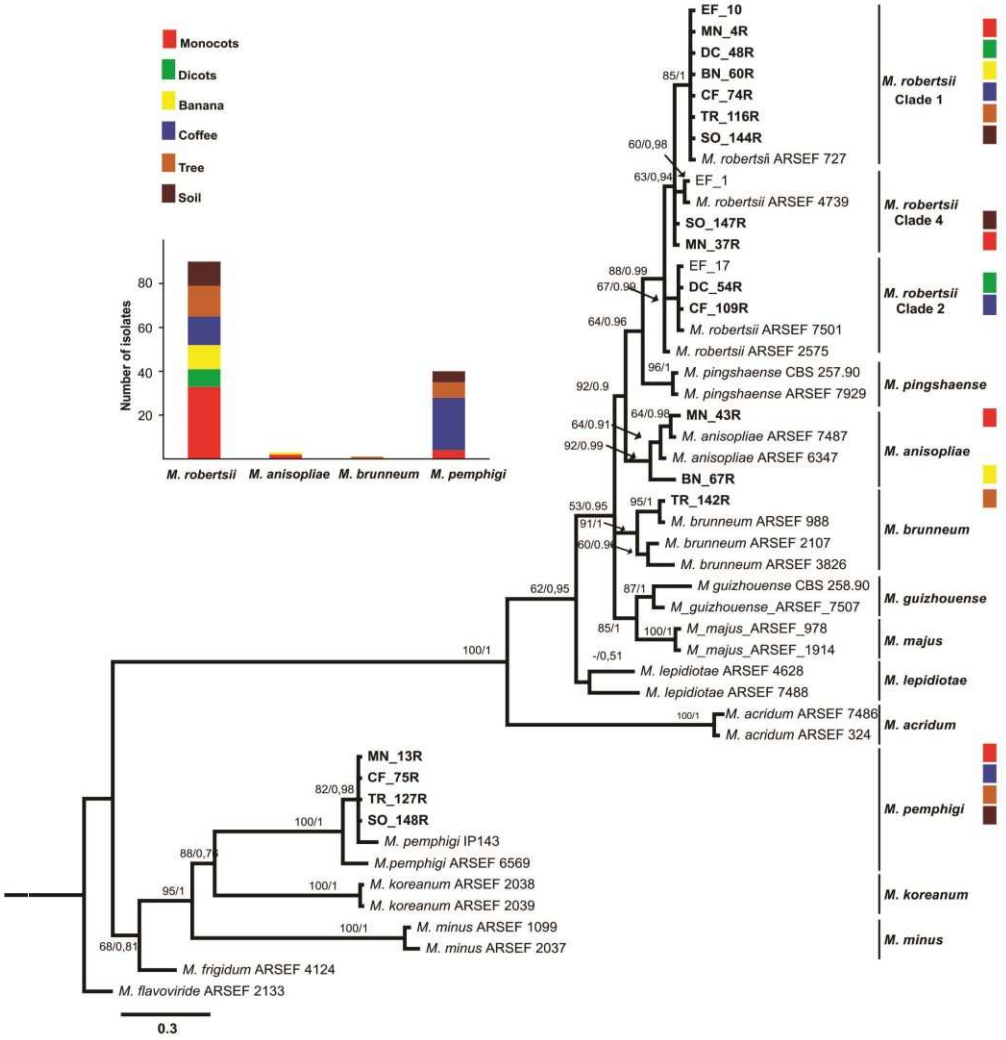

Figure 3. Bayesian consensus tree showing the phylogenetic relationships of *Metarhizium* lineages isolated from different groups of plant roots based on the 5'TEF (Translation elongation factor) sequence alignment. Bayesian consensus tree and the tree obtained from maximum likelihood (ML) analyses showed very similar topologies. Number at the tree nodes are posterior probabilities support values from Bayesian analyses (fraction to 1) and Maximum likelihood (ML) bootstrap support value (integer 10 to 100), respectively. The black line scale bar shows 0.6 expected changes per site. The tree was rooted with *Metarhizium flavoviridae*. Colored boxes indicated the group of plant where fungal isolate was recovered. Plot indicated the number of isolates belonging to each *Metarhizium* species and the color also indicates the precedence of the isolate.

***Metarhizium* species and plant hosts**

Metarhizium colonies were recovered from all the plant groups of plants sampled. In coffee rhizosphere *Metarhizium* was isolated from 73% of the samples and 37 isolates were obtained (Fig. 2). *Metarhizium pemphigi* was the most frequently found species in coffee samples (24 isolates), followed by *M. robertsii* clade 1 (12 isolates) and a single isolate of *M. robertsii* clade 2 (Fig. 4). *Metarhizium* were recovered from 53% of the root samples from tree hosts, that was not different from coffee and spontaneous monocotyledons samples (P= 0.55; Fig.2). A total of 22 isolates were recovered from tree hosts belonging to *M. robertsii* clade 1(14 isolates), *M. pemphigi* (7 isolates) and *M. brunneum* (1 isolate) (Fig. 4). *Metarhizium robertsii* clade 1 was found in 7 of the 8 sampled *Inga* sp. (Fabaceae) trees (Table 1). The single *M. brunneum* isolate was also found in an *Inga* sp. sample (Table 1).

Spontaneous monocotyledons root samples also harbored 73% of *Metarhizium* occurrence, and 39 isolates were recovered (Fig. 4). Spontaneous monocotyledon roots also presented the greatest variety of *Metarhizium* lineages: *M. robertsii* clade 1(32 isolates), *M. robertsii* clade 4 (1 isolate), *M. anisopliae* (2 isolates) and *M. pemphigi* (4 isolates) (Fig 4.). Between the sampled plants in this category 27 of then belonged to the species *Commelina benghalensis* (Commelinaceae) and 20 of them yielded *Metarhizium* colonies with all the clades listed above (Table 1). The remaining monocotyledon spontaneous samples belonged to the species *Euleusine indica* (Poacea) and presented association with *M. robertsii* clade 1 and *M. pemphigi* (Table 1). Banana and spontaneous dicotyledon root samples presented the same frequency of *Metarhizium* positive samples, 23%, that was not different from tree samples (P= 0.12; Fig. 2). Twelve isolates were recovered from banana samples including *M. robertsii* clade 1 (11) and *M. anisopliae* (1) (Fig. 4). Spontaneous dicotyledon samples harbored 8 isolates including *M. robertsii* clade 1 and *M. robertsii* clade 2 (Fig. 4). Of the 11 different spontaneous dicotyledonous taxa sampled 6 yielded *Metarhizium* colonies (Table 1).

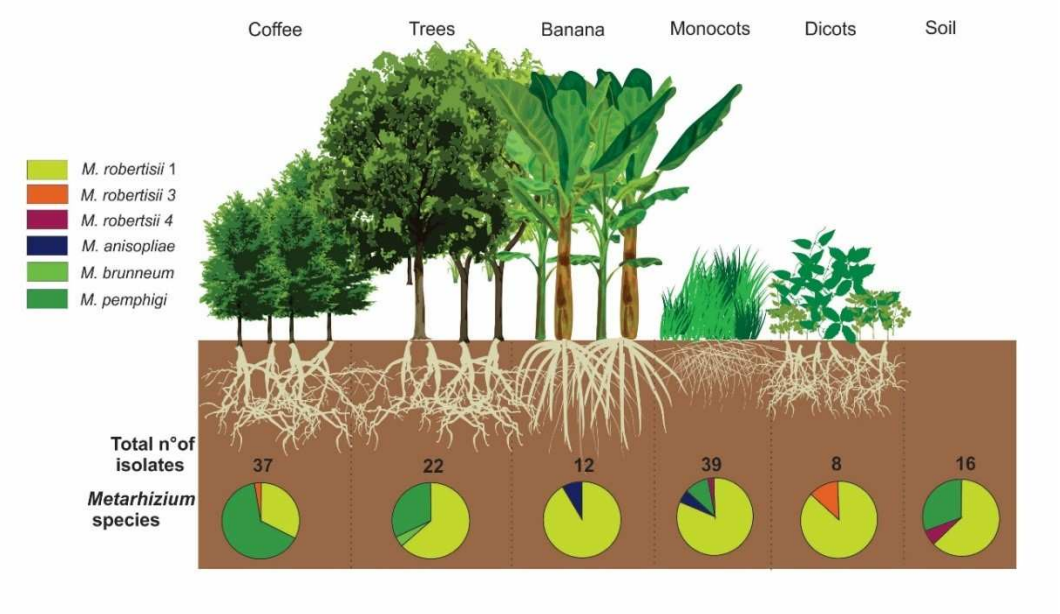

Figure 4. The sampled groups of plants and the *Metarhizium* species recovered. Numbers indicate the number of *Metarhizium* isolates recovered from each group of plant roots and soil. The pie charts represented the proportion of each *Metarhizium* species that was recovered from each plant group or soil.

Discussion

The development of strategies to exploit the potential of naturally occurring *Metarhizium* species in the ecological intensification approach may provide great benefits to cultivated plants. However, to achieve this goal it is crucial to understand the association between these fungi and plants in agricultural environments in order to obtain a realistic picture of their distribution and abundance in particular agricultural systems and their association with plants. Here, we describe the abundance and diversity of *Metarhizium* species associated with the soil and the rhizosphere of plants in a coffee crop managed under organic agroforestry conditions.

Entomopathogenic fungi were found in the rhizosphere of all plants and in the soil with both media types. The fungicide dodine (dodecyl guanidine monoacetate) is the most common selective agent used to isolate those fungi from soil (Chase *et al.* 1986; Liu *et al.* 1993), however this active ingredient is frequently difficult to find or expensive. CTAB is a aliphatic nitrogen-containing compound with a structure close to that of dodine, Posadas et al. (2012) proposed and tested its use to replace dodine in selective media. Kepler et al. (2015) tested both substances to isolate *Metarhizium* from soil and they present closely equivalent results. Here we found that the quarter strength CTAB concentration were more efficient than half strength media to isolate *Metarhizium* and other hypocrealean entomopathogens. Despite this, the differences in the total amount of colonies yielded in both media, the total number of colonies presented by each individual plant type followed the same pattern. Discrepancies were observed in the number of *Metarhizium* colonies from monocotyledonous samples in half strength medium yielded more colonies than in quarter strength. When using selective media a small amount of sample is used and this sample is still diluted, decreasing the chances of finding entomopathogenic fungi, in this way, the discrepancies observed could be attributed to those characteristics. However, the method has the advantages of providing quantitative data (number of CFU), that can be submitted to parametrical statistical analysis. Despite

the disadvantages of the small diluted samples it still can provide evidence of how widespread these fungi are in the samples, because even in a small diluted sample a considerable amount and variety of entomopathogenic fungi colonies can be isolated. In this study, we plated 200 µl of the homogenate, prepared from the roots and soil dilute in water, representing only 66 mg of sample.

Coffee, trees and spontaneous monocotyledons root samples presented the greatest amount of *Metarhizium* positive samples; banana and spontaneous dicotyledons presented the smallest amounts. *Metarhizium* colonies recovered in the root samples could came from inside the root, the root surface or from fine soil particles closely adhered to the root, however *Metarhizium* can associate with plants as an endophyte and through its rhizosphere competence, which includes the fungal growing in the rhizosphere. The rhizosphere establishes a zone of specific climatic conditions and nutrient supply in the soil and different plant species have specific rhizosphere exudates that and can harbor different microbial communities (Bais *et al.* 2006; Broeckling *et al.* 2008). The differences observed in the frequency of the samples colonized by *Metarhizium* can be related to the differences in the rhizosphere of each plant.

Four *Metarhizium* species were recovered, with *M. robertsii* being the prevalent species; *M. pemphigi*. *Metarhizium anisopliae* and *M. brunneum* were isolated in low frequencies in the coffee system. *Metarhizium robertsii* branch was divided in three different clades, with clade 1 the most abundant and clades 2 and 4 also classified as rare. In a previous study conducted in the same area (Area 1) using *Tenebrio mollitor* as bait insect in soil samples*, M. robertsii* was the most frequent species, representing 38 of 41 isolates (92.8%), and both *M. anisopliae* and *M. guizhouense* were found in smaller frequencies (Moreira *et al.* in prep.). In the present study *M. robertsii* is still the most abundant species, however at a lower frequency than previously described. *Metarhizium guizhouense* was absent and *M. anisopliae* was recovered at the same frequency as presented in the previous study. The great difference here is the presence of *M. pemphigi*

in a considerable number; this difference can be attributed to both the different isolation method and to the preference of this species to plant roots. This is the second record of this species in Brazil (Rocha *et al.* 2013), however this is the first study to report this species in a great abundance in a single environment in plant rhizosphere. *Metarhizium pemphigi* belongs to the *M. flavoviridae* species complex and was formally recognized as species in 2014 (Kepler *et al.* 2014), being named previously as *M. flavoviridae var. pemphigi*. The presence of *M. brunneum* is another unexpected finding, as its distribution was considered restricted to temperate areas, this species was not reported until now in surveys in tropical areas (Sanchez-Pena *et al.* 2011; Rocha *et al.* 2013; Rezende *et al.* 2015) and was abundant in surveys in temperate environments in North Hemisphere (Bidochka *et al.* 2001; Wyrebek *et al.* 2011; Steinwender *et al.* 2014). Metarhizium brunneum can grow under low temperatures and has the ability to survive freezing conditions. Bidochka et al. (2001) demonstrated that this specie is cold active, presents reduced growth at high temperatures and is more sensitive to UV radiation. Our results shows that this species has a more wide range of distribution than expected and more surveys in South hemisphere applying the same isolation method can reveal if this species is coomom in those environments.

In Brazil, surveys from *Metarhizium* in soils from Brazilian savanna and sugar cane fields found that the predominant species was a clade close to *M. anisopliae sensu stricto*, represented in the present study by the 2 isolate (MN_43R and MN_44R) from spontaneous monocotyledons samples, and *M. robertsii* was the second most abundant species. In North hemisphere temperate environments, *M. robertsii* and *M. brunneum* seems to dominate soil communities. Using selective media for isolation from soil suspensions Kepler et al. (2015) reported *M. robertsii* being the most abundant species followed by *M. brunneum* in agricultural fields under different managements in Maryland, USA. In Denmark, *M. brunneum* was reported as the most abundant species in agricultural fields in soil samples using *Tenebrio mollitor* as bait insect (Steinwender *et*

al. 2014) and in cultivated plants roots using dodine selective media (Steinwender *et al.* 2015).

Considering the association of *Metarhizium* species to plant types, *M. robertsii* was present in all the plant types and comprised the majority of the isolates recovered from trees, banana, spontaneous monocotyledons plants and soil. In spontaneous dicotyledons, *Metarhizium robertsii* was the single species recovered, with most of the isolates belonging to clade 1 and one to clade 2. Wyrebek and colleagues (2011) also reports *M. robertsii* as the most frequently found species in the rhizosphere of a wider range of plant species, however it was most frequently found in roots of grasses and wildflowers. In the present study, this species was recovered in two of the sampled grasses and was mostly found in the rhizosphere of spontaneous monocotyledons, which might reflect a preference of *M. robertsii* for this plant type. Despite the small frequency, this is the first time that naturally occurring *M. anisopliae* has been recovered from plant roots. The isolate BN_67R, belonging to the clade close to *M. anisopliae*, was already reported in soils from the same area and was very frequent in soil surveys in Brazil (Rocha *et al.* 2013; Rezende *et al.* 2015). The isolates MN_44R and MN_43R, belonging to *M. anisopliae s. s.*, were recovered from monocots spontaneous plants and the isolates from the group close to *M. anisopliae* was recovered from banana roots. The single isolate of *M. brunneum* was isolated from an Inga tree (*Inga* sp.) roots. This species was previously isolated solely from roots of trees and shrubs in Canada (Wyrebek *et al.* 2011). Fisher et al. (2011) also report this species as the most prevalent in strawberry roots and Steinwender et al. (Steinwender *et al.* 2015) reported this species as widespread in roots of oats, rye and cabagge.

Metarhizium pemphigi was isolated from the rhizosphere of coffee, trees, spontaneous monocotyledons and soil, being the second most frequently found *Metarhizium species*. This species was mostly isolated from coffee roots and present in trees, monocots spontaneous plants and soil in a small frequency. Its absence in our

previous study in the same area (Moreira *et al.* in prep.) might indicate its low frequency in soil samples or can be an artefact of the different isolation method used. Coffee roots harbored the majority of the *M. pemphigi* isolates followed by tree samples that also harbored a considerable amount of the isolates. *Metarhizium pemphigi* was already reported in the rhizosphere of Christmas Trees and strawberry in small frequencies (Fisher *et al.* 2011).

The amount of colonization by *M. pemphigi* in coffee roots could mean that this species presents specificity to coffee plants. Specificity to hosts is a questionable subject in *Metarhizium* species; considering its specificity to insect hosts some species in the genus are accounted as specialists to an insect group (*M. acridum*), while most of the them are considered generalists, presenting a broad host range. Regarding plant specificity, Wyrebeck et al. (2011) reported plant-rhizosphere specificity of *M. robertsii* to grasses by its frequency in their roots and complemented by *in vitro* experiments where *M. robertsii* conidia germinated better in grass exudate. In the same study, they also reported that *M. guizhouense* and *M. brunneum* are more frequent in roots of trees and shrubs, and absent in grasses and wildflowers. In the present study trees were colonized by *M. robertsii*, *M. pemphigi* and *M. brunneum*. Endophytic and nutrient transfer capability seems to be widespread in the genus and different species also show varying levels of endophytic association and nutrient transfer in different plant species (Behie & Bidochka 2014b). The specificity of a given species of *Metarhizium* to a specific plant group or its prevalence in a certain ecosystem of a geographic region could be referred as "ecological specialization" (Futuyma & Moreno 1988), wherein a species show a preferential host in the ecological context while retaining the physiological ability to associate with other hosts. As ecological context we refer to all the biotic (e.g. the different neighboring host species, interspecific competition, attraction to root exudates and ability to overcome host defenses) and abiotic conditions (e.g. soil characteristics,

climatic conditions and the environment created by rhizosphere). This phenomenon is commonly observed in arbuscular mycorrhiza (McGonigle & Fitter 1990).

Of the sampled plants, coffee plants, Inga trees (*Inga* sp.) and Benghal dayflower (*Commelina bengalhesis*) showed distinctive levels of colonization by *Metarhizium*. Few studies have addressed fungal diversity in coffee rhizospheres. However it is known that the presence of trees, especially leguminous trees, can influence quantity and quality of coffee root exudates, consequently stimulating microbial activity and nutrient cycling (Munroe *et al.* 2015). Muleta et al. (2007) observed higher abundance of arbuscular mycorrhizal spores in the rhizosphere of coffee associated with trees than in monoculture, with highest abundance in leguminous trees. Inga trees are leguminous nitrogen-fixing trees that can provide higher rates of N mineralization in coffee systems (Hergoualc'h *et al.* 2008) and also can enhance natural pest control in coffee crops due the attraction of insect natural enemies by its extrafloral nectaries (Rezende *et al.* 2014). *Commelina benghalensis* is a common weed in coffee plantations and is reported as an alternative host for nematodes (Wilson 1981), here we reported that this plant can also act as a reservoir of entomopathogenic fungi.

The association of *Metarhizium* with the plant rhizosphere can increase its persistence (Hu & St Leger 2002), population levels in soil (Bruck 2010; Wang *et al.* 2011; Klingen *et al.* 2015) and also influence the control of soil pests (Bruck 2005; 2010; Batta 2013; Keyser *et al.* 2014; Peña-Peña *et al.* 2015). Our study presented great abundance and diversity of *Metarhizium* species associated to plants roots, especially in coffee, trees and monocotyledonous spontaneous plants, and these fungi may play important roles in this system. Taking into account the apparent preference of *M. pemphigi* for coffee roots, the particular effects of this species on coffee pests and its potential benefits to coffee, as plant growth promoter, nutrient transfer and antagonist of microbial pathogens, can give important insights into how to explore ecosystem services provided by this particular species. Studying *Metarhizium* from coffee roots from

different regions and different management types could show if the preference of this species to coffee roots is widespread, and may enable the development of seed treatments for coffee seeds with *M. pemphigi* boosting the establishment of plants and its own populations in the field. Future studies also should explore how the different species of *Metarhizium* found in the plant roots can contribute to mortality of coffee insect pests, which could give important information regarding the management of non-crop plants in agroforestry coffee systems. Coffee agroforestries are management systems that can contribute to the recuperation of soils and the restoration of deforested areas. Improving the productivity and the sustainability of those areas may guarantee that more farmers could adopt the strategies. Our results show the diversity of a genus of insect pathogenic and plant symbiont fungus in a single coffee agroforestry ecosystem in the Atlantic Forestry biome, where strategies which couple productivity and conservation are urgent and essential. Future research considering naturally occurring *Metarhizium* species in agricultural ecosystems could lead to a better understanding and exploitation of their roles as ecosystem services providers and therefore to a more sustainable food production.

Supplementary table 1. Information about the isolates from Genbank sequences used to construct 5'TEF phylogeny

Species	Voucher	Genbank Number 5'TEF	Reference
M. acridium	ARSEF 324	EU248844	Bischoff *et al.*, 2009
M. acridium	ARSEF 7486*	EU248845	Bischoff *et al.*, 2009
M. anisopliae	ARSEF 6347	EU248881	Bischoff *et al.*, 2009
M. anisopliae	ARSEF 7487*	DQ463996	Bischoff *et al.*, 2009
M. brunneum	ARSEF 2107*	EU248855	Bischoff *et al.*, 2009
M. brunneum	ARSEF 3826	EU248874	Bischoff *et al.*, 2009
M. brunneum	ARSEF 988	EU248890	Bischoff *et al.*, 2009
M. flavoviride	ARSEF 2133*	DQ463988	Bischoff *et al.*, 2009
M. frigidum	ARSEF 4124*	DQ463978	Bischoff *et al.*, 2009
M. guizhouense	CBS 258.90*	EU248862	Bischoff *et al.*, 2009
M. guizhouense	ARSEF 7507	EU248858	Bischoff *et al.*, 2009
M. koreanum	ARSEF 2038	KJ398805	Kepler et al.,2014
M. koreanum	ARSEF 2039	KJ398806	Kepler et al.,2014
M. lepidiotae	ARSEF 4628	EU248863	Bischoff *et al.*, 2009
M. lepidiotae	ARSEF 7488	EU248865	Bischoff *et al.*, 2009
M. majus	ARSEF 978	EU248889.1	Bischoff *et al.*, 2009
M. majus	ARSEF 7488	KJ398801.1	Kepler et al.,2014
M. minus	ARSEF 1099	KJ398799	Kepler et al.,2014
M. minus	ARSEF 2037	DQ463979	Bischoff *et al.*, 2009
M. pemphigi	IP 143	JQ061180.1	Rocha et al. 2013.
M. pemphigi	ARSEF 6569	KJ398813.1	Kepler et al.,2014
M. pingshaense	ARSEF 7929	EU248847	Bischoff *et al.*, 2009
M. pingshaense	CBS 257.90*	EU248850	Bischoff *et al.*, 2009
M. robertsii	ARSEF 4739	EU248848	Bischoff *et al.*, 2009
M. robertsii	ARSEF 727	DQ463994	Bischoff *et al.*, 2009
M. robertsii	ARSEF 7501*	EU248849	Bischoff *et al.*, 2009
M. robertsii	ARSEF 2575		Bischoff *et al.*, 2009
M. robertsii	-	-	Moreira et al.in prep.
M. robertsii	-	-	Moreira et al.in prep
M. robertsii	-	-	Moreira et al.in prep

References

Bais H.P., Weir T.L., Perry L.G., Gilroy S. & Vivanco J.M. (2006). The role of root exudates in rhizosphere interactions with plant and other organisms. *Annual Review of Plant Biology*, 57, 233-266.

Bali A., Kumar A. & Krishnaswamy J. (2007). The mammalian communities in coffee plantations around a protected area in the Western Ghats, India. *Biological Conservation*, 139, 93-102.

Batta Y.A. (2013). Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota: Hypocreales) against larvae of *Plutella xylostella* L. (Yponomeutidae: Lepidoptera) infesting *Brassica napus* plants. *Crop Protection*, 44, 128-134.

Behie S.W. & Bidochka M.J. (2014a). Nutrient transfer in plant–fungal symbioses. *Trends in Plant Science*, 19, 734-740.

Behie S.W. & Bidochka M.J. (2014b). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and Environmental Microbiology*, 80, 1553-1560.

Behie S.W., Zelisko P.M. & Bidochka M.J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science*, 336, 1576-1577.

Berendsen R.L., Pieterse C.M.J. & Bakker P.A.H.M. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, 17, 478-486.

Bidochka M.J., Kamp A.M., Lavender T.M., Dekoning J. & De Croos J.N.A. (2001). Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: Uncovering cryptic species? *Applied and Environmental Microbiology*, 67, 1335-1342.

Bischoff J.F., Rehner S.A. & Humber R.A. (2006). *Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. *Mycologia*, 98, 737-745.

Bischoff J.F., Rehner S.A. & Humber R.A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 101, 512-530.

Bommarco R., Kleijn D. & Potts S.G. (2013). Ecological intensification: harnessing ecosystem services for food security. *Trends in Ecology & Evolution*, 28, 230-238.

Broeckling C.D., Broz A.K., Bergelson J., Manter D.K. & Vivanco J.M. (2008). Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology*, 74, 738-744.

Bruck D.J. (2005). Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. *Biological Control*, 32, 155-163.

Bruck D.J. (2010). Fungal entomopathogens in the rhizosphere. In: *The Ecology of Fungal Entomopathogens* (eds. Roy H, Vega F, Chandler D, Goettel M, Pell J & Wajnberg E). Springer Netherlands, pp. 103-112.

Cardoso I.M., Guijt I., Franco F.S., Carvalho A.F. & Neto P.S.F. (2001). Continual learning for agroforestry system design: university, NGO and farmer partnership in Minas Gerais, Brazil. *Agricultural Systems*, 69, 235-257.

Caudill S.A., Vaast P. & Husband T.P. (2013). Assessment of small mammal diversity in coffee agroforestry in the Western Ghats, India. *Agroforest Syst*, 88, 173-186.

Chase A.R., Osborne L.S. & Ferguson V.M. (1986). Selective Isolation of the Entomopathogenic Fungi Beauveria bassiana and Metarhizium anisopliae from an Artificial Potting Medium. *The Florida Entomologist*, 69, 285-292.

Coleman-Derr D. & Tringe S.G. (2014). Building the crops of tomorrow: advantages of symbiont-based approaches to improving abiotic stress tolerance. *Frontiers in Microbiology*, 5, 283.

Crawley M.J. (2007). *The R Book*. Wiley Publishing.

De la Mora A., García-Ballinas J.A. & Philpott S.M. (2015). Local, landscape, and diversity drivers of predation services provided by ants in a coffee landscape in Chiapas, Mexico. *Agriculture, Ecosystems & Environment*, 201, 83-91.

Elliot S.L., Sabelis M.W., Janssen A., Van der Geest L.P.S., Beerling E.A.M. & Fransen J. (2000). Can plants use entomopathogens as bodyguards? *Ecology Letters*, 3, 228-235.

Fisher J.J., Rehner S.A. & Bruck D.J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of Invertebrate Pathology*, 106, 289-295.

Futuyma D.J. & Moreno G. (1988). The Evolution of Ecological Specialization. *Annual Review of Ecology and Systematics*, 19, 207-233.

Gaba S., Bretagnolle F., Rigaud T. & Philippot L. (2014). Managing biotic interactions for ecological intensification of agroecosystem. *Frontiers in Ecology and Evolution*, 2.

Hacquard S., Garrido-Oter R., González A., Spaepen S., Ackermann G., Lebeis S., McHardy Alice C., Dangl Jeffrey L., Knight R., Ley R. & Schulze-Lefert P. (2015). Microbiota and Host Nutrition across Plant and Animal Kingdoms. *Cell Host & Microbe*, 17, 603-616.

Hepperle D. (2010). DNA Dragon 1.4. 1–DNA Sequence Contig Assembler Software. URL Available at: www. dna-dragon. com.

Hergoualc'h K., Skiba U., Harmand J.-M. & Hénault C. (2008). Fluxes of greenhouse gases from Andosols under coffee in monoculture or shaded by *Inga densiflora* in Costa Rica. *Biogeochemistry*, 89, 329-345.

Hu G. & St Leger J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology*, 68, 6383-6387.

Isbell F., Calcagno V., Hector A., Connolly J., Harpole W.S., Reich P.B., Scherer-Lorenzen M., Schmid B., Tilman D., van Ruijven J., Weigelt A., Wilsey B.J., Zavaleta E.S. & Loreau M. (2011). High plant diversity is needed to maintain ecosystem services. *Nature*, 477, 199-202.

Kepler R.M., Humber R.A., Bischoff J.F. & Rehner S.A. (2014). Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia*, 106, 811-829.

Kepler R.M., Ugine T.A., Maul J.E., Cavigelli M.A. & Rehner S.A. (2015). Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology*.

Keyser C.A., Thorup-Kristensen K. & Meyling N.V. (2014). *Metarhizium* seed treatment mediates fungal dispersal via roots and induces infections in insects. *Fungal Ecology*, 11, 122-131.

Khan A., Hamayun M., Khan S., Kang S.-M., Shinwari Z., Kamran M., ur Rehman S., Kim J.-G. & Lee I.-J. (2012). Pure culture of *Metarhizium anisopliae* LHL07 reprograms soybean to higher growth and mitigates salt stress. *World J Microbiol Biotechnol*, 28, 1483-1494.

Klingen I., Westrum K. & Meyling N.V. (2015). Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres. *Biological Control*, 81, 1-7.

Leakey R.R.B. (2014). The Role of Trees in Agroecology and Sustainable Agriculture in the Tropics. *Annu. Rev. Phytopathol.*, 52, 113-133.

Liu Z.Y., Milner R.J., McRae C.F. & Lutton G.G. (1993). The use of dodine in selective media for the isolation of *Metarhizium* spp. from soil. *Journal of Invertebrate Pathology*, 62, 248-251.

Loureiro M.L. & Lotade J. (2005). Do fair trade and eco-labels in coffee wake up the consumer conscience? *Ecological Economics*, 53, 129-138.

Lozada T., de Koning G.H.J., Marché R., Klein A.-M. & Tscharntke T. (2007). Tree recovery and seed dispersal by birds: Comparing forest, agroforestry and abandoned agroforestry in coastal Ecuador. *Perspectives in Plant Ecology, Evolution and Systematics*, 8, 131-140.

McGonigle T.P. & Fitter A.H. (1990). Ecological specificity of vesicular-arbuscular mycorrhizal associations. *Mycological Research*, 94, 120-122.

Mendes R., Kruijt M., de Bruijn I., Dekkers E., van der Voort M., Schneider J.H.M., Piceno Y.M., DeSantis T.Z., Andersen G.L., Bakker P.A.H.M. & Raaijmakers J.M. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, 332, 1097-1100.

Moreira C.C., Celestino D., Guerra T.G., Cardoso i.M. & Elliot S.L. (submitted). Ecosystem services offered by entomopathogenic fungi: Agroforestry coffee soils are more insect-suppressive than Full-sun soils. *Submitted to Journal of Invertebrate Pathology*.

Moreira C.M., Mizubuti E.S.G., Hora Júnior B.T. & Elliot S.L. (in prep.). *Metarhizium* associated to coffee based agroforestry and full sun systems: molecular community characterization and population genetics.

Muleta D., Assefa F., Nemomissa S. & Granhall U. (2007). Composition of coffee shade tree species and density of indigenous arbuscular mycorrhizal fungi (AMF)

spores in Bonga natural coffee forest, southwestern Ethiopia. *Forest Ecology and Management*, 241, 145-154.

Munroe J.W., Soto G., Viginio Filho E.d.M., Fulthorpe R. & Isaac M.E. (2015). Soil microbial and nutrient propertioes in the rhizosphere of coffee under agroforestry and management. *Applied Soil Ecology*, 40-46.

Nehra V. & Choudhary M. (2015). A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture *Journal of Applied and Natural Science*, 7, 540 – 556.

Nylander J.A.A. (2004). MrModeltest v2. Program distributed by the author. In. Uppsala University 2 Evolutionary Biology Centre,.

Pellegrino E. & Bedini S. (2014). Enhancing ecosystem services in sustainable agriculture: Biofertilization and biofortification of chickpea (*Cicer arietinum* L.) by arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry*, 68, 429-439.

Peña-Peña A.J., Santillán-Galicia M.T., Hernández-López J. & Guzmán-Franco A.W. (2015). *Metarhizium pingshaense* applied as a seed treatment induces fungal infection in larvae of the white grub *Anomala cincta*. *Journal of Invertebrate Pathology*, 130, 9-12.

Perfecto I., Vandermeer J., Mas A. & Pinto L.S. (2005). Biodiversity, yield, and shade coffee certification. *Ecological Economics*, 54, 435-446.

Posadas J.B., Comerio R.M., Mini J.I., Nussenbaum A.L. & Lecuona R.E. (2012). A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate *Beauveria bassiana*, *Metarhizium anisopliae* sensu lato and *Paecilomyces lilacinus* from soil. *Mycologia*, 104, 974-980.

R Development Core Team (2015). R: A Language and Environment for Statistical Computing. In. R Foundation for Statistical Computing Vienna, Austria.

Ratnadass A., Fernandes P., Avelino J. & Habib R. (2012). Plant species diversity for sustainable management of crop pests and diseases in agroecosystems: a review. *Agron. Sustain. Dev.*, 32, 273-303.

Rehner S.A. & Buckley E. (2005). A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia*, 97, 84-98.

Rezende J.M., Zanardo A.B.R., Lopes M.D., Delalibera I. & Rehner S.A. (2015). Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. *Biocontrol*, 60, 495-505.

Rezende M.Q., Venzon M., Perez A.L., Cardoso I.M. & Janssen A. (2014). Extrafloral nectaries of associated trees can enhance natural pest control. *Agriculture, Ecosystems & Environment*, 188, 198-203.

Roberts D.W. & St. Leger R.J. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological Aspects. In: *Advances in Applied Microbiology*. Academic Press, pp. 1-70.

Rocha L.F.N., Inglis P.W., Humber R.A., Kipnis A. & Luz C. (2013). Occurrence of *Metarhizium* spp. in Central Brazilian soils. *Journal of Basic Microbiology*, 53, 251-259.

Sanchez-Pena S.R., Lara J.S.J. & Medina R.F. (2011). Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, Mexico, and their virulence towards thrips and whiteflies. *Journal of Insect Science*, 11, 1-10.

Sasan R.K. & Bidochka M.J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany*, 99, 101-107.

Sasan R.K. & Bidochka M.J. (2013). Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*, 35, 288-293.

Sayyed R.Z., Reddy M.S., Kumar K.V., Yellareddygari S.K.R., Deshmukh A.M., Patel P.R. & Gangurde N.S. (2012). Potential of plant growth-promoting rhizobacteria for sustainable agriculture. In: *Bacteria in Agrobiology: Plant Probiotics* (ed. Maheshwari DK). Springer Berlin Heidelberg, pp. 287-313.

Smith C., Barton D., Johnson M.D., Wendt C., Milligan M.C., Njoroge P. & Gichuki P. (2015). Bird communities in sun and shade coffee farms in Kenya. *Global Ecology and Conservation*, 4, 479-490.

Steinwender B.M., Enkerli J., Widmer F., Eilenberg J., Kristensen H.L., Bidochka M.J. & Meyling N.V. (2015). Root isolations of *Metarhizium* spp. from crops reflect diversity in the soil and indicate no plant specificity. *Journal of Invertebrate Pathology*, 132, 142-148.

Steinwender B.M., Enkerli J., Widmer F., Eilenberg J., Thorup-Kristensen K. & Meyling N.V. (2014). Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. *Journal of Invertebrate Pathology*, 123, 6-12.

Swofford D.L. (2003). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*.

Tilman D., Cassman K.G., Matson P.A., Naylor R. & Polasky S. (2002). Agricultural sustainability and intensive production practices. *Nature*, 418, 671-677.

Tscharntke T., Bommarco R., Clough Y., Crist T.O., Kleijn D., Rand T.A., Tylianakis J.M., Nouhuys S.v. & Vidal S. (2007). Conservation biological control and enemy diversity on a landscape scale. *Biological Control*, 43, 294-309.

Tscharntke T., Klein A.M., Kruess A., Steffan-Dewenter I. & Thies C. (2005). Landscape perspectives on agricultural intensification and biodiversity – ecosystem service management. *Ecology Letters*, 8, 857-874.

Tscharntke T., Milder J.C., Schroth G., Clough Y., DeClerck F., Waldron A., Rice R. & Ghazoul J. (2015). Conserving biodiversity through certification of tropical agroforestry crops at local and landscape scales. *Conservation Letters*, 8, 14-23.

Wang S., O'Brien T.R., Pava-Ripoll M. & St. Leger R.J. (2011). Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations. *Proceedings of the National Academy of Sciences*, 108, 20449-20454.

Williams-Guillén K., McCann C., Martínez Sánchez J.C. & Koontz F. (2006). Resource availability and habitat use by mantled howling monkeys in a Nicaraguan coffee plantation: can agroforests serve as core habitat for a forest mammal? *Animal Conservation*, 9, 331-338.

Wilson A.K. (1981). Commelinaceae-A review of the distribution, biology and control of the important weeds belonging to this family. *Tropical Pest Management*, 27, 405-418.

Wyrebek M., Huber C., Sasan R.K. & Bidochka M.J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology*, 157, 2904-2911.

Yang C., Ellouze W., Navarro-Borrell A., Taheri A.E., Klabi R., Dai M., Kabir Z. & Hamel C. (2014). Management of the arbuscular mycorrhizal symbiosis in sustainable crop production. In: *Mycorrhizal fungi: Use in sustainable sgriculture and land restoration* (eds. Solaiman ZM, Abbott LK & Varma A). Springer Berlin Heidelberg, pp. 89-118.

GENERAL CONCLUSIONS

The evidences presented in the following chapters provide support for the following conclusions:

- The qPCR method developed to detect and quantify *Metarhizium* association in plant roots is consistent and reproducible (Chapter 1);

- This method is very valuable experimental tool to study plant-*Metarhizium robertsii* association and can contribute to further studies to clarify all the mechanisms engaged in this partnership and also to detect *Metarhizium robertsii* in environmental samples (Chapter 1);

- *Metarhizium* is able to establish long term association with bean roots (Chapter1);

- *M. robertsii* is the most abundant species in the soil of the surveyed areas (Chapter 2);

- Besides the presence of diverse multilocus genotypes there is strong evidence for clonal structure in *M. robertsii* populations (Chapter 2);

- The clonal population structure is a common strategy to retain virulence in pathogens and also could be a strategy to maintain association to plant hosts (Chapter 2);

- *Metarhizium robertsii* is also the most frequent species associated with the plant rhizosphere in the coffee agroforestry system (Chapter 3); *Metarhizium pemphigi* was prevalent in coffee rhizosphere, besides its lower occurrence in spontaneous monocotyledonous samples, trees and soil (Chapter 3); The preference of *M. pemphigi* for coffee plants may be reflected in high population levels in coffee

rhizosphere that could mean more protection of the radicular system and nutrient transfer (Chapter 3); *Metarhizium pemphigi* could be a candidate to the development of a seed treatment for coffee, boosting the development of seedlings and protecting the seedlings (Chapter 3);

- Overall, in this thesis we took the first steps to understand the ecology of *Metarhizium* in an agricultural promising field of study regarding the *Metarhizium* in agroforestry coffee system which emphasize the importance of natural control in sustainable agriculture as well as the development of biotechnological products that could be more effective than the use of pesticide and fertilizers.