GUSTAVO LEÃO ROSADO

COMPOSIÇÃO DA COMUNIDADE MICROBIANA DO INTESTINO MÉDIO E CARACTERIZAÇÃO CINÉTICA DE PROTEASES INTESTINAIS DE *Anticarsia gemmatalis* ALIMENTADAS COM DIFERENTES GENÓTIPOS DE SOJA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de Doctor Scientiae.

VIÇOSA
MINAS GERAIS – BRASIL
2016

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

Rosado, Gustavo Leão, 1981-
R788c Composição da comunidade microbiana do intestino
2016 médio e caracterização cinética de proteases intestinais de *Anticarsia gemmatalis* alimentadas com diferentes genótipos de soja / Gustavo Leão Rosado. - Viçosa, MG, 2016.
v, 83f. : il. (algumas color.) ; 29 cm.

Orientador : Maria Goreti de Almeida Oliveira.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. *Anticarsia gemmatalis* - Intestino - Microbiologia. 2. Interação inseto-planta. 3. Simbiose. 4. Cinética enzimática. 5. Inibidores de protease. 6. Soja - Doenças e pragas - Controle . I. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Program de Pós-graduação em Bioquímica Aplicada. II. Título.

CDD 22. ed. 596.78

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COMPOSIÇÃO DA COMUNIDADE MICROBIANA DO INTESTINO MÉDIO E CARACTERIZAÇÃO CINÉTICA DE PROTEASES INTESTINAIS DE *Anticarsia gemmatalis* ALIMENTADAS COM DIFERENTES GENÓTIPOS DE SOJA

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APROVADA: 30 de junho de 2016.

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Maria Goreti de Almeida Oliveira
(Orientadora)

“Disciplina é liberdade”
(Legião Urbana)

SUMÁRIO

RESUMO

ROSADO, Gustavo Leão, D.Sc., Universidade Federal de Viçosa, junho de 2016. **Composição da comunidade microbiana do intestino médio e caracterização cinética de proteases intestinais de Anticarsia gemmatalis alimentadas com diferentes genótipos de soja.** Orientadora: Maria Goreti de Almeida Oliveira.

O estudo da interação inseto-planta tem se mostrado um caminho promissor no controle de pragas. No entanto para um controle efetivo de pragas, tornou-se necessário o conhecimento da relação entre os insetos e sua microbiota associada. Esta interação entrou em foco quando os primeiros estudos provaram a dependência direta do hospedeiro de sua microbiota. Este estudo teve como objetivos caracterizar a diversidade microbiológica associada simbioticamente com o inseto herbívoro Anticarsia gemmatalis e suas consequências em termos de atividade proteolítica digestiva quando alimentado com diferentes dietas de soja suscetíveis e resistentes. Foram encontradas diferenças para a microbiota simbionte alimentadas com os diferentes cultivares de soja, (suscetível controle, estresse hídrico suscetíveis e resistência a insetos (IAC 17 e IAC 24)). Observamos a predominância dos filos Firmicutes e Proteobactérias para as bactérias e Ascomycota e Chytridiomycota para fungos. Em todos os grupos, foram observadas 210 unidades operacionais taxonômicos (OTUs) para bactérias e 110 OTUs para fungos. Os dados mostram que a dieta é determinante na composição da microbiota, sendo agrupadas em diferentes quadrantes, quando analisados por Escalonamento Multidimensional não paramétrico (nMDS), existindo OTUs bem estabelecidos para cada tipo de dieta. No entanto, não foram observadas diferenças para a atividade proteolítica das enzimas isoladas do intestino de Anticarsia gemmatalis alimentada com os diferentes cultivares de soja. Foi verificado a predominância de uma classe de enzimas para a atividade amidolítica e esterolítica, uma vez que não foi observado diferenças nos valores de Km entre os tratamentos. Análise de componente principal agrupou os tratamentos em três grupos distintos, apesar das diferentes dietas não ser determinante no perfil enzimático. Apesar da dieta ser determinante na pressão seletiva sobre a microbiota, ela não foi capaz de modular a resposta das proteases digestivas de Anticarsia gemmatalis. Estudos posteriores poderão acrescentar subsídios no entendimento do papel adaptativo de cada um.

ABSTRACT

ROSADO, Gustavo Leão, D.Sc., Universidade Federal de Viçosa, June 2016. **Midgut microbial community composition and kinetic characterization of intestinal proteases Anticarsia gemmatalis fed with different soybean genotypes.** Adviser: Maria Goreti de Almeida Oliveira.

The plant-insect interaction study has proven to be a promising way to control pests. However, for effective pest control, it has become necessary to know the relationship between insects and their associated microbiota. This interaction came into focus when the first studies proved the direct dependence of the host of their microbiota. This study aimed to characterize the microbial diversity associated symbiotically with herbivorous insect Anticarsia gemmatalis and its consequences in terms of digestive proteolytic activity when fed with different soybean diets susceptible and resistant. Differences were found for the symbiont microbiota fed with different soybean cultivars (susceptible control, susceptible water stress and insect resistance (IAC 17 and IAC 24)). We observed the prevalence of phyla Firmicutes and Proteobacteria for bacteria, and Ascomycota and Chytridiomycota for fungi. In all groups, we observed 210 taxonomic units (OTUs) for bacteria and 110 OTUs for fungi. The diversity and richness of data shows that the diet is critical in the composition of the microbiota, being grouped in different quadrants when analyzed by non-Metric Multidimensional Scaling ($_N$MDS), there are OTUs well established for each type of diet. However, no differences were observed for the proteolytic activity of enzymes isolated from the intestine Anticarsia gemmatalis fed with different soybean cultivars. It was found the prevalence of a class of enzymes for amidolytic and esterolytic activity, since it was not observed differences in their Km values between treatments. Analysis principal component grouped the treatments on three separate groups, despite different diets not be determinative in the enzymatic profile. Although the diet is crucial in the selective pressure on the microbiota, she was not able to modulate the response of digestive proteases Anticarsia gemmatalis. Further studies may add subsidies on understanding the adaptive role of each.

INTRODUÇÃO

O Brasil é segundo maior produtor mundial de soja e este grão representa um papel de destaque em diversos seguimentos da sociedade. No setor econômico, a sojicultora apresentou uma produção em 100 milhões de toneladas na safra de 2015, em uma área que ultrapassou os 36 milhões de hectares.

Os números para a soja seriam ainda melhores se não fossem um dos principais fatores que restringem a agricultura no Brasil, as pragas agrícolas. Hoje no Brasil insetos-praga da soja são responsáveis por uma perda em torno de 15%.

Neste contexto a lagarta da soja, Anticarsia gemmatalis Hübner, é considerada uma das principais pragas da cultura da soja. Esse lepidóptero, na fase larval, causa danos severos à soja que vão desde o desfolhamento até a destruição completa da planta, atuando em áreas cultivadas em todo o país. Os danos causados pelo ataque deste inseto associado à relevância econômica do cultivo da soja para o Brasil fomentam a busca por alternativas no controle de insetos-praga.

Estratégias de controle de insetos-praga baseados no conhecimento do processo adaptativo da lagarta em resposta as defesas de planta, principalmente os que se referem as proteases digestivas e de bactérias endosimbiontes tem se mostrado fundamental. Os métodos mais utilizados de controle de pragas para prevenir danos à cultura de interesse econômico são baseados principalmente no uso de inseticidas químicos. No entanto, cresce a procura pelo desenvolvimento de novas estratégias de menor custo, que sejam mais específicos e menos danosos ao meio ambiente. Assim alternativas como cultivares transgênicos expressando inibidores específicos ou altos níveis de inibidores de proteases tem se mostrado uma saída promissora. Os inibidores de proteases são uma das estratégias mais bem sucedidas das plantas em resposta ao ataque de insetos herbívoros, pois agem principalmente como compostos antinutricionais nos insetos.

Os inibidores de proteases têm se mostrado efetivo na inibição da atividade proteolítica de insetos in vitro, no entanto, esses inibidores não têm causado bons resultados in vivo, apresentando apenas um pequeno retardamento no desenvolvimento de insetos herbívoros. Isso se deve ao fato principalmente dos mecanismos dos insetos em contornar essa barreira evolutiva imposta pelas plantas. Dentre os mecanismos adaptativos, estudos mostram o agrupamento de genes de proteases em diversas famílias multigênicas e o acesso de proteases digestivas ofertadas pela microbiota associada, aumentando evolutivamente o arsenal dos insetos contra as defesas impostas pelas plantas.

Com o desenvolvimento de ferramenta genômicas, especialmente o sequenciamento em larga escala, as análises metagênomicas e de diversidade, cresce a oportunidade de explorar os recursos metabólicos dos microrganismos simbióticos e do seu inseto hospedeiro e assim conhecer o papel adaptativo desempenhado por cada organismo.

Nesse sentido, os estudos sobre os mecanismos adaptativos desenvolvidos por esses insetos herbívoros através de genes de proteases presentes no seu genoma que poderiam estar atuando na adaptação aos inibidores e/ou através da associação com sua microbiota para contornar essa adversidade, em resposta as defesas naturais impostas pelas plantas em especial a soja, representa importante alternativa a ser mais explorada.

Baseado nessas informações, o presente trabalho teve como objetivos, 1) a caracterização da estrutura da comunidade microbiana de lagartas da soja alimentadas com três diferentes cultivares; 2) perfil da atividade enzimática de proteases digestivas isoladas do intestino médio de lagartas da soja alimentadas com cinco diferentes dietas.

REVISÃO DE LITERATURA

Anticarsia gemmatalis Hübner

A lagarta da soja, Anticarsia gemmatalis, Hübner 1818 (Lepidoptera: Noctuidae) é uma das principais pragas desfolhadora da cultura da soja presente no Brasil, sendo encontrada em todos os locais de produção, representando um grande risco à sojicultura. Este inseto causa sérios danos à lavoura de soja, que vão desde o desfolhamento até a destruição completa da planta. A lagarta da soja é um inseto que tem preferência alimentar pelas folhas jovens. Quando a folhagem é removida, ataca outras partes da planta, como pecíolos e haste. O desfolhamento compromete o enchimento das vagens, devido à diminuição da área foliar responsável pela fotossíntese, com consequente redução na produção de grãos. Inicialmente a lagarta da soja costuma atacar as lavouras nas regiões setentrionais no final de novembro e início de dezembro e a partir de janeiro no extremo sul do país e chega a ocasionar 100% de destruição foliar (Praça et al. 2006; De Bortoli et al. 2012; Mehrkhou et al. 2012).

O inseto adulto é uma mariposa de coloração pardo-acinzentada com hábito noturno, que mede em torno de 40 mm de envergadura e pousa com as asas abertas (Figura 1). Essas mariposas são frequentemente encontradas durante o dia em locais sombreados, principalmente, na base das plantas, no entanto, todo o processo reprodutivo ocorre durante o período noturno (Praça et al. 2006).

Figura 1. Anticarsia gemmatalis, inseto adulto.
(Fonte: http://www.discoverlife.org/mp/20q?guide=Caterpillars)

As fêmeas de A. gemmatalis fazem as posturas à noite, depositando os ovos de forma agrupada ou isolada nos caules, ramos e pecíolos e na face abaxial das folhas. Os ovos após a eclosão têm um período de incubação de três a cinco dias. Após o período de incubação dos ovos, as lagartas recém eclodidas alimentam-se das folhas. As lagartas geralmente apresentam coloração verde, com cinco estrias brancas longitudinais sobre o dorso (Figura 2), mas em condições de alta população assumem a coloração negra, mantendo as estrias brancas. A capacidade de consumo da A. gemmatalis cresce com a idade da lagarta e, ao final desta fase, que dura de 12 a 14 dias, um indivíduo pode consumir em média 90 cm^2 de folhas até completar seu desenvolvimento larval (Praça et al. 2006).

Figura 2. Anticarsia gemmatalis, larva do quinto instar
(Fonte: http://www.programamri.com/uy)

A fase de pré-pupa dura em torno de dois dias. Nesta fase, a lagarta para de se alimentar, ficando encolhida com aspecto umedecido. São encontradas formando um casulo, unindo partículas de fezes por uma espécie de teia. A fase de pupa (Figura 3) ocorre no solo, a pouca profundidade, e após uma semana emerge o adulto. As pupas apresentam cor verde com um dia de formada e logo depois coloração marrom avermelhada, ficando quase preta próxima a emergência do adulto. O período de pupa pode durar de sete a nove dias no solo e em torno de oito dias em condições de laboratório. O ciclo biológico é de aproximadamente 30 dias, dependendo das condições ambientais, apresentando normalmente três gerações por ciclo da cultura da soja (Praça et al. 2006).

Figura 3. Pupas de Anticarsia gemmatalis
(Fonte: http://www.esalq.usp.br/noticia/)

Os métodos mais utilizados para o controle de pragas para prevenir danos às culturas de interesse econômico são baseados principalmente no uso de inseticidas químicos. Contudo, cresce a procura pelo desenvolvimento de compostos químicos ou biológicos mais específicos e menos abrasivos (George 2013).

Entretanto, muitos problemas associados ao uso indiscriminado destes inseticidas de amplo-espectro foram evidenciados ao longo dos anos tais como:

produtos de elevada toxicidade; os insetos têm adquirido resistência a estas substancias, sendo necessárias doses cada vez mais elevadas de aplicações; possuem um amplo-espectro de ação, eliminando os predadores naturais e sua persistência no meio ambiente, pois se trata de substancias que não se degradam com facilidade, acumulando-se nos ecossistemas (Finkler 2013; George 2013).

A utilização indiscriminada dos defensivos químicos e suas consequências danosas ao meio ambiente impulsionam o desenvolvimento de técnicas de controle biológico no combate a insetos prejudiciais em culturas economicamente importantes. Dentre os procedimentos empregados para o manejo integrado de pragas, o controle biológico se destaca pelas vantagens que apresenta, particularmente, especificidade e inocuidade para o homem (Finkler 2013).

Segundo Finkler (2013) o controle biológico possui uma definição bastante abrangente, e compreende todos os métodos que tem como objetivo biológico principal limitar o desenvolvimento de pragas, insetos vetores, doenças e plantas invasoras. Estes métodos podem ser divididos em quatro tipos: i) Controle biológico clássico: envolve a utilização de inimigos naturais; ii) Controle microbiano: utiliza micro-organismos capazes de causar doenças em insetos ou de prevenir o estabelecimento de micro-organismos causadores de doenças em plantas; iii) Modificadores do comportamento da praga: que exploram o comportamento específico da praga de forma a confundi-las ou alterá-las; iv) Manipulação genética: técnica que possui a capacidade de interferir na reprodução do inseto e na imunização da planta ao ataque de insetos selecionados.

Para dar suporte principalmente ao último método, o estudo genômico e metagenômico da lagarta da soja para identificação de proteases digestivas “escondidas” no seu genoma e a identificação de bactérias associadas a lagarta, dariam ainda mais suportes no desenvolvimento de metodologias específicas de controle desta praga.

Evolução Adaptativa

Os insetos são os animais mais abundantes da Terra, e a convivência com as plantas já ocorre há mais de 400 milhões de anos e desde então tem exercido uma corrida armamentista: enquanto os insetos têm que enfrentar as defesas da planta e evoluir estratégias para superá-los, as plantas tendem a reduzir a herbivoria através de diversos mecanismos. No meio dessa corrida, plantas e insetos estabeleceram diferentes tipos de relações associativas microbianas que podem influencia os resultados das interações (Jamal et al. 2013; Yun et al. 2014; Sugio et al. 2015). A interação planta-inseto-microrganismo pode mostrar mecanismos extremamente refinados que proporcionaram a evolução dos três. Micróbios podem modular o metabolismo primário e/ou secundário de plantas atuando no seu sistema de defesa contra insetos em benefício de ambos, da planta ou do inseto (Lin et al. 2015; Sugio et al. 2015).

Os insetos e microrganismos patogênicos usam enzimas proteolíticas para penetrar e se alimentar do tecido da planta hospedeira. Como consequência do processo evolutivo, as plantas desenvolveram um engenhoso mecanismo de proteção contra fitopatógenos e insetos-pragas. Entre eles destaca-se o uso de proteínas específicas com atividade tóxica e/ou antimetabólica destinada a esses patógenos (Jamal et al. 2013).

Ao estudarmos os meios adaptativos de defesa das plantas aos insetos herbívoros, vemos que um dos mais eficientes são os inibidores de proteases (IPs). Os IPs estão distribuídos em todo o reino Plantae e sua atuação em insetos baseia-se principalmente como antimetabólicos, interferindo na digestão dos insetos. Esse IPs induzidos em planta, atualmente estão agrupados em 13 famílias, onde principalmente têm como alvo proteases de herbívoros e patógenos de planta (Jongsma & Beekwilder 2011; Jamal et al. 2013).

A função de defesa dos IPs ficou evidente quando Green & Ryan 1972 demonstraram que folhas de planta induziam altos níveis de IP quando submetidos a estresse mecânico e ao ataque de insetos. Somente com o avanço das técnicas de modificação genética permitiu-se a superexpressão de IPs sendo possível assim demonstrar pela primeira vez o papel do IPs na defesa de plantas contra insetos (George 2013). A ingestão crônica de inibidores de proteases limita a disponibilidade de aminoácidos essenciais para o crescimento, desenvolvimento e reprodução do inseto, ocasionando uma hiperprodução de proteinases digestivas (Moreira et al. 2011).

Uma ideia óbvia para a proteção de plantas com o objetivo de aumentar o seu espectro de defesa, seria a superexpressão de inibidores de proteases em plantas transgênicas com o objetivo de conferir resistência a insetos (George 2013). Assim, os inibidores de proteases tornaram-se alvo para estudo de controle biológico através de plantas modificadas geneticamente expressando esses IPs. Segundo Jongsma e Beekwilder (2011), apesar dos níveis aumentados de IP na defesa contra insetos herbívoros, o efeito na mortalidade e no desenvolvimento desses insetos é relativamente pequeno ou até mesmo inexistente. No entanto, a observação de como os insetos contornam os efeitos dos inibidores através de mecanismos evolutivos, demostra um intrigante “cabo de guerra evolutivo” entre inibidores e proteases a ser investigado.

Os mecanismos capazes de contornar os efeitos de inibição desenvolvido pelos insetos herbívoros já descritos, se resumem em três estratégias bem definidas: Aumentar os níveis de expressão de proteases no intestino médio, uma vez que se houver mais oferta de protease do que inibidores, um saldo positivo é atingido no sentido de aproveitamento de substrato; induzir a expressão de proteases insensíveis aos inibidores de proteases; e promover a associação simbionte com bactérias do intestino médio. Recentemente duas novas estratégias têm sido investigadas, sendo elas: a associação entre proteases na formação de complexos multimericos e a indução da expressão de proteases específicas para inibidores de proteases (Jongsma & Beekwilder 2011; Yao et al. 2012).

Os esforços para identificar os mecanismos de defesa dos insetos frente aos impostos pelas plantas, levaram ao reconhecimento da notável diversidade e plasticidade do processo digestivo do inseto. Os insetos podem possuir diversas proteases digestivas pertencendo ao mesmo ou a diferentes famílias mecanicistas (Chi et al. 2009; George 2013).

Devido a essas descobertas recentes, tanto em IP quanto em proteases, muito se tem esclarecido a respeito do jogo adaptativo de ambas as partes. No entanto, pouco se avançou efetivamente no controle de insetos, seja por favorecimento da defesa da planta ou seja pelo desfavorecimento dos insetos herbívoros (Jongsma & Beekwilder 2011).

Acredita-se que o sucesso da estratégia de controle de pragas baseada no uso de inibidores de proteases depende de uma análise caso a caso, envolvendo necessariamente o conhecimento mais detalhado das enzimas digestivas presentes no intestino do inseto alvo (Mendonça et al. 2012).

A ligação entre a capacidade de adaptação do inseto e a diversidade de genes de proteases digestivas é um estudo interessante do ponto de vista evolutivo. Deste modo, o significado da expressão de diferentes tipos de proteases digestivas nunca pode ser subestimado. Estudos sobre as respostas de insetos que envolvam diferentes possibilidades de expressão e identificação de proteases são necessários para identificar moléculas fundamentais e que eventualmente ajudarão na compreensão dos complexos enzimáticos que, são responsáveis pelo acompanhamento e coordenação de absorção de nutrientes e atividade proteolítica intestinal.

Para que técnicas como as descritas anteriormente tenham efeito, torna-se necessário conhecer os genes

proteases digestivas contidas no genoma da A. gemmatalis, para inferir até que ponto poderia ir a sua adaptação proteolítica frente aos diferentes inibidores e identificar sua microbiota associada que contribui efetivamente para sua adaptação.

Proteases de insetos

As proteases são enzimas responsáveis pela hidrólise de proteínas, agindo em ligações peptídicas. De acordo com Enzyme Commission of the International Union of Biochemistry and Molecular Biology - IUBMB, as proteases são classificadas dentro do grupo três (hidrolases), subgrupo quatro (peptidases). Segundo Barett (1994) e Jongsma e Beekwilder (2011), as proteases também são classificadas com base em três critérios: i) tipo de reação catalisada, ii) natureza química do sítio catalítico (família) e iii) relação evolutiva de acordo com a estrutura (Clans).

As proteases são subdivididas em dois grupos principais ((i) tipo de reação catalisada)), os das exopeptidases e os das endopeptidases, dependendo do seu sítio de ação. As exopeptidases clivam as ligações peptídicas próximas ao grupamento amino ou carboxi-terminal no substrato, enquanto, as endopeptidases clivam ligações peptídicas distante do grupo terminal do substrato. Com base no grupo funcional (ii) presente no sítio ativo, as proteases são classificadas dentro de nove grupos, denominados famílias: serino-proteases (família S), aspartil-proteases (família A), cisteíno-proteases (família C), metalo-proteases (família M), treonina-protease (família T), asparagina-protease (família N), glutâmico-protease (família G), “mixed”-protease (família P) e “unknown” (família U). Finalmente, agrupadas em parentesco, filogeneticamente baseada na estrutura primaria da proteína, sempre partindo de um ancestral comum (iii) (MEROPS, 2014).

As proteases digestivas de insetos, responsáveis pela quebra dos alimentos proteicos no intestino, são caracterizadas em serino-proteases, cisteíno-proteases, aspartil-proteases e metalo-proteases. As serino-proteases possuem um resíduo de serina em seu centro ativo, enquanto as aspartil-proteases têm duas unidades de ácido aspártico no seu centro catalítico. Cisteíno-proteases apresentam um aminoácido cisteína e as metalo-proteases usam um íon metal no seu mecanismo catalítico (Yao et al. 2012; Paixão et al. 2013).

O intestino das larvas de lepidópteros representa um ambiente com um complexo proteolítico contendo principalmente serino-proteases (tripsina, quimotripsina e elastases) e cisteíno-proteases envolvidas na digestão, sendo que tripsina contribui com mais de 95% desse processo em lepidópteros (Yao et al. 2012; Paixão et al. 2013). Muitos insetos exibem uma surpreendente flexibilidade na adaptação a diferentes plantas hospedeiras por alterar as proteases específicas do seu intestino em resposta a mudanças qualitativas no teor de proteína na dieta e também quando as proteases existentes são ineficazes e ou ineficientes para a digestão (George 2013).

Genes que codificam diversas proteases já foram identificados, principalmente aqueles que respondem a presença de inibidores proteicos SBTI (soybean trypsin inhibitor). Como descrito por Yao e colaboradores (2012), dois diferentes tipos de tripsina, HzTrypsin-C e HzTrypsin-S de Helicoverpa zea, estão envolvidas diretamente no processo de digestão do material vegetal, no entanto, HzTrypsin-C é sensível aos inibidores de plantas e a HzTrypsin-S foi insensível, sugerindo que HzTrypsin-S está envolvida no mecanismo de defesa de IPs contra insetos. Yao et al., (2012) identificou dois genes que codificam tripsina de Spodoptera frugiperda. Um desses genes foi constitutivamente expresso antes e depois da exposição do inibidor de trypsina SBTI e o outro foi induzido após a ingestão desse inibidor. Resultados similares foram obtidos por Pilon (2012), quando identificou três genes de serino-proteases, Agem 1, 2 e 3, envolvidos na resposta adaptativa de A. gemmatalis a inibidores não proteicos. Assim, esses estudos sugerem uma diversidade de serino-proteases de insetos envolvido na adaptação a inibidores induzidos em planta.

Em muitos lepidópteros, genes que codificam tripsina e quimiotripsina sofreram duplicação, e esse processo já foi encontrado em vários insetos-pragas como Helicoverpa zea, Manduca sexta, Choristoneura fumiferana, Plodia interpunctella, Helicoverpa armigera, Agrotis ipsilon, Sesamia nonagrioides, Tineola bisselliella, Bombyx mori, Spodoptera litura, Bombyx mandarina, S. frugiperda, Mamestra configurata, O. nubilalis, e Heliothis virescens (Zhao et al. 2010; Erlandson et al. 2010; Zhu et al. 2011; Zhan et al. 2011; Yao et al. 2012). Isso demostra que a duplicação gênica de proteases é um processo proposto em resposta à pressão de seleção evolutiva dos inibidores de plantas. Estudo detalhado do processo e dos genes envolvidos na adaptação do sistema digestivo dos insetos frente a defesa de plantas torna-se necessário para o desenvolvimento biotecnológico de estratégias de controle de insetos herbívoros em plantas geneticamente modificadas expressando inibidores de maior espectro de atuação (Petek et al. 2012).

O mapeamento de serino-proteases em genomas tem-se mostrado uma excelente arma no estudo de adaptação por insetos, Zhao et al. (2010) mapearam o genoma completo do bicho-da-seda (Bombyx mori) com o objetivo de identificar serino-protease (SP) e serino-proteases homologas (SPH). Segundo esses autores, foram identificadas 51 SPs e 92 SPH agrupadas em seis famílias gênicas, todas essas proteínas tiveram maior expressão quando o B. mori foi desafiado com diferentes dietas. Ross et al. (2003), quando mapearam serino-proteases e proteínas homologas no genoma recém sequenciado de Drosophila melanogaster encontraram 147 SPs e 57 SP.

O conhecimento sobre proteases digestivas de insetos é vital para o controle de pragas, principalmente com o uso de IPs, sendo as serino-proteases a classes de maior expressão nesse processo fisiológico incluindo nos Lepdopteros como a A. gemmatalis (Moreira et al. 2011; Paixão et al. 2013).

Bactérias Associadas

A microbiota associada aos insetos desempenha papéis importantes, tanto em interações benéficas quanto em interações patológicas com estes hospedeiros. Recentes pesquisas que empregam tecnologias avançadas têm destacado uma infinidade de propriedades associativas microbianos que anteriormente eram ocultos. Estudos genômicos recentes confirmam que os animais não possuem o repertório metabólico completo que possibilite extrair com máximo de eficiência os nutrientes dos alimentos, e que por isso dependem enormemente da microbiota associada (Priya et al. 2012; Yun et al. 2014).

É bem conhecido que os herbívoros não têm a capacidade enzimática necessária para degradar metabólitos vegetais, particularmente celulose, e em vez disso contam com comunidade de microrganismos que têm essa capacidade. Contudo, muito pouco se sabe sobre o potencial genético e as relações estrutura-função intrínseca a estes microbiomas (Xu et al. 2015).

A dimensão da ecologia microbiana associada a nutrição de insetos é cada vez mais reconhecida principalmente quando relacionada ao interesse na pesquisa de insetos saudáveis. Os insetos entram em contato com micro-organismos em suas superfícies e associado a alimentos, mas somente uma pequena minoria desses micro-organismos são patogênicos. A maioria dos micro-organismos não possuem nenhum significado para o inseto, pois não aderem a sua superfície corporal ou intestinal ou passam diretamente pelo intestino junto com o alimento, sendo assim transientemente associado a ele. No entanto, outros microrganismos são significativos para a nutrição dos insetos contribuindo para a sua dieta, promovendo a disponibilidade, a aquisição e o processamento de alimentos. Além disso, estas comunidades microbianas, atualmente são consideradas essenciais para a reprodução e sobrevivência do hospedeiro, podendo afetar consideravelmente em muitos aspectos da ecologia do seu anfitrião, como comportamento e fisiologia, colonização, proteção contra inimigos naturais e até mesmo respostas às mudanças climáticas (Russell et al. 2014; Sugio et al. 2015).

Os insetos possuem necessidades nutricionais muito uniformes, no entanto, têm uma dieta bastante diversificada (Douglas 2009). Dentro da classe Insecta podemos encontrar organismos predadores, herbívoro e catadores específicos especialistas que utilizam néctar, pólen, seiva de plantas, fungos, pele e sangue (Douglas 2009; Hansen & Moran 2014). A capacidade dos insetos em adotar certos estilos de vida nutricionais não se deve somente a ele, mas a uma relação simbiótica entre o inseto e micro-organismos associados com diferentes capacidades biossintéticas e degradativas (Douglas 2009; Jia et al. 2013).

Como descrito por Hansen e Moran (2014), muitas bactérias endosimbiontes desempenham papel importantes na síntese de nutrientes essenciais para o inseto, como exemplo o aproveitamento de resíduos nitrogenados por cupins, baratas e hemípteros em consequência do metabolismo de Bacteroides e Citrobacter. Algumas bactérias são eficientes em fixar nitrogênio atmosférico, micro-organismos associados a insetos que tem essa capacidade permitem ao hospedeiro uma dieta com oferta de nitrogênio, como em Ceratitis capitata (mosca de fruta) em associação com enterobacteria no intestino (Suen et al. 2010).

A interação simbionte entre insetos sugadores de seiva e micro-organismos tornou-se indispensável, devido ao seu metabolismo limitado, em particular por sua incapacidade em sintetizar nove aminoácidos. No entanto esses insetos demandam esses aminoácidos, mas de onde devem ser obtidos? Em muitas plantas, o nitrogênio proveniente das seivas é essencialmente aminoácidos não essenciais, com uma porcentagem menor que 20% para os aminoácidos essenciais. Isso demonstra uma grande evidencia da associação dos insetos sugadores e micro-organismos em suprir suas necessidades nutricionais (Douglas, 2009). Evidencias experimentais apontam que Buchnera aphidicola sintetiza aminoácidos essenciais e os fornece para o pulgão da ervilha (Acyrthosiphon pisum). Esses resultados foram confirmados por anotação completa do genoma de B. aphidicola, revelando que essa bactéria tem a capacidade genética de sintetizar esses nutrientes (Hansen e Moran, 2014).

Além dos aminoácidos, muitos trabalhos descrevem a produção e disponibilidade de vitaminas do complexo B por parte de bactérias endosimbiontes e dados genômicos são consistentes com essa expectativa. O genoma de Wigglesworthia, uma bactéria endosimbiontes de Glossina brevipalpis, mostrou a presença de genes que codificam para a síntese de pantotenato (vitamina B_5), biotina (vitamina B_7), tiamina (vitamina B_1), riboflavina (vitamina B_2), piridoxina (vitamina B_6), nicotinamida (vitamina B_3) e ácido fólico (vitamina B_9) (Shi et al. 2010).

Os esteroides também podem ser disponibilizados por fungos endosimbiontes, uma vez que os insetos não podem produzir esteroides devido a falta da enzima necessária para a formação do núcleo esteroidal. Nos insetos, os esteroides são aproveitados como precursores hormonais e na estrutura celular (Douglas, 2009; Jia et al., 2013).

Muitos estudos têm descrito a síntese de nutrientes essenciais por micro-organismos, no entanto, esses micro-organismos simbiontes também podem atuar no processamento de alimentos (Douglas, 2009). A celulose é uma abundante fonte de carbono mas em contrapartida por ser um polímero estável, formado por uma rede de microfibras, relativamente inacessível a enzimas digestivas. Assim, a quebra mecânica realizada por insetos mastigadores, aumenta a viabilidade da celulose pelas enzimas. Douglas (2009) evidenciou a relação simbiótica entre cupins (Nasutitermes takasagoensis e N. walkeri) e bactérias do intestino com capacidade celulolíticas (Douglas, 2009). Dados de análise metagenômica identificaram genes de celulases e xilanases de membros da microbiota do intestino de Nasutitermes sp. (Hansen e Moran, 2014).

Scully et al. (2013) demonstrou através da análise metagenômica do Anoplophora glabripennis, no qual encontraram diversos genes de enzimas específicas ligada a degradação de biomassa. Assim como já definido por Hungate (1990), insetos que se alimentam de material derivado de planta ricos em celulose são considerados “mamíferos herbívoros em miniatura”.

A maioria dos alimentos contêm compostos (ou elementos) em concentrações que são potencialmente deletérios. Os perigos são particularmente maiores para os herbívoros, pois muitos tecidos vegetais contêm agentes antinutricionais que funcionam como defesa contra consumidores. Os insetos podem se proteger de tais compostos por várias vias, incluindo a eliminação, desintoxicação e sequestro desses metabólitos. Esses recursos são geralmente intrínsecos do inseto, independentemente de qualquer associação microbiana (Hansen & Moran 2014).

No entanto, microrganismos do intestino de insetos herbívoros também poderia desempenhar um papel desintoxicantes de compostos da planta hospedeira. Estudos sobre a lagarta da soja, Anticarsia gemmatalis (Lepidoptera: Noctuidae) e outros herbívoros de soja, mostraram que a sobrevivência e crescimento foram melhores quando as comunidades bacterianas intestinais estavam intactas, sugerindo um papel benéfico da microbiota (Visôtto et al. 2009; Chu et al. 2013). Esses insetos se alimentam de plantas leguminosas que são relativamente ricas em nitrogênio, no entanto, inibidores de protease (IPs) são capazes de prevenir a digestão de proteínas. As bactérias isoladas do intestino de A. gemmatalis secretam proteases insensíveis aos inibidores presente no tecido das plantas (Pilon et al. 2013; Hansen & Moran 2014). Estas observações estão aquém de provar que as bactérias do intestino são cruciais para a superação das defesas de planta, mas nos fornecem evidências sugestivas do seu papel neste processo.

A partir de uma perspectiva ecológica, as interações nutricionais entre insetos e microrganismos são consideradas dinâmicas. Estudos com Helicoverpa armigera, uma potente praga da cultura da soja, milho e algodão, mostraram que além de auxiliar na eficiência alimentar, a diversidade da sua comunidade bacteriana é induzida pela planta hospedeira (Xiang et al. 2006; Gayatri Priya et al. 2012). Jia e colaboradores (2013) avaliaram o impacto da mudança de temperatura na comunidade associada ao besouro escaravelho-vermelho (Rhynchophorus ferrugineus). Mudanças extremas provocadas por diferentes estações do ano provocaram uma drástica mudança no perfil de diversidade microbiana desse besouro.

A literatura sobre a diversidade bacteriana associada a insetos está crescendo rapidamente, mas ainda é carente de informações de alguns grupos importantes de insetos como leptopteros por exemplo. Além disso, todos esses dados nos remete a necessidade de analisarmos o organismo como um todo e não deixando de lado também os micro-organismos associados a ele. Muitos organismos, como principalmente as bactérias que fazem parte dessas associações simbiontes, possui um genoma extremamente pequeno, geralmente < 1 Mb, quando comparado com E. coli, com 4-5 Mb (Shivaji et al. 2010; Segata et al. 2013; Sugio et al. 2015).

Estudos da diversidade e identidade das comunidades microbianas complexas nativas do intestino de insetos tem sido possível devido aos avanços recentes nas técnicas de biologia molecular, incluindo sequenciamento de nova geração (NGS). Assim, esses estudos têm contribuído para formar o conceito de Microbioma, no qual esses micro-organismos formam uma parte fundamental do repertório genômico funcional do inseto devido a sua influência na fisiologia do hospedeiro (Priya et al., 2012).

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CAPÍTULO I

Anticarsia gemmatalis-microorganism interaction under the influence of different soybean genotypes

ABSTRACT

Ecosystems are shaped by complex communities of mostly unculturable microbes. The microbiota plays an important role in insect development and fitness. The gastrointestinal tract of animals harbors a diversity microbial community, and the composition of this community ultimately reflects the co-evolution of microorganisms with their animal host and the diet adopted by the host. Understanding the gut microbiota composition is essential for the development of pest management strategies. Midgut microbiota were isolated from Anticarsia gemmatalis from four different compositions of species from different soy diets. This study aimed to characterize the microbiota of the midgut of A. gemmatalis under the influence of different cultivars resistant and susceptible soybean. There were differences (P> 0.05) for the symbiont microbiota fed with different soybean cultivars (Control susceptible, susceptible water stress and insect resistance (IAC 17 and IAC 24)). It noted the predominance of phyla Proteobacteria Firmicutes for bacteria and to Ascomycota and Chytridiomycota for fungi, what to fungi has few reports. In all groups, were observed 210 operational taxonomic units (OTUs) to bacteria and 110 OTUs for fungi, with an average of 52 and 27 OTUs for bacteria and fungus, respectively. The diversity and richness of data show that the diet is determining the composition of the microbiota, being grouped into different quadrants, when analyzed by non-Metric Multidimensional Scaling (nMDS), well established OTUs for each type of diet.

Keywords: herbivores, host–microbe interactions, symbionts, gut microbiota, Next-generation sequencing (NGS)

INTRODUCTION

The velvetbean caterpillar, Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae), is a key soybean pest species in warm climates and is able to bypass the effects imposed by soybeans [Glycine max (L.)] in Brazil. In order to complete their development, each caterpillar consumes about 90 cm^2 of leaves, thus, high infestations of this insect in soybean crops may compromise yield depending on the level of defoliation and physiological stage of the crop. [1,2]

This destructive power is due mainly reported in insects largely is due to symbiotic interaction between there insect and with their microbiota. [3–6] The insect gastrointestinal tract contains a complex community of microbes, which reflects the co-evolution of microorganisms with their insect host and the diet adopted by the host.[7,8] Although the importance of gut microbiota of humans has been well demonstrated, there is a paucity of research regarding in insects.[5,9,10]

Arthropods are known to harbour various endosymbionts that have close interactions either intracellularly or extracellularly. The bacterial endosymbionts play an important role in host nutrition, development, fitness, survival, modulation of immune responses and communication. A vast range of gut bacteria have been isolated and identified from different insect orders.[4,11] Abundant and diverse populations of microbes are shaped by complex communities of mostly uncultivable microbes. It is estimated that less than 1% of environmental microbes are cultured and resistant to physiological conditions laboratories. [12] The proteolytic activity of gut bacteria associated with the velvetbean caterpillar seems to play a role in this species adaptation to natural proteinase inhibitors.[10]

Recently, next-generation sequencing technologies have been used to characterize the microbial diversity and functional capacity of a range of microbial communities in the gastrointestinal tracts of several animal species.[9,12–14] In Drosophila melanogaster, for example, the sequence reads were dominated by 5 operational taxonomic units (OTUs) at ≤ 97% sequence identity that could be assigned to Acetobacter pomorum, A. tropicalis, Lactobacillus brevis, L. fructivorans and L. plantarum. Featuring low-diversity bacterial community in the gut fruitfly.[7,15]

The search on function of different gut inhabiting bacteria of Helicoverpa Armigera Hübner (Lepidoptera: Noctuidae) revealed their role in nutrition, detoxification of lethal insecticide molecules, and defensive action against pathogens besides insecticidal toxin producing bacterial species were also found associated with the H. armigera gut.[6]

In the way to improve our knowledge, in the present work, high-throughput sequencing has been used to characterize the microbiome associated with a major Lepidopteran pest in Brazil, the soybean caterpillar, Anticarsia gemmatalis. This study try to see whether velvetbean caterpillars fed with different soybean cultivars differ in the composition of the microbial community. In order to define the relationship between diet and microbial diversity, we have focused on four different population feeding with natural diet: a group fed with soybean susceptible to insects, two groups fed with two different strains of soybeans resistant to insects and finally, a food group is susceptible soybean under water stress. Here we present the results of the first diversity characterization of a Lepidopterous pest, which may shed light on the poorly understood host-microbiome-environment interactions.

MATERIALS AND METHODS

Rearing and Damage by Insects

The A. gemmatalis eggs obtained from the Soybean National Research Center of the Brazilian Agricultural Research Corporation (EMBRAPA Soja, Londrina, PR, Brazil), and kept in the Laboratory of Insects (LI) of the Department of Biochemistry and Molecular Biology (DBB) of the Federal University of Viçosa (UFV) at 25 ± 2 °C temperature and 70 ± 10% relative humidity. The A. gemmatalis larvae were incubated at 25 °C with a relative humidity of 60 ± 10% and photoperiod of 14 h and fed witch natural diet soybean plants, at the five instar larvae of A. gemmatalis. After 24 h, the caterpillars were collected, and their midguts were removed for analysis.[2]

Soybean Plants

Seeds of the soybean cultivars were obtained from the Agronomic Institute of Campinas (IAC), São Paulo, Brazil and the Germplasm Bank of the Department of Plant Science Federal University of Viçosa (UFV). Soybean cultivars [Glycine max (L.) Merrill], IAC 17 (insect resistance), and IAC 24 (insect resistance), UFV-16 (under hydric stress = S) and UFV-16 (susceptible control = C), were grown without application of any products. Soybean plants were placed per pot containing 4.0 kg of soil in a greenhouse. The cultivate UFV-16 (stress susceptible) was subjected to a water stress on average -0.8 Mpa. Each variety of plants were used to feed one different groups of larvae until the fifth instar, for two generations. [16,17]

DNA Extraction

Twenty larvae of A. gemmatalis from each group in five instar were disinfected with 75% alcohol and the intestine was removed under aseptic environment. In 1.5 mL centrifuge tube on clean bench and then added in 500 μL lysis solution (0,21 mol/L Tris-HCl, pH=8.0, 0,05 mol/L sodium EDTA, 0,1 mol/L NaCl and 0,5% SDS dissolved in deionized water, with 20 μL (20 mg/mL) lysozyme proteinase K added before use) dealing with water bath at 55 ◦ C for "overnight" (12h - 16h). Then exact DNA solution with the same volume of extraction solvent (Phenol-chloroform-isoamyl alcohol 25 : 24 : 1) was centrifuged at 12 000 g for 10 min at 4 ◦ C twice to remove impurities. The supernates were transferred into new sterilized centrifuges tube with precooled isopropyl alcohol overnight, after centrifuged the DNA were washed twice with 75% alcohol and last each DNA sample dissolved in 50 μL sterile water stored at −20 ◦ C.

PCR amplification of 16S rRNA, ITs and sequencing

DNA samples were quantified and quality checked with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The V3–V4 hypervariable regions of 16S rRNA gene were PCR amplified from bacterial genomic DNA using the following universal primers: MiSeq V3-4F (5'-CCTACGGGNGGCWGCAG-3') and MiSeq V3-4R (5'-GACTACHVGGGTATCTAATCC-3').[18] The fungal internal transcribed spacer (ITS) was PCR amplified using the ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primer pair.[19] The variable regions V6-V8 of the 16S rRNA gene of the Archaea, were PCR amplified using archaea-specific primers Ar915aF (5´-AGGAATTGGCGGGGGAGCAC-3') and Ar1386R (5'-GCGGTGTGTGCAAGGAGC-3').[20] DNA samples were sent for PCR amplifications, purification and sequencing on the Illumina MiSeq platform (Macrogen, Korea).

Bioinformatics analysis

The sequences were processed using the software MOTHUR v.1.36.0 (Schloss et al., 2009) and clustered into operational taxonomic units (OTUs) defined by 97% similarity. Chimera detection and removal was performed, and sequences were classified using the Greengenes database to Bacteria and Archaea and using the UNITE ITS database to Fungi with a bootstrap cut-off of 80.[21,22]

Mothur was also used to calculate the coverage (rarefaction curves), the number of species in a sample (species richness; Chao1 and ACE) and abundance-based coverage, as well as the number of equally abundant species (species diversity) with Shannon-Wiener and Simpsons index.

Differences between overall bacterial community and fungal were visualized using Non-metric multidimensional scaling (n-MDS) was carried out using the Bray-Curtis dissimilarity[23] to plot the distances between the samples, considering the OTU distribution calculated in software Past.[24] Hierarchal dendogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and Euclidean distance method and Heatmap were created using CIMMiner program package (www.discover.nci.nih.gov/cimminer).

Analysis of similarities (ANOSIM) was used to test the significance of the differences observed between caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean, based on the Bray-Curtis distance, considering the OTU composition of the samples and Similarity percentage (SIMPER) analysis was used to identify the taxa that were mainly responsible for the differences observed between caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean were calculated in software

R (vegan package; R Core Team, 2015).[25] Venn diagrams were constructed using the Mothur software (Schloss et al., 2009).[26]

RESULTS

It was used next-generation Illumina MiSeq platform to sequence the V3–V4 hypervariable regions of 16S rRNA gene evaluate the diversity associated with bacterial communities in four groups (C, S, IAC 17 and IAC 24; n = 80) A. gemmatalis. Also using the same sequencing platform was used for the ITS region sequence fungi and V6-V8 Archaea region to identify diversity. An avarage of 120,815 raw sequences yielded in average 725.5±114.7 and 618±218.8 high-quality sequences after filtering in MOTHUR, in bacteria and fungus, respectively (Table 1). After filtering the sequences of Archaea were not obtained sufficient coverage for subsequent analysis.

Bacterial communities caterpillar midgut in all groups (C, S, IAC 17 and IAC 24) was dominated by phylum Proteobacteria (C: 50%, S: 14.3%, IAC17: 33.4% IAC24: 60%) and Firmicutes (C: 50%, S: 85.7%, IAC17: 66.6% IAC24: 40%) (Table S1). For fungi, caterpillar midgut communities in both groups were dominated by phyla Ascomycota (C: 50%, B: 40% IAC17: 0% IAC24: 10%), Chytridiomycota (C: 28.5%, S: 14.2% IAC17: 42.8% IAC24: 14.2%) and Zygomycota (C: 100% S: 0% IAC17: 0% IAC24: 0%), with a number of unclassified phyla (Table S2).

A total of 4 bacterial classes were identified and among them Bacilli was the most dominant group followed by Alphaproteobacteria, Gammaproteobacteria and Clostridia (Figure 1A). A total of 5 bacterial orders, 8 families, 4 genera, and 5 species were identified from the sequence analysis. Analysis at species level showed similarity to the sequences deposited in Greengenes Database with Enterococcus casseliflavus, Lactobacillus plantarum, Magnetospirillum magnetotacticum, Enterococcus haemoperoxidus and Leuconostoc mesenteroides.

Table 1. Number of observed OTUs at 3% cutoff, for unique OTUs, richness estimates and diversity indices to Bacteria and Fungi of the midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean.

	Bacteria				**Fungi**			
	C	**S**	**IAC 17**	**IAC 24**	**C**	**S**	**IAC 17**	**IAC 24**
Number of Sequence	547	819	703	833	363	684	941	484
Coverage (%)	0,99	0,99	0,99	0,99	0.92	0.89	0.96	0.97
Total # of Unique OTUs	59	50	50	51	25	27	27	31
Richness Estimate								
Chao1	8	7	4	11	47	155	54	155
ACE	8	7	1	1	92	527	106	403
Diversity Indices								
Shannon-Weiner	1.51	1.73	0.19	0.38	2.10	2.66	2.14	2.47
Simpson’s	0.29	0.19	0.92	0.84	0.18	0.06	0.19	0.12

ACE: abundance coverage estimator. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

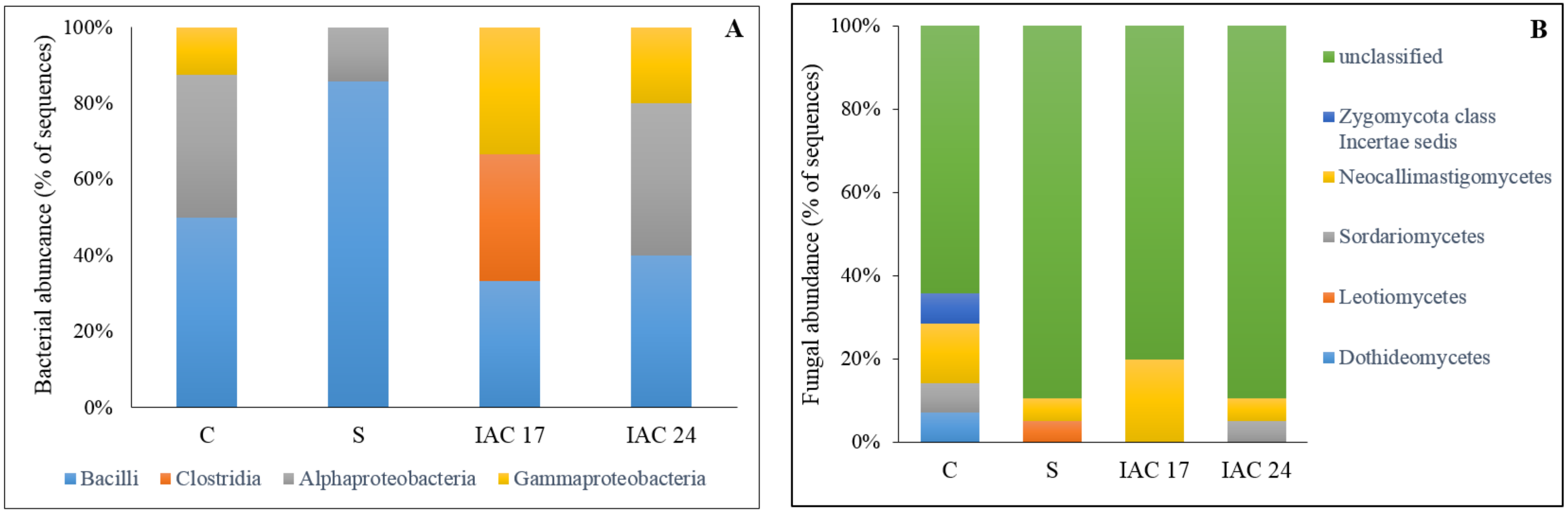

Figure 1. Classes-level composition of midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean. A Color-coded bar plot showing the bacterial-classes and (A) fungal-classes (B) distribution of the caterpillar fed with different diets. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

For fungi, a total of 5 classes were identified and among them Neocallimastigomycetes was the most dominant group followed by Sordariomycetes, Dothideomycetes, Leotiomycetes and Zygomycota class Incertae sedis (Figure 1B). A total of 6 fungal orders, 5 families, 6 genera, and 4 species were identified from the sequence analysis. Analysis at species level showed similarity to the sequences deposited in the UNITE ITS database with Caecomyces communis, Cyllamyces aberensis, Leohumicola minima and Clonostachys divergens.

To estimate and compare diversity in each groups (C, S, IAC 17 and IAC 24) bacterial and fungal diversity indices were calculated from OTUs of each library. Sequence coverage was considered sufficient for a good coverage, bacteria were found 99% and fungi ranging from 89-97% for all samples and leveling rarefaction curves (Table 1; Figure 2A and 2B). In all groups, were observed 210 operational taxonomic units (OTUs) to bacteria and 110 OTUs for fungi, binned 97% similarity were identified in all groups (Table 1). To estimate species richness of the contents of Chao1 and ACE were used. For the Chao index, wealth estimator based on abundance, observed C = 8, S = 7, IAC 17 = 4 and IAC 24 = 11 for bacteria and C = 47, S = 155, IAC 17 = 54 and IAC 24 = 155 to fungus. In ACE index, based on the concept of coverage sample, obtained C = 8, S = 7, IAC 17 = 1 and IAC 24 = 1 and C = 92, S = 527, IAC 17 = 106 and IAC 24 = 403 for bacteria and fungus respectively (Table 1). Now to estimate diversity, they were also used two indices. Diversity estimated by Shannon index, which takes into account the number of species more starting from the premise that all species have the same abundance, ranges from 0.19 to 1.73 between groups for bacterial community and ranged from 2.10 to 2.66 in groups for fungal community. For Simpson’s index, measuring the dominance, ranges from 0.19 to 0.92

between groups for bacterial community and ranged from 0.06 to 0.19 in groups for fungal community (Table 1).

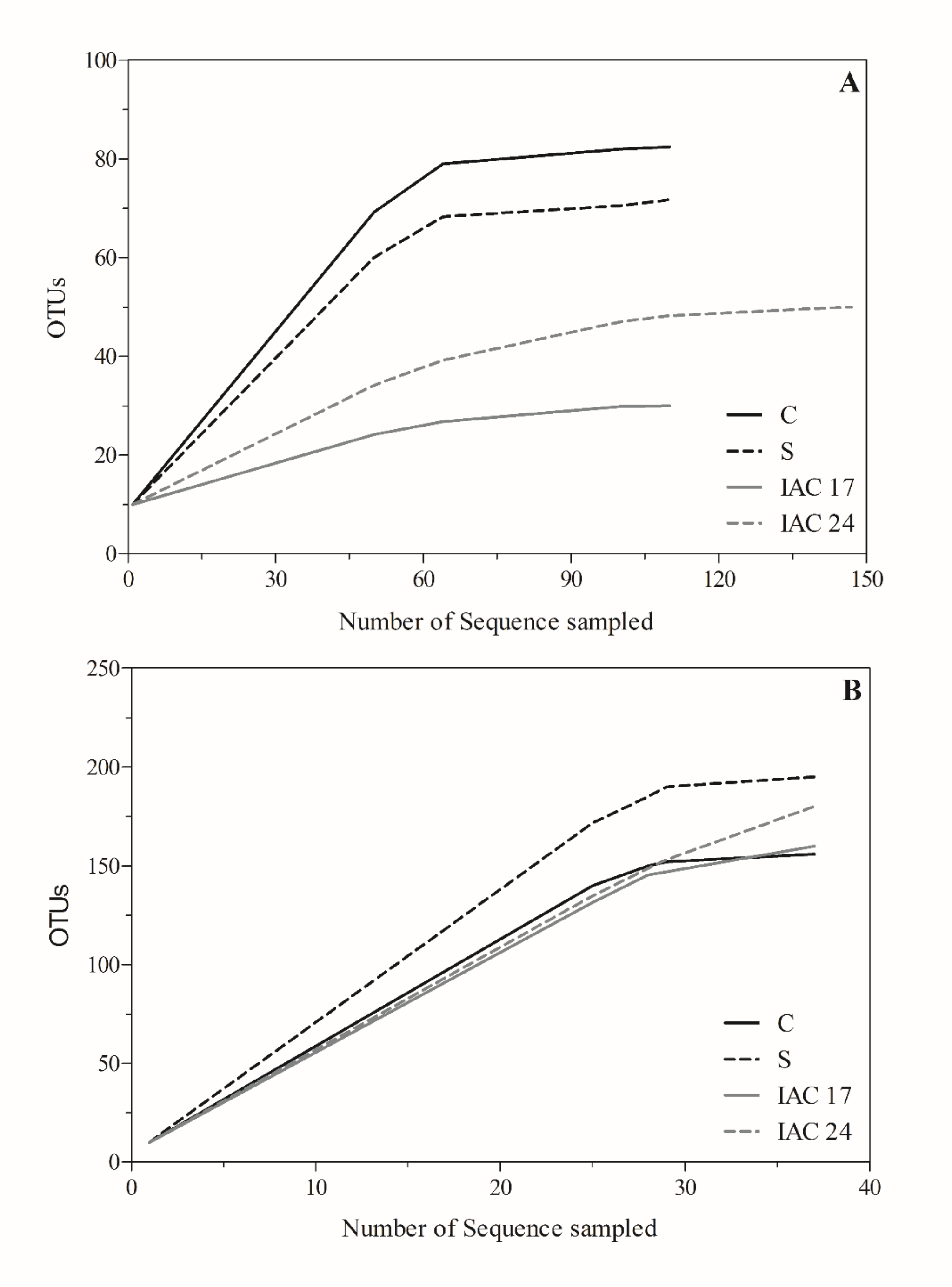

Figure 2. Sample-based rarefaction analysis to bacterial composition (A) and fungal composition (B). Species-accumulation curves showing the increase in OTU numbers as a function of the number of individuals sampled of midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

For a better understanding of the microbial community structure, the data were submitted to analysis of beta diversity. To visualize changes in the composition of the caterpillar community fed with different diets, it was used non-metric multidimensional scaling (nMDS), based on Bray-Curtis distances. Caterpillars fed varieties IAC 17 and IAC 24 (diet-resistant plants) groups are grouped together in bacteria, distant from C and S groups (Figure 3A). For fungi, a dispersion of the all groups was observed (Figure 3B).

The analysis of similarities (ANOSIM) to bacteria and fungi, which considered the OTU compositions, revealed significant differences between caterpillar fed with diet different (bacteria, $p = 0.04$ and fungus, $p = 0.03$).

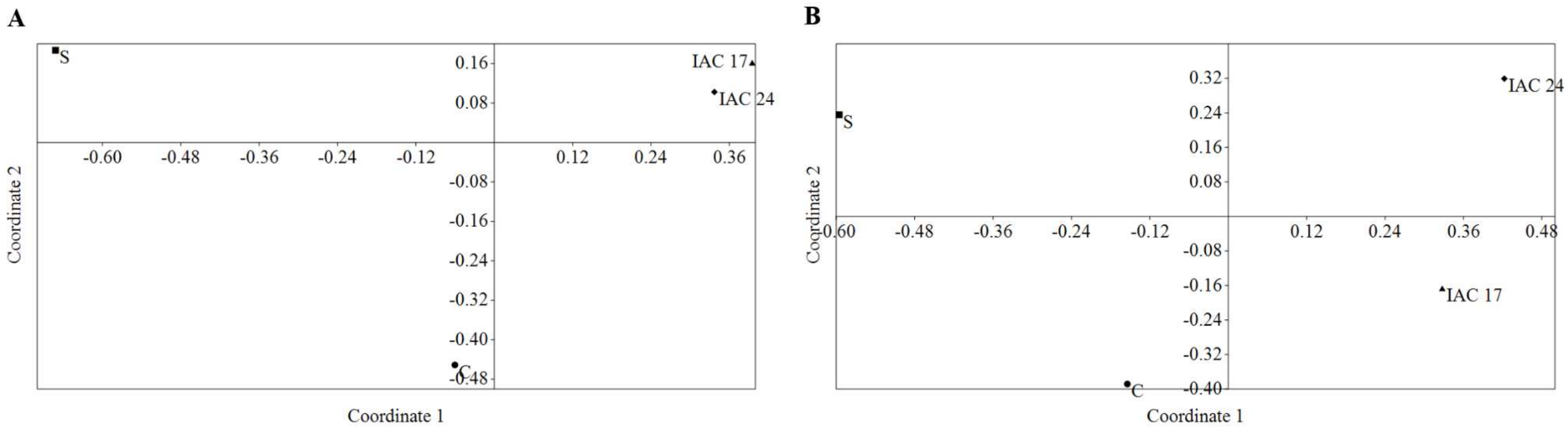

Figure 3. Distance between of midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean, based on similarity in OTU composition (OTU similarity ≥ 0.97%) of each caterpillar calculated using the Bray–Curtis dissimilarity index and plotted using non-metric multidimensional scaling (nMDS) to Bacteria (A) and Fungus (B). Each point represents a different sample plotted according to their OUTs composition and abundance (Bacteria, stress value = 0.08 and Fungus, stress value = 0.05). A greater distance between two points infers a lower similarity between them, whereas samples with a more similar composition and abundance cluster closer together. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

The heatmap colors indicate the relative percentage of bacteria and the hierarchical heatmap showed that four groups could be organized into three main groups: The first group was composed of two groups, IAC 17 and IAC 24, the second group was composed of one group, C and the third group was composed of one group, S (Figure 4). Statistical differences similarity percentage between the bacterial community caterpillars fed with different diets ((SIMPER), contribution to overall Dissimilarity) were driven by the Enterococcus casseliflavus, Lactobacillus sp, Enterobacteriaceae, Rickettsiales, Lactobacillus sp., Lactobacillus sp. and Christensenellaceae (Table 2). For fungi, were used the top 20 OTUs for heatmap analysis. The hierarchical heatmap showed that four groups could be organized into three main groups different of the bacteria: The first group was composed of two groups, S and C, the second group was composed of one group, IAC 17 and the third group was composed of one group, IAC 24 (Figure 5). Statistical differences similarity percentage between the fungal community caterpillars fed with different diets (SIMPER), were driven by the Unclassified Fungi, Caecomyces communis, Cyllamyces aberensis and Neocallimastigaceae (Table 3).

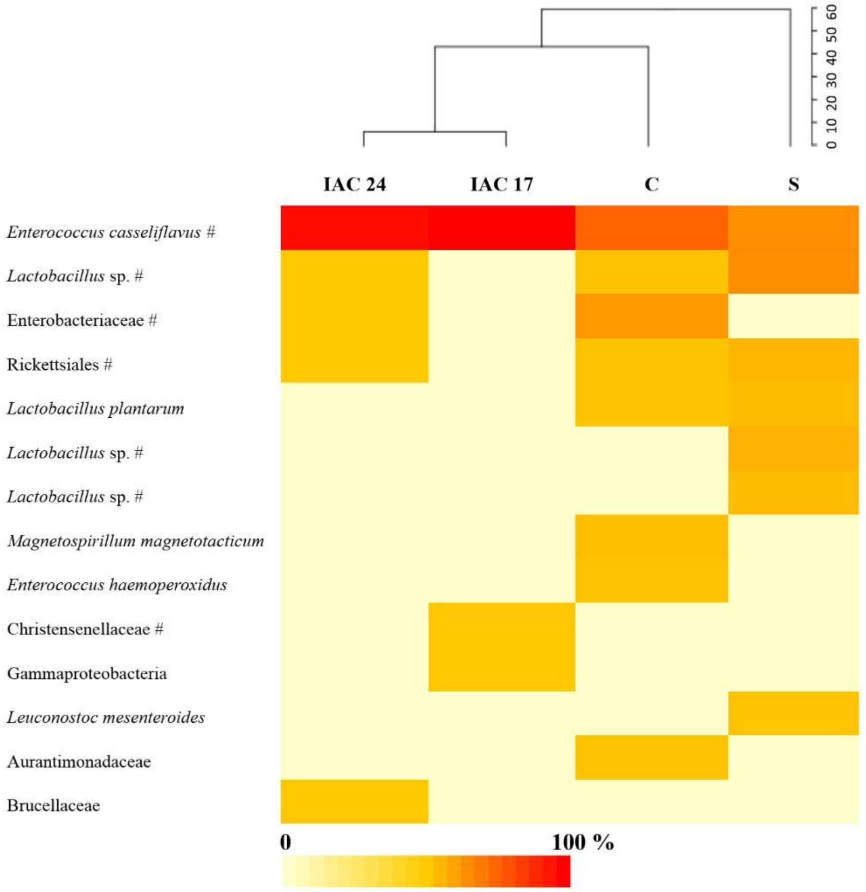

Figure 4. Hierarchal dendogram to evaluate bacterial distribution of midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean, using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and Euclidean distance method. The heat map colors represent the relative percentage of the OTUS within each treatment with the legend indicated at the lower left of the figure. The OTUs that significantly contributed to differences seen between midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties of soybean (SIMPER) are indicated by #. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

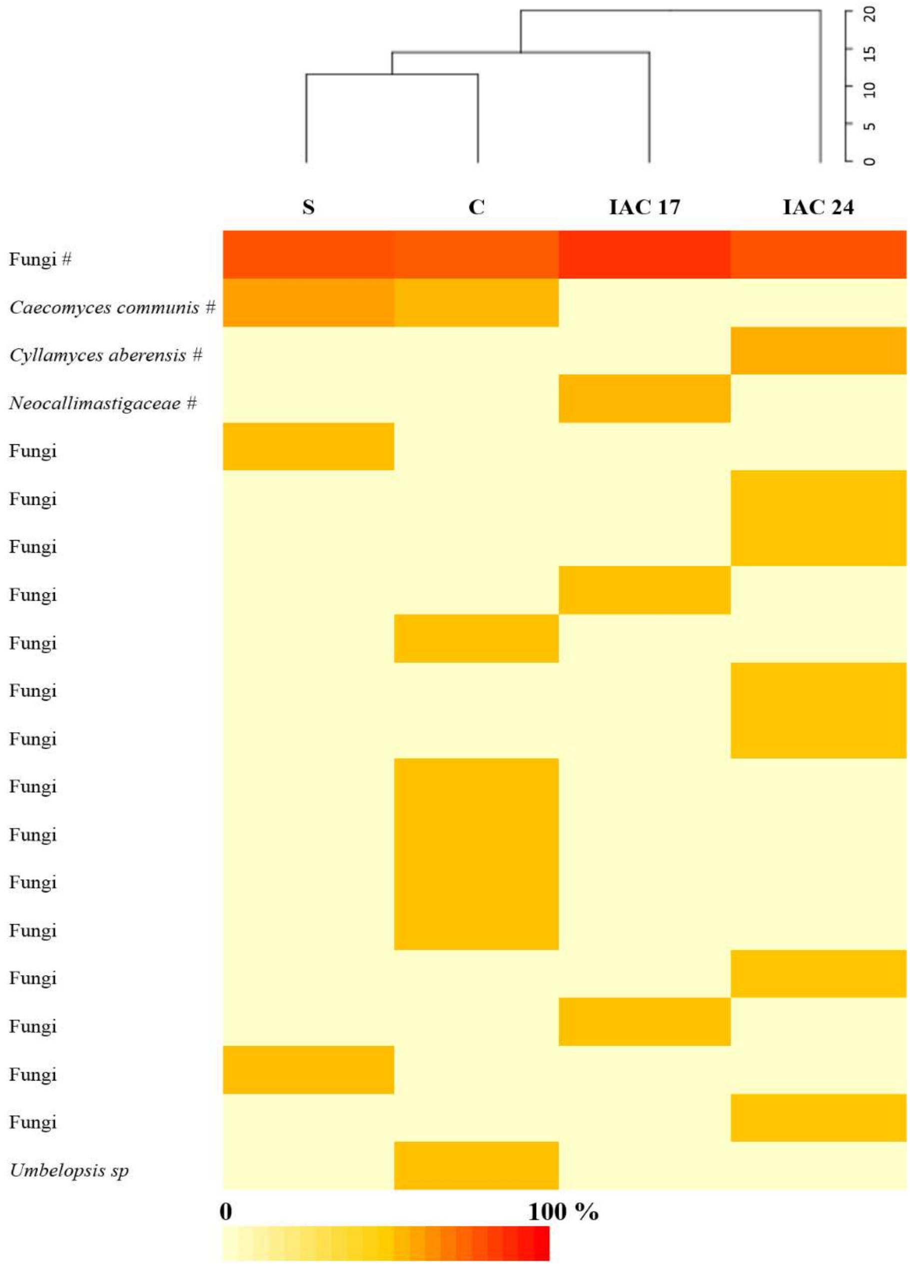

Figure 5. Hierarchal dendogram to evaluate fungal distribution of midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean, using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and Euclidean distance method. The heat map colors represent the relative percentage of the OTUS within each treatment with the legend indicated at the lower left of the figure. Only OTUs at 3% relative abundance or greater in at least one sample are shown. The OTUs that significantly contributed to differences seen between midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties of soybean (SIMPER) are indicated by #. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

Table 2. Principal OTUs bacterial responsible for differences between midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean. Values represent dissimilarity contribution in percentage (%).

OTU #	**Lowest classification**	**C x S**	**C x IAC 17**	**C x IAC 24**	**S x IAC 17**	**S x IAC 24**	**IAC 17 x IAC 24**
Otu01	Enterococcus casseliflavus	0.17	0.47	0.50	0.47	0.47	0.14
Otu02	Lactobacillus sp.	0.22			0.21	0.21	0.14
Otu03	Enterobacteriaceae	0.2	0.23	0.24			0.14
Otu04	Rickettsiales						0.15
Otu06	Lactobacillus sp.	0.1			0.08	0.08	
Otu07	Lactobacillus sp.	0.07					
Otu0010	Christensenellaceae						0.14

C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

Table 3. Principal OTUs fungal responsible for differences between midgut of caterpillars (Anticarsia gemmatalis) fed with four different varieties (C, S, IAC 17 and IAC 24) of soybean. Values represent dissimilarity contribution in percentage (%).

OTU #	**Lowest classification**	**C x S**	**C x IAC 17**	**C x IAC 24**	**S x IAC 17**	**S x IAC 24**	**IAC 17 x IAC 24**
Otu01	Unclassified Fungi	0.09	0.09	0.12	0.14	0.16	0.05
Otu02	Caecomyces communis	0.03	0.03	0.05	0.07	0.06	
Otu03	Cyllamyces aberensis			0.10		0.08	0.10
Otu04	Neocallimastigaceae		0.06		0.05		0.06

C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

Venn diagram aims to facilitate relations of union and intersection between the groups. In bacteria, only a single OTUs was shared among all groups (C, S, IAC 17 and IAC 24). The number of unique bands varied between caterpillar groups fed with different varieties of soybeans. For the C group (fed susceptible soybean) presented three unique bands; S (fed susceptive stressed soybean) three single bands; in IAC 17 and IAC 24 (resistant cultivars) two and one unique bands were observed, respectively. To fungi, indicated that only a single OTUs was shared among all groups (C, S, IAC 17 and IAC 24). The number of unique bands varied considerably between caterpillar groups fed different varieties of soybeans. For the C group presented 12 unique bands; S 17 unique bands; in IAC 17 and IAC, 14 and 18 unique bands were observed, respectively (Figure 6).

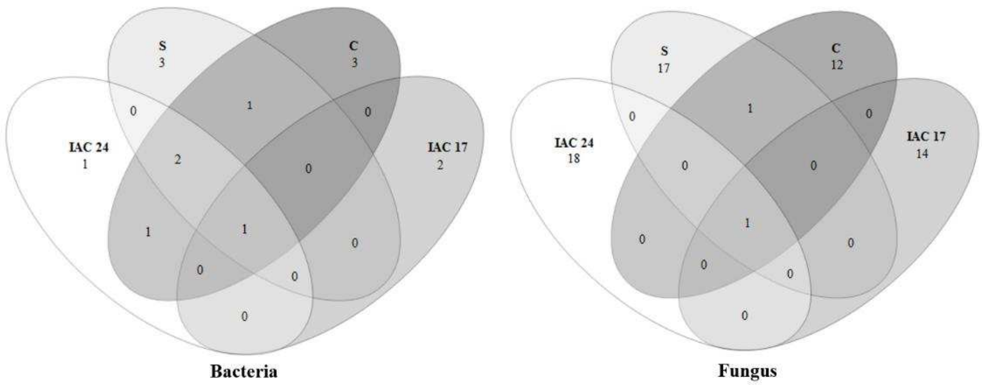

Figure 6. Venn diagram of the composition bacterial community (A) and composition fungal community (B) of midgut of caterpillars (Anticarsia gemmatalis). The 4 circle Venn diagram shows caterpillar fed with four different varieties of soybean. C = susceptible control; S = under hydric stress; IAC 17 = insect resistance; IAC 24 = insect resistance. Each circle represents gut caterpillar taxa within overlapping areas were common to the corresponding diets.

DISCUSSION

Data related to microbial diversity associated with insects is growing rapidly, but still need information from some important groups of herbivorous insects (eg Lepidoptera). In general, the herbivorous insects harbor microbial limited communities diversity, dominated by some specific groups. Two recent studies analyzing the insect microbial communities species of different orders, is conclusive to say that the diversity of associated microbial species is low (10-15 OTUs on average, insects) and this is reflected mainly by specific food habits, which selects its microbial community, and also for its evolutionary history.[14,27]

Diet is a controlling factor for microbial diversity. In the mammalian gut, the diversity of bacteria increases according to host diet, from carnivores to omnivores to herbivores.[28] The intestinal bacteria of insects such as termites and aphids produce compounds that are essential and otherwise inaccessible for the host (e.g., detritus, phloem, sap, wood, and xylem).[29,30]

Currently most studies on the relationships direct between insect diet and the distribution of gut microbiota have tended to focus on specific microbes or in a single insect group; however, a recent, wider-ranging study based on previously published and newly generated 16S rRNA gene sequences from 62 insect species from seven orders reported that both host diet and taxonomy affect insect gut bacterial communities.[31] This study also have shown that in insects, their microbiota is dominant by phyla Proteobacteria, and in particular Enterobacteriaceae, are the most prevalent microbial associates of herbivore insects, what exactly was found in our work (supplementary data). It has been show that0 Proteobacteria and in particular Enterobacteriaceae, are the most prevalent microbial associates of herbivore insects.[14]

Diversity and richness of the results show that A. gemmatalis is associated with a small bacteria number, but with high dominance of some species, especially in resistant varieties (IAC 17 and IAC 24). In fungi, which for us was a surprise related to fungus data, since there are few reports of mutualistic relationship between insect and fungi, we found a great richness and diversity, compared to bacteria, but with low dominance. Of the few existing studies on the interaction of insects and fungi so far, all are related to termites. Showing fungal function to degrade cellulose derivatives for the use of these products by termites and the type of fungus phylogenetically varies with the species of termites.[32] The same could happen to Anticarsia gemmatalis since this insect herbivore be rodent, can attack any plant.

As we have previously shown by Vissôtto et al. (2009) Anticarsia gemmatalis is extremely dependent on its microbiota, and now with these results showing that in addition to being dependent on the microbiota, this microbiota has a limited core. We can infer that the association between this herbivore insect and their microbial community have is mutualism and that helps not only to withdraw the benefit maximum from your diet but also to overcome the adversities imposed by plants in response to herbivory.

The high dominance and low diversity of the microbiota, when related to bacteria, may be related to a selective pressure imposed by the host according to your diet, which will cause changes according to every nutritional need. Results obtained in shrimp clarify that different local individuals shared similar bacterial niches and that this is due to a selective pressure imposed by the host according to the nutritional need.[33]

Here for us to selective pressure imposed by diets is evident when we represent the data by NMDS and Venn diagram. By nMDS was created distinct groups by diets.

In bacteria, diets composed of resistant cultivars (IAC 17 and IAC 24) were grouped in the same quadrant, giving both the same influences, and other susceptible quadrant. For fungi, similar result was observed with clusters of resistant diets distinct side susceptible. This may be due to biochemical constitution of soybean varieties, since the resistant varieties containing higher levels of metabolites that interfere with the survival of the insects, which directly interferes with their microbial composition.

This analysis was reflected in Venn diagrams. In bacteria and fungi, different unique and specific bands were found in each individual diet, and a single shared bandwidth for bacteria and fungus. This clearly shows the selective pressure dictated by the diet.

The insects have the ability to modulate their microbiota in order to get the best out of your diet, since according to the literature this is only possible with the contribution of its microbiota in symbiosis with the host. So for effective control of herbivorous insects becomes necessary modulation of the microbial community that could even be acting in induced plant response.[14]

ACKNOWLEDGMENTS

This study was supported by grants by the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”, “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)”, “Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)”, Instituto Agronômico de Campinas (IAC), Dapartamento Fitotecnia, and “Instituto Nacional de Ciência e Tecnologia em Interações Planta−Praga (INCTIPP)”, Brazil.

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Table S1. Count and classification of all midgut-associated bacterial OTUs of caterpillars (Anticarsia gemmatalis) fed with four different varieties of soybean.

OTU	Size	Phylum	Class	Order	Family	Genus	Species
Otu01	289	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus casseliflavus
Otu02	20	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	unclassified
Otu03	17	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unclassified	unclassified
Otu04	11	Proteobacteria	Alphaproteobacteria	Rickettsiales	unclassified	unclassified	unclassified
Otu05	7	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	Lactobacillus plantarum
Otu06	6	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	unclassified
Otu07	4	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	unclassified
Otu08	4	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Magnetospirillum	Magnetospirillum magnetotacticum
Otu09	3	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus haemoperoxidus
Otu10	2	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	unclassified	unclassified
Otu11	2	Proteobacteria	Gammaproteobacteria	unclassified	unclassified	unclassified	unclassified
Otu12	2	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	Leuconostoc mesenteroides
Otu13	2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	unclassified	unclassified
Otu14	2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	unclassified	unclassified

Table S2. Count and classification of all midgut-associated fungal OTUs of caterpillars (Anticarsia gemmatalis) fed with four different varieties of soybean.

OTU	Size	Phylum	Class	Order	Family	Genus	Species
Otu01	49	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu02	5	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Caecomyces	Caecomyces communis
Otu03	4	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Cyllamyces	Cyllamyces aberensis
Otu04	2	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Unclassified	Unclassified
Otu05	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu06	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu07	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu08	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu09	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu10	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu11	1	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu12	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu13	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu14	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu15	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu16	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu17	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu18	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu19	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus

Otu20	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu21	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu22	1	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Unclassified	Unclassified
Otu23	1	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Unclassified	Unclassified
Otu24	1	Chytridiomycota	Neocallimastigomycetes	Neocallimastigales	Neocallimastigaceae	Caecomyces	Caecomyces communis
Otu25	1	Ascomycota	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu26	1	Ascomycota	Sordariomycetes	Unclassified	Unclassified	Unclassified	Unclassified
Otu27	1	Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	Paraphaeosphaeria	Unclassified
Otu28	1	Ascomycota	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu29	1	Ascomycota	Leotiomycetes	Leotiomycetes Order Incertae Sedis	Leotiomycetes Family Incertae Sedis	Leohumicola	Leohumicola minima
Otu30	1	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	Clonostachys divergens
Otu31	1	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu32	1	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu33	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu34	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu35	1	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Otu36	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu37	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu38	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu39	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu40	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu41	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified

Otu42	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu43	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu44	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu45	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu46	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu47	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu48	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu49	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu50	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu51	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu52	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu53	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu54	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu55	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu56	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu57	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu58	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu59	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified
Otu60	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu61	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Uncultured Fungus
Otu62	1	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified Fungi	Unclassified

Otu63	1	Zygomycota	Zygomycota Class Incertae Sedis	Mucorales	Umbelopsidaceae	Umbelopsis	Unclassified

CAPÍTULO II

Comparative metabolic responses of Anticarsia gemmatalis (Lepidoptera: Noctuidae) fed with different soybean genotypes.

ABSTRACT

Evolutionary, the insect provides mechanisms which allowed them adjust to different diets. The mechanisms to overcome the inhibitory developed by herbivorous insects already described. This study aimed to enzymatic characterization of digestive proteases Anticarsia gemmatalis under the influence of different cultivars resistant and susceptible soybean. No differences (P>0.05) were observed for proteolytic activity of enzymes isolated of midgut Anticarsia gemmatalis fed with different soybean cultivars (susceptible control, hydric stress susceptible and insect resistance (IAC 17 and IAC 24)). The predominance of a class of enzymes for amidolytic activity were observed, as it was not observed difference (P>0.05) in the Km values. The principal component analysis grouped the treatments in three different groups, despite the treatments were not decisive in the enzymatic profile. We can conclude that in our study digestive proteases larvae were efficient in the adaptive process, ensuring more a study of compounds entomotoxic produced by these cultivars and their involvement in the plant in response to insects.

Keywords: Plant−insect interaction, activity protease, proteases kinetics, enzymatic profile.

INTRODUCTION

The plants usually have a refined repertoire to fight defensively against phytophagous insects. However, the need to increase agricultural productivity compromises this potential defense against phytophagous insects in many cases. For favor high yield economically important species (eg. Soybean, corn, cotton), it becomes necessary to use alternative pest control, leaving the inefficient control based on chemical pesticides. [1,2] These trends have aroused interest in new insecticidal compounds, especially bioinsecticides and bio-rational insecticides. [3–5]

Proteins derived from pathway lipoxygenase metabolism, in particular plant protection products, such as inhibitors of digestive enzymes may be used in pest control because these proteins may change the intake of food, causing changes in the digestive profile insects. The protease inhibitors (PI) including antinutritional effects may decrease the growth and survival of the herbivore. The intake IP reduces degradation of the protein in the insect midgut and the availability of amino acids for growth, development and reproduction. [6,7]

Among the techniques that involve Integrated Pest Management, the resistance of plants is pointed as a tool of great value and can contribute to the reduction of populations of insects. [8,9] Plant proteins have been evaluated as potential biopesticides, but many, including proteinase inhibitors, have had minimal success as insect control proteins in transgenic plants [5,9].

However, as a "tug of war" evolutionary, the insect provides mechanisms which allowed its adjustment to different diets. The mechanisms to overcome the inhibitory developed by herbivorous insects already described, are summarized in three well-defined strategies: Increasing protease expression levels in the midgut, because if there is more protease supply than inhibitors, one balance positive effect is achieved in substrate utilization; inducing the expression of protease insensitive to protease inhibitors; and promote the symbiotic association with midgut bacteria. Recently two new strategies have been investigated. The association between proteases in the formation of multimeric complexes; induction of expression of specific proteases to protease inhibitors. [2,3,10,11]

The ability to detoxify or metabolize plant defense mechanisms, including IP within the intestine has become one of the essential mechanisms for the evolution of insects in the management of phytotoxins diversity in their diet. So all the proteolytic activity of insects is part of this adaptive system. [2]

However, whereas expression of genes is studied in detail as digestive enzyme activities can respond to diet composition, the protein level was not considered in depth. Especially in termite, Reticulitermes flavipes, studies show that the change in diet composition, especially in the lignocellulose content there was a change in the profile enzymatic responsible for the degradation of diet and this change was influenced by the change in microbiota.[12] More recent study in lepidopterans showed substantial changes in physiological expression levels in the midgut of proteins related to various functions such as detoxifying, carriers, immunity and especially digestive enzymes.[2]

The underlying molecular mechanisms that allow insects herbivores to circumvent both nutritional and diverse deficit chemical defense are still poorly understood, but the proteolytic activity of your digestive enzymes play an important role. Knowledge of the enzymatic profile of digestive proteases, serine and cysteine proteases are the main digestive enzymes of Lepidoptera, including Anticarsia gemmatalis, a major pest of soybean is vital to pest control. Our hypothesis is that the digestive enzyme activities of A. gemmatalis vary between diets as an adaptive mechanism which enables the caterpillar soybeans and their use microflora available resources. This study proposed to compare the effects on the profile serine and cysteine proteases A. gemmatalis through the diet change.

MATERIALS AND METHODS

Rearing and Damage by Insects

The A. gemmatalis eggs obtained from the Soybean National Research Center of the Brazilian Agricultural Research Corporation (EMBRAPA Soja, Londrina, PR, Brazil), and kept in the Laboratory of Insects (LI) of the Department of Biochemistry and Molecular Biology (DBB) of the Federal University of Viçosa (UFV) at 25 ± 2 °C temperature and 70 ± 10% relative humidity. The A. gemmatalis larvae were incubated at 25 °C with a relative humidity of 60 ± 10% and photoperiod of 14 h and fed witch natural diet soybean plants, at the in five instar larvae of A. gemmatalis. After 24 h, the caterpillars were collected, and their midguts were removed for analysis .[6] The larvae reared on the artificial diet described by Hoffman-Campo,[13] and maintained under controlled conditions of 25 ± 5ºC, 70 ± 10% r.h., and 14:10 h (L:D) photoperiod. Fourth and fifth-instar larvae were used in the experiments. All reagents were purchased from Sigma-Aldrich Química Brasil (São Paulo, SP, Brazil).

Soybean Plants

Seeds of the soybean varieties were obtained from the Agronomic Institute of Campinas (IAC), São Paulo, Brazil and the Germplasm Bank of the Department of Plant Science Federal University of Viçosa (UFV). Soybean cultivars [Glycine max (L.) Merrill], IAC-17 (insect resistance), and IAC-24 (insect resistance), UFV-16 (hydric stress susceptible = S) and UFV-16 (susceptible control = C), were grown without application of any products. Soybean plants were placed per pot containing 4.0 kg of soil in a greenhouse. The cultivate UFV-16 (stress susceptible) was subjected to a water stress on average -0.8 Mpa. Each variety of plants were used to feed one different groups of larvae until the fifth instar, for two generations.[14–16]

Enzyme Extract from the Larval Midgut

Velvetbean caterpillars were dissected, and their midguts were removed and placed in 10^{-3} mol.l^{-1} HCl at 4 °C in 1,5 mL plastic tubes. The enzyme extract was obtained by cell disruption during 9 cycles of freezing in liquid nitrogen and thawed in a water bath at 37 °C. Next, 1 mL fractions of the extracts were centrifuged in 1,5 mL plastic microtubes at 10000g for 45 min at 4 °C. The supernatant, containing soluble material, was removed and maintained at −80 °C for analysis.

Determination of the Protein Concentration

Using 0.2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) as the standard, we obtained the protein concentration of the enzyme extract of A. gemmatalis. [17]

Determination of Protease Activity in A. gemmatalis Midgut

Protease activities were determined using the substrate 2% (w/v) azocasein (Sigma-Aldrich, St. Louis, MO) in 0.1 mol.l^{-1} Tris-Hcl buffer at pH 8.0. The reaction mixture, with 50 µL of substrate and 60 µL of enzyme extract, was incubated for 30 min at 37 °C. Then, with the addition of 240 µL of trichloroacetic acid (TCA) at 10% (w/v), the reaction was stopped. Next, the samples were homogenized by vortexing, maintained on ice for 15 min, and centrifuged at 8000 x g for 5 min at 25°C to remove the precipitated protein. An aliquot of 240 µL of supernatant was poured into tubes containing 280 µL of 1 mol.l^{-1} NaOH. The protease activity was monitored in a spectrophotometer at 440 nm. The experiment was conducted with three replicates, in triplicates.

Determination of Amidolytic Activity in A. gemmatalis Midgut

The amidolytic activity was evaluated by employing the chromogenic substrate N-benzoyl- L-arginyl-ρ-nitroanilide (L-BapNA) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 0.5 mM at 25 °C in 0.1 mol.L^{-1} Tris-HCl buffer at pH 8.2 containing 20 mmol.L^{-1} $CaCl_2$. The reaction mixture consisted of 0.5 mL of substrate, 0.5 mL of buffer, and 100 µL of enzyme extract. The initial rates of trypsin-like serine proteases were determined by the formation of the product ρ-nitroanilide, by measuring the increase in absorbance at 410 nm versus time (2.5 min) and using the molar extinction coefficient of 8800 M^{-1} cm^{-1} for the calculations. The experiments were performed with three replicates, in triplicates.

Determination of Esterolytic Activity in A. gemmatalis Midgut

The esterolytic activity was determined with the substrate N-α-ρ-tosyl- L -arginine methyl ester (L-TAME) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 0.1 mM at 25 °C in 0.1 mol.L^{-1} Tris-HCl buffer at pH 8.2 containing 20 mmol.L^{-1} $CaCl_2$. The initial rates of trypsin-like serine proteases were determined by the absorbance at 247 nm versus time (2.5 min) and using the molar extinction coefficient of 540 M^{-1} cm^{-1} for the calculations. The experiment was conducted with three replicates, in triplicates.

Kinetic assays

The kinetic parameters Km and Vmax were determined at the pH and temperature conditions indicated above for each enzyme. The concentrations of L-BApNA ranged from 0.1 to 1.6 mM for amidolytic activity. The concentrations of L-TAME ranged from 0.01 to 1.0 mM for esterolytic activity. Kinetic parameters were estimated by non-linear regression (Michaelis–Menten equation) with the curve-fitting procedure of GraphPad Prism 7 (GraphPad Software 2016). The Km and Vmax estimate of each triplicate for both substrates and proteinase activities of each of the diets were subjected a multivariate analysis of variance and principal component analysis (PCA) to determine whether there were significant overall differences in kinetic parameters between treatments (maintaining total $P>0.05$) and group these treatments according to their similarity with respect to estimated parameters (PAST 3.12; 2016).

RESULTS

The adaptation of A. gemmatalis to different diets was evaluated by proteolytic caterpillars profile fed with different diets consisting of susceptible cultivars (C and S), insect resistant (IAC 17 and IAC 24) and also fed with artificial diet made in the laboratory in order to verify the adaptive differences in digestive proteolytic terms, one of its main mechanisms of survival imposed by different diets.

Total protease activity was detected with azocasein, amidolytic activities proteolytic and esterolytic were determined with L-BApNA and L-TAME respectively. The activity of total protease midgut of A. gemmatalis larvae fed with different diets (C, S, IAC 17, IAC 24 and Diet) it has significantly different for only the C and Diet treatments ($p < 0.05$). However, the proteolytic activity on larvae fed plants was 18% higher on average compared with larvae fed a diet (Figure 1).

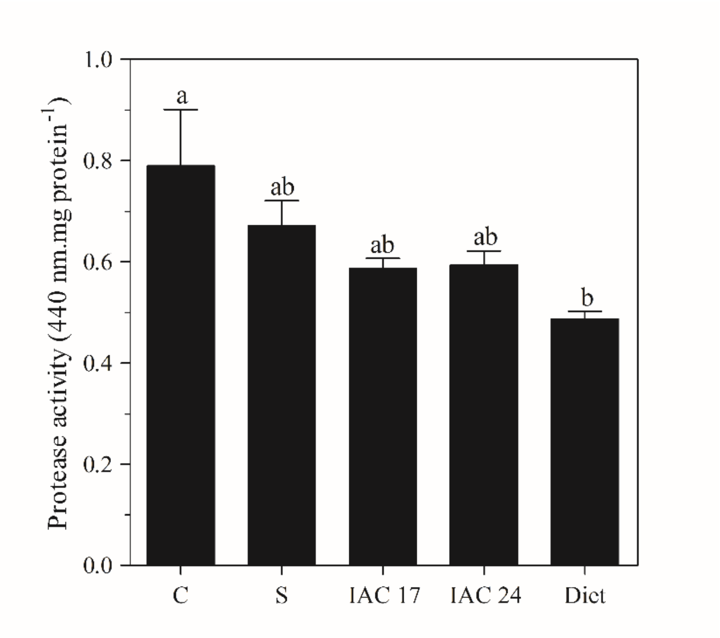

Figure 1. Protease activity in the midgut of A. gemmatalis (Lepidoptera: Noctuidae). The larvae were fed soybean leaves of four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance. The same letters show no difference at 5% probability by Tukey's test between cultivars (n = 45).

The amidolytic activity in the midgut of A. gemmatalis larvae fed with different cultivars (C, S, IAC 17 and IAC 24) were different but differentiated statistically ($P < 0.05$) for caterpillars fed Diet, which has a activity on average 50% higher (Figure 2). For esterolytic activity in the midgut of A. gemmatalis fed different cultivars were similar to the treatments C, IAC 17 and IAC 24, differentiating statistically caterpillars fed with stressed plants (S) and diet (Figure 3).

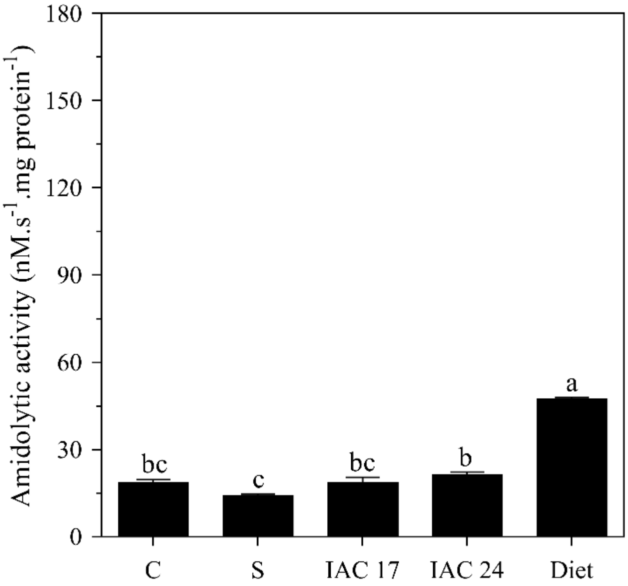

Figure 2. Amidolytic activity in the midgut of A. gemmatalis (Lepidoptera: Noctuidae). The larvae were fed soybean leaves of four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance. The same letters show no difference at 5% probability by Tukey’s test between cultivars (n = 45).

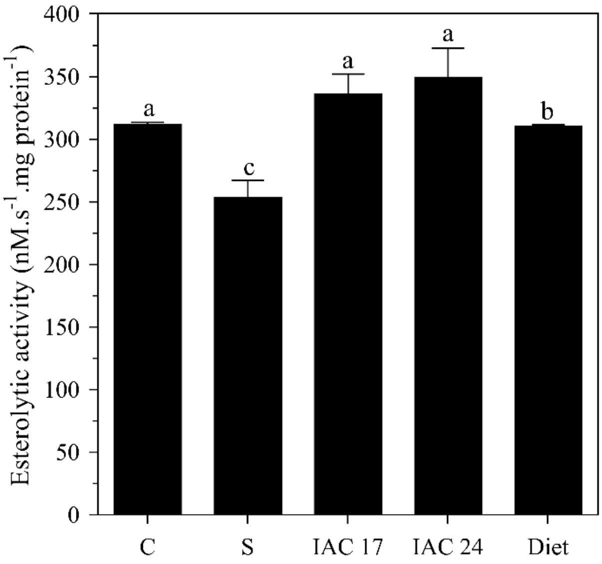

Figure 3. Esterolytic activity in the midgut of A. gemmatalis (Lepidoptera: Noctuidae). The larvae were fed soybean leaves of four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance. The same letters show no difference at 5% probability by Tukey's test between cultivars (n = 45).

The kinetic parameters of esterolytic and amidolytic activities of proteases from the intestine of the crude extract were estimated with L-BApNA and L-TAME (Table 1). L-BApNA concentrations ranged from 0.1 to 1.6 mM for amidolytic activity and L-TAME concentrations ranged from 0.01 to 1.0 mM for esterolytic activity. Kinetic parameters were estimated by non-linear regression (Michaelis–Menten equation) with the curve-fitting procedure of GraphPad Prism 7 (GraphPad Software 2016). The Km values for enzyme kinetics for amidolytic activity (L-BApNA) did not change statistically. For the kinetic parameter, Vmax, diet had greater value (Figures 4A-4E and Table 1). For enzyme kinetic esterolytic (L-TAME), the kinetic constant Km was different for Diet in relation to other treatments. In Vmax diet treatment was higher (Figure 5A-5E and Table 1).

Table 1. Kinetic parameters (Km and Vmax; ± SD) of midgut proteases activity of velvet bean caterpillar (Anticarsia gemmatalis).

Treatments	L-BApNA		L-TAME	
	K_m (mM)	V_{max} (nM.s^{-1})	K_m (µM)	V_{max} (µM.s^{-1})
Diet	0.4108	262.40^{a}	391.73^{a}	3.45^{a}
Control	0.4693	74.81^{bc}	99.63^{b}	0.77^{b}
Stress	0.4468	44.60^{c}	102,66^{b}	0.70^{b}
IAC 17	0.3596	76.45^{bc}	73.36^{b}	0.58^{c}
IAC 24	0.4002	82.43^{b}	105,63^{b}	0.80^{b}

Proteinase activity was determined with two substrates (L-BApNA and L-TAME) for each class of proteases. Means followed by the same letter for each kinetic parameter for each diet are not significantly different by Tukey's test ($p<0.05$).

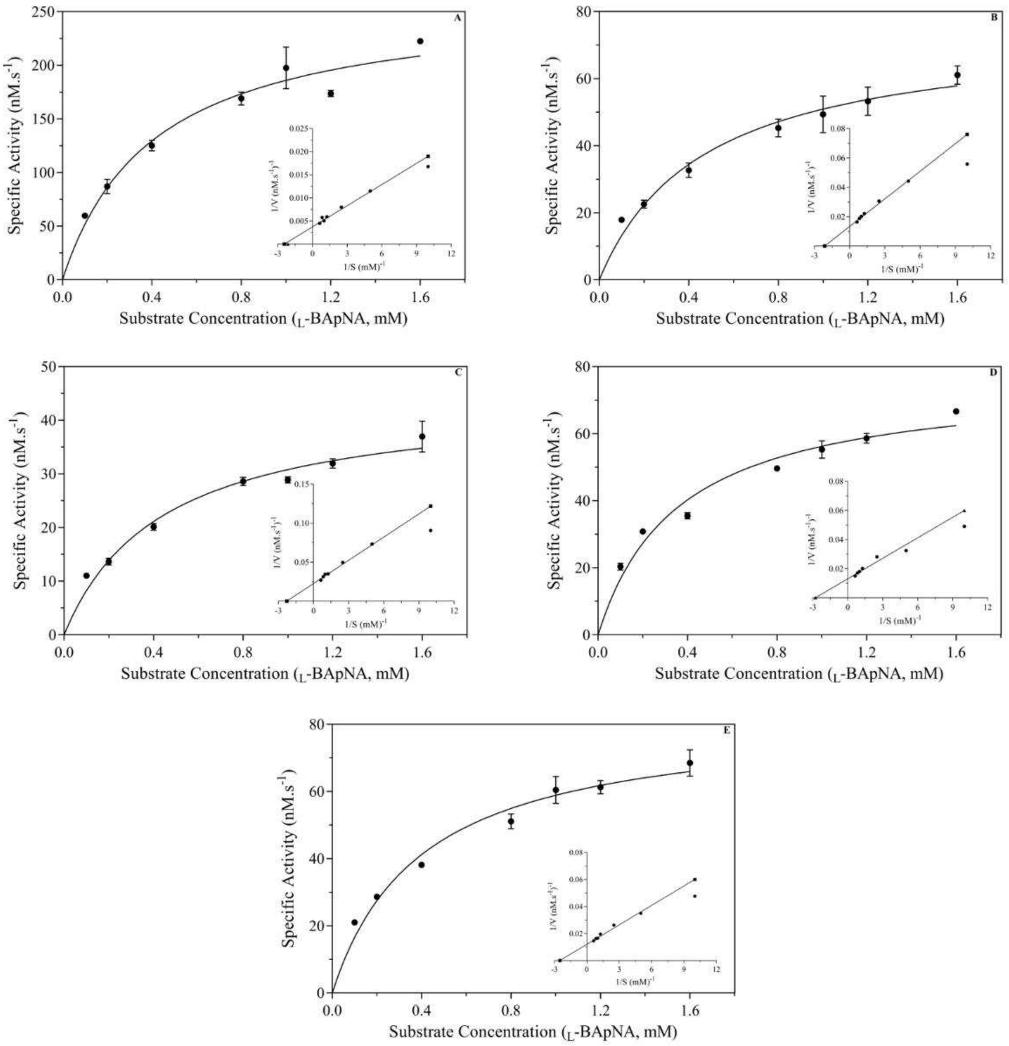

Figure 4. Representative Michaelis–Menten plots depicting the protease amidolytic activities from the velvet bean caterpillar *Anticarsia gemmatalis* from four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance. A= Diet, R^2= 0.94; B= susceptible control, R^2= 0.93; C= hydric stress susceptible, R^2- 0.95; D= IAC 17, R^2= 0.94 and E= IAC 24, R^2= 0.95. Insert: Lineweaver–Burk (double reciprocal). Each symbol represents the mean and standard error of three replicates.

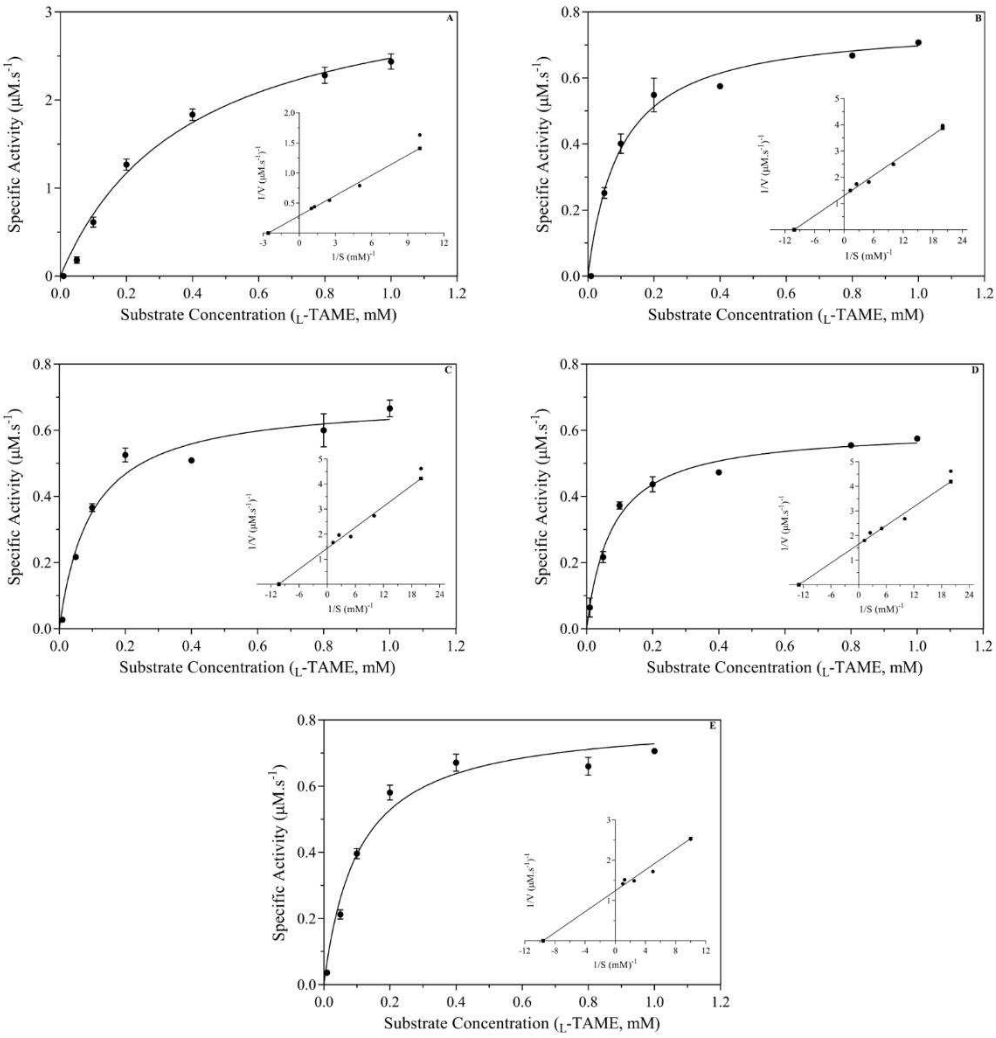

Figure 5. Representative Michaelis–Menten plots depicting the protease esterolytic activities from the velvet bean caterpillar Anticarsia gemmatalis from four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance. A= Diet, R^2= 0.97; B= susceptible control, R^2= 0.95; C= hydric stress susceptible, R^2- 0.94; D= IAC 17, R^2= 0.97 and E= IAC 24, R^2= 0.96. Insert: Lineweaver–Burk (double reciprocal). Each symbol represents the mean and standard error of three replicates.

The Km and Vmax estimate of each triplicate for both substrates and proteinase activities of each of the diets were subjected a multivariate analysis of variance and principal component analysis (PCA) to determine whether there were significant overall differences in kinetic parameters between treatments (maintaining total P> 0.05) and group these treatments according to their similarity with respect to estimated parameters (PAST 3.12; 2016). Principal component analysis was performed to represent the variables (Km, Vmax, proteases activity, esterolytic and amidolytic activity) in relation to the treatments used in this job. Principal component analysis explained 91.12% of accumulated variance in the first two principal components (Figure 6). The vectors indicate which parameters are influencing the treatments with greater intensity in each quadrant. The first component (PC1) explained 65.34% of the variance and the second component (PC2) explained 25.758% of data variance (Figure 6).

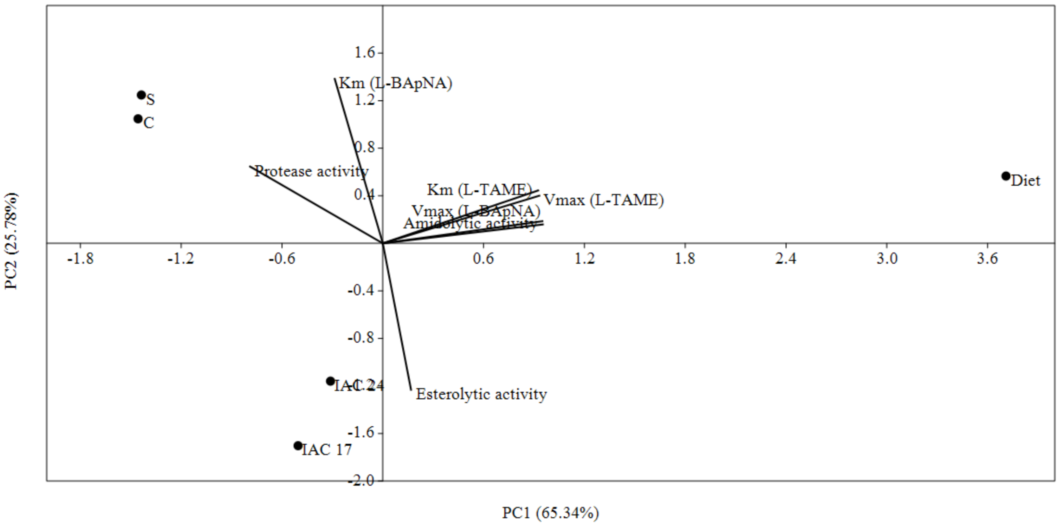

Figure 6. Analysis of principal components to evaluate the effect of different diets in the proteolytic activity of Anticarsia gemmatalis. Parameters assessed by principal component analysis were: Km, Vmax, amidolytic activity, esterolytic activity and protease activity. Treatments correspond to the larvae fed soybean leaves of four different varieties (C, S, IAC 17 and IAC 24) and Diet. C = susceptible control; S = hydric stress susceptible; IAC 17 = insect resistance; IAC 24 = insect resistance.

Vmax of both, Km (L-TAME) and amidolytic activity had a positive correlation with PC1. The susceptible diets (C and S), resistant (IAC 17 and IAC 24) and Diet artificial were grouped in different quadrants, showing his great influence.

DISCUSSION

As a defense mechanism, plants have the ability to modify their effectors expression after injury by abiotic and biotic factors. For the production of defense proteins against insects, large biomolecules related plants lesions are stimulated to alleviate the damage caused by attacks. [18] One of the main defense mechanisms is the increase of anti-nutritional factors that directly affect the digestibility of insects, inducing in turn, increased levels of digestive proteases by the insects. [9,19] High total protease activity levels of caterpillars fed with soybean cultivars, is due precisely to this.

The same does not happen when caterpillars are fed with artificial diet, which doesn't induce the accumulation of proteases. Similar results were obtained by Paixão et al., 2014, where with A. gemmatalis fed natural diet the proteolytic activity levels are high. When adding artificial protease inhibitors, these levels drop. [6]

Two synthetic substrates were used to recognize both serine- and cysteine-proteinase activities: L-BApNA and L-TAME. Both the molecules are substrates of these two enzyme classes. The A. gemmatalis protease activity for two L-BApNA and L-TAME substrates had conflicting results. This is due to the catalytic model of each substrate. As the L-BApNA has a lower affinity for the active site of the enzyme, specific protease inhibitors (PI) occupy the active site irreversibly at low levels of amidolytic activity, what did not happen in diet, possibly by the absence of protease inhibitors. Alternatively, L-TAME has greater affinity for the active site, promoting greater activity and competing intensely by the active center, esterolytic activity. [20]

The most significant difference occurred with larvae fed on plants under stress (S), still analyzing esterolytic activity, which leads us to think of two hypotheses. First, that plants under these conditions respond more efficiently to herbivores attack or, second, by not being at his best, the caterpillar ingested some of that food, only necessary for their survival, which does not demand higher levels of proteases. Further studies could clarify this issue.

Concentration–velocity curves with hyperbolic profiles that followed the Michaelis–Menten kinetic model were obtained (Figure 4A-5E). The kinetic parameters of gut protease activities vary with the type and class of proteases. [1,21] Using L-TAME as substrate, different classes of proteases may be acting in the digestive process which explains the variation in Km for artificial diet in relation to other diets. This change was accompanied by changes in Vmax (Figure 5A-5E and Table 1).

In the case of L-BApNA, it appears that only serine-proteases participate in the digestive process, since the Km is the same for all the treatments, varying only Vmax for artificial diet (Figure 4A-4E and Table 1). It is known that serine proteases are the largest group of digestive enzymes lepidopterans adaptation imposed by plants in their adaptive mechanism, such as protease inhibitors (PIs). [2,9,22,23]

The principal component analysis was performed with the aim of presenting an overview of all the variables considered and treatments that most influenced. Analysis explained 91.12% of the accumulated variance (Figure 6). The vectors indicate which parameters are influencing more intensity the treatment in each quadrant.

Different diets are the factors that most influence the discussed parameters (Km, Vmax, proteolytic activity), it made that were separated into four distinct quadrants. Caterpillars fed resistant plants (IAC 17 and IAC 24) did not suffer any influence of parameter settings, proving to be most effective in resistance to insects. [8] In caterpillars fed varieties considered susceptible (C and S), there was influence mainly of total proteases which leads us to infer the accumulation of different proteases for their effectiveness and Km amidolytic. Finally, caterpillars fed diet, which is pretty much influenced by all parameter setting, due to not challenging requirement to induce a significant number of digestive proteases.

The plants have the ability to develop defense mechanisms to reduce damage by herbivores. However, the resistance mechanisms IAC-17 and IAC-24 (resistant) do not differ from each other in effective terms, which affect the proteolytic activity of larvae Anticarsia gammatalis. As this is the main mechanism that bypasses the barriers imposed by plants, we can conclude that in our study digestive proteases larvae were efficient in the adaptive process, ensuring more a study of compounds entomotoxic produced by these cultivars and their involvement in the plant in response to insects. Digestive enzymes tested A. gemmatalis were not sensitized resistant varieties.

ACKNOWLEDGMENTS

This study was supported by grants by the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”, “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)”, “Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)”, Instituto Agronômico de Campinas (IAC), Departamento de Fitotecnia and “Instituto Nacional de Ciência e Tecnologia em Interações Planta−Praga (INCTIPP)”, Brazil.

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CONCLUSÕES GERAIS E CONSIDERAÇÕES FINAIS

- A alimentação de larvas de Anticarsia gemmatalis com diferentes dietas altera significativamente a sua microbiota;

- A microbiota associada a lagarta de maneira geral tem uma baixa diversidade e uma alta dominância para bactérias;

- A microbiota associada a lagarta de maneira geral tem uma alta diversidade e uma baixa dominância para fungos;

- O tipo de dieta é determinante na microbiota de Anticarsia gemmatalis;

- Dietas compostas por diferentes variedades de sojas, suscetíveis e resistentes não alterou o perfil de proteases totais digestivas de Anticarsia gemmatalis;

- Dietas compostas por diferentes variedades de soja não alterou o perfil de atividade de proteases do tipo serino-proteases, quando testado para os substratos L-BApNA e L-TAME;

- Diferentes dietas de soja alterou a constante cinética, Km, e não alterou o parâmetro cinético, Vmax;

- Apesar das dietas serem determinante na microbiota, as diferentes variedades de soja não foram suficientes para alterar o perfil enzimático de proteases digestivas.

Os resultados do presente trabalho sugerem que a microbiota associada ao inseto herbívoro Anticarsia gemmatalis é determinado pelo tipo de dieta que esse hospedeiro adota. No entanto essa microbiota não altera os perfis bioquímicos de resposta adaptativa de proteases digestivas.

Assim, o interesse em estudar os efeitos adaptativos a diferentes dietas está relacionado a microbiota associada ao hospedeiro. É importante que este trabalho de pesquisa tenha contribuído para melhor entendimento das características adaptativas de insetos, da ecologia e dinâmica dos microrganismos no intestino, visando melhores resultados frente as barreiras impostas pelas plantas e consequente entendimento a respeito do controle biológico de insetos.