CAMILA COSTA MOREIRA

SUPPRESSIVENESS MEDIATED BY ENTOMOPATHOGENS IN AGROFORESTRY AND CONVENTIONAL SOILS

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

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APROVADA: 17 de fevereiro de 2012.

Eduardo Seiti Gomide Mizubuti

Madelaine Venzon

Tathiana Guerra Sobrinho

Simon Luke Elliot
(Orientador)

“Tudo, aliás, é a ponta de um mistério. Inclusive os fatos. Ou a ausência deles. Duvida?
Quando nada acontece, há um milagre que não estamos vendo”
(João Guimarães Rosa)

" By proper ventilation and illumination of the mind it is possible to cultivate tolerance,
poise and real courage.″
(Elie Metchnikoff)

Aos agricultores familiares de Araponga,
pelo cuidado no uso da terra.
Dedico.

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RESUMO

MOREIRA, Camila Costa M.Sc., Universidade Federal de Viçosa, fevereiro de 2012. **Supressividade mediada por entomopatógenos em solos agroflorestais e convencionais.** Orientador: Simon Luke Elliot.

Sistemas agroflorestais são agroecossitemas caracterizados por apresentarem condições mais próximas a ecossistemas naturais, mantendo assim muitas funções presentes nestes. A paisagem diversificada e as condições microclimáticas presentes agroflorestas podem incrementar a ação de inimigos naturais de insetos-praga. Fungos entompatogênicos pertencentes à ordem Hypocreales são importantes inimigos naturais de insetos. No entanto, os efeitos de sistemas agroflorestais nunca foram medidos em relação ao seu desempenho. Nesse sentido revisamos aspectos da ecologia desses fungos e os relacionamos ao contexto da paisagem diversificada presente em agroflorestas. Nós também comparamos os efeitos dos manejos agroflorestal e convencional em cultivos de café sobre o potencial supressivo desses fungos a insetos isca e sua abundância. Amostras de solo dos dois tipos de manejo foram coletadas e submetidas à metodologia de "isca viva". Através da mortalidade das iscas, curvas de sobrevivências foram construídas e os fungos obtidos infectando-os foram isolados, identificados e quantificados para cada sistema. Esses fungos foram mais ativos em agroflorestas apresentando maior potencial para controle mais rápido de insetos praga. Observou-se que agroflorestas apresentam comunidades mais abundantes em relação a esses entomopatógenos que cultivos convencionais. Através desses resultados podemos inferir que os serviços prestados por fungos entomopatogênicos aos ecossistemas provavelmente são incrementados pelas características encontradas em sistemas agroflorestais, como: paisagem diversificada, estabilidade microclimática e solos mais conservados, no entanto, estes fatores ainda precisam ser investigados. Além, disso nossos resultados fornecem uma nova abordagem à metodologia de "isca viva" pela inferência do potencial supressivo a insetos através da mortalidade das iscas.

ABSTRACT

MOREIRA, Camila Costa M.Sc., Universidade Federal de Viçosa, February, 2012. **suppressiveness mediated by entomopathogens in agroforestry and conventional soils**. Adviser: Simon Luke Elliot.

Agroforestry systems are agroecosystems characterized by a similarity to natural ecosystems and the maintenance of many functions presented in these ecossystems. The diversified landscape and microclimatic conditions in these agroecosystems can increase the action of natural enemies of pests. Entomopathogenic fungi of the order Hypocreales are important insect natural enemies but the effects of agroforestry systems on them have not been measured. In this context, we review important aspects of the ecology of entomopathogenic fungi and relate them to the diversified landscape context present in agroforestry systems. We also compare the effects of agroforestry and conventional farming systems on the pest suppressive potential of these fungi and on their abundance. Soil samples from both farming systems (in this instance, coffee) were taken and the insect bait method was used to isolate fungi. We examined bait insect mortality with survival analyses while the fungi found infecting bait insects were isolated, indentified and quantified in both systems. We show that these fungi are more active in the agroforestry soils, probably indicative of greater potential for insect pest control. We also observed that the agroforestry system had more fungal abundant communities than the conventional system. We suspect that ecosystem services provided by entomopathogenic fungi can be increased by characteristics of agroforestry systems such as: diversified landscape, microclimatic stability and reduced soil disturbance. Furthermore, our results provide a new approach to the "bait insect method" enabling us to estimate the pest suppressive potential through bait insect mortality.

INTRODUÇÃO GERAL

Fungos entomopatogênicos pertencentes à ordem Hypocreales (Ascomycota), na sua fase anamórfica (assexuada), são comumente encontrados no solo de ecossistemas naturais e agroecossistemas (Keller & Zimmerman 1989; Bidochka *et al.* 1998) e podem atuar no controle de populações de insetos. Esses fungos, na maioria das vezes, têm ampla gama de hospedeiros e podem permanecer no solo fora do corpo destes por longos períodos.

As pesquisas com esses organismos ao longo de muitas décadas se concentraram na obtenção de isolados para utilização no controle biológico inundativo de pragas, isto é, como bioinseticidas. Todavia, na última década, alguns trabalhos foram conduzidos na tentativa de compreender o papel desempenhado por esses fungos ocorrendo naturalmente em solos de agroecossistemas (Klingen *et al.* 2002; Meyling & Eilenberg 2006b; Meyling & Eilenberg 2007; Jabbour & Barbercheck 2009; Goble *et al.* 2010). Alguns destes trabalhos ainda mostraram a influência positiva de práticas agrícolas sustentáveis, como o cultivo orgânico, na abundância e diversidade desses fungos (Klingen *et al.* 2002; Meyling & Eilenberg 2006b; 2007; Jabbour & Barbercheck 2009; Meyling *et al.* 2009; Goble *et al.* 2010).

Contudo, os estudos citados acima foram desenvolvidos em regiões temperadas, sendo que o papel desse grupo de microrganismos ainda é desconhecido em áreas tropicais. Entretanto, tudo indica que tenham papel mais importante nos trópicos que o já demonstrado em sistemas equivalentes em regiões de clima temperado. Entre as razões para se acreditar nessa diferença está o fato de que florestas tropicais serem reconhecidas como as maiores detentoras de diversidade desses fungos, uma vez que são os locais onde as fases sexuadas são encontradas (Hughes & Boomsma 2006; Evans *et al.* 2011a, b). De acordo com o exposto, as fases anamórficas desses fungos encontradas em agroecossistemas podem representar uma expansão desses ecossistemas naturais colonizando habitats simplificados (Hughes & Boomsma 2006).

Supõe-se que em agroecossistemas em regiões tropicais exista um maior potencial supressivo de populações de insetos por fungos entomopatogênicos, ou seja, eles estariam prestando melhor os seus serviços de ecossistema atuando como agentes de controle biológico (Meyling & Eilenberg 2007). Porém, esses serviços podem ser perdidos com a utilização cada vez mais intensiva dos agroecossistemas visando maior produtividade agrícola (Matson *et al.* 1997) e conseqüente deterioração do solo. Assim, esquemas agrícolas sustentáveis podem favorecer a ação desses fungos (Altieri 1999), como é o exemplo de sistemas agroflorestais, os quais são considerados mais favoráveis à manutenção de organismos benéficos e ao incremento das funções desempenhadas por eles (Peng *et al.* 1993; Tylianakis *et al.* 2005; Bianchi *et al.* 2006; Tylianakis *et al.* 2006; Tscharntke *et al.* 2008). Dentre essas funções está o controle biológico de pragas principalmente em uma abordagem conservativa, onde a comunidade natural de inimigos naturais exerce pressão na população de uma determinada praga (Tscharntke *et al.* 2007). No entanto, para fungos entomopatogênicos da ordem Hypocreales os efeitos de sistemas agroflorestais nunca foram mensurados.

Nesse contexto, é importante avaliar os efeitos de sistemas de cultivos sobre a comunidade de fungos entomopatogênicos do solo de áreas tropicais, principalmente no que diz respeito ao seu potencial para regulação de insetos pragas, isto é, os serviços prestados por estes aos agroecossistemas.

Uma dificuldade em avaliar o papel dos microrganismos em questão no solo é o estabelecimento de técnicas apropriadas. Não existem muitas técnicas disponíveis e é necessário avaliar se a técnica utilizada atende aos objetivos do estudo e se os dados obtidos serão adequados para responder as hipóteses levantadas. Dentre as metodologias utilizadas estão incluídas aquelas mais simples como o uso de meios de cultura seletivos para os organismos de interesse, os quais estão disponíveis para fungos e bactérias (Elad *et al.* 1981; Hagedorn *et al.* 1987; Goettel & Inglis 1997; Meyling & Eilenberg 2006a), ou fracionamento sucessivo do solo por peneiração e ressuspensão de esporos em gradiente de densidade descontínuo utilizada para fungos entomopatogênicos da ordem Entomophtorales (Hajek & Wheeler 1994) ou fungos arbusculares micorrízicos (Kucey & McCready 1982); até técnicas moleculares mais elaboradas e modernas como "*pyrosequencing*" (Urich *et al.* 2008; Feinstein *et al.* 2009), PCR em tempo real (Hermansson & Lindgren 2001; Filion *et al.* 2003) ou eletroforese em gel de gradiente desnaturante (DGGE) (Bridge & Spooner 2001; Oros-Sichler *et al.* 2006).

Todas as técnicas citadas acima seriam adequadas para a caracterização da comunidade de fungos entomopatogênicos da ordem Hypocreales. Todavia, para esses fungos, os métodos mais utilizados são meios seletivos (Erland 1991; Goettel & Inglis 1997) e a metodologia de "isca-viva" (Zimmermann 1986). Essa última apresenta a vantagem adicional de explorar a capacidade desses fungos em matar e infectar insetos.

Dentro do exposto, esta dissertação é o primeiro estudo em uma linha de pesquisa visando entender a ecologia de fungos entomopatogênicos pertencentes a ordem Hypocrelaes no solo de agroecossistemas tropicais. O objetivo geral foi compreender os efeitos dos manejos agroflorestal e convencional sobre a comunidade de fungos entomopatogênicos ocorrendo naturalmente nesses sistemas. Assim, levantamos a hípotese de que sistemas agroflorestais são mais favoráveis à manutenção dos serviços de ecossistemas prestados por esses fungos e que apresentem níveis populacionais mais altos que sistemas de cultivo convencional.

No capítulo 1 da dissertação revisamos aspectos importantes da ecologia desses fungos no contexto de paisagems agrícolas mais complexas, como as agroflorestas. Nós também consideramos como esses agroecossistemas diversificados como poderiam influenciar a diversidade e as multiplas relações estabelecidas por esses fungos em um agroecossistema diversificado.

No capítulo 2, comparamos os efeitos dos sistemas agroflorestal e convencional em solos sobre cultivos de café na atividade e na abundância de fungos entomopatogênicos. Amostras de solo coletadas nos dois tipos de sistemas foram submetidas a metodologia de "isca-viva" para quantificação da atividade e da abundância desses fungos. Como esses mostraram maior atividade e abundância em solos agroflorestais, discutimos os efeitos deste sistema sobre a comunidade de fungos.

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CAPÍTULO 1:

Agricultural landscape context and ecosystem services of entomopathogenic fungi

1.1 Introduction

Natural enemies contribute to pest regulation, providing environmental and economic benefits. They can reduce yield loss without the negative environmental consequences that result from chemical pesticide use (Bianchi *et al.* 2006). In this way farming systems that mimic natural habitats (Altieri 1999; Lin 2007) can support a diverse and active community of natural enemies that can suppress pests. Agroforestry systems shade tree cover protect the system against extreme variations in microclimate and soil moisture (Lin 2007; Jose 2009), contributing to higher biological and genetic diversity (Atta-Krah *et al.* 2004; Dawson *et al.* 2009)

Empirical evidence indicates that diverse communities of natural enemies are effective in regulating herbivore populations (Wilby & Thomas 2002; Cardinale *et al.* 2003). For arthropod natural enemies, greater regulation of herbivores is obtained in more complex landscapes where crop and non-crop habitats are mixed or where crops have conserved field margins (Dennis & Fry 1992; Settle *et al.* 1996; Stamps & Linit 1997; Gardiner *et al.* 2009). With entomopathogenic fungi of the order Entomophthorales (Zygomycota), emphasis had been given to natural epizootics occurring in aphid populations and greater insect control is also influence by crop and non-crop mixed areas and conserved field margins (Ekesi *et al.* 2005; Roy *et al.* 2009; Pell *et al.* 2010).

With entomopathogenic fungi of the order Hypocreales (Ascomycota), however, we only know that diverse or more sustainable agricultural systems, such as organic agriculture or semi-natural habitats, support more abundant (Klingen *et al.* 2002; Meyling & Eilenberg 2006b; Goble *et al.* 2010) or diverse fungal populations (Meyling *et al.* 2009; Fisher *et al.* 2011).

Thus, we know nothing about pressures exerted by the indigenous hypocrealean entomopathogenic fungi on insect pest populations. Although it is already recognized that these fungi play different roles beyond infecting and killing insect hosts (Vega *et al.* 2009), little is known about the dynamics of these relationship in ecosystems. Here, we review a recent change in how hypocrealean fungi are viewed and connect the new paradigms in entomopathogenic fungus ecology with an agricultural landscape context. Next we propose how the agricultural landscape in agroforestry systems can influence entomopathogenic fungal diversity and the multiple roles that they establish when they are not inside the insect host body.

1.2 Hypocrealean Entomopathogenic Fungi: an overview

At the end of the 18th century, the Russian scientist Elie Metchnikoff observed for the first time the "green muscardini", an insect disease caused by the fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (first described by him as *Entomophthora anisopliae*), when he was seeking a means to control the cereal cockchafer *Anisoplia austriaca* Hbst. (Coleoptera: Rutelidae) (Zimmermann *et al.* 1995). Since this, the microbial control of insects using entomopathogenic fungi has been explored in studies ranging from the naturally occurring disease in insect populations (epizootics) to genetic engineering of the fungi.

Since the beginning of insect pathology studies in the early 19th century, the most investigated entomopathogenic fungi have been *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vullemim. The idea of using pathogens to combat pest insects emerged from the initial recognition that they were contagious under both laboratory and natural conditions. The mortality occurring in natural epizootics was also recognized to contribute to the control of pests (Lord 2005).

Over the last 200 years, we have gained a huge amount of information about the hypocrealean entomopathogens, but mostly directed at their use as bioinsecticides in inundative biological control (Vega *et al.* 2009). They are safe for other organisms, some are considered selective for a specific target pest and they also can help to reduce the use of chemical insecticides (Zimmermann 2007a, b). Another important advantage is that they can be formulated and applied in extensive areas, facilitated by the possibility to grow these fungi in artificial media. Faria and Wraight (2007) listed at least 13 fungal species or isolates used as bioinsecticides that already had been formulated and registered as commercial products in agricultural, urban, forest, livestock and aquatic environments. However, in many cases where these fungi have been used as biological control agents, they have had inconsistent performance. This can often be attributed to an incomplete understanding of the ecological constraints of the biological system in which they are used (Bruck 2005). A number of studies have therefore been directed at understanding the reasons for successive failures in the application of entomopathogenic fungi as biological control agents, focusing on, for example: the influence of biotic and abiotic factors, environmental persistence (Vänninen *et al.* 2000), the role of toxins produced by these fungi in the host body (Samuels *et al.* 1988; Huxham *et al.* 1989; Quesada-Moraga & Vey 2003) and genetic manipulation of the fungi to enhance virulence (Screen *et al.* 2001; Bagga *et al.* 2004;

Pava-Ripoll *et al.* 2008; Fang & St Leger 2010; St Leger & Wang 2010; Fang *et al.* 2011).

Despite advances in understanding these fungi, information such as fungal ecology in naturally occurring habitats had been neglected for many decades. Over the past two decades, though, some studies have been conducted on the isolation of insect-pathogenic fungi from soils, being considered the most common habitat of these organisms (Keller & Zimmerman 1989). These studies usually aim to understand the distribution of these fungi and also to obtain isolates that could ultimately be used in biopesticides (Vanninen 1996; Chandler *et al.* 1997; Bidochka *et al.* 1998; Ali-Shtayeh *et al.* 2003; Keller *et al.* 2003; Quesada-Moraga *et al.* 2007; Sun & Liu 2008) while a few studies concern the obtention of isolates for immediate use against a target pest (Sookar *et al.* 2008; Imoulan *et al.* 2011).

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1.3 Ecosystem services and diversity in an agricultural landscape context

More recently, a new ecological approach has been considered in research on entomopathogenic fungi. The aim has been to understand the factors that influence their natural occurrence on a small scale, i.e. in single fields or adjacent areas, comparing the influence of the farming systems on the distribution and occurrence of these fungi (Klingen *et al.* 2002; Meyling & Eilenberg 2006b; Jabbour & Barbercheck 2009; Goble *et al.* 2010; Meyling *et al.* 2011)

Currently, besides the importance and applicability of the biotechnological traits obtained in research with hypocrealean fungi, there is an interest in conservation and means of sustainable agricultural production. These fungi are recognized as ecosystem service providers controlling insect pest populations (Meyling & Eilenberg 2007) although next to nothing is known about how this may occur. Meanwhile, knowledge of the roles of these fungi in ecosystems and their fundamental ecology are still at an early stage.

Agroecossystems may be managable in such a way as to favor the activity of entomopathogenic fungi. Multistratum agroforestry systems have been recognized to be by themselves pest-suppressive systems (Staver *et al.* 2001); this can reduce the need for direct pest control measures and their associated costs and impacts (Altieri 1999). Entomopathogens may contribute to this, even if their action has so far remained hidden. We hypothesize that agroforestry systems with a multistrata environment could create conditions for the establishment of more diverse insect-pathogenic fungi community and that this could improve the establishment of their different roles in this system, in particular suppression of herbivorous pest insects.

With the increase of plant diversity, fungal species have more chances to establish different symbiotic relationships with different plant species, increasing the permanence of these fungal in the absence of their insect hosts. The increase of plant diversity also increases the diversity of compartments that these fungi can occupy and consequently the interaction with different insect hosts. Different insect hosts can mediate fungal dispersion to other insects and other compartments, generating a highly interconnected food web (Meyling *et al.* 2009). Furthermore, plant diversity can contribute to a more diverse community of arthropods such as predators and parasitoids and these insects can also contribute to the dispersal of fungi between compartments (Meyling *et al.* 2006).

Another important factor present in agroforestry landscape is the level of shade, as this mitigates the direct effects of solar radiation and microclimatic extremes (Lin 2007). Solar radiation reduces conidial germination of insect-pathogenic fungi, while shade favors fungi by extending the viability of conidia and providing sufficiently high humidity for their germination (Staver *et al.* 2001).

1.4 Entomopathogenic fungi and plants

Another question that has emerged from the natural occurrence of these fungi in soils is how they can survive in the environment for extended periods without infecting an insect host. It remains unclear if the propagules recovered in soil are conidia, surviving mycelial remains of fungus-killed hosts (i.e. sclerotia or parts of sclerotia), or saprophytes on non-insect substrates (St Leger 2008). More recent studies have added another possibility: symbiotic associations between plants and entomopathogenic fungi.

According to Vega *et al* (2009) the Hypocreales include a wide range of fungal species with highly variable lifestyles, including pathogens of plants, insects and other animals, endophytes and mycoparasites. These lifestyles could have arisen independently several times within the order and multiple host changes have probably occurred through the evolutionary history of the group (Vega *et al.* 2009). It is further hypothesized that the physical proximity between fungi, insect and plants on which these insects feed may provide the hypocrealeans with the opportunity to move from plant to insect (or even fungal) host and *vice-versa.* However, these fungi share a phylogenetic basal root with the grass endossymbionts *Claviceps* and *Epichloe*, suggesting that the ancestral lifestyle was as plant-associated fungi (Spatafora *et al.* 2007; Sasan & Bidochka 2012).

Beauveria bassiana has been reported as an endophyte in at least 15 plant taxa, whether naturally occurring or inoculated (Vega 2008), and can have multiple effects on plant performance like herbivore defense, growth promotion or disease antagonists (Ownley *et al.* 2008). Meanwhile, one of the nine phylogenetic species that compose the *M. anisopliae* species complex, *M. robertiisi* J. F. Bisch., Rehner & Humber has recently been shown to have endophytic capacity in grass roots, even promoting the proliferation of root hairs (Sasan & Bidochka 2012). This new result reinforces the strong relationship between *M. anisopliae* and plant root system that was previously reported as rhizosphere competent (Bruck 2005; St Leger 2008; St. Leger *et al.* 2011). The idea that some fungi can show population growth on the plants rhizosphere is not new, but Hu and St Leger (2002) were the first to register an entomopathogenic fungus that had this ability (Bruck 2005). After this initial report in cabbage, *M. anisopliae* was found in the same association with a variety of other cultivated plants (Fisher *et al.* 2011).

These associations are far from being completely understood, although it seems clear that these fungi can protect plants from herbivore insects, either above ground as with endophytic *B. bassiana* or below ground as rhizosphere-competent or endophytic *M. anisopliae* (Meyling *et al.* 2011). In fact these fungi could work as plant “bodyguards”, this hypothesis asserts that plants could have evolved mechanisms to favor natural enemies of their herbivore pests and still protect themselves (Elliot *et al.* 2000).

1.5 Hidden diversity

Conventionally, studies of genetic diversity of entomopathogenic fungi *B. bassiana* and *M. anisopliae* have sought to establish links between genetic differences and distinct insect host groups (Meyling *et al.* 2009). However, it appears that that these fungi are generalist pathogens with limited host specificity (Bidochka et al. 2002; Coates et al. 2002; Wang et al. 2003; Rehner & Buckley 2005), and it may be genetic divergences can mostly be attributed to environment survival rather than to host specificity (Meyling *et al.* 2009; Fisher *et al.* 2011; Sasan & Bidochka 2012).

One of the difficulties of studying the diversity of these fungi is that *B. bassiana* and *M. anisopliae* are actually species complexes whose members are morphologically indistinguishable. *Beauveria bassiana sensu strictu* has been shown to contain five phylogenetically distinguishable species that can be related to some degree to the habitats in which they were found: insect pests and natural enemies, soil from field and hedgerow, and phylloplanes; all this in a study of a single agroecosystem in Denmark (Meyling *et al.* 2009). In this study, all of these phylogenetic species were shown to occupy different ecosystem compartments, with diversity highest in hedgerows (semi-natural habitats). The authors also tried to build a food web with these species recovered from different ecosystem compartments and different insect hosts, showing how pathogen, insects and the different compartments are interconnected.

In the same vein, Bischoff (2009) described nine phylogenetic species of *Metarhizium*, and Fisher (2011) was subsequently able to separate these species according to their competence in the rhizospheres of plant species belonging to different families.

These result shows the importance of taking the landscape structure into account in surveys of entomopathogenic fungi and their diversity and also when proposing actions to improve ecosystem services they may provide.

2 Conclusions

The above shows a change of paradigm in research on entomopathogenic fungi. This change is highly relevant to global changes of view about agricultural intensification, and the drive to find sustainable means of agricultural production. There is the potential to include a consideration of the action of insect-pahogenic fungi in the management of more complex agri-environmental schemes. These systems also offer useful contexts in which to study the ecology of entomopathogenic fungi.

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CAPÍTULO 2:

Suppressiveness mediated by entomopathogens in agroforestry and conventional soils

2.1 Introduction

Agricultural intensification increases land productivity with the intensive use of chemical fertilizers and pesticides, irrigation and mechanization and with the utilization of high-yielding crop varieties (Matson *et al.* 1997). This intensification leads to extensive areas under monoculture, characterized by increasingly simplified agroecosystems. Fifty years on from the Green Revolution, which propelled agricultural intensification (Conway & Barbie 1988), there is an increasing realization that more suitable means of agricultural productions are needed. Futhermore, it is now acknowledged that global-scale conversion of tropical rainforests to agricultural areas and agricultural intensification are major causes of biodiversity loss (Hoekstra *et al.* 2005; Steffan-Dewenter *et al.* 2007).

Meanwhile, the loss of biodiversity in simplified landscapes erodes ecosystem functions (Bianchi *et al.* 2006). One response to this is the adoption of agroecological systems and technologies that emphasize the conservation and regeneration of biodiversity, along with similar considerations for soil, water and other resources (Altieri 1999). Farming systems such as agroforestry mimic natural habitats (Altieri 1999; Lin 2007) promoting shaded soil, reduced microclimatic variation, moisture conservation, and less ecosystem disturbance (Jose 2009), improving the maintenance of biodiversity and ecosystem functions.

One of the most important ecosystem services that has been associated with biodiversity is pest control (Wilby & Thomas 2002), yet paradoxically pest control is one of the factors most responsible for chemical releases and biodiversity loss in conventional agriculture. As a natural means of pest regulation, this ecosystem function is especially important within a conservational biological control approach (Tscharntke *et al.* 2007; Pell *et al.* 2010), and is mainly accomplished by the action of natural enemies of pests such as arthropod predators and parasitoids, birds and pathogenic microorganisms. To date, the effects of agri-environmental schemes (such as agroforestry systems) on conservational biological control potential has been measured mainly for arthropod predators and parasitoids (Tylianakis *et al.* 2005; Klein *et al.* 2006; Philpott & Armbrecht 2006; Tylianakis *et al.* 2006; Letourneau *et al.* 2009). Meanwhile, the potential of soil microorganisms such as insect-pathogenic fungi as providers of ecosystem services has largely been overlooked.

Asexual forms of insect-pathogenic fungi of the order Hypocreales (Ascomycota) have a cosmopolitan distribution and soil is considered their typical habitat (Keller & Zimmerman 1989; Bidochka *et al.* 1998). Over recent decades, occasional efforts have been made to characterize the soil-associated community of these fungi. These organisms have been surveyed in several localities around the world with an emphasis on sampling large geographical areas, but with few soil samples. The intention in most of these studies was to determine the species diversity and distribution of these fungi (Vanninen 1996; Bidochka *et al.* 1998; Ali-Shtayeh *et al.* 2003; Quesada-Moraga *et al.* 2007; Sookar *et al.* 2008; Sun & Liu 2008; Sun *et al.* 2008; Sevim *et al.* 2010). In many of these cases, a prime objective is to obtain isolates for use as biopesticides, with little consideration of their ecology. Recently the effects of agricultural practices and farming systems on communities of entomopathogenic fungi at local scales have been taken into consideration in surveys of species richness, occurrence and distribution (Klingen *et al.* 2002; Meyling & Eilenberg 2006; Meyling & Eilenberg 2007; Jabbour & Barbercheck 2009; Meyling *et al.* 2009; Goble *et al.* 2010; Meyling *et al.* 2011).

The main method used in these studies is the “bait insect method”, where laboratory reared insect larvae are used as baits to isolate fungi from soil (Zimmermann 1986; Meyling 2007). The insect acts as a selective medium for entomopathogenic fungi: if they are present in the soil sample they can infect the insect bait and be isolated. The method can provide basic information such as species richness, distribution and abundance. However, the link between these variables and ecosystem services (i.e. the control of pest insects) has not yet been made.

Incorporated within this technique, yet infrequently or never explored, is the speed with which the fungi kill the bait insect hosts. This variable has the potential to reveal the degree to which entomopathogenic fungi provide ecosystem services and consequently the potential for conservative biological control of pests.

Recently, Evans *et al* (2011) related that is very common to find sexual forms of insect-pathogenic hypocrealean fungi in tropical Atlantic rainforest, so we suspect that agricultural areas within this biome may also support a great abundance of the asexual forms. In this sense, some crop management systems may be favorable to the maintenance of these entomopathogenic fungi forms on agricultural soils. Specifically, we consider that agroforestry coffee plantions soils harbored more diverse and abundant insect-pathogenic community.

In this study we use the insect baiting method described above and explore for the first time how long it takes for the fungi in the soil to kill the bait insects; this should correlate, we suspect, with the capacity to kill insects in the field and can be used as a measure of the biological control potential of the soils (their pest suppressive potential). We suspect that fungi in agroforestry soils will kill bait insects faster than fungi from conventionally managed soil, and this variable could be taken as measure of the ecosystem services provided by this fungi. We further believe that agroforestry soils support more abundant and more diverse insect-pathogenic fungi; differences in fungal abundance between different agricultural management systems have been tested before, but this is the first study to compare agroforestry and conventional management systems. Therefore the objectives of the present study is to test the hypothesis that (i) the speed of kill of the bait insect by entomopathogenic fungi is faster in agroforestry soils and (ii) the abundance of these fungi is greater in soils from the agroforestry system than in conventional soil.

2.2 Material and Methods

2.2.1 Study area

The study was conducted in the municipality of Araponga, Minas Gerais, southeastern Brazil (20° 48’ S e 42° 32’ W). This municipality is in the ‘Zona da Mata’ region, within the Atlantic Coastal Rainforest biome. It is characterized by a tropical highland climate with average temperature and precipitation of 18°C and 1,500 mm. The landscape consists of characteristically steep slopes (20-45%) and altitudes ranging from 200 to 1,800 m (Cardoso *et al.* 2001). The dominant soil type is Oxisoils, that are acidic and poor in nutrients (Cardoso *et al.* 2001; Mendonça & Stott 2003) (Appendix 1).

Sampling was conducted on smallholdings under coffee (*Coffea arabica* L.) cultivation. Fields were either organic agroforestry systems (shade trees planted between coffee rows; fertilization provided by green manure and organic fertilizers; no pesticide use) or conventional systems (unshaded open cultivation with inorganic fertilizers and also no pesticide use) (Appendix 2).

2.2.2 Soil samples

We collected soil samples from six paired areas, i.e. from three sites where samples could be taken from both systems. Sampling was conducted such that all soil samples from a given pair of areas were collected on the same day (Appendix 2). The numbers of samples were thus: Area 1, 6th June 2010: 97 agroforestry samples & 97 conventional; Area 2, 10th July: 78 agroforestry & 76 conventional; Area 3, 17th September: 70 agroforestry & 72 conventional). These 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 soil sampler to 20cm depth, from the base of bushes, beneath the canopy but ca. 75cm from the plants' trunks. Samples were transferred to individual polyethylene bags for transport to the laboratory. The core sampler was washed in water and then 70% ethanol between each sample.

Once in the laboratory, each sample was mixed and homogenized manually. A subsample of soil was transferred from each of the plastic bags to a 200ml transparent cup, these subsequently moistened with 10 ml of distilled water to maintain humidity (Appendix 3). In line with previous studies (Klingen *et al.* 2002; Meyling & Eilenberg 2006b; Goble *et al.* 2010), we did not use controls as it is nearly impossible to use a substrate that is similar to soil and which will not affect the bait insects' survival (even sterilized soil properties are totally modified by high temperatures) (Richard J 2004).

2.2.3 Insect-bait method

Larvae of *Tenebrio molitor* L. (Coleoptera:Tenebrionidae) were used as bait insects. This method was initially proposed by Zimmerman (1986), and named the "Galleria bait method" as larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) are used as the bait insect. The procedure has been adapted using other insect larvae such as *T. molitor* (Vanninen 1996; Vänninen *et al.* 2000; Sanchez-Pena *et al.* 2011). It has been used successfully (with one bait insect or another) to isolate entomopathogenic fungi from soil in a number of studies (Vanninen 1996; Chandler *et al.* 1997; Keller *et al.* 2003; Meyling & Eilenberg 2006b; Quesada-Moraga *et al.* 2007; Sun & Liu 2008). The insect acts as a selective medium for entomopathogenic fungi: if they are present in the soil sample they can infect the insect bait. Incorporated within the technique is additional advantage of exploring the fungal ability to kill the hosts.

Here, larvae of *T. molitor* were obtained from a stock rearing maintained on wheat bran and chayote (a cucurbit). When used, the insects were ca. two months old and were of similar size (ca. 1.3cm); it is difficult under these circumstances to determine the exact instar (Morales-Ramos *et al.* 2010). For each cup containing soil, 4 larvae were added. The cups were sealed with perforated lids. During the first week all cups were shaken daily, inverted and left upside down (to force the insects to traverse the substrate). Cups were inspected every three days and dead insects were surface-sterilized with 70% ethanol, 5% sodium hypochlorite and two washes in sterile distilled water. Insects were then incubated in moisture chambers (1.5 µl microtubes half-filled with moistened cotton wool) to promote fungal growth.

2.2.4 Isolation and identification of fungi

Dead insects were inspected under a stereomicroscope (40x) for external fungal growth and for preliminary fungal identification. All fungi detected were isolated on to plates with PDA (20% Potato, 2% Dextrose and 1.5% Agar) and rifampycin and incubated at 24°C. Slides were made from these culture plates for microscopic (400x) observation and identification according to morphological characteristics in taxonomic keys (Humber 1997a). Isolates were stored and preserved in silica gel (Humber 1997b).

2. 2.5 Statistical procedures

All statistical analyses in this study were performed in R software version 2.13.0 (R Development Core 2008). In all analyses, the farming system (Agroforestry *vs.* Conventional) was used as an explanatory variable to construct full models. Except where specified, the area (i.e. which of the three pairs of fields was being considered) was also used to construct the full model, as a fixed effect, i.e. as a blocking factor. The first analyses were survival regression analyses, and time was also used to construct the full models. The remaining analyses all used generalized linear models (GLM). Dependent factors are specified below. Model simplification was attempted by excluding non-significant terms; in practice only the area was ever non-significant but this was generally kept in the models as it represented blocks. Following all analyses, we checked for data overdispersion and residual analyses were carried out to determine model acceptability and error distribution suitability (Crawley 2007).

Survival regression analyses were carried out with bait insect survival as the dependent factor. Data from all three areas (i.e. pairs of fields) were analyzed based on a censored Weibull distribution and were compared by ANOVA and χ^2 tests (Crawley 2007). Following this, an analysis was conducted of data from all three areas together, to explore whether the result was consistent across areas (although we recognize that the areas should not strictly speaking be used in the same analysis). Finally, we divided the evaluation time in two periods (0 to 20 and 24 to 40 days) and conducted a survival analysis in each period separately, i.e. two analyses for each area for detect in which of the both periods the survival differences is more accentuate.

Eight further analyses were conducted. Independent variables and full model construction (GLM) were as described above, while the response variables were: (1) numbers of soil samples positive for at least one insect-associated fungus; (2) numbers of fungal genera per soil sample; (3) numbers of fungal isolates per soil sample; (4) numbers of insects that had mixed infections (soil samples were not identified here); (5-8) numbers of soil samples positive for each fungal genus individually (i.e. four analyses for each of the four fungal genera found). All dependent variables were accorded quasipoisson distributions. Analyses were all ANOVAs with χ^2 tests (these are used in place of F-tests for count data; Crawley 2007). For the last analyses (5-8) model simplification incorporated a model contrast amalgamating areas to find the final model.

2. 3 Results

2.3. 1 Bait insect mortality

The survival of bait insects exposed to agroforestry soils was shorter than that of bait insects exposed to conventional soils (Agroforestry *vs.* Conventional, Area 1: 15.2±0.40 mean±SE *vs.* 23.8±0.51 days, $\chi^2_{[773]}$=154.5; P<0.001; Area 2: 23.8±0.80 *vs.* 29.9±0.85 days, $\chi^2_{[613]}$=21.53, *P*<0.001; Area 3: 22.2±0.71 *vs.* 25.6±0.68 days, $\chi^2_{[565]}$=9.43; *P*=0.002; Fig.1A-C). When area was included as an independent variable in an analysis of all data combined, it was eliminated during model simplification as it did not contribute significantly to the observed variation in the data (and the general result was maintained: survival of bait insects was shorter in agroforestry than in conventional soils; 19.8±0.38 (mean±SE) *vs.* 26.2±0.39 (mean±SE) days respectively; $\chi^2_{[1952]}$=323.571; *P*<0.001). Furthermore, when we analyzed survival in the two periods of evaluation (0-20 and 24-40 days) we found that the first period (0-20 days) was responsible for the differences in bait insect survival in all three areas (0-20 days, Agroforestry *vs.* Conventional, Area 1: 12.3±0.19 mean±SE vs. 14.4±0.28 days, $\chi^2_{[508]}$=154.5, P<0.001; Area 2: 13.2±0.26 *vs.* 14.7±0.35 days, $\chi^2_{[290]}$=13.19, *P*=0.002; Area 3: 13.8±0.35 *vs.* 15.3±0.44 days, $\chi^2_{[319]}$=8.24, *P*=0.004), while no such differences were found for the second period (20-40 days, Agroforestry *vs.* Conventional, Area 1: 31.4±0.76 mean±SE *vs.* 32.18±0.40 days, $\chi^2_{[262]}$=1.66, *P*=0.202; Area 2: 30.36±0.73 *vs.* 31.38±0.71 days, $\chi^2_{[185]}$=0.68, *P*=0.40; Area 3: 36.25±0.53 *vs.* 36.23±0.40 days, $\chi^2_{[243]}$=0.27, *P*=0.60).

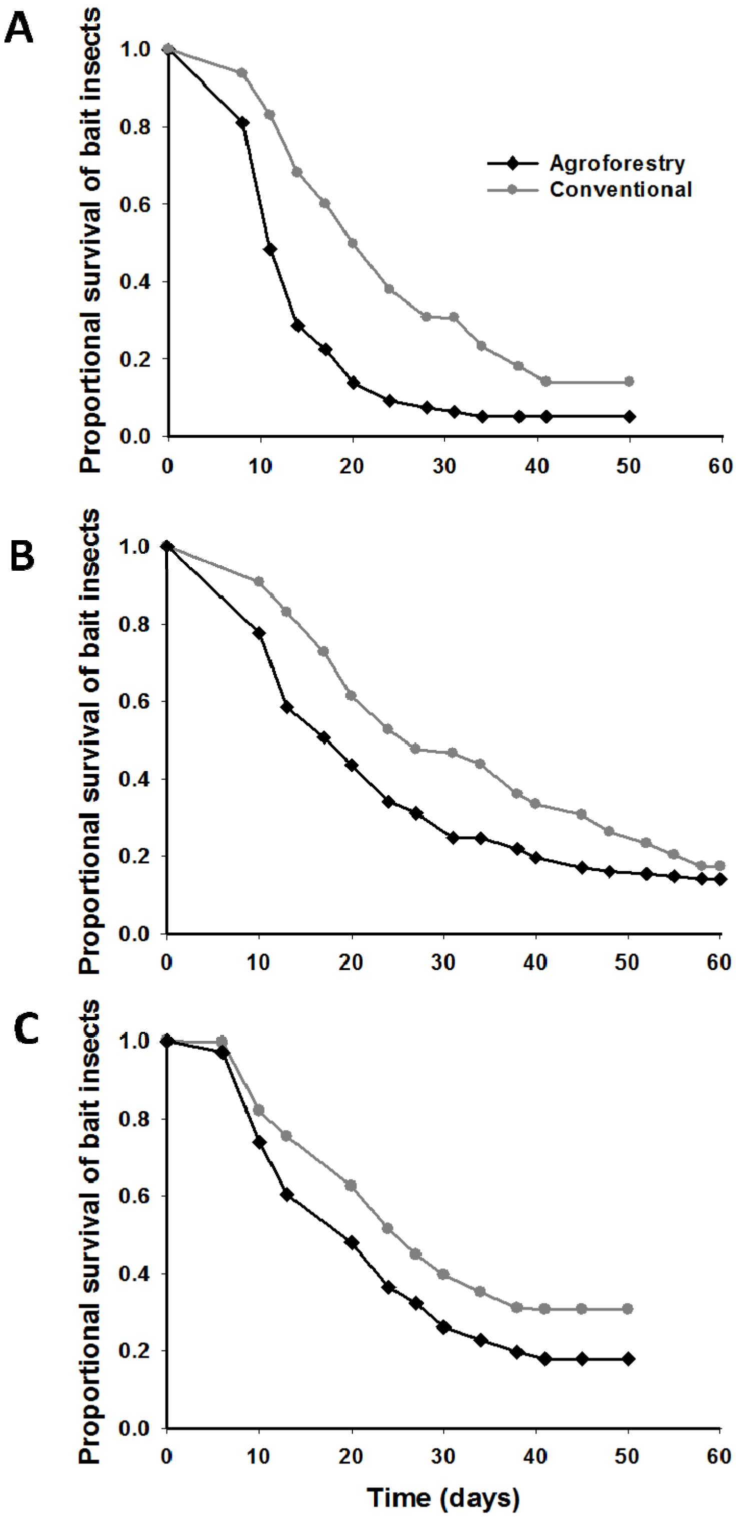

Figure 1. Differential survival of *Tenebrio molitor* bait insect larvae in soils from Agroforestry *vs.* Conventional coffee farming systems. Soils from six areas were sampled and were taken in pairs, each pair containing one area for each management type (Agroforestry x Conventional). Shown is proportional insect survival for (A) Area 1; (B) Area 2 and (C) Area 3. Survival analyses are presented in the text.

2.3.2 Fungal abundance and farming system

Frequencies of occurrence of insect-associated fungi (i.e. the number of samples that harbored at least one insect-associated fungus) were similar in agroforestry (96.7±0.01%, mean±SE) and conventional soil (93.8±0.01%, mean±SE) (ANOVA generalized linear models: $\chi^2_{[488]}$=183.34, *P*=0.1344). However, the numbers of fungal genera found per soil sample were greater in agroforestry (1.98±0.05, mean±SE) than conventional soils (1.79±0.06) (ANOVA, generalized linear models: $\chi^2_{[488]}$=257.37; *P*=0.02386; Fig. 2A). The number of isolates per soil sample was also greater in agroforestry (3.41± 0.11, mean±SE) than conventional soils (3.07± 0.10, mean ±SE) (ANOVA, generalized linear models: $\chi^2_{[488]}$=551.32, *P*=0.03895; Fig. 2B).

Sometimes, the fragment of the same bait insect placed at Petri dish yielded more than one fungal taxon, probably indicating a mixed infection. This happened in 16.2±0.009% (mean±SE) of the bait insects, but it was not affected by farming system (ANOVA, generalized linear models: $F_{[974]}$=1415.1; *P*=0.4334).

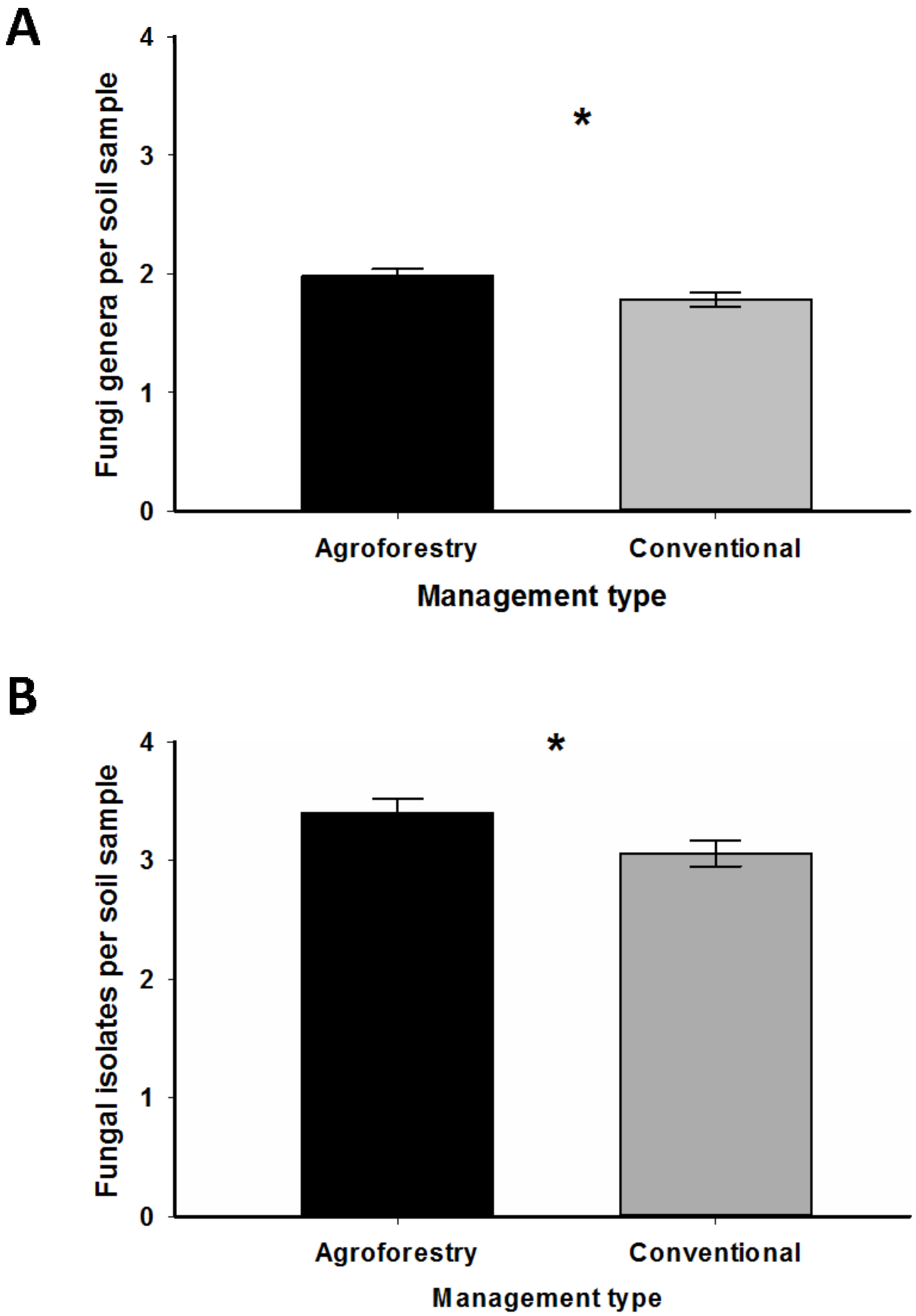

Figure 2. Mean (± SE) numbers of insect-associated fungi infecting bait insects (*Tenebrio molitor*) per sample of soil from two coffee management systems: Agroforestry and Conventional. (A) Mean (± SE) numbers of fungal genera per soil sample. (B) Mean (± SE) number of fungal isolates per soil sample. Soil samples were taken from six paired coffee fields, i.e. from three sites where samples could be taken from both systems, Araponga, Minas Gerais, Southeastern Brazil. * means $P < 0.05$ (see text).

2.3.3 Fungal species

Four fungal taxa were found. These were *Beauveria bassiana*, *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Isaria (=Paecilomyces) fumosoroseus* (Wise) and *Fusarium* spp. The latter includes a wide range of species and life styles, so we do not treat it as an entomopathogen here, although it can be a weak pathogen and is often reported in insects in opportunistic infections (Teetorbarsch & Roberts 1983). The other three taxa are well-known as entomopathogens.

Metarhizium anisopliae was found more in soils from agroforestry farming systems than conventional systems, while no such differences were found for the other species (ANOVA, generalized linear models: $\chi^2_{[488]}$=326.52, P=0.01) (Table 1). Of the fungi with proven entomopathogenic activity (i.e. excluding *Fusarium* spp.), this was also the most frequently found fungus species recovered (51.4±0.02 % mean+SE). Area 3 harbored a lower frequency of *M. anisopliae* than the other areas (ANOVA, generalized linear models: χ^2= 304.78, P= 0.766; Table 1) while, this frequency did not differ between areas 1 and 2 (ANOVA, generalized linear models: $\chi^2_{[488]}$= 304.78, P= 0.1369).

In area 3 *B. bassiana* frequency, in both management systems, was much higher than in the other areas (ANOVA, generalized linear models: $\chi^2_{[488]}$= 310.89, P=0.001; Table 1). In the other two areas, *B. bassiana* frequencies ranged from 4.1 to 15.9 % but there were no significant differences (ANOVA, generalized linear models: $\chi^2_{[488]}$=236.00, P= 0.7922).

For *I. fumosoroseus* there was no difference between the farming systems (ANOVA, generalized linear models: $\chi^2_{[488]}$=332.16, P=0.5237) or in relation to sampled areas (ANOVA, generalized linear models: $\chi^2_{[488]}$=328.67, *P*=0.3443). The same was observed for *Fusarium* spp. (Table 1)

Table 1. Fungi recovered from live bait insects (*Tenebrio molitor* larvae) in soils from agroforestry and conventional coffee plantations. Shown are frequencies of occurrence (% positive soil samples) (*$P<0.05$; ***$P<0.001$; *n.s.*= not significant).

Fungi	Agroforestry			Conventional			Mean	χ^2	P^1		Agroforestry	Conventional	χ^2	P^2	
	Area 1 n=97	Area 2 n=78	Area 3 n=70	Area 1 n=97	Area 2 n=76	Area 3 n=72					n=245	N=245			
Metarhizium anisopliae	70.1	70.5	30	59.8	46	31.9	51.4	303.73	<0.001	***	58.7	47.3	326.52	0.01169	*
Beauveria bassiana	4.1	15.9	48.9	6.2	7.9	45.9	21.5	234.89	<0.001	***	20.4	18.4	311.44	0.57	*n.s.*
Isaria fumosoroseus	29.9	24.3	17.1	23.7	18.4	23.6	22.8	328.67	0.3443	*n.s.*	24.48	22.04	332.16	0.5237	*n.s.*
Fusarium spp.	83.5	84.6	90	89.6	85.5	77.7	85.1	131.93	0.1868	*n.s.*	85.7	84.8	132.85	0.7998	*n.s*

$^{1,2}P$- values are from the analyses comparing frequencies of occurrence of the each fungal taxa between ^{1}the sampled areas & ^{2} farming systems

2.4 Discussion

Our study design enabled us to detect apparently positive effects of the agroforestry systems on the activity of insect-pathogenic fungi and their abundance, when compared with conventional systems. This is the first time that an interaction between farming management systems and the activity of soil-borne insect-pathogenic fungi has been demonstrated in agroecossystems.

2.4.1 Speed of kill of bait insects

Entomopathogenic fungi infecting an insect host invariably expend time until complete the process of disease development up to host death, and the outcome of an infection depends on the pathogen's potential to grow rapidly, to penetrate host-induced barriers and resist insect defenses (Hajek & Stleger 1994). If we consider that the bait insects here were put in contact with the soil, where the concentration of fungal conidia (the infective stage) is probably low and the insects need to move through the soil to enter in contact with these, the mortality shown with agroforestry soils samples could be considered high, and the time to death quick. In experiments with the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleptera: Scolytidae), one of the most important pest insect of coffee crop cultivation, *B. bassiana* and *M. anisopliae* took about 6 to 10 days to kill around 40 to 90% of insects when inoculated directly at high concentrations (Samuels *et al.* 2002; Neves & Hirose 2005). This is not too different from a mortality of 67.8 % of the bait insects in agroforestry soils after 20 days (the present study).

Although insect mortality was concentrated in the first 20 days in agroforestry soils insect hosts continued to be killed by fungi until the end of the evaluation period (ca. 40 days), even for bait insects in contact with conventionally farmed soils, and despite of the low rates of mortality. This is crucial when we consider the persistence and maintenance of the functionality of these fungi in the field, mainly when pest control potential is considered (Vänninen *et al.* 2000), indicating that these fungi can survive in soil in the absence of insect hosts, until they find a suitable host.

2.4.2 Speed of kill as an indicator of greater ecosystem services in agroforestry - a link to biodiversity?

Our finding of greater activity of insect-pathogenic fungi in organically managed agroforestry soils supports the hypothesis that these systems have a positive effect on insect-associated fungi and promote insect biological control. Agroforestry might contribute to the improvement and maintenance of the viability and virulence of these fungi, i.e. the ability to infect and kill an insect host, because these systems can provide a more constant environment, with low levels of disturbance (Jose 2009) where solar radiation and microclimatic stability increase as a function of the shade cover (Lin 2007). These characteristics can directly influence the persistence of entomopathogenic fungi in soils (Chandler *et al.* 1997; Vänninen *et al.* 2000). Thus it seems that agroforestry systems offer more suitable conditions that are close to the characteristics found in natural and semi-natural habitats.

The occurrence (in terms of genera and isolates) of insect-pathogenic fungi was also greater in agroforestry soils. This may help to explain the more rapid death of bait insects in these soils. Meyling and Eilenberg (2006b) found a greater abundance of entomopathogenic fungi in hedgerow soil than in neighbouring cultivated soil and this correlated with more rapid death of bait insects.

If we take bait insect survival times as an indicator of the ecological service provided by these fungi in agroecosystems, we can compare this variable with services provided by other organisms. In more heterogeneous agroecosystems there is evidence of greater provision of ecological services such as biological control of insect pests provided by insects predators and parasitoids (Altieri 1999; Bianchi *et al.* 2006; Tscharntke *et al.* 2007; Gardiner *et al.* 2009; Garrido-Jurado *et al.* 2011; Winqvist *et al.* 2011). Many studies report the increase of ecosystem services with the increase of biodiversity in agroecossystems under organic management (Benton *et al.* 2003; Tscharntke *et al.* 2005; Bianchi *et al.* 2006; Letourneau & Bothwell 2007; Tscharntke *et al.* 2007), and agroforestry (Tscharntke *et al.* 2011). Here we cannot yet comment on fungal diversity, because for entomopathogenic fungi like *B. bassiana* e *M. anisopliae*, it is very difficult to measure diversity. These two species actually contain a number of cryptic species that are morphologically indistinguishable and have distinct roles in a single ecosystem (Meyling *et al.* 2009; Fisher *et al.* 2011). If the diversity of the fungi we study here relates to ecosystem services they provide, then there is much work to be done to elucidate how, and it is early to draw any firm conclusions.

According to Tscharntke et al. (2011) shaded tree agriculture can reduce pesticide spraying and protect functional agrobiodiversity such as antagonists of pests, diseases and pollinating. When landscape perspective is considered, natural forest alongside agroforestry allows non crop to crop spillover of a diversity of functionally important organisms. This perspective is important mainly considering conservational biological control (Tscharntke *et al.* 2007).

Another important characteristic of the agroforestry system is elevated plant biodiversity. This has been shown to affect biodiversity maintenance, ecosystem functions and services, promote natural enemy populations and reduce crop damage by herbivores (Winqvist *et al.* 2011). Insect-pathogenic fungi have been reported in an extensive range of associations with plants during the periods that these fungi are not infecting insects. These feature is attribute to a versatile lifestyle that is characteristic of Hypocreales (Vega *et al.* 2009). Thus, plant diversity in agroforestry systems could promote insect-pathogenic fungi.

2.4.3 Numbers of samples positive for fungi

The majority of our soil samples were positive for fungi (96.7% for agroforestry and 93.8% for conventional soils) but there were no statistically significant differences between the two types of soil. Similarly high frequencies were found by Bidochka et al (1998) and Keller et al (2003) but most studies yielded much lower frequencies of 19% to 55.5% (Ali-Shtayeh *et al.* 2003; Bruck 2004; Sookar *et al.* 2008; Sun & Liu 2008; Jabbour & Barbercheck 2009; Goble *et al.* 2010). Most studies that used this measure to compare farming systems showed no differences between systems (Meyling & Eilenberg 2006b; Jabbour & Barbercheck 2009; Goble *et al.* 2010). Only one study has shown a difference in frequencies of fungal occurrence in baited soils from different farming systems (higher frequencies in organic soils) (Klingen *et al.* 2002). It is possible that the very high frequencies found in our study masked any differences between areas. It is also possible that the lack of a difference between the two areas is due to fact that neither system included the use of chemical pesticides – Goble et al. (2010) used this to explain a similar lack of difference between organic and conventional farming system in citrus crops in South Africa. Overall, it is possible that the studies that used only this variable to quantify fungi in soil might more profitably have used other variables such as those discussed above.

2.4.2 Fungal taxa

We found *M. anisopliae* to be the more common of the insect-pathogenic fungi and we also found it to be more frequent in the organically managed agroforestry soils. In previous studies from temperate regions, this fungus has been reported to be more abundant in cultivated soils than in surrounding non-cultivated soils (Vanninen 1996; Bidochka *et al.* 1998; Meyling & Eilenberg 2007; Sun & Liu 2008; Jabbour & Barbercheck 2009). It is possible that this fungus is quite well-adapted to survive in agricultural soils but very little is known about its ecology in the soil, except that some strains (at least) are rhizosphere-competent (Hu & St Leger 2002) and can promote root development (Sasan & Bidochka 2012).

In our study, *B. bassiana* was generally found at low frequencies. This is in line with previous studies that report high abundance and diversity of this fungus in semi-natural areas like hedgerows and refugia (Meyling & Eilenberg 2006b; Sun *et al.* 2008; Meyling *et al.* 2009; Goble *et al.* 2010) but low abundance and diversity in cultivated soils (Meyling *et al.* 2009). We did find high levels of this fungus in area 3. Curiously, this area was sampled last, after the coffee harvest (in September). *Beauveria bassiana* is extensively reported in coffee plantations due to its occurrence as a natural mortality factor in the coffee berry borer *H. hampei* (De La Rosa *et al.* 2000). This coleopteran pest bores galleries into the endosperm of the coffee seed, and subsequently these infested berries fall to the ground (Damon 2000). If these insects are infected with *B.*

bassiana, they can act as inoculum in the soil. Thus, our elevated levels of *B. bassiana* in area 3 could be derived from fallen berries. This fungus has low conidial persistence in soils (Vänninen *et al.* 2000) and some studies suggest that its persistence depends directly on the presence of the host insects to maintain high conidial levels in soil (Meyling & Eilenberg 2006b). Bustillo et al. (1999) reported that field levels of infection of *H. hampei* by *B. bassiana* increased 25 days after the first infection of *H. hampei* in fallen berries, this increase may be caused by the conidiation of fungi on cadavers.

The high levels of *B. bassiana* in area 3 may also explain the low *M. anisopliae* levels in this area if there is competition between these fungi for the bait insect host. It does not mean that *M. anisopliae* is at low levels in soil, but that it is competitively excluded by *B. bassiana* in this season or only in our soil samples.

The genus *Fusarium* has previously been recovered with the bait insect method in Palestine (7.9%) (Ali-Shtayeh *et al.* 2003), in Norway (62%) (Klingen *et al.* 2002), in China from crop fields (76.9%) and orchard (80.6) and in China again from different parts of the country (14.1%). The high frequencies that we found have not previously been reported. *Fusarium* species have been reported infecting the insect orders Coleoptera, Diptera, Isoptera, Lepidoptera and Orthoptera (Teetorbarsch & Roberts 1983). This genus is widespread in nature and can switch between parasitism (although predominantly of plants) and saprophytism during cycles. It is possible that it can also switch between insect parasitism and other lifestyles (Teetorbarsch & Roberts 1983).

We speculate that its high frequency in our study may be due to secondary infections following primary infections by an entomopathogenic fungus – *B. bassiana* and *M. anisopliae* are poor competitors for organic resources compared to opportunistic and saprophytic fungi that are ubiquitous in the soil (Goble *et al.* 2010) like *Fusarium*. It remains to be seen whether the *Fusarium* isolates we found are in fact insect pathogens or derive from secondary infections of the bait insects; this will require assays in the laboratory.

2.4.3 Conclusions and perspectives

Our findings suggest that agroforestry systems improve the action of insect-pathogenic fungi as has previously been shown for other functional groups such as hymenoptera parasitoids (Peng *et al.* 1993; Lopez-Llorca *et al.* 1999; Tylianakis *et al.* 2005; Klein *et al.* 2006) and processes such as decomposition (Schroth *et al.* 1992; Martius *et al.* 2004). The finding is novel for insect-pathogenic fungi, however, and we suggest that we have taken the first steps to reveal and explore a very important ecosystem service, one requiring, ultimately, conservation in order to exploit it to the fullest.

Although our method of using survival analyses to measure the effect of soil management on the bait-insect mortality is innovative, it is necessary to improve and refine the technique if we wish to use it to study insect pathogen as providers of ecosystem services. It appears that insects need to be accompanied for only twenty days to reveal differences in survival, but other parameters that we need to establish are: how many insects and how many soil samples to use. We also need to make the link between survival of bait insects in the laboratory an survival of pest insects in the field, whih promises to be challenging.

A number of other questions arise from this study. Among the most relevant we can ask: Is fungal genetic diversity responsible for high mortality rates in agroforestry systems? Could this diversity be derived from diversity of ecosystem compartments? Do these entomopathogens maintain a symbiotic relationship with plants when they are not infecting insects?

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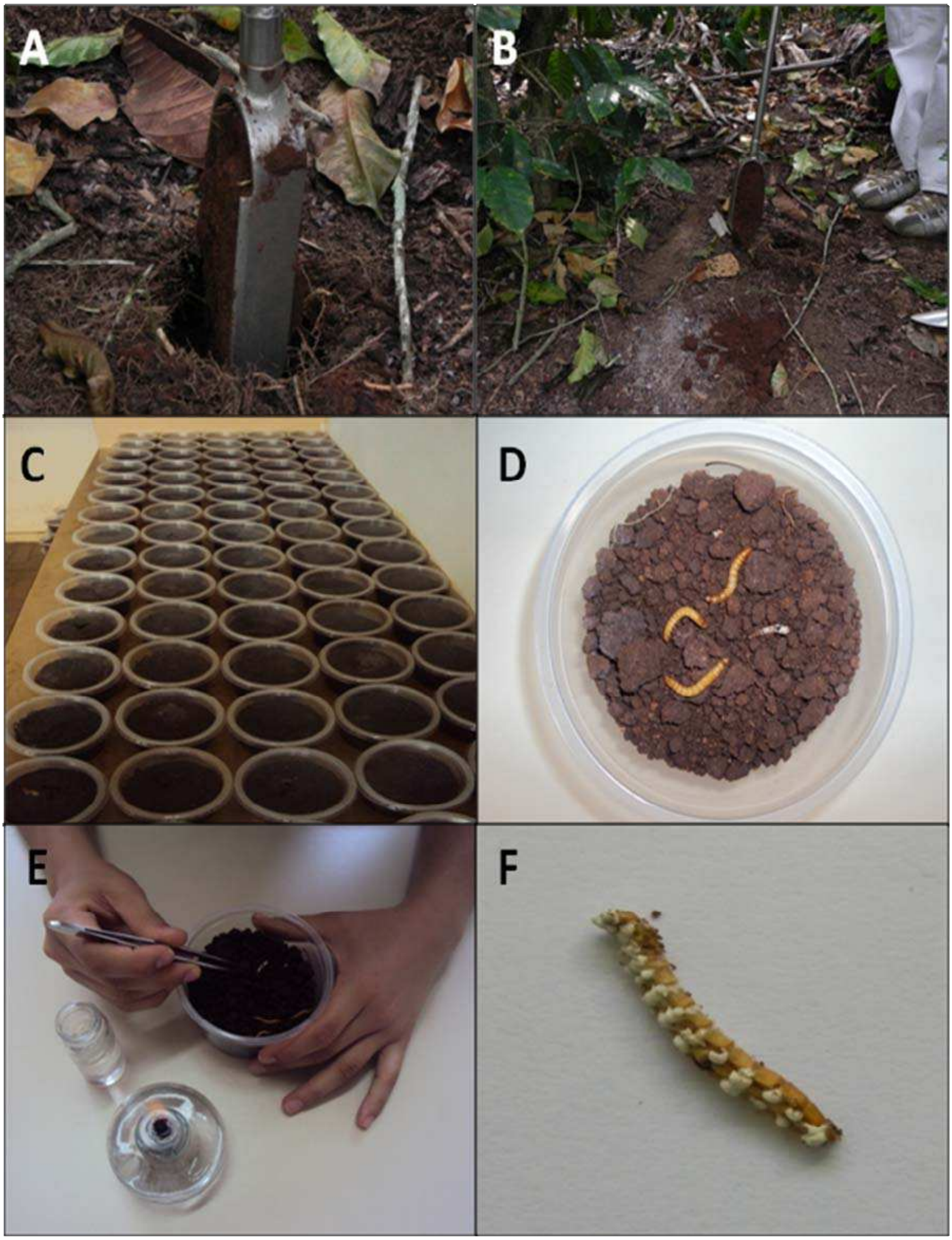
Appendix 1. Soil characteristics of each sampled area in agroforestry and conventional coffee systems in Araponga, Zona da Mata, Southest Brazil

	Agroforestry			Conventional		
Soil characteristics	F1	F3	F5	F2	F4	F6
Depths (cm)	20	20	20	20	20	20
Clay (%)	52	48	46	59	47	46
Sand (%)	43	44	39	33	39	59
pH (H2O)	4.86	6.03	6.02	5.36	5.36	5.60
P Mehlich-1 (mg/dm3)	0.7	3,3	3.9	2.7	1.8	3.1
Organic Matter (dag/Kg)	5.87	4.61	5.72	5.2	2.94	4.92

Appendix 1. Sampled areas informations in coffee systems, Araponga, Minas Gerais, Southestern Brazil.

Area	Farming System	Locality	Farming characteristics	Number of soil samples
Area 1	Agroforestry	Pedra Redonda	Organic fertlizers, Green manure, no pesticides use, shaded soil	97
	Conventional	Pedra Redonda	Inorganic fertlizers, no pestices use, unshaded soil	97
Area 2	Agroforestry	Araponga-Canaã	Organic fertlizers, Green manure, no pesticides use, shaded soil.	78
	Conventional	Araponga-Canaã	Inorganic fertlizers, no pestices use, unshaded soil	76
Area 3	Agroforestry	Pedra Redonda	Organic fertlizers, Green manure, no pesticides use, shaded soil	70
	Conventional	Pedra Redonda	Inorganic fertlizers, no pestices use, unshaded soil	72



Appendix 3. (A,B) Soil sampling with core soil sampler. (C) Soil samples in transparent cups. (D) Bait insects *Tenebrio molitor* in soil sample. (E) Evaluation procedure (F) Dead bait insect with entomopathogenic fungus external growth

CONCLUSÃO GERAL

Nós concluímos que fungos entomopatogênicos apresentam maior atividade e abundância em solos de sistemas agroflorestais comparados a solos convencionais. Esses resultados corroboram com resultados anteriores mostrando que sistemas agrícolas com condições mais próximas às encontradas em ecossistemas naturais são mais supressivos a insetos pragas. Acreditamos que esse tipo de sistema pode proporcionar melhores condições favoráveis a maior atividade e maior abundância de fungos entomopatogênicos, todavia os efeitos dessas condições ainda precisam ser investigados. Nós também geramos uma nova abordagem da metodologia de "inseto-isca", explorando a habilidade desses fungos em infectar e matar insetos, construindo curvas de sobrevivência com os dados de mortalidade. Essa abordagem nos permitiu inferir sobre os serviços de ecossistemas prestados por esses fungos no controle biológico de insetos praga através do tempo de sobrevivência dos "insetos-isca".