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Entomopathogenic fungi as promoters of ecosystem services for pest control in coffee crop management systems.

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Doctor Scientiae

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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A mim, por seguir firme e não desistir.

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ABSTRACT

REIS, Lorene Carla dos, D.Sc., Universidade Federal de Viçosa, February, 2025. **Entomopathogenic fungi as promoters of ecosystem services for pest control in coffee crop management systems.**. Adviser: Simon Luke Elliot. Co-advisers: Thairine Mendes Pereira, Elenir Aparecida Queiróz and Madelaine Venzon.

The concept of ecosystem services has been discussed due to its relevance to the sustainability of agricultural systems, especially in relation to food security, environmental protection and the promotion of human health. There is increasing evidence that ecosystem services are achieved as a function of ecosystem biodiversity. Considered the main reservoir of biological diversity, soils are home to a variety of microorganisms, which are responsible for a significant proportion of ecosystem services. A fundamental regulatory ecosystem service is the biological control of naturally occurring pests, in which entomopathogenic fungi play a central role. The population dynamics and effectiveness of entomopathogenic fungi are directly related to soil conditions and the agricultural management adopted. Practices that favor the maintenance of soil biodiversity can enhance biological control, reducing dependence on chemical inputs and promoting a more sustainable balance in agroecosystems. This thesis aims to analyze the diversity and functionality of entomopathogenic fungi in different coffee cultivation systems (agroforestry, organic and conventional), evaluating the impact of management practices and the availability of macro and micronutrients in the soil on fungal composition. For this purpose, molecular methodologies were used to identify and quantify the species present in the soil, in addition to the live bait technique to evaluate the effectiveness of fungi in suppressing pests. Principal Component Analysis, which aimed to identify whether there is evidence that the types of management differ in terms of the type of nutrition and the composition of the fungi, revealed that there are significant differences in fungal diversity between the agricultural management systems. The results indicate that the organic and agroforestry systems have greater fungal diversity compared to conventional crops, evidencing the effect of agricultural management on the composition of soil fungal communities. In addition, we evaluated the ecosystem service of pest control according to the type of coffee management systems, and through the mortality of the baits, survival curves were constructed and the fungal isolates from the insects were isolated, identified and quantified for each system. These fungi were more active in organic and agroforestry management systems, showing greater potential for the control of Hypothenemus hampei, through the survival curves, and for Tenebrio molitor, the collections in different coffee management systems had the greatest influence on its survival. The research aims to reinforce the importance of the biodiversity of entomopathogenic fungi for the resilience of agricultural systems and provide fundamental information for the development of management strategies that maximize the ecosystem services provided by these organisms. Thus, the results obtained may contribute to the development of sustainable approaches, thus promoting the conservation of soil biodiversity, aligning with the demands for more efficient and environmentally responsible agricultural production.

Keywords: Keywords: Soil-microorganism interaction, microbial diversity, agroecosystems, pest suppression, agricultural sustainability

RESUMO

REIS, Lorene Carla dos, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Fungos entomopatogênicos como promotores de serviços ecossistêmicos para controle de pragas em sistemas de manejo da cultura cafeeira.**. Orientador: Simon Luke Elliot. Coorientadores: Thairine Mendes Pereira, Elenir Aparecida Queiróz e Madelaine Venzon.

O conceito de serviços ecossistêmicos tem sido atualmente discutido devido à sua relevância para a sustentabilidade dos sistemas agrícolas, especialmente em relação à segurança alimentar, à proteção do meio ambiente e à promoção da saúde humana. Há cada vez mais evidências de que os serviços ecossistêmicos são atingidos em função da biodiversidade dos ecossistemas. Considerado o principal reservatório de diversidade biológica, os solos abrigam uma diversidade de microrganismos, sendo esses responsáveis por uma proporção significativa dos serviços ecossistêmicos. Um serviço ecossistêmico regulador fundamental é o controle biológico de pragas que ocorre naturalmente. Nele, os fungos entomopatogênicos desempenham um papel central. A dinâmica populacional e a eficácia dos fungos entomopatogênicos estão diretamente relacionadas às condições edáficas e ao manejo agrícola adotado. Práticas que favorecem a manutenção da biodiversidade do solo podem potencializar o controle biológico, reduzindo a dependência de insumos químicos e promovendo um equilíbrio mais sustentável nos agroecossistemas. Esta tese tem como objetivo analisar a diversidade e a funcionalidade dos fungos entomopatogênicos em diferentes sistemas de cultivo de café (agroflorestal, orgânico e convencional), avaliando o impacto das práticas de manejo e da disponibilidade de macro e micronutrientes do solo sobre a composição fúngica. Para isso, foram empregadas metodologias moleculares para a identificação e quantificação das espécies presentes no solo, além da técnica de iscas vivas para avaliar a eficácia dos fungos na supressão de pragas. Análise de Componentes Principais que teve como objetivo identificar se há indícios de que os tipos de manejos se diferenciam quanto ao tipo de nutrição e a composição dos fungos, revelou que, existe diferenças significativas na diversidade fúngica entre os sistemas de manejo agrícola. Os Resultados indicam que os sistemas orgânico e agroflorestal apresentam maior diversidade fúngica em comparação aos cultivos convencionais, evidenciando o efeito do manejo agrícola na composição das comunidades fúngicas do solo. Além disso, avaliamos o serviço ecossistêmico de controle de pragas em função do tipo de sistemas de manejo do cafeeiro, e através da mortalidade das iscas, curvas de sobrevivências foram construídas e os isolados fúngicos provenientes dos insetos foram isolados, identificados e quantificados para cada sistema. Esses fungos foram mais ativos em sistemas de manejo orgânico e agroflorestais apresentando maior potencial para o controle *Hypothenemus hampei*, através das curvas de sobrevivências, e para *Tenebrio molitor* as coletas em diferentes sistemas de manejo do café que tiveram maior influência em sua sobrevivência. A pesquisa busca reforçam a importância da biodiversidade de fungos entomopatogênicos para a resiliência dos sistemas agrícolas e fornecem informações fundamentais para o desenvolvimento de estratégias de manejo que maximizem os serviços ecossistêmicos prestados por esses organismos. Dessa forma, resultados obtidos poderão contribuir para o desenvolvimento de abordagens sustentáveis, promovendo assim a conservação da biodiversidade do solo, alinhando-se às demandas por uma produção agrícola mais eficiente e ambientalmente responsável.

Palavras-chave: Palavras-chave: Interação solo-microrganismo, diversidade microbiano, agroecossistemas, supressão de pragas, sustentabilidade agrícola

Summary

GENERAL INTRODUCTION

1. Ecosystem Services, Biological Control and the Role of Entomopathogens

Anthropogenic phenomena such as economic growth, demographic changes, advances in science and technology, together with changes in political behavior, have resulted in significant changes in ecosystems. These changes can affect the capacity of ecosystems to generate services essential to life. In recent centuries, humans have significantly altered the cycles of several key nutrients for supporting life (Kumar et al., 2013; Sukhdev et al., 2008; Costanza et al., 1993). Interest in ecosystems has gained special importance due to the growing concern about the interconnections between the state of ecosystems, the well-being of human landscapes, and the negative impacts that drastic changes in the flows of essential services provided by ecosystems can have on the well-being of societies (Andrade et al., 2010). According to Millennium Ecosystem Assessment - MEA (2003; 2005), a comprehensive assessment included information on the state and condition of the world's ecosystems, concluding that there was a 60% loss between 1960 – 2000.

Ecosystems encompass complex, dynamic and continuous interactions between biotic and abiotic resources occurring at different spatial and temporal scales. Following the economic-ecological perspective, an ecosystem represents “natural capital” that corresponds to the set and stock of ecosystem functions, which give rise to the benefits provided by ecosystems to human well-being (Costanza & Daly, 1992; Andrade & Romeiro, 2009).

The set of biotic and abiotic resources that make up ecosystems, the ecosystem structure, is heterogeneous (Andrade et al. 2010). Each ecosystem has thousands of structural elements, exhibiting varying degrees of complexity (Costanza et al., 1993; Costanza et al., 1997). These structural elements exhibit evolutionary and non-mechanistic behaviors (Costanza et al., 1993), so ecosystems can be characterized by non-linear behaviors, that is, it is not possible to make predictions of interventions based solely on knowledge about each component that makes up ecosystems (Turner; Daily, 2008; Daly; Farley, 2004).

The constant interactions that exist between the structural elements of an ecosystem, including energy transfer, nutrient cycling, gas regulation, climate regulation and the water cycle, are called ecosystem functions (De Groot et al., 2002; MEA, 2003; Daily & Farley, 2004). These functions are a subset of the ecological processes and ecosystem structures, thus allowing a systematic relationship within the ecosystem itself (De Groot et al., 2002).

Ecosystem functions can be classified as: regulatory function, responsible for the self-regulation of essential ecological resources and maintenance of life through biogeochemical cycles and other biosphere processes. Among the processes of regulation of biota, maintained by biogeochemical processes, is the chemical composition of the atmosphere, oceans and biosphere – balance between oxygen and carbon dioxide, maintenance of the ozone layer; habitat function, responsible for providing refuge and habitat for the reproduction of wild plants and animals, cooperating for the preservation (in situ) of biological and genetic diversity, supporting ecological succession, ensuring evolutionary processes; production function: responsible for providing ecosystem goods for consumption by autotrophic and heterotrophic organisms; information function, responsible for contributing to cognitive and spiritual development, cultural, aesthetic and artistic experiences, in addition to offering historical, cultural and scientific information (De Groot et al. 2002). The regulatory and habitat functions are crucial in maintaining natural elements, thus, the production and information functions are conditional (De Groot et al. 2002).

Ecosystem function is considered an ecosystem service (ES) when it presents the possibility and/or potential to be used for human purposes (Costanza et al., 1989; MEA, 2005). Thus, services and goods provided by ecosystem functions are tangible (material) or intangible (brands, licenses, patents) resources originating from natural capital and can be consumed by the inhabitants of a region (Daily, 1997; Hueting et al., 1998).

The relationship between ecosystem functions and ES becomes relevant when we consider that ES can be understood as specific functions performed by ecosystems (Hueting et al., 1998; Andrade & Romeiro, 2009). Not all ecosystem functions generate ES for use by humans, but they are as important for the balance and maintenance of ecosystems as those that play a role as ES for the natural environment (Mononen et al., 2016).

The concept of ES was introduced in the mid-1980s with the aim of raising public awareness about the negative impacts of the loss of biological diversity on human quality of life (Ehrlich and Ehrlich, 1996; Mooney et al., 1997). Several authors suggest different definitions to describe ES (Santos et al., 2014). Numerous studies have investigated the relationship between natural processes (biogeochemical cycles, biodiversity and natural resources) and economic systems, highlighting how natural resources provide a continuous flow of goods and services essential to human well-being (De Groot, 1987; Costanza et al. 1997).

The most commonly used approach, according to the MEA (2005), to the multiple benefits of ecosystem services for humans can be classified in a similar way to ecosystem functions into: 1) regulating services – referring to the benefits obtained from ecosystem processes that regulate the environmental conditions that sustain human life, such as air quality, pest and disease control, climate regulation, purification and regulation of the water cycle, flood and erosion control, waste treatment and detoxification; 2) provisioning services – responsible for the outputs of material or energy that can be obtained directly from ecosystems, for example, food, raw materials for energy generation, fibers, phytopharmaceuticals, genetic and biochemical resources, ornamental plants, water; 3) supporting services –the natural processes necessary to maintain other ecosystem services such as: soil formation, nutrient cycling, pollination and seed dispersal; and 4) cultural services – the important roles the ecosystem plays in education, recreation or conservation.

Ecosystems and their services (provision, support, cultural and regulatory) have (economic) value to the extent that humans, directly or indirectly, derive utility from their actual or potential use. One example of a regulatory ecosystem service is biological control. Biological control is a natural phenomenon involving the regulation of one population by another. In this service, the control of population density is regulated by natural enemies, biotic agents of mortality. When biological control is interfered with by humans, for use in agriculture, for example, with the aim of increasing the antagonistic interactions that occur between living beings, this is called Applied Biological Control. Applied Biological Control acts to regulate the population of agricultural pests by introducing a natural enemy, also called biological control agents, which can be parasitoids, predators or pathogens, keeping the pest population below the level of non-economic damage, and consequently reducing the need for pesticide application and production costs (Fiedler et al., 2008; Bengtsson, 2015). Applied Biological Control can be Classical, Inundative, Inoculative or Conservative (Parra, 2014; Hajek & Eilenberg, 2018).

Classical Biological Control occurs with the introduction of exotic natural enemies, from one country to another or from one biogeographic region to another. In Inundative Biological Control, natural enemies come from mass rearing in the laboratory and are periodically released in large numbers to obtain an immediate pest control effect for one or two generations, that is, these organisms are used as biological insecticides, acaricides, nematicides, fungicides, and herbicides (Schrank & Vainstein, 2010). Inoculative Biological Control consists of the release of a small number of natural enemies with the aim of suppression of the pest population over the next few generations (Greathead & Greathead, 1992; Parra, 2014; Gielen et al., 2024). Both Inundative and Inoculative Biological Control benefit from the resources provided by the ecosystem through provisioning ES, as they become products with economic value obtained directly from the exploration or management of ecosystems for human use (Bengtsson, 2015).

Conservation Biological Control (CBC) involves strategies that can increase the effectiveness of ecosystem services (Mattias et al. 2008) because this preserves the natural enemies of an agroecosystem, i.e., habitat management is carried out through agricultural practices that favor the maintenance of natural enemies in the field. In addition, CBC requires in-depth knowledge of the ecology of natural enemies and the regulatory ES of the ecological communities of which they are part (Mattias et al. 2008; Bengtsson, 2015).

Among the various biological control agents, EPF are recognized for promoting the ecosystem service of natural regulation of arthropods and pest biocontrol (Quesada-Moraga et al., 2023). They stand out for their applicability and their ability to cause infections in their hosts without requiring the ingestion of fungal propagules (Schrank & Vainstein, 2010). In addition to acting as an insect pest control agents, their ability to colonize plants and exist as fungal endophytes is unique and adds a new dimension to their use as biological agents for crop management (Bamisile et al., 2018; Vega et al., 2009). Entomopathogenic fungi have a phylogenetically diverse variety of species, and are capable of infecting and causing diseases in insects and other arthropods at different stages of development and with the most varied life habits (Valadares-Inglis et al., 2020; Gielen, 2024).

Currently, microbiological control has stood out as an inundative and sometimes classical biological control, since it is possible to produce microorganisms on an industrial scale. Here, mass production systems have been designed to provide large quantities of inocula that can be formulated and applied as sprays, granules and wettable powder (Shah; Pell, 2003; Brownbridge, 2006; Charnley; Collins, 2007). The adoption of CBC involves the implementation of agricultural practices or habitat management to increase the activities of entomopathogen populations (Fuxa, 1998). To adopt CBC, a greater knowledge of the natural enemies existing in the target area needs to be achieved. This can aid in the subsequent adoption of management practices that can help to conserve or promote the activities of natural enemies (Gurr et al., 2018).

The main advantages of using entomopathogenic microorganisms for pest control are the specificity and selectivity of these control agents, as well as the ease of multiplication, dispersion and production in artificial media and the absence of environmental pollution and toxicity to humans and other non-target organisms, among others. Thus, it is clear that microbial control of insects, as an arm of biological control, is the basis for supporting the natural balance of insect species potentially considered pests.

2. Entomopathogenic Fungi

The Kingdom Fungi is a group of heterotrophic organisms well known for providing a wide range of ecosystem functions that indirectly or directly affect human life (Heilmann-Clausene et al., 2014; Zedda & Rambold 2015; Willis, 2015; Pérez-Moreno 2021). Indirect ecosystem functions include nutrient cycling and recycling of organic matter, and transport, storage, and release of nutrients such as carbon (C), phosphorus (P), and nitrogen (N) (Niego et al., 2023). Fungi play a key role in C sequestration, with mycorrhizal symbioses functioning as C sinks, increasing the use of atmospheric carbon dioxide (CO2) (Wang et al., 2016). Saprotrophic fungi are responsible for the degradation of organic matter and assimilation of degradation products rich in C, also acting as a C sink, retaining C in the soil (He et al., 2019). Fungi have the capacity to remediate environmental pollutants through the production of enzymes with the capability to degrade these pollutants (Ali et al., 2017; Uddin et al., 2020; Yadav et al., 2021). They enhance soil formation by producing organic compounds that bind soil particles, thus improving soil structure and porosity. In addition, they directly contribute as a source of food, bioactive compounds, and raw material (Niego et al., 2023).

Fungi also exhibit a wide spectrum of lifestyles, for example in lichens or as ecto- or endomycorrhizae, endophytes, or mutualists or parasites of plants, of insects or of other fungi. Their interaction with other organisms can indicate their role and the service they provide to the ecosystem in which they live (Pringle et al., 2011; Heilmann-Clausen et al., 2014; Naranjo-Ortiz & Gabaldón, 2019; Niego et al., 2023; Quesada-Moraga et al., 2023). This is the case of entomopathogenic fungi, a group that forms hemibiotrophic interactions with insects, acting as natural regulators of their host populations (David, 1967; Meyling and Eilenberg, 2007; Jaramillo et al., 2015; Brunner-Mendoza et al., 2019).

The entry route for most EPF occurs through penetration of the host cuticle (Sharma et al., 2023). In contrast to other entomopathogens, such as viruses and bacteria, these fungi do not depend on ingestion to perform their pathogenic function, giving them an advantage (Rustiguel et al., 2017). The beginning of the fungal infection process begins with the attachment of the spore to the insect cuticle (Kershaw & Talbot, 1998), promoting germination and the formation of a germ tube that gives rise to the appressorium. This is a site of intense metabolic activity, favoring penetration into the host, or results in the formation of a mucilaginous mass around the germ tube, facilitating adhesion and the production of extracellular enzymes (Hajek & Leger, 1994;

Alves, 1998). During the penetration stage, the hyphae apply pressure to the insect's tegument, breaking it with the help of enzymes such as proteases, lipases and chitinases. These enzymes facilitate the mechanical invasion of the fungus (Alves, 1998). The cuticle represents a significant barrier, both mechanically and chemically, and can complicate or prevent the fungus from entering the insect's hemocoel. This resistance is due to structural characteristics, such as sclerotization, which blocks penetration by making the tegument more rigid, and the presence of tyrosinases that produce melanins with antimicrobial properties (Charnley, 2003). The fungus generates hyphae that multiply inside the insect after penetration, invading structures such as the procuticle, epidermis and hemocoel. During this process, various parts of the body, such as muscle tissue, adipose tissue and internal organs, are colonized by the vegetative growth of the fungus and the production of toxins (Pucheta et al., 2006). When nutritional resources are exhausted, hyphae emerge on the surface, initiating the generation of spores under appropriate moisture conditions (Gillespie & Claydon, 1989).

Generalist entomopathogenic fungi, such as *Beauveria bassiana*, *Metarhizium* spp., and *Cordyceps* spp., are primarily recognized for promoting the ecosystem service of natural regulation of arthropod populations and pest biocontrol (Quesada-Moraga et al., 2023). They are widely used in commercial formulations to regulate the populations of a wide range of pests in crop fields, forests, greenhouses, and storage environments (de Faria and Wraight, 2007; Mascarin and Jaronski, 2016; Jaronski and Mascarin, 2017; Mascarin et al., 2019; Quesada-Moraga et al., 2023). The formulations are produced with fungal strains isolated from naturally infected soil-dwelling insects (Islam et al., 2021). However, only a few genera of entomopathogenic fungi (e.g., *Beauveria*, *Cordyceps*, *Hirsutella* and *Metarhizium*) are being used for biocontrol strategies, although Order Hypocreales harbors over 700 species known to be insect pathogens (Shin et al., 2020; Sinha et al., 2016; Shapiro-Ilan, Hazir & Glazer, 2017). Despite their use for biocontrol, the other functions and services promoted by entomopathogenic fungi in ecosystems are still not fully understood.

Many fungal species are naturally found in the soil environment, therefore soil serves as a natural reservoir and shelter for a vast number of species of entomopathogenic fungi and a potential source for the isolation of novel and functional strains (Jaronski, 2007; Bridge & Spooner, 2001). Factors such as soil moisture, temperature, pH, solar radiation and other microorganisms that inhabit the soil can influence the potential of the soil for new strains of entomopathogenic fungi (Moore et al., 1993; Alves et al., 1998).

3. Soil

Soil can be defined as the layer of unconsolidated material that covers the Earth's surface, being a physical substrate for plant and animal life and for most human activities (Lepsch, 2013). Composed of three interfaces, solid (dead geological and biological materials), liquid (water) and gaseous (air in the soil pores) (Aislabie, Deslippe and Dymond, 2013), soil provides several benefits related to climate change, pollutant stabilization, in addition to being a reservoir of biological diversity (MEA, 2005; Adhikari & Hartemink, 2016).

Soil is considered a natural habitat for a wide diversity of microorganisms, including (EPF) (Sharma et al., 2018). Among the EPF successfully isolated from soils for arthropod control and widely used, the following stand out: *Beauveria* spp. (Hypocreales: Cordycipitaceae), *Metarhizium* spp. (Hypocreales: Clavicipitaceae), *Cordyceps* spp. (Hypocreales: Cordycipitaceae) (Faria & Wraight, 2007; Medo and Cagán, 2011, Vega et al. 2012). This particular group of fungi is recognized for the ecosystem service of regulating arthropods, especially insects. This ecosystem service is essential for the sustainability of natural and anthropogenic ecosystems (Barrios, 2007; Correa et al., 2022).

The growth of entomopathogenic fungi is conditioned by an organic source of carbon, oxygen, water, inorganic or organic nitrogen and additional elements, such as minerals and growth factors. The essential macronutrients are phosphorus, potassium, magnesium and sulfur (Rath et al., 1992). Different factors, such as soil moisture, temperature, pH and solar radiation interfere with the effectiveness of EPF in the soil, in addition to other microbes and organisms that inhabit the soil and influence their persistence and survival (Johansson et al., 1999; Jaronski, 2007; Meyling & Eilenberg, 2007; Vega et al., 2012). To allow the conservation and transmission of fungal infections in the soil, it is necessary to understand the effects of these factors on the effectiveness, for example, of spore germination and mycelial growth of these fungi (Lacey & Mercadier, 1998).

Several studies have investigated the occurrence and abundance of EPF in natural and cultivated soils worldwide (Vanninen, 1996; Bidochka et al., 1998; Klingen et al., 2002; Ali-Shtayeh et al., 2003; Meyling & Eilenberg, 2006; Quesada-Moraga et al., 2007; Goble et al., 2010; Moreira et al., 2019). The occurrence and distribution of EPF in soil includes many species belonging to the order Hypocreales that deposit their infectious spores and reside in the soil for part of their life cycle, when they are not parasitizing their arthropod host (Faria; Wraight 2007; Medo and Cagán, 2011, Vega et al. 2012).

Soil management is essential for the sustainability of agricultural production, as it involves a set of practices that are external to the maintenance and improvement of its chemical, physical, and biological properties. The main objective of these practices is to minimize manipulation processes and ensure long-term productivity, reconciling agricultural exploitation with the conservation of natural resources (Melo, 2021). Within this context, different management systems have been adopted, each with different impacts on the ecosystem and agricultural production.

Agroforestry systems represent a sustainable land use strategy based on the integration of agricultural crops with tree species. This approach increases the structural and functional complexity of agroecosystems, providing benefits such as greater biodiversity and expansion of ecosystem services, including pollination (Klein et al., 2003) and natural pest control (Kellermann et al., 2008). However, the structure of these systems varies according to the level of management intensification. More intensive systems that prioritize agricultural productivity tend to reduce tree diversity and, consequently, the complexity of the environment (Beer et al., 1998). Organic management, in turn, seeks to minimize the environmental impacts of agriculture by replacing synthetic fertilizers and chemical pesticides with practices that promote the health of the soil and agricultural ecosystems. Among the strategies adopted, crop rotation, the use of legumes for biological nitrogen fixation, the integration of crops and livestock, in addition to the use of organic ester and biological control of organic matter stand out. These practices not only harm soil, water and air contamination, but are also reduced in order to maintain the fertility and resilience of the agroecosystem (Ormond et al., 2002; Köpke, 1997; Vogt, 2000). In contrast, conventional management intensifies agricultural production through the massive use of external inputs, such as synthetic fertilizers and pesticides (Souza, 2005). Although this approach allows for high yields in the short term, its long-term environmental impacts are worrying. Soil manipulation, contamination of water resources and reduction of biodiversity are some of the neglected consequences of conventional agriculture (Caporal & Costabeber, 2004).

Soils have different qualities, so not all soils can provide satisfactory ecosystem services. Soil quality is understood to refer to the soil's ability to perform its functions within the natural or managed limits of an ecosystem, thus ensuring the sustainability of both plant and animal productivity, of air and water quality, and the guarantee of conditions for human habitation (Doran and Jones, 1997; Karlen et al., 1997). Although knowledge about soils has been widely disseminated and classified in terms of physical and chemical characteristics, knowledge of soil biodiversity and function is far from complete (Wall and Virginia, 2000, Brussaard et al., 2004).

This gap is due, in part, to limited knowledge of the conservation of ecosystem service performed by soil microbiota (Brusaard et al., 1997, Coleman et al., 2017).

4. Sampling of entomopathogenic fungi in soil

Entomopathogenic fungi are generally heterogeneously distributed in soil, presumably on or near insect corpses (Inglis et al., 2012). The occurrence of entomopathogenic fungi in soil may depend on the soil type (Tkaczuk & Mietkiewski, 1996), the cultivated plant species (Krysa et al., 2012; Mantzoukas et al., 2020) or agricultural practices (Tkaczuk, 2008; Jabbour & Barbercheck, 2009; Quesada-Moraga et al. 2007; Oliveira et al., 2013).

To isolate fungi present in the soil, such as actively growing or dormant hyphae, several methods have been devised, for example: a) dilutions of suspensions of soil spread on plates: this consists of the direct isolation of entomopathogenic fungi from the soil on plates. In general, the soil is homogenized in sterile water or sterile distilled water with 0.01% Tween to release propagules from the soil matrix and an aliquot is spread on an appropriate medium with the aid of a glass or plastic spreader (Drigalski loop). The sample can be diluted again in cases of high density of microorganisms (serial dilution). This method is conditioned by the use of a selective medium suitable for germination/growth of the fungus. In general, entomopathogenic fungi are considered weak competitors in the soil in relation to other soil-borne fungi, so the densities of entomopathogenic fungi in the soil are generally low. b) Direct plating: this consists of plating the soil directly onto the plate; in relation to the soil dilution plating method, direct plating is faster. In this method, soil particles are sprinkled on the surface of an agar medium, allowing fungal hyphae to radiate outward from the particles. If a selective medium is not used, the isolation of entomopathogenic fungi may be unsatisfactory due to the excessive growth of contaminating fungi. c) Live baits (insects): this consists of the principle of inducing the passage of a susceptible insect through a substrate containing pathogenic microorganisms. In general, entomopathogenic Hypocreales are considered weak saprotrophs, but because they have the ability to infect live insects, they can gain access to a live insect relatively free of competitors (Inglis et al., 2012; Zimmermann, 1986). Unlike other insect pathogens, entomopathogenic fungi directly infect their host through the exoskeleton.

The bait method was originally developed for the isolation of entomopathogenic nematodes and is currently adopted for the isolation of entomopathogenic fungi in soil samples. The bait method offers an elementary measure of the activity of these entomopathogenic fungi in the soil, helping to determine how crop management systems can favor or hinder the ecosystem services of these microorganisms (Moreira et al., 2019). The main advantage of using the insect bait method in soil samples is that this method selectively isolates entomopathogenic fungi (Klingen et al., 2002; Inglis et al., 2012). It is important to note that the susceptibility to different entomopathogenic fungi may vary among the different insect species used as bait (Klingen et al., 2002).

Commonly, wax moth (*Galeria mellonella*) larvae are the most used in the baiting method, while larvae of other insects such as *Tenebrio molitor* (Coleoptera) and *Diabrotica virgifera* (Coleoptera: Chrysomelidae), large flour beetle (*Tribolium destructor*) and pine bark beetle (*Acanthocinus aedilis*) can also be used (Zimmermann, 1986, Goble et al., 2010; Oddsdottir et al., 2010; Medo and Cagán, 2011, Moreira et al., 2019). Using Delia floralis larvae, Klingen et al., (2002) found that they isolated *Tolypocladium cylindrosporum* more frequently than from *G. mellonella*. Adults of stored grain pests such as *Prostephanus truncatus* (Coleoptera: Bostrychidae), *Sitophilus zeamais* (Coleoptera: Curculionidae), *Sitophilus oryzae* (Coleoptera: Curculionidae), *Rhyzopertha dominica* (Coleoptera: Bostrychidae), *Tribolium confusum* (Coleoptera: Tenebrionidae) and *Cryptolestes ferrugineus* (Coleoptera: Laemophloeidae) were used as baits to isolate entomopathogenic fungi from soil because they are easy to handle, fast in their development and economical to mass rear(Mantzoukas, et al. 2020).

The fungi *M. anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *B. bassiana* (Bals. -Criv.) Vuill. (Hypocreales: Cordycipitaceae) are the most frequently recorded fungi in several studies with insect baits (Brooks 1984; Zimmermann, 2007a; Zimmermann, 2007b; Moreira et al., 2019; Cardoso et al., 2023 Mantzoukas et al., 2020). Other fungi, *Aspergillus insuetus* (Bainier) Thom and Church (Eurotiales: Trichocomaceae); *Aspergillus* sp. Micheli (Eurotiales: Trichocomaceae); *Chaetomium acropullum* X. Wei Wang (Sordariales: Chaetomiaceae); *Chaetomium globosum* Kunze (Sordariales: Chaetomiaceae); *Chaetomium truncatulum* Kunze (Sordariales: Chaetomiaceae); *Trichoderma gamsii* Samuels and Druzhinina (Hypocreales: Hypocreaceae) are also reported, but less frequently (Mantzoukas et al., 2020).

Moreira et al., (2019) adapted the insect baiting method together with survival analysis to evaluate the insect suppression potential of entomopathogenic fungi. Thus, the first steps were taken to evaluate an important ecosystem service provided by entomopathogenic fungi in agricultural soils.

5. Coffee crop

Coffee is a eudicotyledonous plant, class Angiosperms, Family Rubiaceae, belonging to the genus Coffea (Carvalho,1946; Bridson, 1987), and has 124 species cataloged in the literature (Davis et al., 2011). Of these, only Coffea arabica and Coffea canephora are of economic importance. Other species, such as Coffea liberica and Coffea racemosa, are important in genetic improvement programs, being used in the hybridization and transfer of genes and alleles responsible for agronomic characteristics such as drought tolerance and resistance to pests and diseases, for the two commercially produced species (Carvalho, 1946; Krug; Carvalho, 1951).

Originating from intertropical regions characterized by a hot and humid climate, coffee has had its successful development in Brazil. These climatic factors, combined with the development of new technologies such as genetic improvement aimed at greater resistance to pests and diseases, higher productivity, and suitable size for different cultivation systems, make Brazil a global leader in coffee production and export (Vegro and Almeida, 2020; Volsi et al. 2019; Nicikava and Junior, 2022; CONAB, 2023).

One of the major challenges in maintaining coffee production and quality is pest control, as pests can cause severe losses. Among the most prevalent pests in the Brazilian scenario, the following stand out: coffee berry borer (*Hypothenemus hampei* - Coleoptera: Curculionidae), coffee leaf miner (*Leucoptera coffeella* - Lepidoptera: Lyonetiidae), scale insect species (coccidia, pseudococcidia and diaspids), cicadas (Hemiptera) and mites (Acari) (Dantas et al. 2021).

The coffee berry borer, *H. hampei*, requires continuous monitoring due to the systematic damage it has caused to coffee. This pest requires continuous technological development to improve management techniques, mainly because chemical control is the main method to reduce infestations in field conditions (Ferrão et al., 2019). *Hypothenemus hampei* is the main pest of coffee crops worldwide (Le Pelley, 1968, Dantas et al. 2021). It directly attacks coffee fruits, causing a reduction in production. Females lay eggs in galleries inside the beans, where the larvae feed on the seeds, compromising not only productivity but also the quality of the beans. Controlling this pest is somewhat challenging due to the cryptic life cycle of the insect inside the bean, thus making control measures difficult (Le Pelley, 1968, Posada et al., 2007). Several control measures have been used to control these pests, including the use of chemical insecticides (Godoy et al., 1984; Villalba et al., 1995), the application of entomopathogenic fungi (Posada et al., 2004, Góngorra et al., 2023), the release of parasitoids (Quintero et al., 1998; Aristizabal et al., 2004, Góngorra et al., 2023) and practices aimed at interrupting the pest's life cycle, such as frequent harvesting of ripe and infested fruits (Bustillo et al., 1998, Jaramillo et al, 2015; Góngorra et al., 2023).

Increased production, reduced biological imbalances, and more efficient control of pests and diseases that infest coffee crops are a result of the adoption of integrated practices and planning of tactics (Posada et al., 2004; CONAB, 2021; Góngorra et al., 2023). These practices include the characterization of coffee lots and identification of areas where *H. hampei* aggregates. Cultural control measures, such as the collection of coffee beans that remain on the soil or retained on the plant after harvest, also play a fundamental role. In addition, chemical insecticides should be applied at the right time and only in areas where *H. hampei* aggregates. The critical period for controlling the borer generally occurs 120 days after the main incident, when infestation exceeds 2% and more than 50% of the borers are in the entry positions, exclusively piercing the coffee fruits (Benavides, 2012). Is necessary to continuously and systematically develop research that can continue to support the correct phytosanitary management implemented in coffee crops (Ferrão et al., 2019).

The entomopathogenic fungus *Beauveria bassiana*(Balsamo) Vuillemin, is being examined as a biological agent to control insects (Posada et al. 2007; Góngorra et al., 2023). Natural control of *H. hampei* populations by the fungus *B. bassiana* has been observed in infested coffee beans, indicating that this fungus has promise in the biological control of the borer (Vélez & Benavides, 1990; Jaramillo et al, 2015; Góngorra et al., 2023). *Metarhizium anisopliae* also infects the borer but is better adapted to soil conditions than B. bassiana and is widely used in pest control at the rhizosphere level (Milner & Lutton, 1976). Currently, the coffee borer can be controlled with the use of the fungus *B. bassiana*, available in commercial formulations (Agrofit, 2025).

The emergence of chemically resistant pesticides and the need to reduce dependence on chemical pesticides in favor of more, environmentally friendly methods encourage the investigation of sustainable strategies for controlling pests in coffee crops through biologically based alternative control methods.

6. This Thesis

To evaluate the possible ecosystem services provided by entomopathogenic fungi naturally present in agroforestry, organic and conventional soils, we raised the hypothesis that agricultural management practices directly influence soil health and ecosystem sustainability, and more conserved systems, with greater richness and functional diversity of entomopathogenic fungi, provide more efficient ecosystem services in pest control.

In Chapter 1, we investigated which species of entomopathogenic fungi occur in coffee soils and how soil management, combined with the availability of micro and macronutrients, influences their diversity. To this end, we collected soil samples from nine different locations in a coffee-growing south-eastern region of Brazil, following a sampling plan in each of these. We then isolated the fungi using the serial dilution propagation technique and conducted analyses to identify the present species and evaluate the relationship between soil management, chemical composition and fungal diversity in different study systems. The results indicate that organic (n=23) and agroforestry (n=12) systems present greater fungal diversity compared to Conventional crops (n=11). Soil organic matter content was analyzed using a generalized linear model (GLM) with a Gaussian distribution. The results indicated that more conservation-oriented management practices, such as agroforestry and organic systems, promote greater accumulation and stability of soil organic matter, in addition to enhancing the diversity of entomopathogenic fungi. The results of this study demonstrate that the diversity and composition of entomopathogenic fungi in coffee soils are influenced by agricultural management practices and soil chemical characteristics. These findings reinforce the importance of microbial biodiversity for the provision of essential ecosystem services, such as indirect biological control, and suggest that more diversified agricultural systems may promote conditions that are beneficial to the persistence and functionality of these fungi.

In Chapter 2, We investigated how bait insect mortality varies as a function of soil management systems and how this variation impacts the diversity and abundance of fungal isolates captured by bait insects. To this end, we used a 'live bait technique', which allowed us to evaluate the activity and efficacy of these fungi as biological control agents. We monitored the survival of *Tenebrio molitor* larvae and female coffee berry borer (*Hypothenemus hampei*) exposed to soils under different management systems (agroforestry, organic and conventional). In addition, we applied molecular techniques to identify entomopathogenic fungi captured by bait insects and analyzed their abundance and functional diversity. The results indicate that, for *H. hampei*, survival times were influenced by the type of soil management, while for *T. molitor*, the determining variable was the collection date, even after data simplification. These results highlight the relevance of sustainable practices in coffee farming for biological pest control, promoting the conservation of soil biodiversity and reducing dependence on chemical inputs. Furthermore, they reinforce the importance of entomopathogenic fungi as ecosystem services in natural pest control.

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CHAPTER 1

Agricultural management systems influence the composition of entomopathogenic fungi in coffee soils

Article 1: Agricultural management systems influence the composition of entomopathogenic fungi in coffee soils

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ABSTRACT

The intensification of agricultural practices has led to significant changes in the physical, chemical, hydric, and biological properties of soils. These alterations directly affect the biodiversity of soil microbial communities, reducing their capacity to provide essential ecosystem services. This study evaluated the effects of different soil management practices on entomopathogenic fungal communities in coffee-growing areas. From May to July 2023, soil samples were collected from nine coffee farms under three distinct management systems: agroforestry, organic, and conventional. The study areas are located in the Atlantic Forest biome, in Minas Gerais, southeastern Brazil, with soils classified as Red-Yellow Latosols. Fungal diversity was assessed using a molecular approach, revealing a predominance of isolates from the order Hypocreales, with emphasis on the families Clavicipitaceae (n = 21) and Ophiocordycipitaceae (n = 29). A generalized linear model (GLM) with a Gaussian distribution was used to analyze soil organic matter content, with management type and sampling period as explanatory variables. Residual analysis confirmed the adequacy of the distribution and model fit. Subsequently, the data were subjected to analysis of variance (ANOVA), followed by mean comparison using Tukey's test. The results suggest that more conservation-oriented management systems, such as agroforestry and organic practices, promote higher accumulation and stability of soil organic matter and enhance the diversity of entomopathogenic fungi. These findings reinforce the role of these systems in maintaining soil quality and providing biological control ecosystem services in coffee agroecosystems. The evidence generated highlights the importance of sustainable agricultural strategies for conserving ecological processes and the functional biodiversity of soils.

Key words: Soil fungal composition, microbial diversity, agricultural management, soil nutrients, coffee growing soils

INTRODUCTION

Intensive soil management that predominates in agricultural ecosystems has the potential to affect a wide range of plant and animal species, as well as ecosystem processes that support production (Emmerson et al., 2016). As the use of intensive agricultural practices, such as expansion, chemical inputs, and mechanization, intensifies, the physical, chemical, water, and biological attributes of the soil undergo changes. These are generally adverse to plant growth, which becomes clearer when soils are compared with the soils still under natural vegetation (Abd-Elmabod et al., 2017; Kopittke et al., 2019; Niego et al., 2023). These inadequate practices cause loss of productivity and the occurrence of pests and diseases, in addition to the degradation of natural resources which includes the basis for food production: soil (Bommarco et al., 2013).

Changes in vegetation cover modify the soil's capacity to provide many of its ecosystem services (Pulleman et al., 2012) because soils host an enormous diversity of organisms, in terms of abundance, number of species and functions. This biodiversity provides the soil with greater resistance and resilience against disturbances and stress (Wall et al., 2015). Thus, soil organisms and their interactions are fundamental to many soil formation processes and functions, including organic matter decomposition, nutrient cycling, soil structure formation and pest regulation. These processes and functions are related to ecosystem services that are essential for humans (Matson et al., 1997; Pulleman et al., 2012; Moreira et al., 2019).

Among these essential ecosystem services present in the soil, biological control of pests by natural enemies stands out. This is an important ecosystem service that contributes substantially to agricultural and forestry production worldwide (Pimentel et al., 1997; Hill & Greathead, 2000; Oerke, 2006). Among biological control agents, the entomopathogenic fungi (EPF) *Metarhizium* spp. (Hypocreales: Clavicipitaceae), *Beauveria* spp. and *Isaria* spp. (Hypocreales: Cordycipitaceae) stand out for playing important roles in the natural regulation of many species of insect and mite pests, due to their ability to cause epizootics that lead to rapid declines in host populations (Pell et al., 2010).

The regularity and intensity of epizootics can be improved through conservation biological control, which involves agricultural practices that ensure the permanence and of these agents in the soil and their capacity to function. To support EPF communities present in the soil, it is important to identify environmental and soil conditions that affect their functioning within agricultural systems. The diversity and abundance of EPF in agricultural habitats are influenced by physical and chemical parameters of the soil, by management practices, and by climate (Lacey et al., 2015; Moreira et al., 2019). Within physical parameters of the soil, soil texture and structure influence the availability of nutrients, oxygen, water, shelter and food for EPF (Eilenberg and Hokkanen, 2006). In addition, the quality and extent of soil organic matter are important drivers for the diversity, number, and activity of a variety of soil biota (Moreira et al., 2019; Uzman, et al., 2019). Nutritional conditions and the carbon:nitrogen ratio (C:N ratio) have also been shown to be important for sporulation, growth, propagule yield and virulence of different EPF species (Uzman, et al., 2019; Gao et al., 2007).

Organic farming systems and agroforestry systems have higher soil organic matter contents and greater soil microbial diversity and biomass, as well as greater soil biota richness compared to conventional systems (Uzman, Deniz et al., 2019; Moreira et al., 2019). This implies that the management system also affects the habitat conditions for EPF. Thus, although soil disturbance is an inevitable aspect of agricultural production, it is possible to use management practices that have similarities to natural systems to reduce negative environmental impacts. In this chapter, we investigate how agricultural management practices and soil nutrients affect the species composition of entomopathogenic fungi. We use molecular biology techniques to identify which fungal species are present in soils subjected to different management practices, thus allowing us to advance in the understanding of the presence of these species in different coffee cultivation systems. Through principal coordinates analysis (PcoA), we investigated whether there is evidence that different management types differ in terms of the type of nutrition and composition of entomopathogenic fungi.

2. MATERIALS AND METHODS

2.1 Study area

The study was conducted in the microregion of Viçosa, in the municipalities of Araponga, Paula Cândido São Miguel do Anta and Coimbra, located in the Zona da Mata region of Minas Gerais, in the Atlantic Forest biome, a region where deep, well-drained, acidic soils with low nutrient availability predominate (Ker, 1995) (Figure A).

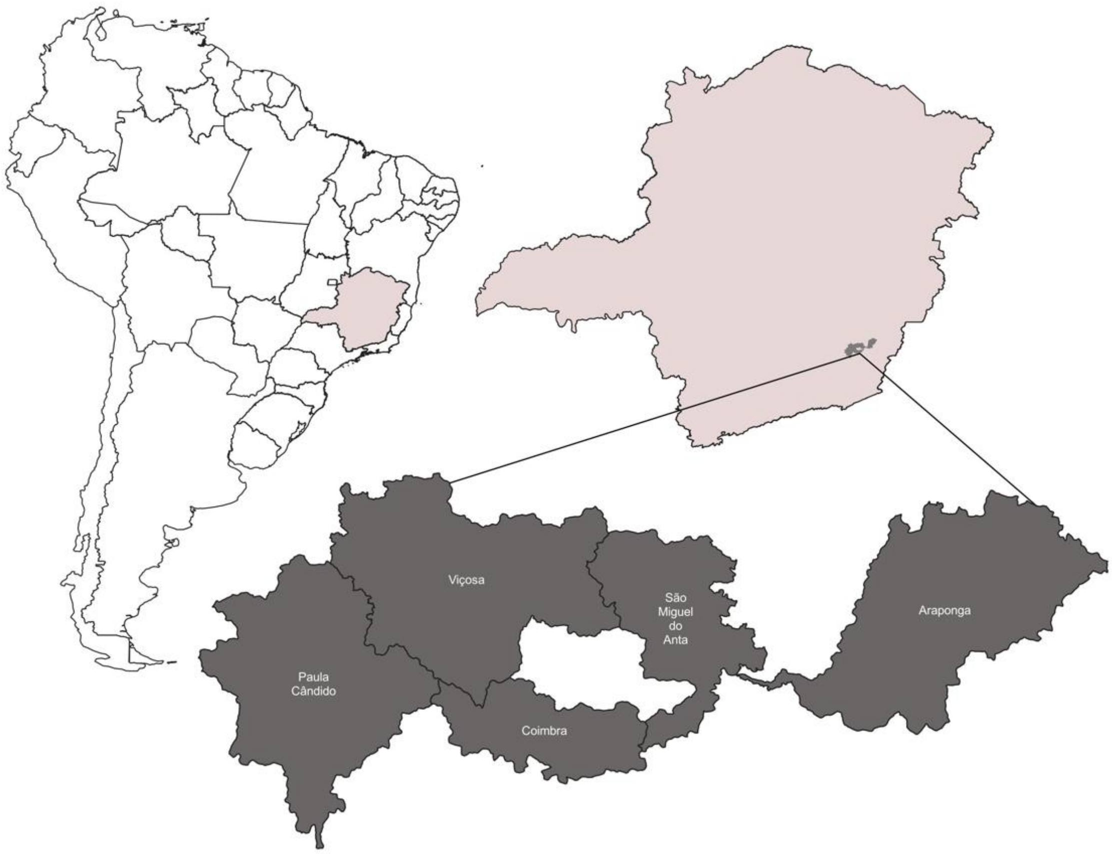


Figure 1: Location of the study areas in the state of Minas Gerais, Brazil. The region highlighted in the enlarged map includes the microregion of Viçosa with the municipalities where we conducted soil sampling from coffee farms: Paula Cândido, Coimbra, São Miguel do Anta and Araponga. The larger map shows the location of the state of Minas Gerais in the context of South America and Brazil.

The Zona da Mata Mineira stands out as one of the principal regions of coffee production in Brazil (Souza et al. 2009). Currently, family farming predominates in the area; this is characterized by long-term sustainable land use, as well as small-scale production and the adoption of traditional agricultural practices (Cardoso et al. 2001).

We considered three different management systems: agroforestry, organic and conventional. In agroforestry management systems, native or planted shade trees are integrated between the coffee rows; chemical fertilizers are applied, but no pesticides are used. Organic management involves the use of monoculture (without intercropped trees or other plants) and exclusively organic fertilizers and, again, no pesticides are applied. In contrast, conventional management involves the use of monoculture (without intercropped trees or other crops) and the use of chemical fertilizers and pesticides. We selected three properties that use agroforestry systems (identified from now on as ED, IRO, IAQ), three organic properties (WA, RO, CA) and three conventional properties (GI, AT, JF) (Table 1). For logistical reasons, collections from the field were carried out on six dates over two months.

The characterization of the sampling sites is presented, including geographic coordinates (latitude and longitude), altitude and climatic variables (mean annual temperature and precipitation), which influence soil dynamics and the associated microbiota. The age of the coffee plants and the main tree species present are also reported, factors that can affect nutrient cycling, the structure of the agroecosystem and the interaction between soil microorganisms (Table 2). This information is essential to understand the environmental conditions of the coffee systems evaluated.

Table 1: Soil sample collection dates and sites in different municipalities of Minas Gerais, Brazil, including the coffee management system (organic, agroforestry and conventional) for each site.

collection	Municipality	Site	Management systems
19.06.23	Araponga	Pedra Redonda (ED)	Organic
19.06.23		Serra das Cabecas (WA)	Agroforestry
03.07.23		Romualdo (RO)	Organic
03.07.23		Reinaldo (RE)	Agroforestry
22.05.23	Coimbra	Sítio da Graça (CA)	Organic
22.05.23		Instituto Alba Quercus (IAQ)	Agroforestry
23.06.23	São Miguel do Anta	Capivara (GI)	Conventional
05.07.23	Paula Cândido	Antenor (AT)	Conventional
17.05.23		José Fernandes (JF)	Conventional

Table 2: Characterization of the sampling sites, including geographical coordinates (latitude and longitude), altitude, climatic variables (mean annual temperature and precipitation), coffee plant age, and main associated tree species.

Characteristics of the sampling sites	**ED**	**WA**	**RO**	**RE**	**CA**	**IQA**	**GI**	**AT**	**JF**
Location	Araponga	Araponga	Araponga	Araponga	Coimbra	Coimbra	São Miguel do Anta	Paula Cândido	Paula Cândido
Latitude	20° 38’44,7”	-20,704815	-20,693775°	20° 41’53,9”	20° 844273°	20,8225040	-20,77645°	-20,871448º	20º51’37,8”
Longitude	42° 30’02,0448”	-42,48694°	-42,528677°	42° 31’45,4	42° 888860°	42,8110610	-42,704520°	-43,008240º	42º00’ 203,0”
Altitude (m)	1254	1360	1065	1040	720	730	750	744	758
Mean annual temperature (°C)	18	18	18	18	22	22	20	22	22
Mean annual precipitation (mm)	1300	1300	1300	1300	99,08	99,08	99,08	97,11	97,11
Ages of coffee plants (Years)	3 - 25	2 - 40	8	20	20	5	5	29	30
Main tree species present	*Musa*, *Tibouchina granulosa*, *Arachis pintoi*, *Persea americana*	*Musa*, *Persea americana*, Zea mays, *Citrus sinensis*, palms, *Euterpe edulis*	*Ingá subnuda*, *Musa*	*Inga subnuda*	*Musa, Persea americana, Eucalyptus globulus, Bertholletia excelsa*	*Piptadenia gonoacantha*, *Senna occidentalis*, *Ingás*, *Cecropia*, *Musa* cedars.	-	-	-

2.2 Sampling design

Soil sampling is the most critical step in the entire soil analysis process, as it involves everything from collection to evaluation of samples (chemical and physical characteristics). Sampling is essential in this process, as the quality of the results depends on the representativeness of the sample in relation to the area analyzed, i.e., a small portion of land can represent several hectares (Furtini Neto et al., 2001). Therefore, samples were collected following the BON Field Soil Sampling Protocol (Potapov, 2022) with adaptations. In each coffee plantation, four sample plots were planned, 50 meters apart from each other, starting at the edges, in order to cover as much of the planted area as possible. The layout of the plots varied according to the layout of the planted area

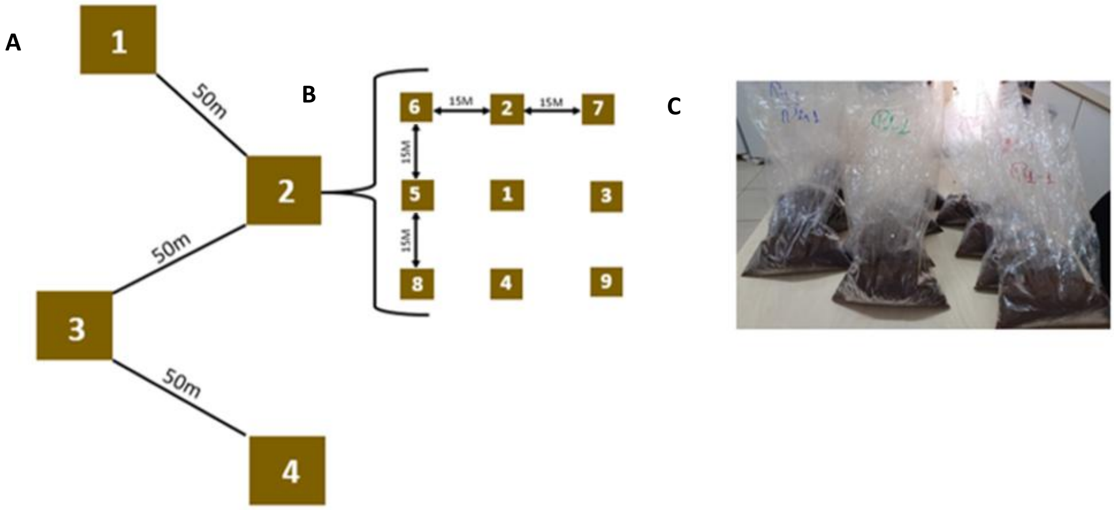


Figure 2: Design used for soil sampling. **A.** The area was subdivided into four main plots (1, 2, 3 and 4), with distances of 50 meters between them, taken from the endpoints. **B.** Focusing on plot 2, the subsamples within each plot are represented, totaling nine collection points (1 to 9), spaced 15 meters apart. **C.** Each subsample was stored individually in polyethylene bags to be transported to the Insect-Microorganism Interactions Laboratory.

It is important to emphasize that the sampling units were collected exclusively in a single type of habitat, which means that they were not collected in transition areas between different types of habitat. This care is relevant to ensure the homogeneity of the samples and the consistency of the results obtained.

2.3 Soil samples

Soil was collected using a 65S auger, to a depth of 10 cm and with a diameter of 5 cm. Each subsample was transferred to individual polyethylene bags for transport to the laboratory.

Between each collection, the auger was washed sequentially in water, 70% ethanol, then distilled water to limit cross-contamination (Moreira et al 2019).

Once in the laboratory, each sample was mixed with its respective plot and homogenized manually. In line with previous studies (Klingen et al. 2002; Meyling & Eilenberg 2006b; Goble et al. 2010), we did not use controls, as it is almost impossible to use a substrate similar to soil, even the properties of sterilized soil are completely modified by high temperatures (Ellis, R.J., 2004; Moreira et al., 2019).

After assembling all experiments the soil from each subsample of its respective plot was combined into a single sample, which was later sent to the Soil Analysis Laboratory of the Federal University of Viçosa to determine the physical and chemical properties of the soil (Teixeira et al., 2018) (Appendix 2).

2.4 Fungal isolation

For fungal isolation from each soil sample, 5 g of soil was diluted in 45 ml of distilled water containing 0.01% Tween in 50 ml Falcon® tubes. These tubes were shaken for one hour on a rotary shaker at 150 revolutions per minute (Lacey, 2012). The soil suspensions were then serially diluted five times to reduce the concentration of microorganisms. The dilutions were then shaken for 15 seconds and plated onto a selective culture medium for hypocrealean fungi (10 g peptone, 20 g dextrose, and 15 g agar per liter of distilled water, with 0.05 g/L cycloheximide and tetracycline, 0.6 g/L streptomycin, and 0.175 g/L cetyltrimethylammonium bromide added after sterilization of the medium (Kepler et al., 2015). We plated 100 microliters of each soil suspension onto 9 cm diameter Petri dishes and spread this with a sterile Drigalski loop. The plates remained open for a few minutes to allow the medium to completely absorb the suspension, and were then closed, sealed with PVC plastic film (Magipack®), inverted and kept in the dark at 25 °C for 14 days until colony forming units (CFU) could be distinguished.

To obtain pure colonies, fungi recovered from the soil were individually subcultured on PDA plates (39 gl^{-1} of potato dextrose agar). When isolates of the same genus with distinct morphological characteristics were found in the same sample unit, both were purified. After fungal growth, the isolates were screened and grouped into morphotypes. At this point, fungal isolates were mounted on slides for microscopic observation (400×) and identification based on micromorphological characteristics, analyzing the size and arrangement of hyphae, conidiophores and conidia (Samson et al., 1988, Humber, 2005, 2012). Isolates were preserved in cryogenic tubes with 20% glycerol and kept in a freezer at -80ºC in the Insect Microorganism Interaction Laboratory in Viçosa, Minas Gerais.

2.5 DNA extraction, amplification and sequencing

A total of 57 isolates were recovered from the soil. We macerated the fungal material using liquid nitrogen and 0.12 mL microtubes prefilled with acid-washed 2.8 mm stainless steel homogenizing beads (Sigma-Aldrich) at 400 rpm for 60s at the BeadBug™ homogenizer. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega®, Madison, USA), following the manufacturer's recommendations. The concentration and final quantity of DNA extraction was determined using a NanoDrop® spectrophotometer (Thermo Scientific®, Waltham, USA).

We amplified the genomic region of translation elongation factor 1-α (TEF) using the primers 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 2218R (5'-ATGACACCRACRG-CRACRGTYTG-3') (Castlebury et al. 2004). The 25 µL-PCR reaction contained 12.5 µL KAPA Taq ReadyMix (KAPA Biosystems™), 1 µL of each forward and reverse primer (10 ng/µL), 5 µL of DNA template, and 5.5 µL of DNAse/RNAse Free Water (ZymoBIOMICS™). The PCR reactions were placed in an Eppendorf™ thermocycler under the following conditions: TEF (1) 2 min at 94 °C, (2) 10 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 1 min, and extension at 72 °C for 1 min, followed by (3) 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min and (4) 3 min at 72 °C. For RPB1(1) 2 min at 95 °C, (2) 10 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 1 min, and extension at 72 °C for 1 min, followed by (3) 35 cycles of denaturation at 95 °C for 30 s, annealing at 56.6 °C for 1 min, and extension at 72 °C for 1 min and (4) 3 min at 72 °C. After amplification, the PCR products was purified and sequenced by Macrogen® (Seoul, South Korea). The generated sequences were assembled into contigs using Geneious Prime® 2024.0.5 (Geneious Prime, 2024) (https://www.geneious.com) and compared to sequences of related species deposited in GenBank using BLASTn. Sequences from previous studies were retrieved from NCBI databases to perform a phylogenetic analysis along with the sequences we obtained. Bayesian Inference with 68 sequences was performed in MrBayes, using 2 Markov Chain Monte Carlo until the convergence of split frequence (<0.01). Analysis was conducted to 5 million generations.

2.6 Statistical analysis

Statistical analysis was conducted to evaluate the differences in soil organic matter (OM, dag/kg) content between three coffee management systems (Agroforestry, Conventional and

Organic). We built a GLM with Gaussian distribution using organic matter as the response variable, and type of coffee management and collection as the explanatory variable. Residuals were analyzed to check for distribution suitability and fit in the model. Data were submitted to analysis of variance (ANOVA) with subsequent comparison of means by Tukey's F test.

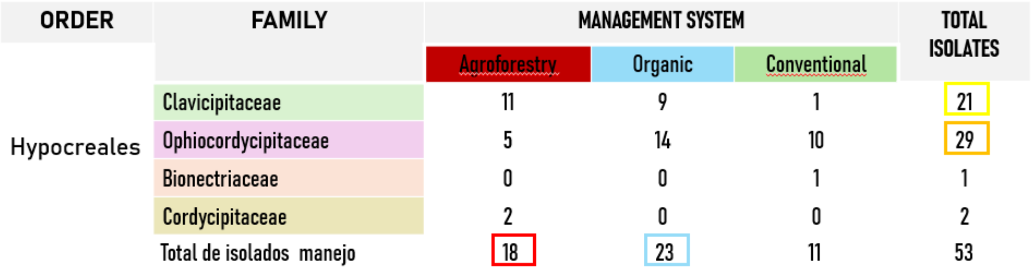
All statistical analyses adopted a significance level of α = 0.0001 and were performed in R software (version 4.2.2), using the emmeans package. The graphical representation of the results was prepared with the ggplot package.

3. RESULTS

3.1 Phylogenetic analyses of soil Hypocreales species

In total, 53 fungal isolates from the three different coffee management soils were sequenced for the TEF region. The isolates were predominantly from two families of the order Hypocreales: Clavicipitaceae (n=21), Ophiocordycipitaceae (n=29), with a few isolates from two other hypocrealean familes: Cordycipitaceae (n=2), and Bionectriaceae (n=1) (Table 3).

Table 3: Distribution of fungal isolates from the order *Hypocreales* across different management systems (Agroforestry, Organic, and Conventional). The values represent the number of isolates found in each fungal family for each system. The families with the highest number of isolates are highlighted in yellow and orange, while the total number of isolates per management system is indicated in red (Agroforestry), blue (Organic), and green (Conventional).



ORDER	FAMILY	MANAGEMENT SYSTEM			TOTAL ISOLATES
		Agroforestry	Organic	Conventional	
Hypocreales	Clavicipitaceae	11	9	1	21
	Ophiocordycipitaceae	5	14	10	29
	Bionectriaceae	0	0	1	1
	Cordycipitaceae	2	0	0	2
	Total de isolados manejo	18	23	11	53

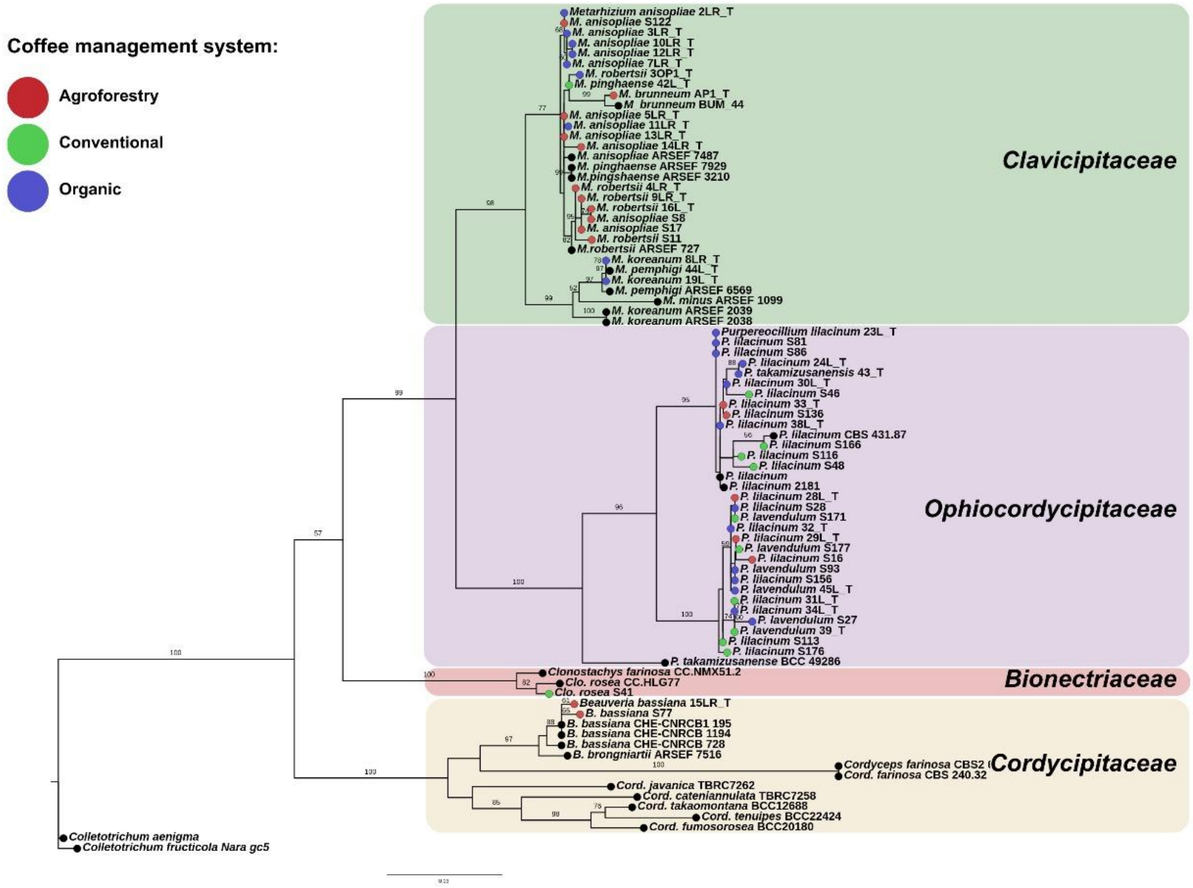


Figure 3: Diversity of entomopathogenic fungi isolated from soils of three different coffee management systems in the microregion of Viçosa (Araponga, Coimbra, São Miguel do Anta and Paula Cândido). Management systems are: agroforestry (red), conventional (green), organic (blue) and samples deposited in Genbank (black). Phylogenetic reconstruction was constructed using the maximum likelihood (ML) method based on the translation elongation factor (TEF) genomic region. The values at the nodes represent the bootstrap support, indicating the robustness of the phylogenetic groupings. The horizontal scale (0.03) reflects the evolutionary distance, calculated based on the number of nucleotide substitutions per site.

For the family Clavicipitaceae, we obtained a strongly bootstrap-supported clade (BS = 87%), subdivided into two subclades. The first subclade presented a bootstrap support of 58% and included isolates from soils under agroforestry and organic management. In agroforestry soils, one isolate of *Metarhizium brunneum* was identified (n = 1). In soils under organic management, four isolates were recorded, represented by the species *M. pemphigi* (n = 1), *M. anisopliae sensu lato* (n = 1) and *M. koreanum* (n = 2). The second subclade presented bootstrap support of 66% and also included isolation from soils under agroforestry and organic management. In agroforestry soils, 12 isolates were recorded, distributed among the species *M. anisopliae s.l* (n = 7), *M. robertsii* s.l (n = 4) and *M. pemphigi* (n = 1). In soils under organic management, 15 isolates were identified, represented by the species *M. anisopliae s.l* (n = 5), M. *robertsii* s.l (n = 6) and *M. pinghaense* (n = 4).

For the family Ophiocordycipitaceae, a strongly bootstrap-supported clade (BS = 100%) was identified, which was subdivided into two subclades. The first subclade presented a bootstrap support of 92% and included isolates from soils under agroforestry, organic and conventional management. In agroforestry soils, *Purpureocillium lilacinum* (=*Paecilomyces lilacinus*) was identified (n = 4). In organic management systems, the isolates identified were *P. lilacinum* (n = 3) and *P. lavendulum* (n = 2). In soils under conventional management, isolates of *P. lilacinum* (n = 3) and *P. lavendulum* (n = 4) were recorded. The second subclade presented a bootstrap support of 83% and also included isolates from soils under the three management systems. In agroforestry soils, two isolates of *P. lilacinum* (n = 2) were identified. In organic management systems, the isolates recorded were *Purpureocillium* sp. (n = 1), *P. lilacinum* (n = 5) and *P. takamizusanensis* (n = 1). Finally, in soils under conventional management, isolates of *P. lilacinum* (n = 4) were found.

For the family Bionectriaceae, we obtained a strongly supported bootstrap clade (BS = 99%). In this clade, one isolate from conventional soil was observed, identified as *Clonostachys rosea* (n = 1).

For the family Cordycipitaceae, we obtained a strongly supported bootstrap clade (BS = 100%). Within this clade, the two isolates came from soil under agroforestry management and these were both identified as *Beauveria bassiana* (n = 2).

3.2 Statistical analysis of soil organic matter (MO) content under different management systems

Soil organic matter (OM) contents showed significant variations between management systems and across different collections (Figure 1).

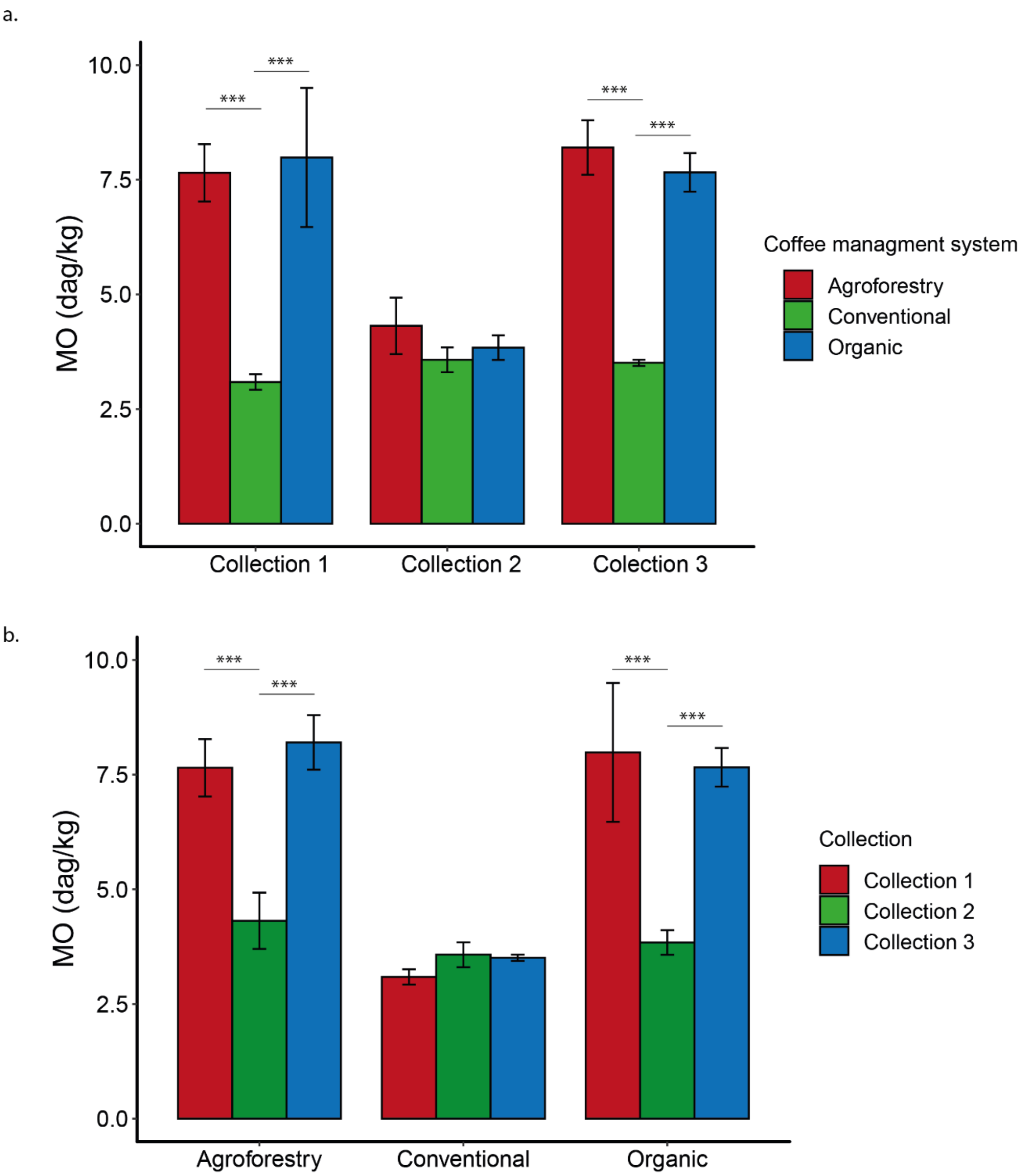


Figure 3: Soil organic matter (OM) content (dag/kg) across different coffee management systems and sampling periods**.** (a) Comparison of OM content among management systems: agroforestry (red), conventional (green), and organic (blue), over the three sampling periods. (b) Comparison of OM content among sampling periods within each management system. Bars represent mean values ± standard error (n = X). Statistically significant differences between treatments were determined using Tukey's test ($p < 0.001$), indicated by "***".

As shown in Graph (a), in Collection 1, the agroforestry and organic systems presented significantly higher OM levels than the conventional system. The comparison between the agroforestry and conventional systems resulted in a value of $t = 6.283$ ($p < 0.0001$), and between conventional and organic in t = -8.443 ($p < 0.0001$). There was no significant difference between agroforestry and organic (t = -2.160; p = 0.0967). In Collection 2, OM levels did not differ significantly between management systems ($p > 0.57$ for all comparisons). In Collection 3, the pattern observed in Collection 1 was repeated: agroforestry presented higher values than conventional (t = 6.469; $p < 0.0001$), as did organic in relation to conventional (t = -5.722; $p < 0.0001$). Again, there was no significant difference between the agroforestry and organic systems (t = 0.747; p = 0.7377).

Graph (b) shows the variation in organic matter levels over time within each system. In the agroforestry system, significant differences were observed between Collections 1 and 2 (t = 4.595; p = 0.0003) and between Collections 2 and 3 (t = -5.356; $p < 0.0001$), while Collections 1 and 3 did not differ from each other (t = -0.761; p = 0.7295). In the conventional system, no significant differences were detected between the three collections ($p > 0.78$). In the organic system, organic matter levels were higher in Collection 1 compared to Collection 2 (t = 7.409; $p < 0.0001$) and in Collection 3 compared to Collection 2 (t = -5.263; $p < 0.0001$). The comparison between Collection 1 and Collection 3 did not show any significant difference (t = 2.146; p = 0.0994).

These results suggest that agroforestry and organic systems provide greater accumulation and stability of organic matter in the soil over time, while the conventional system presents low and constant levels, evidencing a lower capacity for maintenance or accumulation of organic matter.

4. DISCUSSION

Our findings enabled us to identify the specific entomopathogenic fungal isolates present in soils from distinct coffee production systems, including agroforestry, organic, and conventional management. We examined the apparent effects of fungal diversity and community composition on soil health and ecosystem functioning across these management systems. Notably, our results revealed that fungal communities from conventionally managed soils were compositionally distinct when compared to those from agroforestry and organic systems.

Through phylogenetic inference based on molecular data, we identified representatives from several entomopathogenic fungal families within the order Hypocreales, including Clavicipitaceae, Ophiocordycipitaceae, Cordycipitaceae, and Bionectriaceae. Specifically, we detected 29 isolates in soils under organic management, 17 isolates from agroforestry systems, and 11 from conventionally managed soils. The application of molecular techniques proved essential for detecting microbial populations from diverse habitats, many of which might remain undetected using traditional isolation methods (Papke & Ward, 2004).

Among the predominant species recovered from organic and agroforestry soils were *Metarhizium anisopliae* and *M. robertsii* (Clavicipitaceae). This predominance aligns with previous reports suggesting that *Metarhizium* spp. preferentially colonize the rhizosphere of plants and exhibit a certain degree of host specificity (Fisher et al., 2011; Wyrebek et al., 2011; Liao et al., 2014). Our data further corroborate the dominance of *M. anisopliae* and *M. robertsii* in tropical soils, consistent with the findings of Carrillo-Benítez et al. (2013) and Rezende et al. (2015). Notably, in both agricultural and forested environments, *Metarhizium* remains one of the most frequently encountered entomopathogenic fungal genera (Gottwald & Tedders, 1984; Meyling & Eilenberg, 2006). The distribution and abundance of *Metarhizium* spp. are shaped by a combination of soil physicochemical characteristics and biotic and abiotic factors that vary across landscapes (Bruck et al., 2010).

Additionally, we detected the presence of *Purpureocillium lilacinum* (Ophiocordycipitaceae) across all management systems investigated. This fungus, which can be isolated from soil, plant material, insect hosts, nematodes, and even indoor environments, is also recognized as an opportunistic pathogen in vertebrates and immunocompromised humans (Luangsa-Ard et al., 2011). Its survival strategies in nature are diverse and highly plastic, ranging from entomopathogenic and mycoparasitic lifestyles to saprophytic and nematophagous modes of existence (Medeiros et al., 2018). This remarkable ecological versatility grants *P. lilacinum* significant biotechnological relevance, particularly as a biocontrol agent against phytoparasitic nematodes—an application that has been commercially established for several years (Dahlin et al., 2019).

Agricultural intensification has been widely associated with reductions in biodiversity across multiple taxonomic groups within agroecosystems (Benton et al., 2003). The findings of the present study reinforce this pattern by demonstrating significant variation in soil fungal community composition among the different coffee management systems evaluated. Conventional systems exhibited markedly different fungal assemblages compared to organic and agroforestry systems, a trend likely driven by differences in input regimes, nutrient availability, and microclimatic conditions.

In summary, soil functions as a dynamic and multifactorial matrix in which the diversity and abundance of entomopathogenic fungi are shaped by a complex array of edaphic and environmental factors (Jaronski, 2007). Soil texture and organic matter content, in particular, have emerged as key determinants influencing fungal ecology (Medo & Cagáň, 2011; Quesada-Moraga et al., 2007). Organic matter plays a critical role in nutrient cycling, soil structural stability, water retention capacity, and erosion control, creating a favorable environment for the persistence of biological control agents (Lehmann & Kleber, 2015).

Furthermore, microclimatic factors—especially shading—exert direct control over fungal dynamics. Solar radiation has been shown to impair conidial viability and germination, whereas shaded environments sustain fungal activity by preserving soil moisture and reducing desiccation stress (Staver et al., 2001). Conventional agricultural systems, characterized by intensive chemical and mechanical management (Letourneau & van Bruggen, 2006), tend to impose distinct selective pressures on soil microbial communities. In contrast, organic farming, guided by holistic ecological principles (Altieri, 1986; Letourneau & van Bruggen, 2006), and agroforestry systems, which provide structural and microclimatic heterogeneity (Lin, 2007), offer more favorable conditions for the maintenance and proliferation of entomopathogenic fungi.

Collectively, these results highlight the ecological significance of sustainable management practices, such as organic agriculture and agroforestry, in conserving entomopathogenic fungi and promoting broader soil biodiversity. By fostering favorable microhabitats and enhancing soil ecological functions, these systems contribute to the sustainability and resilience of agroecosystems. Understanding the intricate linkages between soil management and microbial diversity is therefore critical for advancing ecologically sound biological control strategies and for ensuring the long-term health and productivity of agricultural landscapes.

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CHAPTER 2

Pest control and entomopathogenic fungi: impact of soil management in coffee

Article 2: Pest control and entomopathogenic fungi: impact of soil management in coffee

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ABSTRACT

Biological control is an essential ecosystem service for agricultural production, consisting of the use of living organisms, such as predators, parasitoids, and pathogens, to regulate pest populations. Here, we investigate the functionality of biological control as an ecosystem service in soils subjected to different management systems (agroforestry, organic, and conventional), seeking to understand how these practices affect the diversity and abundance of entomopathogenic fungi. To this end, we used the live bait technique, exposing *Tenebrio molitor* larvae and *Hypothenemus hampei* females to soil samples from the different cultivation systems. The survival of *H. hampei* was significantly influenced by the soil management system, while that of *T. molitor* varied according to the sampling expedition, even after simplifying the model. In addition, we applied molecular techniques to identify entomopathogenic fungal isolates from baits. In total, 113 fungal isolates were sequenced for the TEF region (translation elongation factor), including two from bait insects exposed to soils under different coffee management systems. Seven fungal families were identified, distributed in seven genera: *Fusarium*, *Purpureocillium*, *Metarhizium*, *Trichoderma*, *Clonostachys*, *Beauveria* and *Cordyceps*. The results of this study contribute to a better understanding of the interactions between soil management, fungal microbiota and biological pest control, providing support for sustainable management strategies in coffee production.

Keywords: biological control, soil fungal microbiota, *Hypothenemus hampei*, *Tenebrio molitor*, molecular technique,

INTRODUCTION

Biological control is an ecosystem service supported by biodiversity and of great importance for agricultural production (Iverson et al., 2014; Pell, Hannam, & Steinkraus, 2010; Tscharntke et al., 2015; Wilby & Thomas, 2002; Moreira et al., 2019). Considered an alternative to chemical control, biological control consists of the use of living organisms such as predators, parasitoids and pathogens to regulate pest population densities, preventing them from reaching levels capable of causing economic damage (Parra, 2014).

Among biological control agents, entomopathogenic fungi stand out, with more than 750 species spread throughout the environment. More than 75% of entomopathogenic fungi belong to the order Hypocreales being capable of causing disease and death in insects (David, 1967; Meyling and Eilenberg, 2007; Sung et al., 2008; Jaramillo et al., 2015; Brunner-Mendoza et al., 2019)). These fungi are responsible for approximately 80% of the diseases found in the Class Insecta. They differ from other groups of agents in that they have the ability to infect all stages of host development (Souza, 2019). Currently, entomopathogenic fungi of the genera *Metarhizium* and *Beauveria* are the most studied in pest control.

Soil constitutes an important reservoir for the diversity of entomopathogenic fungi, being the habitat where a large part of the life cycle of entomopathogenic fungi occurs, including multiplication within hosts, growth in saprophytic, endophytic and rhizospheric forms (Morgan et al., 2005; Meyling and Eilenberg, 2007; Hesketh et al., 2010; Pell et al., 2010). These fungi can also occur naturally in agricultural soils (Kepler et al., 2015; Hernández-Domínguez et al., 2016; Sharma et al., 2018; Moreira et al., 2019) and can play a role in the natural regulation of many arthropods that spend part or all of their life in the soil (Townsend et al., 1995; Pell et al., 2010). Infection by these fungi begins when hosts are exposed to fungal conidia, a propagule responsible for fungal reproduction. Fungal propagules can persist outside the host in the soil and phylloplane, serving as reservoirs for the fungal inoculant (Hesketh et al., 2010).

Knowledge of the species composition and distribution of entomopathogenic fungi is essential to assess the potential for biological control in a specific ecosystem. The bait method was developed to detect the presence of these fungi in the soil. The principle of this method is to force the presence of the susceptible insect (bait) through a substrate that possibly contains pathogenic microorganisms (Aguilera Sammaritano et al., 2016; Kim et al., 2018; Sanchez-Pena, Lara, & Medina, 2011; Moreira et al., 2019). The main advantage of this method is the identification and selection of biologically active entomopathogenic fungi (Moreira et al., 2019).

Different types of management can affect the presence of fungal species, and the response of each species to these conditions varies. Thus, in this chapter, the objective was to evaluate the function of the ecosystem service of pest control in relation to soils under different agricultural management systems (agroforestry, organic and conventional), allowing an understanding of how management strategies may or may not influence the biological control of pests. We adopted the “bait survival technique” approach as described by Moreira et al. (2019), in which we evaluated the suppressive potential of fungal strains present under different soil management systems. By monitoring the mortality of baits (female coffee berry borer *Hypothenemus hampei* and *Tenebrio molitor* larvae), survival curves were constructed, and fungal isolates obtained from the baits were identified and quantified for each system.

2. MATERIALS AND METHODS

2.1 Study area

Soil samples were collected between May and July 2023, from various coffee-producing farms located in the Zona da Mata region of Minas Gerais, within the Atlantic Forest biome, southeastern Brazil. This region is characterized by deep, well-drained, acidic soils with low nutrient availability (Ker, 1995) (Figure 1).

Figure 1: Geographic location of the soil collection areas for the study. The enlarged map highlights the microregion of Viçosa, Minas Gerais, SE Brazil, and the location (municipalities) of the study areas: Paula Cândido, Coimbra, São Miguel do Anta and Araponga, where collections were carried out in coffee growing areas. The main image contextualizes the location of the state of Minas Gerais in relation to Brazil and South America.

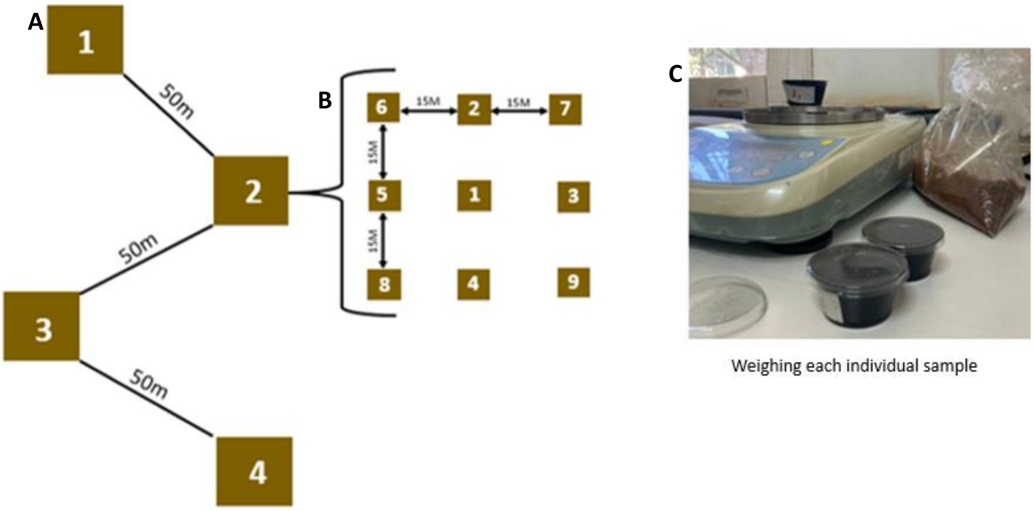
Soil samples were collected from agroforestry, organic, and conventional management systems. In the agroforestry system, shade trees, either native or planted, are integrated between coffee rows, with fertilizers being applied, but no pesticides are used. The organic management system, in turn, is characterized by a high concentration of decomposing organic matter, without the use of pesticides or synthetic fertilizers. In contrast, the conventional system involves the intensive use of agricultural pesticides. Nine coffee farms managed under the different systems were selected. The study included three farms utilizing agroforestry systems, identified as ED, RE, and IAQ; three organic farms, identified as WA, RO, and CA; and three conventional management farms, identified as GI, AT, and JF.

2.2 Sample design

The sampling protocol was designed to allow comparisons between the different coffee cultivation systems. During each expedition cycle, all management systems were included. The sampling followed the Soil BON Field Sampling Protocol (Potapov, 2022), with adaptations (Appendix 1).

The arrangement of the plots varies according to the layout of the plantation. Each sample plot was composed of nine individual samples arranged in a 3 x 3 class, covering an area of 30 x 30 meters, to ensure sample homogeneity. Thus, each sample plot was composed of nine individual samples, distributed in a 3 x 3 grid, covering an area of 30 m x 30 m, in order to ensure sample homogeneity. Thus, each site had a total of 36 individual samples (4 plots x 9 samples) (Figure 2). During each collection cycle, the three management systems were considered (Table 1). In each of the management systems — agroforestry, organic and conventional — four sample plots were determined 50 meters apart from the edges, covering as much of the planted area as possible.

The soil was collected with a 65S probe at a depth of 10 cm and a diameter of 5 cm. Between each collection, the drill was washed sequentially in water, 70% ethanol and distilled water to limit cross-contamination (Moreira et al. 2019).



Weighing each individual sample

Figure 2: Design used for soil sampling. **A.** The cultivation area was divided into four main plots (1, 2, 3 and 4), each separated by 50 meters, measured from their ends. **B.** A closer view of how the division occurred within the plots, for example plot 2 shows the arrangement of the subsamples within each plot, with nine collection points (1 to 9) spaced 15 meters apart. **C.** Each subsample being weighed for the "Insect bait" bioassay, we weighed 2 grams of each individual soil sample to expose *Hypothenemus hampei* to the soil and weighed 4 grams of each individual soil sample to expose *Tenebrio molitor*.

It is important to emphasize that the sampling units were collected exclusively from a single habitat type, meaning that no samples were taken from transition areas between different vegetation types. This precaution is crucial to ensure sample homogeneity, and the consistency of the results obtained.

2.3 Insects

To assess the potential of ecosystem services provided by entomopathogenic fungi naturally present in different management systems, we employed the "bait survival technique," as proposed by Moreira et al. (2019). This methodology represents a simplified approach to measure the activity of these fungi in the soil, contributing to the understanding of how agricultural management practices can positively or negatively impact this process (Moreira, 2019).

For *H. hampei*, coffee fruits with boreholes were collected directly from coffee trees, planted in the Coffee Nursery at the Federal University of Viçosa, Minas Gerais, Brazil, which encompasses a 15-hectare planting area. These beans were then surface-sterilized (to prolong their preservation) by sequential immersion in 70% alcohol, 5% sodium hypochlorite and rinsing in distilled water. They were kept in an air-conditioned room at 25°C (± 1°C) and 70% (± 10%) relative humidity until they were cut open to extract *H. hampei* females for the experiments.

Tenebrio molitor (Coleoptera: Tenebrionidae) larvae were obtained from a mass rearing at the Laboratory of Insect-Microorganism Interactions in Viçosa, Minas Gerais, Brazil. The insects are reared in opaque plastic boxes (21×17×9 cm) with a ventilated lid at room temperature 25±1°C, 12:12 L:D photocycle, and 65 ± 10% relative humidity. Larvae are supplied *ad libitum* with a diet composed of a mix of oat bran, wheat fiber and wheat bran (1:1:1). Five slices of carrot are provided every 4 days. Boxes with larvae are checked twice a week and individuals which have pupated are manually transferred to new plastic boxes and covered with a sheet of A4 paper to allow emerged adults to hide. Adults that emerge are transferred and maintained in plastic containers (41×26×7 cm) with the same diet plus carrot slices. Once a month, newly emerged larvae and eggs are collected.

2.4 Insect Baiting

Each insect was subjected to the following methodological approaches: *(i) Hypothenemus hampei*: From each of the nine individual sample, using a spatula, we randomly weighed 2 grams of soil and placed them in sterile 30ml plastic containers (59 mm x 38 mm x 29 mm) with ventilated lids. Subsequently, one adult female of *H. hampei* was added per container and this was stored in an incubator at 25 ± 1°C and 70-80% relative humidity in the dark. The containers were moistened when necessary, and manually agitated daily, ensuring that the insects were exposed to the soil. In sterile conditions, insect mortality was assessed every two days. *(ii) Tenebrio molitor:* We selected two-month-old larvae of similar size and placed one larva per transparent 30 ml plastic container (59 mm x 38 mm x 29 mm) containing 4 grams of soil, with a plastic lid featuring ventilation holes. They were stored in an incubator at 25 ± 5°C with a 12:12 photoperiod. The containers were moistened when necessary, and inverted daily, ensuring that the insects had greater soil contact. Every two days, insect mortality was assessed under sterile conditions.

Dead insects were surface-sterilized by immersion in 70% alcohol followed by 5% sodium hypochlorite, then rinsed in distilled water and dried on sterile filter paper. Subsequently, the insects were incubated in sterile humid chambers, consisting of 1.5ml microtubes with cotton wool soaked in sterile distilled water maintained in a climate-controlled chamber (25 ± 5°C) to promote fungal growth. Incubated insects were inspected daily. Some of the bait insects did not show evidence of fungal infection and colonization after death, and a few sporulated while inside the plastic container with soil. Hardened or mummified insect cadavers, where the interior of the body was colonized by fungal hyphae, appear to be the main indicator of death resulting from entomopathogenic fungal infection.

Following the methodology established by previous studies (Klingen et al., 2002; Meyling & Eilenberg, 2006; Goble et al., 2010; Moreira et al., 2019), control groups were not used, as it is nearly impossible to find a substrate similar to soil that does not affect the survival of the bait insects; even sterilized soil is altered by the high temperatures used in the process (Ellis, 2004). The insects used as bait showed no signs of infection and were therefore used as experimental controls.

2.5 DNA extraction, amplification and sequencing

We amplified the translation elongation factor 1-α (TEF) genomic region using primers 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 2218R (5'-ATGACACCRACRG-CRACRGTYTG-3') (Castlebury et al. 2004) from 114 isolates recovered from insect baits. We macerated the fungal material using liquid nitrogen and 0.12 mL microtubes containing and prefilled with acid-washed 2.8 mm stainless steel homogenizing beads (Sigma-Aldrich) at 400 rpm for 60 s in the BeadBug™ homogenizer. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega®, Madison, USA) following the manufacturer’s recommendations. DNA concentrations were determined using a NanoDrop® spectrophotometer (Thermo Scientific®, Waltham, USA).

PCR amplifications were performed in a total volume of 25 µL, consisting of: 12.5 µL of KAPA Taq ReadyMix (KAPA Biosystems™), 1 µL of each forward and reverse primer (10 ng/µL), 5 µL of DNA template and 5.5 µL of DNAse/RNAse-free water (ZymoBIOMICS™). PCR reactions were placed in an Eppendorf™ thermal cycler under the following conditions: TEF (1) 2 min at 94 °C, (2) 10 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 1 min, and extension at 72 °C for 1 min, followed by (3) 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min, and (4) 3 min at 72 °C. For RPB1 (1) 2 min at 95 °C, (2) 10 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 1 min, and extension at 72 °C for 1 min, followed by (3) 35 cycles of denaturation at 95 °C for 30 s, annealing at 56.6 °C for 1 min, and extension at 72 °C for 1 min, and (4) 3 min at 72 °C. 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 by EXO-IT® (Affymetrix) and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The generated sequences were assembled into contigs using Geneious Prime® 2024.0.5 (Geneious Prime, 2024) (https://www.geneious.com) and compared with sequences from related species deposited in GenBank using BLASTn. Sequences from previous studies were retrieved from NCBI databases to perform a phylogenetic analysis together with the sequences we obtained. Bayesian inference with sequences was performed in MrBayes, using 2 Markov Chain Monte Carlo until convergence of the split frequency (<0.01). The analysis was conducted for 5 million generations.

2.6 Statistical Analysis

All statistical analyses were conducted using R software version 4.2.3 (R Core Team, 2023). For the survival assay female of *H. hampei* and larvae *T. molitor*, we performed a Kaplan-Meier survival analysis with the "survival" R package (Therneau, 2023). This analysis aimed to compare the effect of soil from three different coffee management systems — agroforestry, organic, and conventional — on the survival of the larvae. If significant differences are observed, this would allow us to estimate the contribution of ecosystem services related to pest control provided by different coffee cultivation systems. The full model included categorical variables blocking terms: expedition order, city sample location, farm identity and plot identity (representing one of the four quadrants within farms and each treatment area). These factors were incorporated to account for the inherent variability in soil characteristics due to the origin of the samples.

Model simplification was performed using backward stepwise deletion, assessing changes in deviance between models (Crawley, 2015). After retaining only statistically significant terms in the model, further simplification involved amalgamating levels within categorical variables based on the increasing mean survival time of the larvae.

3. RESULTS

3.1 Survival analysis of bait insects

We analyzed the survival of two species of insects used as bait, *H. hampei* (coffee borer) and *T. molitor* (mealworm), exposed to soils from different coffee management systems. These results were obtained considering only the insects that died and presented fungal sporulation. The results indicated that: For *H. hampei (*coffee borer), only the type of soil management ("management") significantly influenced survival after excluding fungi that were not identified; For *T. molitor (*tenebrio*),* only the collection expedition ("Collection") significantly affected survival, even after simplifying the model.

Table 4. Results of the statistical analysis to evaluate the effects of agricultural management (Management), collection period (Collection) and location (City) on the mortality of the bait insects *Hypothenemus hampei* and *Tenebrio molitor*. The values presented include the Akaike Information Criterion (AIC), chi-square statistic (χ^2) and significance value (p-value). Significant p-values (p < 0.01) are highlighted with "**".

Bait insects	Factor	AIC	χ^2	p-value
Hypothenemus hampei	Management	508.80	22.240	1.481e-05**
	Collection	501.89	0.813	0.6660
	City	502.33	2.992	0.3929
Tenebrio molitor	Management	430.59	0.775	0.6788
	Collection	441.68	52.319	4.355e-12**
	City	421.13	0.664	0.8817

3.2 *Hypothenemus hampei* (coffee berry borer)

Females of *H. hampei* exposed to soils from organic management systems exhibited the shortest survival time, with a mean of 9 days. In contrast, individuals exposed to soils from agroforestry systems survived significantly longer (mean = 12 days; df = 2; $p = 0.0079$). The longest survival was observed in females exposed to soils from conventional management systems (mean = 19 days), with a significant difference compared to the organic system (log-rank test: $\chi^2 = 20$; df = 2; $p = 1.9 \times 10^{-5}$). However, no significant difference was found between the agroforestry and conventional systems ($p = 0.1209$). Overall, survival times ranged from the second to the 29th day after exposure, and the analysis confirmed significant variation in survival among the different management systems.

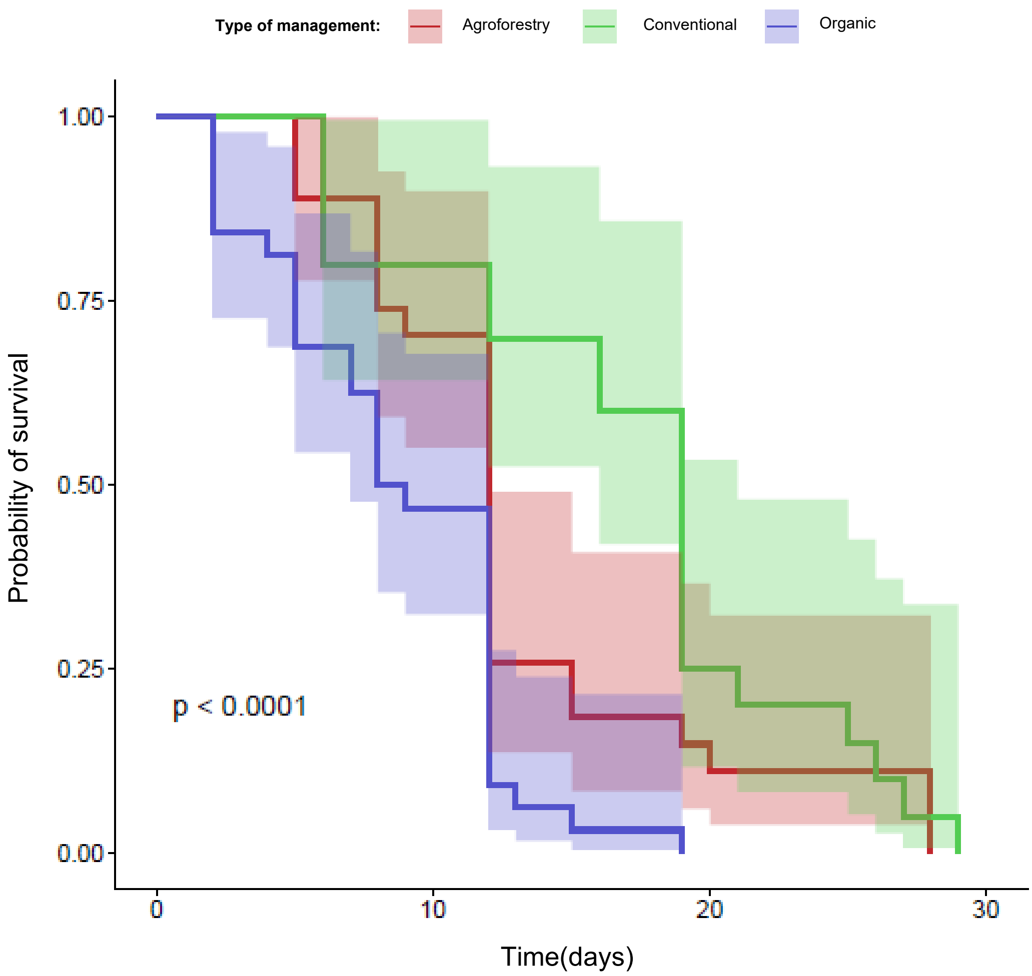


Figure 4: Survival curves *of Hypothenemus hampei* in different agricultural management systems. The conventional system showed higher survival of borers over time, compared to the agroforestry and organic systems. The shadows represent the confidence intervals of the estimates. The difference between treatments was statistically significant.

3.3 Survival analysis of Tenebrio molitor

The exposure of *T. molitor* larvae to soils from agroforestry, organic, and conventional coffee systems did not show significant differences in survival time between management types. However, survival time varied significantly between soil collection periods (log-rank test: $\chi^2 = 46.8$; df = 2; $p = 7 \times 10^{-11}$). Larvae exposed to soils from collection period 2 showed the highest mean survival time (61 days), significantly exceeding those from collection periods 1 (37 days; p = 0.0057) and 3 (33 days; $p = 3 \times 10^{-11}$). Furthermore, survival was also significantly higher in collection period 1 compared to collection period 3 (p = 0.0054).

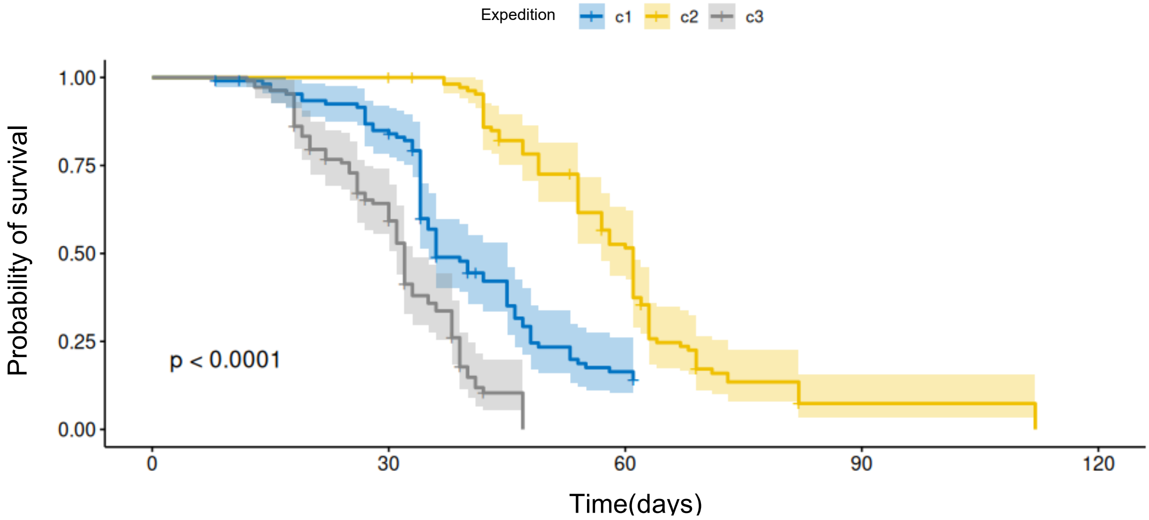


Figure 5: Survival curves of *Tenebrio molitor* in different collection periods. Expeditions c1 and c2 presented a faster mortality rate compared to expedition c3. The shadows represent the confidence intervals of the estimates, and the difference between the groups was statistically significant ($p < 0.0001$).

3.4 Phylogenetic analyses of fungal species captured by bait insects

In total, 113 fungal isolates were sequenced for the TEF region, including two bait insect isolates that were exposed to soils from different coffee management systems, agroforestry (n = 39), organic (n = 44) and conventional (n = 30) used in the study. Among the bait insect isolates, 6 families were identified: Nectriaceae (n = 44), Ophiocordycipitaceae (n = 25), Clavicipitaceae (n = 17), Hypocreaceae (n = 5), Bionectriaceae (n = 15), Cordycipitaceae (n = 7), distributed in seven genera *Fusarium, Purpureocillium, Metarhizium, Trichoderma, Clonostachys, Beauveria, Cordyceps*.

Table 5: Distribution of fungal isolates of the order Hypocreales obtained from bait insects exposed to soils from different management systems is indicated in red (Agroforestry), blue (Organic) and green (Conventional). The isolates were classified by taxonomic family based on the sequenced TEF region.

ORDER	FAMILY	MANAGEMENT SYSTEM			TOTAL ISOLATES
		Agroforestry	Organic	Conventional	
Hypocreales	Nectriaceae	15	18	11	44
	Ophiocordycipitaceae	5	7	13	25
	Clavicipitaceae	10	6	1	17
	Hypocreaceae	4	1	0	5
	Bionectriaceae	4	7	4	15
	Cordycipitaceae	0	6	1	7
	Total number of isolates by management	39	44	30	113

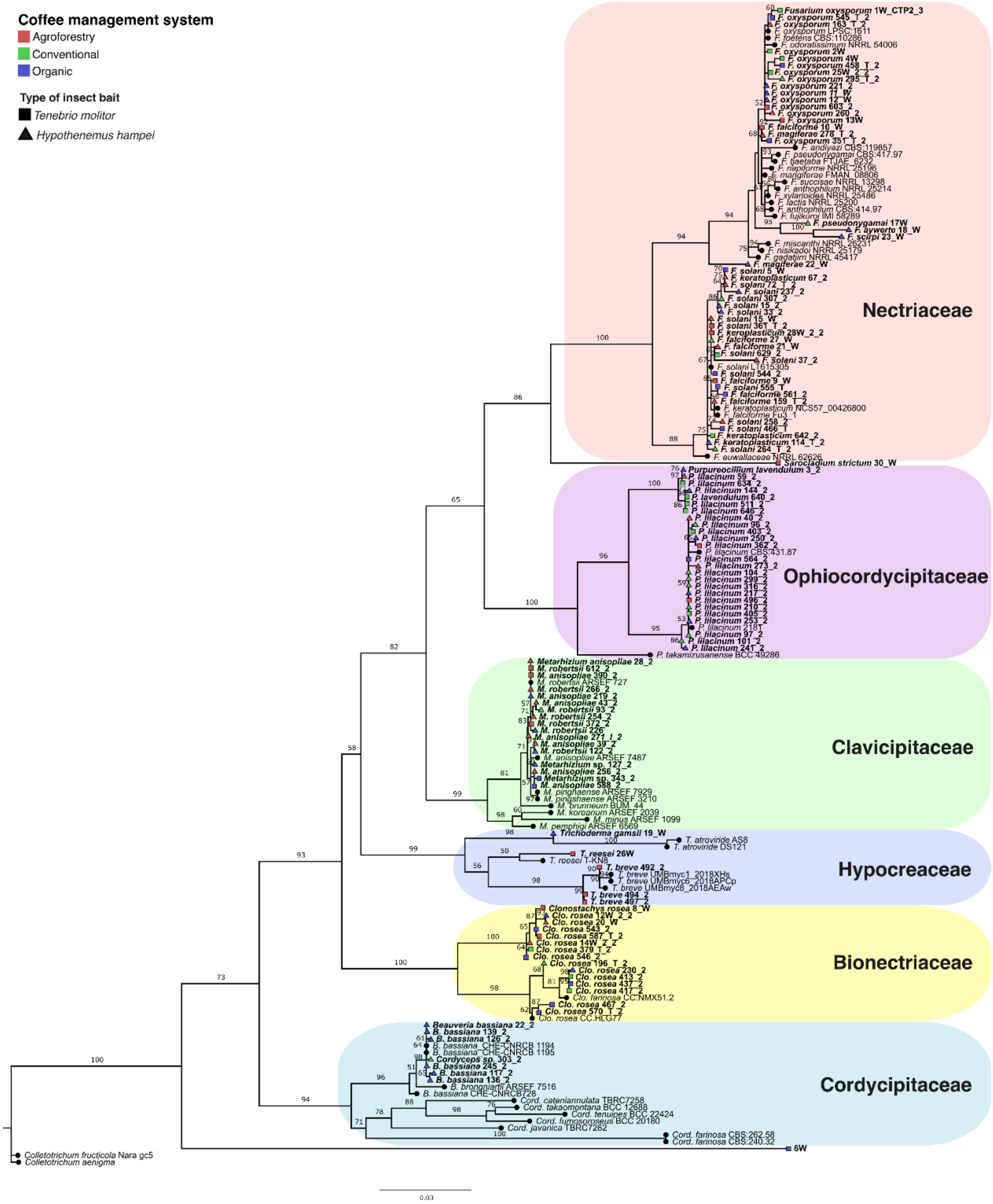


Figure 6: Diversity of fungal isolates from bait insects that were exposed to soils from different coffee management systems in the microregion of Viçosa (Araponga, Coimbra, São Miguel do Anta and Paula Cândido). The management systems are: agroforestry (red), conventional (green) and organic (blue) and the bait insects *Hypothenemus hampei* represented by triangle (black) and Tenebrio molitor represented by square (black). The phylogenetic reconstruction was constructed using the maximum likelihood (ML) method based on the genomic region of the translation elongation factor (TEF). The values at the nodes represent the bootstrap support, indicating the robustness of the phylogenetic groupings. The horizontal scale (0.03) reflects the evolutionary distance, calculated based on the number of nucleotide substitutions per site.

For the family Nectriaceae, we obtained a clade strongly supported by bootstrap (BS = 100%), subdivided into two subclades. The first subclade presented a bootstrap support of 94% and included isolates from soils under agroforestry, organic and conventional management. In agroforestry soils, isolates from the host coffee berry borer (*H. hampei*) were identified as *Fusarium oxysporum* (n = 2) and *F. magiferae* (n=1). Furthermore, isolates from the host *T. molitor* were recorded as *F. oxysporum* (n=2) and *F. falciforme* (n=1). In organic soils, isolates from the host *H. hampei* were identified as *F. oxysporum* (n=3), *F. aywerte* (n=1), *F. scirpi* (n=1) and *F. magiferae* (n=1) and isolates from the host *T. molitor* were recorded as *F. oxysporum* (n=3). In conventional soils, isolates from the host *H. hampei* were recorded as *F. oxysporum* (n=2), *F. magiferae* (n=1) and from the host *T. molitor,* isolates of *F. oxysporum* (n=2) and *F. falciforme* (n=1) were observed. The second subclade presented a bootstrap support of 88% and included isolates from soils under agroforestry, organic and conventional management. In agroforestry soils, isolates from the host *H. hampei* were identified as *F. keratoplasticum* (n=1), *F. solani* (n=4) and *F. falciforme* (n=2). In addition, isolates from the host *T. molitor* were recorded as *F. solani* (n=1), F. *keratoplasticum* (n = 1) and *F. falciforme* (n = 1). In organic soils, isolates from the host *H. hampei* were identified as *F. keratoplasticum* (n=1), *F. solani* (n=3). In isolates from *T. molitor, F. solani* (n=4) and *F. falciforme* (n=1) were recorded. In conventional soils, isolates from the host *H. hampei* were identified as *F. solani* (n = 2) and *F. falciforme* (n = 1) and isolates from *T. molitor* were identified as *F. solani* (n = 4) and *F. keratoplasticum* (n = 1).

For the family Ophiocordycipitaceae, a clade strongly supported by bootstrap (BS = 96%) was identified, which was subdivided into 2 subclades. The first subclade presented a bootstrap support of 100% and included isolates from soils under agroforestry, organic and conventional management. In agroforestry soil, an isolate from the host *H. hampei* was identified as *Purpureocillium lilacinum* (n=1). In organic soils, isolates from the host *H. hampei* were identified as *P. lavendulum* (n=1), *P. lilacinum* (n=1). In soils under conventional management, isolates from the host *T. molitor* were recorded as *P. lavendulum* (n=1) and *P. lilacinum* (n=3). The second subclade presented a bootstrap support of 95% and also included isolates from soil under the three management systems. In agroforestry soils, isolates from the host *H. hampei* were identified as *P. lilacinum* (n=2) and from the host *T. molitor P. lilacinum* (n=2). In organic management systems, isolates from the host *H. hampei* were identified as *P. lilacinum* (n=4) and from the host *T. molitor, P. lilacinum* (n=1). Finally, in soils under conventional management, isolates from the host *H. hampei* were identified as *P. lilacinum* (n=7) and from the host *T. molitor* were identified as *P. lilacinum* (n=2).

Clavipitaceae family, the isolates identified in this study formed a clade supported by 81% of bootstraps and encompassing isolates from soils under agroforestry, organic and conventional management. In agroforestry soils, isolates from the host *H. hampei* were identified as *Metarhizium anisopliae* (n=5) and *M. robertsii* (n=2). In addition, isolates from the host *T. molitor* were recorded as *M. anisopliae* (n=1) and *M. robertsii* (n=2). In organic management systems, isolates from the host *H. hampei* were identified as *M. anisopliae* (n=1), *M. robertsii* (n=2) and Metarhizium sp. (n=1) and isolates from the host *T. molitor* were recorded as *M. anisopliae* (n=1) and *Metarhizium* sp. (n=1). Finally, in soils under conventional management, we observed an isolate from the host *H. hampei* identified as *M. robertsii* (n=1).

For the family Hypocreaceae, a clade strongly supported by bootstrap (BS = 99%) was identified, subdivided into 3 subclades. The first subclade (BS = 98%) included an isolate from the host *H. hampei* in soil under organic management, identified as *Trichoderma gamsii* (n = 1). The second subclade (BS = 50%) isolated from the *T. molitor* host identified as *T. reesei* (n = 1). Finally, the third subclade presented a bootstrap support of 98% and included isolates from the *T. molitor* host identified as *T. breve* (n = 3).

For the family Bionectriaceae, only a single species has been identified, *Clonostachys rosea*, forming a clade (BS = 100%), subdivided into two subclades. The first subclade (BS = 100%) included isolates from soils under agroforestry, organic and conventional management. In agroforestry soils, four isolates of *C. rosea* (n= 4) were identified, two isolates from the host *H. hampei* and two isolates from the host *T. molitor.* In soils under organic management, three isolates of *C. rosea* (n = 3) were recorded, one isolate from the host *H. hampei* and two isolates from the host *T. molitor*. In soil under conventional management, one isolate of *C. rosea* (n = 1) from the host *T. molitor* was identified. The second subclade (BS = 98%) included isolates from organic and conventional soil. In soil under organic management, isolates of *C. rosea* (n= 4) were recorded, one from the host *H. hampei* and three from the host *T. molitor*. In soils under conventional management, isolates of C. rosea (n= 3) were recorded, one from the host *H. hampei* and two from the host T. molitor.

For the Cordycipitaceae family, a clade was formed (BS = 98%), composed of isolates from the coffee berry borer host under organic and conventional management. In the soils under organic management, *Beauveria bassiana* was identified (n = 6), while in the soil under conventional management, *Cordyceps* sp. was isolated (n = 1), both obtained from coffee berry borer.

4. DISCUSSION

Biological pest control is an essential tool to promote more sustainable and efficient agriculture. Many species belonging to the order Hypocreales include entomopathogenic fungi that deposit their infectious spores and reside in the soil for part of their life cycle, when they are not parasitizing their host (de Faria; Wraight 2007; Medo and Cagán, 2011, Vega et al. 2012). In our experiments, the baits were exposed to soils subjected to different management systems (agroforestry, organic and conventional). Larvae of *T. molitor*, a model insect widely used in entomopathogenicity studies, and females of the coffee berry borer (*Hypothenemus hampei*), considered one of the main pests of coffee plants, were used.

In experiments with *T. molitor* larvae exposed to agroforestry and conventional soils, Moreira et al. (2019) reported a mortality of 67.8% of insects in a 20-day interval in agroforestry soils and 40 days in conventional soils. The results of the present study show a similar pattern in soils with different management systems, with the death of 43.46% *T. molitor* larvae occurring in a period of approximately 10 to 110 days. For the coffee berry borer (*Hypothenemus hampei)*, we observed a relatively fast death time, ranging from 2 to 29 days for a total of 76.6 % females. Previous studies have reported that coffee berry borer mortality can occur within 6 to 10 days, affecting 40 to 90% of insects when directly exposed to high concentrations of entomopathogenic fungi (Samuels et al., 2002; Neves & Hirose, 2005). The ability of the fungus to kill insects is a more relevant measure to infer its pest suppression potential, as it reflects a broader range of its activity (Moreira et al., 2019). Environmental factors, whether biotic or abiotic, are determinants of the occurrence, distribution, and abundance of entomopathogenic fungi in the most diverse biomes. If we consider that the density of fungal spores in the various management systems studied is probably lower than in laboratory conditions, and that insects need to move through the soil to come into contact with these spores, we can infer that the survival and time to death of the bait insects presented in our results can be considered short. This probably occurred due to the variation in the intensity of the virulence factors of the different entomopathogenic fungi present in the soil.

Our results indicated that soil under organic management presented greater activity of insect pathogenic fungi when compared to the other systems evaluated. On the other hand, no significant difference in activity was observed between the soils of the agroforestry and conventional systems, indicating that, despite the expected differences in microclimatic stability between these two management systems, this factor did not translate into greater mortality of *H. hampei* in the agroforestry soil. These findings demonstrate that the relationship between the type of management, the microclimate and the ecosystem service of entomopathogenic fungi control is more complex than expected, and that factors other than microclimatic stability may be influencing the activity of these microorganisms in the soil. In environments that offer greater microclimatic stability and lower levels of disturbance, they can favor the maintenance of the viability and virulence of entomopathogenic fungi, ensuring their ability to infect and kill host insects (Jose, 2009), in addition to providing greater shading, which reduces solar radiation and contributes to the regulation of soil moisture and temperature (Lin, 2007). These conditions can favor the longevity and effectiveness of fungi as biological control agents (Vänninen et al., 2000).

More heterogeneous agroecosystems tend to provide greater ecosystem services, including biological control of pests mediated by predators and parasitoids (Altieri, 1996; Bianchi et al., 2006; Gardiner et al., 2009; Garrido-Jurado et al., 2011). Several studies indicate that biodiversity favors the provision of ecosystem services, especially in systems under organic management (Benton et al., 2003; Tscharntke et al., 2005; Bianchi et al., 2006; Letourneau & Bothwell, 2007) and agroforestry (Tscharntke et al., 2011). It is not yet possible to draw conclusions about functional diversity, since the characterization of some entomopathogenic genera, such as *Beauveria, Metarhizium,* and *Fuasrium*, is challenging. These genera encompass complexes of cryptic species, morphologically indistinguishable, but with distinct ecological functions within the same environment (Meyling et al., 2007; Fisher et al., 2011). If the diversity of these fungi is in fact related to the ecosystem services they provide, there is still a long way to go to understand how this relationship is established, making it premature to draw definitive conclusions.

The evolutionary success of Hypocreales fungi is largely attributed to their ecological plasticity, which allows them to adapt to diverse environmental conditions and establish interactions with a wide range of hosts. In Brazil, *Beauveria bassiana* stands out as the most widely used biological agent for the control of *H. hampei* in coffee plantations (Lopes, 2004). Recent studies demonstrate the pathogenicity of different isolates of this fungus on the coffee berry borer, highlighting its potential as a control agent in the field (Souza, 2019). This entomopathogenic fungus is frequently associated with high levels of natural infection in insect populations and is considered a promising biological control agent within integrated pest management programs (Mota, 2017). In parallel, the fungus *Metarhizium anisopliae* also infects the coffee berry borer, but it is better adapted to soil conditions than *B. bassiana*. Both fungi, *B. bassiana* and *M. anisopliae,* are distinguished by their operational advantages, such as ease of mass production of infectious propagules, simplicity of application in the field, low cost associated with their use and, above all, their reduced environmental impact, consolidating themselves as a viable and sustainable solution in the biological control of insects (Orlandelli & Pamphile, 2011).

Biodiversity in agroecosystems has been widely recognized for its role in providing essential ecosystem services, including biological pest control, which contributes to the sustainability of agricultural production. In our results, fungal isolates from insects used as bait *H. hampei and T. molitor* play fundamental ecological roles in agroecosystems, contributing both to the regulation of insect populations and to the maintenance of soil health. Increasing evidence indicates that the structural diversity of the agricultural landscape plays a fundamental role in maintaining biodiversity and preserving ecological functional groups essential for pest management(Benton et al., 2003 , Weibull and Ostman , 2003) More heterogeneous environments favor the coexistence of natural enemies and beneficial microorganisms, promoting ecological interactions that strengthen biological control and reduce dependence on chemical inputs, contributing to the sustainability of agriculture (Benton et al., 2003 , Weibull and Ostman , 2003).

Among the genera identified in our study, *Beauveria, Cordyceps, Metarhizium* and *Purpureocillium* stand out for their recognized entomopathogenic potential, playing an essential role in the biological control of agricultural pests. In addition, the genera *Fusarium, Trichoderma* and *Clonostachys* were identified. These microorganisms can be found in soil, in insect bodies or even in the internal tissues of plants. Insect corpses, as well as still infected individuals, represent an important source for the isolation of entomopathogenic fungi (Qayyum et al., 2021). However, colonization of these corpses can also occur by opportunistic fungi, which take advantage of favorable conditions in the final stages of the host's life. Furthermore, studies suggest that the distribution of entomopathogenic fungi in soil is more influenced by the physicochemical characteristics of the soil than by the surrounding vegetation. However, some species may be more strongly associated with host plants (Farooq et al., 2021; Zahid et al., 2020; Saeed et al., 2019; Nishi & Sato, 2019; Steinwender et al., 2014). The distribution of fungal species among different management systems suggests that agricultural practices can influence the diversity and abundance of these organisms, highlighting the importance of microbial biodiversity in providing ecosystem services essential for the sustainability of coffee production.

Our study showed high diversity of fungal isolates in several coffee management systems, which were selected based on the assumption that different soil management practices can mediate important ecosystem services. The provision of ecosystem services is influenced by the composition, diversity and elements of the systems (Santos et al., 2019). Few studies have quantified the differences between these systems regarding the provision of ecosystem services (Torralba et al., 2016). Identifying effective management strategies that allow the simultaneous provision of several ecosystem services is a scientific issue of great relevance in different types of ecosystems (Lafond et al., 2017) and has been the subject of discussion by several authors (Bugalho et al., 2016; Lafond et al., 2017). Although we have evidence of insect suppressive potential in several coffee management systems, the complexity of the soil and the numerous interactions that occur in this environment may provide different explanations for the contribution to ecosystem services, especially with regard to the suppression of subterranean insect pests.

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CONCLUSIONS AND FUTURE PROSPECTS

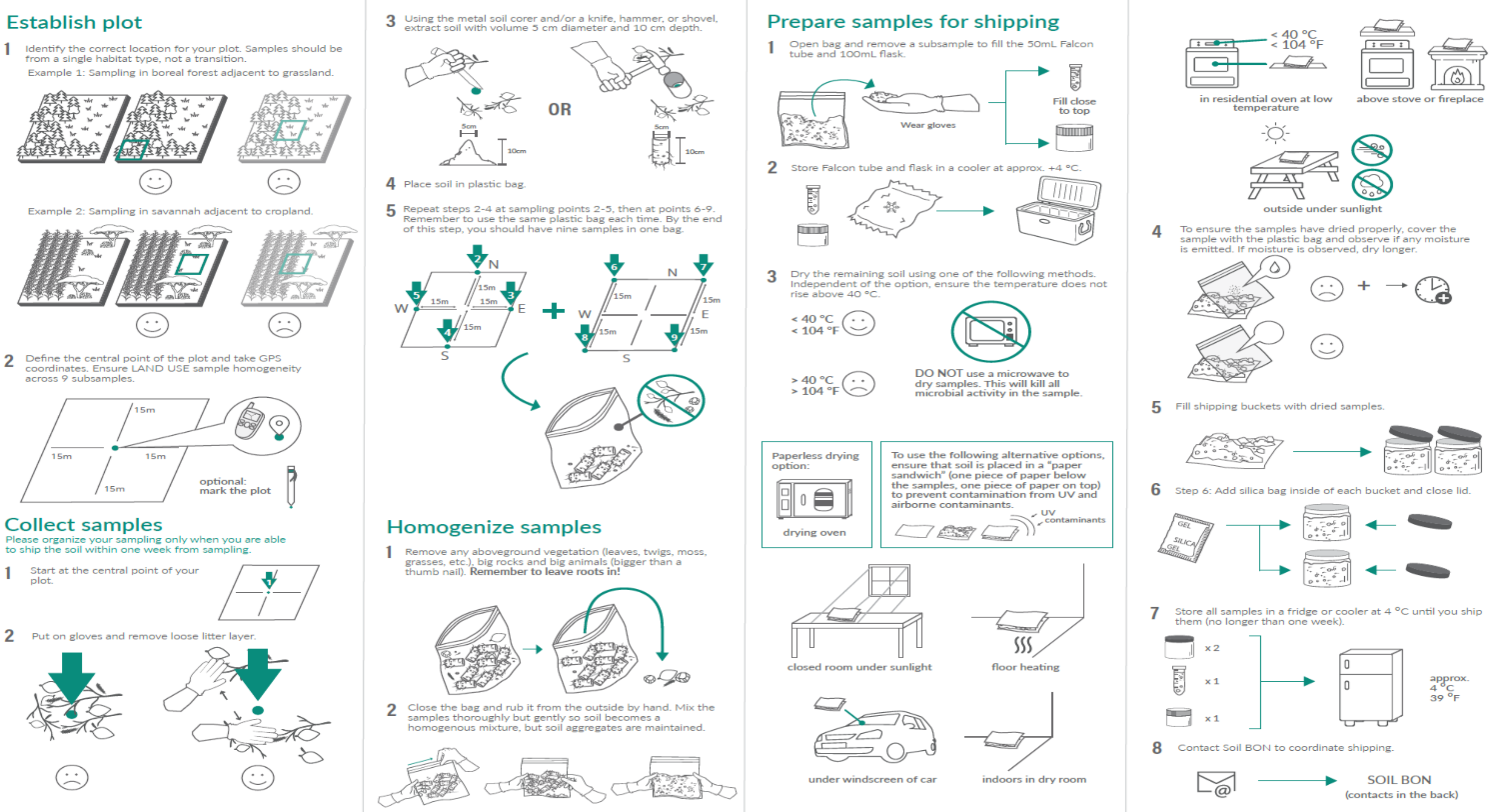
This study demonstrated that agricultural management systems significantly influence the diversity, composition and functionality of entomopathogenic fungi in soil, directly affecting the provision of the ecosystem service of biological pest control. The results revealed that soils under organic and agroforestry management present greater diversity and activity of these fungi compared to conventional systems. This greater diversity can be attributed to more stable microclimatic conditions, greater availability of organic matter and lower impact of chemical inputs, which favor the persistence and virulence of entomopathogenic fungi.

Hypothenemus hampei, used as bait, is noteworthy , reinforcing the impact of management on the effectiveness of biological control. We observed that mortality occurred in a shorter time in soils under organic and agroforestry management. These results indicate that soils managed in a sustainable manner favor the occurrence and activity of entomopathogenic fungi, making the environment more conducive to the infection and suppression of insect pest populations.Although our results provide robust evidence of the influence of agricultural management on the diversity and activity of entomopathogenic fungi, there is still a long way to go to fully understand their ecological interactions and their impact on ecosystem services. Future studies should adopt more integrated approaches, combining genetic, ecological and functional analyses, in addition to advancing molecular identification analyses. The exploration of other regions, such as ITS, and the search for specific markers that are resolutive for the genera found can significantly contribute to a deeper understanding of the relationship between microbial biodiversity, agricultural management and sustainable biological control. Thus, this study reinforces the importance of soil biodiversity in the provision of ecosystem services and highlights the need for management strategies that reconcile productivity and environmental conservation.

Several other questions arise from this study. Among the most relevant, we can ask: What environmental and management factors influence the persistence and effectiveness of entomopathogenic fungi in soil, and how can these factors be modeled to optimize biological control of insect pests in agricultural systems? How do different agricultural management systems (conventional, organic, and agroforestry) influence the persistence and effectiveness of entomopathogenic fungi in soil? Which environmental and chemical factors in the soil are most decisive for conidial viability and the efficiency of biological control? Is it possible to predict the survival and infection rate of entomopathogenic fungi under different environmental conditions through mathematical and computational models? Is there a correlation between the genetic diversity of entomopathogens and their capacity for persistence and infection in soil?

APPENDICES

Appendix 1: Soil BOM Field Sampling Protocol (Potapov, 2022)



Establish plot

1 Identify the correct location for your plot. Samples should be from a single habitat type, not a transition.

Example 1: Sampling in boreal forest adjacent to grassland.

Example 2: Sampling in savannah adjacent to cropland.

2 Define the central point of the plot and take GPS coordinates. Ensure LAND USE sample homogeneity across 9 subsamples.

Collect samples

Please organize your sampling only when you are able to ship the soil within one week from sampling.

1 Start at the central point of your plot.

2 Put on gloves and remove loose litter layer.

3 Using the metal soil corer and/or a knife, hammer, or shovel, extract soil with volume 5 cm diameter and 10 cm depth.

4 Place soil in plastic bag.

5 Repeat steps 2-4 at sampling points 2-5, then at points 6-9. Remember to use the same plastic bag each time. By the end of this step, you should have nine samples in one bag.

Homogenize samples

1 Remove any aboveground vegetation (leaves, twigs, moss, grasses, etc.), big rocks and big animals (bigger than a thumb nail). **Remember to leave roots in!**

2 Close the bag and rub it from the outside by hand. Mix the samples thoroughly but gently so soil becomes a homogenous mixture, but soil aggregates are maintained.

Prepare samples for shipping

1 Open bag and remove a subsample to fill the 50mL Falcon tube and 100mL flask.

2 Store Falcon tube and flask in a cooler at approx. +4 °C.

3 Dry the remaining soil using one of the following methods. Independent of the option, ensure the temperature does not rise above 40 °C.

< 40 °C
< 104 °F

> 40 °C
> 104 °F

DO NOT use a microwave to dry samples. This will kill all microbial activity in the sample.

Paperless drying option:

To use the following alternative options, ensure that soil is placed in a "paper sandwich" (one piece of paper below the samples, one piece of paper on top) to prevent contamination from UV and airborne contaminants.

4 To ensure the samples have dried properly, cover the sample with the plastic bag and observe if any moisture is emitted. If moisture is observed, dry longer.

5 Fill shipping buckets with dried samples.

6 Step 6: Add silica bag inside of each bucket and close lid.

7 Store all samples in a fridge or cooler at 4 °C until you ship them (no longer than one week).

8 Contact Soil BON to coordinate shipping.

Appendix 2: Table of soil variables and geographic characteristics of the fields in each sample field

Samples	pH	Ca^{2+}	Mg^{2+}	K^+	Al^{3+}	H+Al	SB	t	T	V	m	P	P rem	MO	Micronutrients			
															Cu	**Mn**	**Fe**	**Zn**
	H_2O	$cmol_c/dm^3$								%		mg/kg	mg/L	dag/kg	mg/dm^3			
Agroforestry																		
AGF1	6.1	5.95	1.77	0.21	0.00	5.85	7.93	7.93	13.78	57.8	0.0	19.43	10.98	7.65	0.58	50.90	43.28	5.24
AGF2	6.6	7.78	1.22	0.26	0.00	1.15	9.26	9.26	10.41	88.7	0.0	41.68	32.68	4.32	2.50	70.63	27.43	13.68
AGF3	5.8	5.66	2.22	0.52	0.05	8.20	8.40	8.45	16.60	50.6	0.8	59.53	10.68	8.20	0.53	17.73	87.48	5.74
Organic																		
ORG1	5.9	4.22	1.80	0.36	0.05	8.95	6.38	6.43	15.33	41.6	1.0	12.38	9.80	9.22	0.57	52.78	30.98	3.76
ORG2	6.0	5.19	1.22	0.43	0.00	3.45	6.84	6.84	10.29	66.6	0.0	81.13	35.33	3.84	1.67	58.83	38.00	12.34
ORG3	5.5	7.83	1.84	0.62	0.00	6.85	10.29	10.29	17.14	60.4	0.0	20.65	20.18	7.66	0.73	89.68	36.90	6.55
Conventional																		
CNV1	5.5	2.22	0.87	0.22	0.05	3.33	3.30	3.35	6.63	49.9	1.7	2.80	30.95	3.09	1.41	29.95	73.95	1.16
CNV2	5.7	4.00	0.92	0.34	0.09	4.18	5.25	5.34	9.43	54.8	2.2	20.50	25.73	3.58	2.60	19.23	63.93	4.17
CNV3	6.9	5.89	1.95	0.27	0.00	1.15	8.10	8.10	9.25	87.6	0.0	9.43	20.80	3.51	0.78	29.08	26.73	2.65

AGF1 Agroforestry collects 1
AGF2 Agroforestry collects 2
AGF3 Agroforestry collects 3

ORG1 Organic collects 1
ORG2 Organic collects 2
ORG3 Organic collects 3

CNV1 Conventional collects 1
CNV2 Conventional collects 2
CNV3 Conventional collects 3