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ANA PAULA DE ALMEIDA PORTELA DA SILVA

POTENCIAL BIOTECNOLÓGICO DA ASSOCIAÇÃO DE FUNGOS ENTOMOPATOGÊNICOS EM FORMULAÇÕES COM PRODUTOS VEGETAIS NO CONTROLE DE *Diatraea saccharalis* (LEPIDOPTERA: CRAMBIDAE)

RECIFE - PE

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Recife, 21 de fevereiro de 2014.

“As pessoas fazem seus planos, porém é o Deus Eterno quem dá a última palavra.” Provérbios 16:1 (BLH)

Ao meu esposo e filho, dedico.

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SUMÁRIO **pág.**

LISTA DE FIGURAS **pág.**

CAPÍTULO I

CAPÍTULO II

CAPITULO III

CAPITULO IV

CAPITULO V

CAPÍTULO VI

LISTA DE TABELAS **pág.**

CAPÍTULO III

CAPÍTULO IV

CAPÍTULO V

CAPÍTULO VI

RESUMO

O Brasil é o maior produtor mundial de cana-de-açúcar, porém as condições ambientais favorecem o aparecimento de pragas, dentre elas, *Diatraea saccharalis*, conhecida como broca da cana. Este trabalho teve por objetivo avaliar o efeito de um óleo adjuvante emulsionável e de extratos de *Indigofera suffruticosa* e de *Myrciaria cauliflora* sobre *Metarhizium anisopliae* e *Beauveria bassiana*, bem como a patogenicidade de formulações a *D. saccharalis*. Foram utilizadas as linhagens *M. anisopliae* PL43, *M. anisopliae* IBCB425, *B. bassiana* ESALQ447 e *B. bassiana* ARSEF1398. O efeito fungitóxico foi avaliado por meio da incorporação de Veget'oil® e dos extratos vegetais das folhas e sementes de *Indigofera suffruticosa* e dos frutos de *Myrciaria cauliflora* ao Batata-Dextrose-Ágar (BDA) em diferentes concentrações. O grupo controle foi estava isento de óleo e extrato. Foram analisados os parâmetros biológicos: crescimento vegetativo, produção e germinação de conídios. Os efeitos do armazenamento das formulações (fungo + óleo) foram avaliados levando em consideração o percentual de germinação dos conídios formulados, em dois ambientes (25 ± 1ºC e -7 ± 1ºC). As larvas de *D. saccharalis*, do 3º estágio, foram imersas em suspensões contendo conídios fúngicos, formulação e extratos vegetais. O grupo controle foi inoculado com água destilada autoclavada e óleo. Os bioensaios foram realizados em cinco repetições e observados diariamente, por 10 dias. Veget'oil é compatível com *B. bassiana* ESALQ447, moderadamente tóxico para *M. anisopliae* PL43 e IBCB425 e tóxico para *B. bassiana* ARSEF1398, na menor concentração. Os fungos formulados permaneceram viáveis por até 90 dias, a 25 ± 1ºC. Os conídios formulados estocados a -7 ± 1ºC permaneceram viáveis por 11 meses. Todas as linhagens testadas foram patogênicas a *D. saccharalis*. A formulação em óleo mais eficiente foi a de *Beauveria bassiana* ESALQ447 que causou mortalidade de 74% das larvas. Os extratos vegetais analisados são compatíveis com os fungos e sua associação resultou num maior número de insetos mortos (96%) para *M. anisopliae* IBCB425 e 94% para *M. anisopliae* PL43 ambos com o extrato das sementes de *I. suffruticosa*. O extrato dos frutos de *M cauliflora* associado a *B. bassiana* ESALQ447 matou 84% das larvas. O extrato das folhas de *I. suffruticosa* foi o menos eficiente, atingindo 70% de mortalidade, somente quando associado à formulação. Os resultados demonstram o potencial das formulações e associação dos fungos com os extratos vegetais, que constituem uma alternativa viável para o controle de *D. saccharalis*.

Palavras-chave: *Metarhizium anisopliae*; *Beauveria bassiana*; Óleo Adjuvante Emulsionável; Formulação; Extratos Vegetais.

ABSTRACT

Brazil is the largest producer of cane sugar, but the environmental conditions favor the emergence of pests, among them, *Diatraea saccharalis*, known as the sugarcane borer. This study is aimed to evaluate the effect of an emulsifiable adjuvant oil and extracts of *Indigofera suffruticosa* and *Myrciaria cauliflora* on *Beauveria bassiana* and *Metarhizium anisopliae*, as well as the pathogenicity of the formulations *D. saccharalis*. *M. anisopliae* PL43, *M. anisopliae* IBCB425, *B. bassiana* ESALQ447 and *B. bassiana* ARSEF1398 strains were used. The antifungal effect was assessed by incorporation of Veget'oil® and plant extracts from the leaves and seeds of *Indigofera suffruticosa* and *Myrciaria cauliflora* fruit on Potato-Dextrose-Agar (PDA) at different concentrations. The control group consisted free of oil and extract. Biological parameters analyzed: vegetative growth, production and germination of conidia. The effects of storage of the formulations (fungus + oil) were evaluated taking into consideration the percentage of germination of conidia formulated in two rooms (25 ± 1ºC and -7 ± 1ºC). Third-instar larvae of *D. saccharalis* were immersed in suspensions containing conidia of the studied fungi, formulation and plant extracts. The control group was inoculated with autoclaved distilled water and oil. Bioassays were performed in five replicates and observed daily for 10 days. Veget'oil is compatible with *B. bassiana* ESALQ447, moderately toxic to *M. anisopliae* PL43 and IBCB425 and toxic to *B. bassiana* ARSEF1398 in lower concentration. The formulated fungi remained viable for up to 90 days at 25 ± 1ºC. The formulated conidia stored at -7 ± 1ºC remained viable for 11 months. All strains tested were pathogenic to *D. saccharalis*. The most efficient oil formulation was *Beauveria bassiana* ESALQ447 that caused 74% larval mortality. The plant extracts analyzed are compatible with the fungi and their association resulted in an increased number of dead insects (96%) for *M. anisopliae* IBCB425 and 94% for *M. anisopliae* PL43, both with seed extract of *I. suffruticosa*. The fruit extract of *M cauliflora* associated with *B. bassiana* ESALQ447 killed 84% of the larvae. The extract of I. *suffruticosa* was less efficient, reaching 70% mortality only if associated with the formulation. The results demonstrated the potential of formulations and association of fungi with plant extracts as a viable alternative for control of *D. saccharalis*.

Keywords: *Metarhizium anisopliae; Beauveria bassiana*; Emulsifiable Adjuvant Oil; Formulation; Plant Extracts.

1. INTRODUÇÃO

O Brasil é o maior produtor mundial de cana-de-açúcar, ocupando o primeiro lugar na produção de açúcar e etanol e cada vez mais conquista o mercado externo com o uso desse vegetal para fornecimento de biocombustível, como alternativa energética (MAPA, 2013). Devido às extensas áreas de cultivo distribuídas por todo território brasileiro, bem como as condições climáticas dessas áreas, esta cultura propicia um agroecossistema favorável ao desenvolvimento de pragas, dentre estas, a broca da cana-de-açúcar *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) (PINTO et. al., 2006).

Diatraea saccharalis é uma das principais pragas da cana que causa grandes prejuízos à produção final e seu controle implica diretamente no aumento da produtividade (POLANCZYK et al., 2004). A mariposa fêmea dessa espécie deposita seus ovos na fase dorsal das folhas e ao eclodirem ,as larvas alimentam-se de folhas jovens; posteriormente, deslocam-se até o colmo, perfurando-o. Ao penetrarem, abrem galerias longitudinais e transversais ocasionando danos diretos e indiretos à planta, que podem levar à morte da gema apical (“coração morto”) ou ainda a entrada de microrganismos fitopatógenos que irão comprometer o rendimento do açúcar e do álcool (BOTELHO & MACEDO, 2002; GALLO et al., 2002; PARRA et al., 2002).

Com o incentivo do governo para o aumento do cultivo orgânico da cana no País, faz-se necessário a adoção de medidas mais eficazes para o controle de pragas. Uma alternativa viável ao controle químico é o Controle Biológico utilizando fungos entomopatogênicos, pois os mesmos podem infectar diferentes estágios de desenvolvimento do hospedeiro, tais como ovo, larva, pupa e adulto, sendo esta

característica desejável e peculiar desse grupo de organismo (ALVES, 1998). Os fungos que crescem dentro dos tecidos do hospedeiro, têm a característica de matar o inseto em poucos dias; contudo, o seu emprego com sucesso no campo depende, dentre outros fatores, da qualidade do isolado a ser aplicado, da quantidade de inóculo, da viabilidade e virulência dos esporos, das condições climáticas favoráveis e da correta forma de aplicação (MACCHERONI JR. et al., 2004; MENDONÇA, 2005).

Após a deposição do fungo sobre o inseto ocorre a adesão e posterior penetração pela cutícula do hospefeiro. Nesta fase, estão envolvidos processos físicos e mecânicos, devido à pressão exercida pelas hifas ao romperem as áreas membranosas ou esclerosadas e também o processo químico ou enzimático, resultante da elaboração de enzimas, principalmente proteases, quitinases e lipases (ALVES, 1998). Destas, a protease e a quitinase são as principais enzimas envolvidas na infecção, pois alteram a superfície do tegumento liberando peptídeos que servirão de nutrientes para o fungo (ST. LEGER et al., 1998; MACCHERONI JR et al., 2004).

Entre os entomopatógenos, destacam-se as espécies fúngicas *Metarhizium anisopliae* e *Beauveria bassiana* que são utilizadas com sucesso no controle de pragas, tanto no Brasil quanto em outros países, entretanto o tempo de preservação e a viabilidade dos conídios são um dos principais obstáculos para sua utilização em larga escala. Os componentes presentes nas formulações contribuem para o incremento da estabilidade, virulência e eficácia do agente entomopatogênico. Dentre esses produtos, as formulações à base de óleos emulsionáveis são amplamente estudadas devido a facilidade de estocagem a temperatura controlada (25°C), proteção dos conídios dos raios UV com consequente aumento da persistência no campo, e facilidade de aplicação, utilizando equipamentos convencionais para pulverização manual ou aplicação em grandes áreas (MARQUES et al., 1999; ALVES et al., 2002; ALMEIDA et al., 2008).

Plantas com potencial inseticida estão sendo estudadas, visando a resolução de problemas como o desenvolvimento de resistência e a poluição ambiental, causada pelo uso indiscriminado de agroquímicos. Os compostos bioativos das plantas são capazes de agir como inseticidas naturais (FAROOQ et al., 2011) e seus extratos demonstram repelência e embriotoxicidade contra insetos-praga de diferentes ordens (VIEIRA et al., 2009; ANDRADE-FILHO et al., 2010; VIEIRA et al., 2012).

O estabelecimento de um método de controle mais eficiente para as pragas da cana-de-açúcar, entre elas *D. saccharalis,* irá refletir no aumento da produtividade da cultura, além de assegurar o crescimento sustentável do País, reduzindo o uso de agrotóxicos. O estudo da viabilidade e da patogenicidade de formulações utilizando fungos entomopatogênicos, além da associação destes com extratos vegetais pode indicar a eficiência da aplicação no campo, com vistas ao controle do inseto-alvo.

CAPITULO I

2. REVISÃO DE LITERATURA

2.1 *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) (broca da cana-de-açúcar)

A cana-de-açúcar (*Sacharum officinarum* L.) é uma gramínea, da família Poaceae, originária do Sudeste Asiático que chegou ao Brasil em 1532. Nas capitanias de Pernambuco e da Bahia os engenhos se fixaram e a partir daí se multiplicaram pelo País, devido às condições propícias para o desenvolvimento da cultura. Atualmente, o Brasil é o maior produtor mundial de açúcar, com uma área de 8.485,000 mil hectares cultivados e a lavoura ainda continua em expansão (FERRO et al., 2009; CONAB, 2013).

O efeito do clima adverso, no final do ano de 2012, prejudicou o desenvolvimento das lavouras fazendo muitas usinas postergarem o início da safra por não terem cana na idade ideal para o corte. Com o início das chuvas, a colheita teve início e a produção foi recuperada. Na safra 2012/2013, a produção nacional alcançou aproximadamente 588.915,700 mil toneladas e a Região Centro-sul contribuiu com cerca de 532.986,00 mil toneladas de cana-de-açúcar (CONAB, 2013). São Paulo destaca-se com a maior produção nacional (52,07%, equivalente a 4.419,48 mil ha.), seguido por Goiás (8,55%, equivalente a 725,91 mil ha.). No nordeste, Alagoas, Pernambuco, Paraíba e Rio Grande do Norte são os maiores produtores (ÚNICA, 2013), como mostra a Figura 1.

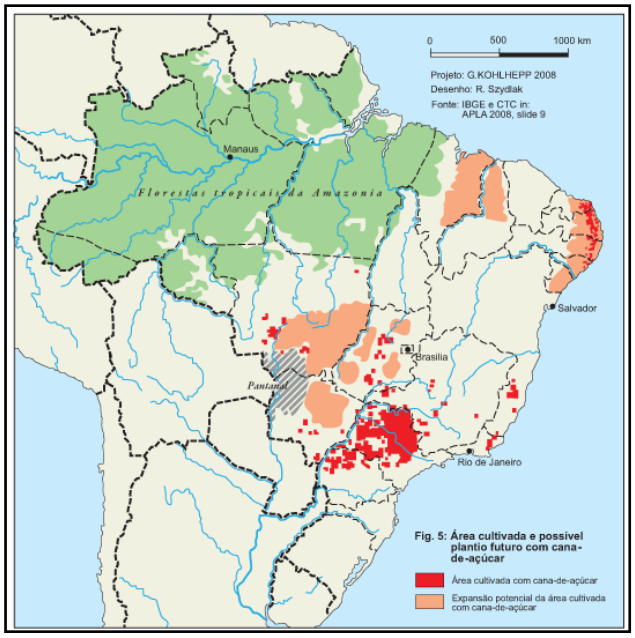

Figura 1. Área cultivada e possível plantio futuro de cana-de-açúcar (Fonte: KOHLHEPP, 2013).

Os problemas fitossanitários constituem fatores limitantes para a produção agrícola e o rendimento industrial. A cana-de-açúcar é atacada por mais de 80 espécies de pragas, que contabilizam aproximadamente 20% de perda por ano, sendo a broca da cana (*D. saccharalis* F.) uma das pragas responsáveis pelas perdas econômicas mais severas (ALMEIDA et al., 1988; ROSETTO & SANTIAGO, 2007).

As lagartas de *D. saccharalis* provocam danos diretos e indiretos durante a infestação na planta (Figura 2). Os danos diretos são ocasionados devido ao hábito de perfurar a cana nova, formando galerias, causando a morte da gema apical (“coração morto”). Na cana adulta, provoca brotação lateral, enraizamento aéreo, atrofia de entrenós e canas quebradas, reduzindo a quantidade de cana e consequentemente, afetando a produção final (MENDONÇA, 1996). Por meio dos orifícios formados pelas lagartas, ocorre a penetração de microrganismos, geralmente fungos (*Fusarium*

moniliforme e *Colletotrichum falcatum*), que causam a podridão vermelha do colmo, ocasionando queda no rendimento industrial pela inversão da sacarose, diminuindo a pureza e a qualidade do caldo, acarretando perda no rendimento do açúcar e do álcool (BOTELHO & MACEDO, 2002; GALLO et al., 2002; PARRA et al., 2002).

Figura 2. Danos diretos e indiretos causados por *Diatraea saccharalis* em cana-de-açúcar: a) orifício deixado pela lagarta; b) galeria no interior do colmo da cana; c) podridão vermelha do colmo da cana (Fonte: http://www.agrolink.com.br/culturas/milho/broca-do-colmo_375.html, 2013).

A mariposa fêmea de *D. saccharalis* coloca os ovos na face dorsal das folhas da cana, com um agrupamento característico, com aparência de escama de peixe, contendo de cinco a 50 ovos. Cada fêmea pode colocar de 300 a 600 ovos. As larvas eclodem entre o quarto e o nono dia, apresentam coloração amarela clara, com cabeça marrom e passam por cinco *instars* dentro de nove a 14 dias. No 1º *instar*, pode atingir de 2 a 4mm; no 2º *instar*, 6 a 9mm; no 3º *instar*, 10 a 15mm; no 4º *instar*, 15-20mm; e no 5º *instar*, 20 a 30mm. As lagartas de 1º *instar* alimentam-se do parênquima das folhas e após a primeira ecdise, deslocam-se para a bainha e penetram pela parte mais mole do colmo, perfurando-o. Ao penetrarem na região do entrenó abrem galerias, até a fase de pupa (GALLO et al., 2002).

A duração da vida da broca está entre 53 e 60 dias, passando por quatro gerações anuais (excepcionalmente, 5) dependendo do clima (Figura 3). Na primeira geração (outubro e novembro), os adultos ovopositam em canas jovens; a segunda geração (acontece entre dezembro e fevereiro); a terceira geração (entre fevereiro e abril) e em maio e junho, verifica-se a quarta geração, que se prolonga por cinco a seis meses (MENDONÇA, 1996; BARRIGOSSI et al., 2004; POLANCZYK et al., 2004; TRUMPER et al., 2004). Essas gerações podem acontecer tanto na cana quanto no milho e no arroz.

No interior da planta, em um túnel criado pela larva e fechado com fios de seda e resto de alimento, acontece a transformação em pupa, permanecendo assim por seis a 14 dias. A pupa é alongada e fina, de cor marrom e mede entre 16 e 20mm. O adulto sai do interior da planta pelo furo deixado pela lagarta. É de hábito noturno e apresenta coloração amarela-palha, com 25mm de envergadura. As fêmeas apresentam abdômen dilatado e nos machos, verificam-se cerdas, no último par de patas. Os adultos vivem, em média, oito dias (AFOCAPI/COPLACANA, 2013).

Devido as grandes perdas anuais provocadas pela ocorrência desta praga, alguns métodos de controle são utilizados como o controle cultural, o controle químico com inseticidas e o controle biológico com inimigos naturais e entomopatógenos. O controle cultural pode ser realizado por meio da rotação de culturas, plantio de variedades resistentes ou tolerantes e ainda pela eliminação de plantas hospedeiras próximas ao canavial. O controle químico é considerado inviável porque além de aumentar o custo da produção, pode deixar resíduos dos produtos nos alimentos, restringindo a exportação (ALVES, 1998).

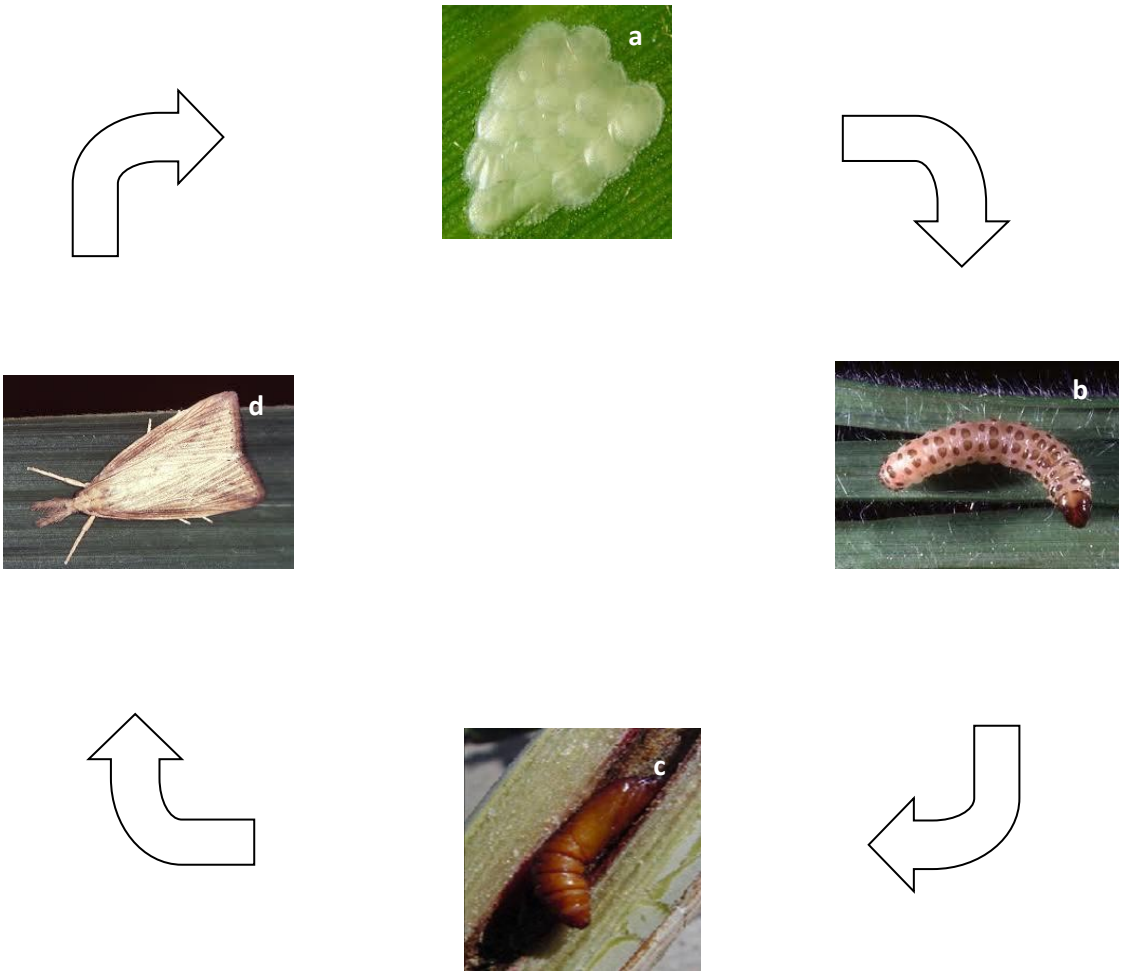

Figura 3. Ciclo de vida de *Diatraea saccharalis*. a) ovos; b) larva; c) pupa; d) adulto (Fonte:MACEDO, 2005).

Estudos com o parasitóide de ovos, *Trichogramma galloi* Zucchi, foram desenvolvidos com sucesso no controle de *D. saccharalis*, mas as pesquisas com o parasitóide *Cotesia flavipes* contribuiram significativamente para a diminuição das perdas ocasionadas pelas brocas e consolidaram esta vespinha como o principal biocontrolador da praga (POLANCZYK et al., 2004; BROGLIO- MICHELETTI et al., 2007). O himenóptero, originário de Trinidad, na América central, foi introduzido no Brasil na década de 70 e em 1973, quando foi iniciado um programa de controle da

broca pelo Instituto do Açúcar e do Álcool (IAA/PLANALSUCAR). A facilidade de criação massal e a sua capacidade de localizar o hospedeiro são algumas das vantagens da sua utilização. A liberação deve ocorrer de forma a cobrir toda a área infestada, sendo que o número de vespinhas liberadas deve estar entre 2.500 e 10.000 (adultos). Posteriormente, o controle deve ser transferido para outra área (MARUCCI, 2006; PINTO, 2006; ZAPPELINI et al., 2010).

O controle biológico utilizando fungos entomopatogênicos veio incrementar o controle da broca da cana. Em condições climáticas favoráveis, estes patógenos podem causar epizootias e enzootias naturais em populações de insetos das ordens Hemiptera, Lepidoptera e Coleoptera. Dentre os fungos entomopatogênicos destacam-se *Metarhizium anisopliae* e *Beauveria bassiana*, devido à ampla distribuição geográfica e a variedade de hospedeiros (ALVES, 1998; WENZEL et al., 2006; OLIVEIRA et al., 2008; ZAPPELINI et al., 2010).

2.2 Aspectos Taxonômicos e Biológicos de *Metarhizium anisopliae*

Metarhizium anisopliae (Metsch.) Sorokin foi classificado pela primeira vez como *Entomophora anisopliae*, quando Metschnikoff o isolou da larva de *Anisopliae austriaca*, besouro do trigo, em 1879, na Rússia. Um ano depois, o mesmo pesquisador o reclassificou como *Isaria destructor*. A partir daí, a espécie recebeu várias denominações, até que em 1883, na França, Delacroix o registrou como *Oospora destructor* (Metsch). No mesmo ano, Sorokin o pôs em sinonímia, conferindo ao isolado a denominação de *M. anisopliae* (LUNA-ALVES LIMA, 1985).

Gams & Rozsypal (1973) descreveram pela primeira vez a espécie *M. flavoviride* que foi isolada de larvas e pupas de curculionídeos e de solos cultivados, na Europa. Os autores separaram *M. flavoviride* de *M. anisopliae* pelo tamanho do conídio e tipo de fiálide, pois concluíram que *M. flavoviride* apresenta conídios elipsóides, variando de 7,0-9,0 x 4,5-5,5 µm e fiálides clavadas, enquanto *M. anisopliae* apresenta fiálides e conídios cilíndricos, variando de 3,0-5,0 x 2,0-3,0 µm (Figura 4).

Tulloch (1976) reconheceu duas espécies para o gênero *Metarhizium* (*M. anisopliae* e *M. flavoviride*) e propôs, com base no tamanho dos conídios, duas variedades para *M. anisopliae*: *M. anisopliae* (Metsch.) Sorokin var. *anisopliae* (3,5-9,0 µm) e *M. anisopliae* (Metsch.) Sorokin var. *major* (Johnston) Tulloch (9,0-18,0 µm). Mais tarde, uma nova variedade foi proposta para *M. flavoviride*: *M. flavoviride* Gams & Rozsypal var. *minus* Rombach, Humber & Roberts, esta apresentando conídios que variam de 4,5-7,0 x 2,0-3,0 µm (ROMBACH et al., 1986).

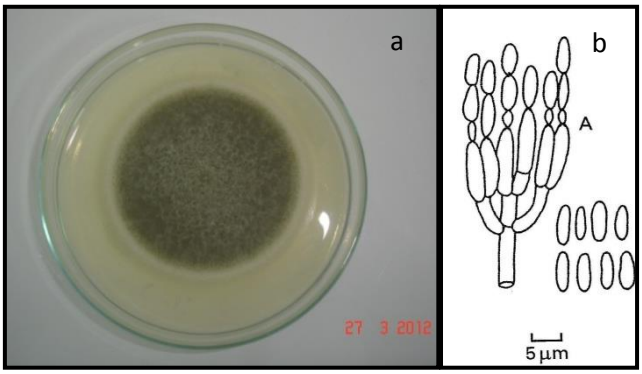

Figura 4. *Metarhizium anisopliae* PL43 em BDA (27 ± 1ºC). a) Colônia com 15 dias de desenvolvimento; b) Conidióforos e conídios. Fonte: a) PORTELA-SILVA, A.P.A., 2013; b) ALVES, 1998.

Driver et al. (2000) revisaram e caracterizaram as espécies *M. anisopliae*, *M. album* e *M. flavoviride*, utilizando RAPD e dados das regiões ITS e 28S do rDNA. O dendrograma gerado revelou 10 grupos distintos, dentre estes, dois foram descritos como variedades novas de *M. anisopliae*: *M. anisopliae* var. *lepidiotum* (conhecida anteriormente como *M. anisopliae* var. *anisopliae*) e *M. anisopliae* var. *acridum* (conhecida anteriormente como *M. flavoviride* var. *minus*). A partir daí, duas espécies de *M. flavoviride* var. *minus*, isoladas de gafanhotos (Acrididae) e uma espécie de *M. anisopliae* var. *anisopliae*, passaram a sinonímia de *M. anisopliae* var. *acridum*, (MAGALHÃES et al., 2000; ARTHURS & THOMAS, 2001; OUEDRAOGO et al., 2003).

Durante muitas décadas, os fungos que não apressentavam fase sexuada conhecida pertenciam à subdivisão Deuteromycota. Em 1992, passaram a ser denominados Anamorfos (KENDRICK, 1992; ALEXOPOULOS et al., 1996) visto que não apresentam relação filogenética com os demais fungos, considerados perfeitos, são fases conidiais de Ascomycota ou mais raramente, de Basidiomycota. Nesse sentido, Liu et al. (2002) encontraram em coletas na China, uma espécie fúngica desconhecida que atacava a larva de um besouro subterrâneo (Coleoptera: Scarabaeidae). Descreveram-na como C*ordyceps brittlebankisoides* Zuo Y. Liu, Z. Q. Liang, Whalley, Y. J. Yao & A. Y. Liu, teleomorfo de *Metarhizium*. Estudos de filogenética molecular apoiaram esta classificação e atualmente, a fase sexual de espécies de *Metarhizium* é denominada *Metacordyceps* (HUANG et al., 2005; SUNG et al., 2007; BISCHOFF et al., 2009).

Os Anamorfos, apresentam uma alternativa de sexo para garantir sua diversidade genética: o ciclo parassexual. (MESSIAS & AZEVEDO, 1980). No ciclo parassexual, são originados conídios diplóides que em condições favoráveis dão origem a colônias

também diplóides. Quando ocorre variação no ciclo parassexual, como a formação de haplóides sem aparente formação do diplóide ou este é altamente instável, diz-se que ocorreu a parameiose (PACCOLA-MEIRELLES & AZEVEDO, 1991). A partir da descoberta da parassexualidade, surgiu a possibilidade de recombinação entre linhagens com características diversas que poderiam ser unidas em uma nova linhagem (AZEVEDO, 2000).

A espécie *M. anisopliae* pode ser isolada de insetos ou do solo, sendo considerada uma das espécies fúngicas entomopatogênicas mais importantes, nas condições climáticas brasileiras, capaz de infectar mais de 300 espécies de insetos (ALVES, 1998). O tempo de preservação e a viabilidade dos conídios são um dos principais obstáculos para sua utilização como agente de controle microbiano (MARQUES & ALVES, 1996). Seu limite de temperatura, para crescimento, varia de 5 a 40ºC, mas a temperatura ótima é de aproximadamente 28°C, porém após 30 dias, na temperatura de 30 ± 1ºC o micélio fúngico tem sua viabilidade reduzida (HALLSWORTH & MAGAN,1999; MARQUES et al., 1999); entretanto, isolados da região sub-Antártica germinaram a 2,5º C, após 49 dias (RODDAM & RATH, 1997).

2.3 Aspectos Taxonômicos e Biológicos de *Beauveria bassiana*

O gênero *Beauveria* (Vuillemin) foi reorganizado em 1912, mas a espécie *Beauveria bassiana* (Bals.) Vuill. foi descrita em 1835, pela primeira vez, por Agostino Bassi como *Botrytis bassiana,* que encontrou este fungo causando a doença muscardine branca que atacava *Bombyx mori*, o bicho da seda (BENHAM & MIRANDA, 1953). De Hoog (1972) estudou o gênero e considerou três espécies: *B.*

bassiana (Balsamo) Vuillemin, *B. brongniartti* (Saccard) Petch. e *B. alba* (Limber) Saccas. Em 1982, Samson & Evans citaram duas espécies novas: *B. velata* Samsom & Evans e *B. amorpha* (Hohn) Samsom & Evans. Em 2001, foi encontrado sobre cadáver de Lepidoptera, o teleomorfo de *Beauveria bassiana*, denominado *Cordyceps bassiana*. Este fungo é cultivado com sucesso, *in vitro*, para uso comercial e muitos estudos vem sendo desenvolvidos buscando elucidar nos corpos de frutificação, peritécios, compostos químicos com ação antinflamatória, antioxidante, entre outras (LI et al., 2001).

A conidiogênese de *B. bassiana*, ocorre em modelo simpodial onde o núcleo migra da célula conidiogênica para o primórdio de conídio, após mitose. Essas células apresentam formato de garrafa, que se posicionam com terminações em forma de zig-zag. Os conídios são uninucleados e variam de tamanho, com diâmetro de 1,5-2,0 µm. O micélio apresenta hifas delgadas, hialinas e septadas, colônias levemente pulverulentas e esbranquiçadas (Figura 5). Estruturas leveduriformes uni ou binucleadas podem ser visualizadas em meio sólido ou líquido, ou ainda na hemolinfa do inseto, apresentando-se multinucleadas. Estas estruturas estão relacionadas com a fase de insfestação do fungo no interior do inseto e se originam a partir da germinação de conídios ou da diferenciação micelial (DE HOOG, 1972; LUNA-ALVES LIMA & TIGANO, 1989).

As condições favoráveis para que *B. bassiana* cause a doença no inseto são umidade relativa em torno de 90% e temperatura na faixa de 23 a 28ºC, sendo o limite de crescimento de 5 a 35ºC, dependendo do isolado (ALVES, 1998). Temperatura muito alta ou muito baixa retarda o desenvolvimento da doença que levará a morte do inseto.

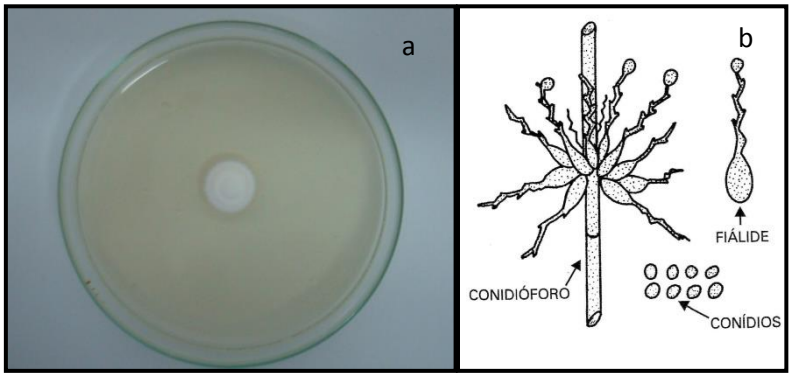

Figura 5. *Beauveria bassiana* ESALQ447, em BDA (27 ± 1ºC). a) Colônia com oito dias de desenvolvimento; b) Conidióforos e conídios. Fonte: a) PORTELA-SILVA, A.P.A., 2013; b) ALVES, 1998.

2.4 *Metarhizium anisopliae* e *Beauveria bassiana* no Controle Biológico de Pragas

Dentre os fungos entomopatogênicos, os gêneros *Metarhizium, Beauveria, Isaria, Nomuraea, Aschersonia* e *Entomophthora* são considerados os mais importantes (ONOFRE et al., 2002). O registro fóssil de patógenos microbianos de insetos é quase inexistente, consiste apenas de três amostras em âmbar, onde o fungo está se exteriorizando pela cutícula, tórax e abdômen de mosquitos adultos da ordem Diptera (famílias Culicidae e Mycetophilidae); após análise, os fungos foram identificados como pertencentes à classe Trichomycetes (POINAR JR & POINAR, 2005), fungos que estão associados ao trato digestivo de artrópodes.

O agente *M. anisopliae* é um patógeno, comprovadamente virulento para diversas ordens de insetos-praga. É testado e empregado com sucesso no controle de

importantes pragas agrícolas no Brasil e no exterior. O controle da cigarrinha da cana-de-açúcar (*Mahanarva posticata* Stal) (Hemiptera: Cercopidae), no Nordeste do Brasil, constitui o programa mais bem sucedido utilizando este fungo como biocontrolador, onde 474 mil hectares de canaviais infestados por essa praga foram tratados com aproximadamente 38 mil quilos de conídios de *M. anisopliae*, no período de 1970-1991 (MARQUES & ALVES, 1996; ALVES, 1998; MENDONÇA, 2005).

A infecção de larvas de *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) por *M. anisopliae* reduziu o consumo diário de alimento dos insetos entre 70-85%, quando estas foram tratadas com $1x10^8$ conídios/mL. Os autores sugerem que a redução no consumo de alimento pode compensar a velocidade lenta de morte ocasionada pelos fungos (TEFERA & PRINGLE, 2003). *M. anisopliae* quando testado contra operárias de *Atta sexdens sexdens* Linnaeus (Hymenoptera: Formicidae) apresentou TL_{50} de 2,5 dias na concentração de $1x10^9$ conídios/mL e mortalidade total de 34% após 3 dias de infecção (LOUREIRO & MONTEIRO, 2004).

Albuquerque et al. (2005) estudaram a ação de *M. anisopliae* sobre o cupim do montículo *Nasutitermes coxipoensis* Holmgren (Isoptera: Termitidae) e constataram que esse fungo tem potencial para o Controle Biológico desse cupim, pois ao 3º dia pós-tratamento, 100% dos insetos tratados estavam mortos. No mesmo sentido, Wright et al. (2005) relataram o primeiro registro de um agente biocontrolador para cupins alados (*Coptotermes formosanus* Shiraki), onde a concentração de 10^6 conídios/mL de *M. anisopliae* matou 100% dos insetos em três dias, sugerindo seu potencial para controlar cupins no campo.

Loureiro et al. (2005) selecionaram isolados de *M. anisopliae* virulentos a *Mahanarva fimbriolata* (Stal) e verificarm que no 4º dia após a exposição ao fungo

causaram baixa mortalidade, porém no 6º dia pós-pulverização, a mortalidade confirmada variou de 70-88%.

Avaliando o efeito de *M. anisopliae* sobre ninfas dos pulgões *Aphis gossypii* Glover e *Myzus persicae* Sulzer (Hemiptera: Aphididae), em laboratório, 100% de mortalidade dos insetos ocorreu logo após cinco dias da infecção, no tratamento com $1x10^8$ conídios/mL. A espécie mais suscetível à infecção foi *Myzus persicae* com TL_{50} de 1,76 dia, enquanto para *Aphis gossypii* o TL_{50} foi alcançado com 1,98 dia (LOUREIRO & MOINO JR, 2006). Por outro lado, França et al. (2006) avaliaram os efeitos de *M. anisopliae* sobre o percevejo predador *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) e verificaram mortalidade de 72% de ninfas, com tratamento tópico, mas não houve confirmação de mortalidade de adultos. Isso indica que a variabilidade existente entre linhagens fúngicas, implica em diferentes respostas do hospedeiro ao entomopatógeno.

Athayde et al. (2006) analisaram a ação patogênica de *M. anisopliae* sobre teleógenas de *Boophilus microplus* Canestrini, carrapato bovino (Acari: Ixodidae). A mortalidade superior a 90% demonstrou o potencial do fungo para o controle desse carrapato e possibilitou pesquisas posteriores com larvas da mesma espécie (QUINELATO et al., 2012). Os autores selecionaram três isolados ideais para o seu controle (CG46, IBCB481 e CG32) porque causaram mortalidade em 100% das larvas, em 20 dias, na concentração de 10^8 con./mL.

Metarhizium anisopliae também foi virulento à barata-do-coqueiro (*Coraliomela brunnea* Thumb) em todas as suspensões t estadas. A mortalidade máxima chegou a 85%, na maior concentração ($1x10^8$ conídios/mL) e as concentrações $5x10^7$ conídios/mL e $1x10^7$ conídios/mL também tem potencial para serem testadas em campo porque a

maior concentração pode não ser economicamente a mais indicada (CUNHA et al., 2008).

Lopes & Alves (2011) avaliaram a susceptibilidade de ninfas e adultos de *Blattella germanica* Linnaeus (Blattodea: Blattellidae), à infecção por *M. anisopliae*. Testando três metodologias diferentes de infecção (aplicação tópica, contato direto em superfície pulverizada com pó molhável e conídios em iscas), concluíram que os adultos foram mais suscetíveis à infecção pelo fungo nos três testes e que o teste com pó molhável causou maior mortalidade nos adultos (100%) e nas ninfas (83%), com tempo médio de sobrevivência de 5 dias. Estudos anteriores de virulência por transmissão horizontal de infectadas para não-infectadas (na razão de 1:10) contra adultos da mesma espécie mostraram que a mortalidade foi de 87,5% e o TL_{50} de 12,2 dias, indicando a eficiência desse fungo em ser transmitido horizontalmente e em espalhar infecção na população do inseto (MORAGA et al., 2004).

Svedese et al. (2012) analisaram a suscetibilidade da mosca do figo (*Zaprionus indianus* Gupta) a *M. anisopliae* e a *B. bassiana*. Não houve mortalidade larval, mas o estágio de pupa foi aumentado em até três dias quando se utilizou *B. bassiana*. A emergência de adultos diminuiu em relação ao grupo controle e a mortalidade de adultos atingiu 98,7% com *B. bassiana* e 100,0% com *M. anisopliae*.

Beauveria bassiana é de ocorrência generalizada em todos os países, sendo mais frequente sobre insetos e no solo, onde pode subsistir por longo tempo, em saprogênese. Tornou-se conhecida internacionalmente pelo produto soviético Boverin, formulação que contém 6×10^9 conídios/g, sendo recomendado para controle de *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (ALVES, 1998). Na China, esse fungo é utilizado de forma significativa para o controle de *Ostrinia nubialis*, reduzindo

progressivamente os danos a 2% desde a implantação do programa de controle (HUSSEY & TINSLEY, 1981). Desde então, outras formulações são desenvolvidas na ESALQ/USP (Boveriol e Boveril) para o controle do cupim do montículo com resultados promissores (ALVES, 1998).

Em laboratório, De La Rosa et al. (2002) mostraram a ação de diferentes linhagens de *B. bassiana* sobre larvas e fêmeas adultas da mosca mexicana das frutas (*Anastrepha ludens* Loew) (Diptera: Tephritidae). Os autores verificaram que o fungo causou baixa mortalidade em larvas (2-8%), nenhuma mortalidade em pupas (0%) e elevada mortalidade em adultos (82-100%), na concentração de 1,2 - 1,6 x 10^8 conídios/mL. Dimbi et al. (2003) avaliaram a eficiência de *B. bassiana* e *M. anisopliae* no controle de adultos de moscas das frutas (*Ceratitis* sp.) (Diptera: Tephritidae), em condições de laboratório. Segundo os autores, todos os isolados testados foram patogênicos para as espécies testadas, causando mortalidade que variou de 7 a 100% em *C. capitata*, de 11,4 a 100% *em C. rosa* var. *fasciventris* e de 72–78% em *C. Cosyra*, após quatro dias de inoculação.

Estudos realizados por Silva et al. (2003) selecionaram linhagens *de B. bassiana* e *M. anisopliae* que foram patogênicas à traça das crucíferas *Plutella xylostella* (Lepidoptera: Plutellidae). Os autores testaram cinco linhagens de cada fungo na concentração de 10^8 conídios/mL. A mortalidade causada por *B. bassiana* variou de 78% a 90% decorridos oito dias após a inoculação. Com base nesses estudos, os autores selecionaram os isolados ESALQ634 e ESALQ447 como os mais virulentos a este lepidóptero.

Linhagens de *B. bassiana* foram avaliadas também contra a broca do café *Hypothenemus hampei* (Ferrari) e apresentaram alto potencial para ser utilizada em

programas de Controle Biológico de pragas, principalmente a linhagem *B. bassiana* CG425 que apresentou CL_{50}= 2,5x10^6 con./mL e *B. bassiana* CB102 com a maior taxa de esporulação em cadáveres da broca (NEVES & HIROSE, 2005). Almeida et al. (2005) verificaram maior mortalidade de insetos *Anthonomus grandis* (Boheman) quando inoculados por *B. bassiana* reisolada de ovos (96,7%), larvas (83,4%) e adultos (91,1%) do próprio inseto.

Rhode et al. (2006) selecionaram o isolado *B. bassiana* UNIOESTE04 como o mais virulento contra larvas e adultos do cascudinho *Alphitobius diaperinus* (Panzer), praga da avicultura. Os autores destacaram ainda que este isolado foi originalmente encontrado em insetos mortos do cascudinho e que é muito frequente a presença de *B. bassiana* nas populações do inseto, em aviários nas Américas.

Almeida et al. (2009) testaram 10 linhagens de *B. bassiana* contra adultos de *Cosmopolites sordidus* (Germar, 1824), broca da bananeira. A linhagem que apresentou melhores resultados atingiu 66% de mortalidade confirmada. Posteriormente, LÓPEZ et al. (2010), testaram diferentes meios de inoculação na mesma espécie e constataram que a imersão foi o método que apresentou maior resultado na mortalidade total, matando 28,8% dos insetos. A aplicação tópica ventral foi a que apresentou menor mortalidade confirmada, provavelmente porque os insetos têm o hábito de se "limparem" enquanto estão passeando pelo pseudocaule.

Wraight et al. (2010), testaram 43 isolados de *B. bassiana* de diferentes origens geográficas contra oito espécies de lepidópteros que atacam várias culturas vegetais. A virulência variou entre os isolados, contudo a maioria foi patogênica contra os insetos testados. Na busca de linhagens que ataquem o maior número de pragas, os estudos reafirmaram a importância de bioensaios para a seleção de linhagens.

Boyle & Cuther (2012) testaram *B. bassiana* contra o percevejo *Blessus leucopterus* Hirtus e verificaram que as ninfas de 1º, 2º e 3º *instars* foram mais suscetíveis ao fungo do que as ninfas do 4º e do 5º *instar* e os adultos, matando mais de 80% dos insetos após quatro dias da exposição. Esses resultados foram favoráveis à região do estudo (Canadá) porque o controle de estágios mais jovens seria favorecido no campo pela aplicação entre os meses de junho e julho, época chuvosa.

Svedese (2012) avaliou a eficiência de três métodos de inoculação fúngica contra a broca da cana-de-açúcar *D. saccharallis* e concluiu que os métodos de imersão e pulverização conidial causaram elevada mortalidade (70-91%), enquanto que larvas alimentadas com colmos infectados com a suspensão fúngica apresentaram mortalidade variando de 26 a 42%. *B. bassiana* foi eficazmente transmitida entre os indivíduos da broca, causando mortalidade significante e essa capacidade pode representar uma nova estratégia de controle.

2.5 Formulações com óleo no Manejo Integrado de Pragas (MIP)

Formular um entomopatógeno é acrescentar a ele determinados compostos para melhorar seu desempenho no campo, que facilitem o manuseio e a aplicação e, principalmente, permitam o armazenamento sob condições que minimizam os custos, com perda mínima das qualidades do produto. Esses componentes devem também aumentar a persistência do produto, a adesividade sobre o inseto e a atratividade para a praga (BATISTA FILHO et al., 1998; ALVES & LOPES, (2008)).

Além do ingrediente ativo (patógeno) e do inerte/veículo, as formulações podem conter adjuvantes, componentes que são utilizados para otimizar a atividade do ingrediente ativo e melhorar as características do produto formulado. Dentre outras propriedades, eles têm função fotoprotetora, fagoestimulante e antievaporante. Os adjuvantes mais importantes são os surfactantes, compostos que facilitam a dispersão do organismo na calda de pulverização (RHODES, 1993; ALVES, 1998).

Segundo Michereff-Filho et al. (2009), a maior parte dos micopesticidas brasileiros não têm registro no Ministério da Agricultura. Dentre os produtos, 2,5% são comercializados como conídios puros e 72,5%, concentrados técnicos (substratos líquidos ou sólidos, colonizados por fungos) e apenas 25% são, de fato, formulações do tipo dispersão oleosa. Entretanto, há expectativa de crescente adoção de fungos no Controle Biológico em razão do mercado emergente, da agropecuária orgânica, dos cultivos protegidos, da expansão do agronegócio da cana-de-açúcar e bovinocultura, dentre outros.

O Veget'oil® (Oxiquímica Agrociência Ltda, SP/Brasil) é um óleo vegetal que possui em sua composição 93% de ésteres de ácidos graxos e 7% de emulsificante. Nesse sentido, pesquisas com formulações á base de outros óleos vegetais e emulsificantes estão em crescimento e vêm produzindo resultados interessantes (MARQUES, 1993; ALVES & BATEMAN, 2000).

Realizando testes de compatibilidade com posterior eficiência sobre o inseto, *Triatoma infestans* (Klug), vetor da doença de Chagas, Luz et al. (1999) concluíram que foi necessário um número menor de conídios para matar 50% dos insetos (CL_{50}) após a exposição ao fungo formulado em óleo mineral. Em experimentos posteriores, com *B. bassiana* formulada contra *Triatoma sordida*, confirmou-se alta eficiência (85%) em

causar mortalidade no inseto; entretanto, no campo a mortalidade não foi tão elevada (33%) (LUZ et al., 2004).

Alves et al. (2002) avaliaram os efeitos de diferentes formulações na viabilidade e no armazenamento (10ºC e 27ºC) de conídios de *M. anisopliae* var. *acridum* (umidade próxima a 4,9%). Os óleos Ethoken e Ethoken C12 causaram perda de viabilidade dos conídios e os demais óleos analisados, no máximo, retardaram a germinação, mas com 48 horas, foi superior a 94%G. A viabilidade dos conídios da formulação com Natur'oil® só começou a cair a partir de 25 semanas (a 27ºC), entretanto a 10ºC, até o final de 40 semanas, ainda estava com viabilidade acima de 90%G. O Penault'oil® manteve a viabilidade dos conídios até o final de 40 semanas, nos dois ambientes.

A viabilidade deve ser considerada como o parâmetro mais importante a ser avaliado em testes de compatibilidade por ser o passo inicial para o processo de infecção. Assim, em estudos com *B. bassiana* e inseticidas usados em plantações de café, para o controle de *Hypothenemus hampei* foram selecionados os inseticidas com formulações de Alfa Cipermetrina e Tiametoxan como compatíveis com o fungo e recomendados para o MIP do cafeeiro (OLIVEIRA et al., 2003).

Analisando os produtos fitossanitários, Silva et al. (2006) detectaram que em calda de pulverização, o fungo *B. bassiana* foi o que apresentou a viabilidade menos afetada pelos óleos testados, seguido de *M. anisopliae*; por fim, *Paecilomyces* sp. foi o mais sensível aos óleos. Em experimentos de campo, foi analisada a eficiência do controle da cigarrinha da raiz da cana-de-açúcar (*Mahanarva fimbriolata* Stal, 1854) utilizando *M. anisopliae* com adjuvaantes. Concluiu-se que o fungo mais AgRho DEP775 (150L/ha) manteve-se eficiente (73%) por até 90 dias, mantendo a população abaixo do nível de dano por todo o período (ALMEIDA et al., 2007).

Araújo Jr et al. (2009) mostraram que os isolados *B. bassiana* CG001 e *M. anisopliae* CG30 foram os mais virulentos para *Lipaphis erysimi* (Kalt) porém, tiveram seu crescimento colonial e sua viabilidade foram alterados quando expostos a concentrações de nim maiores que 0,25%. Mesmo assim, os valores de IB (Índice Biológico) indicaram que as emulsões nas concentrações testadas foram compatíveis com os isolados. Adicionalmente, o tratamento com o Neemseto® proporcionou mortalidade de 90% dos pulgões.

Buscando selecionar agrotóxicos, utilizados na cultura do arroz, compatíveis com *M. anisopliae* para o controle do percevejo-do-colmo-do-arroz (*Tibraca limbativentis*) foram realizados experimentos de toxicidade *in vitro*. Constatou-se que Fenitrotiona, Carbofurano, Glifosato e Azoxixistrobina afetaram parâmetros biológicos do isolado *B. bassiana* CG891 (RAMPELOTTI-FERREIRA, et al., 2010).

A cultura do tomate é conhecida como uma das que mais se utiliza defensivos agrícolas para o controle de pragas e doenças. Assim, foram selecionados inseticidas compatíveis com *B. bassiana* e/ou *M. anisopliae* para o controle de *Tuta absoluta* (Meyrick) traça do tomateiro. *M. anisopliae* foi compatível com a maioria dos insetidas testados, exceto com Abamectina e Nim, nas maiores concentrações. A associação desse entomopatógeno com inseticidas compatíveis pode vir a aumentar sua eficiência de controle (PIRES et al., 2010).

Os detergentes pertencem ao grupo dos surfactantes e podem ser usados como adjuvantes para melhorar a dispersão dos conídios durante a pulverização. Deste modo, o detergente comercial Ipê quando testado em três diferentes concentrações associado a *M. anisopliae* IBCB425, em meio BDA, interferiu no crescimento vegetativo do fungo, mas a menor concentração (0,01%) não alterou a produção de conídios, sendo

considerada compatível com o fungo, visando futuras aplicações em campo (HARTERREITEN et al., 2011).

Bukhari et al. (2011) desenvolveram formulações com *M. anisopliae* e *B. bassiana* em óleo, para o controle do mosquito transmissor da malária (*Anopheles* sp.). Os conídios dos fungos, quando formulados, foram mais eficientes em controlar as larvas do que os não formulados; além disso, os conídios não formulados se aglomeraram na superfície da água e perderam viabilidade em cinco dias enquanto que conídios formulados aumentaram sua persistência no campo. De modo semelhante, larvas de *Plutella xylostella* (L.) – traça das crucíferas, foram mortas (acima de 70%) tanto por contato quanto por ingestão de folhas pulverizadas com óleo de mamona adicionado de *B. bassiana* (RONDELLI et al., 2011).

2.6 O Uso de Plantas no o Manejo Integrado de Pragas (MIP)

A busca por métodos alternativos de controle de pragas também inclui pesquisas utilizando extratos de plantas com potencial inseticida. Segundo Farooq et al. (2011) a alelopatia envolve a síntese de compostos bioativos de plantas, conhecidos como aleloquímicos, capazes de atuar como pesticidas naturais, podendo assim resolver problemas como o desenvolvimento de resistência da praga e poluição ambiental causada pelo uso indiscriminado de agroquímicos sintéticos. Assim sendo, pesquisas vem sendo desenvolvidas com extratos de diversas espécies vegetais, contra insetos-praga de diferentes ordens.

Azadirachta indica A. Juss, conhecida como nim, é uma espécie bastante estudada e seus extratos tem ação inseticida semelhante a alguns inseticidas químicos comerciais, contra lepidópteros (MARTINEZ, 2002; LIMA et al., 2008). O nim é conhecido há mais de 5.000 anos e apresenta ação contra mais de 430 espécies de pragas, em diversos países; causa repelência, interrupção do desenvolvimento, redução da fertilidade e fecundidade, alterações comportamentais e fisiológicas, até levar o inseto à morte. Apresenta uma série de compostos liminóides, dentre os quais a Azadiractina, que está em maior concentração, possui maior atividade tóxica contra insetos e ácaros (SCHMUTTERER, 1990; BERNARDI et al., 2013).

Hirose et al. (2001) estudaram o efeito do óleo de nim (*Azadirachta indica* A. Juss) em meio de cultura (2%) sobre os fungos *M. anisopliae* (CB38) e *B. bassiana* (CG252). Os autores constataram que o óleo foi tóxico a *B. bassiana* e moderadamente tóxico a *M. anisopliae* por reduzir a germinação dos conídios, o diâmetro das colônias e a esporulação. Porém, a alta toxicidade *in vitro* nem sempre irá se repetir no campo, onde vários outros fatores estão atuando em conjunto. Outras pesquisas comprovam que muitos produtos estimulam o crescimento vegetativo e a produção de conídios dos fungos, proporcionando efeito sinérgico e auxiliando a infecção sobre o inseto (TAMAI et al., 2002). O óleo de Nim quando testado, *in vitro*, em diferentes concentrações em BDA também não afetou a viabilidade dos conídios, contudo afetou o crescimento vegetativo e a esporulação dos fungos *M. anisopliae*, *B. bassiana e P. farinosus* (*Isaria farinosa*) (MARQUES et al., 2004).

Determinou-se em laboratório o efeito de extratos aquosos de *Melia azedarach* L. sobre o desenvolvimento da traça do tomateiro *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Foi constatado que as folhas foram a estrutura vegetal com maior bioatividade sobre a traça, seguida dos frutos verdes, ramos e frutos maduros. O

extrato de folhas reduziu a viabilidade larval e pupal em 51% e 47%, respectivamente, demonstrando potencial inseticida contra a praga (BRUNHEROTTO & VENDRAMIM, 2001).

Estudos foram realizados com jurubeba *Solanum fastigiatum* var. *acicularium* contra o pulgão *Brevicoryne brassicae* (Hemiptera: Aphididae), praga da couve (*Brassica oleracea* var. *acephala*) e confirmaram ação repelente do extrato dos frutos quando pulverizado sobre a cultura e ação inseticida do extrato das folhas quando aplicado sobre o inseto (LOVATTO et al., 2004).

A ação de extratos aquosos de frutos verdes, de pecíolos com caule, de folíolos e de casca de *Melia azedarach* var. *azedarach* (cinanomo) foi avaliada no controle de adultos de *Diabrotica speciosa* (Coleoptera: Chrysomelidae), em cultivos de pepino, (*Cucumis sativus*) e de feijão (*Phaseolus vulgaris*). Os resultados mostraram que todos os extratos foram promissores no controle do inseto, em cultivos de pepino. Entretanto, no cultivo de feijão, apenas o extrato de frutos verdes foi eficiente. Os autores recomendam esses extratos como alternativa ao uso de inseticidas químicos nas culturas citadas (SEFFRIN et al., 2008).

Foi avaliado o efeito dos extratos de *Aristolochia lagesiana* sobre aspectos biológicos da lagarta-da-soja *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Os extratos alongaram o período larval, reduziram o peso de pupas e diminuíram a viabilidade larval (VIEIRA et al., 2009), demonstrando efeito indireto sobre o ciclo de vida da praga. De modo semelhante, o extrato aquoso de folhas de *Anacardium humile* (cajuzinho-do-cerrado) foi testado sobre *Bemisia tuberculata* (Bondar, 1923) (Hemiptera: Aleyrodidae) (mosca branca da mandioca) e provocou mortalidade parcial

em ninfas e pupas, aumento da fase ninfal nos insetos sobreviventes e mortalidade entre 73-100% dos insetos no final do ciclo (ANDRADE-FILHO et al., 2010).

Tagliari et al. (2010) avaliaram o efeito de 20 extratos de plantas sobre a mortalidade larval de *Spodoptera frugiperda* (J.E. Smith), (Lepidoptera: Noctuidae) praga do milho. Dentre as espécies avaliadas, *Petiveria alliacea*, *Malva silvestris* e *Artemisia verlotorum* foram as que causaram maior mortalidade de larvas: 98%, 98% e 88%, respectivamente.

Indigofera suffruticosa Mill. é uma leguminosa, da família Fabaceae, encontrada nas regiões tropicais e subtropicais. O gênero *Indigofera* L. é constituído por cerca de 700 espécies, representadas por plantas herbáceas e arbustivas. No nordeste do Brasil é bem conhecida e utilizada na medicina popular contra infecções, inflamações e epilepsia (WONG et al., 1999; LEITE et al., 2006; BARROS & TEIXEIRA, 2008). Posteriormente, foi relatada também sua atividade antitumoral. A análise fitoquímica de extratos de folhas de *I. suffruticosa* revelou a presença de alcalóides, flavonóides, esteróides, proteínas, carboidratos e Indigo, compostos esses que tem atividade biológica antinflamatória e antimicromiaba (VIEIRA et al., 2007).

Apesar de seu emprego com fins medicinais, esta espécie apresenta toxicidade para animais. RIBEIRO et al. (1991) encontraram efeitos hepatotóxicos após o consumo de pequena quantidade de extrato aquoso do fruto de *I. suffruticosa* por ratos, além de efeitos citotóxicos após o consumo de quantidades maiores. As partes aéreas e as sementes de *I. suffruticosa* foram consideradas tóxicas em experimentos com bovinos, resultando em anemia hemolítica nos mesmos, além, de outras patologias encontradas no fígado e rim por intoxicação pelo consumo da planta (BARBOSA-NETO et al., 2001).

Panizzi (1992) avaliou a atividade inseticida de quatro espécies de *Indigofera*: *I. endecaphylla* Jacq., *I. suffruticosa* Mill., *I. hirsuta* L. e *I. truxilliensis* H. B. K. contra *Piezodorus guildin*ii (Heteroptera: Pentatomidae), praga da soja. *I. suffruticosa* foi eficiente em reduzir a sobrevivência ninfal para 15%, a sobrevivência de adultos foi menor que 20% e a ovoposição foi reduzida para menos de 30%. Vieira et al. (2012) verificaram atividade inseticida de *I. suffruticosa* contra *Aedes aegypti* (Diptera: Culicidae) (mosquito transmissor da dengue), afetando também a ovoposição e com efeito embriotóxico nos primeiros estágios de desenvolvimento do inseto.

Myrciaria cauliflora Berg., pertence a família Myrtaceae, conhecida popularmente como jabuticabeira, é uma árvore nativa brasileira e o Brasil é o seu maior produtor mundial. Seu fruto pode ser consumido ao natural ou processado, na forma de geléia, vinho, licor e vinagre (LIMA et al., 2008). Na medicina popular, a jabuticabeira é utilizada no tratamento de diarréias, irritações da pele, asma e hemoptise (PEREIRA et al., 2000; OLIVEIRA et al., 2003). Polo et al. (2006) também relatou atividade antimicrobiana do extrato de folhas de *M. cauliflora* contra *Streptococcus*. Pesquisas com extrato metanólico de frutos citam na análise fitoquímica a presença de compostos fenólicos, incluindo flavonóides, ácidos orgânicos, antocianinas e alta atividade anti-radicais, além de compostos antioxidantes e antitumorais. Estudos subsequentes de fracionamento resultaram no isolamento de um novo composto: Jaboticabin (REYNERTSON et al., 2006; DUARTE et al., 2010; SANTOS et al., 2010), que tem um possível papel na prevenção de doenças relacionadas ao *stress* oxidativo (CAVALCANTI et al., 2011).

Myrciaria. cauliflora ainda não possui atividade inseticida relatada, entretanto a família Myrtaceae tem ação inseticida conhecida contra insetos de diversas ordens, como por exemplo: *Ecalyptus citriodora* Hook contra *Tribolium castaneum* Herbst

(besouro de grãos armazenados) (MAZZONETTO & VENDRAMIM, 2003) e extratos de folhas e óleo essencial de *Eugenia uniflora* L. (pitangueira) e *Melia azedarach* L. (cinanomo) contra *Atta laevigata* Smith (Hymenoptera: Formicidae) (formiga cortadeira) (JUNG et al., 2013). Isto indica a possibilidade dos extratos de *M cauliflora* também atuarem no controle de insetos-praga.

Assim sendo, percebe-se o potencial biotecnológico de formulações com fungos entomopatogênicos e dos extratos vegetais, demonstrando que através de sinergismo, o controle de pragas da agricultura pode ter melhores resultados, em menos tempo, e sem causar danos ao ambiente, contribuindo para o desenvolvimento sustentável.

3. OBJETIVOS

3.1 Geral

Avaliar o efeito de formulações a base de óleo adjuvante emulsionável (OAE), extratos de *Indigofera suffruticosa* e de *Myrciaria cauliflora* na viabilidade de conídios de *Metarhizium anisopliae* e de *Beauveria bassiana*, bem como a patogenicidade dessas formulações a *Diatraea saccharalis*.

3.2 Específicos

- Avaliar o efeito fungitóxico do Veget'oil® sobre *M. anisopliae* e *B. bassiana, in vitro*;
- Avaliar a viabilidade dos conídios de *M. anisopliae* e *B. bassiana* formulados em Veget'oil®;
- Avaliar a patogenicidade de *M.anisopliae* e de *B.bassiana* formulados em Veget'oil® a *D. saccharalis*;
- Avaliar o efeito fungitóxico dos extratos das folhas e das sementes de *Indigofera suffruticosa* e dos frutos de *Myrciaria cauliflora* sobre *M. anisopliae* e *B. bassiana, in vitro*;
- Avaliar o efeito dos extratos das folhas e das sementes de *Indigofera suffruticosa* e dos frutos de *Myrciaria cauliflora* e sua associação com *M.anisopliae* e *B.bassiana* sobre *D. saccharalis*;

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CAPÍTULO II

BIOFORMULATIONS IN PEST CONTROL – A REVIEW

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BIOFORMULATIONS IN PEST CONTROL – A REVIEW

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Review article

ABSTRACT

Biotic and abiotic factors limit the action of entomopathogens and interfer for reaching the expected results. Moreover, the requirement of import and export markets for good quality foods with low content of toxic waste has increased. In this sense,new organic products have been developed in order to improve the stability, virulence and efficacy of entomopathogenic agent in the field.The aim of this paper is to report on the use of formulations with entomopathogenic fungi to control pests. About 12 species or varieties of fungi have been used as active ingredients in formulations of mycopesticides. A formulation can be defined as the combination of an active ingredient (such as entomopathogen), an inert carrier and an adjuvant which will improve the performance of the product, and also will be ease for handling and application. The *Metarhizium anisopliae* and *Beauveria bassiana* are the most used fungi in formulations worldwide. The synergistic effect of fungal interactions with the phytosanitary product has attracted the attention of several researchers due to their potential to cause high mortality of the target insect, becoming a tool for deployment in integrated pest management.

KEY-WORDS: Formulations with Entomopathogens; Biological Control; Metarhizium anisopliae; Beauveria bassiana.

1. INTRODUCTION

Over several decades, the widespread use of chemical insecticides to control pests caused side effects such as environmental imbalance, toxic residues in foods, diseases in humans and other animals and the development of resistance mechanisms in insects [1]. A

viable alternative to chemical control is the use of natural enemies, a practice known as biological control. This method enables the maintenance of insect populations in balance, limiting their rapid multiplication without causing harm to other organisms [2,3].

The entomopathogenic fungi are widely used in Biological Control because they are the main pathogens of insects, causing more than 80% of their diseases [4]. Under favorable conditions, they can cause outbreaks and enzootic diseases in natural species of Hemiptera, Lepidoptera and Coleoptera. They specialize in penetration via the tegument and may infect different stages of host development [5,6,7].

In relation to the Integrated pest management, which recommends the combination of different techniques and resources to maintain a population of insect pests below the economic injury level [8,9], various researches have been designed to enhance the action of entomopathogenic formulations, which contribute to the development of stability, virulence and efficacy of the entomopathogenic agent in the field [10,11]. This study aimed to present a review of the use of formulations containing entomopathogenic fungi to control pests.

2. BIOLOGICAL CONTROL WITH ENTOMOPATHOGENIC FUNGI

Among the entomopathogenic fungi, *Metarhizium*, *Beauveria*, *Paecilomyces*, *Lecanicillium*, *Nomurea*, *Aschersonia*, *Hirsutella* and *Entomophthora* are considered the most important [4] genus. Most of the entomopathogenic fungi have been distributed through out many decades in the Hyphomycetes class.These organisms were called anamorphic (group of fungi that have no phylogenetic relationship with others, being considered as perfect) [12]. The anamorph has its counterpart in sexual teleomorph, which corresponds to the sexual phase of Ascomycota or more rarely, Basidiomycota. Subsequently, the *Metarhizium* genus was described as the anamorph of Ascomycota *Cordyceps brittlebankisoides* [13], since the anamorph *Metarhizium anisopliae* var. *majus* (= M. *anisopliae*) [12, 13, 14, 15] was isolated from the larvae of Coleoptera (Scarabaeidae). Molecular phylogenetic studies have supported

this classification and currently the teleomorph of *Metarhizium* was assigned to *Metacordyceps* [16,17].

Among the entomopathogenic fungi, *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuilemin stand out due to their wide geographic distribution and host range. The most successful biological control program with *M. anisopliae* was from 1970-1991, when the fungus was applied in several acres of sugar cane fields infested by *Mahanarva posticata* Stal (Hemiptera: Cercopidae), sugar cane leafhopper in the northeast of Brazil [4]. Since then, several researches have been conducted proving the pathogenicity of *M. anisopliae* to other species of agricultural importance such as *Coptotermes formosanus* Shiraki (termites) [18], *Schistocerca gregaria* Forck (grasshoppers) [19], *Hylobius abietis* (pine weevil) [20], *Rhipicephalus (Boophilus) microplus* Canestrini (cattle tick) [21] and *Anthonomus grandis* Boheman (boll weevil) [22].

B. bassiana, an anamorph of *Cordyceps* Fr [23], is one of the most established entomopathogenic fungi taxonomic, and it has widespread occurrence in all countries, being more frequent on insects and soil samples, in which it can survive for long time in saprogenesis [24]. The *B. bassiana* fungus has become internationally known by the Soviet product called Boverin, a wettable powder formulation [25] recommended for the control of *Leptinotarsa decemlineata* Say (potato beetle) and other species. Research conducted with different species showed that *B. bassiana* is pathogenic and virulent to various pests and parasites as *Haematobia irritans*L. (horn fly) [26]; *Psoropets ovis* Hering (rabbit parasite) [27]; *Laniifera cyclades* Druce (cactus pest) [28]; *Tribolium castaneum* Herbst (red flour beetle) [29]; *Atteva sciodoxa* Meyrick (medicinal plants caterpillar) [30]; *Hyalomma anatolicum* Koch [31] and *Rhipicephalus (Boophilus) microplus*(ticks) [32], and *Zaprionus indianus* (fig wasp) [33] .

The mechanism of action of entomopathogenic fungi involves several processes until the insect is completely colonized and killed. First, the mechanical force exerted by the pressure of the hyphae, by breaking the membranous or sclerotic areas of the cuticle of the host, followed by the start of the enzymatic process that results from the release of enzymes, especially proteases, chitinases and lipases, which alter the surface tegument, releasing peptides that serve as nutrients for the fungus and facilitate the penetration into the insect

[6,34]. These features are unique to fungi and puts them at an advantage compared to other pathogens that depend on the intake of their propagules to initiate infection [35,7]. Thus, the fungi can infect different host development stages including the stages, which are not fed such as eggs and pupae. After infection, the externalization of the fungus appears on the body surface of the parasitized insect, and the morphologic appearance of the colonization depend on the specie of the entomopathogenic fungi, an example is shown in Figure 1 for larvae of *Diatraea saccharalis*, the sugarcane borer, killed by *M. anisopliae* (Figure 1a) and *B. bassiana* (Figure 1b) colonization. The sick insect dies of a set of modifications on the hemocele, tissues and internal organs. The cycle is completed when sporulation occurs in the body of the parasitized insect allowing horizontal transmission of the pathogen by spreading the propagules among the insect population, as well as to the environment, so that resulting in spreading infection [36,37].

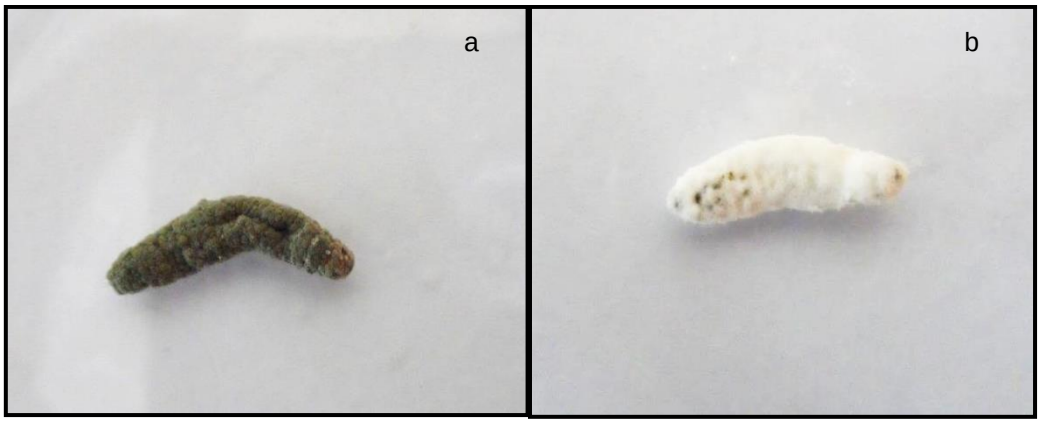

Figure 1. Larvae of *Diatraea saccharallis* colonized by *Metarhizium anisopliae* (a) and *Beauveria bassiana* (b).

3. FORMULATIONS OF ENTOMOPATHOGENIC FUNGI

The preservation time and viability of conidia are the main obstacles to its use in a large scale. Generally, biotic and abiotic factors (temperature, solar radiation, moisture, predation competition, among others) limit the fungi action on the field and can have a direct effect on the growth, germination and infective potential of entomopathogenic fungi [38,39,40,41,42,43,44]. In this sense, several products have been developed in order to increase stability, virulence and

efficacy of the entomopathogenic agent (Figure 2). Among these products, formulations based on emulsifiable adjuvant oil have been widely studied due to the facility for storage under controlled temperature and relative humidity (25 ± 1 °C; 70 ± 10 %), and protection of conidia against UV rays, with consequent increase persistence in the field and ease of implementation [10].

Several aspects should be considered prior to develop a formulation with entomopathogenic fungi. Firstly, it is necessary the addition of certain compounds that improve the performance of the fungus in the field. Secondly, that the formulation be easier to handle and apply; and finally, that it allows a longer time of storage under conditions that minimize the cost, and also with the minimal loss of the quality of the product. These components should also increase the persistence of the product, adhesiveness on the insect and attractiveness to the pest [45] (Figure 2). In addition to the active ingredient (pathogen) and inert/vector, the formulations can contain adjuvant, components that are used to optimize the action of the active ingredient and improve the characteristics of the formulated product (Figure 2), for example, their ability to spread on hydrophobic surfaces. Among other properties, they have photoprotective, phagostimulant and anti-evaporation functions .

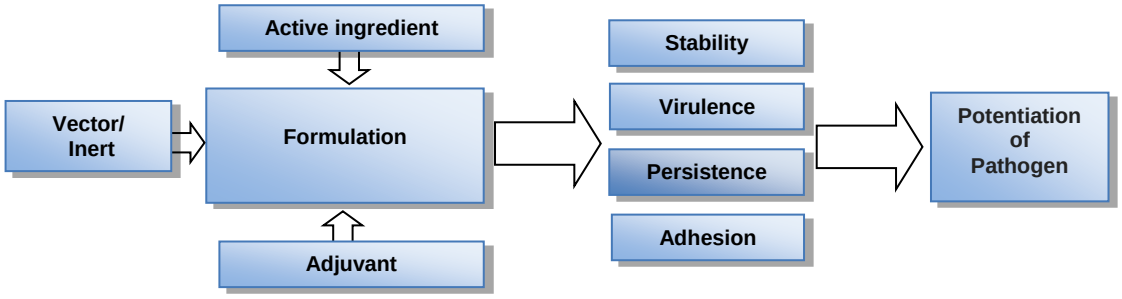

Figure 2. Schematic representation of developing formulations and their advantages.

About 12 species or subspecies (varieties) of entomopathogenic fungi have been used as active ingredients in mycopesticides, for the control of insects and mites, *such as M. anisopliae var. anisopliae, M. anisopliae var acridum, M. flavoviride, B. bassiana, B. brongniartii, Verticillium lecanii, Paecilomyces fumosoroseus, Isaria farinosa, Sporothrix insectorum, Hirsutella thompsonii, Nomuraea rileyi* and *Cladosporium cladosporioides*. Among the most common products developed worldwide containing entomopathogenic fungi as the active

ingredients, *M. anisopliae* and *B. bassiana* are in first place, each one representing 33.9% of the total, followed by *Lecanicillium* spp. (9.4%), *Isaria fumosorosea* (5.8%) and *B. brongniartii* (4.1%). In the inventory of products and formulations, around 26% are substrate colonized by the fungi, 20.5% are wettable powders and 15.2% oil dispersions [45,46].

The most used types of formulation containing entomopathogenic fungi are wettable powder, granules, water dispersible granule, bait, sprinkle powder, powder for contact, oil dispersion, suspension concentrate, miscible suspension concentrate in oil, and suspension in ultra-low volume. The formulation of wettable powder type is applied after dilution with water, such as Boveril®, a commercial product which is used for control of pests in crucifers [47]. The sprinkle powder is applied by dusting, whilst powder for contact is by direct application on the pest [48]. The granules are a kind of solid formulation of uniform size, but cereal grains such as rice are not included in this type of formulation, because they are considered as technical concentrated or non-formulations [49] .On the other hand, water dispersible granule disintegrates in water before application, such as PFR97 TM® having *Paecilomyces fumosoroseus* as the active ingredient [50].

The type bait formulation was developed to attract the pest and be consumed by it, as traps Termitrap® used for subterranean termites Water based and sugar cane molasses [51]. The oily dispersion contains the active ingredient (entomopathogenic fungus) in surfactant for using after dilution in water [52]. In this type of formulation are included suspensions in oil emulsion, for example Met52 EC®, a commercial product (Novozymes Biologicals, Inc., USA) that must be diluted prior to use in the laboratory or in the field, e.g. against the sweet potato beetle *Cylas formicarius* [53]. The suspension concentrate is already an active ingredient in water, and may be diluted further prior to be applied [54]. The miscible suspension concentrate in oil is a suspension containing the active ingredient in a fluid for dilution in organic liquid, in this formulation it is included the Metarril SP Organic® produced by Koppert Biological Systems [55]. The suspension in ultra-low volume comes ready to use or may need small dilution, but it requires special sprayers equipment for application [56], in order to avoid blockage in the outlet nozzle of the applicators; most of ultra-low volume sprayers utilize a small electric pump that

can be very finely adjusted to vary droplet size and flow rate, so that meet the desired specific spray application

4. GLOBAL SCENARIO OF FORMULATIONS

In Brazil, products based on *M. anisopliae* represent 55% of commercially available products or in the registration process, followed by *B. bassiana* (30%), *Lecanicillium* spp. (7.5%) and *Sporothrix insectorum* (7.5%). Most of these Brazilian mycopesticides have no record; of these, 2.5% are marketed as pure conidia, 72.5% are technical concentrates (liquid or solid substrates, colonized by fungi) and only 25% are in fact oil dispersion formulations. In this sense, studies with formulations based on vegetable oils and emulsifiers have being carried out in several countries and have produced interesting results for example, when the neem oil (*Azadirachta indica*) is associated with the fungus *B. bassiana*, causing over 90% mortality, on nymphs of *Bemisia tabaci* (whitefly), in the United States of America [57]. Experiments in the South Africa with *Tetranychus urticae* Koch exposed to *B. bassiana* in an oil emulsion obtained 61% of mortality of mites after seven days [58], demonstrating the potential of the formulation for field-testing.

Test formulations of the fungus *M. anisopliae* and *B. bassiana* in oil, for the control of malaria mosquito (*Anopheles* sp.) have showed that formulated conidia were more effective in controlling larvae than non-formulated ones, in addition to persisting longer under field conditions, in the Netherlands [59]. Other studies have also confirmed the high mortality of insects (*Plutella xylostella* (L.)) when in contact with castor oil (*Ricinus communis* L.) added with *B. bassiana* [60].

Therefore, one can notice that the synergism of the association between fungus and oil, indicate its potential against the target pest in the field. The availability of products on the market formulated with high concentration and viability of infective structures, easy handling and application, greater efficiency and with competitive price is essential for establishing the use of entomopathogenic fungi for pest control in large-scale [61,62].

5. CONCLUSION

Among the main advantages of using bioformulations with entomopathogen fungi for biological control of insect pests we can point out the easiness of production of its infective units on a commercial scale, the simplicity of usage in field conditions, the low cost of its utilization, and mainly, the reduction on environmental impact [63, 64, 65, 66,67]. Interactions between entomopathogenic fungi with phytosanitary products, such as chemical insecticides (e.g. Decis OC), fungicides (e.g. Manzate 800) or herbicides (e.g. Granoxone) are important to evaluate new formulations, since it can be positive when an additive or synergistic action occurs with the entomopathogen and the product. However, a negative interaction may appear when an antagonistic effect is caused by the inhibition of one of the components, which usually is the active ingredients or entomopathogen. Therefore, prior to consider joint implementation as an effective formulation, there is a need for compatibility testing, seeking more selective products and able to promote the conservation of the pathogen in the field for a longer period of time [68,69].

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COMPETING INTERESTS

The authors declare that there are no conflicts of interest.

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CAPÍTULO III

EFFECTS OF EMULSIFIABLE ADJUVANT OIL ON THE BIOLOGICAL PARAMETERS OF ENTOMOPATHOGENIC FUNGI

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EFFECTS OF EMULSIFIABLE ADJUVANT OIL ON THE BIOLOGICAL PARAMETERS OF ENTOMOPATHOGENIC FUNGI

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ABSTRACT:

Background: Studies on the stability and toxicity of fungi enthomopatogen with emulsifiable oil are important and deserve investigation with a view ofachieving a better

effect against pests. In this work it was evaluated the effects of Veget'oil® on the biological parameters of *Metarhizium anisopliae* and *Beauveria bassiana* strains.

Results: The viability of conidia was unaffected, neither the sporulation remained unchanged, except for *B. bassiana*ARSEF1398, at the highest oil concentration. The vegetative growth of fungi was adversely impacted by incubation with emulsifiable oil resulting in reduced colony diameters. The formulation with *B. Bassiana* ESALQ447 remained viable for more than 5 months at 25 ± 1 ºC, as well as all the fungal formulations stored at -7 ± 1 º C. The other fungi formulation remained alive for until 90 days at room. There was no significant difference among strains of *M. anisopliae*, however *B. bassiana* ESALQ447 was better than *B. bassiana* ARSEF1398, when stored.

Conclusion: Formulations of *M. anisopliae* and *B. bassiana* fungi emulsionable oil based may be storage at control room temperature and action will increased because protection for conidia and persistence increases in the field, providing pest control more effectively.

Key words: *Beauveria bassiana*; Compatibility; Emulsifiable oil; Viability of Conidia; Toxicity.

1 INTRODUCTION

Fungal development in biological control of insect pests has received special attention since they are the main insect pathogens[1]. *Metarhizium anisopliae* and *Beauveria bassiana* stand out among the entomopathogens and have been successfully applied in pest control, both in Brazil and abroad. [2-5] Shelf life and conidia viability remain two of the main obstacles to large scale fungal production.[6]

Formulations are a mixture of the active ingredient (live propagules of the fungus) with adjuvants to provide greater stability for the biological agent, ease in handling and application, environmental protection and incremented action mechanism. Myco-insecticides based on emulsifiable oils are a good alternative for use in the field because they mix with water thus reducing the hydrophobic nature of conidia and increase the spread and adhesion on the insect cuticle.[7,8]

Research shows that formulated products enable preservation of fungi stability during storage, in addition to the combined chemical and biological action promoting increased host susceptibility, through the synergistic effect of this association. Emulsifiable oil-based formulations are widely studied because of their easy storage at room temperature (25 ± 1 º C), and protection of conidia from UV rays, with the consequent increased persistence in the field and the ease of application using conventional equipment such as powered sprayers or aerial application.[9-11] However, little information is available to indicate the stability or fungitoxic effects of emulsifiable oils on microorganisms, requiring appropriate scientific research to introduce new knowledge to help in pest management.[12, 13]

Organic farming has been increasing in Brazil, making mandatory the adoption of more effective measures in order to ensure crop sustainability by minimizing losses from insect pests. [7, 14-16] The aim of this study was to evaluate the effect of a commercially available emulsifiable oil-based adjuvant formulations, on different biological parameters of *M. anisopliae* and *B. bassiana*.

2 MATERIAL AND METHODS

2.1 Fungal Strains

The strains used in the experiments were *Metarhizium anisopliae* PL43, *Beauveria bassiana* ESALQ447 and *Beauveria bassiana* ARSEF 1398 from the collection of entomopathogens, maintained at the Laboratory of Microbial Control of Insects and Pathology, Department of Entomology, Plant Pathology and Agriculture, College of Agriculture "Luiz de Queiroz", São Paulo University (USP-ESALQ/SP). The strain *Metarhizium anisopliae* IBCB425 provided by the Department of Biological Control of Biology Institute, Campinas / SP. The fungi were cultured on a PDA (potato-dextrose-agar) medium (Merck).

2.2 Compatibility Test

Veget'oil (Oxiquímica Agrociência Ltda, Jaboticabal,Brazil) was the natural insecticide tested. The oil is composed of fatty acid esters (93% m/v) in a refined soybean oil preparation with an emulsifier (7% m/v), suitable for formulations of the emulsifiable concentrate (EC) type. The *in vitro* effect on the entomopathogenic fungi was evaluated at three concentrations (FR = average field recommendation (0,5%); 0.5 x FR (0,25%) and 2 x FR (1%)) for the purpose of analyzing the following biological parameters: vegetative growth, sporulation and conidia germination. The oil was incorporated into soft, unsolidified PDA culture medium at 40 ± 5° C. After mixing, the medium was poured onto Petri dishes (9cm diameter) and inoculated with fungus.[17] Vegetative growth and sporulation tests were performed by inoculating fragments culture in the center of Petri dishes with the aid of a platinum loop. Eight days after incubation at 25 ± 1° C, the colony diameter was measured with a millimeter scale ruler. After that, a central disc (4mm) of the colony was taken in order to measure conidia production. Each disc was placed in a glass tube containing 10mL of water plus agral (0.02%) and vigorously agitated until conidia were totally released from the surface of the medium.[18] The suspension obtained was diluted to 10^{-1} and the conidia were

counted using a Neübauer chamber. A volume of 0.1ml of this suspension was seeded on PDA and 20 hours after incubation (25 ± 1ºC), the percentage of germination was quantified by observation using an optical microscope. 500 conidia germinated and non-germinated were counted.[19] The experiment had 5 replicates. The product toxicity level (T) was calculated using: T = (20 [VG] + 80 [SPO]) / 100, where VG = percentage of vegetative growth in relation to the control; SPO = percentage of sporulation in relation to the control.[1] The biological index was used for comparing toxicity values. The biological index includes conidia germination in the calculation: BI = 47 [VG] + 43 [SPO] +10 [GERM] / 100, where VG = percentage of vegetative growth in relation to the control; SPO = percentage of sporulation in relation to the control; GERM = percentage of conidia germination as compared to the control. The values for T are classified according to the following limits: 0-30 (very toxic), 31-45 (toxic), 46-60 (moderately toxic) and 60 (compatible); the values for BI can vary within the range: 0-41 (toxic), 42-66 (moderately toxic) and 66 (compatible) .[17]

2.3 The effects of Veget'oil® on conidia germination on medium-term storage

The effects of storage on the fungal formulations were evaluated through the percentage of conidia germination. Conidia were produced using autoclaved parboiled rice (100g) in polypropylene bags. The bags were inoculated with a 10mL of a suspension containing $1x10^8$ conidia/mL. After 12 days at 25±1ºC for fungal development and sporulation, the plastic bags were opened, the rice with conidia was spread out on clean plastic trays and then, for about three days, kept under controlled conditions (T = 17 ± 1 ° C, RH = 20 ± 3%) with dehumidifiers (Thermomatic do Brazil, São Paulo, Brazil, model Desidrat D4), until achieving a moisture level of around 5%.[8] Conidia were extracted from the rice using metallic sieves (300mm mesh) and assessed using an infrared moisture analyzer (Ind. e Com. Eletro-Eletrônica Gehaka Ltda, São

Paulo, Brazil, model IV2000/IV2002). One gram of dry conidia from each strain to be tested was sorted and mixed with 10 mL of water plus Agral (0.02%) in order to calculate the number of conidia using a Neübauer chamber. The number of conidia per gram of dry material ranged from 1.6 $x10^{10}$ conidia/g for *M. anisopliae* IBCB425 to 6.3 $x10^{10}$ conidia/g for *B. bassiana* ESALQ447. After that, one gram of pure dry conidia was mixed with 10mL of oil and the resulting formulation placed in plastic bottles and stored at two different environments (at 25° C and at -7° C). The viability of stored formulations was assessed at every fifteen days, for ninety days, by taking 0.1mL and diluting it in saline solution plus Agral (0.1%). After vortexing for 1 minute to break up conidial chains, a new aliquot was removed and spread over the PDA medium surface in acrylic plates (6 cm). The plates were incubated at 25 ± 1º C and assessed after 24 hours. Five replicates were prepared for each formulation.[11]

2.4 Statistical analysis

The software used for statistical calculations was ASSISTAT 7.5 beta.[20] Significant differences in the analysis of variance were compared by the Tukey test, with a 5 % error margin.

3 RESULTS AND DISCUSSION

The growth of fungi was significantly impacted by different concentrations of tested oil, with a reduction in colony diameter as compared to the control (Figure 1). All doses of oil influenced the fungal behavior. As for the number of conidia, there was no significant difference between strains of *M. anisopliae* and *B. bassiana* ESALQ447 and the control. However, with *B. bassiana* ARSEF1398, all doses applied negatively influenced sporulation (Table 1 e 2).

In similar studies, Neem oil promoted a larger negative effect on *B. bassiana*, inhibiting germination, colony diameter and conidiogenesis. [7]. Previous studies showed the negative effect of biofertilizers on conidiogenesis. [15, 21-23] In this paper, any of the concentrations tested no effect on conidia viability was observed, however the parameters evaluated change for each strain depending on the product and concentration used, showing the importance of selection of plant protection products.[18, 24]

The germination percentage of *M. anisopliae* and *B. bassiana* showed no significant change as compared to control. Even at the highest oil concentration, conidia viability remained above 99% germination (Tables 1 and 2). These results corroborate existing research on the effects of agrochemicals based on mineral and vegetable oil added in tank-mixes for spraying entomopathogenic fungi, in the field, given that conidia germination levels also showed no change when in contact with products.[19] However, the *in vitro* results germination of *B. bassiana* ESALQ447was stimulated in contact with oil, indicating the variability among fungal strains resulting in different responses to various products.

Based on the values obtained for toxicity of the formulations (Table 3), Veget'oil is compatible with *M. anisopliae* PL43 and *B. bassiana* ESALQ447 but is moderately toxic to *M. anisopliae* IBCB425 at the highest concentration (2 x FR) and toxic to *B. bassiana* ARSEF1398 at the lowest concentration (0.5 x FR). Furthermore, the use of Biological Index (BI) showed that the product is toxic to *B. bassiana* ARSEF1398 only at the highest concentration. When the inclusion of conidia germination in the formula was done it was observed an increase in the value of BI because the conidia viability was not affected when the fungus were in contact with the product; for the other strains, Veget'oil is, at most, moderately toxic. Other authors also found changes in pesticide

classification when they employed both formulas, recommending the use of the most current mathematical model for selection of pesticide products. [15, 17, 25]

As for the storage of the formulations, the initial viability of the conidia was higher than 90% and fell off over time. This effect was noted over the first month, at 25 ± 1°C (Table 4). Moreover, after the first 30 days, germination was delayed and only started after 44 hours of incubation. There was no significant difference between strains of *M. anisopliae* in the environment or in a cold chamber any.

Beauveria bassiana ESALQ447 stood out in keeping germination percentage above 70% after 90 days and remained viable even after five months at 25 ± 1ºC. This strain was statistically better than *B. bassiana* ARSEF1398 in all tests, during the entire period (Table 5). All formulations stored at -7 ± 1ºC maintained high viability during the three months of study and even after 11 months, still displayed over 70% viability (data not shown). On the other hand, *B. bassiana* ARSEF1398, *M. anisopliae* PL43 and *M. anisopliae* IBCB425 died out at between 75 and 90 days at 25 ± 1ºC.

Studies showed that *M. anisopliae* and *B. bassiana* conidia have greater longevity at low relative moisture levels (around 10%);[24] and may reach 40 weeks at 5% humidity in a formulation with peanut oil, which caused minor adverse effects on the *Isaria fumosorosea*, while Natur'l® oil caused loss of viability after 25 weeks of storage at 27°C. [11] In this research, formulation with *B bassiana* remained viable for more than 20 weeks, at 25ºC, for one desirable characteristic mycoinsecticide because the temperature is a determining factor for spore germination and low temperature is needed to maintain the viability for long periods. Then the farmer will have a product easy to use, with the option to keep at room temperature, reducing costs.

One major challenge for Biological Control is bioinsecticide storage capability at ambient conditions, similar to conventional insecticides, without suffering adverse

effects with possible loss of product viability. The results obtained in this work showed the possibility of storage of the formulations based oil adjuvant emulsionable, at environment, and thus increase the integrated pest management in sugarcane crop.

4 CONCLUSIONS

The results indicate that *Beauveria bassiana* ESALQ447 and *Metarhizium anisopliae* PL43 and IBCB425 formulated with Veget'oil remain viable when they are stored at control temperature (25 ± 1ºC) or under refrigeration (-7 ± 1ºC) and will have their action optimized in Integrated Pest Management Program (IPM). Nevertheless, the fungal formulations with Veget'oil has to be tested to investigate the fungi pathogenicity to insect pests.

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8. APPENDIX

Table 1. Colony diameter, sporulation and viability of conidia of *Metarhizium anisopliae* on PDA containing different concentrations of oil emulsionable.

Treatments	Strains					
	Colony diameter (cm)		**Sporulation** ($x10^7$ spores)		**Viability** (%G)	
	PL43 **	IBCB425**	PL43 ns	IBCB425 ns	PL43 ns	IBCB425*
Control	3.93aA[1]	3.88aA	4.78a	6.26a	99.80aA	99.36abA
0,5xFR[2]	1.44bA	1.34bA	3.62a	4.32a	99.94aA	99.88aA
FR	1.40bA	1.04cB	3.12a	4.76a	99.80aA	99.68abA
2xFR	0.97cA	1.01cA	3.98a	4.18a	99.80aA	99.18bB
Average	-	-	3.87	4.88	99.83	-
Δ (5%)	0.20	0.20	2.80	2.80	0.62	0.62
	CV%= 6.37		CV%= 37.43		CV%= 0.37	

ns Not significant;

** significant by Tukey Test (P=0,05)

[1]Average of five replicates. Means followed by the same small letter within the same column and means followed by the same capital letter within the same row are not significantly different by Tukey Test (P = 0.05) within each parameter.

[2] FR = average field recommendation (0,5%); 0.5 x FR (0,25%) and 2 x FR (1%).

Table 2. Colony diameter, sporulation and viability of conidia of *Beauveria bassiana* on PDA containing different concentrations of oil emulsionable.

Treatments	**Strains**					
	Colony diameter (cm)		**Sporulation** ($x10^7$spores)		**Viability** (%G)	
	ESALQ447**	ASEF1398**	ESALQ447ns	ARSEF1398**	ESALQ447**	ARSEF1398ns
Control	3.15aA[1]	2.15aB	50.56aA	39.08aB	96.86bB	99.33aA
0,5xFR[2]	1.52bA	1.32bB	45.90aA	12.00bB	99.73aA	99.50aA
FR	1.40bA	1.19bB	65.08aA	10.62bB	99.26aA	99.80aA
2Xfr	1.30bA	1.10bB	51.74aA	6.62bB	99.73aA	99.64aA
Average	-	-	53.32	-	-	99.56
Δ (5%)	0.25	0.25	24.50	24.50	1.17	1.17
	CV%= 9.13		CV%= 40.61		CV%= 0.69	

ns Not significant;

** significant by Tukey Test (P=0,05)

[1]Average of five replicates. Means followed by the same small letter within the same column and means followed by the same capital letter within the same row are not significantly different by Tukey Test (P = 0.05).

[2] FR = average field recommendation (0,5%); 0.5 x FR (0,25%) and 2 x FR (1%).

Table 3. Value of toxicity (T) and Biological Index (IB)-rated Veget'oil® as the antifungal effect of *Metarhizium anisopliae* and *Beauveria bassiana*.

TRATMENT	STRAINS	VALUE T[1]	CLASSIFICATION	VALUE IB[2]	CLASSIFICATION
0,5XFR[3]	PL43	73.36	C	59.79	MD
	IBCB425	68.82	C	55.95	MD
	ESALQ447	85.75	C	72.01	C
	ARSEF1398	37.19	T	52.07	MD
FR	PL43	63.78	C	54.80	MD
	IBCB425	76.46	C	55.32	MD
	ESALQ447	124.51	C	86.48	C
	ARSEF1398	26.92	VT	47.74	MD
2xFR	PL43	73.96	C	57.40	MD
	IBCB425	55.92	MD	50.94	MD
	ESALQ447	100.77	C	73.69	C
	ARSEF1398	25.77	VT	41.35	T

[1]According to Alves et al., 2008; [2]According Rampelotti-Ferreira et al., 2010.

[1]The values for T are classified according to the following limits: 0-30 (very toxic), 31-45 (toxic), 46-60 (moderately toxic) and 60 (compatible); [2]the values for IB can vary within the range: 0-41 (toxic), 42-66 (moderately toxic) and 66 (compatible); .

[3] FR = average field recommendation (0,5%); 0.5 x FR (0,25%) and 2 x FR (1%).

Table 4. Conidial viability (%G) of *Metarhizium anisopliae* formulations stored at two different temperatures.

TIME (days)	T= 25 ± 1ºC		T= -7± 1ºC	
	PL43	**IBCB425**	**PL43**	**IBCB425**
0	92.09aB[1]	93.89aAB	98.54aA	96.76aAB
15	82.37bB	94.09aA	97.80aA	96.74aA
30	81.71bB	84.52bB	98.18aA	97.90aA
45	90.98aB	91.02aB	98.00aA	97.48aA
60	92.82aB	89.76abB	98.50aA	98.18aA
75	73.32cB	77.98cB	98.38aA	98.50aA
90	7.00dB	7.06dB	97.06aA	97.46aA
msd column= 5.7179			msd row= 4.9751	
CV%= 3.47				

[1]Average of five replicates. Means followed by the same small letter within the same column and means followed by the same capital letter within the same row are not significantly different by Tukey Test (P = 0.05).

Table 5. Conidial viability (%G) of *Beauveria bassiana* formulations stored at two different temperatures.

	T= 25 ± 1ºC		T= -7± 1ºC	
TIME (days)	**ESALQ447**	**ARSEF1398**	**ESALQ447**	**ARSEF1398**
0	99.02aA[1]	94.29aB	99.23aA	95.00aB
15	97.73aA	91.57bB	98.92aA	92.22bB
30	84.43bD	89.86cC	98.02aA	91.52bcB
45	81.91cC	82.12dC	96.54bA	90.09cdB
60	81.11cC	67.79eD	92.30cA	89.81dB
75	81.03cC	45.01fD	92.34cA	90.12cdB
90	75.67dB	0.18gC	90.90cA	90.00cdA
msd column= 1.6679			msd row= 1.4512	
CV%= 1.03				

[1]Average of five replicates. Means followed by the same small letter within the same column and means followed by the same capital letter within the same row are not significantly different by Tukey Test (P = 0.05).

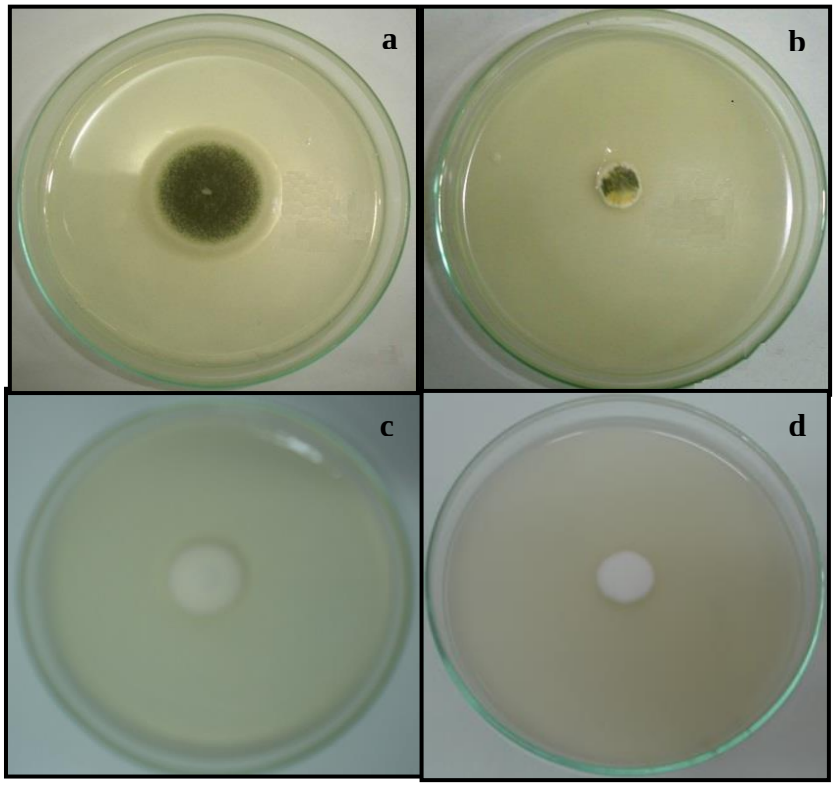

Figure 1. Macroscopic colony of *Metarhizium anisopliae* PL43 and *Beauveria bassiana* ESALQ447 after 8 days of development (26 ± 1 ° C) on PDA (a,c) and in medium containing adjuvant oil (recommendation field) (b,d), respectively.

CAPÍTULO IV

EFEITO DE FORMULAÇÕES DE *BEAUVERIA BASSIANA* E *METARHIZIUM ANISOPLIAE* À *DIATRAEA SACCHARALIS*

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O artigo será submetido a Biological Control

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Efeito de formulações de *Beauveria bassiana* e *Metarhizium anisopliae* à *Diatraea saccharalis*

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Resumo

Diatraea saccharalis (Lepidoptera: Crambidae) é uma importante praga da cana-de-açúcar. Causa danos ao desenvolvimento da planta e consequentemente, grandes prejuízos nos produtos finais: etanol e açúcar. Este trabalho teve por objetivo avaliar a virulência de fungos entomopatogênicos formulados em óleo adjuvante emulsionável (Veget'oil) à broca da cana, sob condições de laboratório. Foram avaliadas as linhagens *Metarhizium anisopliae* PL43, *Metarhizium anisopliae* IBCB425, *Beauveria bassiana* ESALQ447 e *Beauveria bassiana* ARSEF1398. As lagartas, de 3º *instar*, foram imersas em suspensões contendo diferentes concentrações de formulações (fungo + óleo), variando de 0% a 2%. O grupo controle foi inoculado com suspensões isentas de fungo ou de óleo. Os bioensaios foram examinados diariamente, por 10 dias. Todas as linhagens testadas foram patogênicas a *D. saccharalis*. O tratamento mais eficiente foi o que continha 2% de formulação, porém não existiu diferença significativa a partir de

0,25%, nas duas espécies avaliadas. *M. anisopliae* não foi tão eficiente quanto *B. bassiana* em reduzir a população de brocas, mesmo na maior concentração. A CL_{50} de *B. bassiana* ARSEF1398 foi 1% de formulação, enquanto que para *B. bassiana* ESALQ447 a CL_{50} foi 0,2% de formulação. *B. bassiana* ESALQ447 foi mais virulenta do que *B. bassiana* ARSEF1398, com mortalidade confirmada de 74% e 52%, respectivamente. Os resultados comprovam a relevância da seleção de isolados em laboratório, visando a formulação de entomopatógenos mais virulentos para o controle da praga no campo.

Palavras-chave: Fungos Entomopatogênicos; Formulação; Manejo Integrado de Pragas.

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1 Introdução

A cana-de-açúcar (*Saccharum officinarum*), planta semiperene de origem asiática, é considerada uma das culturas mais lucrativas para a economia brasileira. O Brasil é o maior produtor mundial de cana e o primeiro país do mundo na produção de açúcar e etanol, movimentando milhões de reais ao ano com o consumo interno e também com as exportações (IEA, 2013).

Grandes prejuízos são causados por *Diatraea saccharalis* (Lepidoptera: Crambidae), conhecida como a broca da cana-de-açúcar, uma das principais pragas que atacam a cultura causando danos diretos e indiretos. A abertura de galerias pela larva, ocasiona perda de peso da cana e provoca a morte das gemas ou ainda o secamento dos ponteiros, sintoma conhecido como "coração morto", além de facilitar a entrada de fungos fitopatógenos por meio dos orifícios e canais, caracterizando o complexo broca-podridão (HUANG et al., 2012; ZAPPELINI et al., 2010).

Vários métodos de controle são utilizados para minimizar as perdas decorrentes da ação da praga, como o controle cultural, por meio da rotação de culturas e do plantio de variedades resistentes; além destes, o controle biológico com o parasitóide *Cotesia flavipes*, com a bactéria *Bacillus thuringiensis* (*Bt*) ou ainda, com fungos entomopatogênicos como *Metarhizium anisopliae* e *Beauveria bassiana* (GUO et al., 2012; OLIVEIRA et al., 2008a,b; ZHANG et al., 2013). Diversos estudos de compatibilidade com produtos fitossanitários são realizados, em condições de laboratório, para avaliar sua seletividade a microrganismos e potencializar sua ação no Manejo Integrado de Pragas (MIP) (BLANFORD et al., 2011; ISLAM et al., 2010; SABBOUR et al., 2013; ZAHRAN et al., 2013).

Os componentes presentes nas formulações contribuem para a manutenção da estabilidade, virulência e eficácia do agente entomopatogênico. Dentre esses produtos, formulações à base de óleos emulsionáveis são amplamente estudadas devido à facilidade de estocagem em ambiente (25°C), proteção dos conídios dos raios UV, com consequente aumento da persistência no campo, e facilidade de aplicação, utilizando equipamentos convencionais como pulverizadores manuais ou mesmo aplicação aérea (ALVES et al., 2002a; ALMEIDA et al., 2008; BUKHARI et al., 2011; LOPES et al., 2013).

Este trabalho teve por objetivo avaliar o efeito de formulações de *M. anisopliae* e *B. bassiana* com um óleo adjuvante emulsionável disponível comercialmente (Veget'oil®) a *D. saccharalis*.

2 Material e Métodos

2.1 Linhagens fúngicas: foram utilizadas *M. anisopliae* PL43, *B. bassiana* ESALQ447 e *B. bassiana* ARSEF1398 provenientes da coleção de entomopatógenos, mantida no Laboratório de Controle Microbiano e Patologia de Insetos, no Departamento de Entomologia, Fitopatologia e Agricultura, da Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (ESALQ-USP); *M. anisopliae* IBCB425 foi fornecida pelo setor de Controle Biológico, do Instituto Biológico (Campinas/SP/Brasil).

2.2 Obtenção e manutenção de Diatraea saccharalis: as lagartas de 3º *instar* de *D. saccharalis* utilizadas nos bioensaios foram procedentes da criação experimental da Empresa Biotech Controle Biológico (Maceió/Alagoas/Brasil), mantidas a T= 25 ± 1ºC e U= 70 ± 10 %. Os insetos foram alimentados com dieta artificial (Hansley and Hammond, 1968).

2.3 Quantificação do inóculo: os conídios foram produzidos em arroz parboilizado autoclavado em sacos de polipropileno. Após 12 dias, mantidos a 27 ± 1ºC, para o desenvolvimento do fungo e a esporulação; o arroz colonizado foi espalhado em bandejas e estas transferidas para ambiente controlado (T= 17 ± 1ºC, UR= 20 ± 1%), contendo aparelhos desumidificadores (Thermomatic do Brasil, São Paulo, Brasil, model Desidrat D4), por cerca de três dias, até que a umidade ficasse em torno de 5% (MOORE et al., 1996). Os conídios foram então extraídos do arroz, com peneiras metálicas (300 mm de malha) em seguida, submetidos a um analisador de umidade por infra-vermelho (Ind. e Com. Eletro-Eletrônica Gehaka Ltda, São Paulo, Brazil· model IV2000/IV2002). Foi pesado 1g de conídios secos de cada linhagem a ser testada e misturados a 10 mL de água + Agral (0,02%), para quantificação de conídios em

câmara de Neübauer. A formulação foi composta por 1g de conídios puros secos adicionados a 10 mL de Veget'oil® (Oxiquímica Agrociência Ltda, Jaboticabal,Brasil).

2.4 Viabilidade dos conídios: a viabilidade dos conídios foi confirmada retirando-se 0,1mL da formulação e diluindo em 10 mL de solução salina + Agral (0,1%). Após agitação em Vortex, por 1 minuto, para desagregação dos conídios, uma nova alícota foi retirada e espalhada na superfície de placas de Petri de acrílico (06 cm) contendo BDA. As placas foram incubadas a 27 ± 1ºC e observadas após 20h. Foram realizadas cinco repetições para cada formulação (ALVES et al., 2002a).

2.5 Bioensaios: os bioensaios foram realizados com cinco grupos tratados com solução aquosa de 0%, 0,25%, 0,5%, 1% e 2% de formulação (Veget'oil + fungo). Para cada tratamento, havia um grupo controle respectivo, isento de fungo ou de óleo. As lagartas foram imersas nas suspensões por 30 segundos e o excesso de suspensão foi removido em papel filtro (QUINELATO et al., 2012). Posteriormente, as lagartas foram transferidas individualmente para placas de Petri de acrílico contendo dieta artificial. Foram utilizados 10 tratamentos, com cinco repetições, contendo 10 lagartas cada, totalizando 50 insetos/tratamento. O experimento foi mantido em condições apropriadas de temperatura e umidade (25 ± 1ºC, U= 70 ± 10 %) e 12 horas de fotofase. As observações foram realizadas diariamente, por 10 dias, transferindo-se as lagartas mortas para câmara úmida para confirmação do agente causal, por meio de exame microscópico de conidióforos e conídios na superfície do cadáver. Ao final dos 10 dias de observação foi calculada a mortalidade corrigida aplicando-se a fórmula: % Mortalidade Total - % Mortalidade da Testemunha x 100 / 100 - % Mortalidade da Testemunha (ABBOTT, 1925).

2.6 Análise estatística: os dados foram analisados utilizando-se o programa ASSISTAT 7,5 Beta e sujeitos à análise de variância (ANOVA). O teste de Tukey foi usado para separar as médias (P= 0,05) e a regressão polinomial para análise da CL_{50} (SILVA & AZEVEDO, 2002).

3 Resultados e Discussão

O número de conídios obtidos de cada linhagem foi ajustado conforme segue: $1,8x10^{10}$ con./g para *M. anisopliae* PL43, $1,6x10^{10}$ con./g para *M. anisopliae* IBCB425, $1,3x10^{10}$ con./g para *B. bassiana* ESALQ447 e $1,0x10^{10}$ con./g para *B. bassiana* ARSEF1398. A viabilidade inicial dos conídios formulados, no dia da realização dos experimentos, foi superior a 92%G, após 20 horas.

Todos os isolados testados foram patogênicos a *D. saccharalis* matando entre 16 e 74% dos insetos. Na superfície do corpo dos insetos mumificados observou-se a proliferação de micélio e conídios de coloração típica das espécies estudadas (Figura 1). A mortalidade total do grupo controle foi de no máximo 4%, entretanto, não ocorreu crescimento fúngico nos insetos quando estes foram colocados em câmara úmida, sugerindo que a manipulação dos insetos durante a montagem do experimento pode ter causado a morte.

Existiu uma correlação positiva entre o aumento da concentração da formulação e o aumento da mortalidade dos insetos, conforme pode ser observado na Figura 2. O tratamento mais eficiente foi aquele que continha 2% de formulação, embora não tenha sido significativa a diferença a partir de 0,25%. Isto foi detectado nas duas espécies estudadas. No grupo tratado apenas com fungo, isento de óleo, a mortalidade confirmada não ultrapassou 18%.

Nos experimentos com *M. anisopliae*, a mortalidade foi maior entre o 6º e o 9º dia de análise (Tabela 1), aumentando ao longo do tempo e à medida que a concentração da formulação também aumentava, porém o máximo obtido foi de 30% de insetos mortos. Não foram observadas diferenças significativas entre as diversas concentrações nem entre as linhagens (Tabela 2).

As linhagens de *B. bassiana* diferiram significativamente entre si quanto à mortalidade das larvas, independentemente da concentração da formulação utilizada (Tabela 3). As lagartas parasitadas por este fungo apresentaram coloração rósea, típica da infecção por *Beauveria*, e ocorreru em maior número do 4º ao 9º dia. A CL_{50} de *B. bassiana* ARSEF1398 foi 1% de formulação, enquanto que para *B. bassiana* ESALQ447 a CL_{50} foi de 0,2% de formulação (Figura 2). *B. bassiana* ESALQ447 foi mais virulenta à broca da cana do que *B. bassiana* ARSEF1398, com mortalidade confirmada de 74% e 52%, respectivamente (Tabela 4).

Várias pesquisas são desenvolvidas na busca por linhagens mais virulentas contra a broca da cana-de-açúcar todavia, experimentos utilizando óleo vegetal para o controle da praga são escassos. Alguns fatores devem ser analisados num bioensaio de patogenicidade para que a praga seja controlada de maneira mais eficiente, tais como: método de exposição das larvas ao patógeno, a umidade e a temperatura ideais para que ocorra a infecção. Avaliações realizadas por Svedese et al (2012) revelaram que *B. bassiana* foi mais patogênica para larvas de *D. saccharalis* à 26ºC, alcançando 100% de mortalidade. Além desses, outros fatores interferem no processo de infecção no campo, como a imunização social de colônias, que inibe a proliferação da doença dos insetos que vivem em sociedade (KONRAD et al., 2012) e mecanismos de resistência específicos desenvolvidos pelos insetos contra alguns fungos entomopatogênicos (DUBOVSKIY et al., 2013).

O processo de infecção dos fungos entomopatogênicos no corpo do hospedeiro envolve várias etapas até que o inseto seja colonizado por completo. Em primeiro lugar, está a força mecânica exercida pelas hifas rompendo a cutícula; associada a essa, o aparato enzimático que altera a superfície do tegumento, liberando nutrientes que servirão de alimento para o fungo e facilitando a penetração no inseto (DONATTI et al.,

2008). Os fungos apresentam essa vantagem em relação aos outros patógenos, que dependem da ingestão via oral de seus propágulos para dar início à infecção (FANG et al., 2009; KIM et al., 2010). Deste modo, os fungos podem infectar diferentes fases de desenvolvimento do hospedeiro, inclusive aquelas em que eles não se alimentam, como ovo e pupa. O inseto doente para de se alimentar e finalmente morre, devido a um conjunto de modificações na hemocele, tecidos e órgãos internos. O ciclo é completado quando ocorre a exteriorização do fungo no inseto completamente colonizado, permitindo a transmissão horizontal do patógeno na população e a disseminação de suas estruturas reprodutivas (GARCÍA-MUNGUÍA et al., 2011; QUESADA-MORAGA et al., 2008).

Alguns fungos entomopatogênicos possuem uma fase leveduriforme no interior do inseto, que tem o papel de disseminar rapidamente o patógeno no corpo do hospedeiro (LUNA-ALVES LIMA & TIGANO, 1989). Alves et al. (2002b) verificaram que a fase leveduriforme de *B. bassiana* ESALQ447 causou maior mortalidade às larvas de *D. saccharalis* (70%) do que a suspensão de conídios (30%); porém não houve diferença significativa entre a suspensão de estruturas leveduriformes e a suspensão de conídios sobre *Tetranychus urticae*, com mortalidade entre 74 e 77% dos insetos. Os autores indicaram estruturas leveduriformes para aplicação no campo contudo, ressaltam a importância dos testes de tolerância à adversidade ambiental, testes de adesão e penetração na cutícula do hospedeiro e mecanismos de produção massal.

Os fungos *B. bassiana* e *M. anisopliae* além de serem patogênicos às larvas de *D. saccharalis*, também interferem negativamente nas características biológicas do inseto. Segundo Oliveira et al., (2008a) a viabilidade larval foi reduzida à 56,6%, o período pupal foi aumentado em 1 dia e a viabilidade pupal foi reduzida para 53,3%. Em relação aos adultos tratados, os dois fungos foram eficientes em reduzir a

longevidade de machos e fêmeas e a viabilidade dos ovos. Nesse sentido, Oliveira et al., (2008b) observaram a dinâmica hemocitária de larvas de *D. saccharalis* desafiadas imunologicamente por entomopatógenos. Geralmente, ocorreu redução do número de plasmócitos nas primeiras 36 horas após a infecção fúngica por *B. bassiana* e aumento dos granulócitos, nas primeiras 36 horas. Essas células estão presentes na hemolinfa do inseto e estão envolvidas no processo de infecção inicial e final. Os autores concluíram que *B. bassiana* tem ação mais efetiva sobre a dinâmica populacional dos granulócitos e plasmócitos do que *M. anisopliae*.

Bioensaios anteriores de patogenicidade com *D. saccharalis* foram realizados com avaliação de mortalidade durante 12 ou até 15 dias após o contato das larvas com os conídios fúngicos, consequentemente os valores de mortalidade foram mais elevados chegando até 100% de insetos mortos (ACEVEDO et al., 2007; ZAPPELINI et al., 2010). Neste trabalho, o maior número de insetos mortos foi observado durante o 4º e o 9º dia, demonstrando a eficiência da formulação em matar os insetos.

A interação entre fungos entomopatogênicos e produtos fitossanitários diz-se positiva quando ocorre um sinergismo entre o patógeno e o produto, resultando no controle da praga de forma eficaz. Pesquisas utilizando óleo emulsionável em formulações comprovam que conídios formulados matam mais insetos do que os não-formulados, em menos tempo, e além disso, permanecem por longos períodos no campo, devido a proteção fornecida pelo óleo contra os raios UV e as condições adversas (BLANFORD et al., 2011; BUKHARI et al., 2011; FARENHORST et al., 2010; GATARAYIHA et al., 2010; ISLAM et al., 2010; VIDAL et al., 2011; ZAHRAN et al., 2013). A disponibilização dos produtos formulados no mercado poderá estabelecer definitivamente o emprego de fungos entomopatogênicos no controle das pragas.

Beauveria bassiana ESALQ447 associada ao óleo adjuvante emulsionável foi a linhagem mais eficiente no controle de *D. saccharalis*, sob condições de laboratório, e *M. anisopliae* PL43 foi a menos eficiente. A aplicação de conídios formulados melhorou a ação de *M. anisopliae* e de *B. bassiana*.

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6. Apêndice

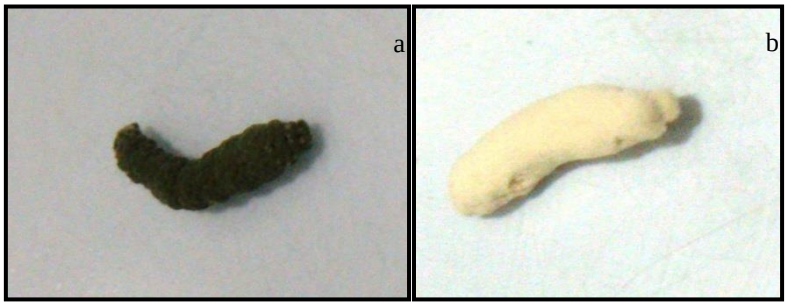

Figura 1. Larvas de *Diatraea saccharallis* colonizadas por a) *Metarhizium anisopliae* PL43 e b) *Beauveria bassiana* ESALQ447. Fonte: PORTELA-SILVA, A.P.A., 2014.

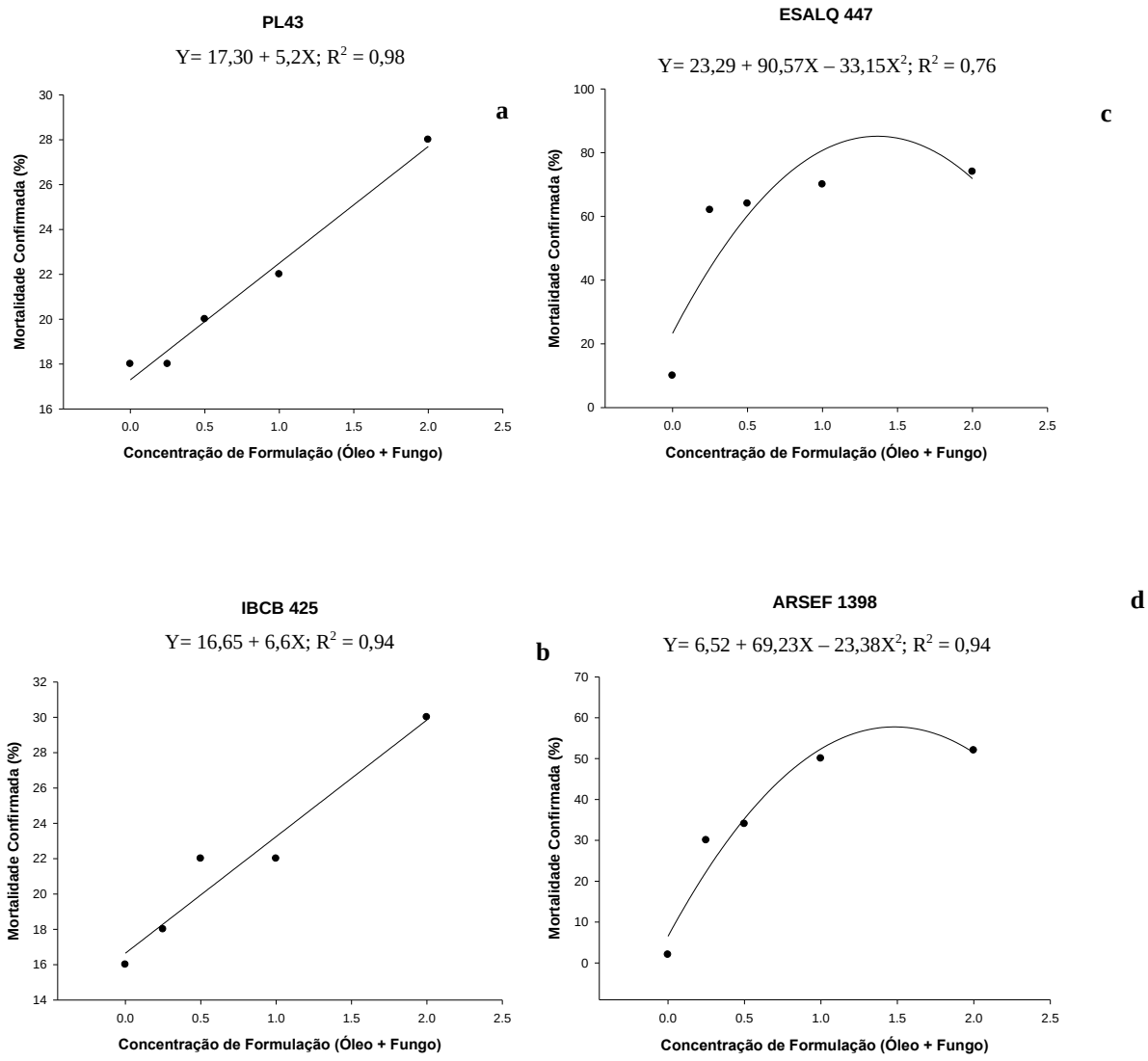

Figura 2. Mortalidade confirmada de *Diatraea saccharallis* após exposição às formulações de *Metarhizium anisopliae* (PL43, IBCB425) (a,b) e *Beauveria bassiana* (ESALQ447, ARSEF1398) (c,d) com óleo adjuvante emulsionável.

Tabela 1. Mortalidade acumulada (%), ao longo do tempo, após o contato das larvas de *Diatraea saccharalis* com diferentes concentrações de formulação de *Metarhizium anisopliae* (25 ± 1ºC, 70 ± 10% de umidade e 12 horas de fotofase).

	03 dias		**06 dias**		**09 dias**	
Concentração	**PL43**	**IBCB425**	**PL43**	**IBCB425**	**PL43**	**IBCB425**
Conídios- 0% formulação	0.00aA	4.00aA	4.00bA	12.00aA	12.00aA	12.00aA
0,25% formulação	0.00aA	2.00aA	16.00abA	18.00aA	16.00aA	18.00aA
0,50% formulação	0.00aB	6.00aAB	20.00abA	22.00aA	20.00aA	22.00aA
1,0% formulação	0.00aB	0.00aB	14.00abAB	18.00aAB	22.00aA	20.00aA
2,0% formulação	0.00aB	12.00aAB	26.00aA	28.00aA	26.00aA	28.00aA
	dms p/ colunas = 17.67 dms p/ linhas = 18.48					
	CV% = 76.00					

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 2. Mortalidade total, confirmada e corrigida (%) 10 dias após o contato das larvas de *Diatraea saccharalis* com diferentes concentrações de formulação de *Metarhizium anisopliae* (25 ± 1ºC, 70 ± 10% de umidade e 12 horas de fotofase).

Concentração	Mortalidade Total		Mortalidade Confirmada		Mortalidade Corrigida	
	PL43	IBCB425	PL43	IBCB425	PL43	IBCB425
Grupo 01*						
Branco	4.00bcdA	4.00bcA	0.00bA	0.00bA	0.00bA	0.00bA
0,25% óleo	2.00cdA	2.00cA	0.00bA	0.00bA	0.00bA	0.00bA
0,50% óleo	2.00cdA	2.00cA	0.00bA	0.00bA	0.00bA	0.00bA
1,0% óleo	0.00dA	0.00cA	0.00bA	0.00bA	0.00bA	0.00bA
2,0% óleo	2.00cdA	2.00cA	0.00bA	0.00bA	0.00bA	0.00bA
Grupo 02**						
Conídios- 0% formulação	18.00abcdA	16.00abcA	18.00aA	16.00abA	14.44abA	12.22abA
0,25% formulação	18.00abcdA	18.00abcA	18.00aA	18.00aA	16.44abA	16.00abA
0,50% formulação	20.00abcA	22.00abA	20.00aA	22.00aA	18.00abA	20.44aA
1,0% formulação	22.00abA	22.00abA	22.00aA	22.00aA	22.00aA	22.00aA
2,0% formulação	28.00aA	30.00aA	28.00aA	30.00aA	26.22aA	28.22aA
	dms p/ colunas = 18.12 dms p/ linhas = 11.08 CV% = 75.24		dms p/colunas=17.04 dms p/ linhas=10.41 CV% = 77.35		dms p/ colunas = 18.50 dms p/ linhas = 11.31 CV% = 91.70	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

*Grupo 01: corresponde ao controle, isento de fungo, com diversos percentuais de óleo; branco= água; **Grupo 02: corresponde ao tratamento com diversos percentuais de formulação (óleo + fungo); conídios= suspensão de conídios com água e agral, isento de óleo.

Tabela 3. Mortalidade acumulada (%), ao longo do tempo, após o contato das larvas de *Diatraea saccharalis* com formulações de *Beauveria bassiana* (25 ± 1ºC, 70 ± 10% de umidade e 12 horas de fotofase).

Linhagem	**03 dias**	**06 dias**	**09 dias**
ESALQ447	3,60Ca	43,60Ba	55,20Aa
ARSEF1398	0,00Ba	6,00Bb	32,00Ab

dms p/ colunas = 8.51 dms p/ linhas = 7.09

CV% = 54.01

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 4. Mortalidade total, confirmada e corrigida (%) 10 dias após o contato das larvas de *Diatraea saccharalis* com diferentes concentrações de formulação de *Beauveria bassiana* (25 ± 1ºC, 70 ± 10% de umidade e 12 horas de fotofase).

Concentração	Mortalidade Total		Mortalidade Confirmada		Mortalidade Corrigida	
	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398	ARSEF1398	ESALQ447
Grupo 01*						
Branco	4.00bA	4.00bA	0.00bA	0.00bA	0.00bA	0.00bA
0,25% óleo	2.00bA	2.00bA	0.00bA	0.00bA	0.00bA	0.00bA
0,50% óleo	2.00bA	2.00bA	0.00bA	0.00bA	0.00bA	0.00bA
1,0% óleo	0.00bA	0.00bA	0.00bA	0.00bA	0.00bA	0.00bA
2,0% óleo	2.00bA	2.00bA	0.00bA	0.00bA	0.00bA	0.00bA
Grupo 02**						
Conídios- 0% formulação	12.00bA	2.00bA	10.00bA	2.00bA	10.00bA	2.00bA
0,25% formulação	62.00aA	32.00aB	62.00aA	30.00aB	61.55aA	30.00aB
0,50% formulação	64.00aA	34.00aB	64.00aA	34.00aB	64.00aA	32.44aB
1,0% formulação	70.00aA	50.00aB	70.00aA	50.00aB	70.00aA	50.00aB
2,0% formulação	76.00aA	52.00aB	74.00aA	52.00aB	75.55aA	50.66aB
	dms p/ colunas = 25.92 dms p/ linhas = 15.84 CV% = 53.12		dms p/ colunas = 25.22 dms p/ linhas = 15.41 CV% = 54.68		dms p/ colunas = 26.06 dms p/ linhas = 15.93 CV% = 56.74	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

*Grupo 01: corresponde ao controle, isento de fungo, com diversos percentuais de óleo; branco= água; **Grupo 02: corresponde ao tratamento com diversos percentuais de formulação (óleo + fungo); conídios = suspensão de conídios com água e agral, isento de óleo.

CAPÍTULO V

COMPATIBILIDADE DE *METARHIZIUM ANISOPLIAE* E *BEAUVERIA BASSIANA* COM EXTRATOS VEGETAIS DE *INDIGOFERA SUFFRUTICOSA* E SEU EFEITO SOBRE *DIATRAEA SACCHARALIS*

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Compatibilidade de *Metarhizium anisopliae* e *Beauveria bassiana* com extratos vegetais de *Indigofera suffruticosa* e seu efeito sobre *Diatraea saccharalis*

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Resumo

A compatibilidade de extratos aquosos de *Indigofera suffruticosa* com os fungos entomopatogênicos *Metarhizium anisopliae* e *Beauveria bassiana* foi avaliada *in vitro*. O efeito dos extratos vegetais e da sua associação com os fungos foi verificado sobre *Diatraea saccharalis*. Foi preparada uma solução estoque (30%) do extrato aquoso de folhas e de sementes de *I. suffruticosa* e a partir desta, os extratos foram incorporados a Batata-Dextrose-Ágar (BDA) em volumes diferentes, de forma a se obter concentrações de1%, 2%, 4%, 8% e 16%. Fragmentos fúngicos foram inoculados no centro da placa

de Petri e após seis dias de incubação (T=27 ± 1ºC) avaliou-se o crescimento vegetativo, a produção e a germinação de conídios. Foi calculado o valor do Índice Biológico (IB) dos extratos. Conídios desidratados foram formulados em óleo adjuvante emulsionável para avaliação da virulência sobre as brocas. No bioensaio de patogenicidade, as larvas foram imersas por 30 segundos em diferentes suspensões de conídios, conídios + extrato vegetal e formulação + extrato. Para cada tratamento havia um grupo controle respectivo, tratado com água ou óleo (2%). Foram utilizadas cinco repetições contendo 10 lagartas cada, totalizando 50 insetos por tratamento. As observações foram realizadas diariamente, por 10 dias. O extrato de folhas de *I. suffruticosa* reduziu a viabilidade dos conídios de todas as linhagens, exceto de *B. bassiana* ARSEF1398 que teve aumento no percentual de germinação. O extrato das folhas e das sementes de *I. suffruticosa* estimularam a produção de conídios de *B. bassiana* ESALQ447. De acordo com o IB todos os extratos foram compatíveis com os fungos. O maior percentual de mortalidade foi obtido quando as larvas foram expostas ao extrato de sementes de *I. suffruticosa* (30%) e deste associado aos conídios de *M. anisopliae* IBCB425 (96%) e de *M. anisopliae* PL43 (94%). A associação do extrato de sementes de *I. suffruticosa* com a formulação de *B. bassiana* ESALQ447 causou 82% de mortalidade das larvas. O extrato das folhas de *I. suffruticosa* quando associado às formulações dos fungos *M anisopliae* IBCB425 e *B. bassiana* ESALQ447 causou mortalidade máxima de 70%. Estes resultados indicam o potencial inseticida desses extratos quando associados aos fungos testados, formulados ou não, para o controle de *D. saccharalis*.

Palavras-chave: Formulação; Extrato Vegetal; Fungos Entomopatogênicos; Broca-da-Cana; Manejo Integrado de Pragas (MIP).

1. Introdução

Diatraea saccharalis Fabricius (Lepidoptera: Crambidae), conhecida como a broca da cana é considerada uma praga comum na cultura da cana-de-açúcar. A mariposa fêmea ovoposita na folha da cana e as larvas ao eclodirem se deslocam até o colmo, perfurando-o. Ao abrirem galerias causam grandes prejuízos, reduzindo a qualidade do produto final (açúcar e álcool). Além disso, causam danos indiretos, facilitando a entrada de fitopatógenos (Polanczyk., 2004; Pinto, 2006).

O controle químico deve ser evitado por deixar resíduos nos alimentos, desenvolver resistência nos insetos, destruir os inimigos naturais da praga e ainda, desencadear doenças em humanos que trabalham diretamente com os produtos. O controle biológico é uma alternativa promissora para a redução do uso de inseticidas químicos. Programas de controle de *D. saccharalis* demonstram alta eficiência no campo, utilizando parasitóides (*Cotesia flavipes* Cameron e *Trichograma galloi* Zucchi), entomopatógenos como *Bacillus thuringiensis* Berliner e os fungos *Metarhizium anisopliae* (Metsh.) Sorok. e *Beauveria bassiana* (Bals.) Vuill. (Botelho e Macedo, 2002; Broglio-Micheletti et al., 2007; Guo et al., 2012).

Pesquisas desenvolvidas com extratos vegetais atuando como bioinseticidas no controle de pragas (Dabrowski e Seredynska, 2007; Duso et al., 2008) obtiveram eficiência comprovada, como os extratos de *Leucaena leucocephala* L. contra *Bemisia tabaci* (mosca branca) (Vasconcelos et al., 2006), de *Trichilia pallida* Swartz contra *Spodoptera frugiperda* Smith (lagarta do cartucho) (Roel e Vendramim, 2006), de *Ruta graveolens* L. contra *Menacanthus stramineus* Nitzsch (parasita de aves) (Pablo et al., 2009) e de *Azadirachta indica* A. Juss contra *Tetranychus urticae* Koch (ácaro rajado) (Bernardi et al., 2013), dentre outros.

Indigofera suffruticosa Mill é uma espécie da família Fabaceae, encontrada nas regiões tropicais e subtropicais, é bem adaptada para crescer no semi-árido, em solos pouco férteis. No Nordeste do Brasil é conhecida como anileira e usada na medicina popular no tratamento de infecções, inflamações e doenças como epilepsia (Wong et al., 1999; Leite et al., 2004; Leite et al., 2006); posteriormente foi relatada também a atividade antitumoral do extrato de folhas de *I. suffruticosa* (Vieira et al., 2007). Panizzi (1992) avaliou a atividade inseticida de quatro espécies de *Indigofera* contra *Piezodorus*

*guildin*ii Westood (Heteroptera: Pentatomidae) (praga da soja) e descreveu a eficiência de *I. suffuticosa* ao reduzir a sobrevivência de ninfas, de adultos e a ovoposição. Mais tarde, Vieira et al. (2012) relataram o efeito embriotóxico do extrato das folhas de *Indigofera suffruticosa* Mill para larvas de *Aedes aegypti* (mosquito transmissor da dengue).

O objetivo do trabalho foi avaliar a compatibilidade dos extratos de folhas e sementes de *I. suffruticosa* com os fungos entomopatogênicos *M. anisopliae* e *Beauveria bassiana* e seu efeito sobre *D. saccharalis*.

2. Materiais e Métodos

2.1 Linhagens fúngicas: foram utilizadas *M. anisopliae* PL43, *B. bassiana* ESALQ447 e *B. bassiana* ARSEF1398 provenientes da coleção de entomopatógenos mantida no Laboratório de Controle Microbiano e Patologia de Insetos, no Departamento de Entomologia, Fitopatologia e Agricultura, da Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (ESALQ-USP); a linhagem *M. anisopliae* IBCB425 foi fornecida pelo setor de Controle Biológico do Instituto Biológico, Campinas/SP.

2.2 Obtenção e manutenção de *Diatraea saccharalis*: as lagartas de 3º *instar* de *D. saccharalis* utilizadas nos bioensaios foram provenientes da criação experimental da Empresa Biotech Controle Biológico, mantidas a 25 ± 1ºC, U= 70 ± 10 % de umidade. Os insetos foram alimentados com dieta artificial (Hansley e Hammond, 1968) que é composta basicamente de germe de trigo, levedo de cerveja e caseína.

2.3 Quantificação do inóculo: os conídios foram produzidos em arroz parboilizado autoclavado, em sacos de polipropileno. Após 12 dias, mantidos a 27 ± 1ºC, para desenvolvimento do fungo e da esporulação, o arroz mais o fungo foram espalhados em bandejas e mantidos em sala climatizada (T= 17 ± 1ºC, UR= 20 ± 1%), contendo aparelhos desumidificadores (Thermomatic do Brasil, São Paulo, Brasil, model Desidrat

D4), por cerca de três dias, até que a umidade do material ficasse em torno de 5% (Moore et al., 1996). Os conídios foram então extraídos do arroz com peneiras metálicas (300 mm de malha) e então submetidos ao analisador de umidade por infra-vermelho (Ind. e Com. Eletro-Eletrônica Gehaka Ltda, São Paulo, Brazil· model IV2000/IV2002). Foi pesado 1g de conídios secos de cada linhagem a ser testada e misturados a 10 mL de água + Agral (0,02%); após diluições sucessivas foi realizada a quantificação de conídios, em câmara de Neübauer. A formulação foi composta por 1g de conídios puros secos adicionados a 10 mL de Veget'oil® (Oxiquímica Agrociência Ltda, Jaboticabal,Brasil).

2.4 Viabilidade dos conídios: a viabilidade dos conídios foi verificada retirando-se 0,1mL da formulação e diluindo em 10 mL de solução salina + Agral (0,1%). Após agitação em Vortex, por 1 minuto, para desagregação dos conídios, uma nova alícota foi retirada e espalhada na superfície de placas de Petri de acrílico (6 cm) contendo BDA. As placas foram incubadas a 27 ± 1ºC e observadas após 20 horas. Foram realizadas cinco repetições para cada formulação (Alves et al., 2002).

2.5 Obtenção dos extratos vegetais: foram coletados ramos de *Indigofera suffruticosa*, no município de Igarassu/Pernambuco/Brasil. Todo o material vegetal foi lavado e selecionado, de modo que apenas as folhas e as sementes sadias passaram para a etapa de secagem. O material foi seco em estufa a 45ºC, por 48 horas, e em seguida triturado em liquidificador. Os extratos aquosos, na concentração de 10% (v/v) foram obtidos pela adição de 35g de pó seco a 350mL de água destilada. A solução permaneceu de 14 a 16 horas à temperatura ambiente, em mesa agitadora. A fase líquida foi removida, por decantação, e o resíduo foi submetido a uma nova extração. Ao final, os extratos foram filtrados, à vácuo, liofilizados e armazenados em recipientes hermeticamente fechados, em freezer (-14ºC) (Vasconcelos et al., 2006).

2.6 Teste de compatibilidade: o efeito fungitóxico de cada extrato sobre os fungos foi avaliado *in vitro*, a partir de uma solução estoque (30%). O extrato foi incorporado, ao BDA fundido, não solidificado, à temperatura de 40 ± 5ºC, em determinados volumes, de modo a se obter cinco concentrações (1%, 2%, 4%, 8% e 16%). Após homogeneização, o meio foi distribuído em placas de Petri (9cm de diâmetro) e em seguida procedeu-se a inoculação. O grupo controle foi inoculado em meio de cultura isento de extrato vegetal (Depieri et al., 2005). Os testes de esporulação e crescimento vegetativo foram realizados, inoculando-se fragmentos fúngicos no centro da placa de Petri com o auxílio de uma alça de platina; após seis dias de incubação (27 ± 1ºC), o diâmetro da colônia foi mensurado, com uma régua milimetrada. Em seguida, foi retirado um disco central (4mm) da colônia para quantificação da produção de conídios. Cada disco foi individualizado num tubo de ensaio contendo 10mL de água mais Agral (0,02%) e agitado vigorosamente para desagregação dos conídios da superfície do meio de cultura (Oliveira et al., 2003). A suspensão obtida foi diluída a 10^{-1} e então se procedeu a contagem dos conídios em câmara de Neübauer. 0,1 mL desta suspensão foi semeado em meio BDA; após 20h de incubação (27 ± 1ºC) o percentual de germinação foi quantificado por meio de observação sob microscópio óptico, contando-se os conídios germinados e não germinados (Alves e Pereira, 1998). O efeito fungitóxico foi avaliado sobre o crescimento vegetativo, a produção e a germinação de conídios dos fungos testados. Os experimentos foram feitos em quintuplicata. O índice biológico foi obtido com base no cálculo: IB= 47[CV]+43[ESP]+10[GERM]/100, onde CV: porcentagem de crescimento vegetativo com relação à testemunha; ESP: porcentagem de esporulação com relação à testemunha; GERM: porcentagem de germinação dos conídios em relação à testemunha. O valor do IB pode variar de: 0-41 (tóxico), 42-66 (moderadamente tóxico) e >66(compatível) (Rossi-Zalaf et al., 2008).

2.7 Quantificação de conídios para formulação: foi pesado 1g de conídios secos (desidratados como descrito anteriormente) de cada linhagem a ser testada e misturados a 10 mL de água + Agral (0,02%); após diluições sucessivas foi realizada a quantificação de conídios em câmara de Neübauer. A formulação foi composta por 1g de conídios puros secos adicionados a 10 mL de Veget'oil® (Alves et al., 2002).

2.8 Bioensaios: os bioensaios foram realizados com um grupo tratado com conídios fúngicos ($1,0x10^{10}$ conídios/ml) das quatro linhagens analisadas, extrato vegetal (20%) (dois extratos analisados), conídios + extrato vegetal, formulação (conídios + óleo), formulação + extrato vegetal. Para cada tratamento, havia um grupo controle respectivo, tratado com água destilada autoclavada e óleo (2%). As lagartas foram imersas nas suspensões por 30 segundos e o excesso de suspensão foi removido em papel filtro (Quinelato et al., 2012). Posteriormente, as lagartas foram transferidas individualmente para placas de Petri de acrílico contendo dieta artificial. Foram utilizadas cinco repetições, contendo 10 lagartas cada, totalizando 50 insetos/tratamento. O experimento foi mantido em condições apropriadas de temperatura e umidade (25 ± 1ºC, U= 70 ± 10 %) e 12 horas de fotofase. As observações foram realizadas diariamente, durante 10 dias, transferindo-se as lagartas mortas para câmara úmida para confirmação do agente causal, por meio do exame microscópico, analisando-se hifas e conídios, na superfície do inseto mumificado.

2.9 Análise estatística: os dados foram analisados utilizando-se o programa ASSISTAT 7,5 Beta e sujeitos à análise de variância (ANOVA). O teste de Tukey foi usado para separar as médias (P= 0,05) e a análise de regressão polinomial para avaliação do Tempo Letal (TL_{50}) (Silva e Azevedo, 2002).

3. Resultados e Discussão

3.1 Compatibilidade dos extratos vegetais com os fungos

O extrato das folhas de *I. suffruticosa* não afetou o crescimento vegetativo das linhagens, exceto para *M. anisopliae* IBCB425, que teve o diâmetro da colônia reduzido à medida que a concentração do extrato fora aumentada; a produção de conídios não foi

influenciada pelas diferentes concentrações do extrato, não existindo diferenças significativas entre as linhagens analisadas, porém as linhagens *M. anisopliae* IBCB425 e *B. bassiana* ESALQ447 foram levemente estimuladas a produzir mais conídios. A viabilidade dos conídios, após seis dias de exposição dos fungos ao extrato foi reduzida significativamente em relação ao controle, porém não foi menor que 92%, em nenhuma concentração; contrariamente, *B. bassiana* ARSEF1398 apresentou percentual de germinação mais alto nos tratamentos com extrato do que o grupo controle (Tabelas 1; 2).

O extrato das sementes de *I. suffruticosa* reduziu discretamente o crescimento vegetativo de *M. anisopliae* IBCB425, nas duas menores concentrações, enquanto que o crescimento das demais linhagens não sofreu influência da presença do extrato; a produção dos conídios da linhagem *B. bassiana* ESALQ447 foi estimulada na presença do extrato; o percentual de germinação de *B. bassiana* ARSEF1398 foi aumentado em relação ao controle, enquanto que nas outras linhagens a germinação sofreu discreta redução, mas não foi menor que 94% (Tabelas 3; 4).

De acordo com o valor do IB (Índice Biológico) todos os extratos foram considerados compatíveis com os fungos, nas concentrações testadas (Tabela 5).

Hirose et al. (2001) relataram que o óleo de nim foi considerado tóxico a *B. bassiana* CG252, causando maior efeito negativo do que biofertilizantes. Por outro lado, Araujo Jr et al. (2009) demonstraram que *B. bassiana* CG001 e *M. anisopliae* CG30 podem ter seu crescimento colonial e viabilidade alterados quando expostos a concentrações de óleo de nim maiores que 0,25%, mas ambos tem potencial para o controle do pulgão da couve *Lipaphis erysimi* (kalt.). Esses resultados sugerem que as

linhagens se comportam de maneira diferente e salientam a importância dos estudos de compatibilidade entre fungos e produtos fitossanitários.

Pesquisas com fungos e extratos vegetais das folhas e sementes de nim (*Azadirachta indica*) mostraram que embora o extrato das folhas tenha afetado um pouco os parâmetros biológicos do fungo (crescimento vegetativo, esporulação e germinação), ainda se manteve compatível com *B. bassiana* (Depieri et al., 2005), podendo haver a associação dos extratos com o fungo para o controle da broca do café *Hypothenemus hampei* (Ferrari). Assim sendo, os extratos das folhas e das sementes de *I. suffruticosa* foram utilizados nos bioensaios com larvas de *D. saccharalis* porque segundo os valores de IB não são tóxicos aos fungos.

3.2 Efeito dos extratos vegetais sobre *Diatraea saccharalis*

O maior percentual de mortalidade foi obtido quando as larvas foram expostas ao extrato das sementes de *I. suffruticosa* associado aos fungos, alcançando 96% de mortalidade com *M. anisopliae* IBCB425e 94% com *M. anisopliae* PL43. *B. bassiana* ESALQ447 matou mais insetos quando o extrato das sementes de *I. suffruticosa* foi adicionado à formulação (fungo + óleo) (82%) ou quando a formulação foi aplicada sozinha (76%), porém não houve diferença significativa entre as linhagens (Tabela 6).

O extrato das folhas de *I. suffruticosa* quando em contato com as larvas de *D. saccharalis* não causou mortalidade significativa dos insetos (20%), entretanto quando associado ao fungo *M. anisopliae* causou mortalidade de 58% e de 60% dos insetos, com a linhagem *M. anisopliae* PL43 e *M. anisopliae* IBCB425, respectivamente. Ao ser adicionado o extrato à formulação (fungo + óleo), a mortalidade aumentou para 70% com *M. anisopliae* IBCB425. A formulação feita com a linhagem *B. bassiana* ESALQ447 e óleo causou mortalidade de 76%, mas não diferiu significativamente da

associação da formulação com o extrato (70%). No entanto, *B. bassiana* ARSEF1398 causou mais mortalidade quando formulada em óleo do que associada ao extrato (Tabela 7).

As larvas de *D. saccharalis* que foram expostas aos fungos, em formulação ou em associação com extratos, ao serem encontradas mortas, eram transferidas para câmara úmida. Cerca de cinco dias depois, a superfície do seu corpo estava coberta por micélio, conidióforos e conídios (Figura 1); já nas larvas que foram imersas apenas em suspensão de extratos, isentos de conídios, não ocorreu qualquer exteriorização de estruturas fúngicas.

Ao se analisar a mortalidade das larvas, ao longo do tempo, percebeu-se que aquelas imersas no extrato das sementes de *I. suffruticosa* associado a *M. anisopliae* (PL43 e IBCB425) morreram mais cedo (TL_{50}= 5,5 dias) do que as tratadas com *B. bassiana* ESALQ447 (TL_{50}= 6,0 dias) (Figura 1). Mesmo o extrato das folhas de *I. suffruticosa* sendo o que causou menor mortalidade de insetos ao final do experimento, quando este foi associado à formulação de *B. bassiana* ESALQ447, o TL_{50} foi reduzido para 5,0 dias (Figura 2).

Os resultados são relevantes para a comunidade científica que busca o controle da broca-da-cana, visto que são escassos os trabalhos utilizando extratos vegetais no controle de *D. saccharalis*, tanto associados a fungos entomopatogênicos quanto individualmente. Embora os extratos das duas espécies testadas não tenham causado mortalidade elevada das larvas, quando estes foram associados aos fungos ou às formulações com óleo, ocorreu uma resposta positiva, refletida pelo aumento do percentual de mortalidade, indicando que houve ação sinérgica do fungo + extrato. Neste sentido, Islam et al., (2010) também demonstraram que a ação combinada de *Beauveria bassiana* e nim contra *Bemisia tabaci* Gennadius (mosca branca) causou perda significativa da viabilidade dos ovos (29,5%) e maior mortalidade de ninfas (97,2%) e TL_{50} de 2,08 dias, menor do que cada componente aplicado individualmente. Os autores destacaram que a aplicação combinada de um extrato e um fungo entomopatogênico pode promover o controle efetivo da praga.

Panizzi et al. (2004) avaliaram diversas plantas hospedeiras para *Anticarsia gemmatalis* Hubner (Lepidoptera: Noctuidae), conhecida como lagarta-da-soja. Dentre as espécies não-cultiváveis, *I. suffruticosa* foi considerada a mais adequada para o

desenvolvimento larval, pois 70% dos insetos atingiram a fase pupal, sem diferença significativa em relação à planta da soja, demonstrando que o vegetal não apresentava qualquer atividade inseticida sobre a lagarta. Por outro lado, estudos posteriores com o extrato aquoso de folhas de *I. suffruticosa* demonstraram a sua atividade de repelência e embriotoxicidade contra *Aedes aegypti*, reduzindo significativamente a ovoposição das fêmeas, causando alterações morfológicas no trato digestivo das larvas e retardo no crescimento (Vieira et al., 2012), sugerindo que os insetos desenvolveram diferentes mecanismos de resistência quando sujeitos a condições adversas.

Debonsi et al. (2009) avaliaram a letalidade de dois compostos (Isobutil amidas) isolados do extrato de sementes de *Piper tuberculatum* Jacq. aplicados de forma tópica sobre *D. saccharalis*. Os resultados revelaram a resposta letal dos compostos, matando mais de 50% das larvas, após 48 horas da exposição, na concentração de 100µg. inseto^{-1}. Os valores da DL_{50} e DL_{90} para o composto 1 (4-5 dihydropiperlongumenine) foram 92,83 e 176,50µg. inseto^{-1} e para o composto 2 (Pellitorine), foram 91,19 e 184,56µg. inseto^{-1}. Apesar de resultados promissores, ainda são poucas as pesquisas de tais extratos vegetais contra a broca-da-cana.

Três extratos vegetais foram testados contra *Menacanthus stramineus* Nitzsch (parasita de aves), com três exposições, em intervalos de 48 horas. Após a terceira exposição aos extratos, o extrato de *Azadirachta indica* causou 93,6% de mortalidade, o de *Ruda graveolens* L. 85,2% e o de *Ardisia solanaceaea* Roxb. 98,2%, sugerindo um método alternativo de biocontrole (Pablo et al., 2009). Azamax é um produto comercial à base de Azadirachtina que ao ser testado contra *Tetranychus urticae* (Koch), principal praga do morango, reduziu a população do ácaro (94%), sem causar mortalidade significante ao seu predador natural, após sete dias da aplicação (Bernardi et al., 2013), demonstrando a especificidade do produto, característica esta desejável num bioinseticida.

4. Conclusões

Os extratos das folhas e das sementes de *Indigofera suffruticosa* são compatíveis com os fungos *Metarhizium anisopliae* e *Beauveria bassiana*; quando associados

fungos e extratos, ou formulações e extratos, controlam de forma mais eficaz *Diatraea saccharalis*. De modo geral, os tratamentos com o extrato das sementes de *Indigofera suffruticosa* são mais eficientes contra o inseto, seguido do extrato das folhas de *I. suffruticosa*. Os maiores valores de mortalidade são obtidos quando o extrato das sementes de *I. suffruticosa* é associado aos conídios das linhagens de *M. anisopliae*. Os resultados indicam o potencial inseticida dos extratos vegetais associados aos fungos para o controle de *D. saccharalis*.

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ANEXOS

Tabela 1. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Metarhizium anisopliae* em BDA, contendo diferentes concentrações do extrato das folhas de *Indigofera suffruticosa*.

Tratamento	Crescimento vegetativo		Produção de conídios		Germinação de conídios	
	PL43	IBCB425	PL43	IBCB425	PL43	IBCB425
Controle	2.69aA	2.56aA	2.01	2.39	99.15aA	99.11bB
1%	2.60aA	2.42aB	1.22	1.72	97.45cB	99.35aA
2%	2.62aA	2.56aA	1.35	1.50	99.13aA	98.47dB
4%	2.48aA	2.38abA	1.61	2.42	97.40dB	98.80cA
8%	2.50aA	2.16bcB	1.60	2.77	97.88bB	98.80cA
16%	2.58aA	2.06cB	1.79	2.75	96.60eB	99.10bA
	dms p/ colunas= 0.2384 dms p/ linhas= 0.1617 cv%= 5.15		cv%= 27.27		dms p/ colunas= 0.0297 dms p/ linhas= 0.0201 cv%= 0.02	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 2. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Beauveria bassiana* em BDA, contendo diferentes concentrações do extrato das folhas de *Indigofera suffruticosa*.

Tratamento	Crescimento vegetativo		Produção de conídios		Germinação de conídios	
	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398
Controle	1.86bA	1.74aA	1.08	0.30	98.73aA	90.76fB
1%	2.16aA	1.72aB	0.78	0.44	95.88dA	92.05eB
2%	1.74bA	1.72aA	1.20	0.23	96.47bB	96.71aA
4%	1.82bA	1.66aB	1.18	0.41	92.05fB	95.25cA
8%	1.78bA	1.68aA	1.23	0.15	92.29eB	96.49bA
16%	1.84bA	1.70aB	1.01	0.27	96.23cA	92.76dB
	dms p/ colunas= 0.1780 dms p/ linhas= 0.1207 cv%= 5.31		cv%= 63.14		dms p/ colunas= 0.0297 dms p/ linhas= 0.0201 cv%= 0.02	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 3. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Metarhizium anisopliae* em BDA, contendo diferentes concentrações do extrato das sementes de *Indigofera suffruticosa*.

Tratamento	Crescimento vegetativo		Produção de conídios		Germinação de conídios	
	PL43	IBCB425	PL43	IBCB425	PL43	IBCB425
Controle	2.69aA	2.56aB	2.01	2.39	99.15cA	99.11aB
1%	2.64aA	2.44abB	1.84	3.21	99.21bA	97.88bB
2%	2.63aA	2.44abB	2.54	2.77	96.59fA	94.06fB
4%	2.62aA	2.56aA	1.45	3.22	97.88eA	95.91eB
8%	2.66aA	2.54aB	2.89	2.59	99.45aA	96.08cB
16%	2.68aA	2.36bB	1.66	2.58	99.05dA	96.00dB
	dms p/ colunas= 0.1640 dms p/ linhas= 0.1113 cv%= 3.40		cv%= 32.93		dms p/ colunas= 0.0305 dms p/ linhas= 0.0207 cv%= 0.02	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 4. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Beauveria bassiana* em BDA, contendo diferentes concentrações do extrato das sementes de *Indigofera suffruticosa*.

Tratamento	Crescimento vegetativo		Produção de conídios		Germinação de conídios	
	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398
Controle	1.86	1.74	1.08bA	0.30aA	98.73aA	90.76fB
1%	1.81	1.82	2.97abA	0.33aB	98.57bA	97.61bB
2%	1.88	1.76	3.88aA	0.24aB	98.18cA	97.88aB
4%	1.84	1.76	3.54aA	0.33aB	96.77fA	96.55dB
8%	1.84	1.74	3.35aA	0.29aB	97.50dA	95.30eB
16%	1.68	1.66	4.20aA	0.24aB	96.80eB	97.48cA
	cv%= 4.59		dms p/ colunas= 2.1053 dms p/ linhas= 1.4278 cv%= 64.71		dms p/ colunas= 0.0297 dms p/ linhas= 0.0201 cv%= 0.02	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 5. Valor do IB (Índice Biológico) com classificação dos extratos das folhas e das sementes de *Indigofera suffruticosa* quanto ao efeito fungitóxico sobre *Metarhizium anisopliae* e *Beauveria bassiana*.

TRATAMENTO	LINHAGEM	VALOR IB/ CLASSIFICAÇÃO	
		Folha	Semente
		I. suffruticosa	*I. suffruticosa*
1%	PL43	81,35 C*	95,49 C
	IBCB425	85,40 C	113,16 C
	ESALQ447	95,34 C	173,97 C
	ARSEF1398	117,63 C	105,68 C
2%	PL43	84,65 C	110,03 C
	IBCB425	83,92 C	104,12 C
	ESALQ447	101,91 C	212,32 C
	ARSEF1398	89,01 C	91,61 C
4%	PL43	87,60 C	86,67 C
	IBCB425	97,20 C	114,61 C
	ESALQ447	102,29 C	132,83 C
	ARSEF1398	112,20 C	103,95 C
8%	PL43	87,78 C	118,33 C
	IBCB425	99,45 C	102,92 C
	ESALQ447	103,29 C	190,14 C
	ARSEF1398	76,82 C	97,72 C
16%	PL43	93,11 C	92,32 C
	IBCB425	97,29 C	99,43 C
	ESALQ447	97,45 C	219,87 C
	ARSEF1398	93,59 C	88,87 C

*Os valores do IB podem variar: 0-41 (tóxico - T), 42-66 (moderadamente tóxico -MT) e 66 (compatível - C), segundo Rampelotti-Ferreira et al. (2010).

Tabela 6. Percentual de mortalidade de larvas de *Diatraea saccharalis* expostas aos fungos *Metarhizium anisopliae* e *Beauveria bassiana*, à formulações (fungo + óleo), ao extrato das sementes de *Indigofera suffruticosa* e associações.

Tratamento	***Metarhizium anisopliae***		***Beauveria bassiana***	
	PL43	**IBCB425**	**ESALQ447**	**ARSEF1398**
Grupo 1*				
Branco	4.00	4.00	4.00	4.00
Óleo emulsionável	2.00	2.00	2.00	2.00
Grupo 2**				
Extrato	30.00	30.00	30.00	30.00
Conídios	18.00	16.00	12.00	2.00
Formulação	28.00	30.00	76.00	52.00
Conídios + extrato	94.00	96.00	56.00	46.00
Formulação + extrato	72.00	58.00	82.00	76.00
	cv%= 34.31		cv%= 37.44	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

*Grupo 1: corresponde ao controle, isento de fungo; branco = água;

**Grupo 2: corresponde aos tratamentos; conídios = suspensão de conídios com água e agral; formulação (fungo + óleo).

Tabela 7. Percentual de mortalidade de larvas de *Diatraea saccharalis* expostas aos fungos *Metarhizium anisopliae* e *Beauveria bassiana*, à formulações (fungo + óleo), ao extrato das folhas de *Indigofera suffruticosa* e associações.

Tratamento	***Metarhizium anisopliae***		***Beauveria bassiana***	
	PL43	**IBCB425**	**ESALQ447**	**ARSEF1398**
Grupo 1*				
Branco	4.00	4.00	4.00cdA	4.00cdA
Óleo emulsionável	2.00	2.00	2.00dA	2.00dA
Grupo 2**				
Extrato	20.00	20.00	20.00cA	20.00bcA
Conídios	18.00	16.00	12.00cdA	2.00dA
Formulação	28.00	30.00	76.00aA	52.00aB
Conídios + extrato	58.00	60.00	40.00bA	18.00bcdB
Formulação + extrato	58.00	70.00	70.00aA	30.00bB
			dms p/ colunas= 17.2084	
			dms p/ linhas= 11.2864	
	cv%= 43.54		cv%= 35.41	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

*Grupo 1: corresponde ao controle, isento de fungo; branco = água;

**Grupo 2: corresponde aos tratamentos; conídios = suspensão de conídios com água e agral; formulação (fungo + óleo).

Figura 1. Larvas de *Diatraea saccharalis* colonizadas por *Beauveria bassiana* associada ao extrato das folhas de *Indigofera suffruticosa*, após o oitavo dia. a) *Beauveria bassiana* ESALQ447; b) *Beauveria bassiana* ARSEF1398.

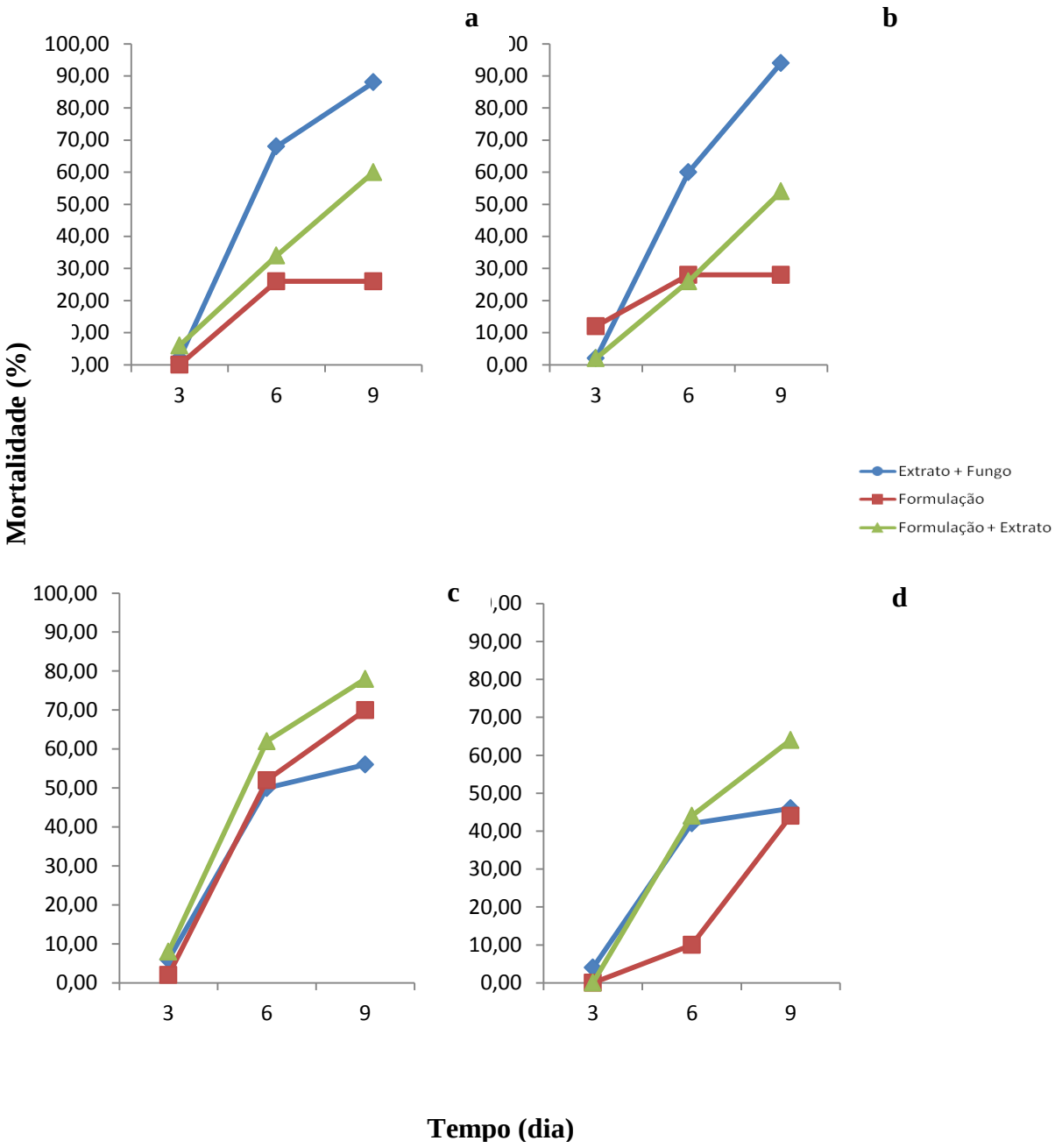

Figura 2. Mortalidade de larvas de *Diatraea saccharallis*, ao longo do tempo, após exposição à formulações com óleo adjuvante emulsionável e associações com extrato das sementes de *Indigofera suffruticosa*. a) *Metarhizium anisopliae* PL43; b) *Metarhizium anisopliae* IBCB425; c) *Beauveria bassiana* ESALQ447; d) *Beauveria bassiana* ARSEF1398).

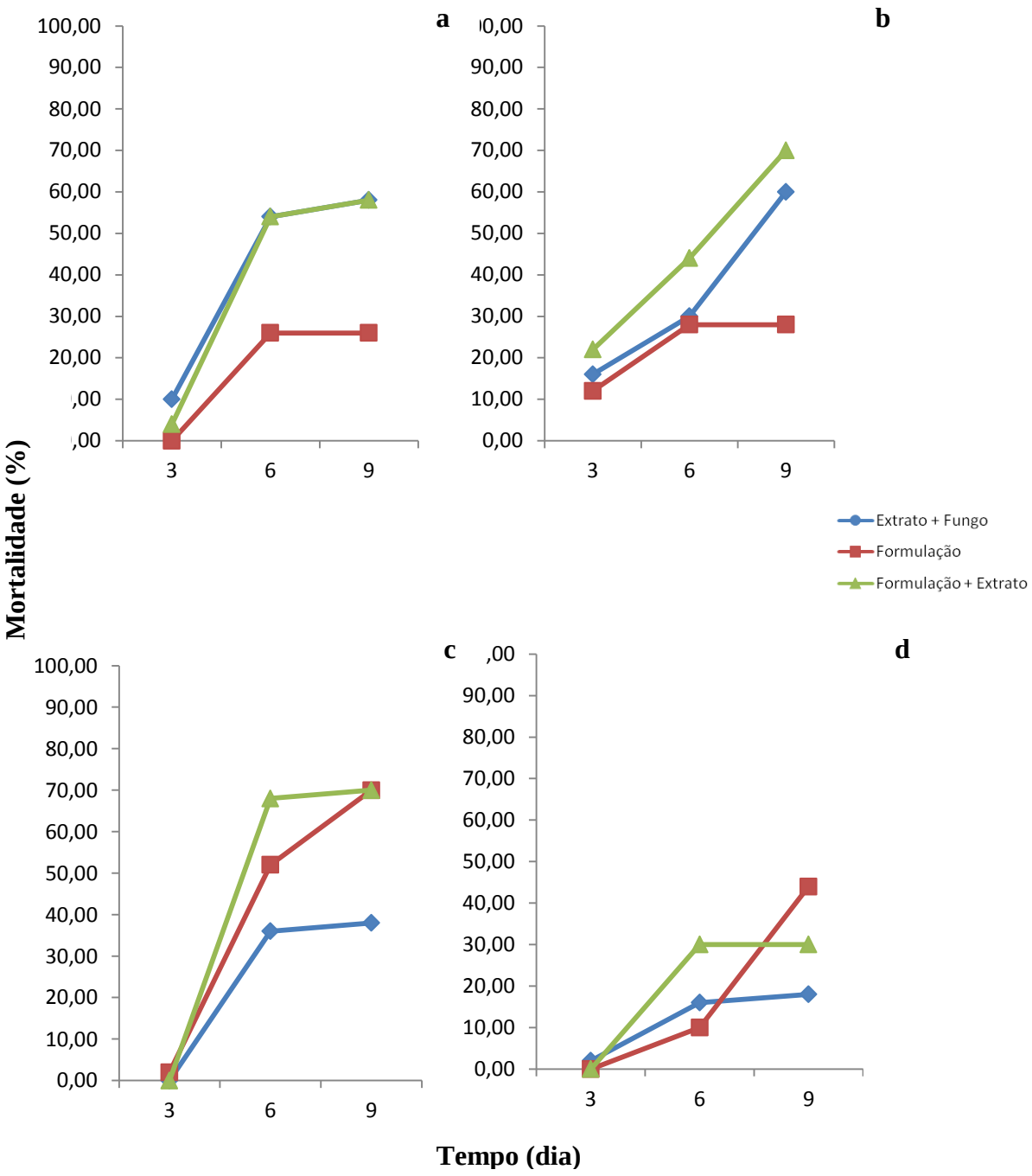

Figura 3. Mortalidade de larvas de *Diatraea saccharallis*, ao longo do tempo, após exposição à formulações com óleo adjuvante emulsionável e associações com extrato das folhas de *Indigofera suffruticosa* (a) *Metarhizium anisopliae* PL43; b) *Metarhizium anisopliae* IBCB425; c) *Beauveria bassiana* ESALQ447; d) *Beauveria bassiana* ARSEF1398).

CAPÍTULO VI

ATIVIDADE INSETICIDA DE *MYRCIARIA CAULIFLORA* SOBRE *DIATRAEA SACCHARALIS* E SEU EFEITO SOBRE FUNGOS ENTOMOPATOGÊNICOS

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ATIVIDADE INSETICIDA DE *MYRCIARIA CAULIFLORA* SOBRE *DIATRAEA SACCHARALIS* E SEU EFEITO SOBRE FUNGOS ENTOMOPATOGÊNICOS

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RESUMO

A compatibilidade do extrato aquoso de frutos de *Myrciaria cauliflora* com os fungos entomopatogênicos *Metarhizium anisopliae* e *Beauveria bassiana* foi avaliada *in vitro*. O efeito do extrato vegetal e da sua associação com os fungos foi verificado sobre *Diatraea saccharalis*. Foi preparada uma solução estoque (30%) do extrato aquoso de cascas de frutos de *M. cauliflora* e a partir desta, o extrato foi incorporado à Batata-Dextrose-Ágar (BDA) em quantidades diferentes, de forma a se obter concentrações de 1%, 2%, 4%, 8% e 16%. Fragmentos fúngicos foram inoculados no centro da placa de Petri e após seis dias de incubação (T=27 ± 1ºC) avaliou-se o crecimento vegetativo, a

produção e a germinação de conídios. Foi calculado o valor do Índice Biológico (IB) dos extratos. Conídios desidratados foram formulados em óleo adjuvante emulsionável para avaliação do seu efeito sobre as brocas. No bioensaio de patogenicidade, as larvas foram imersas por 30 segundos em diferentes suspensões de conídios, conídios + extrato vegetal, formulação + extrato vegetal. Para cada tratamento havia um grupo controle respectivo, tratado com água ou óleo (2%). Foram utilizadas cinco repetições contendo 10 lagartas cada, totalizando 50 insetos por tratamento. As observações foram realizadas diariamente, por 10 dias. O crescimento vegetativo e a produção de conídios não foram afetados significativamente na presença do extrato. O extrato reduziu discretamente a germinação dos conídios porém, estimulou a germinação de *B. bassiana* ARSEF1398 nas duas menores concentrações. De acordo com o IB todos os extratos foram considerados compatíveis com os fungos analisados. O extrato vegetal quando associado ao fungo *M. anisopliae* causou mortalidade de 84% e 76% dos insetos, *M anisopliae* PL43 e *M. anisopliae* IBCB425 respectivamente. O extrato vegetal associado às formulações em óleo de *M. anisopliae* (PL43 e IBCB425) e *B. bassiana* ESALQ447 causou a morte de mais de 70% dos insetos. Os resultados indicam a propriedade inseticida do extrato de frutos de *M. cauliflora*, em associação aos fungos entomopatogênicos, para o controle de *D. saccharalis*.

Palavras-chave: Jabuticabeira; Fungos entomopatogênicos; Formulação; Broca-da-cana; Manejo Integrado de Pragas (MIP)

INTRODUÇÃO

Myrciaria cauliflora Berg, conhecida popularmente como jabuticabeira é uma árvore nativa do Brasil, pertencente à família Myrtaceae. Essa espécie é bastante utilizada na indústria alimentícia, principalmente, na fabricação de geléia, licor, vinho e vinagre, podendo ser consumida também *in natura* (Lima et al., 2008). A sua eficiência também está relatada na medicina popular, no tratamento de diarréias, irritações da pele, asma e hemoptise (Pereira et al., 2000; Oliveira et al., 2003). Posteriormente, Polo e Iha (2006) relataram a atividade antimicrobiana do extrato de folhas de *M. cauliflora* contra *Streptococcus*. A análise fitoquímica de extrato metanólico de frutos de jabuticabeira detectou a presença de flavonoides, ácidos orgânicos, antocianinas e alta atividade anti-radicais, além de compostos antioxidantes e antitumorais (REYNERTSON et al., 2006; DUARTE et al., 2010; SANTOS et al., 2010). Pesquisas posteriores de fracionamento

permitiram o isolamento do composto Jaboticabin, que possivelmente prevenirá doenças relacionadas ao *stress* oxidativo (CAVALCANTI et al., 2011).

A família Myrtaceae tem ação conhecida contra várias pragas, a exemplo de *Eugenia uniflora* L. e *Melia azedarach* L. contra *Atta laevigata* Smith. (formiga cortadeira) (Jung et al., 2013); *Eucalyptus citriodora* Hook contra *Tribolium castaneum* Herbst **(**besouro castanho de grãos armazenados) (Mazzonetto e Vendramim, 2003). Essas evidências indicam também o potencial de M. *cauliflora* para o controle de insetos- praga, porém nenhuma atividade inseticida é demonstrada para esta espécie, na literatura consultada.

Diante da possibilidade de métodos alternativos de controle de pragas, o trabalho teve por objetivo avaliar a compatibilidade do extrato de frutos de *M. cauliflora,* com os fungos entomopatogênicos *M. anisopliae* e *Beauveria bassiana* e seu efeito sobre *D. saccharalis*.

2. Materiais e Métodos

2.1 Linhagens fúngicas: foram utilizadas *M. anisopliae* PL43, *B. bassiana* ESALQ447 e *B. bassiana* ARSEF1398 provenientes da coleção de entomopatógenos mantida no Laboratório de Controle Microbiano e Patologia de Insetos, no Departamento de Entomologia, Fitopatologia e Agricultura, da Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (ESALQ-USP); a linhagem *M. anisopliae* IBCB425 foi fornecida pelo setor de Controle Biológico do Instituto Biológico, Campinas/SP.

2.2 Obtenção e manutenção de *Diatraea saccharalis*: as lagartas de 3º *instar* de *D. saccharalis* utilizadas nos bioensaios foram provenientes da criação experimental da Empresa Biotech Controle Biológico, mantidas a 25 ± 1ºC, U= 70 ± 10 % de umidade.

Os insetos foram alimentados com dieta artificial (Hansley e Hammond, 1968) que é composta basicamente de germe de trigo, levedo de cerveja e caseína.

2.3 Quantificação do inóculo: os conídios foram produzidos em arroz parboilizado autoclavado, em sacos de polipropileno. Após 12 dias, mantidos a 27 ± 1ºC, para desenvolvimento do fungo e esporulação, o arroz mais o fungo foram espalhados em bandejas e mantidos em sala climatizada (T= 17 ± 1ºC, UR= 20 ± 1%), contendo aparelhos desumidificadores (Thermomatic do Brasil, São Paulo, Brasil, model Desidrat D4), por cerca de três dias, até que a umidade do material ficasse em torno de 5% (Moore et al., 1996). Os conídios foram então extraídos do arroz com peneiras metálicas (300 mm de malha) e então submetidos a um analisador de umidade por infra-vermelho (Ind. e Com. Eletro-Eletrônica Gehaka Ltda, São Paulo, Brazil. model IV2000/IV2002). Foi pesado 1g de conídios secos de cada linhagem a ser testada e os conídios misturados a 10 mL de água + Agral (0,02%); após diluições sucessivas, foi realizada a quantificação de conídios, em câmara de Neübauer. A formulação foi composta por 1g de conídios puros secos adicionados a 10 mL de Veget'oil® (Oxiquímica Agrociência Ltda, Jaboticabal,Brasil).

2.4 Viabilidade dos conídios: a viabilidade dos conídios foi verificada retirando-se 0,1mL da formulação e diluindo em 10 mL de solução salina + Agral (0,1%). Após agitação em Vortex, por 1 minuto, para desagregação dos conídios, uma nova alícota foi retirada e espalhada na superfície de placas de Petri de acrílico (6 cm) contendo BDA. As placas foram incubadas a 27 ± 1ºC e observadas após 20 horas. Foram realizadas cinco repetições para cada formulação (Alves et al., 2002a).

2.5 Obtenção dos extratos vegetais: foram coletados frutos maduros de *Myrciaria cauliflora* na cidade de Limoeiro/Pernambuco/ Brasil. Todo o material vegetal foi

lavado e selecionado, de modo que apenas os frutos sadios passaram para a etapa de secagem. A polpa da jabuticaba foi descartada e somente a casca foi utilizada nos extratos. O material foi seco em estufa a 45ºC, por 48 horas, e em seguida triturado em liquidificador. Os extratos aquosos, na concentração de 10% (v/v) foram obtidos pela adição de 35g de pó seco a 350mL de água destilada. A solução permaneceu de 14 a 16 horas à temperatura ambiente, em mesa agitadora. A fase líquida foi removida, por decantação, e o resíduo foi submetido a uma nova extração. Ao final, os extratos foram filtrados, à vácuo, liofilizados e armazenados em recipientes hermeticamente fechados, em freezer (-14ºC) (Vasconcelos et al., 2006).

2.6 Teste de compatibilidade: o efeito fungitóxico do extrato sobre os fungos foi avaliado *in vitro*, a partir de uma solução estoque (30%). O extrato foi incorporado, ao BDA fundido, não solidificado, à temperatura de 40 ± 5ºC, em determinados volumes, de modo a se obter cinco concentrações (1%, 2%, 4%, 8% e 16%). Após homogeneização, o meio foi distribuído em placas de Petri (9cm de diâmetro) e em seguida procedeu-se a inoculação. O grupo controle foi inoculado em meio de cultura isento de extrato vegetal (Depieri et al., 2005). Os testes de esporulação e crescimento vegetativo foram realizados, inoculando-se fragmentos fúngicos no centro da placa de Petri com o auxílio de uma alça de platina; após seis dias de incubação (27 ± 1ºC), o diâmetro da colônia foi mensurado, com uma régua milimetrada. Em seguida, foi retirado um disco central (4mm) da colônia para quantificação da produção de conídios. Cada disco foi individualizado num tubo de ensaio contendo 10mL de água mais Agral (0,02%) e agitado vigorosamente para desagregação dos conídios da superfície do meio de cultura (Oliveira et al., 2003). A suspensão obtida foi diluída a 10^{-1} e então procedeu a contagem dos conídios em câmara de Neübauer. Em seguida, 0,1 mL da suspensão foi semeado em BDA; após 20h de incubação (27 ± 1ºC) o percentual de germinação foi

quantificado por meio de observação sob microscópio óptico, contando-se os conídios germinados e não germinados (Alves e Pereira, 1998). O efeito fungitóxico foi avaliado sobre o crescimento vegetativo, a produção e a germinação de conídios dos fungos testados. Os experimentos foram feitos em quintuplicata. O índice biológico foi obtido com base no cálculo: IB= 47[CV]+43[ESP]+10[GERM]/100, onde CV: porcentagem de crescimento vegetativo com relação à testemunha; ESP: porcentagem de esporulação com relação à testemunha; GERM: porcentagem de germinação dos conídios em relação à testemunha. O valor do IB pode variar de: 0-41 (tóxico), 42-66 (moderadamente tóxico) e >66(compatível) (Rossi-Zalaf et al., 2008).

2.7 Quantificação de conídios para formulação: foi pesado 1g de conídios secos (desidratados como descrito anteriormente) de cada linhagem a ser testada e misturados a 10 mL de água + Agral (0,02%); após diluições sucessivas, foi realizada a quantificação de conídios em câmara de Neübauer. A formulação foi composta por 1g de conídios puros secos adicionados a 10 mL de Veget'oil®.

2.8 Bioensaios: os bioensaios foram realizados com um grupo tratado com conídios fúngicos ($1,0x10^{10}$ conídios/ml) das quatro linhagens analisadas, extrato vegetal (20%), conídios + extrato vegetal, formulação (conídios + óleo), formulação + extrato vegetal. Para cada tratamento, havia um grupo controle respectivo, tratado com água destilada autoclavada e óleo (2%). As lagartas foram imersas nas suspensões por 30 segundos e o excesso de suspensão foi removido em papel filtro (Quinelato et al., 2012). Posteriormente, as lagartas foram transferidas individualmente para placas de Petri de acrílico contendo dieta artificial. Foram utilizadas cinco repetições, contendo 10 lagartas cada, totalizando 50 insetos/tratamento. O experimento foi mantido em condições apropriadas de temperatura e umidade (25 ± 1ºC, U= 70 ± 10 %) e 12 horas de fotofase. As observações foram realizadas diariamente, durante 10 dias, transferindo-se as

lagartas mortas para câmara úmida para confirmação do agente causal, por meio do exame microscópico analisando-se hifas e conídios, na superfície do inseto mumificado.

2.9 Análise estatística: os dados foram analisados utilizando-se o programa ASSISTAT 7,5 Beta e sujeitos à análise de variância (ANOVA). O teste de Tukey foi usado para separar as médias (P= 0,05) e a análise de regressão polinomial para avaliação do Tempo Letal (TL_{50}) (Silva e Azevedo, 2002).

3. Resultados e Discussão

3.1 Compatibilidade dos extratos vegetais com os fungos

O crescimento vegetativo e a produção de conídios dos fungos não foram influenciados pelo extrato dos frutos de *M. cauliflora*; a germinação dos conídios foi reduzida à medida que a concentração do extrato foi aumentada, exceto para *B bassiana* ARSEF1398, que teve a viabilidade dos conídios aumentada nas duas menores concentrações (1% e 2%) de extrato no meio de cultura, enquanto que nas três maiores concentrações a germinação foi retardada, chegando a 83% (Tabelas 1; 2).

Frequentemente, se observa produtos vegetais e fitossanitários inibindo o crescimento da colônia fúngica e a conidiogênese (HIROSE, et al., (2001); Marques et al., (2004)) porém, o extrato dos frutos de *M. cauliflora* não causou efeito significativo sobre esses parâmetros, o que é uma característica desejável na seleção de diferentes tipos vegetais; entretanto, os parâmetros avaliados podem variar de um isolado para o outro, dependendo do produto e da concentração utilizada (PIRES, et al., 2010; RAMPELOTTI-FERREIRA et al., 2010), demonstrando a relevância de testes prévios de compatibilidade.

De acordo com o valor do IB (Índice Biológico) o extrato foi considerado compatível com os fungos, nas concentrações testadas (Tabela 3); indicando que ambos poderiam ser aplicados em conjunto, sem a perda das características dos entomopatógenos. Assim sendo, o extrato dos frutos de *M. cauliflora* foi utilizado nos bioensaios com larvas de *D. saccharalis*.

3.2 Efeito dos extratos vegetais sobre *Diatraea saccharalis*

O extrato dos frutos de jabuticaba associado ao fungo *M. anisopliae* PL43 matou 84% dos insetos e associado ao *Metarhizium anisopliae* IBCB425 matou 76% dos insetos, mas não diferiu significativamente do último tratamento, formulação + extrato. *B. bassiana* ESALQ447 foi mais eficiente em causar a morte dos insetos do que *B. bassiana* ARSEF1398 quando foi aplicada apenas a suspensão de conídios; porém, não houve diferença significativa entre a formulação de *B. bassiana* ESALQ447 (fungo + óleo) (76%) e o extrato adicionado à formulação (74%) (Tabela 4).

O menor TL_{50} do extrato dos frutos de *M. cauliflora* foi 6,0 dias, quando associado a *M. anisopliae* PL43. Os maiores TL50 foram obtidos nos tratamentos com a linhagem *B. bassiana* ARSEF1398, e mesmo a associação fungo e extrato não chegou a matar metade da população de brocas (Figura 1).

Tendo em vista o efeito sinérgico da associação do extrato dos frutos da jabuticabeira com os fungos entomopatogênicos, formulados ou não, os resultados obtidos são promissores para o controle da broca-da-cana. Islam et al., (2010) também demonstraram que *B. bassiana* combinada ao nim causou redução na viabilidade dos ovos e maior mortalidade de ninfas de *Bemisia tabaci* Gennadius (mosca branca),

destacando que a aplicação combinada de um extrato vegetal e um entomopatógeno pode promover um controle efetivo da praga.

Debonsi et al., (2009) avaliaram a letalidade de dois compostos (Isobutil amidas) isolados do extrato de sementes de *Piper tuberculatum* Jacq. aplicados de forma tópica sobre a broca-da-cana; após 48 horas da aplicação, metade da população de larvas havia morrido, demonstrando a ação letal dos compostos. Entretanto, são poucas as pesquisas com extratos vegetais contra *D. saccharalis,* praga que ainda causa grandes prejuízos às plantações de cana-de-açúcar.

Conclusões

Diante dos resultados obtidos, o extrato dos frutos de *Myrciaria cauliflora* é compatível com os fungos *Metarhizium anisopliae* e *Beauveria bassiana*; quando o extrato é associado aos fungos ou às formulações, causa maior mortalidade de lagartas de *Diatraea saccharalis*. Estes resultados indicam o potencial inseticida do extrato de *M. cauliflora* associado aos fungos para o controle eficaz de *D. saccharalis*.

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ANEXOS

Tabela 1. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Metarhizium anisopliae* em BDA, contendo diferentes concentrações do extrato dos frutos de *Myrciaria cauliflora*.

Tratamento	**Crescimento vegetativo**		**Produção de conídios**		**Germinação de conídios**	
	PL43	IBCB425	PL43	IBCB425	PL43	IBCB425
Controle	2.69aA	2.56aA	2.01	2.39	99.15aA	99.11aB
1%	2.62aA	2.44abB	1.74	2.69	94.61eB	97.40bA
2%	2.68aA	2.52aB	2.25	2.70	92.87fB	94.62dA
4%	2.66aA	2.50aB	2.36	2.47	97.28bA	93.95fB
8%	2.68aA	2.50aB	2.65	3.49	96.56cA	95.99cB
16%	2.68aA	2.24bB	2.48	3.20	95.05dA	94.32eB
	dms p/ colunas= 0.2158 dms p/ linhas= 0.1463 cv%= 4.49		cv%= 23.50		dms p/ colunas= 0.0297 dms p/ linhas= 0.0201 cv%= 0.02	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 2. Crescimento vegetativo, produção e germinação de conídios de linhagens de *Beauveria bassiana* em BDA, contendo diferentes concentrações do extrato dos frutos de *Myrciaria cauliflora*.

Tratamento	Crescimento vegetativo		Produção de conídios		Germinação de conídios	
	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398	ESALQ447	ARSEF1398
Controle	1.86	1.74	1.08	0.30	98.73abA	90.76eB
1%	1.84	1.70	1.34	0.31	99.09aA	94.57bB
2%	1.92	1.62	1.83	0.28	98.50bB	99.98aA
4%	1.82	1.72	1.41	0.23	98.34bA	83.14dB
8%	1.74	1.70	1.39	0.27	93.23dA	90.18cB
16%	1.74	1.68	2.45	0.39	96.60cA	83.03dB
					dms p/ colunas= 0.5866	
	cv%= 9.58		cv%= 60.80		dms p/ linhas= 0.3978	
					cv%= 0.33	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

Tabela 3. Valor do IB (Índice Biológico) com classificação do extrato dos frutos de *Myrciaria caulifrora* quanto ao efeito fungitóxico sobre *Metarhizium anisopliae* e *Beauveria bassiana*.

TRATAMENTO	**LINHAGEM**	**VALOR IB/ CLASSIFICAÇÃO**
		Fruto
		M. cauliflora
1%	PL43	92,54 C
	IBCB425	103,02 C
	ESALQ447	110,28 C
	ARSEF1398	102,11 C
2%	PL43	104,54 C
	IBCB425	104,39 C
	ESALQ447	126,69 C
	ARSEF1398	96,38 C
4%	PL43	106,97 C
	IBCB425	99,82 C
	ESALQ447	112,00 C
	ARSEF1398	90,29 C
8%	PL43	113,47 C
	IBCB425	118,37 C
	ESALQ447	109,15 C
	ARSEF1398	95,29 C
16%	PL43	109,46 C
	IBCB425	108,21 C
	ESALQ447	151,29 C
	ARSEF1398	112,18 C

*Os valores do IB podem variar: 0-41 (tóxico - T), 42-66 (moderadamente tóxico -MT) e 66 (compatível - C), segundo Rampelotti-Ferreira et al. (2010).

Tabela 4. Percentual de mortalidade de larvas de *Diatraea saccharalis* expostas aos fungos *Metarhizium anisopliae* e *Beauveria bassiana*, à formulações (fungo + óleo), ao extrato dos frutos de *Myrciaria cauliflora* e associações.

Tratamento	***Metarhizium anisopliae***		***Beauveria bassiana***	
	PL43	**IBCB425**	**ESALQ447**	**ARSEF1398**
Grupo 1*				
Branco	4.00cA	4.00cA	4.00	4.00
Óleo emulsionável	2.00bcA	2.00bcA	2.00	2.00
Grupo 2**				
Extrato	20.00cA	20.00cA	20.00	20.00
Conídios	18.00bcA	16.00bcA	12.00	2.00
Formulação	28.00bA	30.00bA	76.00	52.00
Conídios + extrato	84.00aA	76.00aA	48.00	28.00
Formulação + extrato	74.00aA	74.00aA	74.00	52.00
	dms p/ colunas= 22.4548			
	dms p/ linhas= 14.7274			
	cv%= 35.99		cv%= 45.12	

Médias seguidas pela mesma letra minúscula, na coluna, e maiúscula, na linha, não diferem estatisticamente entre si pelo Teste de Tukey ao nível de 05% de probabilidade.

*Grupo 1: corresponde ao controle, isento de fungo; branco = água;

**Grupo 2: corresponde aos tratamentos; conídios = suspensão de conídios com água e agral; formulação (fungo + óleo).

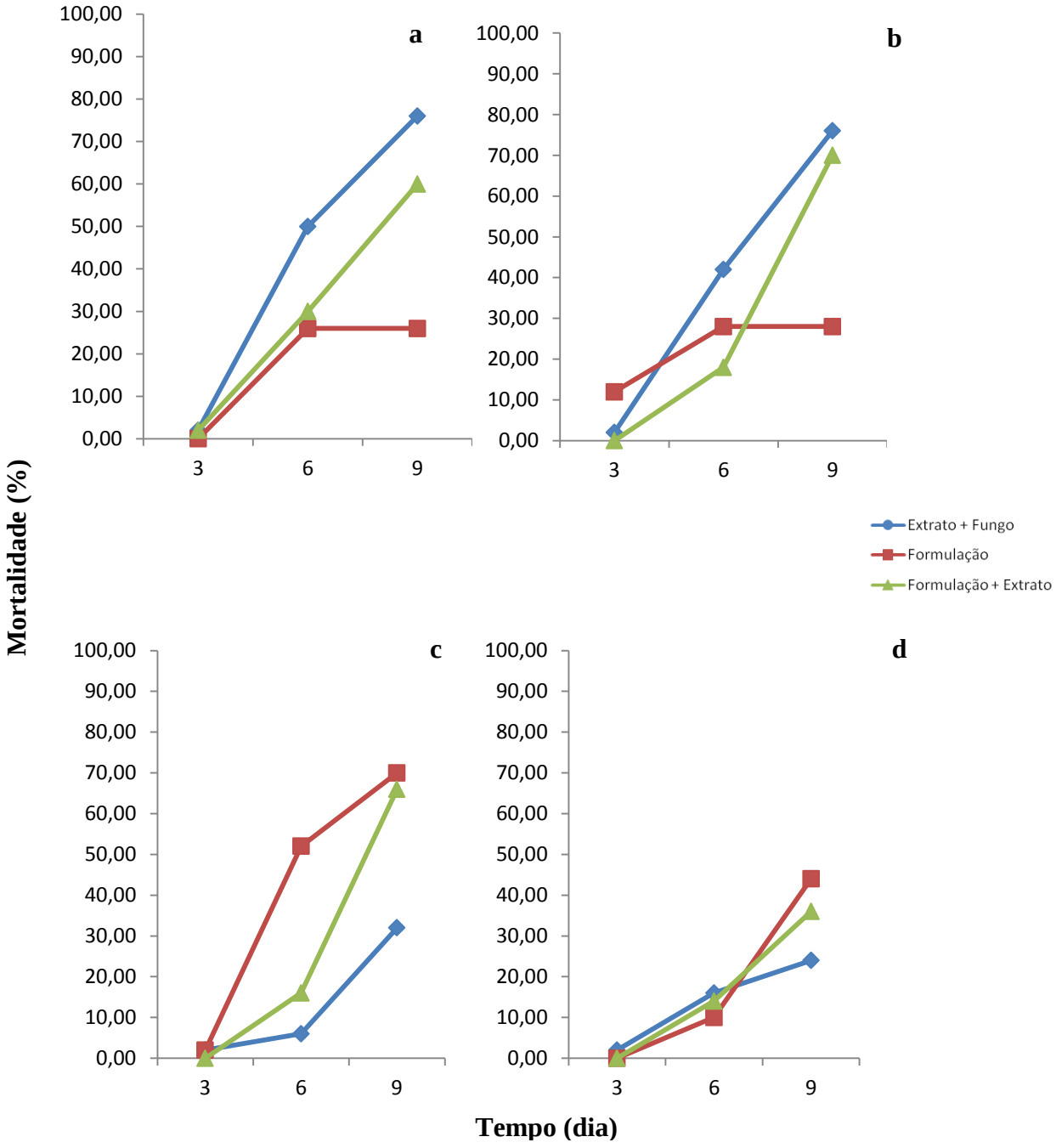

Figura 1. Mortalidade de larvas de *Diatraea saccharallis*, ao longo do tempo, após exposição à formulações com óleo adjuvante emulsionável e associações com extrato dos frutos de *Myrciaria cauliflora* (a) *Metarhizium anisopliae* PL43; b) *Metarhizium anisopliae* IBCB425; c) *Beauveria bassiana* ESALQ447; d) *Beauveria bassiana* ARSEF1398).

9. CONCLUSÕES GERAIS

- O óleo adjuvante emulsionável Veget'oil é compatível com *Metarhizium anisopliae* (PL43 e IBCB425) e *Beauveria bassiana* ESALQ447;
- As forrmulações de *Metarhizium anisopliae* e *Beauveria bassiana* com óleo adjuvante emulsionável permanecem viáveis ao serem estocadas em temperatura ambiente controlada ou sob refrigeração;
- As formulações de *Metarhizium anisopliae* e *Beauveria bassiana* com óleo adjuvante emulsionável tem ação potencializada na mortalidade de *Diatraea saccharalis*;
- A formulação de *Beauveria bassiana* ESALQ447 com óleo adjuvante emulsionável (2%) é recomendada para utilização no controle de *D. saccharalis*;
- Os extratos das folhas e sementes de *Indigofera suffruticosa* e dos frutos de *Myrciaria cauliflora* são compatíveis com *M. anisopliae* e *B. bassiana*;
- Os extratos das folhas e sementes de *I. suffruticosa* e dos frutos de *M. cauliflora* ao serem associados com *M. anisopliae* e *B. bassiana,* ou com formulações em óleo adjuvante emulsionável provocam alta mortalidade de *D. saccharalis;*
- O extrato das sementes de *I. suffruticosa* associado a *M. anisopliae* IBCB425 é recomendado para utilização no controle de *D. saccharalis;*
- Os extratos das folhas e sementes de *I. suffruticosa* e dos frutos de *M. cauliflora* são descritos pela primeira vez causando mortalidade em *D. saccharalis*, associados ou não a *M. anisopliae* e *B. bassiana*.

ANEXOS

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Annual Review & Research in Biology

General Guideline for Authors

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Type of papers

1. Original research papers:
Papers that include original empirical data that have not been published anywhere earlier (except as an abstract). Null/negative findings and replication/refutation findings are also welcome.

2. Review papers:
These papers will not have empirical data acquired by the authors but will include discussion of papers published and data acquired in a specific area.

3. Minireview papers:
Minireviews are brief historical perspectives, or summaries of developments in fast-moving areas covered within the scope of the journal. They must be based on published articles; they are not outlets for unpublished data. They may address any subject within the scope of the journal. The goal of the Minireviews is to provide a concise summary of a particular field in a manner understandable to all readers.

4. Policy Papers:
The purpose of the policy paper is to provide a comprehensive and persuasive argument justifying the policy recommendations presented in the paper, and therefore to act as a decision-making tool and a call to action for the target audience.

5. Short communications:
Short Communications are urgent communications of important preliminary results that are very original, of high interest and likely to have a significant impact on the subject area of the journal. A Short Communication needs only to demonstrate a 'proof of principle'. Authors are encouraged to submit a Original Research Paper to the journal following their Short Communication. There is no strict page limit for a Short Communication; however, we advise a length of 2500-3500 words, plus 2-3 figures and/or tables, and 15-20 key references.

6. Commentaries / Opinion Articles:
An opinion-based article on a topical issue of broad interest which is intended to engender discussion.

7. Short Research Articles:
These may be small single-result findings. Such articles can be brief but need to include enough information, particularly in the methods and results sections, that a reader could understand what was done.

8. Study Protocols and pre-protocols: (Mainly for bio-medical journals)
SDI journals welcome protocols for any study design, including observational studies and systematic reviews. All protocols for randomised clinical trials must be registered and follow theCONSORT guidelines; ethical approval for the study must have been already granted.
Study pre-protocols (i.e. discussing provisional study designs) may also be submitted and will be clearly labelled as such when published. Study protocols for pilot and feasibility studies may also be considered.

9. Method Articles:
These articles describe a new experimental or computational method, test or procedure, and should have been well tested. This includes new study methods, substantive modifications to existing methods or innovative applications of existing methods to new models or scientific questions.
We also welcome new technical tools that facilitate the design or performance of experiments and data analysis such as software and laboratory devices, or of new technologies to assist medical treatment such as drug delivery devices.

10. Data Articles (Mainly for bio-medical journals):
A dataset (or set of datasets) together with the associated methods/protocol used to create the data. No analysis of the data, results or conclusions should be included.

11. Case reports / Case studies (Mainly for bio-medical journals):
Case reports describe patient cases which are of particular interest due to their novelty and their potential message for clinical practice. While there are several types of case reports, originality and clinical implications constitute the main virtues by which case reports are judged. (Ref: http://www.ncbi.nlm.nih.gov/pubmed/18677298). Case studies are an invaluable record of the clinical practices of a profession. While case studies cannot provide specific guidance for the management of successive patients, they are a record of clinical interactions which help us to frame questions for more rigorously designed clinical studies. Case studies also provide valuable teaching material, demonstrating both classical and unusual presentations which may confront the practitioner. (Ref: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2597880/)

(Note:
1. Case Reports should follow the structure of Abstract, Introduction, Presentation of Case, Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables. Abstract (not more than 250 words) of the Case reports should have the following sections: Aims, Presentation of Case, Discussion and Conclusion. Only Case Reports have word limits: Papers should not exceed 2000 words, 20 references or 5 figures. Other Type of papers have no word limits.
2. Review papers may have different headings of the sections and are exempted from following these suggestions.

3. Research Papers and Short Notes should follow the structure of Abstract, Introduction, Methodology, Results and Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables.*)*

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Manuscript structure
The manuscript should be written in English with simple lay out. The text should be prepared in single column format. Bold face, italics, subscripts, superscripts etc. can be used.
The text, excluding the abstract, if required, can be divided into numbered sections with brief headings. Starting from introduction with section 1. subsections should be numbered (for example 2.1 (then 2.1.1, 2.1.2, 2.2, etc.), up to three levels.

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2. Review papers may have different headings of the sections and are exempted from following these suggestions.
3. Research Papers and Short Notes should follow the structure of Abstract, Introduction, Methodology, Results and Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables.)

Brief guidelines
Title Page
The title page should contain a brief title, name(s) of author(s) and their affiliations. The title should be without any abbreviations and it should enlighten the contents of the paper. All affiliations should be provided with a lower-case superscript letter just after the author's name and in front of the appropriate address.
The name of the corresponding author should be indicated along with telephone and fax numbers (with country and area code) along with full postal address and e-mail address.

Abstract
The abstract should be concise and informative. It should not exceed 300 words in length. It should briefly describe the purpose of the work, techniques and methods used, major findings with important data and conclusions. Different sub-sections, as given below, should be used. No references should be cited in this part. Generally non-standard abbreviations should not be used, if necessary they should be clearly defined in the abstract, at first use.
SAMPLE ABSTRACT:
Aims: Here clearly write the aims of this study. Sample: To correlate platelet count, splenic index (SI), platelet count/spleen diameter ratio and portal-systemic venous collaterals with the presence of esophageal varices in advanced liver disease to validate other screening parameters.
Study design: Mention the design of the study here.
Place and Duration of Study: Sample: Department of Medicine (Medical Unit IV) and Department of Radiology, Services Institute of Medical Sciences (SIMS), Services Hospital Lahore, between June 2009 and July 2010.
Methodology: Please write main points of the research methodology applied. Sample: We included 63 patients (40 men, 23 women; age range 18-75 years) with liver cirrhosis and portal hypertension, with or without the medical history of gastrointestinal bleeding. Clinical as well as hematological examination (platelet count) and ultrasonography (gray as well as color Doppler scale including splenic index and splenorenal/ pancreaticoduodenal collaterals) was done besides upper GI endoscopy for esophageal varices. Platelet count/spleen diameter ratio was also calculated.
Results: Kindly make sure to include relevant statistics here, such as sample sizes, response rates, P-values or Confidence Intervals. Do not just say "there were differences between the groups". sample: Out of 63 patients, 36 patients with small varices (F1/F2) and 27 with larger (F3) varices were detected on endoscope. Significant increase in mean splenic index from low (86.7 +/- 27.4) to high (94.7 +/- 27.7) grade varices was documented. Opposite trend was found with platelets (120.2 +/- 63.5 to 69.8 +/- 36.1) and platelets/ splenic diameter ratio (1676.7 to 824.6) declining significantly. Logistic regression showed splenic collaterals and platelets are significantly but negatively associated with esophageal varices grades.
Conclusion: Non-invasive independent predictors for screening esophageal varices may decrease medical as well as financial burden, hence improving the management of cirrhotic patients. These predictors, however, need further work to validate reliability.

Keywords
Immediately after the abstract, about 4-8 keywords should be given. Use of abbreviations should be avoided, only standard abbreviations, well known in the established area may be used, if appropriate. These keywords will be used for indexing.

Abbreviations
Non-standard abbreviations should be listed and full form of each abbreviation should be given in parentheses at first use in the text.

Introduction
Provide a factual background, clearly defined problem, proposed solution, a brief literature survey and the scope and justification of the work done.

Material and methods

Give adequate information to allow the experiment to be reproduced. Already published methods should be mentioned with references. Significant modifications of published methods and new methods should be described in detail. This section will include sub-sections. Tables & figures should be placed inside the text. Tables and figures should be presented as per their appearance in the text. It is suggested that the discussion about the tables and figures should appear in the text before the appearance of the respective tables and figures. No tables or figures should be given without discussion or reference inside the text.

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Each figure should have a caption. The caption should be concise and typed separately, not on the figure area. Figures should be self-explanatory. Information presented in the figure should not be repeated in the table. All symbols and abbreviations used in the illustrations should be defined clearly. Figure legends should be given below the figures.

Some guidelines for Medical papers:

Randomized controlled trials should follow the CONSORT (Consolidated Standards of Reporting Trials) guidelines (http://www.consort-statement.org).

Case reports, case series, cross-sectional and other observational studies should follow the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines (http://www.strobe-statement.org). If the detailed methods are explicitly stated in the manuscript for single case studies, STROBE may be avoided.

Authors producing systematic reviews and meta-analyses should follow the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (http://www.prisma-statement.org).

Results & Discussion

Results should be clearly described in a concise manner. Results for different parameters should be described under subheadings or in separate paragraph. Table or figure numbers should be mentioned in parentheses for better understanding.

The discussion should not repeat the results, but provide detailed interpretation of data. This should interpret the significance of the findings of the work. Citations should be given in support of the findings. The results and discussion part can also be described as separate, if appropriate.

Tables & Figures

Tables & figures should be placed inside the text. Tables and figures should be presented as per their appearance in the text. It is suggested that the discussion about the tables and figures should appear in the text before the appearance of the respective tables and figures. No tables or figures should be given without discussion or reference inside the text.

Tables should be explanatory enough to be understandable without any text reference. Double spacing should be maintained throughout the table, including table headings and footnotes. Table headings should be placed above the table. Footnotes should be placed below the table with superscript lowercase letters.

Each figure should have a caption. The caption should be concise and typed separately, not on the figure area. Figures should be self-explanatory. Information presented in the figure should not be repeated in the table. All symbols and abbreviations used in the

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Guideline for Reporting *P* values:
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v) Reporting actual *P* values avoids this problem of interpretation. *P* values should not be listed as not significant (NS) since, for meta-analysis, the actual values are important and not providing exact*P* values is a form of incomplete reporting.
vi) Do not use 0 before the decimal point for statistical values *P*, alpha, and beta because they cannot equal 1.

Conclusions
This should briefly state the major findings of the study.

Acknowledgements
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AUTHORS’ CONTRIBUTIONS
Authors may use the following wordings for this section: “ ‘Author A’ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. ‘Author B’ and ‘Author C’ managed the analyses of the study. ‘Author C’ managed the literature searches…… All authors read and approved the final manuscript.”

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Only published or accepted manuscripts should be included in the reference list. Articles submitted for publication, unpublished findings and personal communications should not be included in the reference list but may be mentioned in the text (e.g., T Nelson, Purdue University, USA, Unpublished results or personal communication). Avoid citing a “personal communication” unless it provides essential information not available from a public source, in which case the name of the person and date of communication should be cited in parentheses in the text. For scientific articles, obtain written permission and confirmation of accuracy from the source of a personal communication. Unpublished result which has been accepted for publication in any journal should be cited as "in press". Journal name abbreviations should be those found in the NCBI databases (Link:http://www.ncbi.nlm.nih.gov/nlmcatalog/journals).

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1. Hilly M, Adams ML, Nelson SC. A study of digit fusion in the mouse embryo. Clin Exp Allergy. 2002;32(4):489-98.

Note: List the first six authors followed by et al.
Note: Use of DOI number for the full-text article is encouraged. (if available).
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1. Saha M, Adams ML, Nelson SC. Review of digit fusion in the mouse embryo. J Embryol Exp Morphol. 2009;49(3): (In press).

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For Articles not in English
Forneau E, Bovet D. Recherches sur l'action sympathicolytique d'un nouveau dérivé du dioxane. Arch Int Pharmacodyn. 1933;46:178-91. French.

Reference to a book:

Personal author(s)
Rang HP, Dale MM, Ritter JM, Moore PK. Pharmacology. 5th ed. Edinburgh: Churchill Livingstone; 2003.

Editor(s) or compiler(s) as authors
Beers MH, Porter RS, Jones TV, Kaplan JL, Berkwits M, editors. The Merck manual of diagnosis and therapy. 18th ed. Whitehouse Station (NJ): Merck Research Laboratories; 2006.

Authored chapter in edited publication
Glennon RA, Dukat M. Serotonin receptors and drugs affecting serotonergic neurotransmission. In: Williams DA, Lemke TL, editors. Foye's principles of medicinal chemistry. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2002.

Reference to Web-resource or Electronic articles.

Hugo JT, Mondal SC. Parallels between tissue repair and embryo morphogenesis: a conceptual framework. Global Health. 2006;16:4. Accessed 29 March 2012. Available: http://www.globalizationandhealth.com/content/1/1/14.
Anonymous. Parallels between tissue repair and embryo morphogenesis: a conceptual framework. Global Health. 2006;16:4. Accessed 29 March 2012. Available: http://www.globalizationandhealth.com/content/1/1/14.

Reference to Organization as author
Diabetes Prevention Program Research Group. A study of digit fusion in the mouse embryo. J Embryol Exp Morphol. 2009;49(2):259–276.

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For biological nomenclature, the conventions of the *International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria*, and the *International Code of Zoological Nomenclature* should be followed.

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Pest Management Science

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2 FRONT MATTER

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A Perspective is a lightly referenced scholarly opinion piece about current or future directions in the field. A Perspective can serve to assess the science directly concerned with a particular topic or report on relevant issues that may arise from the discipline (for example, policy, effects on society, regulatory issues and controversies). Perspectives that address interdisciplinary research areas or experimental results with significance to a broader audience are of particular interest to the Editors. The Perspective should be accompanied by an abstract and generally range from 6 to 12 double-spaced manuscript pages (including tables and figures).

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A Mini-review is a sharply focused summary and assessment of the relevant literature concerning any topic covered within the Aims and Scope of the Journal. These reviews are particularly effective when discussing cutting-edge advancements in the discipline. Mini-reviews should be accompanied by an abstract, are generally no longer than 14 double-spaced manuscript pages (including tables and figures), and are selectively referenced.

Review

A full-length critical Review provides a summary and discussion of the relevant literature about any topic covered within the Aims and Scope of the Journal. Reviews should be accompanied by an abstract and should be a maximum of 6000 words excluding references and tables.

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The *In Focus* section presents a collection of articles (full papers and/or other article types) by different research groups on a theme of interest to the Journal's readership. These themes will be linked to the Journal's Aims and Scope, as well as to novel subjects or techniques. *In Focus*themes and articles are generally solicited by the Journal's Editors or by a guest editor with particular expertise, but ideas are also welcome.

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BIOLOGICAL CONTROL

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 1049-9644

BIOLOGICAL CONTROL

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DESCRIPTION

Biological controlis an environmentally sound and effective means of reducing or mitigating pests and pest effects through the use of natural enemies. The aim of Biological Controlis to promote this science and technology through publication of original research articles and reviews of research and theory. The journal devotes a section to reports on biotechnologies dealing with the elucidation and use of genes or gene products for the enhancement of biological control agents.

The journal encompasses biological control of viral, microbial, nematode, insect, mite, weed, and vertebrate pests in agriculture, aquatic, forest, natural resource, stored product, and urban environments. Biological control of arthropod pests of human and domestic animals is also included.

Ecological, molecular, and biotechnological approaches to the understanding of biological control are welcome.

This multidisciplinary journal covers:

• Entomology-parasitoids, predators, and pathogens and their use through importation, augmentation, and/or habitat management strategies

• Plant Pathology-antagonism, competition, cross-protection, hyperparasitism, hypovirulence, and soil suppressiveness through naturally occurring and introduced agents

• Nematology-predators, parasitoids, and pathogens in biological control through augmentation and/ or habitat management strategies and suppressive soils through naturally occurring and introduced agents

• Weed Science-vertebrates, invertebrates, and pathogens and their use through classical, augmentative, or bioherbicidal tactics The following sections are included:

• Molecular Technology-advances in the understanding of biological control agents and their mechanisms

• Forum-theoretical and special topics Letters to the Editors-serving as an avenue for debate.

AUDIENCE

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Pest control scientists, ecologists, agricultural scientists, entomologists

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GUIDE FOR AUTHORS

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INTRODUCTION

Biological Control promotes the science and technology of biological control through publication of original research articles and reviews of research and theory. The focus includes new and emerging trends in this field. Biological control is defined as the reduction or mitigation of pests and pest effects through the use of natural enemies. Biotechnologies dealing with the elucidation and use of genes or gene products for the enhancement of biological control agents are also of interest.

The journal encompasses biological control of viral, microbial, nematode, insect, mite, weed, and other invertebrate and vertebrate pests in agricultural, aquatic, forest, natural resource, stored products, and urban environments. Biological control of arthropod pests of human and domestic animals is also included. Ecological, behavioral, molecular, and biotechnological approaches to advancing the understanding of biological control agents are welcome.

Types of paper

The following types of original papers only will be considered.

Regular research papersare hypothesis-driven projects in biological control as defined above under the "Subject areas of the journal". These papers constitute the majority of the articles published in the journal. However, papers which report on routine results of host specificity studies on new biological control agents, surveys for known or unknown biological control agents, or screening of natural enemies against a pest

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Research papers should have the following sections: a brief Abstract that contains a concise statement of the results obtained; Keywords listed immediately after the Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgments; and References. Further details are provided below under "Preparation of manuscript".

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JOURNAL OF INVERTEBRATE PATHOLOGY

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TABLE OF CONTENTS

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AUTHOR INFORMATION PACK 16 Feb 2014 www.elsevier.com/locate/jip 9

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Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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AUTHOR INFORMATION PACK 16 Feb 2014 www.elsevier.com/locate/jip 10

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AUTHOR INFORMATION PACK 16 Feb 2014 www.elsevier.com/locate/jip 11