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Agroecosystem management strategies and trophic interactions among herbivores, pathogens and predators

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APROVADA: 28 de maio de 2016

Cleide Maria Ferreira Pinto

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Madelaine Venzon
(Orientadora)

"While the farmer holds the title to the land,
actually it belongs to all people because civilization rests upon the soil".

Thomas Jefferson

"The soil is the great connector of lives, the source and destination of all. It is healer and restorer and resurrector, by which disease passes into health, age into youth, death into life. Without proper care for it we can have no community, because without proper care for it we can have no life"

Wendell Berry, 1977

A minha família, meu porto seguro, meu retiro:
A meu pai Domingos e minha Carmem, por todo o amor e dedicação
Às minhas irmãs Virgínia e Amanda
À minha sobrinha Olívia, o novo sopro de alegria na família
À Elem, o meu amor, minha alegria e luz dos meus dias,
dedico todos os esforços desta caminhada
que sem o amor de vocês soariam em vão

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BIOGRAFIA

ANDRÉ LAGE PEREZ, filho de Domingos Perez Vidal e Maria Carmem Lage Perez, nasceu em Governador Valadares – MG, no dia 04 de abril de 1985. Em 2003 iniciou o curso de Ciências Biológicas na Universidade Vale do Rio Doce (MG), concluindo-o em dezembro de 2006. Em março de 2012 concluiu o curso de mestrado em Entomologia na Universidade Federal de Viçosa, sob a orientação da pesquisadora Madelaine Venzon. Em 2012 iniciou o doutorado em Entomologia na Universidade Federal de Viçosa, sob a orientação da pesquisadora Madelaine Venzon.

SUMÁRIO

RESUMO

PEREZ, André Lage, D.Sc. Universidade Federal de Viçosa, maio de 2016. **Estratégias de manejo de agroecossistemas e interações tróficas entre herbívoros, patógenos e predadores.** Orientadora: Madelaine Venzon. Coorientadores: Simon Luke Elliot e Angelo Pallini

A redução da complexidade ambiental e o aumento da produção de plantas oferecem condições favoráveis ao desenvolvimento de espécies consideradas pragas nos sistemas convencionais de cultivo. Entre estratégias de controle biológico de pragas, a restauração da diversidade vegetacional do agroecossistema e a adoção de estratégias de manejo que reduzam o impacto sobre populações de inimigos naturais são práticas que podem ser empregadas em diferentes tipos de sistemas de cultivo. Grande parte dos esforços de controle biológico conservativo é voltada a manutenção de populações de parasitoides e predadores. No entanto, patógenos representam fatores de mortalidade cruciais na regulação de populações de insetos pragas dentro das lavouras. A manutenção da vegetação nativa voltada ao incremento de populações predadores e parasitoides também pode promover a preservação de estruturas de resistência e propágulos de fungos entomopatogênicos no solo. Adicionalmente, a redução dos distúrbios no solo causados pelas práticas de manejo pode reduzir os impactos sobre a comunidade de entomopatógenos no solo de agroecossitemas. A compreensão das interações ecológicas entre predadores, patógenos e herbívoros representa um fator fundamental para o entendimento do papel do controle biológico de pragas em agroecossitemas. Como modelo de estudo das práticas de manejo de cultivos como estratégias de controle biológico conservativo foram utilizados cultivos de pimenta-malagueta (*Capsicum frutescens*) e melão (*Cucumis melo* var *cantalupensis*) caracterizadas por pequenas áreas de plantio e alta susceptibilidade de pragas several a produção. As interações entre inimigos naturais foram feitas em microcosmos em casa de vegetação. Portanto, o objetivo desta tese foi avaliar o papel de práticas de manejo de agroecossistemas como estratégias de controle biológico de pragas em cultivos anuais. Objetivo-se também estudar de forma detalhada das interações ecológicas entre inimigos naturais, herbívoros e planta. Desta forma abordadas as seguintes hipóteses: a) plantas espontâneas promovem a modificação de

fatores bióticos e abióticos afetam positivamente a virulência, ocorrência e abundância de fungos entomopatogênicos no solo; b) o emprego do plantio direto em culturas anuais garante a preservação mais eficaz de entomopatógenos no solo refletindo na virulência e ocorrência dos microorganismos em agroecossitemas; c) a exposição de herbívoros a esporos de fungo entomopatogênico promovem a redução na herbívora sobre a planta hospedeira; d) a exposição do herbívoro aos esporos do patógeno propicia uma facilitação na predação; e) herbívoros reduzem e modulam comportamento alimentar na presença de um predador; f) a ação conjunta de patógeno e predador resulta em uma redução adicional nos danos causados pelos herbívoros às plantas. Como resultados verificou-se que a manutenção de plantas espontâneas em cultivos de pimenta-malagueta não representa uma estratégia eficaz de preservação de fungos entomopatogênicos em curto prazo. A virulência de fungos entomopatogênios em áreas duas área de estudo com plantas espontâneas foi superior à monocultura, no entanto em outra área de estudo foi observado um padrão inverso. O plantio direto em cultivos de melão não afetou a virulência de entomopatógenos no solo. A ocorrência e virulência de entomopatógenos no solo foram afetadas pela época de amostragem durante o tempo de cultivo, demonstrando um padrão temporal da dinâmica de entomopatógenos em agroecossitemas. Em experimentos em laboratório e casa de vegetação observou-se que aranhas não demonstram uma preferência na predação por presas expostas ao patógeno. A exposição ao patógeno não reduziu a herbívora de *D. undecimpunctata* sobre as plantas de melão, no entanto a presença do predador levou a uma redução no dano causado pelo herbívoro, tanto pela predação quanto pelo menos tempo alocado por besouro na planta. Observou-se que herbívoros expostos a patógenos e na presença do predador causaram danos às plantas semelhantes aos besouros na ausência de qualquer inimigo natural. Em conclusão, a partir dos estudos e resultados obtidos nesta tese, conclui-se que o emprego de estrégias de manejo de habitat deve atentar não somente para a composição da paisagem e a redução de distúrbios, mas principalmente o fator tempo para a recuperação dos serviços de ecossistema desempenhados por entomopatógenos. Conclui-se também que patógenos, predadores, herbívoros e plantas apresentam interações tróficas que afetam diferencialmente a dinâmica de inimigos naturais e seus serviços de ecossistema.

ABSTRACT

PEREZ, André Lage, D.Sc. Universidade Federal de Viçosa, May 2016. **Agroecosystem management strategies and trophic interactions among herbivores, pathogens and predators.** Adviser: Madelaine Venzon. Co-advisers: Simon Luke Elliot e Angelo Pallini

Reducing the environmental complexity and increasing biomass of cultivated plants offer favorable conditions for pest species in conventional systems. Among biological control strategies, the restoration of vegetation diversity in agroecosystems and the adoption of management strategies aiming to reduce impacts on natural enemy populations are practices that can be employed in different types of farming systems. Much of the conservative biological control efforts are focused on maintaining populations of parasitoids and predators. However, pathogens represent key mortality factors in the regulating pest populations in crops. The maintenance of indigenous plants aiming to increment populations of predators and parasitoids, may also promote the preservation of propagules of entomopathogenic fungi in the soil. In addition, the reduction of soil disturbance may reduce impacts on the entomopathogenic community in agroecosystems soil. Studiyng the ecological interactions between predators, pathogens and herbivores is a key factor in understanding the role of biological control of pests in agroecosystems. As a model of crop management practices and conservative biological control strategies we used chili pepper crops (*Capsicum frutescens*) and melon (*Cucumis melo* var *cantalupensis*) represented by small fields and high susceptibility to pests. Interactions between natural enemies were studied in microcosms in greenhouse. Therefore, the goal of this thesis was to evaluate the role of agro-ecosystems management practices as biological control strategies of pests in annual crops. Aim is also to study in detail the ecological interactions between natural enemies, herbivores and plants. From this framework we addressed the following hypothesis: a) Non-crop plants promote the modification of biotic and abiotic factors affecting virulence, occurrence and abundance of entomopathogenic fungi in the soil; b) the use no-tillage in annual crops performs the preservation of entomopathogens in the soil resulting in

higher virulence and occurrence of microorganisms in agroecosystems; c) herbivores exposed to spores of entomopathogenic fungi reduce their herbivory on the host plant; d) the exposure of herbivore to pathogen spores affect predation; e) herbivores reduce and modulate feeding behavior in the presence of a predator; f) joint action of pathogen and predator results in a further reduction in the damage caused by herbivores to plants. As a result we found that the maintenance of non-crop plants in chili pepper crops is not an effective as a short-term strategy for preserving entomopathogenic fungi. The fungal virulence in two experimental fields with non-crop plants was higher than in chili pepper monoculture, but in one field we observed an inverse result for fungi virulence. The tillage regime in melon crops did not affect the virulence of soil-borne entomopathogens. The occurrence and virulence of entomopathogens was affected by sampling time during the growing season, showing a time-scale pattern of entomopathogenic dynamics in agroecosystems. In laboratory assay and in the greenhouse we observed that spiders do not show preferential predation for prey exposed to the pathogen. The exposure to spores of pathogen did not reduce the herbivory *D. undecimpunctata* on melon plants, however the presence of predator reduced the damage caused by the herbivore, both due predation and reduce time allocated by beetle on the plant. Also, we observed that herbivores exposed to pathogen and in the presence of predator damaged the plants in a similar rate compared to beetles in the absence of any natural enemy (predators or pathogen). In conclusion, from the results obtained in this thesis, it is concluded that the use of habitat management strategies should pay attention not only to the landscape composition and reduced disturbances but especially the time factor restore ecosystem services performed by entomopathogens. We also concluded that pathogens, predators, herbivores and plants have trophic interactions that differentially affect the dynamics of natural enemies and their ecosystem services.

Introdução Geral

Sistemas convencionais de cultivo empregam diversas práticas agronômicas visando à otimização da produção agrícola em áreas cultivadas. Em geral, esses sistemas baseiam-se na simplificação da paisagem local e no uso de insumos agrícolas voltados a redução de fatores limitantes à produção das espécies cultivadas. No entanto, as mesmas práticas voltadas ao aumento da produção em agroecossistemas resultam no aumento de fatores negativos às plantas cultivadas. A redução da complexidade ambiental e o aumento da produção agrícola oferecem condições favoráveis ao desenvolvimento de espécies consideradas pragas para o sistema de cultivo (Root 1973). Neste contexto, práticas agrícolas adicionais são aplicadas com o objetivo de reduzir os impactos negativos de pragas. No entanto, estratégias convencionais de controle de pragas elevam os custos da produção agrícola e acarretam impactos ambientais indesejáveis. Contudo, estratégias alternativas ao controle convencional de pragas promovem a redução do impacto de artrópodes indesejáveis em agroecossistemas.

O controle biológico conservativo de pragas baseia-se na manipulação do ambiente e/ou no planejamento de práticas agrícolas visando beneficiar inimigos naturais de pragas (Eilenberg *et al* 2001). O incremento de populações de inimigos naturais emprega estratégias que fornecem recursos alimentares, abrigo e condições favoráveis para a manutenção e reprodução de populações de predadores, parasitoides e patógenos de pragas. A restauração da complexidade ambiental através do aumento da diversidade de plantas em agroecossistemas representa uma prática de controle biológico conservativo empregada na manutenção de predadores e parasitoides em níveis populacionais capazes de controlar surtos de pragas (Landis *et al* 2000; Wilkinson & Landis 2005). Grande parte das estratégias de controle biológico

conservativo é empregada visando beneficiar populações de artrópodes predadores e parasitoides em agroecossistemas (Altieri 1999; Tscharntke *et al* 2005). No entanto, patógenos desempenham um papel igualmente importante na regulação de populações de insetos praga.

Em agroecossistemas, fungos entomopatogênicos são responsáveis por eventos de epizootia causando a mortalidade de um número elevado de insetos (Meyling & Eilenberg 2007). Eventos de epizootia são resultado da interação entre a dinâmica populacional de hospedeiros, viabilidade de estruturas infectivas do patógeno e diversos fatores ambientais que condicionam o desenvolvimento no microorganismo (Anderson & May 1981; Inglis *et al* 2001). Embora esporos de fungos entomopatogênicos sejam encontrados abundantemente no solo e na superfície de plantas, fatores climáticos tais como a radiação solar e temperatura contribuem para a inativação das estruturas de resistência dos fungos entomopatogênicos no ambiente, reduzindo a sua virulência ao hospedeiro (Meyling & Eilenberg 2007). Neste sentido a manipulação de habitat pode prover condições microclimáticas favoráveis à conservação dos propágulos de fungos entomopatogênicos em agroecossistemas. Adicionalmente muitas espécies de fungos entomopatogênicos encontrados no solo de agroecossistemas apresentam um amplo espectro de hospedeiros. Portanto, práticas de manejo de ecossistema que resultem em um aumento na atividade local de insetos também podem beneficiar aos fungos entomopatogênicos pela maior disponibilidade de hospedeiros.

Regimes de manejo do solo empregados intensivamente em sistemas de cultivo anuais provocam modificação das características abióticas e bióticas do solo. Estes distúrbios podem prejudicar o funcionamento de serviço de ecossistema representado pela ação de entomopatógenos na regulação de comunidades locais de herbívoros (Barrios 2006). Neste sentido, a modificação das práticas de manejo do uso do solo podem representar estratégias de controle biológico conservativo viáveis para a

manutenção da comunidade de entomopatógenos no solo de agroecossistemas. No entanto, a adoção de estratégias de manejo de cultivos menos intensivas deve atentar para a sua efetividade na promoção do controle de pragas como um serviço de ecossistema, bem como garantir a produtividade do agroecossistema.

A diversidade e abundância de patógenos e predadores encontrados em vários agroecossistemas sugere a interferência constante de ambos inimigos naturais nas interações tróficas envolvendo hospedeiros e presas. O solo das áreas de cultivo abriga espécies de predadores generalistas que fazem o controle de diversas espécies de herbívoros associados às plantas cultivadas (Landis *et al* 2000). Ao mesmo tempo, entomopatógenos se desenvolvem nos corpos de diversas espécies de insetos causando a morte do hospedeiro (Meyling & Eilenberg 2007). Portanto, a ação conjunta de ambos os inimigos naturais (predadores e patógenos) representa um fator crucial de mortalidade em comunidades de artrópodes herbívoros em áreas de cultivo. Estratégias de controle biológico conservativo e aumentativo podem ser empregadas visando o incremento da população de predadores e patógenos, resultando no controle mais efetivo de populações de pragas. No entanto, a compreensão dos mecanismos ecológicos envolvendo predadores, patógenos, herbívoros e plantas é fundamental para subsidiar a integração de múltiplas estratégias de controle de pragas.

No presente trabalho estudou-se o papel de práticas de manejo cultural tais como a manutenção de plantas espontâneas e o plantio direto como estratégias de controle biológico conservativo visando à manutenção de entomopatógenos no solo de áreas de cultivo anual. Os cultivos estudados no presente trabalho foram a pimenta-malagueta (*Capsicum frutescens* L.) em Oratórios, Minas Gerais, Brasil e cultivos de melão (*Cucumis melo* L. var. *Cantaloupensis*) no Estado de Kentucky, EUA. Adicionalmente estudou-se as interações ecológicas entre o predador generalista *Tigrosa helluo* (Araneae: Lycosidae), o fungo entomopatogênico *Beauveria bassiana (*Balsamo)

Vuillemin, o besouro herbívoro *Diabrotica undecimounctata howardi* Barber (Coleoptera: Chrysomelidae) e plantas de melão.

O cultivo da pimenta (*Capsicum frutescens*) ocupa espaço importante na produção agrícola nacional, embora ainda seja considerada atividade secundária para a agricultura no Brasil. Os estados de Minas Gerais, Goiás, São Paulo, Ceará e Rio Grande do Sul são os maiores produtores do Brasil (Rufino & Penteado 2006). A alta rentabilidade dos cultivos de pimenta-malagueta, tanto para produtores em larga escala quanto para agricultores familiares, tem resultado em um cenário de crescente produção e interesse comercial (Pinto 2006). No entanto, as áreas de produção de pimental-malagueta enfrentam sérios problemas com o ataque de pragas tais como a broca-dos-frutos da pimenta *Symmetrischema dulce* Povolny (Lepidoptera: Gelechiidae) que atacam os frutos em fase de maturação comprometendo a qualidade e o valor de mercado dos frutos. Soma-se a esse fator o baixo suporte fitossanitário para a cultura da pimenta-malagueta no Brasil (Venzon *et al* 2006, 2011). Os sistemas de cultivo de melão (*C. melo*) nos Estados Unidos ocupam uma área anual de produção de aproximadamente 81 mil hectares com uma produção média de 2,7 milhões de toneladas totalizando US$ 800 milhões por ano (Day-Rubenstein & Heisey 2012). Áreas de cultivo de melão no sudeste dos Estados Unidos são atacadas por besouros herbívoros da família Chrysomelidae, com maior destaque a *D. undecimpunctata,* que transmite a bactéria *Erwinia tracheiplila* causando a mortalidade de muitas plantas nas lavouras (Yao *et al* 1996; Cline *et al* 2008). As culturas da pimenta-malagueta no Brasil e do melão nos Estados Unidos carecem de alternativas de controle cultural que promovam a redução dos danos causados pelas pragas de importância econômica.

No presente trabalho, objetivou-se avaliar o papel de práticas de manejo de agroecossistemas como estratégias de controle biológico de pragas em cultivos anuais. Objetivou-se também estudar de forma detalhada as interações ecológicas entre

inimigos naturais, herbívoros e planta. No capítulo I foi avaliado o papel da manutenção de plantas espontâneas em cultivos de pimenta-malagueta, beneficiando a atividade de fungos entomopatógenicos no solo. No capítulo II estudou-se os efeitos de diferentes formas de manejo do solo em um agroecosssistema de produção orgânica de melão sobre a comunidade de entomopatógenos do solo. Adicionalmente foi estudada a atividade de entomopatógenos no solo dos cultivos de melão em escala temporal. Por fim, no capítulo III, investigou-se as interações tróficas entre um predador (*T. helluo*), um entomopatógeno (*Beauveria bassiana*), um inseto herbívoro (*D. undecimpunctata*) e plantas de melão.

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Chapter I

Can non-crop plants benefit entomopathogenic fungi in chili pepper crops?

Abstract

The maintenance of non-crop plants into cultivated areas may create suitable conditions for the establishment of entomopathogenic fungi, which are known to contribute to the regulation of pest populations. We investigated the role of non-crop plants in preserving entomopathogenic fungi in the soil of chili pepper agroecosystems using insect live-bait method with *Tenebrio molitor* larvae. The occurrence and virulence of entomopathogenic fungi were compared in the soil of chili pepper crops in monoculture or managed with non-crop plants. No significant differences were observed in the occurrence and virulence of entomopathogenic fungi in soil samples from monoculture and from non-crop plant areas. The fungal isolates identified were *Fusarium* sp., *Beauveria* sp. and *Metharizium* sp. The most frequent fungal isolates observed infecting *T. molitor* larvae were *Metharizium* sp and *Fusarium* sp. The results are discussed in terms of fungi-plant interactions and differential suitability of fungal isolates to environmental conditions in agroecosystems.

Keywords: conservation biological control, entomopathogens, ecosystem services, live-bait method

1. Introduction

Conservation biological control is defined as the modification of the environment or existing practices in order to protect and enhance specific natural enemies aiming to reduce the effects of pests on crops (Eilenberg *et al* 2001). Most strategies are based on the provisioning of alternative food resources, shelter and microclimatic conditions favorable to indigenous populations of natural enemies (Landis *et al* 2000; Barbosa, 2003; Gurr *et al*, 2003). Most attentions have being paid to arthropod predators and parasitoids (Altieri 1999; Tscharntke *et al* 2005), but the contribution of entomopathogenic fungi for controlling pest populations is being overlooked. However, entomopathogenic fungi are among the main agents of ecosystem services in agroecosystems and most of the pest regulation roles may be attributed to entomopathogens (Meyling & Eilenberg 2007).

A strategy of conservative biological control commonly used is the maintenance of non-crop plant strips in the crop lands in order to enhance beneficial arthropod populations (Gurr *et al* 2003; Norris & Kogan 2005). The environmental heterogeneity adopted for enhancing the arthropod natural enemy populations may work also for entomopathogenic fungi (Fuxa 1998; Meyling & Eilenberg 2007; Pell *et al* 2010). Increasing the shade area in the crops is decisive for maintaining the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin in several agroecosystems (Bidochka *et al* 1998). The plant diversity thought to manipulate insect dispersion may also enhance the entomopathogenic fungi dispersal in cultivated areas due to increased activity of predators and parasitoids (Meyling & Eilenberg 2006).

Specific mechanisms allow plants and entomopathogenic fungi to participate on mutualistic interactions. *Metarhizium anisopliae* (Metschinikoff) is commonly associated to the rhizosphere of local vegetation, persisting in the agroecosystem even in host scarcity situations (Bruck 2005). Some plants that produce exudates are capable

to activate propagules of *M. anisopliae* increasing its infectivity to herbivores (Wang *et al* 2005; Bais *et al* 2006). Also, *M. anisopliae* and plants engage in a mutualistic interaction via hyphae and roots for nutrient exchange. In such interaction, *M. anisopliae* transfer nitrogen from insect host body to roots in exchange for carbon abundant in plant tissues (Behie *et al* 2012). Plants may also work as endophytic containers for the entomopathogenic fungi, as well as fungi act as plants constitutive defense (Quesada-Moraga *et al* 2014).

The occurrence of entomopathogenic fungi in the soil of agroecosystems is determined by a multifactor set of abiotic and biotic conditions (Braga *et al* 2001; Quesada-Moraga *et al* 2007; Goble *et al* 2010) and a range of farming practices may impair or benefit the local community of entomopathogenic fungi (Filho *et al* 2001; Meyling & Eilenberg 2007; Jaronski 2008). Thus, in order to understand the influence of non-crop plants on the community of entomopathogenic fungi, studies should be conducted on ecological issues involving these organisms and the agroecosystem. In this study, we sought to examine the role of non-crop plants to entompathogenic fungi in chili pepper crops (*Capsicum frutescens* L.). Small farmers cultivate chili pepper and it is economically important in several regions of South America (Ohara & Pinto 2012). In this agroecosystem, several pests may cause direct and indirect losses to the production (Venzon *et al* 2006, 2011) and there are no pesticides registered by Brazilian government for their control. In an ecological study of non-crop plants in chili pepper crop systems, Amaral *et al* (2013) concluded that these plants in fact favor the establishment of natural enemies of aphids into the agroecosystem. Meyling & Eilenberg (2006) demonstrated that insects inhabiting native vegetation were able to disperse inoculums of entomopathogenic fungi by their activity. Thus, the use of non-crop plants as a conservation biological control strategy could also enhance the population of entomopathogenic fungi in cropping areas and promote pest control.

Additionally, one of the most important pests of chili pepper in Brazil is *Symmetrischema dulce* (Lepidoptera: Gelechiidae), a chili-pepper-fruit-borer whose larvae develops into the fruits and pupates in the soil (Perez 2012). Entomopathogenic fungi can be promising to control this pest due to the likelihood of interaction of both organisms in the soil.

In this study, we used the insect baiting method to assess the entomopathogenic fungi from the soil of chili pepper crops aiming to understand the effects of non-crop plants on the abundance of entomopathogenic fungi in the chili pepper fields. Additionally, we evaluated the virulence of these fungi to the insect baited (*Tenebrio molitor* L. Coleoptera: Tenebrionidae) and the occurrence of entomopathogenic fungi in the chili pepper crop soil both in monoculture and in areas with non-crop plants. Because of non-crop plants likely perform the improvement of environmental conditions (e.g shade area, humidity) and allow diverse interactions with entomopathogenic fungi, we predicted that chili pepper plots managed with non-crop plants would register higher virulence, abundance and diversity of entomopathogenic fungi.

2. Material and Methods

We conducted the experiment at Oratórios, state of Minas Gerais, in the Experimental Research Station of Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG), Brazil (20º 24' 03" S, 42º 49' 13" W, elevation ~ 481 m. The landscape consists of soft wavy relief with some steeper slopes. The dominant soil class is the Acrisol red-yellow which is clay-rich and poor in nutrients. The region is characterized by semi intensive agricultural use, with small farm properties ranging from 1 to 10 ha. The experimental area has a history of chili pepper (*C. frutescens*) cultivation for the past four years before the execution of this study. The average

temperature and total rainfall during the study period from March to April was 24.7 ºC and 719 mm.

In July 2013, the experimental area was thouroughly plowed and two days after the chili pepper seedlings were transplanted into 10 x 10 m plots with plants spaced in 1 m (Pinto *et al* 2006). The experiment was arranged in three fields and each field consisted of a pair of plots. Each plot consisted of one replication of one of the two treatments: a) chili pepper crop with non-crop plants in the edges and between lines; b) chili pepper crop without non-crop plants (monoculture). The plots were separated from each other by 20 m of bare ground soil in order to reduce the inter-plot interference. Mechanic suppression of non-crop plants was made whenever necessary for the maintenance of bare ground between plots and into the monoculture treatment. The plots with non-crop plants had strips of 20 cm between crop rows and 3 m strips around the plots where the spontaneous plants were growing freely. The maintenance of non-crop plants occurred from the third month after planting of chili pepper seedlings in order to avoid competition between plants (Santos *et al* 2006). The plants were maintained under standard agronomic practices with organic and mineral fertilization (Pinto & Silva 2006). All plots were irrigated weekly by micro sprinkler system during all the growing season. A compound fertilizer NPK (20-5-20) was applied at the rate of 50 g per plant at monthly intervals. No pesticides were used for controlling any pest.

Soil samplings were made six months after planting the chili pepper seedlings. At this time, the plants were in the fruiting period. We collected 20 soil samples from each one of the six plots, thus a total of 120 soil samples were taken. The samples were collected from points in a grid of five lines with four sampling points. The lines and the sampling points were spaced 2 m from each other (Fig. 1). Samplings were conducted in pairs of plots of different treatments on each date (Area 1 – 11th March; Area 2 – 21th March; Area 3 - 4th April), to ensure that all the samples from a given pair of plots of

different treatments were collected on the same day. The soil samples were collected in 20 cm depth using a core soil sampler and collected soil was disposed in polyethylene bags (2 L). The core sampler was rinsed in water, 70% ethanol and distillated water after each sampling point. Bags were transported to the Laboratory of Entomology in the Experimental Research Station of EPAMIG in Viçosa, Minas Gerais, Brazil.

A subsample of soil was transferred from each polyethylene bag to a 200 ml plastic vial and moistened with 10 ml distilled water. Isolation of the entomophatogenic fungi was made by the *'Galleria'* bait method (Zimmerman 1986) modified to *T. molitor* (Vänninen 1996; Vänninen *et al* 2000; Sanchez-Pena *et al* 2011). The rearing came from a colony reared in the Insect-Microorganism Interactions Laboratory (Universidade Federal de Viçosa - UFV). In November 2013, we started a new colony in the Entomology Laboratory (EPAMIG) maintained on wheat bran and chayote *Sechium edule* (Jacq.) Swartz. We selected two-month-old larvae of similar size (1.3 cm) for the experiment because it is difficult to determine the instar under these rearing conditions (Morales-Ramos *et al* 2010). Four *T. molitor* larvae were added for each plastic vial containing soil, The vials were sealed with plastic lids perforated and were incubated in a climate room at 25±5 ºC, 70±10% U.R and 12:12 (L:D). Throughout the first week, the vials were daily inverted and left upside down in order to stimulate the larvae to be in contact with the soil sample. The mortality of *T. molitor* was assessed by inspected samples every two days, and the live larvae were kept in the vial for the later assessments. The dead larvae with or without visible fungal mycelium were sterilized by rinsing them in 70% alcohol, 5% sodium chloride and distilled water (Meyling & Eilenberg 2006). Subsequently, these larvae were incubated in moist chambers, made of a microtube with a moistened cotton wool. They were kept in a climate chamber (25±5 ºC; 70±10% U.R) to promote the fungal growth.

The incubated larvae were inspected daily for the presence of external fungi growing. When fungal growth was visible, the mycelium were transferred with a sterile microbiology loop to agar media PDA (potato, dextrose and agar) with streptomycin and incubated at 24 ºC. We prepared slides for morphological identification in light microscopy (400 x magnification) with the isolates growing in the PDA.

All data were analysed in the R software version 2.3.5.2 (R Development Core Team, 2014). For the survival regression the analyses were carried out with areas representing blocks. The cropping systems (chili pepper monoculture vs chili Pepper + non-crop plants) were used as explanatory variable for composing the models. The survival of the bait insects, as variable response, was analysed by a censored Weibull distribution and compared by ANOVA χ^2 (Crawley 2007). A Kaplan-Meier plot was used to illustrate the survivorship curves. The number of soil samples positive for at least one insect-associated fungus was used as a response variable and the crop systems (monoculture and non-crop plants) was analysed as the explanatory variable. As the experiment was conducted in blocks these data were analyzed by Generalized Linear Mixed Models (*lme4*) and compared by ANOVA X^2 (Crawley, 2007).

3. Results

In the procedures of simplification of models we observed de influence of the area on the results about the survivorship of *T. molitor*, so the data were analyzed separately for each area. In two fields, the survival of bait insects exposed to soil collected from chili pepper crops with non-crop plants was shorter than those from insects exposed to soil collected from chili pepper monoculture (monoculture *vs* non-crop plant, Field 1: 23.74±0.19 (mean±SE) *vs* 20.25±0.20 days; X^2=6.89, df= 1, P<0.01; Field 2: 23.62±0.10 vs 22.05±0.11 days; X^2= 7.67, P<0.01; Fig. 2a-b). In the Field 3, the bait insects exposed to soil collected from chili pepper monoculture had shorter survivorship

than those exposed to soil from chili pepper crops with non-crop plants (Monoculture *vs* Non-crop plant: 29.34±0.14 (mean±SE) *vs* 34.07±0.12 days; X^2= 6.21, df=1, P<0.05; Fig. 2c). The occurrence of entomopathogenic fungi did not significantly differ between soil samples from plots of chili pepper with non-crop plants and chili pepper in monoculture (31.6 ± 0.06% *vs* 41.6±0.06%, mean±SE) (ANOVA, generalized linear mixed-models: χ^2=0.36, df= 1, P>0.05, Fig. 2).

Three species of entomopathogenic fungi were found associated with the bait-insect. The most frequently isolated fungal species was *Metarhizium* spp., which was recovered from 23.3% of all soil sample baited. This was followed by *Fusarium* spp. which was found to occur in 22.5% of all soil samples. *Beauveria* spp. was isolated with a frequency of 1.6% (Table 1). A total of 5.8% (7 out of 120) of the soil samples yielded two entomopathogenic fungal species in the same sample.

4. Discussion

In our findings, survivorship of *T. molitor* larvae was shorter in soil from two fields (field 1 and field 2) from chili pepper with non-crop plants plots. However in the field 3, the survival of the bait larvae was shorter in soil from chili pepper monoculture samples than in soil from non-crop plant samples. The experimental areas have a long history of preseason plowing, intensively chili pepper cropping and vegetation suppression after growing season. Evidences demonstrate that conventional management practices may impair the community structure and activity of entomopathogenic fungi in the soil (Jaronski 2008; Pell *et al* 2010; Goble *et al* 2010). The tillage, in a large-scale sense, may be considered the most harmful agricultural practice for the persistence of fungal soil-borne infectious structures, especially to Hypocreales fungi (Bing & Lewis 1993; Sosa-Gomez & Moscardi 1994; Sosa-Gomez *et al* 2001; Hummel *et al* 2002).

Virulence of entomopathogenic fungi is a factor depending on the density of viable conidia on the environment and the host ability to defend against the pathogens (Hughes *et al* 2004, Krams *et al* 2013). Environmental conditions are responsible to cause denaturation of most entomopathogenic fungi propagules (Meyling & Eilenberg 2007). Some studies demonstrated the positive effect of vegetation diversification and landscape structure on the entomopathogenic fungi community (Spatafora *et al* 2007; Meyling & Eilenberg 2007; Vega 2008; Sasan & Bidochka 2012). Non-crop plants in chili pepper agroecosystems may have preserved viable conidia preexisting in some of the plots studied by providing favorable environmental conditions. However, restoring the optimal levels of soil-borne conidia in a landscape scale require an increased host population, the main natural media for entomopathogenic fungi sporulation (Hajek and St. Leger 1994)

Perennial crops managed in a long-term vegetation diversification may be more effective for enhancing the virulence of entomopathogenic fungi (Moreira 2010, Fisher 2011). These factors probably make the coffee plantation in agroforestry systems closest to the natural preserved environments (i.e forests and meadows) (Jose 2009) what is difficult to match in annual vegetable crops such as chili pepper plantations. Thus, due to the intricate set of factors driving the activity of entomopathogenic fungi in the soil it is possible that the influence of the non-crop plants on the activity of the entomopathogenic fungi occur in long term (Goble *et al* 2010; Vega *et al* 2009).

The occurrence of entomopathogenic fungi did not differ between both crop management systems of chili pepper. A high frequency of soil samples positive for entomopathogenic fungi may reflect a more uniform distribution of viable conidia on the soil layer. Among studies comparing farming systems, higher occurrence of entomopathogenic fungi are obtained in the soil of organic crops and perennial orchards than in conventionally managed annual crops (Klingen *et al* 2002, Quesada-Moraga *et*

al 2007). Though influenced by the cropping systems, seasonal and geographical factor may influence the occurrence and local distribution (Ali-Shtayeh *et al* 2002, Klingen *et al* 2002, Goble *et al* 2010, Jabbour & Barbercheck 2009). Further, some entomopathogenic fungi show higher persistence in cultivated fields (e.g *M. anisopliae*) whilst other species demonstrate higher occurrence in meadows and forestry environment (e.g *B. bassiana*) (Vänninen *et al* 1989; Rath *et al* 1992; Chandler *et al* 1998; Bidochka *et al* 1998; Keller *et al* 2003; Bruck 2004).

The present study has demonstrated *Metarhizium* spp., *Fusarium* spp. and *Beauveria* spp. occurring in chili pepper crops. The species composition was similar in both crop systems with no significant prevalence of any entomopathogenic fungi species. However *Metarhizium* spp. and *Fusarium* spp. showed higher frequencies, and *B. bassiana* registered very low frequencies in both cropping systems. *Metarhizium anisopliae* is commonly found in arable and sun exposed fields (Steenberg 1995, Keller *et al* 2003). Still, it was stated that the conidia of *B. bassiana* generally persisted very poorly in the soil compared to *M. anisopliae* (Vänninem *et al*, 2000). Both species *B. bassiana* and *M. anisopliae* have the potential to engage in fungi-plant interactions (Elliot *et al* 2000). Hu and St. Leger (2002) demonstrated that the *M. anisopliae* isolate released in cabbage fields persisted better in the soil immediately surrounding the cabbage roots compared with bulk soil.

Among the entomopathogenic fungi isolated from the soil of chili pepper crop systems we observed *Fusarium* spp. occurring in *T. molitor* larvae. Although best known as plant pathogens (Cook 2007; Gordon and Martyn 1997), *Fusarium* species have been reported infecting pest insects (Teetorbarsch & Roberts 1983). Though highly virulent for insects, *Beauveria* spp. and *Metarhizium* spp. are easily defeated on resources competition, allowing saprophityc fungi to invade host body (Keller & Zimmerman 1989; Hajek 1997; Meyling & Eilenberg 2007). Thus, possibly the

Fusarium spp. isolates found in our surveys are saprophytic strains taking advantage of the larvae killed by *M. anisopliae* and *B. bassiana*.

Fungi can be a key factor controlling *S. dulce* populations in chili pepper crops when increasing the virulence, abundance and diversity of entomopathogens through the non-crop plants. The higher capacity of *Metarhizium* spp. and *Fusarium* spp. to persist in the soil of agricultural fields can lead to higher mortality rates for larvae and pupae of *S. dulce* in the chili pepper crop soil (Meyling & Eilenberg 2006). The presence of *B. bassiana* strains in the agroecosystem may be an important mortality factor for the larval stages of *S. dulce* living in the tissue of chili pepper plants, due to suggested ability of *B. bassiana* to act as "bodyguards" for plants in endophytic interactions (Elliot *et al* 2000, Quesada-Moraga et al 2014).

From these results, we suggest that maintaining non crop plants in agroecosystems as a short-term strategy is not effective for incrementing biological control provided by entomopathogenic fungi. In order to obtain more satisfactory mortality rates by the action of entomopathogenic fungi, some agricultural practices should be applied in a rational way, such as reducing or eliminating plowing in the areas of cultivation. Increasing evidence suggest that habitat conditions drives the populations of entomopathogenic fungi (Bidochka *et al* 2001, 2002; Goble *et al* 2010; Moreira, 2010) showing that more efforts may be applied for conservative biological control strategies for maintaining such ecosystem services.

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Figure 1. Sampling points grid for each chili pepper plot. Each area consisted of a pair of plots of distinct treatments.

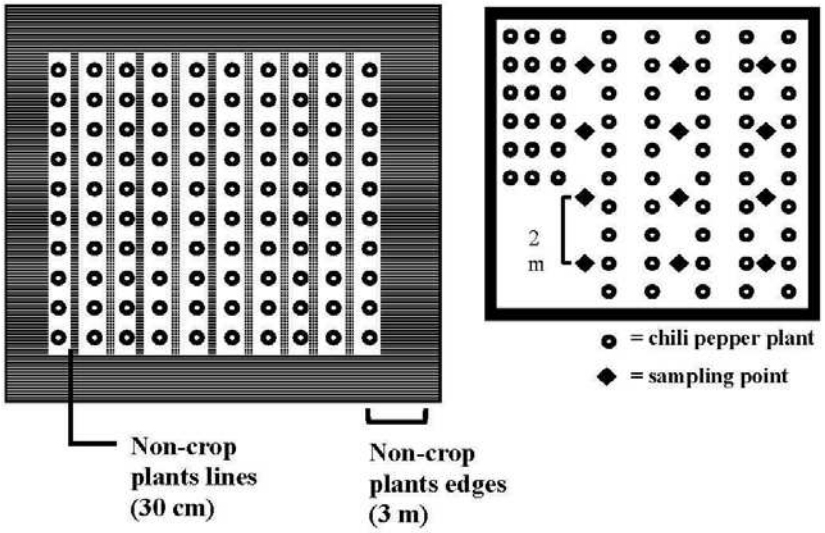

Figure 2. Survivorship of bait insect (*Tenebrio molitor*) in soil from chili pepper monoculture and chili pepper with non-crop plants. Soil samples were taken in pairs of plots of different management system (Monoculture or Non-crop plant). The pairs of plots represented (A) Area 1, (B) Area 2 and (C) Area 3.

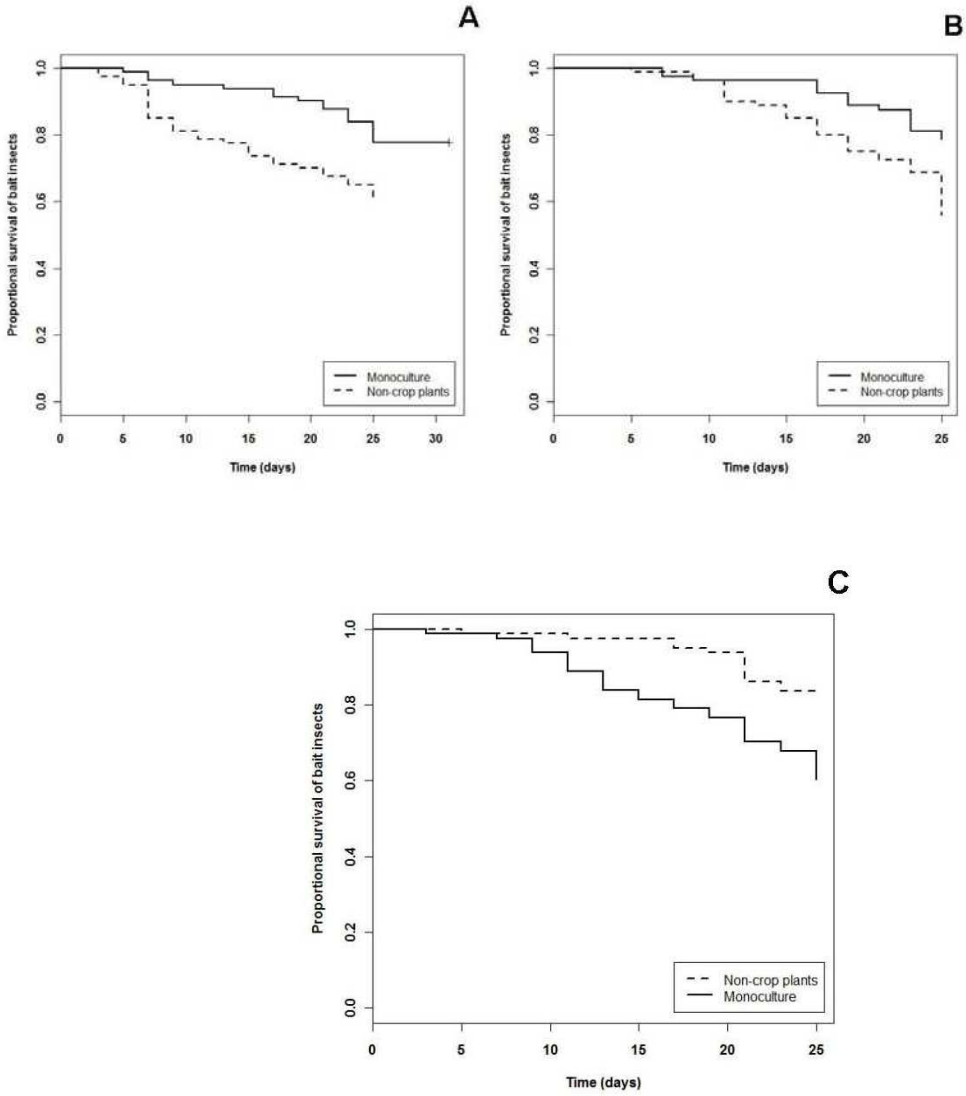

Figure 3. Mean (±SE) frequency of samples with at least one bait insect (*Tenebrio molitor* larvae) positive for entomopathogenic fungi from soil of both chili pepper management systems: Monoculture *vs* Non-crop plants.

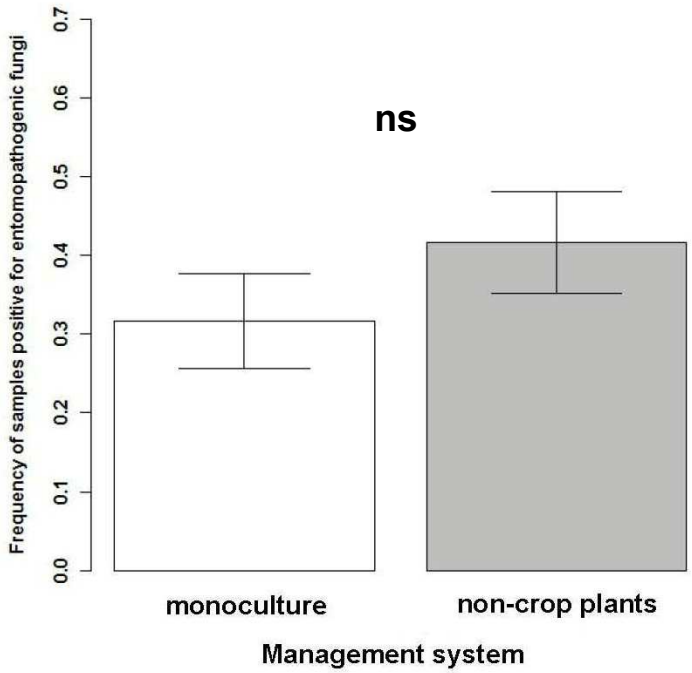

Table 2. Distribution and occurrence frequency of entomopathogenic fungi in 120 soil samples from chili pepper crops managed in monoculture and non-crop plant association systems in Oratórios, state of Minas Gerais, Brazil.

EPF isolates*	Monoculture			Non-crop plants					% F**
	Area 1 *(n=20)*	Area 2 *(n=20)*	Area 3 *(n=20)*	Area 1 (n=20)	Area 2 (n=20)	Area 3 (n=20)	Monoculture (mean)	Non-crop plant (mean)	
Metarhizium sp.	10.0	20.0	35.0	45.0	10.0	20.0	21.6	25.0	23.3
Fusarium sp.	20.0	10.0	10.0	45.0	15.0	25.0	16.6	23.3	22.5
Beauveria sp.	0.0	0.0	5.0	5.0	0.0	0.0	1.6	1.6	1.6

* Percentage frequency of entomopathogenic fungal (EPF) isolates per management system

** Percentage frequency (% F) based on the total number of isolates on all areas/120 soil samples

Chapter II

Occurrence and pathogenicity of soil entomopathogens in no-till and conventional tillage cropping systems

Abstract

Conventional agricultural techniques such as the soil tillage are commonly used in sustainable production systems but are reported to cause losses in soil biodiversity and thus impairing ecosystem services such as the biological control. The naturally occurring soil microbiota contains entomopathogenic organisms that contribute to regulate insect population outbreaks. Environment disturbs like the soil tillage regimes may hinder these ecosystem services, the understanding of the agricultural strategies would help to consort ecosystem conservation and farms productivity. Several pests attack the muskmelon crops and farmers have limited alternatives for the pest management in organic farms. Thus, our goal was to study the effects of the soil tillage regimes (till and no-till) on the entomopathogenic community in the soil of organically managed melon crops. We used *Tenebrio molitor* larvae as live-bait to assess the occurrence and virulence of entomopathogens on both tillage regimes in early and late phase of melon growing phase. We also measured the yield of muskmelon fruits in our experimental plots. The different soil tillage did not affect the occurrence and virulence of soil-borne entomopathogens. However the detection of entomopathogenic fungi increased in the late period of the growing season. The virulence of entomopathogens was higher in the early phase compared to the late phase of the growing season. The yield was higher in the conventional tillage melon fields. We propose that the conventional tillage may not impair the soil entomopathogens managed under organic farming systems and other agricultural practices would contribute to mitigate negative effects.

Keywords: entomopathogenic fungi, ecosystem services, conservation biological control

1. Introduction

The fundamental principle of agroecosystems conservation relies on the maintenance of soil fertility and local biodiversity (Altieri et 2012). Maintaining natural biological processes in agricultural landscapes performs ecosystem services including polinattion, waste assimilation and biological control (Constanza *et al* 1997, Fiedler *et al* 2007). Ecosystem services refers to functions by which ecosystem provides human welfare (Constanza *et al* 1997, Daily 1997). Managing the agroecosystem environment by the optimization of agricultural practices aiming to protect and enhance natural enemies populations is a conservation biological control strategy (Eilenberg et al 2001) addressed to the use of ecosystem services in the control of undesirable organisms. A big deal of studies has focused on the optimization of agricultural strategies to maintain the quality of ecosystem services in sustainable agroecosystems (Gurr et al 2004, Fiedler et al 2007, Zehnder et al 2007). However conventional agricultural practices based on the landscape simplification and land use changes are largely used by farmers disturbing natural enemies communities (Landis et al 2000, Tscharntke et al 2005).

The microbiota are integral part of the soil and include a diverse array of entomopathogenic microorganisms that have wide range of hosts and may contribute to the provision of natural biological control in agroecosystems (Klein 1990, Hajek 1997, Barrios 2007). Endemic entomopathogens are closely related to the biotic and abiotic conditions in the soil, thus any disturbance could impair the activity of these communities (Hummel et al 2002a). The resistance and resilience of soil microbial communities to environmental disturbance depends on several factors, such as community composition and species sensitivity to alterations (Griffiths & Philippot 2012). This complex interaction intricates the understanding of microbial responses to

specific stresses (Bisset et al 2013). Several studies have demonstrated the positive effects of the sustainable farming strategies on the conservation of soil-borne entomopathogens (Meyling et al 2005). However, some agricultural strategies are commonly used in both conventional and organic farming systems (Nielsen *et al* 2015) and a holistic analysis of these effects is essential to improve the organic farming management strategies.

The land-use changes in agroecosystems like the tillage regimes are reported to decrease soil biodiversity and hinder the biological control of pests provided by entomopathogenic microorganisms (Meyling & Eilenber 2006; Quist *et al* 2016). Conversely some evidences suggest that the negative effects of soil management regimes may vary according to other factors like the entomopathogen species (Steenberg 1995; Soza-Gómez *et al* 2001; Campos-Herrera *et al* 2014) and due to the time scale of agroecosystem management (Jabbour & Barbercheck 2009). Further, large-scale factors also have an impact in the soil biota such as climatic conditions, habitat type and cropping system (Chandler *et al* 1997; Bidochka *et al* 1997; Bruck 2004; Meyling & Eilenberg 2007; Quezada-Moraga *et al* 2007). Lastly, specific edaphic factors that are altered by the soil management system (e.g. soil pH, salinity and water potential) may be more decisive shaping the edaphic microflora than the physical disturbs caused by the tillage regimes (Kung *et al* 1990; Nielsen *et al* 2011; Duncan & McCoy 2011).

The goal of the present study was to understand the dynamics of soil entomopathogens in agroecosystems managed under different tillage regimes. Using the live-bait method with larvae of meal-worm (*Tenebrio molitor*, Coleoptera: Tenebrionidae), we assessed the natural occurrence of entomopathogens in the soil of organic muskmelon crops *Cucumis melo* L. var *cantalupensis* under no-tilled and conventionally tilled regimes. We also tested the virulence of endemic soil-borne

entomopathogens from differently managed soils. Because the biotic and abiotic factors vary throughout the growing season the occurrence and virulence of soil-borne entomopathogens was made in two distinct phases of the growing season (early and late phase). In order to assess the feasibility of the different soil management systems to melon crops, we measured the yield of marketable melon fruits in the end of the growing season. We hypothesized that higher occurrence and virulence of entomopathogens would be observed in the no-tilled soil sampled during the early phase due to lower exposition of entomopathogens to disturbing factors Bing & Lewis 1993; Hummel *et al* 2002). We also predicted that the conservation of soil natural processes in no-tilled plots would result in higher production of melon fruits.

Cucurbit crops such as squash, cucumber and melon are attacked by multiple pests, which represent a major concern for organic farmers. The stripped-cucumber beetle *Acalymma vitatta* (Fabricius) and the spotted-cucumber beetle *Diabrotica undecimpuctata* (Barber) (Coleoptera: Chrysomelidae) are economically significant pests causing severe yield losses in cucurbit crops in southeast region of the U.S (Ellers-Kirk & Fleischer 2006). The adult beetles directly feed on the leaves, steams and fruits while the larvae live in the soil feeding on the roots. Additionally the adults of both species are vectors of bacteria wilt caused by *Erwinia tracheiphila* which can cause a substantial mortality of plants (Yao *et al* 1996; Cline *et al* 2008). The organic farming techniques used in the management of the beetles include the use of row covers, use of different mulching techniques and spraying organic safe pesticides (Caldwell *et al* 1999, Cline *et al* 2008, Andino & Motsenbocker 2004). However the scarce alternatives of pest management in organic farms highlights the importance of the optimization of techniques which promotes the conservation of ecosystem services such as the biological control by soil-borne entomopathogens.

2. Material and methods

2.1 Field experiment

Field research was undertaken in melon crops (*Cucumis melo* L.) in the University of Kentucky Horticulture Research Farm (South Farm) near Lexington, KY, USA (37°58'39" N, 84°32'03' W). In April 2015 we planted the 1440 Athena muskmelon plants (*C. melo* L. var. Cantaloupe) in plastic planting trays using organic substrate (Pro-Mix® Mycorrhizae). The trays were kept into greenhouses under controlled conditions (25 ± 5 ºC, 16L:8D) and they were daily watered by micro sprinkler irrigation system. Four weeks after the germination, the plants were transplanted to the plots managed under different farming practices according to the treatment (till and no-till). Previously, all plots (till and no-till) were prepared by planting rye (*Secale cereale* L.) and hairy vetch (*Vicia villosa* Roth) as overwinter cover crops from October (2014) to April (2015) .In May 2015, tilled plots were prepared by mowing the cover crop and plowing the soil one week before planting the melon plants. The plowed soil was pilled into three flat beds (30 cm high, 1.5 m wide, 73.15 m length and 1.5 m spacing from each other) and covered with plastic mulch (Fig 1). In the no-tilled plots, the cover crops were mowed and no plowing was used in the soil. The experimental area was composed by four plots (73.15 m length x 7.62 m wide) divided into 12 subplots (6.10 length x 7.62 wide) (Fig 1). The plots were arranged side by side, interspersing the different treatments. For both treatments (tilled and no-tilled) the melon plants were planted one week after mowing the cover crops to ensure the elimination of allelopathic compounds from the plants (Burgos & Talbert 2000). Organic fertilizer (Nature Safe®) was applied in the tilled and no-tilled plots prior to transplanting the melon plants. We used a water wheel transplanter coupled to a tractor to transplant the melon plants to the soil flat beds. The in-row spacing between plants was 60 cm. In the no-tilled plots, melon plants were transplanted into a hole drilled in the soil (10 cm wide, 20 cm deep).

The edges and the row spacing of the tilled plots were covered with a landscape fabric to control the non-crop plants. For the no-tilled plots, non-crop plants were controlled by mulching the soil with spoiled round-baled hay spread all over the plots (Fig 1).

In order to avoid the attack of several common pests (e.g. spotted and stripped cucumber beetles) we installed row covers made of wire loop hoops (1.50 m wide x 60 cm tall) with a plant netting mesh on top (mesh size 0.35 mm). The fields were irrigated by a dripping irrigation system by which additional organic fertilizer was applied when needed. Because the muskmelon requires pollination by insects, we removed the row cover to allow pollinator to access the flowers during the anthesis period (July 2015) when the flowers are open and receptive. During this period we sprayed pesticides certified for organic production (PyGanic®, Neem seed oil, Surround®) to avoid pests causing several damages to the plants. After the anthesis period, the plants were covered again with the plant netting until the harvest period (September 2015). At the end of the muskmelon-growing season (October 2015) we assessed the yield of the cultivated plots. To avoid any edge effects, we harvested the melon fruits from 4.5 meters of the middle row in each subplot (Fig 1). Melon fruits were sorted by size, ripeness and physical aspects. The fruits that fit the precepts were classified as 'marketable' fruits and those that did not fitted were discarded. We recorded the count and weigh of marketable fruits.

2.2 Soil sampling and laboratory experiment

We aimed to assess the effect of farming practices on the entomopathogenic fungi along the time. Thus, the soil of the tilled and non-tilled melon plots was sampled in two different periods of planting season: 1st sampling – cropping period (June 2015); 2nd sampling – harvesting period (September 2015). We sampled the soil in each subplot using a drill-bit to sample the soil layer of 20 cm deep. The soil from four different points in each subplot was collected with a sterilized spade and placed into transparent

plastic bags and identified according to the subplot number (Fig 1). After sampling each subplot the drill-bit was washed in water, rinsed in sodium hypochlorite (8%) and soaked with 70% ethanol. We took the soil samples to the Invertebrate Ecology Laboratory of the University of Kentucky where the soil was homogenized and placed in transparent plastic containers (350 ml) sealed with a perforated lid. When necessary we added distilled water to the soil to equalize the humidity between the samples.

We added four *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae in each soil sample as live-bait for the entomopahtogenic fungi in the soil (Zimmermann 1986). The *T. molitor* used in the these experiment were reared in laboratory (25 ± 5 ºC, 16:8 L:D) in plastic trays (30 cm x 50 cm x 10 cm) containing rolled oats as food and potato slices as water resource. Because it is difficult to determine the instars of the immatures of *T. molitor,* we used larvae with same developmental time (1 month). The plastic containers were daily inverted upside down to force the larvae to climb and ensure their contact with the soil.

Containers were inspected every two days to record the mortality of the larvae. We used sterilized forceps to dig up the soil and remove the dead larvae while those larvae found still alive were kept into the container with soil for further evaluations. The dead larvae were superficially sterilized by rinsing them in 70% ethanol (1 minute), sodium hypochloride (5%) (1 minute), distilled water (1 minute) and dried in a clean filter paper. We used autoclaved microcentrifuge tubes (1.5 mL) with a humid cotton wool ball in the bottom as a moist chamber where the dead *T. molitor* larvae were kept, under controlled conditions (25 ± 5 ºC, 16:8 L:D) to promote the fungi growth. The larvae in the tubes were inspected periodically to identify the fungi development. The incubated larvae were kept into the tubes until the fungi growing on their bodies started sporulating. The identification of the fungi was made by morphological analysis of the fungi mycelia and the reproductive structures. We used a sterile microbiology loop to

transfer the spores to microscopy slides and the reproductive structures were analyzed in a light microscope (400x magnification).

2.3 Statistical analysis

The mortality data of *T. molitor* larvae were submitted to survivorship analysis. The independent variable consisted of the soil management practice (tilled and no-tilled), and the time to the death of *T. molitor* represented the response variable. The survival regression analysis was made by a censored Weibull distribution and compared the models by Analysis of Variance adjusted to a Chi-square distribution (ANOVA) (Crawley 2007). A Kaplan-Meier plot was performed to illustrate the survival regression curves. In a further analysis, we compared the mortality data of *T. molitor* larvae in the soil of different treatments in both periods of growing season (early and late phase) by censoring the mortality caused by entomopathogenic fungi apart of the other entomopathogens. The survivorship was estimated performing a censored Weibull distribution and the means of treatments and sampling period were compared using a ANOVA adjusted to a Chi-square distribution (Crawley 2007). As a measure of entomopathogenic fungi distribution along the areas, we compared the number of soil samples which at least one dead larva presented entomopathogenic fungi growing on their body. The means of the number of soil samples positive for entomopathogenic fungi were compared by One-way ANOVA. The data of weight of the marketable melon fruits from plots of different soil management were analyzed by Analysis of Variance (ANOVA) using Generalized Linear Models (GLM). The counts of marketable fruits from the different treatments were compared by ANOVA using GLM to adjusted to Poisson error distribution. All the analysis were computed by the R software version 3.2.2 (R Development Team, 2015).

3. Results

The survivorship analysis of the bait insect (*T. molitor*) demonstrated that the farming practices did not influence the virulence of the soil-borne entomopahtogenic fungi ($\chi^{2=}1.24$; P>0.05) (Fig 2). In the first round of soil sampling (July 2015 – planting season), the survivorship of the bait insect in both treatments (till and no-till) was lower than in the second round (August 2015 – harvesting season) indicating that the virulence of the entomopathogens was higher at the early phase (Likelihood ratio test= 161, df=1, P<0.001) (Fig 2). In samples from both treatments (till and no-till) taken during the planting season (earlier), the survivorship of the bait insects reduced to 50% whereas in the soil samples taken during the harvesting season (later) the survivorship was approximately 100% (Fig 2). The bait insects exposed to the soil samples of the early phase registered 100% mortality at the 18th day of experiment whereas the larvae in the soil samples from the harvesting season still had 20% survivorship at the end of the experiment (25th day) (Fig 2). Considering the mortality caused by entomopathogenic fungi separately the soil management system did not affect the mortality of live-baits (χ^2=0.88, P<0.05, Fig 3a), thus we compared the period of sampling by pooling the data together and we obtained that the period of sampling affected the mortality caused by entomopathogenic fungi demonstrating a higher mortality of *T. molitor* in late phase (χ^2=3.93, df=1, P=0.031, Fig 3b)

The occurrence and abundance of fungi associated with the bait insect (*T. molitor*) from each soil sample varied according to the treatment and the phase of sampling as presented in Table 1. A total of six fungi isolates were detected in the larvae of *T. molitor* exposed to the soil samples from both treatments. In the tilled fields two fungi isolates were encountered during the early phase (*Beauveria* sp. and *Penicillium* sp.) and three isolates in the late phase (*Beauveria* spp., *Metarhizium* spp. and *Penicillium* spp.). In the no-till treatment we found four fungi isolates in the early phase and in the

late phase. Although the number of fungi isolates was similar in the no-till treatment for both phases, the composition was different. We encountered *Aspergillus* spp. occurring in two samples of the no-till treatment at the early phase but it did not occur in the late phase. In the no-till treatment there was the occurrence of *Isaria* spp. in one soil sample of the late phase, whereas this fungi isolate did not occur in the early phase. The abundance of *Beauveria* sp. significantly increased in the late phase for both treatments (χ^2= 28.14, d.f= 1, P>0.001) (Table 1). The occurrence of entomopathogenic fungi was significantly higher in the late phase compared to the early phase (18.70% *vs* 63.99%) (χ^2= 18.18, d.f= 1, P<0.001) (Table 1). In contrast, the number of larvae killed by non-fungal pathogens was higher in the early phase than in the late phase (χ^2= 11.03, d.f= 1, P<0.001) (Table 1). Consistently with the survival analysis, the total number of dead larvae was higher in the early phase (190 larvae – 98.95%) compared to the late phase (166 larvae – 86.45%) (Table 1).

The tilled plots produced a higher number of marketable fruits (2.16 ± 0.23, mean±SE) compared to the no-tilled plots (1.25 ± 0.18) (χ^2= 5.97, d.f= 46, P= 0.0164, Fig 3a). The melon plants from the tilled plots produced fruits with higher mass (5.65±0.74, mean±SE) compared to the fruits produced in the no-tilled plots (2.81±0.73) ($F_{1,46}$= 7.33, P= 0.009, Fig 3b).

4. Discussion

We predicted that the no-tilled soil management would perform the soil preservation necessary to an increased mortality of live-bait insects caused by entomopathogens, although the farming practices did not altered the virulence of soil borne entomopathogenic microorganisms in melon crops. The mortality of live bait insects was significantly higher in the soil samples from the early phase compared to the soil samples of the late phase. Further, comparing the action of entomopathogenic fungi separately we observed that the soil tillage regimes did not affect the mortality of *T.*

molitor. The higher mortality of *T. molitor* due to entomopathogenic fungi was observed in the soil sampled in late phase. The virulence of entomopathogens is a response of the density of viable spores and abundance of individuals in infective stage (Peters & Ehlers 1997; Hughes *et al* 2004). The persistence of propagules of entomopathogens is also close related to the biotic and abiotic conditions in the soil. The persistence of some strains of *Bacillus thuringiensis* and *B. cereus* is highly influenced by moisture and the chemical properties of the soil (Polanczyk *et al* 2009). Also more than occurring in the soil surface entomopathogenic bacteria are encountered in the phylloplane microflora (Pedersen *et al* 1995) and in the rhizosphere of some plants (Jensen *et al* 2003). Thus, the increased mortality of the bait insect (*T. molitor*) in the soil from the early phase of muskmelon plots may have reflected the optimal condition of the soil provided by the overwinter cover crops (Susurluk & Ehlers 2008) suppressed just before the planting season.

The long-term organic management of the experimental farm and the farming practices applied to our muskmelon plots (e.g cover crop, mulching, plant netting, organic fertilizer) likely contributed to reduce the negative effects of soil tillage on soil-borne entomopathogens. Although some studies demonstrate that intensively managed soils are less favorable for communities of entomopathogens (Meyling & Eilenberg 2007), the reports about the interference of the soil tillage on the entomopathogens community are highly heterogeneous (Hummel *et al* 2002, Jabbour & Barbercheck 2009, Bing & Lewis 1993, Millar & Barbercheck 2001, Sosa-Gomez & Moscardi 1994). Ecological communities of entomopathogenic fungi may respond to levels of disturbance in the habitat (Steenberg 1995, Bidochka *et al* 1998; Meyling & Eilenberg 2006). Although the soil tillage practices are studied more often as a single effect of soil disturbance, it result in specific changes in the environment that may affect the soil entomopathogens. The solar radiation exposure of entomopathogens may result in a

decreased viability and virulence of propagules and infective structures (Fargues *et al* 1997; Fernandes *et al* 2015; Shapiro-Ilan *et al* 2015).

The higher mortality rates due to general entomopathogens were observed in the early phase whereas the higher mortality of *T. molitor* due to entomopathogenic fungi and frequency of such entomopathogenic fungi was observed in the late phase. The period of anthesis, when the plants netting cover were removed to permit the access of pollinators (July – August), may have represented a window for the colonization of melon plants by diverse insects. Thus the augmented host abundance and the higher rainfall occurrence during the anthesis period (July: 245.35 mm; August: 55.89 mm) compared to the early phase (May: 52.57 mm; June: 145.79 mm) might have provided optimal conditions for the higher entomopathogenic fungi abundance and occurrence in the late phase (Table 1). The cover crops are shown to promote the conservation of propagules of entomopathogenic fungi in the soil (Chandler *et al* 1997; Meyling & Eilenberg 2007). The different mulching techniques and the netting cover applied to the melon plots may have provided a sort of favorable conditions by blocking the solar radiation and maintaining the soil moisture, keeping some viable fungal conidia on the soil surface after the cover crop suppression. Although some entomopathogenic fungi are able to involve in endophytic interactions with plants (Vega *et al* 2008, 2009; Hartley & Gange 2009, Lopez *et al* 2014) and exchange nutrients via rhizosphere (Behie *et al* 2012; Behie & Bidochka 2013) the nutrients from host body is a crucial resource for the fungi reproduction (Gottwald & Tedders 1982; Hajek 1997; Boucias & Pendland 1998). As key factors in entomopathogenic fungi biology the dispersion of propagules and host abundance (Anderson & May 1981; Meyling and Eilenberg 2007) require dispersion forces like wind, rain and hosts movement for spreading propagules in a large scale (Shah & Pell 2003; Ulevicius *et al* 2004). Additionally, physical barriers may impair the fungal dispersion into the crop (Bruck & Lewis 2002). Moreover

augmentative applications of *Metarhizium anisopliae* demonstrated increased conidia production after an intense rainfall period (Boetel et al 2012).

Our goal was to determine how the soil management regimes affected the community of entomopathogens in an organic farm. Although the tillage regimes did not affected the entomopathogen microorganisms, the period of the growing season in which the soil samples were taken (early and late phase) affected the virulence and occurrence of these natural enemies in the soil. The higher virulence of soil-borne entomopathogens was observed in the early phase of the muskmelon and in the late phase the entomopathogenic fungi demonstrated and increased frequency with *Beauveria bassiana* as the predominant species. Our results revealed a short-term process of ecological succession for entomopathogens in the soil which is not affected by the tillage regime in an organic farm. Future studies addressed to understand the specific alterations of biotic and abiotic factors in the soil of agroecosystems along the growing season are necessary for clarifying the role of farming strategies shaping the entomopathogens community.

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Figure 1. Schematic overview of the muskmelon plots managed under different tillage systems in an organic farm. The red squares represent subplots and the red rectangles inside indicate the plants harvested for yield study. The black dots show the soil sampling sites in each subplot.

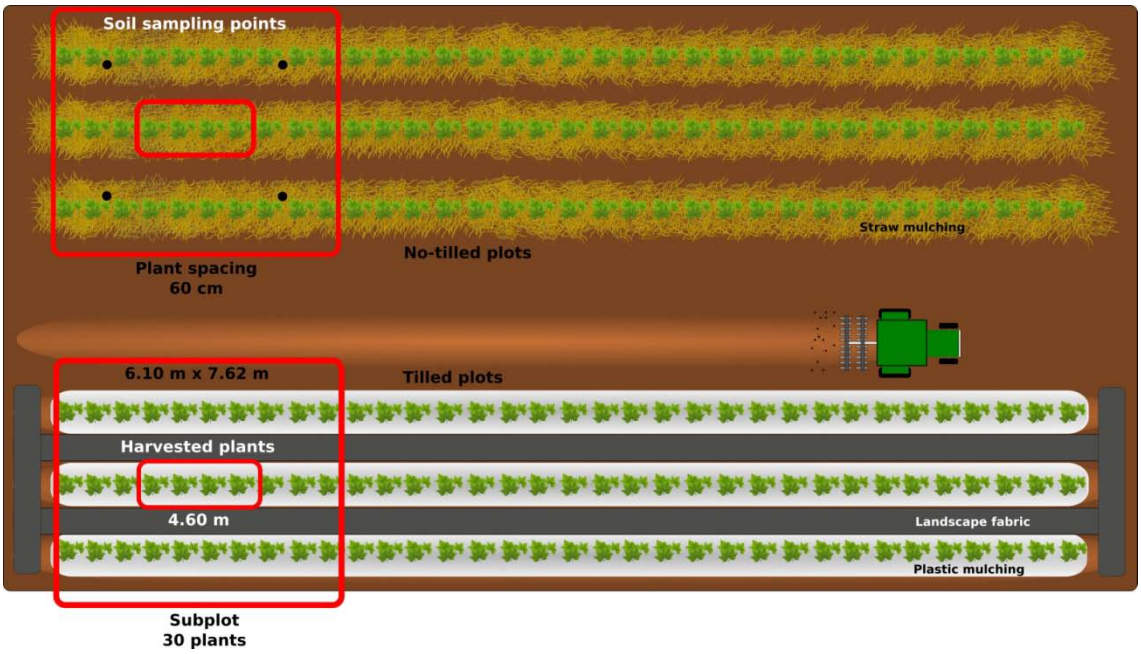
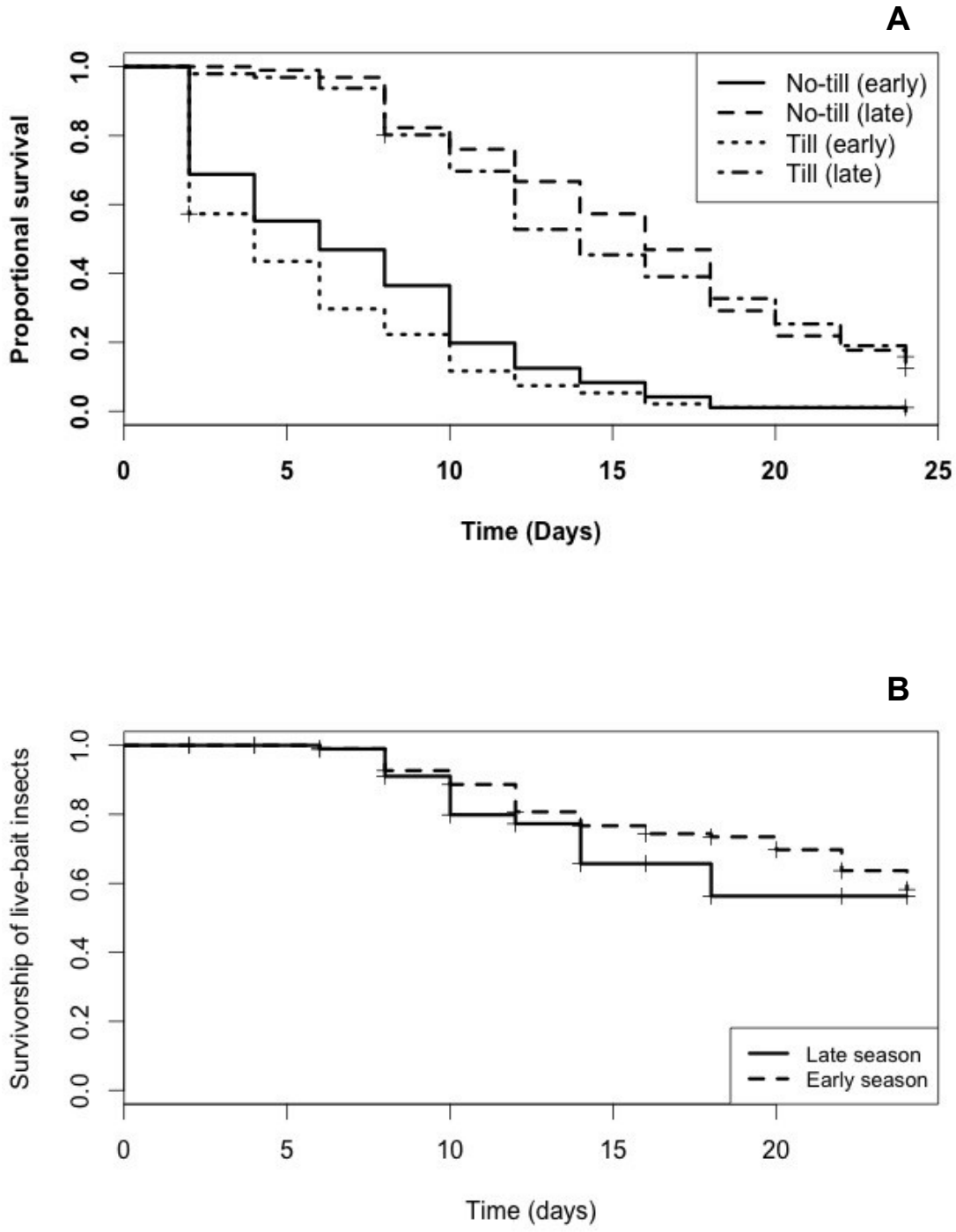


Figure 2. Kaplan-Meier survivorship of *T. molitor* as live-bait for the entomopathogens occurring in the soil of muskmelon plots managed under no-till and conventionally tilled systems sampled in distinct periods of the growing season: early (June 2015) and late (September 2015): A- mortality of *T. molitor* mortality due to general entomopathogens; B- mortality of *T. molitor* caused by entomopathogenic fungi ($P<0.05$).



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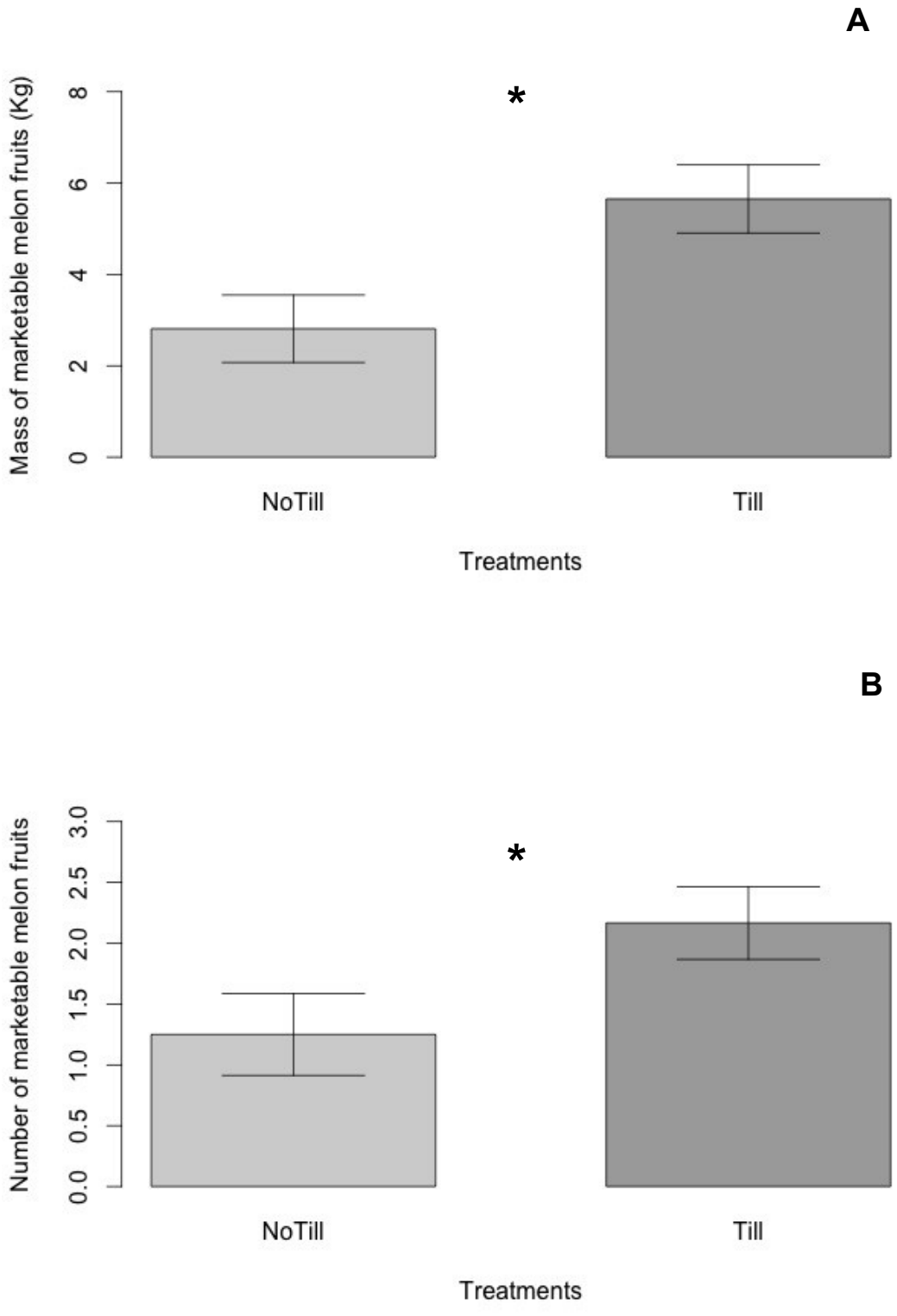

Table 1. Frequencies of occurrence (% positive samples) of entomopathogens in soil samples from no-tilled and conventionally tilled plots assessed in the early season (June 2015) and late season (September 2015)

Entomopathogens isolates	Early season			Late season			χ^2	*P*
	Till ($n = 96$)	No-till ($n = 94$)	Sum	Till ($n = 80$)	No-till ($n = 86$)	Sum		
Beauveria sp.	3.1	3.2	6	30.0	18.60	40	28.14	>0.001
Metarhizium sp.	0.0	0.0	0	1.3	0	1	-	-
Isaria sp.	0.0	2.1	2	0	1.2	1	0.33	0.56
Penicillium sp.	8.3	1.1	9	5.0	4.7	8	0.58	0.80
Aspergillus sp.	0.0	2.1	2	0	0	0	-	-
Trichoderma sp.	0.0	0.0	0	0	1.2	1	-	-
Other entomopathogens	85	86	171	51	64	115	11.03	0.0008
Positive samples for fungi occurence (%)	8.24	10.46	18.70	39.28	24.70	63.99	15.18	>0.0001

Within rows, standard χ^2 tests were made between the total frequencies of both sampling periods (early season and late season). When zero count were present no analysis were made.

Chapter III

Trophic interactions among an epigeal predator, a pest insect and an entomopathogenic fungi

Abstract

The use of different natural enemies combined is desirable for optimizing the pest control efforts. Still, the effective use of multiple natural enemies requires the meticulously understanding of the interactions among natural enemies and the pest intended. We studied the interactions among a wolf spider species, *Tigrosa helluo*, a broad host range entomopathogenic fungi, *Beauveria bassiana* and the spotted cucumber beetle, *Diabrotica undecimpunctata*. By feeding spotted cucumber beetles on melon plants treated with spores of *B. bassiana* we tested the preference of wolf spider for chemical cues of beetles exposed and non-exposed to the pathogen in binary choice test arena. Further, in a microcosm assay we assessed the predation of wolf spiders on exposed and non-exposed fungi beetles. In the microcosms we compared the activity and frequency of beetles on melon plants and we measured the herbivory of exposed and non-exposed cucumber beetles on melon plants with the predator *T. helluo* present or absent. The choice test arena revealed that wolf spiders show no preference for chemical cues of beetles exposed or non-exposed to the pathogen. In the microcosms, the exposition of the beetles to the pathogen did alter the wolf spiders predation. The pathogen as single natural enemy had no effect on herbivory of *D. undecimpunctata*. When the spider was present as single natural enemy there was a reduction of herbivory of beetles on melon plants. In contrast when the beetles were exposed to the pathogen and the predator was present herbivory was similar to the beetles in the complete absence of predator and pathogen. The

implications of these data are discussed in terms of trophic interactions among multiple natural enemies and insect-plant interaction.

Key words: wolf spider, *Beauveria bassiana*, spotted cucumber beetle, *Diabrotica undecimpunctata*, *Tigrosa helluo*

1. Introduction

The joint action of predators and pathogens is a key mortality factor in structuring ecological communities (Hawkins *et al* 1997, Dwyer *et al* 2004). Different strategies of resource exploitation permit predators and pathogens to coexist (MacArthur 1970, Chase and Leibold 2003), but sharing prey/host in the same habitat will consequently result in interferences among predators and pathogens, especially when these natural enemies present a broad host/prey range (Ramirez & Snyder 2009). Ecological traits such as reproduction strategies, size and distribution may also foster predators and pathogens to share their resources. The classical foraging models addressed to predators predict that the population growth relies on prey density (Solomon 1949, Holling 1959). Likewise, the success of pathogens infection depends on host population density to ensure passive propagule transmission (McCallum *et al* 2001). Therefore, syntopic species of predators and pathogens may compete constantly for the prey/host as a critical resource.

Generalist predators are abundant in agroecosystems and their plasticity on resource exploitation enable their persistence in food scarce habitats (Symondson 2002). Wolf spiders (Araneae: Lycosidae) are generalist predators that attain high population densities in crop systems and play an importance role as epigeal insect predators (Sunderland 1999). The species *Tigrosa helluo* Walckenaer (Araneae: Lycosidae) is a ground-dweling spider occurring in agricultural areas, forests and

moist fields (Dondale & Redner 1990, Marshall & Rypstra 1999) feeding on a wide range of arthropod preys including other spiders (Marshall & Rypstra 1999) and eventually feeding on dead arthropods in the soil, demonstrating a scavenger behavior (Knost & Rovner 1975). In the soil, *T. helluo* are in constant contact with indigenous entomopathogenic fungi like *Beauveria bassiana* (Hypocreales: Cordycipitaceae) (Meyling & Eilenberg 2007). This fungi has global distribution occurring in agricultural fields and in forested areas (Bidocka *et al* 1998, Meyling & Eilenberg 2007) infecting a wide range of arthropod hosts and possibly acting as saprophytic fungi (Hajek & Eilenberg 1997, Meyling & Eilenberg 2006, Ownley *et al* 2009). Because of its high virulence to various insects and possibility of mass production, *B. bassiana* has been used as biological control agent worldwide (Shah and Pell 2003). Tests on augmentative applications of *B. bassiana* demonstrated that it may also cause mortality to populations of non-target organisms such as predators (James *et al* 2005) and parasitoids (Lord 2001). However, studies on the effects of *B. bassiana* on wolf spiders are scarce and it is likely that interactions between both natural enemies are important factors shaping populations of pests in agroecosystems.

Spotted cucumber beetles, *Diabrotica undecimpunctata* (Barber) (Coleoptera: Chrisomelidae), are polyphagous herbivores, feeding on a variety of agricultural and old-field plants (Krysan 1986). The larvae feed on roots and develop in the soil, whereas the adults feed on plant foliage and flowers (Godfrey *et al*. 1998). In cucurbit crops (e.g. melon, cucumber, squash) *D. undecimpunctata* are vectors of a bacteria wilt caused by *Erwinia tracheiphila* which can cause a substantial mortality of plants (Yao *et al* 1996; Cline *et al* 2008). The direct contact to the soil exposes *D. undecimpunctata* to a variety of predators and pathogens during their life cycle. Studying the interactions between *T. helluo* and *D. undecimpunctata* Snyder and Wise

(2000) observed that *D. undecimpunctata* was less frequent in squash plants than reduced the herbivory as response to the predation risk of *T. helluo*. Although *T. helluo* has been shown as an important predator of *D. undecimpunctata,* its natural occurrence does not promote sufficient control to the pest. In natural conditions, due to their constant contact with the soil surface, pathogens like *B. bassiana* participate of trophic interactions between *T. helluo* and *D. undecimpunctata* which would cause interferences in the predator/prey interactions. Inundative applications of *B. bassiana* aiming to increment the biological control of *D. undecimpuncatata* would be a promising strategy to reduce yield losses caused by this herbivore, but there is a lack of studies concerning the trophic interactions among predator, pathogen and prey. Also, because of the multiple trophic level approach, the system of interactions presented would shed some light over the understanding of food webs involving predator, pathogen, herbivore and host plant.

Aiming to understand such interactions we conducted a series of laboratory and greenhouse experiments addressed to the following questions: (1) Can *T. helluo* use chemical cues to distinguish non-exposed and exposed prey to *B. bassiana*; (2) Does the exposition of *D. undecimpunctata* to *B. bassiana* spores cause interferences in the trophic interaction between the predator *T. helluo* and the pest *D. undecimpunctata*; (3) Does the joint effect of predator and pathogen over the pest result in reduce plant damage? Classical predation models include prey vulnerability as a channel by which predators select their prey (Emlen 1966; MacArthur & Pianka 1966; Curio 1976; Temple 1987). Hypocrealean fungi are reported to cause the feeding reduction of its hosts early in the infection (Tefera *et al* 2003). Thus, we predicted that *T. helluo* would demonstrate preference for chemical cues of exposed fungi prey due to their weaken condition. We also predicted that the exposition of beetles to *B. bassiana*

would result in an increased predation by *T. helluo,* and the pathogen exposition and the presence of the wolf spider would complementary decrease the herbivory of *D. undecimpunctata* in the melon plants.

2. Material and methods

2.1 Research material collection and arthropod rearing

The spotted cucumber beetles (*D. undecimpunctata*) were collected from the University of Kentucky Horticulture Research Farm (N 37°58′39″ W 84°32'03″, elevation ca. 317 m) and from three other commercial farms near Lexington, Kentucky, USA (September – October 2015). These farms are composed by different crops in which the cucurbitaceae (e.g squash, melon, pumpkin) are predominant. We collected the spotted cucumber beetle using a sweeping net in all the extension of the crops aiming to sample the largest possible area. The adult beetles were sorted and placed in plastic containers (500 ml). Insects were taken to the Invertebrate Ecology Laboratory of the University of Kentucky and kept under controlled conditions (25 ± 2 ºC and L16:D8). The beetles were fed in melon leaves (*Cucumis melo* L. var *cantalupensis*) planted from non-treated seeds. The seeds were planted in pots (500 ml) in organic substrate (Pro-Mix® Mycorrhizae) kept into mesh screen cloth cages (mesh size 5µm) in greenhouse under controlled conditions (25 ± 5 ºC, 12L:12D). No pesticides or fertilizers were used in any plant during the experiment.

The wolf spider species used in this assay *T.* [formely *Hogna*] (Brady 2012) *helluo* Walckenaer 1837 (Aranae: Lycosidae) were collected from the Experimental Farm North of the University of Kentucky (September – October 2015). A total of 150 adult female spiders were collected directly from the foliage in a forest area and placed in plastic containers (50 ml) individually. Each container was kept humid by a

damp filter paper disc to avoid spider dryness. We fed the spiders weekly with a *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae from a laboratory colony. Fortnightly, spiders were fed mixed insects collected from the field including crickets, beetles and bugs. Adults and larvae of *T. molitor* were reared in plastic boxes (45 x 30 x 15 cm) under controlled conditions (25 ± 5 ºC and 16L:8D). The boxes were maintained filled 2/3 with rolled oats and four potatoes (*Solanum tuberosum* L.) slices (2 cm thick) on the top as water resource. The spiders were maintained starving for one week before the assay to increase their foraging activity (Walker et al 1999).

2.2 Cucumber beetles exposure to B. bassiana spores

We planted 30 melon plants from non-treated muskmelon seeds (*C. melo* L. var. *cantalupensis*) in plastic plant pots (500 ml) containing autoclaved plant substrate (Pro-Mix® Mycorrhizae). Three weeks after germination, the plants were treated with a dip suspension of deionized water and *B. bassiana* spores (BotaniGard® 22WP – 2 x 10^{13} viable spores per pound). The plants were immersed in the water-fungi spore solution for 5 seconds and let dry for 1 hour. After treating the plants the soil surface in the plant pot was covered with aluminum foil to prevent the insects to contact any non-exposed water sources. The plants were covered with a transparent plastic cylinder (20 cm diameter, 50 cm height) covered with mesh screen cloth (mesh size 5µm) and 15 adult beetles were released within the microcosm. The beetles were kept within the microcosm for 5 days to ensure the entire consumption of plant tissues. After the period of exposure, the beetles were released into plastic containers (21.5 cm diameter, 6 cm height) with a filter paper disc (21.5 cm) in the bottom, sealed with a plastic cap and kept for 24 hours in laboratory conditions (25 ± 5 ºC and L16:D8) (Walker *et al* 1999). The beetles were removed from the plastic container and the filter paper disc was used in the wolf spider choice test.

2.3 Spider choice test

Tests were undertaken aiming to study the role of chemical cues of exposed and non-exposed beetles for the foraging behavior of *T. helluo* choice tests were undertaken. The tests were performed in the laboratory in an arena consisted of a circular plastic container (21.5 cm diameter, 6 cm height) in which the bottom was covered with two semi-circle halves (21.5 diameter) of filter paper. Previously, the filter paper halves were exposed for 24 hours to cucumber beetles in different treatments (non-exposed beetles, exposed beetles). After the exposure of filter paper halves, the arenas were assembled using two filter paper halves covering the bottom of the arena separated by a clean filter paper strip (1.5 cm width x 21.5 cm long) and a small central clean filter paper circle (5 cm diameter). The spiders used in this experiment had food withheld a week before. The plastic cups (50 ml) containing the spiders were inverted over the central circle and allowed for acclimation for 2 minutes. Then the cups were removed and the spider choice recorded each 20 minutes lasting one hour of total observation. Because of the nocturnal behavior of *T. helluo,* the observations were made under red light (Walker *et al* 1999). We tested the spiders choice between two filter paper halves containing different chemical cues into the arena (control [clean paper] *vs* non-fungi exposed beetles; control *vs* exposed beetles; non-fungi exposed beetles *vs* exposed beetles). The filter paper was changed and the arenas washed with soap and rinsed with distilled water between trials to eliminate any residual cues influencing activity of spiders. Each treatment was replicated 10 times. We considered as a choice made when the spiders moved over the central paper strip and placed their whole body in one of the filter paper halves. Because *T. helluo* is considered a sit-and-wait predator we also count the number of times the spiders

changed between the sides of the arenas to detect any alteration in the behavior due to the choices offered.

2.4 Trophic interaction assay

We performed an experiment to study the trophic interactions between predator (*T. helluo*), herbivore (*D. undecimpunctata*), entomopathogen (*B. bassiana*) and a muskmelon plant. The wolf-spiders used in this assay came from laboratory population, reared as described above and were starved for a week before the experiments began. The adult spotted cucumber beetles were collected from cucurbit crops (pumpkin, squash and melon) from the Horticulture Research Farm South of the University of Kentucky in Lexington, Kentucky, USA. The beetles were kept under controlled conditions (25 ± 5 ºC; 16:8 L:D) in the Invertebrate Ecology Laboratory in the University of Kentucky. The adults were kept into plastic containers (500 mL) and fed with a muskmelon plant grown in green house with autoclaved planting substrate (Pro-Mix® Mycorrhizae). The plants were replaced every 2 days after total consumption of leaves and stems. We added a glass vial (5 mL) with water and a cotton wool ball in each container as a water resource for the beetles.

We planted 28 untreated melon seeds in plastic plant pots using organic planting substrate (Pro-Mix® Mycorrhizae). The pots were kept in green house under controlled conditions (25 ± 5 ºC, 12:12 L:D) and watered daily. After plant germination and growth up to 30 cm (4 weeks after planting), the plants were used in the experiments. For exposing the beetles to the entomopathogenic fungi *B. bassiana,* we fed them melon plants treated with spores of *B. bassiana*. We treated the plants by immersing the aerial parts in a suspension of distillate water and spores of *B. bassiana* (commercial formulation BotaniGard® 22WP GHA – 4.5 x 10^{13} viable spores per kilogram). To achieve the suspension, 3.74 g of Botanigard 22WP was added to 1 L

of distilled water resulting in a concentration of 1.6 x 10^{13} viable/L^{-1} and 0.57 g of methylcellulose (1.5 %) was diluted in the suspension to help adhere the spores in the surface of the plants. The aerial parts of the plants were thoroughly immersed for five seconds and let dry for 30 minutes in the air. The plants treated with *B. bassiana* spores were covered with a plastic cylinder and 15 adult beetles were released for each plant. Four days after the release we observed the total consumption of melon plants. Thus, the beetles were removed from the plastic cylinders and used in the assays. The non-fungi exposed beetles used in these experiment came directly from the laboratory colony.

The experiment consisted of four treatments, that were assembled in microcosms made of a clean potted melon plant covered with a transparent plastic cylinder (20 cm diameter, 50 cm height) covered with mesh screen cloth (mesh size 5µm): I- three beetles exposed to the entomopathogen + one predator (*T. helluo*); II- three non-exposed fungi beetles + one predator; III- three exposed fungi beetles; IV- three non-exposed fungi beetles. Each treatment was repeated 7 times and evaluations were undertaken hourly during 7 hours. During each evaluation we recorded the behavior of beetles itemizing them according to their activity: walking, flying or feeding (active) and resting (inactive). Also, we recorded the position of the beetles in the microcosm (on the melon plant or out the melon plant). For the treatments where the wolf spiders were present, we also recorded the predation of beetles exposed and non-exposed to *B. bassiana*. At the end of the evaluations we used a digital camera to take pictures of the leaves for further analysis of leaf area consumed by the beetles. The calculation of leaf area and proportional damage caused by herbivory was made using the software ImageJ (developed at the National Institutes of Health, USA - http://rsb.info.nih.gov/ij).

2.5 Statistical analysis

We assembled a contingency table to estimate the choice of *T. helluo* in the arenas choice test. The sides in each arena with the beetle cues (exposed fungi beetles or non-exposed fungi beetles) were considered as independent variables and the choice of the spiders between the sides of the arena was considered as the dependent variable. A χ^2-test distribution was performed to compare the difference in the number of spiders choosing the sides of the arenas per time interval. In a further analysis, the different arena compositions (non-exposed beetles *vs* control, exposed beetles *vs* control, exposed *vs* non-exposed beetles) were considered as a categorical predictor variables and the number of changes the spiders performed between both sides of the arenas entered as a response variable. One-way ANOVA χ^2 was performed for calculating the difference in the number of changes between the different arenas composition. A higher rate of choices changes would represent a higher motion activity and a shift in the sit-and-wait foraging behavior of *T. helluo* due to the chemical cues.

Because the predation of spiders reduced the beetle population along the time intervals in the trophic interaction assay, we used the proportion data of beetles activity and position on the microcosm rather than the count data. Thus we compared the proportional frequency of active and inactive beetles on the plant and out of plant using Generalized Linear Mixed-Models (GLMM) discriminating the evaluations in time as random effects fitted to a binomial distribution. The significance of the treatments was computed using ANOVA χ^2 based on estimated mean deviance (Crawley 2007). The mortality of beetles in the treatments where the wolf spiders were present was estimated fitting a Weibull distribution for the censored data of dead and alive beetles at each time interval. We used a Kaplan-Meier plot to reach the

mortality curves. The proportional consumption of foliar area by the beetles was calculated using the ratio of total foliar area and damaged area. The percentage of consumed foliar area was considered as the dependent variable while the treatments were used as the independent variable. We used Generalized Linear Models (GLM) and Analysis of Variance (ANOVA) with χ^2-test to compare the treatments in a pairwise comparison. All the statistical analysis were made using the software R version 2.15.3 (R Development Team 2015).

3. Results

3.1 Arenas choice test

The wolf spiders demonstrated no preferences between both sides of the arena in all time intervals when we offered the choices of non-exposed beetles and clean paper (P>0.05, Figure 1a). There was significant preference for the side of the arenas containing cues from exposed fungi beetles instead of clean paper as a first choice ([0']: χ^2=4, d.f= 1, P=0.040) while in the following time intervals (20', 40' and 60') there was no significant difference in the spiders choice between both options (P>0.05, Fig 1b). When the arenas were composed by exposed fungi and non-exposed fungi beetle cues, the spiders shown preference for the exposed beetles in the first choice ([0']: χ^2= 5, d.f= 1, P=0.025). In the following time intervals, 20 minutes (χ^2= 9.8, d.f= 1, P= 0.001) and 40 minutes (χ^2= 7.2, d.f= 1, P= 0.005) the wolf spiders also demonstrated a preference for the exposed beetles cues. In the last time interval (60 minutes) the spider choices between the side of the arena were not significantly different (P>0.654; Fig. 1c). Although the wolf spiders have showed a distinct behavior in the different treatments (non-fungi exposed beetles *vs* control, fungi exposed beetles *vs* control, fungi exposed *vs* non-fungi exposed beetles) there was no

significant difference in the displacement of spiders performed within among choices between the arenas composition (χ^2= 2.35, df= 2, P=0.308).

3.2 Microcosm assay for trophic interactions

The activity of exposed and non-exposed fungi beetles did not differ in the microcosm either in the presence of the wolf spider or not (χ^2= 4.20, df= 3, P=0.491). The lower frequency of beetles on the melon plant was observed when either exposed or non-exposed beetles were in the presence of the wolf spider (χ^2= 26.84, df= 3, P<0.0001 Fig 2). The survivorship of the exposed and non-exposed beetles due to wolf spider predation did not differ (χ^2= 0.76, df=1, P>0.381; Fig 3). The pairwise comparison of leaf consumption of beetles on melon plants revealed that non-exposed fungi beetles in the presence of *T. helluo* caused less damage (3.18 ±2.05%, mean±SE) to melon plants compared to beetles in the control (16.42±5.99%) where *T. helluo* was absent and had no exposition to the pathogen (χ^2=9.76, df=1, P<0.01; Fig. 5a). Non-exposed beetles in the presence of *T. helluo* were also less damaged when compared to exposed beetles without predator (25.07±10.29, χ^2= 16.19, df= 1, P<0.0001; Fig 5b). The exposed beetles in the presence of *T. helluo* caused less damage (8.79±5.91%) compared to exposed beetles in the absence of predator (25.07±10.29, χ^2= 6.48, df= 1, P<0.05; Fig. 5c). In the absence of predator, the average leaf damage caused by exposed beetles (25.07±10.29%) did not significantly differ from non-exposed beetles (16.42±5.99%, P>0.05; Fig 5d). No significant difference was observed in the leaf damage caused by exposed and non-exposed beetles both in the presence of *T. helluo* (χ^2= 2.24, df= 1, P>0.05; Fig 5e). There was no significant difference between the leaf damage caused by the exposed beetles in the presence of predator and in the control (χ^2= 2.73, df= 1, P=0.098, Fig 5f).

4. Discussion

Our results demonstrated an intricate response of *T. helluo* to the chemical cues associated with *B. bassiana* (Fig 1). The heterogeneity of the responses by *T. helluo* to the cues of infected and healthy preys reflect the complexity of the foraging behavior of wolf spiders. Spiders are known to assess the chemical cues of prey and reject unpalatable ones (Givens 1978, Vasconcellos-Neto & Lewinson 1984). Adults of *D. decempunctata* sequester cucurbitacins by feeding cucurbitaceae plants (pumpkin, squash, cucumber, etc.) and use them as a defense strategy against natural enemies (Webster 1895; Howe *et al* 1976; Ferguson & Metcalf 1985; Nishida & Fukami 1990) and pathogens (Tallamy *et al* 1998). Cucurbitacins are oxygenated tetracyclic triterpenes found in all species of Cucurbitaceae, known as toxic and repellent to most invertebrate and vertebrate herbivores (Metcalf et al 1980; Tallamy et al 1997b). Generally, hypervirulent fungi strains such as *B. bassiana* cause the exhaustion of the defenses of their hosts by depressing the cellular defenses after 3 days of infection (Hung & Boucias 1992, Hajek & St. Leger 1994). The choice of *T. helluo* for infected prey cues instead of non-infected prey might be a response for more palatable prey due to the decrease in chemical defenses of the beetles exposed to *B. bassiana* (Fig 1). However, when *T. helluo* had the options of beetle chemical cues and no prey cues they did not significantly respond for the option (Fig 1 b,c). Because *B. bassiana* and *T. helluo* are natural enemies of insects naturally occurring in the soil (Dondale & Redner 1990, Marshal & Rypstra 1999, Meyling & Eilenberg 2007) it is likely that *T. helluo* constantly consume prey infected with entomopathogenic fungi in their eventual scavenger habit (Knost & Rovner 1975). Also it is known that *B. bassiana* do not cause significant mortality of wolf spiders populations in augmentative

releases (Maketon *et al* 2015) suggesting that prey infected by *B. bassiana* may not represent a limiting factor to foraging behavior of *T. helluo*.

We found that the exposure of *D. undecimpunctata* to *B. bassiana* had no effect on the predation of *T. helluo* since the mortality of beetles did not differ between exposed and non-exposed beetles (Fig 2). Furthermore, the presence of the predator and the exposure to the pathogen did not alter the activity of the beetles within the microcosm. Some entomopathogenic fungi promote behavioral shifts in the host enhancing its vulnerability to predators by reducing their activity (Arthurs & Thomas 2001) or even manipulating the predation risk responses in the host (Roy & Pell 1999, Roy *et al* 2005). However such interactions are observed when predator and pathogen are strictly related to the prey/host. Roy *et al* (1999) observed that the infection of the aphid-specific entomopathogenic fungi *Pandora neoaphidis* decreases the response of pea aphid (*Acyrthosiphon pisum*, Hemiptera: Aphididae) to the alarm pheromone produced by conspecifics (Roy *et al* 1999). Nonetheless, aphids infected with *B. bassiana* demonstrated no changes in sensitivity to alarm pheromone produced by conspecifics but stopped producing it (Roy *et al* 2005). Thus, the selection pressure for the host manipulation is likely to be minimal for broad host range pathogens like *B. bassiana.* Though behavioral alterations in infected prey may happen as a result of fungal damage in internal structures of the host insect (Samuels *et al* 1988; Arthurs & Thomas 2001).

Some predators are able to distinguish and avoid feeding on infected prey reducing their susceptibility to infections via trophic transmission (Pell *et al* 1997, Roy *et al* 1998, Meyling & Pell 2006). However, the trophic transmission can be detrimental to the pathogen if the predator is not a viable host for the pathogen (Roy *et al* 2006). As an epigeal, generalist and scavenger predator (Knost & Rovner 1975),

the wolf spiders are very likely to intake pathogens from trophic interactions. However, evidences demonstrate that the use of *B. bassiana* as biological control agent do not cause negative effects for wolf spiders population (Devotto *et al* 2007 a,b; Maketon *et al* 2015) suggesting that *T. helluo* may not represent a viable host to *B. bassiana*. In this sense *T. helluo* feeding on infected preys would be disadvantageous for *B. bassiana* by reducing the ability to disperse in the prey population reducing the occurrence of disease (Hudson *et al* 1992; Packer *et al* 2003; Duffy *et al* 2005).

The frequency of beetles on the plant was lower in the presence of *T. helluo* than in its absence, but the exposition to *B. bassiana* did not alter the frequency of beetles on plant (Fig 2). The foliar damage caused by *D. decimpunctata* herbivory in the melon plants was lower when *T. helluo* was present regarding beetles exposition to *B. bassiana* (Fig 5). Compared to the control (no spider and no exposition to *B. bassiana*) the natural enemies together (spider present and exposition to the fungi) did not result in a reduction in the leaf damage caused by *D. undecimpunctata*. In accordance with our findings, Snyder and Wise (2003) found that the presence of *T. helluo* reduced the foliar damage and the frequency of *D. undecimpunctata* on the squash plants (*Cucumis pepo* L.). However, our results demonstrate an intriguing interference of *B. bassiana* on the plant consumption by *D. undecimpunctata* (Fig 5). The beetles exposed to *B. bassiana* demonstrated no reduction on the foliar consumption. When exposed beetles were in the presence of the wolf spider, the foliar consumption did no differ from no-spider treatments (Fig 5). Contrary to our findings some studies demonstrate a significant reduction in feeding of insects infected with Hypocrealean fungi (Arthurs & Thomas 2000; France *et al* 2002; Tefera & Pringle 2003). The feeding reduction is assigned to a decreased efficiency of conversion and

digestion of food after insect infection with *B. bassiana* (Mohamed 1982). Though unexpected, this phenomenon may, perhaps, be explained. Interestingly the cucumber beetles are described as pharmacophagous insects because they leave more nutritionally suitable host plants for feeding on cucurbits, aiming the cucurbitacins intake (Nishida & Fukami, 1990). The cucumber beetles are known to invest their stocks of cucurbitacins in the defense against fungal infections (Tallamy *et al* 1998). The 'titer satiation hypothesis' proposed by Tallamy and Halaweish (1993) assumes that *D. undecimpunctata* sequester cucurbitacins seeking a proper quantity for the defense against predators and pathogens. The phagostimulatory effect of cucurbitacins cease when the titers are reached, but once the stocks of cucurbitacins are low the insect back to seek and ingest this compounds. From this framework it is possible that *D. undecimpunctata* exposed to *B. bassiana* have invested their stocks of cucurbitacins fighting the fungal infection, thus increasing the phagostimulatory effect of cucurbitacins contained in the melon plants.

In the current study a trophic cascade effect was observed when the presence of the predator *T. helluo* decreased the herbivory of *D. undecimpunctata* in melon plants directly by decreasing the herbivore population and indirectly by changing the time allocated by the herbivores on the plant, corroborating the results obtained by Snyder and Wise (2003). Another central issue of biological pest control is the applicability of multiple natural enemies aiming the incrementation of the pest control (Sih *et al* 1998, Schmitz 2007). Several studies exploring the subject are based on studies of synergistic and antagonistic relations between natural enemies (Soluk & Collins 1998, Losey & Denno 1998). However as seen in the current study, even if no antagonistic effect occurs between the natural enemies studied it is possible that interaction between pathogen (*B. bassiana*), herbivore (*D. undecimpunctata*) and the host plant

results in a negative trophic effect for biological control, increasing the plant damage caused by the herbivore. These results highlight the importance of including multiple trophic levels in the study of interactions between natural enemies and pests. Also our results highlight the need for an assessment of the trophic interactions from different points of view allowing the integration of biological control different strategies.

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Figure 1. Spiders choice in different time intervals between the sides of arenas containing cues of *D. undecempunctata* exposed or not to *B. bassiana* spores. Control: clean filter paper. A – Fungi exposed prey cues vs non-fungi exposed prey cues; B- Non-fungi exposed prey cues vs control; Fungi exposed prey cues vs control (*n*=20).

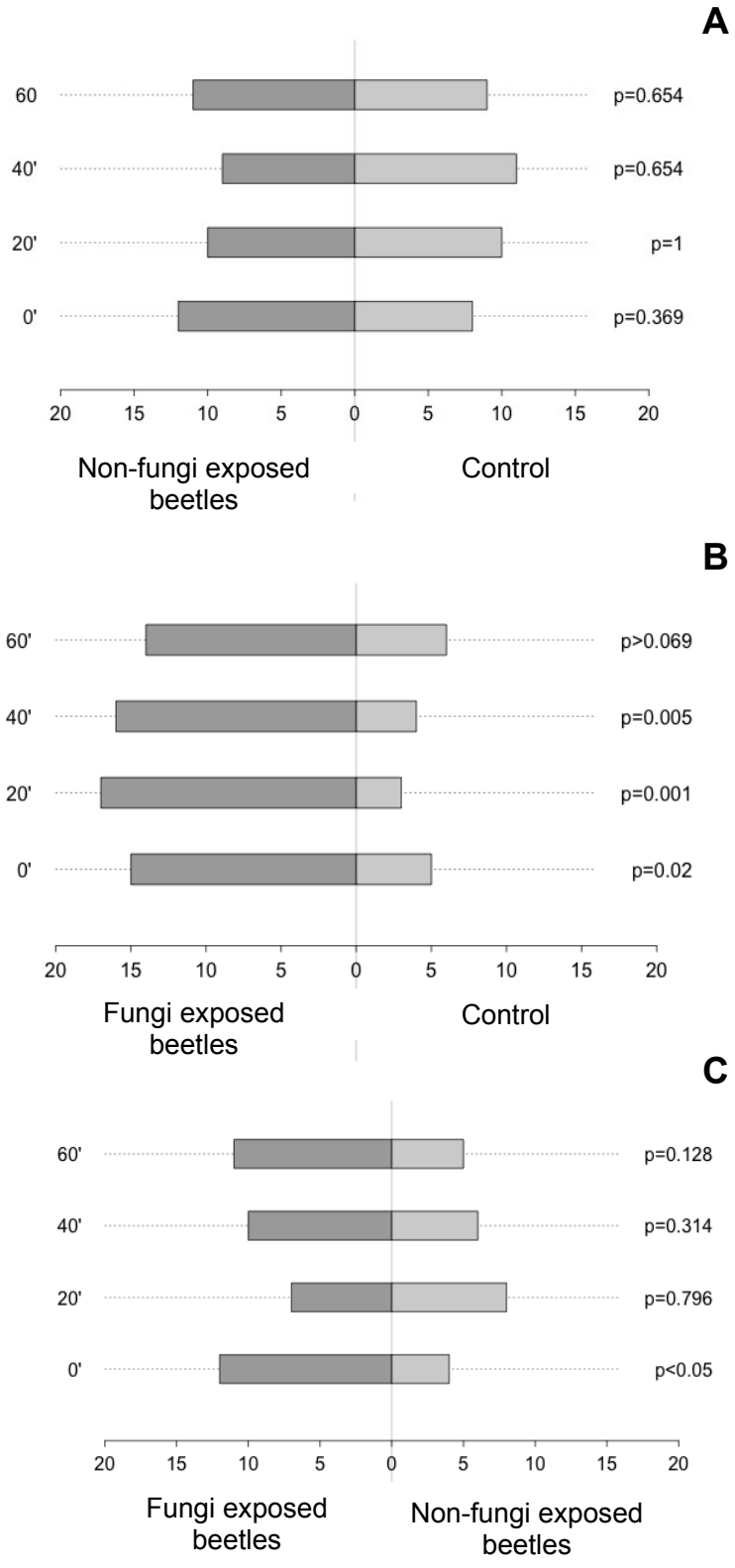


Figure 2. Mean ± SE frequency of *D. undecimpunctata* present on melon plants in the microcosm composed by the different treatments. Different letter over the bars indicate significant differences (P<0.05)

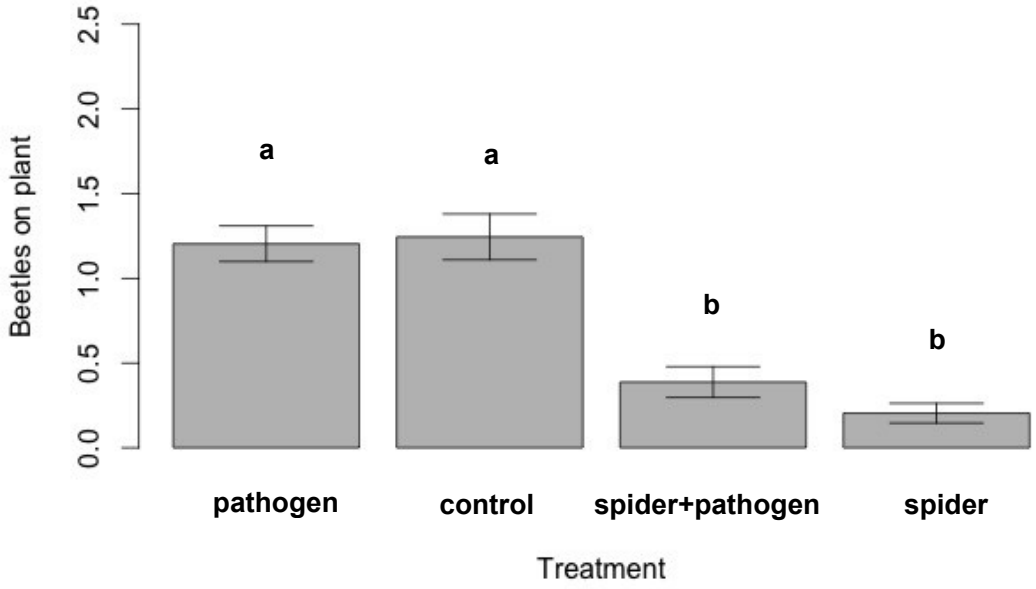


Figure 3. Kaplan-Meier survivorship plot of fungi exposed and non-fungi exposed beetles (*D. undecimpunctata*) due to predation of the wolf spider *T. helluo.*

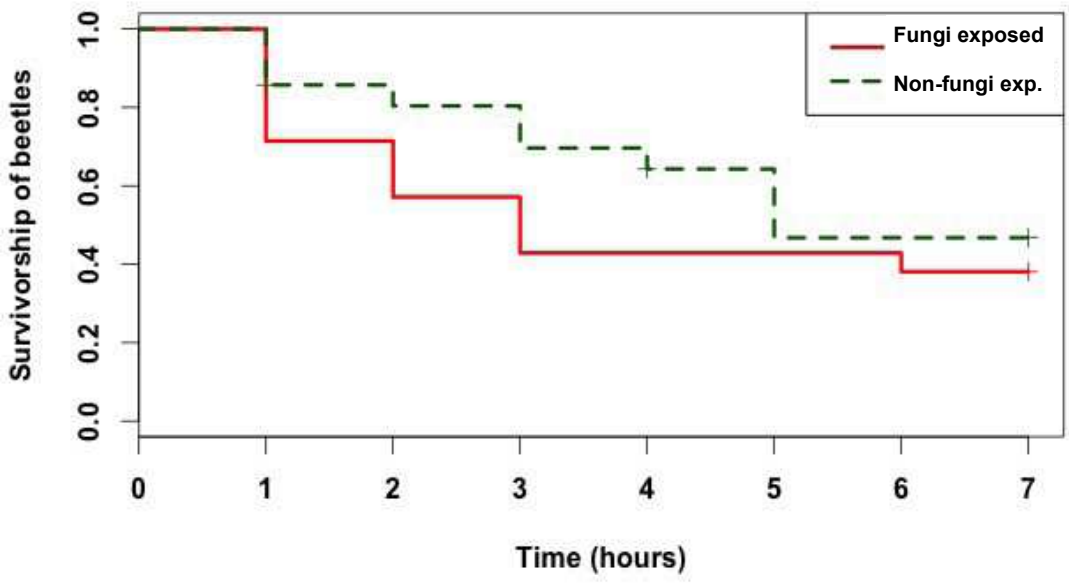
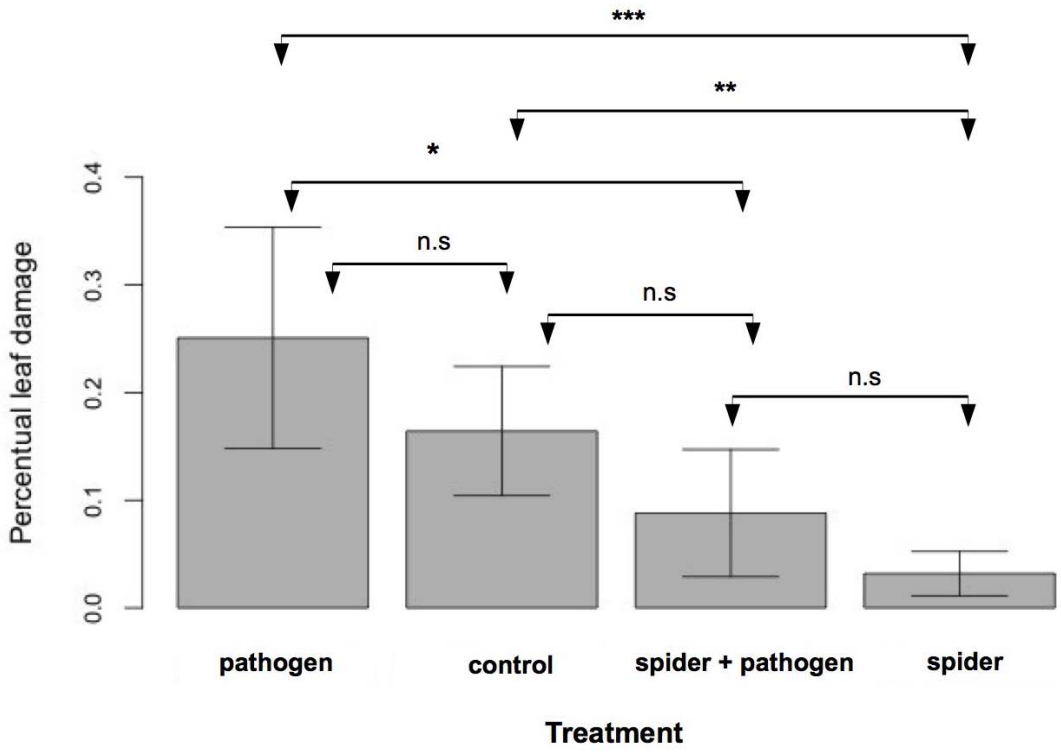


Figure 4. Mean±SE proportional leaf damage caused by the herbivory of *D. undecimpunctata* exposed or no-exposed to the pathogen *B. bassiana* in the presence or absence of the wolf spider *T. helluo.* Arrows over the bars indicate the comparisons among treatments in the pairwise analysis. Significant levels are based on ANOVA Chi-square test: '*' P<0.05; '**'P<0.01; '***'P<0.001



Conclusões Gerais

O objetivo principal desta tese foi estudar a aplicabilidade de práticas de manejo cultural como estratégias de controle biológico conservativo em sistemas de cultivo anual. Além disso, foram estudadas as interações tróficas entre organismos envolvidos nos processos ecológicos dentro de agroecossistemas. Neste sentido os resultados obtidos neste trabalho auxiliam na compreensão da funcionalidade de técnicas de cultivo empregadas como ferramentas no controle biológico de pragas:

i. A manutenção de plantas espontâneas em sistemas de cultivo de pimenta-malagueta não beneficia a atividade de fungos entomopatogênicos de forma efetiva na área cultivada. As modificações bióticas e abióticas promovidas por plantas espontâneas em agroecossistema podem demonstrar padrões variáveis dentro da área cultivada, refletindo em respostas variáveis da atividade de fungos entomopatogênicos para o controle de pragas.

ii. No solo de sistemas de cultivo convencional, os fungos entomopatogênicos *Fusarium* sp. e *Metarhizium* sp. foram isolados com alta frequência enquanto que *Beauveria* sp. demonstra baixa frequência neste ambiente corroborando estudos prévios indicando a maior persistência de *Metarhizium* sp. no solo de sistemas de cultivo convencionais.

A redução dos distúrbios no solo pela prática do plantio direto em sistemas de cultivo orgânico de melão não resultam na maior atividade de entomopatógenos de solo. A escala temporal durante o período de plantio representa fator importante na variação da patogenicidade e ocorrência de entomopatógenos no solo de agroecossistemas orgânicos.

iii. Em sistemas de cultivo orgânico em ambientes temperados a escala temporal

afeta os entomopatógenos de solo de forma diferencial. Fungos entomopatogênicos ocorrem com maior frequência ao final da temporada de plantio e a fase inicial do plantio apresenta maior atividade de outros entomopatógenos não-fungicos. A maior virulência de entomopatógenos em solo de sistemas orgânicos de produção ocorre na fase inicial do cultivo.

iv. A exposição de herbívoros ao esporo do fungo entomopatogênico *Beauveria bassiana* não representa fator limitante ao comportamento de forrageamento e de predação de *Tigrosa helluo*, um predador de solo generalista.

v. A presença de *T. helluo* resulta em redução da herbivoria de *Diabrotica undecimpunctata* em plantas de melão. O besouro herbívoro aloca menos tempo na planta quando na presença do predador. A exposição de *D. undecimpunctata* aos esporos do fungo não resultou na redução dos danos causados por este herbívoro na planta hospedeira.

vi. A exposição do besouro *D. undecimpunctata* aos esporos do fungo entomopatogênico resultam em uma modificação da resposta do besouro a presença do predador, refletida na taxa de herbivoria.