



CAMILA CRAMER FILGUEIRAS

**INDUTORES DE DEFESA DE PLANTAS
EM INTERAÇÕES ENVOLVENDO
CULTIVOS AGRÍCOLAS, PRAGAS DE SOLO
E NEMATOIDES ENTOMOPATOGÊNICOS**

**LAVRAS – MG
2016**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Entomologia, área de concentração em Entomologia, para obtenção do título de Doutora.

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LAVRAS – MG

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DEDICO

A Deus.

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*À Pituquinha, razão do meu viver, sempre companheira, mesmo tão pequena
sempre me apoiando em todos os momentos.*

*Ao companheiro, parceiro e amigo Denis, que faz cada dia de minha vida,
um dia melhor e especial.*

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para apoiar meus sonhos.*

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meus sonhos.*

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RESUMO

As plantas estão presentes em dois ambientes dinâmicos simultaneamente, sob constante ataque de herbívoros foliares na parte aérea e de herbívoros de raiz no subsolo. As vias de defesa da planta desempenham papel importante na mediação de respostas a estes ataques. Visando entender melhor as interações existentes entre estes sistemas e suas interações, examinamos o papel de defesa da planta com estimulação por meio de indutores de defesa (Salicilato de metila e Jasmonato de metila) na parte aérea, sobre as interações tritróficas entre plantas, herbívoros e nematoides entomopatogênicos. Com nossos experimentos, encontramos que a herbivoria na parte aérea do milho, assim como a estimulação das vias de defesa do ácido jasmônico e do ácido salicílico, nas culturas de milho e citros, recrutam nematoides entomopatogênicos no subsolo. Além disso, observamos que plantas produzem voláteis específicos para mediar este recrutamento em citros. Outros fatores observados foram o efeito das diferentes doses de indutores sobre o comportamento dos nematoides entomopatogênicos, bem como o efeito desses indutores nas distâncias de recrutamento dos mesmos. Esses resultados sugerem um papel amplo para a sinalização no subsolo nas interações tritróficas, enfatizando a conexão existente entre a parte aérea e o sistema radicular das plantas mediadas por vias de defesa da planta, e apontam para novas estratégias para melhorar o controle biológico de pragas subterrâneas com nematoides entomopatogênicos.

Palavras-chave: Nematoides entomopatogênicos. Interações tritróficas. Vias de defesa da planta. Inimigos naturais.

ABSTRACT

Plants inhabit two dynamic environments simultaneously, facing attack from foliar herbivores aboveground and root herbivores belowground. Plant defense pathways play a critical role in mediating responses to these attacks. Here, we examine the role of plant defense pathway stimulation aboveground with Methyl Salicylate and Methyl Jasmonate on the tritrophic interactions between plants, herbivores, and entomopathogenic nematode natural enemies belowground. We find that aboveground herbivory in corn and stimulation of the jasmonic acid and salicylic acid pathways in corn and citrus can recruit entomopathogenic nematodes belowground. Further, we implicate a specific induced plant volatile as mediating this recruitment in citrus. Additionally, we explore the effect of elicitor dose and nematode release parameters affecting nematode recruitment to elicitor treated corn seedlings. These results suggest a broad role for belowground signaling in tritrophic interactions, emphasize the connection between the above and belowground parts of plants as mediated by plant defense pathways, and point to novel strategies for enhancing biological control of subterranean insect pests with entomopathogenic nematodes in the field.

Keywords: Entomopathogenic nematodes. Tritrophic interactions. Plant defense pathways. Natural enemies.

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1 INTRODUCTION

Plants, given their immobility, have developed different ways of interacting with their environment and defending against attack. One of the primary means by which plants interact and communicate with their environment is through the release of organic volatiles. These volatiles are often released in response to specific stimuli and can be utilized directly to repel or attract herbivores or indirectly through attraction of natural enemies of those herbivores as a means of defense (BIRKETT et al., 2000; DE MORAES; MESCHER; TUMLINSON, 2001; DICKE; DIJKMAN, 1992; DUGRAVOT; THIBOUT, 2006; HARE, 2011; HEIL, 2008; KESSLER; BALDWIN, 2001; TRIGO; PAREJA; MASSUDA, 2012). Indeed, these indirect defenses can often be efficiently induced; plants can synthesize and release herbivore induced plant volatiles (HIPVs) immediately after damage by an herbivore, or more slowly over time (HEIL, 2008). Additionally, the nature and production of these HIPVs can vary in accordance with the specific herbivore or plant species under attack (ARIMURA; MATSU; TAKABAYASHI, 2009; DICKE; BALDWIN, 2010).

Release of these HIPVs is mediated by plant defense pathways. These plant defense pathways can be induced by herbivory or through the use of synthetic elicitors (FARMER; RYAN, 1990; PARK et al., 2007). While there are many defense pathways that interact within the plant, among the most well known are the salicylic acid pathway, which regulates plant responses to biotrophic pathogens, and the jasmonic acid pathway, which regulates plant responses to herbivory (THALER et al., 2002; THALER; HUMPHREY; WHITEMAN, 2012). Stimulation of these pathways can result in augmentation of direct defenses or indirectly attract natural enemies of herbivores (THALER, 1999).

Plant release of volatiles can be used to attract natural enemies of herbivores above and belowground. Belowground, volatiles released following herbivore attack can attract entomopathogenic nematodes for control of belowground root herbivores (RASMANN et al., 2005; VAN TOL et al., 2001). While these nematodes can be particularly recruited by specific herbivore induced plant volatiles belowground, they are influenced by other plant volatiles (BIRD; BIRD, 1986; CHOO et al., 1989; LEI; RUTHERFORD; WEBSTER, 1992; VAN TOL et al., 2001) and volatiles released by insects such as CO₂ and those from insect feces (BIRD; BIRD, 1986; CHOO et al., 1989; GAUGLER et al., 1980; HALLEM et al., 2011; LEI; RUTHERFORD; WEBSTER, 1992; LEWIS; GAUGLER; HARRISON, 1993; WANG; GAUGLER, 1998). After recruiting to these cues, EPN enter their insect larvae hosts and regurgitate endosymbiotic bacteria (KAYA; GAUGLER, 1993). The bacteria then multiply and kill the host while the nematode completes its lifecycle and feeds on the bacteria (KAYA; GAUGLER, 1993). When resources are sufficiently depleted in the insect host, third instar infective juvenile EPN emerge from the insect cadaver in search of new hosts (KAYA; GAUGLER, 1993).

Given the efficiency and efficacy by which entomopathogenic infective juveniles seek out and kill subterranean herbivores, they are often considered an attractive option for biological control and have been shown to be effective against a variety of insect pests in cryptic and subterranean environments (GAUGLER; KAYA, 1990). This control could be enhanced, however, by using knowledge of the tritrophic interactions between plants, herbivores, and entomopathogenic nematodes to design control strategies involving stimulation of plant defense pathways and release of plant volatiles.

1.1 Objetivo geral

Estudar as interações existentes entre a parte aérea das plantas e o sistema radicular enfatizando o papel da defesa das plantas estimulada por indutores de defesa sobre as interações tritróficas entre plantas, herbívoros e nematoides entomopatogênicos.

1.2 Objetivos específicos

Artigo 1: Investigar o efeito da ativação da via de defesa do ácido salicílico por meio da aplicação foliar do elicitador salicilato de metila no recrutamento de nematoides entomopatogênicos *Steinernema diaprepesi* em plantas de citros.

Artigo 2: Investigar a interação tritrófica entre plantas de milho tratadas com indutores de defesa, adultos de *Diabrotica speciosa* e o nematoide entomopatogênico *Heterorhabditis amazonensis*.

Artigo 3: Explorar o efeito das distâncias de liberação de NEP, danos nas plantas e aplicação exógena de indutores de defesa sobre o comportamento dos nematoides entomopatogênicos *Heterorhabditis amazonensis* em plantas de milho.

2 REFERENCIAL TEÓRICO

2.1 Interações inseto-planta

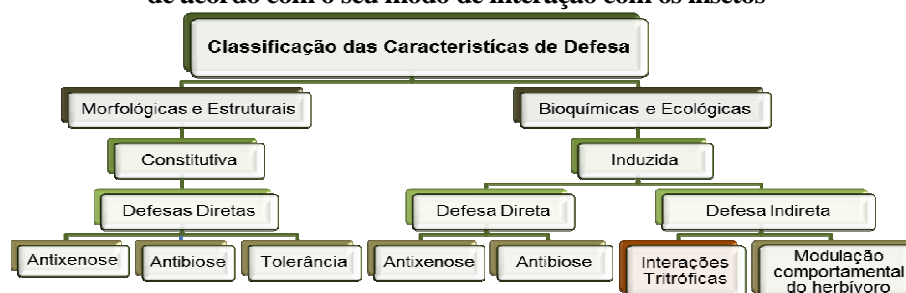
As plantas estão em constante evolução, devido às pressões sofridas pelos fatores bióticos e abióticos do meio. Elas não são simples vítimas passivas do ataque de herbívoros, pois possuem um arsenal de defesas físicas e químicas (RASMANN et al., 2005). Dentre as defesas físicas podemos citar os espinhos, tricomas não glandulares, cristais de oxalato de cálcio, sílica (BAUR et al., 1999; COOPER; GINNETT, 1998; MASSEY; ENNOS; HARTLEY, 2006; RUIZ; WARD; SALTZ, 2002; TRIGO; PAREJA; MASSUDA, 2012). Quanto às defesas químicas, para seu desenvolvimento e sucesso no ambiente onde se encontram, as plantas produzem compostos primários, que atuam diretamente no seu crescimento e desenvolvimento, além dos compostos secundários, que são responsáveis pela defesa da planta (TRIGO; PAREJA; MASSUDA, 2012). Os compostos secundários, também denominados semioquímicos ou infoquímicos, são responsáveis por mediar às interações entre organismos, sendo estes chamados de feromônios quando essas interações ocorrerem entre indivíduos da mesma espécie, e aleloquímicos quando entre indivíduos de diferentes espécies (DICKE; SABELLIS, 1988; NORDLUND; LEWIS, 1976; TRIGO; PAREJA; MASSUDA, 2012).

Os aleloquímicos são classificados de acordo com a forma que influenciam as espécies envolvidas em: alomônios (afeta positivamente o emissor e negativamente o receptor), cairomônios (afeta negativamente o emissor e positivamente o receptor) e sinomônios (afeta positivamente ambos), ressaltando-se a importância de que o efeito desses compostos é dependente do contexto estudado, podendo um mesmo composto apresentar múltiplos efeitos (TRIGO; PAREJA; MASSUDA, 2012). As classes de compostos secundários

importantes em interações inseto-planta são: alcaloides, glicosídeos cianogênicos, glicosinolatos, compostos fenólicos, terpenos e alguns derivados de ácidos graxos (TRIGO; PAREJA; MASSUDA, 2012).

De acordo com Karban e Baldwin (1997) as plantas utilizam diferentes maneiras para diminuir os efeitos ou o próprio ataque dos herbívoros de forma direta (contexto bitrófico), ou indireta, atraindo seus inimigos naturais (contexto multitrófico). Segundo Trigo, Pareja e Massuda (2012) as plantas apresentam dois tipos de defesas químicas (Figura 1): constitutivas, sempre presentes, mesmo na ausência de estresse, ou induzidas, alterações que ocorrem na planta após sofrerem algum tipo de estresse.

Figura 1 Classificação das características de defesa das plantas categorizadas de acordo com o seu modo de interação com os insetos



Fonte: Modificado de Schaller (2008)

Um dos fatores mais importantes das relações entre insetos e plantas é o alto grau de especialização alimentar encontrado entre os insetos, podendo estes serem classificados em: espécies monófagas, que são altamente especializadas, alimentando-se apenas de um gênero ou espécie de planta; espécies oligófagas, especializadas em apenas uma família de plantas; e espécies polífagas ou generalistas que se alimentam de muitas espécies de diferentes famílias (SCHOONHOVEN; VAN LOON; DICKE, 2005; TRIGO; PAREJA; MASSUDA, 2012). Para selecionarem as plantas para alimentação e oviposição,

os herbívoros consideram as características físicas e químicas das mesmas (DETHIER, 1947; FRAENKEL, 1959), desenvolvendo mecanismos para reconhecer e encontrar sua planta hospedeira entre outras plantas não apropriadas. Entre os mecanismos utilizados na interação dos diferentes organismos com as plantas podemos destacar as substâncias voláteis liberadas pela planta (TRIGO; PAREJA; MASSUDA, 2012). Os insetos respondem de diferentes maneiras aos compostos voláteis liberados pelas plantas. De acordo com Dethier, Browne e Smith (1960) os compostos químicos podem ser classificados de forma diferenciada de acordo com o comportamento provocado nos insetos, sendo considerados atraentes quando estimula o inseto a se movimentar em direção à fonte de estímulo, repelente quando o inseto a se afastar da fonte de estímulo, estimulante quando estimula a alimentação ou oviposição e deterrente quando inibe oviposição ou alimentação.

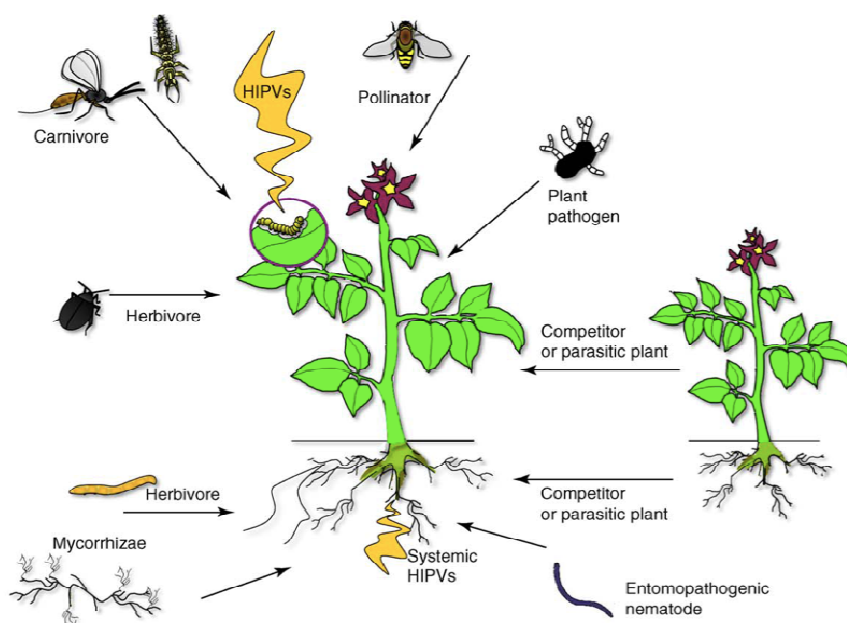
Price et al. (1980) sugeriram que as interações entre plantas e insetos herbívoros não ocorrem de forma isolada e que para entender a evolução dos compostos secundários é necessário considerar o contexto ecológico do local onde essas interações ocorrem. Os compostos secundários das plantas podem influenciar os insetos de diferentes maneiras, herbívoros especialistas ao se alimentarem de plantas tóxicas podem utilizar os compostos produzidos por essas plantas em benefício próprio, os predadores e parasitoides dos herbívoros podem utilizar sinais químicos das plantas para encontrar locais de forrageio com alta probabilidade de encontrar a presa, as plantas podem interagir entre elas por meio de sinais químicos e essa interação pode afetar os herbívoros e os inimigos naturais desses herbívoros (TRIGO; PAREJA; MASSUDA, 2012). Muitos compostos secundários são armazenados e liberados ao ambiente como compostos orgânicos voláteis (COV), esses compostos voláteis liberados por plantas (flores, partes vegetais e raízes) são bastante numerosos ultrapassando 1000 (DUDAREVA et al., 2006; PICHERSKY; NOEL; DUDAREVA, 2006). É

conhecido que a quantidade e a qualidade de COV liberados pelas partes vegetais e raízes podem mudar quando a planta é danificada, enquanto a liberação pelas flores não sofre alterações, por se tratar de uma liberação é ontogeneticamente programada (FARMER, 2001; HEIL, 2008; TUMLINSON; PARÉ; LEWIS, 1990; TURLINGS et al., 1995). Além disso, os COVs induzidos pela herbivoria podem repelir ou atrair os herbívoros além de servir de orientação para as defesas indiretas (BIRKETT et al., 2000; DE MORAES et al., 2001; DICKE; DIJMAN, 1992; DUGRAVOT; THIBOUT, 2006; KESSLER; BALDWIN, 2001). Entre os COVs podemos destacar os voláteis de plantas induzidos pela herbivoria (VPIH), que são os compostos utilizados como sinais para orientação dos inimigos naturais de onde o recurso (presa ou hospedeiro) estará disponível (DICKE; SABELIS, 1988; ELZEN; WILLIAMS; VINSON, 1983; HARE, 2011; TUMLINSON; TURLINGS; LEWIS, 1992; TURLINGS et al., 1991). Essa atração de inimigos naturais é considerada uma defesa indireta da planta, reduzindo o impacto dos herbívoros na planta (HEIL, 2008; TRIGO; PAREJA; MASSUDA, 2012).

Para detectar os sinais químicos liberados pelas plantas, os inimigos naturais enfrentam um conflito entre detectar os sinais e confiar nos mesmos, sendo os odores liberados pelos herbívoros os sinais mais confiáveis do que os liberados pelas plantas (VET; DICKE, 1992). De acordo com Heil (2008), VPIH são mediadores do complexo de interação planta-carnívoros, dando para a planta a oportunidade de uma sintonia fina de defesa no momento que a mesma necessita (Figura 2). A quantidade e a qualidade de VPIH que são liberados pelas partes vegetativas e raízes de plantas podem sofrer grandes mudanças quando as mesmas são danificadas (FARMER, 2001; TUMLINSON; PARÉ; LEWIS, 1990; TURLINGS et al., 1995), variando de acordo com a espécie herbívora, a quantidade atacada, a espécie vegetal e com as condições ambientais locais (HARE, 2011). Zakir (2011) ressalta ainda que, o risco da

herbivoria para a planta pode ser reduzido pela eficiência da mesma em produzir VPIH em resposta ao ataque de herbívoros. Dicke, Van Loon e Soler (2009) sugerem que estudos com VPIH envolvendo interações entre plantas e múltiplos atacantes devem considerar análises de biologia química e molecular para serem melhor explicados.

Figura 2 Voláteis de plantas induzidos pela herbivoria (VPIH), associados às interações entre diferentes organismos (receptores de sinal) ao redor da planta



Fonte: Dicke e Baldwin (2010)

A liberação de COVs pode ser diferente entre o dia e a noite (KUNERT et al., 2002; LOUGHRIN et al., 1994), e a produção desses compostos podem ser limitados tanto pela luminosidade, fertilidade do solo (GOUNGUENÉ; TURLINGS, 2002; SCHMELZ; ALBORN; TUMLINSON, 2003) quanto pelo estresse hídrico (VALLAT; GU; DORN, 2005). Hormônios produzidos pelas

plantas durante os danos por herbívoros tem relação sinérgica e antagônica na interação com os herbívoros, podendo aumentar ou diminuir a expressão dos COVs (ARIMURA; MATSU; TAKABAYASHI, 2009; LEITNER; BOLAND; MITHOFER, 2005; MITHOFER; BOLAND, 2008; ZHANG et al., 2009), que podem ser liberados imediatamente após os danos, enquanto outros compostos são liberados de forma mais lenta (HEIL, 2008). A maioria dos COVs são novamente sintetizados após os danos e seus metabólitos originais são geralmente bem definidos (DUDAREVA et al., 2006; PARÉ; TUMLINSON, 1997; PICHERSKY; NOEL; DUDAREVA, 2006).

2.2 Interações nematoides-planta

O comportamento dos nematoides são governados por quimiosensações, termosensações e mecanosensações para obter informações sobre o ambiente e as estratégias de forrageamento a serem utilizadas, que podem ser Ambush ou Cruise (LEWIS et al., 2006). Nematoides entomopatogênicos com forrageamento Cruise, locomovem-se por meio de movimentos lineares, com típicos intervalos para busca na ausência de pistas associadas ao hospedeiro (LEWIS; GAUGLER; HARRISON, 1993; POINAR JÚNIOR, 1990). Os que apresentam forrageamento Ambush respondem as pistas de voláteis para o contato com o hospedeiro, sendo essas respostas expressas somente quando os nematoides estão parados (LEWIS et al., 2006).

A utilização de nematoides entomopatogênicos para o controle de pragas requer um bom entendimento sobre os agentes de controle biológico e ecológico para otimizar o efeito do nematoide sobre a praga (GAUGLER; LEWIS; STUART, 1997; KOPPENHOFER; FUZY, 2008). Lewis et al. (2006) sugeriram que o comportamento de busca dos nematoides entomopatogênicos pelos hospedeiros é mediado por pistas do hospedeiro ou do ambiente do mesmo.

El Borai et al. (2012) destacaram em seus experimentos a alta complexibilidade do controle biológico e ilustraram a importância do ambiente e das interações entre os vários níveis tróficos para entender a dinâmica de predador-presa, e que embora o alto número de pragas em alguns ambientes possa ser limitante para o controle biológico, as condições físicas locais favoráveis para endemias naturais podem auxiliar na redução de surtos de pragas.

Pistas emitidas por plantas danificadas por insetos geram informações mais específicas sobre a presença do hospedeiro; suas pistas químicas podem mostrar aumento na atração e infecção por nematoides entomopatogênicos (RASMANN et al., 2005; VAN TOL et al., 2001). Estudos comprovaram que pistas emitidas por raízes de plantas podem influenciar o comportamento de nematoides entomopatogênicos (BIRD; BIRD, 1986; CHOO et al., 1989; LEI; RUTHERFORD; WEBSTER, 1992; RASMANN et al., 2011; VAN TOL et al., 2001), sendo os mesmos atraídos não só por danos nas raízes, momento que ocorre a liberação de sinais de alerta pelas plantas (BOFF; VAN TOL; SMITS, 2002; VAN TOL et al., 2001), mas também devido aos seus hospedeiros e por meio da associação de pistas (CO₂ e fezes) (BIRD; BIRD, 1986; CHOO et al., 1989; GAUGLER et al., 1980; HALLEM et al., 2011; LEI; RUTHERFORD; WEBSTER, 1992; LEWIS; GAUGLER; HARRISON, 1993; WANG; GAUGLER, 1998). Estudo realizado por El-Borai et al. (2012) sobre a influência da textura do solo sobre a persistência, eficácia e habilidade dos nematoides entomopatogênicos revelou a forte influência do ambiente e de suas interações no sucesso do controle biológico de *Diaprepes abbreviatus* na cultura do citros, com nematoides entomopatogênicos. Estudos de olfatométria realizado por Van Tol et al. (2001) revelaram que raízes de plantas danificadas por larvas de besouro liberam exudatos que atraem nematoides parasitas com função de pedir ajuda contra a herbivoria. Riemens, Zoon e Van Tol (2003) investigando os sinais de ajuda presentes em exudatos de raízes danificadas por herbívoros,

também evidenciaram que compostos voláteis solúveis em água atraem nematoides entomopatogênicos (*H. megidis*) para raízes (*Thuja occidentalis*) danificadas por herbívoros (*Otiorhynchus sulcatus*).

Soler et al. (2012) em estudo envolvendo interações entre plantas e organismos associados, observaram que raízes atacadas por herbívoros são importantes iniciadores das defesas diretas e indiretas das plantas. Mesmo fato observado por Rasmann et al. (2005) em estudos com *Diabrotica virgifera* na cultura do milho, observaram que, as raízes danificadas liberam sesquiterpenos e atraem nematoides entomopatogênicos que atuam sobre a população dessa praga. Hiltbold et al. (2011) estudaram também na cultura do milho, os voláteis sintetizados e liberados pelo sistema radicular sob o ataque de *Diabrotica*, observaram o recrutamento dos nematoides entomopatogênicos.

Pesquisadores da Flórida-EUA, estudando nematoides entomopatogênicos no controle de larvas de *Diaprepes abbreviatus* em citros, relataram que plantas infestadas pela praga foram mais atrativas aos nematoides do que as larvas isoladas e as plantas não atacadas (ALI; ALBORN; STELINSKI, 2010). Eles também detectaram por meio de análises de GC-MS (cromatografia gasosa acoplada ao espectrômetro de massa) dos compostos voláteis, a presença de alguns terpenos somente em plantas atacadas pela praga, concluindo que as raízes do citros em estudo liberaram voláteis específicos como uma defesa indireta em resposta a herbivoria de *D. abbreviatus*, e que alguns desses voláteis têm a função de atrair nematoides entomopatogênicos (ALI; ALBORN; STELINSKI, 2010, 2011). Estudos publicados por Ali, Alborn e Stelinski (2011) com diferentes variedades de citros, concluíram que as espécies mais vulneráveis a nematoides fitopatogênicos possuem custos de defesa reduzidos por emitirem voláteis somente quando necessário, quando os herbívoros atacam. Por outro lado, espécies não suscetíveis investem mais em defesa constitutiva, apresentando um maior custo associado à atração de patógenos. Trabalho conduzido por Boff, Van

Tol e Smits (2002) com nematoides entomopatogênicos (*Heterorhabditis megidis*) e duas espécies de plantas, demonstrou que os juvenis infectivos são altamente estimulados na presença de raízes atacadas e são fortemente atraídos na combinação entre a larva (herbívoros) e raiz. Os autores também observaram que eles são capazes de distinguir entre danos mecânicos e danos por herbívoros nas raízes, deixando de forma implícita uma forte interação tritrófica envolvendo a planta, o inseto herbívoro e o nematoide entomopatogênico. Estudos envolvendo engenharia genética bioquímica e molecular buscaram cultivares capazes de liberar VPIH conhecidos para o recrutamento de nematoides entomopatogênicos para proteger raízes de danos causados por herbívoros (DEGENHARDT et al., 2009; DEGENHARDT; HALL; LYNSKEY, 2003; HILTPOLD et al., 2011).

2.3 Indutores como potencializadores de defesa das plantas

As plantas pertencem de forma simultânea a dois ambientes distintos, a parte aérea e o solo. Enquanto na parte aérea as plantas necessitam ao mesmo tempo que realiza a fotossíntese se defender de herbívoros, patógenos e outros competidores, no solo ela precisa absorver água e nutrientes além de lidar com os ataques subterrâneos. A evolução da diversidade de plantas também é influenciado pelos ataques sofridos pelas mesmas, tanto na parte aérea (AGRAWAL, 2012) como no sistema radicular (MARON, 1998; VAN DAM, 2009; VAN DAM et al., 2003). A parte aérea e o sistema radicular das plantas são interligados e estão em constante comunicação. Herbívoros se alimentando do sistema radicular induzem defesas na parte aérea (VAN DAM, 2009), ao mesmo tempo em que herbívoros se alimentando da parte aérea da planta influenciam o comportamento de herbívoros do sistema radicular (MASTERS; BROWM, 1992). Esses efeitos não precisam ser recíprocos ou equivalentes, sendo que em algumas situações a herbivoria subterrânea pode estimular respostas tanto na raiz e quanto na parte aérea e, por

outro lado, a herbivoria acima do solo pode promover respostas somente na parte aérea (KAPLAN, 2008).

Os danos na planta, acima ou abaixo do solo, por herbívoros ou patógenos, pode induzir respostas específicas nas plantas mediadas por vias de defesa. Essas vias são inumeráveis, inter-relacionadas e diversificadas sendo as duas vias predominantes, a via do ácido salicílico (via de resistência sistêmica adquirida) e da via do ácido jasmônico (via octodecanoide) (THALER, 2002). A via do ácido salicílico é mediada pelo ácido salicílico, que é um hormônio vegetal que é responsável por induzir as principais defesas contra patógenos que se alimentam de tecidos vivos (THALER; HUMPHREY; WHITEMAN, 2012). Por outro lado, a via do ácido jasmônico, predominantemente mediada pelo ácido jasmônico, é responsável por induzir defesas contra herbívoros (THALER; HUMPHREY; WHITEMAN, 2012). Essas vias defensivas não são independentes e podendo ocorrer cross-talk de forma antagônica entre elas (THALER, 2002). Enquanto essas vias defensivas de plantas podem ser induzidas diretamente com a infecção patogênica ou danos por herbívoros, resposta de defesa semelhante pode ser induzida por compostos orgânicos voláteis produzidos por insetos ou outras plantas ou através da aplicação de indutores de defesa como salicilato de metila e o jasmonato de metila (FARMER; RYAN, 1990; PARK, 2007).

Os produtos produzidos após a indução dessas vias podem conferir maior resistência aos patógenos e aos herbívoros (THALER, 2002) alterando a palatabilidade da planta para os herbívoros ou recrutando inimigos naturais com a liberação de compostos orgânicos voláteis (THALER 1999). A atração de inimigos naturais através da liberação de voláteis de plantas induzidos pela herbivoria é um fenômeno já conhecido tanto na parte aérea (KESSLER; BALDWIN, 2001; TURLINGS; TUMLINSON; LEWIS, 1990) quanto no subsolo (ALI; ALBORN; STELINSKI, 2010; RASMANN, 2005), onde

nematoides entomopatogênicos são recrutados por meio de pistas induzidas pela herbivoria. Em citros, sendo danificado pelo caruncho *Diaprepes abbreviatus*, induz a liberação de pregeijerene que recrutam nematoides entomopatogênicos que se desenvolvem no interior dessa mesma praga (ALI; ALBORN; STELINSKI, 2010, 2011). Da mesma forma, na cultura do milho, sob ataque do besouro *Diabrotica virgifera virgifera* induz a liberação de E- β -cariofileno, que também recrutam os nematoides entomopatogênicos *Heterorhabditis megidis* (RASMANN, 2005).

Os indutores de plantas são ativadores de rotas metabólicas específicas nas plantas para a produção de diferentes substâncias, que influenciam de diferentes maneiras no crescimento e desenvolvimento da planta, além de atuar na interação dessas plantas com o ambiente onde elas se encontram. A utilização desses indutores pode ser uma alternativa para proteção de plantas uma vez que irá ativar rotas de defesa atraindo inimigos naturais para próximo das plantas antes mesmo do aparecimento da praga, como uma forma de prevenção.

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**ARTIGO 1 - Stimulation of the Salicylic Acid Pathway Aboveground
Recruits Entomopathogenic Nematodes Belowground**

(Artigo submetido à revista PLoS ONE)

ARTIGO 1**STIMULATION OF THE SALICYLIC ACID PATHWAY
ABOVEGROUND RECRUITS ENTOMOPATHOGENIC
NEMATODES BELOWGROUND**

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ABSTRACT

Plant defense pathways play a critical role in mediating tritrophic interactions between plants, herbivores, and natural enemies. While the impact of plant defense pathway stimulation on natural enemies has been extensively explored aboveground, belowground ramifications of plant defense pathway stimulation are equally important in regulating subterranean pests and still require more attention. Here we investigate the effect of aboveground stimulation of the salicylic acid pathway through foliar application of the elicitor methyl salicylate on belowground recruitment of the entomopathogenic nematode, *Steinernema diaprepesi*. Also, we implicate a specific root-derived volatile that attracts *S. diaprepesi* belowground following aboveground plant stimulation by an elicitor. In four-choice olfactometer assays, citrus plants treated with foliar applications of methyl salicylate recruited *S. diaprepesi* as compared with negative controls. Additionally, analysis of root volatile profiles of citrus plants receiving foliar application of methyl salicylate revealed production of d-limonene, which was absent in negative controls. The entomopathogenic nematode *S. diaprepesi* was recruited to d-limonene in two choice olfactometer trials. These results reinforce the critical role of plant defense pathways in mediating tritrophic interactions, suggest a broad role for plant defense pathway signaling belowground, and hint at sophisticated plant responses to pest complexes.

Keywords: Entomopathogenic nematodes. Tritrophic interactions.
Plant defense pathways. Natural enemies.

1 INTRODUCTION

Plants adopt constitutive and induced strategies to defend against herbivores and pathogens both aboveground and belowground [1, 2]. These defenses can act directly against the offending herbivore, producing or releasing toxins that deter feeding behavior [3]. Indirectly, these defenses can result in the release of herbivore induced plant volatiles that recruit natural enemies [3]. These tritrophic interactions involving recruitment of natural enemies have been observed aboveground [4, 5] and belowground where feeding by larvae of *Diabrotica virgifera virgifera* results in release of *E-β* caryophyllene and recruits the entomopathogenic nematode *Heterhorabditis megidis* [6]. Similarly, in citrus, feeding belowground by larvae of the weevil *Diaprepes abbreviatus* results in release of pregeijerene which recruits a wide variety of nematodes, including entomopathogenic nematodes that are natural enemies of *D. abbreviatus* [7-9].

These tritrophic interactions between plants, herbivores, and their natural enemies above and belowground are mediated by stimulation of defense pathways within plants [3]. Stimulation of these plant defense pathways can occur through herbivory [10], plant-to-plant communication [11], or application of chemicals that elicit plant defense responses [12]. Among a myriad of plant defense pathways, a prominent pathway that has important roles in plant defense against both pathogens and herbivores is the salicylic acid pathway [13, 14]. It is so called because of the prominent role salicylic acid plays in stimulating plant defense and its known role in recruiting natural enemies aboveground [15].

In addition to its role in recruiting natural enemies aboveground, the salicylic acid pathway also mediates interactions between herbivores and pathogens. Stimulation of the salicylic acid pathway through synthetic elicitors can reduce bacterial lesion development [16] and can affect plant resistance to

herbivores [17]. In addition, the sequence of induction can have ramifications for plant defense pathway stimulation and herbivore-pathogen resistance [16, 18]. Multiple stimulation of plant defense pathways also has tritrophic effects on natural enemies aboveground [19].

Less is known regarding the role the salicylic acid plant defense pathways play in mediating plant responses belowground. While stimulation of plant defenses aboveground has effects belowground, and vice versa, the dynamic nature of plant defense pathways in mediating this communication between the terrestrial and subterranean environments are less well understood [20-22]. Effects of plant defense stimulation aboveground on interactions belowground are varied and occasionally nonexistent [1, 22, 23]. Similarly, the role of plant defense pathways in stimulating production of herbivore induced plant volatiles for the recruitment of natural enemies belowground is not well understood.

Here, we explore the effect of stimulating the salicylic acid pathway aboveground on recruitment of natural enemies belowground. To do so, we applied an elicitor, methyl salicylate, to the leaves of citrus seedlings while monitoring the response of the entomopathogenic nematode *Steinernema diaprepesi* belowground both in the presence and absence of the larval weevil herbivore *Diaprepes abbreviatus*, a prominent polyphagous root pest of citrus and many other crops. The entomopathogenic nematode, *S. diaprepesi*, may be the most effective natural enemy of this cosmopolitan root herbivore and therefore we focused on this particular nematode as part of our multi-trophic investigation [24, 25].

2 MATERIALS AND METHODS

To evaluate the effect of plant defense pathway stimulation on recruitment of natural enemies belowground, particularly in the case of the salicylic acid pathway, methyl salicylate was applied to the aboveground portion of citrus seedlings while nematode response was monitored in olfactometer bioassays belowground. Based on the nematode response, volatiles were collected from the roots of treated and control plants. Volatiles unique to treated plants were then evaluated for activity in two choice bioassays.

2.1 Organisms

Response of the infective juvenile stage of the entomopathogenic nematode *Steinernema diaprepesi* to eight inch citrus Swingle Citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.) seedlings was evaluated in four-choice olfactometers. *S. diaprepesi* infective juveniles were originally collected from sentinel *Diaprepes abbreviatus* larvae in Florida citrus groves and then reared on *Galleria mellonella* larvae and collected on White traps [26, 27]. *S. diaprepesi* infective juveniles were maintained in shallow tissue culture flasks at 14oC and were used within two weeks after emergence. Fifth instar *Diaprepes abbreviatus* larvae used in methyl salicylate bioassay trials were reared on artificial diet from eggs laid by adults collected from Florida citrus groves [28, 29].

2.2 Methyl Salicylate Bioassays

The attraction of the entomopathogenic nematode *S. diaprepesi* to citrus seedlings treated with foliar applications of elicitors in the presence and absence of belowground herbivory by *D. abbreviatus* larvae was evaluated in four-choice

olfactometers filled with clean autoclaved sand adjusted to 12% moisture by volume. Four choice olfactometers were constructed from 4x4x4 inch containers (Tupperware Corporation, Orlando, FL) perforated on each of the four sides to accommodate 2 inch PVC pipe elbows. Connections were sealed with insulation and one citrus seedling was placed in each of the elbows. After allowing 48 hours for acclimatization, plants were treated with elicitor sprays. In each four choice olfactometer, two opposing seedlings received treatment with methyl salicylate (MeSA) and two opposing seedlings were left as untreated, negative controls. Methyl salicylate treated seedlings each received 130 μ l of methyl salicylate by foliar spray in a Tween 20 and ethanol solution at 0.1mL/L and 2.5mL/L respectively. Control seedlings did not receive the elicitor, only the Tween 20 and ethanol solution. For experiments involving *D. abbreviatus* herbivory, five approximately five week old *D. abbreviatus* larvae were placed directly on the roots of methyl salicylate treated and control seedlings. Forty-eight hours after application of the elicitors, approximately 2500 *S. diaprepesi* infective juveniles were released into the center of the olfactometer. After an additional 24 hours, nematodes were extracted from the responding arms using sugar centrifugation, then counted [30].

2.3 Volatile Collection and Analysis

To investigate the potential role of volatile-mediated nematode attraction in the four arm olfactometers, volatiles were collected from the root systems of untreated citrus seedlings and seedlings treated with methyl salicylate. Volatiles were collected 48 hours after application of elicitors for one hour onto 30mg HayesepQ adsorbent filters (Volatile Assay Systems; VAS) at a flow rate of 160ml/min. Extracted volatiles were eluted off of the collection filters with two

aliquots of $75\mu\text{l}$ methylene chloride. Five microliters of $1.5\mu\text{g}/\mu\text{l}$ nonyl acetate was added as an internal standard. A one microliter aliquot of each sample was then injected onto a Clarus 500 gas chromatograph - mass spectrometer (PerkinElmer, Waltham, MA) containing a $30\text{m} \times 0.25\text{mm-ID}$ DB-5 capillary column. The column was held at 35°C for 3 minutes after injection and then increased 10°C per minute until reaching 260°C where it remained for an additional five minutes. Helium was used as a carrier gas at a flow rate of 2 ml per minute. EI spectra were compared with references found in the NIST Mass Spectral Library (2008) and then confirmed with available standards. Differences in volatile profiles between treated and control plants were examined and quantified by comparison to the nonyl-acetate internal standard.

2.4 Volatile Bioassays

To investigate whether d-Limonene, primarily responsible for the differences between volatile profiles of methyl salicylate treated and untreated control plants (see Results), may attract *S. diaprepesi*, two-choice sand-filled assays consisting of inverted 1.5 inch diameter PVC T-Tubes, capped on each end, were used. Individual assay tubes were filled with clean autoclaved sand adjusted to 12% moisture by volume after placing filter paper treated with either a blank control, $10\mu\text{l}$ of water, or $10\mu\text{l}$ aliquots of doses of d-Limonene in water for a total of 17ng , 170ng , $1.7\mu\text{g}$, or $17\mu\text{g}$ at opposing ends of the olfactometer. Approximately 2000 *S. diaprepesi* infective juveniles were applied to the central orifice of each olfactometer. After 24 hours, responding nematodes were extracted from the sand in each PVC cap using Baermann funnels and counted [31].

2.5 Statistical Analysis

S. diaprepesi infective juvenile response to salicylate-treated citrus plants in four choice olfactometers was summed within each replicate for each treatment to avoid aggregation effects then examined for normality by visual inspection with quantile-quantile plots and Shapiro-Wilk's test. Wilcoxon signed rank tests were then used to evaluate preference. Differences in volatile profiles between treated and control plants were quantified through comparison to internal standards. Mean quantities of collected volatiles were calculated and bootstrapped to determine 95 percent confidence intervals. *S. diaprepesi* infective juvenile preference for doses of d-Limonene in two choice olfactometers was evaluated by determining the percentage of infective juveniles responding to d-Limonene in each replicate for each dose. Preference percentages were examined for normality through visual inspection with quantile-quantile plots and interrogation with Shapiro-Wilk's test and subsequently evaluated for differences from a 50% response of no preference through one-sided t-tests with Bonferroni correction. Data were collated in Microsoft Excel 2011 and analyzed using R version 3.2.2 [32] in the R Studio version 0.99.484 development environment [33]. Analysis was facilitated using the packages *xlsx* [34] for interface with Microsoft Excel, *tidyr* [35] and *dplyr* [36] for data arrangement and summary statistics, *ggplot2* [37] for graphics capabilities, and *scales* for visual representation of scaling [38].

3 RESULTS

3.1 Methyl Salicylate Bioassays

The infective juveniles of the entomopathogenic nematode *S. diaprepesi* significantly ($p = 0.01$) preferred (27.7%; 95% CI: 16.4%, 38.9% difference) plants treated with methyl salicylate (MeSA) over control plants in the absence of a weevil pest (Fig. 1). Data were non-normal by visual inspection and interrogation with the Shapiro-Wilk normality test ($W = 0.83$, $p = 0.004$). In the presence of belowground feeding by the insect herbivore *D. abbreviatus* on both the control and treated plants, methyl salicylate treated plants were not significantly ($p = 0.25$) more attractive than controls (Fig. 1).

3.2 Volatile Collection and Analysis

d-Limonene (retention time 14.38) was present in root volatile profiles of methyl salicylate treated plants but not detectable in the controls (Fig. 2). An average of $0.61\text{ng}/\mu\text{l}$ (from 0.04 to $2.22\text{ng}/\mu\text{l}$) d-Limonene was detected in eluted samples from methyl salicylate treated plants; total amount collected averaged 91.5ng .

3.3 Volatile Bioassays

Entomopathogenic nematode *S. diaprepesi* infective juveniles significantly ($p_{adj} = 0.02$) preferred d-Limonene at doses of $17\mu\text{g}$ in two choice olfactometer assays as compared with negative controls (Fig. 3). Data were not significantly different from normal by visual inspection with quantile-quantile plots and interrogation with the Shapiro-Wilk test ($p > 0.28$). Preferences for d-Limonene at other doses were not significantly different from 50% ($p_{adj} > 0.32$).

4 DISCUSSION

Stimulation of the salicylic acid pathway through aboveground application of methyl salicylate results in recruitment of the entomopathogenic nematode *S. diaprepesi*. Herbivory by larvae of the weevil *D. abbreviatus* attenuates this response. Attraction in the absence of the weevil herbivore is likely mediated by belowground root release of the volatile d-Limonene. This result suggests that insect larval feeding induces a competitive plant defense response belowground.

These results highlight, for what we believe to be the first time, the direct role of the salicylic acid pathway in releasing induced plant volatiles for the recruitment of entomopathogenic nematode natural enemies belowground. While previous work has shown that herbivory belowground by the weevil *D. abbreviatus* can induce production of pregeijerene and attract entomopathogenic nematodes [8], the effects of stimulating the salicylic acid pathway on recruitment of subterranean natural enemies suggests a broader role for plant defense signaling for belowground natural enemies of herbivores.

This signaling serves little purpose if no receiver perceives the stimulus. The response of entomopathogenic nematodes to the d-Limonene cue suggests that the entomopathogenic nematodes in this system are highly attuned to the volatiles in their environment. Entomopathogenic nematodes have been shown to respond to herbivory in connection to a variety of plant and herbivore species and to a variety of induced host plant volatiles belowground (e.g., *E-β* caryophyllene and pregeijerene) [6, 8, 39]. In previous work, however, such induced host plant volatiles were produced through herbivory or mechanical damage of a potential host. In our case, the d-Limonene cue was released after stimulation of the salicylic acid pathway aboveground and in the absence of weevil herbivory. This may provide a different and complementary information

pathway for plant defense belowground and does not simply signal presence of a host herbivore feeding on the roots.

Indeed, feeding by the weevil herbivore seems to attenuate the response of belowground entomopathogenic nematodes. In the absence of salicylic acid pathway stimulation, herbivory by *D. abbreviatus* on Swingle Citrumelo citrus seedlings recruits entomopathogenic nematodes through release of the herbivore-induced volatile pregeijerene [8]. In the absence of herbivory, salicylic acid pathway stimulation recruits entomopathogenic nematodes through release of d-Limonene. It was only in the case where herbivory by larvae of the weevil *D. abbreviatus* was coincident with stimulation of the salicylic acid pathway that entomopathogenic nematode response was attenuated in this investigation. This interaction suggests a possible case of crosstalk between plant defense pathways. Insect herbivory has been shown in many instances to stimulate the jasmonic acid pathway [2, 14]. The jasmonic acid pathway, when stimulated, can antagonistically interact with the salicylic acid pathway, in some cases shutting down plant defense response [14].

While the jasmonic acid pathway is traditionally associated with plant responses to herbivory, stimulation of the salicylic acid pathway is often associated with defense against biotrophic pathogens [14]. In this case, its role in recruiting natural enemies may seem counter intuitive. Indeed the evolution and advantages of such attraction remain to be explored. One possible explanation is that the citrus-*D. abbreviatus*-entomopathogenic nematode interaction is not a simple closed system. There is a fourth, and prominent, player. The oomycete *Phytophthora* is frequently found in association with *D. abbreviatus* herbivory. Wounding of plant roots by *D. abbreviatus* opens a passage for infection by *Phytophthora* causing much greater damage to citrus trees and other plants than weevil herbivory alone [40]. The *Phytophthora-Diaprepes* weevil system is a complex that must be considered when developing management strategies for

commercial citrus and plant production [41]. Because *Phytophthora* infections frequently accompany belowground weevil herbivory, recruitment of entomopathogenic nematodes by stimulation of the salicylic acid pathway may be an effective response for defense against attack by both an insect herbivore and a phytopathogen. We are currently exploring this hypothesis.

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FIGURES

Figure 1 *S. diaprepesi* attraction to methyl salicylate (MeSA) treated citrus seedlings. Entomopathogenic nematode *S. diaprepesi* infective juvenile response to citrus seedlings treated aboveground with methyl salicylate in four choice sand filled olfactometers both in the presence and absence of belowground herbivory by *D. abbreviatus* weevil larvae. Bars and error bars denote mean number of respondents and standard error respectively. *S. diaprepesi* infective juveniles significantly ($p = 0.01$) preferred (27.7%; 95% CI: 16.4%, 38.9% difference) plants treated with methyl salicylate (MeSA) over control plants in the absence of weevil feeding damage.

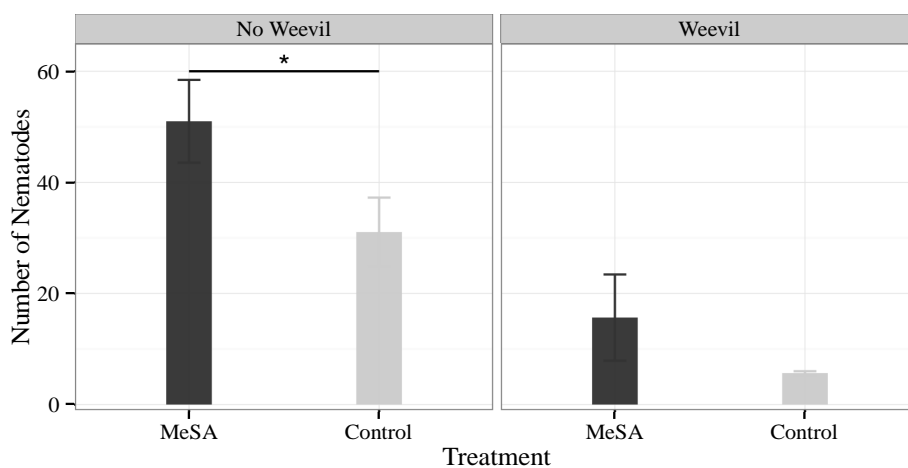


Figure 2 Volatile Profiles of Methyl Salicylate Treated and Control Plants. Sample chromatograms with volatile profiles of methyl salicylate treated (above) and control (below) plants. d-Limonene (retention time 14.38; from 0.04 to 2.22ng) was present in treated plants, but not in controls (n = 10). Nonyl acetate was used as an internal standard. Decane (a) was also recovered in both standards and controls

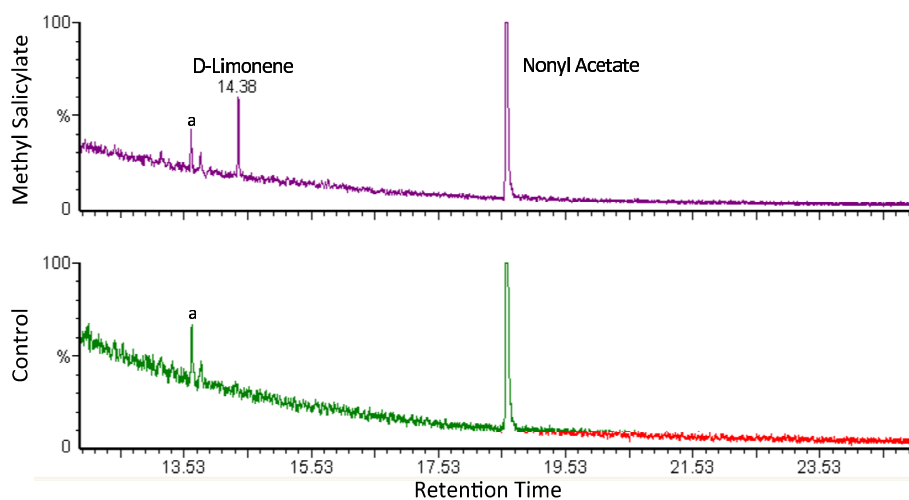
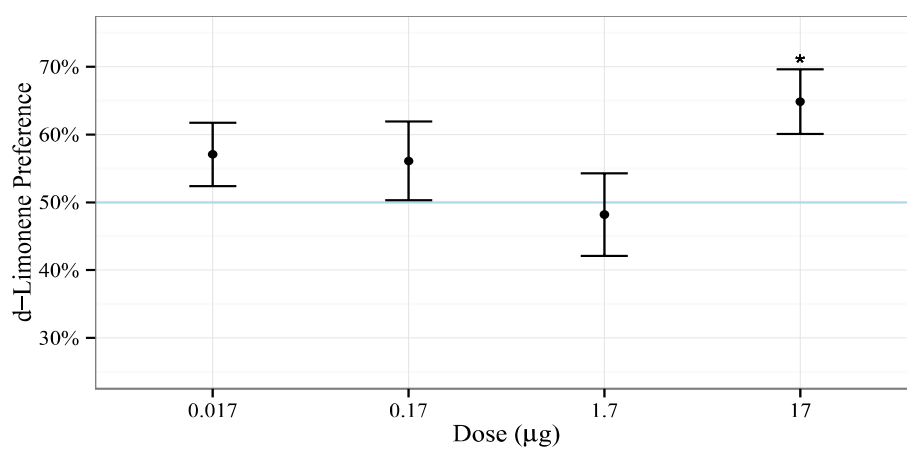


Figure 3 *S. diaprepesi* preference for d-Limonene. Entomopathogenic nematode *S. diaprepesi* infective juvenile preference for doses of d-Limonene as evaluated in two choice sand filled olfactometers. 50% response (horizontal blue line) indicates no preference. Points and error bars denote mean and standard error respectively. *S. diaprepesi* significantly ($p_{adj} = 0.02$) preferred d-Limonene at doses of $17\mu\text{g}$



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**ARTIGO 2 - Elicitors aboveground: an alternative for control of a
belowground pest**

(Artigo revista PLoS ONE)

ARTIGO 2**ELICITORS ABOVEGROUND: AN ALTERNATIVE FOR
CONTROL OF A BELOWGROUND PEST**

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ABSTRACT

Plant defense pathways mediate multitrophic interactions above and belowground. Understanding the connection between plant defense systems above and belowground and the effects of those systems on pests and natural enemies holds great potential for designing effective control strategies. Here we investigate the tritrophic interactions between corn plants treated aboveground with plant defense elicitors (Methyl Salicylate and Methyl Jasmonate), adults of *Diabrotica speciosa*, a polyphagous pest of many crops including corn, and *Heterorhabditis amazonensis*, an entomopathogenic nematode subterranean natural enemy of *D. speciosa* larvae used for biological control. The response of *D. speciosa* and *H. amazonensis* were evaluated independently in olfactometers to all pairwise combinations of corn plants with mechanical damage, pest damage, foliar methyl jasmonate treatment, foliar methyl salicylate treatment, and control (no treatment, no damage). While mechanical damage and methyl jasmonate treatment did not significantly recruit large numbers of either species, both pest damaged plants and those treated with methyl salicylate were more attractive to *D. speciosa* and *H. amazonensis*. The enhanced recruitment to pest damaged and methyl salicylate treated plants suggests that 1) aboveground stimulation of corn defenses holds ramifications for belowground multitrophic interactions, 2) the elicitor Methyl Salicylate may produce induced susceptibility, and 3) provides an option for augmenting volatile mediated crop management.

Keywords: Elicitor; Entomopathogenic nematodes. Tritrophic interactions. Plant defense pathways. Natural enemies.

1 INTRODUCTION

Plants simultaneously inhabit two dynamic environments. In addition to procuring energy through photosynthesis, plant shoots must contend with a variety of aboveground herbivores. Likewise, in addition to procuring water and nutrients, plant roots must contend with attacks from below. Attacks against plants have driven evolution of plant defenses that protect the two critical missions of plant shoots and roots above [1] and belowground [2-4].

Damage to the plant, above or belowground by herbivore or pathogen, can induce specific plant responses mediated by defense pathways within the plant. These pathways are myriad, interrelated, and diverse, but the two most prominent pathways are the salicylic acid pathway and the jasmonic acid pathway [5]. The salicylic acid pathway, mediated predominantly by the plant hormone salicylic acid, is thought to be induced by and primarily responsible for defense against pathogens feeding on living tissue [6]. In contrast, the jasmonic pathway, mediated predominantly by jasmonic acid, is thought to be induced by and predominantly responsible for defense against herbivores [6]. These defensive pathways are not independent; there can be antagonistic cross-talk between the salicylic and jasmonic acid pathway [5]. While these plant defensive pathways may be directly induced through pathogen infection or herbivory, similar defense responses may be induced by volatile organic compounds produced by insects or other plants [7] or through application of methyl salicylate or methyl jasmonate elicitors [8, 9]

Products produced after induction of these pathways can confer increased resistance to pathogens and herbivores [5] by altering plant palatability [10] or recruiting natural enemies through release of volatile organic compounds [11]. Attraction of natural enemies through release of herbivore induced plant volatiles is a well known phenomenon both aboveground [12, 13] and belowground [14,

15] where entomopathogenic nematodes recruit to herbivore induced cues and infect insect larvae feeding on plant roots. In citrus, for example, feeding by the weevil *Diaprepes abbreviatus* induces release of pregeijerene which recruits an array of entomopathogenic nematodes [15, 16]. Similarly, in corn, feeding by the beetle *Diabrotica virgifera virgifera* induces release of E- β caryophyllene which recruits the entomopathogenic nematode *Heterorhabditis megidis* [14].

The effects of root herbivory and belowground release of herbivore induced plant volatiles are not limited to the rhizosphere, however. Belowground herbivory can induce defenses aboveground [3], while herbivory aboveground can have consequences for belowground herbivores [17]. These effects need not be reciprocal or equivalent; in some cases, belowground herbivory can stimulate responses in both the root and the shoot, while aboveground herbivory elicits shoot only responses [18].

Here we investigate the effect of aboveground induction of plant defensive pathways in corn (*Zea mays* L.) on recruitment of pests and natural enemies above and belowground using the South American corn rootworm *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae) and the entomopathogenic nematode *Heterorhabditis amazonensis*. While the adult beetle *D. speciosa* is a widespread, prominent, and polyphagous pest of corn and soybeans in South America, much of the damage to corn results from *D. speciosa* larvae feeding on corn roots [19, 20]. *H. amazonensis* is a natural enemy and parasite of *D. speciosa* present endemically and applied for biological control [21, 22]. The corn, *D. speciosa*, and entomopathogenic nematode *H. amazonensis* represent a tritrophic system; the effects of plant defense pathway stimulation on the multitrophic interactions present in this system hold ramifications and implications not only for our understanding of such systems generally, but also for designing intelligent biological control strategies in the field.

2 MATERIALS AND METHODS

To investigate the effect of aboveground induction of plant defensive pathways in corn on recruitment of pests and natural enemies above and belowground, we presented undamaged, mechanically damaged, pest damaged, and elicitor treated plants to adult beetle *D. speciosa*, and *H. amazonensis* entomopathogenic nematodes in multiple choice and two choice olfactometers above and belowground.

2.1 Plant Materials

Bt transformed Herculex I (Dow AgroSciences, Pioneer Hi-Bred International) corn seedlings expressing the Cry1F gene were used in all experiments. This variety was developed primarily to control Fall Armyworm (*Spodoptera frugiperda*) and is in widespread use in Brazil. Seeds were germinated in moist vermiculite, then grown for twenty days in organic substrate. Prior to use in belowground bioassays with larval *D. speciosa* and entomopathogenic nematodes, corn seedling roots were gently washed to remove substrate before placement in olfactometers.

2.2 Insect Rearing

Adult *D. speciosa* were collected from corn fields maintained by the Federal University of Lavras (Lavras, MG, Brazil) and taken directly to the laboratory for rearing following previously established methodology [23]. Adults were maintained on bean leaves (*Phaseolus vulgaris*) while eggs were collected from black gauze strips placed with the adults to induce oviposition. Prior to use in experiments, adults were starved for 24 hours. Eggs were washed

from the gauze strips every two days then placed in petri dishes with moistened filter paper to maintain humidity until eclosion. Larvae were maintained on recently germinated corn seedlings in vermiculite until pupation.

2.3 Nematode Rearing

Entomopathogenic nematode *H. amazonensis* infective juveniles used in multiple choice olfactometers were obtained from cultures maintained at the Federal University of Lavras where the nematodes were reared on greater wax moth (*Galleria mellonella*) larvae. Wax moth larvae were reared in the laboratory [24] and maintained on artificial media [25]. When nematode infective juveniles were needed, *G. mellonella* larvae were inoculated with entomopathogenic nematodes [26] and subsequently collected on White traps [27]. After collection, nematodes were maintained in culture flasks in aqueous suspension at $16 \pm 1^\circ$ C and used in bioassays within a week of collection.

2.4 Elicitor Preparation

For preparation of elicitors for application to the aboveground parts of maize seedlings, Tween 20 and ethanol were added to water such that final concentrations were 0.1mL/L and 2.5mL/L respectively. Concentrations of methyl salicylate and methyl jasmonate were adjusted to 0.5mM then added to solution such that the amount used per plant per experiment was $65\mu\text{L}$ and $109\mu\text{L}$ respectively. Aliquots of 30mL elicitor solution were applied to the aboveground foliage of corn seedlings using a spray bottle, a quantity sufficient for the corn seedling to become wet and to ensure homogenous application. Contact of elicitor solution with the roots was prevented by an aluminum foil barrier.

2.5 Adult *D. speciosa* Bioassays

The responses of adult *D. speciosa* to treated and untreated corn plants were evaluated in two choice Y-tube glass olfactometers. Aboveground portions of plants with the desired treatments were placed in glass chambers where filtered, humidified air was introduced then pumped via teflon tubes to the olfactometer, one treatment per arm. Adult *D. speciosa* were introduced at the base of the Y-tube olfactometer and allowed five minutes to choose an arm; response time and treatment choice were monitored. Treatments were rotated every three insects to avoid positional effect. Ten replications of each treatment combination were conducted with each replicate consisting of ten insect choices for a total of 100 insects assayed for each contrast. Clean glassware and new plants were used for each replicate.

Treatment combinations consisting of undamaged, mechanically damaged, pest damaged, and elicitor untreated and treated plants were used to determine the effect of plant defense pathway stimulation on *D. speciosa* response. Twenty day old corn seedlings were used in all experiments; undamaged corn seedlings were taken directly from the greenhouse 20 days after germination and immediately used in the experiment. Mechanically damaged corn seedlings were cut with a scalpel using a template replicating foliar damage by adult *D. speciosa* 48 hours prior to use in the experiment. Pest damaged plants were fed upon by five adult *D. speciosa* placed in mesh bags on the foliage of each plant 48 hours prior to use in the experiment. Elicitor treated (either methyl salicylate or methyl jasmonate) plants were treated as described above 48 hours prior to use in experiments.

2.6 Nematode Bioassays

Entomopathogenic nematode *H. amazonensis* response to treated and untreated corn plants was evaluated in sand filled multiple choice olfactometers consisting of a central chamber connected to eight arms into which corn seedlings were inserted. Olfactometers were constructed from 30cm diameter plastic containers (Tupperware) perforated at equally spaced intervals to which eight 4cm diameter PVC elbows were connected. Seventy two hours prior to start of the experiment, olfactometers received corn seedlings and were filled with washed autoclaved sand adjusted to 12% moisture by volume. Treatment contrasts were arranged in alternating fashion around the eight arm olfactometer. After 72 hours of acclimation to the new environment, the aboveground foliage of corn seedlings was treated as described above to evaluate the ability of undamaged, mechanically damaged, pest damaged (above ground feeding by adult *D. speciosa*), and elicitor treated plants to recruit entomopathogenic nematodes belowground. Forty eight hours after treatment application, 2500 *H. amazonensis* infective juveniles were released in the center of each olfactometer. An additional 24 hours later, the olfactometers were disassembled, the responding nematodes extracted via Baermann funnel, and evaluated. Tests of known amount of nematodes placed in Baermann funnels and replicated 20 times yielded an extraction efficiency of $13.1 \pm 1.4\%$. Nematode counts were adjusted accordingly; four replications of each treatment combination were evaluated.

2.7 Statistical Analysis

Response times and preferences for adult *D. speciosa* in two choice Y bioassays were analyzed using multivariate analysis of variance (MANOVA). Mean response time and proportion of adults responding to treatment of interest

were calculated for each replicate; bioassay treatments were used to model variation in those two parameters. *Diabrotica* Time Preference Indices were constructed from the first linear discriminant function and evaluated with Roy's greatest characteristic root test. Conformation to assumptions of normality and homoscedasticity was verified through visual examination of residual plots, the Shapiro-Wilk test, and Levene's test. Significant results from MANOVA were further explored with Dunnett's test, comparing treatments of interest to baseline adult *D. speciosa* responses to Air vs Air trials.

Nematode responses in eight arm sand filled olfactometers were analyzed using repeated G-tests of goodness of fit with Bonferroni corrections. To avoid effects of aggregation (presented with equivalent treatments, nematodes will often aggregate in one treatment arm), responses to individual treatments were summed within replicates. Following procedures for repeated G-tests of goodness of fit [28], individual G-tests of goodness of fit were applied to each replicate, G-tests of independence were applied to each treatment combination to determine heterogeneity of responses, G-tests of goodness of fit were applied to pooled responses for each treatment combination, and G-statistics from each replicate were summed to determine overall significance. Raw numbers of nematodes responding were converted to percentages to facilitate visualizing comparisons between treatment combinations.

All data were collated in Microsoft Excel then read into R version 3.2.2 [29] for analysis. RStudio version 0.99.484 [30] was used as a development environment. Various supplementary packages were used in R for additional functionality: *xlsx* [31] for interface with Microsoft Excel, *tidyr* [32] and *dplyr* [33] for data arrangement and summary statistics, *ggplot2* [34] for graphics capabilities, *RVAideMemoire* [35] for G-tests of goodness of fit, *car* [36] for MANOVA statistics, and *multcomp* [37] for multiple comparisons using Dunnett's test.

3 RESULTS

3.1 Adult *D. speciosa* Bioassays

Multivariate analysis of variance revealed that treatment had a significant ($F = 2.63$; $df = 15,146$; $p = 0.002$) effect on adult *D. speciosa* preference and response time in two choice Y tube bioassays. Data conformed to assumptions of normality (visual inspection; $p > 0.05$ Shapiro Wilk Test) and homoscedasticity ($p = 0.18$, Levene's Test). The linear combination of preference and response time identified by MANOVA and used as the *Diabrotica* Time Preference Index is:

$$D_{TP} = -.992 * Preference - 0.129 * ResponseTime$$

There was a trend towards preference for and faster response to increasing damage of corn plants (Fig. 1). There was significantly ($p = 0.03$) higher preference for and faster response to methyl salicylate (MeSA) treated plants versus corn when compared to a baseline of air versus air (Fig. 1). Additionally, there was significantly ($p = 0.03$) higher preference for and faster response to methyl salicylate treated plants versus mechanically damaged plants when compared to a baseline of air versus air (Fig. 2). Contrasts with methyl salicylate treated plants versus pest damaged plants and methyl salicylate treated mechanically damaged plants seemed to negate that effect ($p > 0.33$). Likewise, methyl jasmonate (MeJA) contrasts (Fig. 3) did not elicit significant effects ($p > 0.29$) as compared to adult *D. speciosa* responses to air versus air.

3.2 Nematode Bioassays

Entomopathogenic nematode *H. amazonensis* infective juveniles preferred corn seedlings damaged aboveground by adult *D. speciosa* over undamaged ($p < 0.001$) and mechanically damaged ($p = 0.0004$) corn seedlings (Fig. 4). Likewise, treatment of corn seedlings with elicitors (methyl jasmonate or methyl salicylate) resulted in recruitment of *H. amazonensis* infective juveniles (Fig. 4). While combination treatments of mechanical damage and application of methyl salicylate increased attraction over mechanically damaged ($p < 0.001$) or methyl salicylate ($p < 0.001$) treated plants (Fig. 5), those same treatments versus undamaged untreated plants and combination treatments of mechanical damage and methyl jasmonate did not have the same effect (significant, $p < 0.001$, heterogeneity between replicates, large ratio between heterogeneity G and pooled G values) (Fig. 6).

4 DISCUSSION

Plant defense pathway stimulation, whether by damage, feeding, or application of elicitors has direct implications for the interactions between corn, *Diabrotica*, and *H. amazonensis* and for control of *D. speciosa* as a pest. The effect of plant defense pathway stimulation aboveground on adult *D. speciosa* response, where increasing damage seems to increase responsiveness, seems to suggest a role for plant defense pathways in mediating adult responses and potentially distributions in the field. Adult *D. speciosa* are well known to monitor and respond to plant volatiles in the lab and in the field [38, 39], responding particularly well to cucurbitacins which may influence progeny fitness [40]. In this case, particularly in regards to treatment with methyl salicylate, stimulation of the salicylic acid pathway may induce release of compounds recognized as favorable by the adult *D. speciosa*. Such recognition may signal presence of other individuals, more available resources, or reduced plant defenses. Adult *D. speciosa* likely recruit to volatiles released by the plant to take advantage of such conditions. Indeed, observations from collections in the field seem to support that hypothesis; adult *D. speciosa* are seldom found individually on corn plants in the field [41, 42]. Corn plants typically host many adult *D. speciosa*, many of them mating pairs [41, 42]. Volatiles emitted by prior feeding or stimulation of plant defense pathways may contribute to their aggregation and mate finding.

Plant defense pathway stimulation aboveground also influences entomopathogenic nematode response belowground; nematodes recruit to corn seedlings fed upon aboveground by *D. speciosa*. While entomopathogenic nematodes are known to respond to plant volatiles induced by herbivores feeding belowground [14, 15], recruitment of *H. amazonensis* to stimulation of plant defenses aboveground by host herbivory and elicitor application suggest a

level of monitoring and adaptation previously unconsidered. Nematodes in this system seem to be highly attuned to the volatile profile of their belowground environment and respond rapidly to changes. In this system, plant volatiles from undamaged plants are attractive to entomopathogenic nematodes (Fig. 4); changes in this volatile profile, as likely occurs in response to aboveground herbivory, can stimulate additional recruitment (Fig. 4). While such recruitment can be variable to combination treatments (Figs. 5–6), additional volatile collection and analysis may shed light on mechanisms behind nematode attraction to plant defense stimulation in this system.

The belowground effects of plant defense pathway stimulation by aboveground herbivory by adult *D. speciosa* can be mimicked through aboveground application of elicitors which can result in recruitment of entomopathogenic nematodes (Figs. 5–6). Monitoring of plant defense pathway stimulation by aboveground herbivory on the part of the entomopathogenic nematode *H. amazonensis* holds adaptive significance; feeding aboveground by the adult *D. speciosa* indicates a greater likelihood of eventually finding suitable larval hosts, especially for cruiser nematodes like *H. amazonensis* which can travel large distances belowground in search of food [43, 44].

While the role of plant defense pathways in mediating multitrophic interactions above and belowground has been noted previously, details of the means and consequences of such mediation are still coming to light [45, 46]. Belowground herbivory has well established influences aboveground on plant defenses and aboveground herbivores [3, 47]. The consequences of aboveground herbivory belowground, however, are just beginning to be understood [3, 48]. What little investigation has occurred in this arena has found varying effects of aboveground herbivory on induced defenses belowground; root chemistry does not change greatly [18], and herbivore effects may be caused by root growth cycles [48]. Our results suggest that the effects of aboveground herbivory

belowground may be indirect. Mediated by plant defense pathways, aboveground herbivory may induce release of volatiles belowground to indirectly control root pests.

Natural means of indirect control of belowground root pests mediated by plant defense pathways, combined with observations of adult response aboveground can be appropriated to augment control in the field. Aboveground, elicitor application can be considered as a means of influencing *D. speciosa* distributions. Belowground, aboveground application of elicitors may also be considered for influencing distribution of entomopathogenic nematodes. Additionally, the volatiles involved in mediating entomopathogenic nematode attraction to aboveground herbivory can be used to augment biological control of larval *D. speciosa* belowground. Balancing aboveground and belowground effects of elicitor application for attraction of herbivores and natural enemies will become critical in developing applied strategies for biological control. We are exploring such possibilities.

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FIGURES

Figure 1 **Adult *D. speciosa* responses to damaged and elicitor treated corn seedlings.** Adult *D. speciosa* choice time and responses in two choice Y tube bioassays. *D. speciosa* Time Preference index is constructed from average response times and preferences for the top treatment in a pair (i.e. for Corn in Corn vs Air); higher numbers indicate a faster response and a greater preference for the top treatment in a pair. Treatment significantly explained response time and preference ($p = 0.002$). Adult *D. speciosa* significantly ($p = 0.03$) preferred and responded faster to Methyl Salicylate treated plants vs Corn when compared to a baseline of Air vs Air. Points and error bars denote mean *D. speciosa* time preference index and standard error respectively.

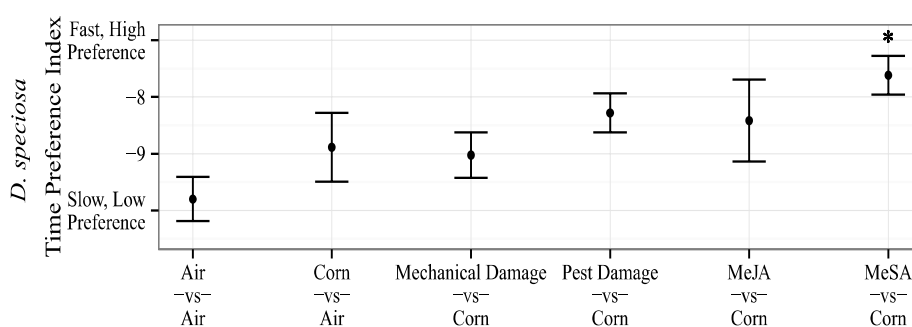


Figure 2 Adult *D. speciosa* responses to Methyl Salicylate treated corn seedlings. Adult *D. speciosa* choice time and responses in two choice Y tube bioassays. *D. speciosa* Time Preference index is constructed from average response times and preferences for the top treatment in a pair (i.e. for MeSA treated plants in MeSA vs Air); higher numbers indicate a faster response and a greater preference for the top treatment in a pair. Treatment significantly explained response time and preference ($p = 0.002$). Adult *D. speciosa* significantly preferred and responded faster to Methyl Salicylate treated plants vs Corn ($p = 0.03$) and Methyl Salicylate treated plants vs mechanically damaged corn ($p = 0.03$ when compared to a baseline of Air vs Air. (MeSA + MD) are mechanically damaged plants treated with methyl salicylate. Points and error bars denote mean *D. speciosa* time preference index and standard error respectively

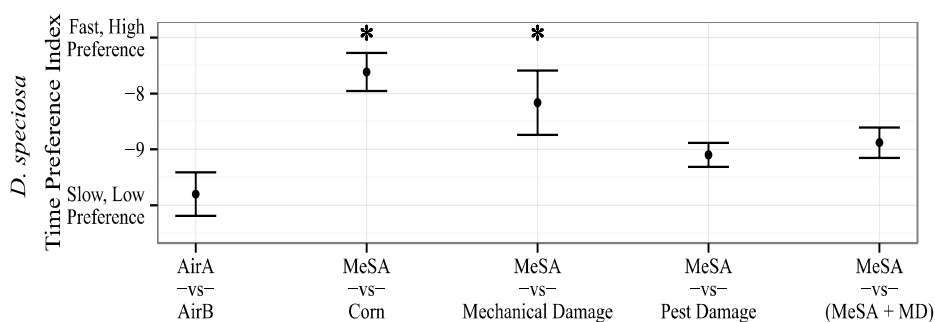


Figure 3 **Adult *D. speciosa* responses to Methyl Jasmonate treated corn seedlings.** Adult *D. speciosa* choice time and responses in two choice Y tube bioassays. *D. speciosa* Time Preference index is constructed from average response times and preferences for the top treatment in a pair (i.e. for MeJA treated plants in MeJA vs Air); higher numbers indicate a faster response and a greater preference for the top treatment in a pair. Methyl jasmonate preferences and response times were not significantly different from those observed in Air vs Air. (MeJA + MD) are mechanically damaged plants treated with methyl jasmonate. Points and error bars denote mean *D. speciosa* time preference index and standard error respectively

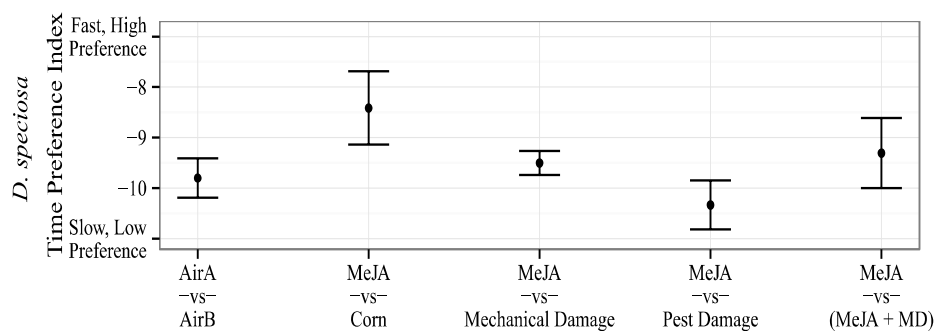


Figure 4 Entomopathogenic nematode *H. amazonensis* infective juvenile responses to aboveground mechanically damaged, pest damaged, and elicitor treated corn plants. Entomopathogenic nematode *H. amazonensis* infective juvenile responses belowground in eight arm sand filled olfactometers to corn plants treated aboveground with mechanical damage, pest damage, methyl salicylate, or methyl jasmonate. *H. amazonensis* infective juveniles preferred corn plants damaged aboveground by adult *D. speciosa* over undamaged ($p \ll 0.001$) and mechanically damaged ($p = 0.0004$) corn seedlings. Bars and errorbars indicate mean percent infective juveniles responding and standard error respectively; errorbars that do not overlap 50% indicate significant differences

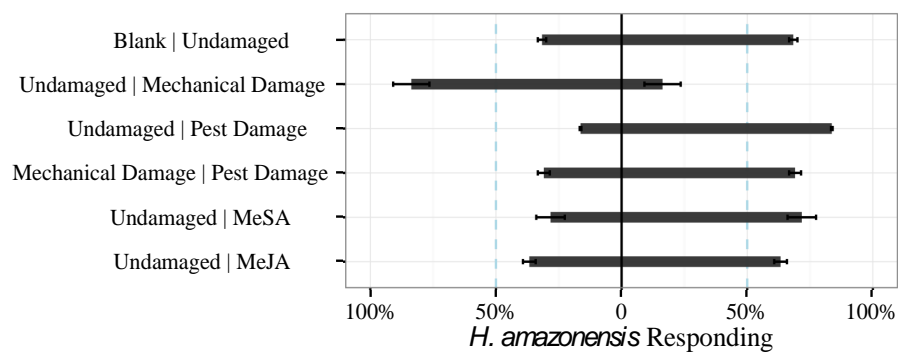


Figure 5 Entomopathogenic nematode *H. amazonensis* infective juvenile responses to corn plants treated aboveground with methyl salicylate. Entomopathogenic nematode *H. amazonensis* infective juvenile responses belowground in eight arm sand filled olfactometers to corn plants treated aboveground with methyl salicylate. (MD + MeSA) treatments indicate a combination of mechanical damage and application of methyl salicylate. Bars and errorbars indicate mean percent infective juveniles responding and standard error respectively; errorbars that do not overlap 50% indicate significant differences

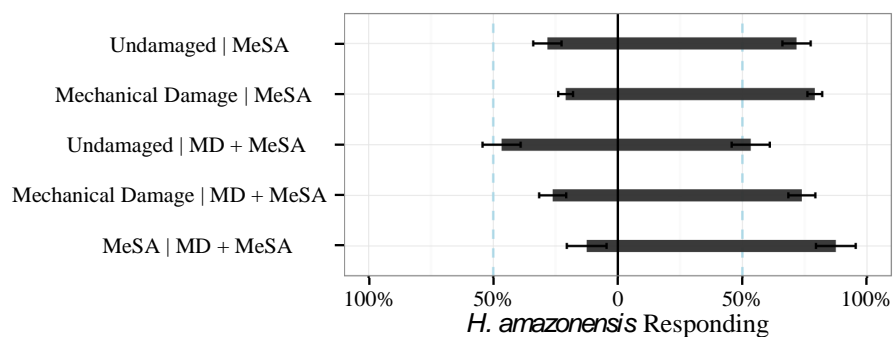
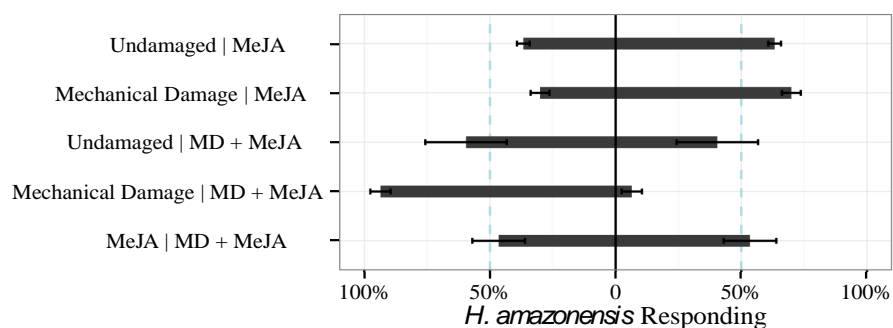


Figure 6 Entomopathogenic nematode *H. amazonensis* infective juvenile responses to corn plants treated aboveground with methyl jasmonate. Entomopathogenic nematode *H. amazonensis* infective juvenile responses belowground in eight arm sand filled olfactometers to corn plants treated aboveground with methyl jasmonate. (MD + MeJA) treatments indicate a combination of mechanical damage and application of methyl jasmonate. Bars and errorbars indicate mean percent infective juveniles responding and standard error respectively; errorbars that do not overlap 50% indicate significant differences



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**ARTIGO 3 - Parameters affecting plant defense pathway mediated
biological control with entomopathogenic nematodes**

(Artigo revista Entomologia Experimentalis et Applicata)

ARTIGO 3**PARAMETERS AFFECTING PLANT DEFENSE PATHWAY
MEDIATED BIOLOGICAL CONTROL WITH
ENTOMOPATHOGENIC NEMATODES**

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ABSTRACT

Entomopathogenic nematodes are natural enemies and effective biological control agents of subterranean insect herbivores. Interactions between herbivores, plants, and entomopathogenic nematodes are mediated by plant defense pathways that can induce release of volatiles that recruit entomopathogenic nematodes. Stimulation of these plant defense pathways for induced defense against belowground herbivory may enhance biological control in the field. Knowledge of parameters affecting entomopathogenic nematode behavior belowground is needed to effectively implement such strategies. To that end, we explore the effect of elicitor, elicitor dose, mechanical damage, and entomopathogenic nematode release distance on recruitment of entomopathogenic nematode infective juveniles to corn seedlings. Increasing doses of methyl jasmonate and methyl salicylate elicitors recruited more entomopathogenic nematodes as did mechanical damage. Recruitment of entomopathogenic nematodes was higher at farther release distances. These results suggest entomopathogenic nematodes are highly tuned to plant status and present a strategy for enhancing biological control using elicitor stimulated recruitment of entomopathogenic nematodes.

Keywords: Tritrophic interactions. Plant defense pathways. Natural enemies.

1 INTRODUCTION

Entomopathogenic nematodes are natural enemies of belowground insect herbivores that have shown promise for biological control in subterranean and cryptic environments [1]. Applications of entomopathogenic nematodes for biocontrol is effective in a variety of cropping systems and new strategies are being developed to extend the abilities of entomopathogenic nematodes to control previously inaccessible pests. Entomopathogenic nematodes have been effective control agents for diptera in mushroom houses [2], for mole crickets and scarab larvae in turf [3, 4], for weevil herbivores in citrus [5, 6], and for corn rootworm *Diabrotica virgifera virgifera* larvae in corn [7, 8]. Extending and enhancing the effectiveness of entomopathogenic nematodes of biological control agents has long been a goal and various means of augmentation including plant genetic manipulation and nematode encapsulation have been pursued [9, 10].

Critical to developing new strategies for enhancing biological control with entomopathogenic nematodes is the use of plant volatiles to manipulate nematode behavior [11, 12]. These strategies many times appropriate existing communication systems in plants that recruit entomopathogenic nematodes to sites of belowground herbivory. In citrus, for example, herbivory by larvae of the weevil *D. abbreviatus* releases the herbivore induced plant volatile pregeijerene which recruits a wide range of entomopathogenic nematodes [13-15]. Similarly, herbivory by weevil larvae on white cedar *Thuja occidentalis* recruits the entomopathogenic nematode *Heterorhabditis megidis* [16]. In corn, too, herbivory by larvae of the western corn rootworm *Diabrotica virgifera virgifera* results in the release of *E-β* caryophyllene which recruits *H. megidis* for control [17].

Recruitment of entomopathogenic nematodes belowground is mediated by plant defense pathways which regulate plant responses to herbivory and pathogen infection [18, 19]. While induction of these pathways can regulate release of plant volatiles belowground as described above, stimulation of these pathways in the

absence of herbivory can also produce effects belowground, potentially regulating responses to pest-pathogen complexes [20]. The two most prominent pathways likely to mediate recruitment of entomopathogenic nematodes belowground are the jasmonic acid and salicylic acid pathways. The jasmonic acid pathway is thought to be stimulated mainly by herbivory and often results in upregulation of plant defenses targeting herbivorous pests [21]. In contrast, the salicylic acid pathway is thought to be stimulated by and mediate resistance to biotrophic pathogen infection [22]. Additionally, both pathways have been implicated in the recruitment of natural enemies aboveground [23, 24] and recent work suggests they could be acting similarly belowground [25].

The role these pathways play in mediating interactions between plants, herbivores, and natural enemies has prompted exploration of the possibility of applying plant defense elicitors for induction of plant resistance in the field. Results of this strategy aboveground have been promising. Stimulation of the jasmonic acid pathway in tomatoes reduces aboveground herbivory with no detrimental effects on yield [26] and is associated with reduction in abundance of many herbivores [27]. Similarly, induced resistance through stimulation of the salicylic acid pathway can reduce bacterial infection [28]. Development of practical applications of elicitors of induced defense for recruitment of entomopathogenic nematodes belowground is being explored and will rely upon knowledge of parameters affecting nematode behavior and application efficacy.

To explore these parameters, we use a corn model system involving the larval herbivore *Diabrotica speciosa*, a polyphagous and ubiquitous pest of corn in South America [29, 30], and the entomopathogenic nematode *Heterorhabditis amazonensis*, a natural enemy of *D. speciosa* with potential for biocontrol [31, 32]. The potential for using elicitor stimulated induced defenses in this system has been raised [25]; here we explore the effect of elicitor, elicitor dose, plant damage, and distance on recruitment of entomopathogenic nematodes belowground.

2 MATERIALS AND METHODS

To explore methods of using elicitors of plant defense to augment biological control of larval *D. speciosa* using entomopathogenic nematodes, we evaluated distance of nematode release and doses of elicitors as possible factors affecting efficacy of entomopathogenic nematode control. Elicitors were applied foliarly while nematode response and infection were evaluated belowground in sand filled arenas.

2.1 Organisms

Corn seedlings were germinated in moist vermiculite and used after cultivation for twenty days in organic substrate. A corn variety in widespread use throughout Brazil, Herculex I (Dow AgroSciences, Pioneer Hi-Bred International), was used in all experiments. *H. amazonensis* entomopathogenic nematodes from cultures maintained at the Federal University of Lavras were reared in larvae of the greater wax moth *Galleria mellonella* and infective juveniles collected using white traps [33, 34]. Collected nematodes were used in experiments within a week of collection. *Galleria* larvae used for rearing entomopathogenic nematodes were likewise reared in the laboratory on artificial diet [35, 36].

2.2 Elicitor Preparations

Methyl jasmonate and methyl salicylate elicitors were applied foliarly to corn seedlings in Tween20 (at 0.1mL/L) and ethanol (at 2.5mL/L) solutions using 30ml calibrated sprays while ensuring homogeneous uniform application. Control plants received spray solutions without elicitors, *i.e.* only Tween20-

ethanol solutions. Applications were prevented from entering the soil using an aluminum foil barrier.

2.3 Dose Response

The effect of different elicitor doses on recruitment of the entomopathogenic nematode *H. amazonensis* was evaluated in eight choice olfactometers filled with sand adjusted to 12% moisture by volume. Eight choice olfactometers were constructed from 30cm diameter plastic containers into which eight 4cm diameter PVC elbows were inserted. Each elbow received one corn seedling which was allowed 72 hours to acclimate to the olfactometer environment prior to receiving elicitor treatment. Methyl salicylate was applied in solutions prepared as described above such that seedlings received either 65 μ L, 130 μ L, or 260 μ L total compound. Similarly, methyl jasmonate was applied in solutions prepared as described above such that seedlings received either 109 μ L, 218 μ L, or 436 μ L. Treatments were arranged in alternating opposition around the eight choice olfactometer. Forty eight hours following treatment application, 2500 *H. amazonensis* infective juveniles were released into the center of each olfactometer. After allowing 24 hours for nematodes to respond, olfactometers were disassembled and nematodes collected from the elbows via Baermann funnels and counted.

2.4 Elicitor Comparison

Contrasts between methyl jasmonate and methyl salicylate were conducted as above for each of the three doses to determine nematode preference for elicitor treatment and dose. As above, experiments were conducted in eight choice sand filled olfactometers observing the same schedule

for seedling planting, application of elicitor treatments, and collection of nematodes. In this case, instead of evaluating effects versus untreated corn seedlings, nematode response to methyl jasmonate treated plants versus methyl salicylate treated plants was evaluated.

2.5 Distance Assays

The effect of distance on recruitment of entomopathogenic nematodes to elicitor treated and mechanically damaged corn plants was evaluated in 20.5 by 20.5 by 75cm rectangular planters. Planters were filled with autoclaved sand adjusted to 12% moisture by volume; corn seedlings were placed at one end of the planter and allowed 72 hours to acclimate to their environment. Following the application period, corn seedlings received elicitor and damage treatment. Corn seedlings either received a control spray containing no elicitors, 65 μ L of methyl salicylate, or 109 μ L of methyl jasmonate prepared as described above. Damage treatment consisted of undamaged control plants or plants receiving mechanical foliar damage with a scalpel mimicking herbivory by adult *D. speciosa*. Forty eight hours after corn seedlings received treatment, entomopathogenic nematode *H. amazonensis* infective juveniles were released either 30 or 60 centimeters from the corn seedling. After allowing twenty four hours for response, nematodes were extracted from the root system of the seedlings using Baermann funnels and counted.

2.6 Statistical Analysis

Entomopathogenic nematode *H. amazonensis* infective juvenile response in eight choice olfactometers to corn seedlings treated with varying doses of methyl jasmonate and methyl salicylate was summed to remove effects

of aggregation then converted to percentages to facilitate comparison across treatments. Percent responses were then examined by visual inspection of quantile-quantile plots and Shapiro-Wilk's test for normality and compared against a null hypothesis of 50% response using a one sample t-test.

Nematode response to methyl jasmonate versus methyl salicylate treated corn seedlings was first summed to obviate effects of nematode aggregation, then converted to percentages to facilitate comparison across treatments. These percent responses were examined for normality by visual inspection with quantile-quantile plots and Shapiro-Wilk's test then evaluated against a null hypothesis of 50% response using a one sample t-test.

The effect of distance on recruitment of the entomopathogenic nematode *H. amazonensis* to damaged and elicitor treated corn seedlings was evaluated with analysis of variance (ANOVA) by using Distance, Damage, and Elicitor to explain nematode response following interrogation with visual inspection of quantile-quantile plots, Shapiro-Wilk's test, and Levene's test to confirm adherence to assumptions of normality and homoscedasticity. Observed significant differences were bootstrapped and confidence intervals reported.

All data were compiled in Microsoft Excel 2011 then analyzed in R version 3.2.2 [37] using the RStudio version 0.99.484 [38] development environment. The following packages were used to facilitate analysis above and beyond base R functionality: *dplyr* [39] and *tidyr* [40] for data management and summary statistics, *ggplot2* [41] for graphics capabilities, *xlsx* [42] for interface with Microsoft Excel, and *car* [43] for ANOVA analysis.

3 RESULTS

3.1 Dose Response

Corn seedlings treated with $109\mu\text{l}$ methyl jasmonate were significantly ($P = 0.01$) more attractive to entomopathogenic nematode *H. amazonensis* infective juveniles versus untreated controls (Figure 1). Similarly, corn seedlings treated with $65\mu\text{l}$ methyl salicylate were significantly ($P = 0.03$) more attractive to infective juveniles versus untreated controls (Figure 1). Increasing doses of methyl jasmonate and methyl salicylate were significantly increasingly more attractive ($P = 0.04$ for methyl jasmonate, $P = 0.001$ for methyl salicylate (Figure 1). Data were not significantly different from normal by visual inspection of quantile-quantile plots and interrogation with Shapiro-Wilk's test ($P > 0.21$).

3.2 Elicitor Comparison

Methyl jasmonate treated mechanically damaged plants were significantly attractive versus methyl salicylate treated mechanically damaged seedlings to nematodes at low doses ($P = 0.01$ at lowest dose, $P = 0.007$ at medium dose)(Figure 2). Methyl salicylate treated undamaged plants were significantly attractive versus methyl jasmonate undamaged plants to nematodes at the highest dose ($P = 0.02$). Data were not significantly different from normal by visual inspection of quantile-quantile plots and interrogation by Shapiro-Wilk's Test ($P = 0.2$).

3.3 Distance Assays

Distance and Damage treatments were significant ($P = 0.002$ for distance, $P = 0.003$ for damage) in explaining *H. amazonensis* response (Figure 3). Elicitor treatment did not show significant effects ($P = 0.535$). An average of 6.27 (95%CI: 2.83, 9.78) more infective juveniles recruited to the seedling when released at 60 centimeters distance than at 30 centimeters. An average of 6.06 (1.97, 10.09) more infective juveniles recruited toward mechanically damaged corn seedlings versus undamaged negative controls. All data conformed to assumptions of normality and homoscedasticity by visual inspection, Shapiro-Wilk's test ($P = 0.62$) and Levene's test ($P = 0.71$).

4 DISCUSSION

Increasing doses of elicitors on aboveground foliage of corn seedlings recruit increasingly more entomopathogenic nematodes belowground. This effect is variable for methyl jasmonate, and particularly strong for methyl salicylate (Fig. 1). In addition, stimulation of the jasmonic acid pathway seems to be attractive in the presence of mechanical damage (Fig 2). These results emphasize the role of plant defense pathways in mediating belowground recruitment of entomopathogenic nematodes and suggest that increased induction of plant defense pathways can potentially augment control. The differential attraction of methyl jasmonate versus methyl salicylate application on mechanically damaged versus undamaged plants suggests that entomopathogenic nematodes can closely monitor differences in plant health, above and beyond simple recognition of herbivore presence as observed previously in corn and citrus [13, 17].

The significant effect of damage in distance assays seems to confirm this observation. Mechanically damaged plants recruited more entomopathogenic nematodes versus undamaged seedlings (Fig 3). In addition to suggesting a broad role for plant signaling belowground, differential recruitment to mechanically damaged seedlings also may present opportunities for adaptive control in the field. Because mechanically damaged seedlings in which the jasmonic defense pathway is stimulated are more attractive to entomopathogenic nematodes, applications of elicitors for induced defense could be tailored for plant health status. Mechanically damaged or physiologically stressed plants could benefit from methyl jasmonate treatment to enhance biological control of root herbivores.

Distance of release also affects recruitment of entomopathogenic nematodes belowground. Interestingly, nematodes released farther away from elicitor treated seedlings responded in greater numbers. While this may seem

counterintuitive, and that greater response is expected closer to the plant, it may be that different signals are at play. The media in which the nematodes and plants are interacting may act as a source or sink of organic volatiles [44]. Closer to the plant, the number and variety of volatiles released by the plant is likely higher; diffusion and adsorption rates of many plant volatiles are variable and higher concentrations of volatiles in specific blends are likely to be present closer in to the plant. Conversely, the number of volatiles permeating and traveling through the pore space up to 60 centimeters is likely to be much lower and limited to smaller, more easily diffusible volatiles. These results could suggest that nematodes are responding to specific blends and, in the absence of those blends, that nematodes fail to orient to host cues. A similar phenomenon has been observed with nematode sex pheromones where previous exposure to sex pheromones disrupts mating [45].

The variable effect of distance on nematode recruitment presents opportunities for further exploration of the basic factors affecting nematode recruitment to volatile signals belowground and suggests that exogenous applications of entomopathogenic nematodes for biological control can be made some distance away from the target area and still have an effect. This long-distance travel of nematodes in response to elicitor treated corn seedlings presents a novel strategy for augmentation biological control of subterranean root herbivores; entomopathogenic nematodes can be applied in a nonspecific manner then called in to areas of herbivory through targeted application of plant defense elicitors. While this strategy remains to be tested in the field, elicitor induced defense could hold promise for enhancing biological control in the belowground environment.

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FIGURES

Figure 1 *H. amazonensis* infective juvenile response to elicitor treatment. Entomopathogenic nematode *H. amazonensis* infective juvenile response to increasing doses of methyl jasmonate and methyl salicylate treated corn seedlings. Elicitor treated plants were more attractive to infective juveniles than untreated controls ($P = 0.01, 0.03$ for methyl jasmonate and methyl salicylate respectively) and increasing doses recruited more infective juveniles ($P = 0.04, 0.001$ for methyl jasmonate and methyl salicylate respectively). Nematode preference represents the percent additional infective juveniles responding to that treatment. Points and error bars denote mean preference and standard error respectively.

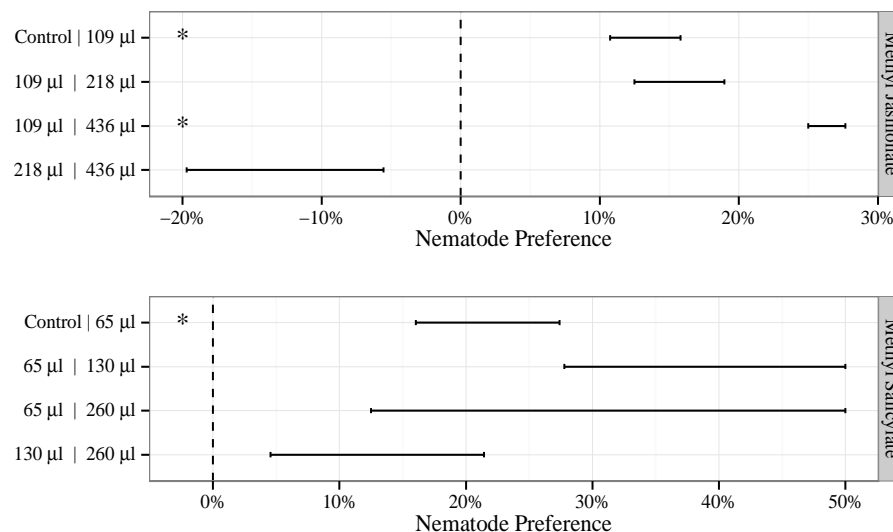


Figure 2 *H. amazonensis* infective juvenile preference for methyl jasmonate versus methyl salicylate treatment. Entomopathogenic nematode *H. amazonensis* infective juvenile response to methyl jasmonate (MeJA) versus methyl salicylate (MeSA) treated corn seedlings at three doses in the presence and absence of mechanical damage. Nematode preference represents the percent additional infective juveniles responding to that treatment. Points and error bars denote mean preference and standard error respectively. * indicates significance at $P < 0.05$; ** indicates significance at $P < 0.01$.

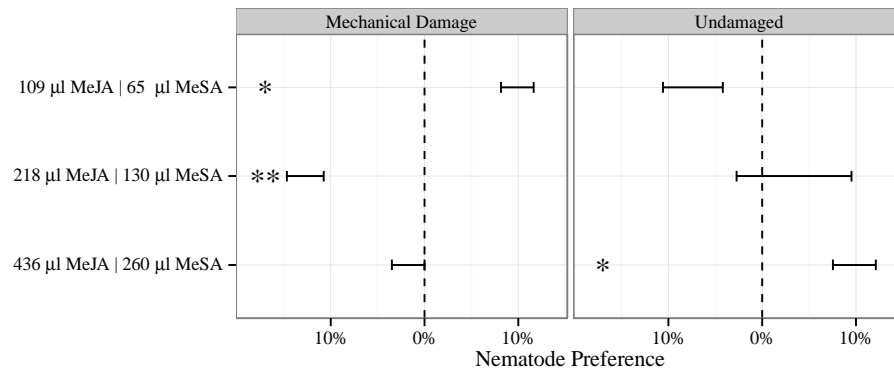
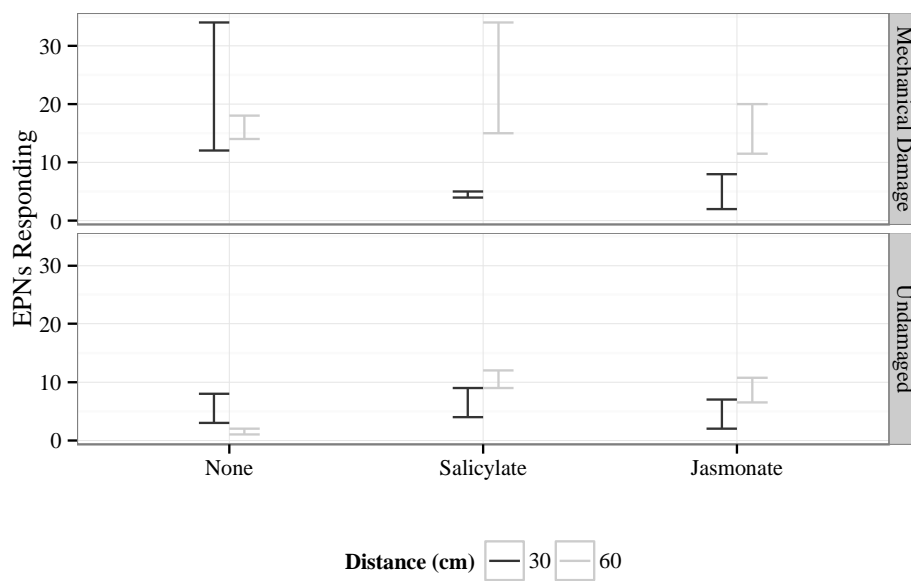


Figure 3 Effect of Distance, Damage, and Elicitor treatment on *H. amazonensis* response. Distance and damage significantly ($P = 0.002, 0.003$ respectively) affected entomopathogenic nematode *H. amazonensis* response to corn seedlings. Points and errorbars denote mean and bootstrapped 95% confidence intervals respectively



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CONSIDERAÇÕES FINAIS

Na agricultura moderna, o melhor aproveitamento dos recursos disponíveis é uma ferramenta imprescindível para alcançar melhores resultados e maiores produtividades. O entendimento detalhado do sistema envolvido torna-se necessário para a melhor utilização das ferramentas disponíveis para alcançar as metas desejadas.

Para as plantas, os fatores que atuam sobre elas são os mais variados, vento, água, nutrição, patógenos, pragas, etc. Esses fatores estão em constante mudança e entender os processos envolvidos no sistema, tornando estes processos favoráveis para um desenvolvimento ótimo das plantas é cada vez mais, uma importante ferramenta para o crescimento agrícola de qualidade. Estudar as interações existentes entre a planta, suas principais pragas e seus inimigos naturais é uma ferramenta importante e estratégica para um bom manejo agrícola.

Uma forma de manipular essa interação de forma indireta, aumentando a capacidade de defesa das plantas, seria a introdução de indutores de defesa.

Neste trabalho, objetivou-se entender sobre as interações que ocorrem na parte aérea e no sistema radicular das plantas, enfatizando o subsolo, suas pragas e os nematoides entomopatogênicos. Investigando o efeito da ativação da via de defesa do ácido salicílico por meio da aplicação foliar do elicitador salicilato de metila no recrutamento de nematoides entomopatogênicos *S. diaprepesi* em plantas de citros. Observou-se que, quando esta via de defesa foi estimulada, as plantas passaram a produzir, em maior quantidade, o composto orgânico volátil d-limonene e que os nematoides entomopatogênicos *S. diaprepesi* são atraídos por este composto.

Nos experimentos realizados para investigar a interação tritrófica entre plantas de milho tratadas com indutores de defesa, adultos de *Diabrotica speciosa* e o nematoide entomopatogênico *Heterorhabditis amazonensis*, observou-se que o elicitor jasmonato de metila não alterou o comportamento de adultos de *D. Speciosa*, bem como o dos nematoides entomopatogênicos.

Nos tratamentos com salicilato de metila, tanto os adultos de *D. speciosa* quanto os nematoides responderam de forma atrativa as plantas tratadas.

Nos experimentos para determinar as doses de aplicação dos indutores de defesa salicilato de metila e jasmonato de metila, observou-se maior atratividade pelos nematoides entomopatogênicos *H. amazonensis* nas maiores doses testadas. Esses resultados sugerem que o aumento da indução de vias de defesa da planta pode, potencialmente, aumentar o controle com a atração de inimigos naturais. A maior atração por plantas danificadas mecanicamente, sugere que os nematoides entomopatogênicos podem perceber diferenças na saúde da planta, não somente o reconhecimento da presença de herbívoros.

Já nos experimentos para estudo da influência das distâncias no recrutamento de NEP nos diferentes tratamentos dos indutores MeJA e MeSA e dos danos na parte aérea, o efeito da variável distância sobre o recrutamento de nematoides apresenta oportunidades para uma maior exploração dos fatores básicos que afetam o recrutamento de nematoides com a sinalização por voláteis no subsolo, sugerindo que as aplicações exógenas de nematoides entomopatogênicos para o controle biológico pode ser feita a uma certa distância da área alvo e ainda ter um efeito. Esta distância mais longa dos nematoides em resposta a plântulas de milho tratadas com elicitor apresenta uma nova estratégia para o aumento do controle biológico de

herbívoros raízes subterrâneas, além disso, sugere que nematoides entomopatogênicos podem ser aplicados de uma região não específica, em seguida serem recrutados para áreas de herbivoria através da aplicação orientada de indutores de defesa nas plantas. Embora esta estratégia dependa de mais estudos, enfatizando o campo, indutores de defesa podem ser uma promessa para melhorar o controle biológico no subsolo.

Estes resultados enfatizam a conexão existente entre a parte aérea e o sistema radicular das plantas mediadas por vias de defesa da planta, e apontam para novas estratégias para melhorar o controle biológico de pragas de insetos subterrâneos com nematoides entomopatogênicos no campo.