EULÁLIO GUTEMBERG DIAS DOS SANTOS

TRIPEPTÍDEO INIBIDOR DE PROTEASES MODELADO A PARTIR DO INIBIDOR NATURAL DE SOJA SKTI ALTERA DIFERENCIALMENTE A EXPRESSÃO GÊNICA E A FISIOLÓGICA DO INTESTINO DE *Anticarsia gemmatalis*

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*.

Orientadora: Humberto Josué de O. Ramos

Coorientadores: Maria Goreti de Almeida Oliveira
Pedro Marcus Vidigal

VIÇOSA - MINAS GERAIS

2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

S237t
2023

Santos, Eulálio Gutemberg Dias dos, 1991-
Tripeptídeo inibidor de proteases modelado a partir do inibidor natural de soja skti altera diferencialmente a expressão gênica e a fisiológica do intestino de *Anticarsia gemmatalis* / Eulálio Gutemberg Dias dos Santos. – Viçosa, MG, 2023.
1 tese eletrônica (131 f.): il. (algumas color.).

Texto em português e inglês.
Inclui apêndice.
Orientador: Humberto Josué de Oliveira Ramos.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Bioquímica e Biologia Molecular, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.779
Modo de acesso: World Wide Web.

1. Inibidores de proteases. 2. *Anticarsia gemmatalis*. 3. Soja - Doenças e pragas - Controle. 4. Transcriptoma. I. Ramos, Humberto Josué de Oliveira, 1969-. II. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Programa de Pós-Graduação em Bioquímica Aplicada. III. Título.

CDD 22. ed. 572.76

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

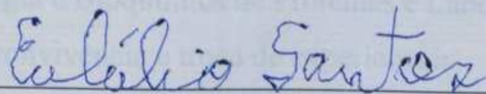
EULÁLIO GUTEMBERG DIAS DOS SANTOS

TRIPEPTÍDEO INIBIDOR DE PROTEASE MODELADO A PARTIR DO INIBIDOR NATURAL DE SOJA SKTI ALTERA DIFERENCIALMENTE A EXPRESSÃO GÊNICA E A FISIOLÓGICA DO INTESTINO DE *Anticarsia gemmatalis*

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*.

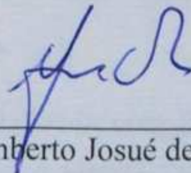
APROVADA: 19/10/2023

Assentimento:



Eulálio Gutemberg Dias dos Santos

Autor



Humberto Josué de Oliveira Ramos

Orientador

AGRADECIMENTOS

Eis que pouco mais de 7 anos atrás, tive a oportunidade de uma segunda chance de viver e que apesar das limitações me imposta daquele momento em diante, eu continuaria a batalhar para conquistar meus objetivos. Mas eu não conseguiria chegar até aqui se não fosse pelo apoio de muitas pessoas que nunca se limitaram a me dizer que não seria possível.

Agradeço imensamente à minha família, que não mediram esforços para tornar toda essa luta possível, em especial minha mãe, que deixou de se dedicar a própria vida para que eu pudesse me dedicar ao doutorado, me dando forças e inspiração. Ao meu pai que se manteve firme, sendo o alicerce e apoio para que não ousasse desistir. Ao Breno, meu irmão, que me deu apoio em todas as decisões que tomei, estando sempre ao meu lado. Tainá, minha namorada, que foi fundamental em toda essa jornada, me proporcionando amor, companheirismo e maturidade, me dizendo sempre que seria capaz. Agradeço aos inúmeros amigos e profissionais da saúde que estiveram presentes nesta caminhada, me dando todo apoio que precisei.

Meu eterno agradecimento à Universidade Federal de Viçosa que me transformou como cidadão e pessoa, que me acolheu e fez do seu campus a minha casa. Agradeço ao Departamento de Bioquímica e Biologia Molecular e ao Bioagro: professores e professoras, técnicos e técnicas de laboratório, e secretários e secretárias pela dedicação e suporte de sempre. Aos membros do laboratório de Enzimologia e Bioquímica de Proteínas e Laboratório de Biologia Molecular de plantas, pela prazerosa convivência e troca de experiencias.

Quero expressar minha sincera gratidão ao meu orientador, Humberto Ramos, coorientador, Pedro Vidigal, pelo apoio, orientação e mentoria ao longo de todo o processo. A dedicação de vocês, paciência e expertise foram fundamentais para minha formação acadêmica e para a qualidade desta tese. Ao Neilier, companheiro de laboratório que me proporcionou fundamental apoio neste trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de estudos. Agradeço à FAPEMIG pelo apoio financeiro. Nubiomol, BIOAGRO e UFV pela estrutura e suporte.

AGRADECIMENTOS

Eis que pouco mais de 7 anos atrás, tive a oportunidade de uma segunda chance de viver e que apesar das limitações me imposta daquele momento em diante, eu continuaria a batalhar para conquistar meus objetivos. Mas eu não conseguiria chegar até aqui se não fosse pelo apoio de muitas pessoas que nunca se limitaram a me dizer que não seria possível.

Agradeço imensamente à minha família, que não mediram esforços para tornar toda essa luta possível, em especial minha mãe, que deixou de se dedicar a própria vida para que eu pudesse me dedicar ao doutorado, me dando forças e inspiração. Ao meu pai que se manteve firme, sendo o alicerce e apoio para que não ousasse desistir. Ao Breno, meu irmão, que me deu apoio em todas as decisões que tomei, estando sempre ao meu lado. Tainá, minha namorada, que foi fundamental em toda essa jornada, me proporcionando amor, companheirismo e maturidade, me dizendo sempre que seria capaz. Agradeço aos inúmeros amigos e profissionais da saúde que estiveram presentes nesta caminhada, me dando todo apoio que precisei.

Meu eterno agradecimento à Universidade Federal de Viçosa que me transformou como cidadão e pessoa, que me acolheu e fez do seu campus a minha casa. Agradeço ao Departamento de Bioquímica e Biologia Molecular e ao Bioagro: professores e professoras, técnicos e técnicas de laboratório, e secretários e secretárias pela dedicação e suporte de sempre. Aos membros do laboratório de Enzimologia e Bioquímica de Proteínas e Laboratório de Biologia Molecular de plantas, pela prazerosa convivência e troca de experiencias.

Quero expressar minha sincera gratidão ao meu orientador, Humberto Ramos, coorientador, Pedro Vidigal, pelo apoio, orientação e mentoria ao longo de todo o processo. A dedicação de vocês, paciência e expertise foram fundamentais para minha formação acadêmica e para a qualidade desta tese. Ao Neilier, companheiro de laboratório que me proporcionou fundamental apoio neste trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de estudos. Agradeço à FAPEMIG pelo apoio financeiro. Nubiomol, BIOAGRO e UFV pela estrutura e suporte.

RESUMO

SANTOS, Eulálio Gutemberg Dias dos, D.Sc., Universidade Federal de Viçosa, outubro de 2023. **Tripeptídeo inibidor de proteases modelado a partir do inibidor natural de soja SKTI altera diferencialmente a expressão gênica e a fisiológica do intestino de *Anticarsia gemmatalis*.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Maria Goreti de Almeida Oliveira, Pedro Marcus Pereira Vidigal.

Anticarsia gemmatalis é uma das principais pragas de insetos que causam desfoliação nas lavouras de soja. Estratégias alternativas têm sido avaliadas para reduzir os ataques de insetos, utilizando inibidores de proteases (IP) que atuam como fatores antinutricionais. Os efeitos fisiológicos dos PIs não se limitam apenas aos danos nutricionais causados aos insetos. Eles desencadeiam respostas bioquímicas que podem variar desde a reorientação da síntese de proteínas para produzir novas proteases até o aumento da produção de enzimas envolvidas em vias de defesa. Um tripeptídeo chamado GORE-2 foi projetado a partir do IP SKTI de soja, com maiores capacidades de inibição de proteases, reduzindo a sobrevivência das lagartas. Portanto, os efeitos do IP natural de soja (SKTI) e do peptídeo projetado racionalmente GORE-2 foram avaliados no nível das mudanças na expressão de genes causadas pelos IPs e sua relação com o estresse oxidativo e as alterações morfológicas das células intestinais. Assim, neste trabalho, avaliamos a reprogramação da expressão gênica por RNA-Seq para verificar as cascatas envolvidas na resposta a esses diferentes IPs. SKTI e GORE-2 desencadearam uma extensa reprogramação da expressão de genes após 24 horas. A presença de IP na dieta de *A. gemmatalis* afetou 1474 genes diferencialmente expressos, dos quais 643 e 831 genes responderam a SKTI e GORE2, respectivamente. Em geral, as respostas foram semelhantes para ambos os PIs, com alterações na expressão dos genes que codificam proteases e proteínas de defesa, principalmente envolvidas na proteção peritrófica da matriz e reconstituição. No entanto, essas respostas foram mais pronunciadas para SKTI, indicando uma cascata de sinalização mais eficiente para acionar os mecanismos de defesa do inseto. Essas diferenças justificam uma ação mais eficaz do GORE-2 na inibição da proteólise e na redução da sobrevivência das lagartas, indicando que a sinalização pode ser devido à privação de aminoácidos, mas também por um sistema geral de percepção desenvolvido para detectar moléculas repelentes da soja. Visto que o GORE-2 é um tripeptídeo mimético do inibidor de proteases SKTI, a cascata de sinalização parece ser menos eficiente. Na verdade, a expressão gênica para processos de desintoxicação e estresse oxidativo foi maior para o GORE-2.

Portanto, é uma vantagem adicional do uso de inibidores de proteases miméticos ou sintéticos. Nesse sentido, o tripeptídeo GORE2 se apresenta como uma alternativa eficiente no controle de *A. gemmatalis.*

Palavras-chave: *Anticarsia gemmatalis*. Transcritoma. *Glycine max*. Manejo de pragas agrícolas.

ABSTRACT

SANTOS, Eulálio Gutemberg Dias dos, D.Sc., Federal University of Viçosa, October 2023. **Protease inhibitor tripeptide modeled from the natural soy inhibitor SKTI differentially alters gene expression and physiology in the intestine of *Anticarsia gemmatalis***. Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Maria Goreti de Almeida Oliveira, Pedro Marcus Pereira Vidigal.

Anticarsia gemmatalis is one of the main insect pests causing defoliation in soybean crops. Alternative strategies have been evaluated to reduce insect attacks, using protease inhibitors (PI) that act as antinutritional factors. The physiological effects of PIs are not limited to nutritional damage to insects; they trigger biochemical responses that can range from reorienting protein synthesis to produce new proteases to increasing the production of enzymes involved in defense pathways. A tripeptide called GORE-2 was designed from soybean SKTI PI, with higher protease inhibition capabilities, reducing caterpillar survival. Therefore, the effects of natural soybean PI (SKTI) and rationally designed peptide GORE-2 were evaluated at the level of gene expression changes caused by PIs and their relationship with oxidative stress and morphological changes in intestinal cells. In this work, we assessed the reprogramming of gene expression by RNA-Seq to examine the cascades involved in the response to these different PIs. SKTI and GORE-2 triggered extensive gene expression reprogramming after 24 hours. The presence of PI in the diet of *A. gemmatalis* affected 1474 differentially expressed genes, of which 643 and 831 genes responded to SKTI and GORE-2, respectively. Overall, the responses were similar for both PIs, with changes in the expression of genes encoding proteases and defense proteins, mainly involved in peritrophic matrix protection and reconstitution. However, these responses were more pronounced for SKTI, indicating a more efficient signaling cascade to trigger insect defense mechanisms. These differences justify a more effective action of GORE-2 in proteolysis inhibition and caterpillar survival reduction, indicating that signaling may be due to amino acid deprivation but also to a general system developed to detect soy-repelling molecules. Since GORE-2 is a tripeptide mimetic of the SKTI protease inhibitor, the signaling cascade seems to be less efficient. Indeed, gene expression for detoxification processes and oxidative stress was higher for GORE-2. Therefore, mimetic or synthetic protease inhibitors are an additional advantage. In this regard, the GORE-2 tripeptide emerges as an efficient alternative in controlling *A. gemmatalis.*

Keywords: *Anticarsia gemmatalis*. Transcriptome. *Glycine max*. pest management.

LISTA DE ILUSTRAÇÕES

REVISÃO DE LITERATURA

Figura 1 - Escala fenológica da soja. Disponível em: Caviness (1977) 21

Figura 2 - *Anticarsia gemmatalis* Hübner,1818 (Lepidoptera: Noctuidae): inseto adulto na forma de mariposa. Disponível em: Sosa-Gómez et al., (2014) 24

Figura 3 - Lagarta da soja *Anticarsia gemmatalis* pequena (a), grande (b) e escura (c). Disponível em Moscardi et al., (2012) 25

Figura 4 - *Anticarsia gemmatalis*, pupa clara (a) e pupa escura (b). Disponível em Mascardi et al., (2012) e Hoffmann-Campo et al., (2000) 26

Figura 5 - Estrutura comum de uma serino-proteases. Os três resíduos catalíticos Asp102 Ser195-His57 estão designados de cor azul. Tripsina PDB1FY8. Na tripsina (A) mostramos também representado em azul o Asp189 (mais a direita) que confere uma maior especificidade. Fonte: disponível em Ribeiro (2009) 30

Figura 6 - Representação de um modelo de inibição entre protease (cinza claro) e inibidor (cinza escuro), representando os aminoácidos catalíticos (S) que sofreram, hidrólise nos respectivos resíduos de aminoácido. Disponível em: Schechter e Berg, (1967) 30

Figura 7 - Representação da estrutura do intestino de um inseto mastigador. Na parte superior as três porções do intestino, anterior (*foregut*), média (*midgut*) e posterior (*hindgut*). Na parte central, compostos que situam e transitam pelo intestino médio e mais abaixo, a estrutura da

membrana peritrófica e as células do intestino. Disponível em Pilon, (2004) 33

Figura 8 - A) dispositivo de sequenciamento Oxford Nanopore MiniON. Poros proteicos acoplados a uma helicase incorporados a uma membrana. B) Sequenciamento SMTR, uso de polimerases guiadas em onda de modo zero, nucleotídeos marcados geram sinais característicos que são registrados. Disponível em: Searli (2023) 38

Figura 9 - Evolução histórica das tecnologias de sequenciamento para biologia molecular. Disponível em: Athanasopoulou, (2022) 38

CAPÍTULO I

Figura 1 - A) Similarity frequency distribution between different species, calculated from the EggNog database by the TrapID tool. B) Functional annotation distribution for similarity using different tools and databases from a total number of identified Unigenes 61

Figura 2 - Distribution of frequency counts identified in the total (de Novo) transcriptome assembly approach A) Major Gene families identified from the EggNog database using the TRAPID tool. B) The main annotations of proteins from the EggNog database by the TRAPID tool. C) The main enzymes annotated by the KAAS tool using the KEEG database as identification 62

Figura 3 - Annotated Unigenes description with the highest normalized average expression rate in transcripts per million (TPM), obtained by the Kallisto and DESeq2 toolset, observing the effect of both treatments with protease inhibitors (GORE-2 and SKTI) 64

Figura 4 - A) Venn diagram showing differentially expressed genes, comparison between Control x GORE-2 treatments, highlighting the up-regulated (GORE-2-UP) with dark green and downregulated (GORE-2-DW) genes with yellow color, and other contrast, Control x SKTI, highlighting the up-regulated (SKTI-UP) with blue and downregulated (SKTI-DW) with pink color. B) The bar graph on the side shows the gene count of the respective contrasts Control x GORE-2 with red and Control x SKTI with blue color subdivided into up-regulated and down-regulated 65

Figura 5 - The most frequent Representation and exclusive gene groups were classified according to GO (gene ontology). A) Main GO assigned to GORE-2-DW treatment, with green color bars representing 'exclusive groups in contrast to SKTI-DW. B) Main GO attributed to SKTI-DW treatment with green color bars represent exclusive groups in contrast to GORE-2-DW C) Main GO assigned to GORE-2-UP treatment, green color bars represent exclusive groups in contrast to SKTI-UP. D) Main GO attributed to the SKTI-UP treatment, with green color bars representing exclusive groups in contrast to GORE-2-UP 68

Figura 6 - Metabolic pathways enriched from differential expression. A) Contrast between Control x GORE-2 group. 4 groups are represented by genes associated with the same pathway functions. First pathway C1 (pink), second C2 (orange), third C3 (blue), fourth C4 (yellow), and a fifth group that did not have common functions. B) Contrast between Control x SKTI group have 4 groups are represented with genes associated with same pathway functions. First C1 (pink), second C2 (orange), third C3 (blue), and the fourth group that did not have common functions 69

CAPÍTULO II

Figura 1 - Distribution of Functional Annotation for Similarity Across Different Species, calculated from the EggNog Database using the TRAPID Tool 89

Figura 2 - The most frequent protein domains represented in the transcriptome sequence of *A. gemmatalis* identified by KEGG 89

Figura 3 - Venn diagram illustrating the differentially expressed genes (DEGs) among the analyzed treatments. The diagram represents the numbers of specific elements shared among 1, 2, 3, and 4 comparisons. The absolute values of up- and down-regulated genes for caterpillars fed with SKTI and GORE-2 are grouped in the column graph 90

Figura 4 - The number of genes encoding proteases with differential expression in the intestines of *Anticarsia gemmatalis* fed with SKTI and GORE-2 90

Figura 5 - Effect of *A. gemmatalis* exposure to protease inhibitors SKTI and GORE-2 on four antioxidants and detoxifying enzymes: (A) Catalase; (B) Peroxidase; (C) Glutathione S-transferase; (D) Superoxide dismutase. Different letters on the bars show a significant difference between treatments ($p < 0.05$) 91

Figura 6 - Light micrographs of the midgut of the Anticarsia gemmatalis caterpillar. [a] Control group epithelium showing the striated border (arrowhead) of digestive cells (DC) with homogeneous cytoplasm and nuclei (N) with decondensed chromatin, goblet cells (GC) with clear striated border, muscle layers (M) and lumen (L). [b] SKTI-fed caterpillar epithelium showing striated border (arrowhead) of digestive cells (DC) with cytoplasm showing vacuolization (black arrows) and goblet cells (GC) with disorganized striated border (white arrows). [c] Caterpillar epithelium fed on GORE-2 showing striated border (arrowhead) of digestive cells (DC) that present strongly vacuolated cytoplasm (black arrows), goblet cells (GC) with disorganized striated border (white arrows) 93

LISTA DE TABELAS

CAPÍTULO I

Tabela 1 - *A. gemmatalis* reference assembly statistics 59

Tabela 2 - Genes overexpression protection of A. gemmatalis peritrophic matrix related when fed with protease inhibitors (GORE-2 or SKTI) compared to the control 64

CAPÍTULO II

Tabela 1 - *A. gemmatalis* reference assembly statistics 88

LISTA DE ABREVIATURAS E SIGLAS

ANOVA – Analysis of Variance

APOX – Ascorbate Peroxidase

AsA – Ascorbatic acid

BGI – Beijing Genomics Institute

BLAST – Basic Local Alignment Search Tool

CAT – Catalase

cDNA – DNA complementar

CDNB – 1-chloro-2,4-dinitrobenzene

CONAB – Companhia Nacional de Abastecimento

DEG – Differential Expressed Genes

DNA – Ácido desoxirribonucleico

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária

FC – Fold Change

GO – Gene Ontology

GST – Glutathione S-transferase

HMM – Hidden Markov Models

IP – Inibidor de protease

KI – Constante de ionização

KO – KEEG Orthology

MIP – Manejo Integrado de Pragas

MP – Matriz Peritrófica

mRNA – RNA mensageiro

NGS – Next Sequence Generation

Nr – Non-redundant

Nt – Nucleotídeos

ORF – Open Read Frame

PCR – Polimerase Chain Reaction

PI – Protease inhibitor

POX – Peroxidase

RNA – Ácido ribonucleico

ROS – Reative Oxigene Species

RT-qPCR – Real-Time quantitative Polimerase Chain Reaction

SBBI – Soybean Bowman-Birk inhibitor

SKTI – Soybean Kunitz Trypsin inhibitor

SMRT – Single Molecule Real-Time

SMS – Single-Molecule Sequencing

SNP – Single Nucleotide Polymorphism

SOD – Superoxide desmutase

TGS – Third Generation Sequence

TPM – Transcripts per Million

UDE – Unigenes Differentially Expressed

SUMÁRIO

1. INTRODUÇÃO GERAL.....18

2. REVISÃO DE LITERATURA.....21

2.1 Cultura de soja.....21

2.2 Inseto-praga da soja *Anticarsia gemmatalis*.....23

2.3 Controle de *Anticarsia gemmatalis*.....26

2.4 Proteases.....27

2.5 Inibidores de proteases.....29

2.6 Estrutura da matriz peritrófica.....32

2.7 Evolução das tecnologias de sequenciamento no estudo da Biologia Molecular.....36

3. REFERÊNCIAS.....41

CAPÍTULO I: Differential gene expression reprograming in *Anticarsia gemmatalis* midgut triggered by a SKTI-derivative tripeptide protease inhibitor compared to natural SKTI protein.....53

ABSTRACT.....53

1. INTRODUCTION.....54

2. METHODOLOGY.....55

2.1. *Anticarsia gemmatalis* Soybean caterpillar.....55

2.2. Preparation of the artificial diet.....56

2.3. RNA extraction and transcriptome sequencing.....56

2.4. Quality analysis and assembly.....57

2.5. ORF prediction.....57

2.6. Differentially expressed transcript analysis.....58

2.7. Functional annotation.....58

3. RESULTS 59
3.1. Data analysis sequencing 59
3.2. Reference transcriptome assembly 59
3.3. Functional annotation 60
3.4. GORE-2 and SKTI inhibitors alter main protease expression 63
3.5. Differentially expressed transcripts were distinct for PIs SKTI and GORE-2 64
4. DISCUSSION 70
5. CONCLUSION 73
6. REFERENCES 75
CAPÍTULO II: Protease inhibitor tripeptide designed from natural SKTI differentially promoted protease expression, oxidative stress response and histological alterations in the midgut of *Anticarsia gemmatalis* 81
ABSTRACT 81
1. INTRODUCTION 82
2. MATERIAL AND METHODS 83
2.1. Insects and preparation of the artificial diet containing PIs 83
2.2. Protein extraction from the caterpillar intestines 84
2.3. RNA Extraction and Sequencing 84
2.4. Quality Analysis and Assembly 84
2.5. Prediction of ORFs 85
2.6. Analysis of Differentially Expressed Transcripts 86
2.7. Functional annotation 86
2.8. Antioxidant and detoxifying enzyme assay 87
2.9. Histological analyzes of the caterpillar midgut 87
2.10. Statistical analysis 88

3. RESULTS 88

3.1. Data analysis sequencing 88

3.2. Reference transcriptome assembly 88

3.3. Functional Annotation 89

3.4. Analysis of Differentially Expressed Genes (DEGs) 90

3.5. Antioxidant Enzyme Activity 92

3.6. Histology 93

4. DISCUSSION 94

5. CONCLUSION 101

6. REFERENCES 102

CONSIDERAÇÕES FINAIS 130

1. INTRODUÇÃO GERAL

Os inibidores de protease (IP) são um grupo de moléculas amplamente distribuídos em toda natureza, que tem como principal ação, inibir a atividade de enzimas proteolíticas em alvos específicos (HABIB e FAZILI, 2007; GUO et al., 2020). Esses inibidores atuam bloqueando o acesso de substratos de interesse ao centro ativo da enzima, formando um complexo estável enzima-substrato que impede sua clivagem (MACEDO et al., 2007; KESSELER e BALDWIN, 2002). Esse processo é extremamente útil, pois regula a atividade de diversas proteases em uma infinidade de processos biológicos (QI et al., 2005). Os IP possuem quatro classificações quanto aos seus resíduos de aminoácidos envolvidos nos centros ativos de clivagem, são eles os inibidores de serino proteases, aspartatil protease, cisteíno proteases e de metaloproteases (KOIWA et al., 1997; ARAÚJO, 2019).

Nos vegetais, os inibidores de protease são moléculas proteicas produzidas de forma constitutiva e induzida, foram descritos primeiramente em plantas leguminosas, quando em 1947 experimentos pioneiros usando farinha de soja (*Glycine max*) evidenciaram a presença de IP em seus tecidos de reserva (DE LEO et al., 2002; MOURA, 2019). Experimentos posteriores identificaram o IP em outras cultivares como amendoim (*Arachis hypogaea*) e feijão comum (*Phaseolus vulgaris*), entre outras (SWATHI et al., 2021). Estes IP são produzidos de forma constitutiva por alguns tecidos de armazenamento nas sementes, uma vez que atuam na regulação de proteases endógenas, presentes em considerável proporção, estando entre 5 e 15% do teor de proteínas totais durante o período de dormência das sementes, além disso podem ser induzidos em tecidos vegetativos, principalmente as folhas, mediante a injúria causada por pragas (SULTANA et al., 2022).

Os ataques de pragas de insetos promovem cerca de 20% de toda perda agrícola no contexto mundial e no Brasil causam em média uma redução próxima a 10% nas principais culturas do país (OLIVEIRA et al., 2014). Este fenômeno tem se tornado cada vez mais frequente em função das mudanças climáticas, com o aumento da temperatura e concentração de CO2, também se elevando a densidade de infestação de insetos em direção às culturas agrícolas (TONNANG et al., 2022). Este prejuízo impacta na segurança alimentar, visto que o aumento da demanda pela produção de alimentos vem crescendo constantemente (A. JOGSMA et al., 2011).

Em geral, o controle de insetos e pragas nas lavouras é realizado em sua grande maioria, por meio de inseticidas sintéticos químicos, o que tem contribuído para o aumento da resistência

de algumas espécies (AGOSTINI et al., 2013). Em face disto, outras alternativas tem emergido no combate de pragas agrícolas, implicando em tecnologias que tragam uma menor contaminação de solos e rios, além de reduzir a agressão a saúde humana, um mercado que vem crescendo mais de 15% ao ano (CAMPOS et al., 2019; FREIRES, 2022). Duas principais linhas de pesquisas envolvendo inseticidas biológicos tem se mostrado promissor, uma é o uso de plantas geneticamente modificadas, expressando proteínas de *Bacillus thuringiensis* (BERLINER, 1911) que são danosas aos insetos, culminando em morte após ingestão (PEZENTI et al., 2021). Esse produto por sinal, já possui registro de uso comercial pela empresa Monsanto (St Louis, MO) desde 2011. No Brasil, essa tecnologia tem resultado em bons níveis de controle de pragas (BERNARDI et al., 2011).

A outra vertente explorada são o uso de IPs como agente de controle. Os IPs agem como um fator antinutricional, impedindo a ação das proteases intestinais dos insetos (SILVA-JUNIOR et al., 2021). Seu mecanismo de ação se dá pelo modelo padrão de Laskowski. Este modelo descreve o inibidor como um pseudo-substrato, formando um complexo que elimina a atividade proteolítica, a interação enzima-inibidor forma um intermediário, com uma alta constante de associação (LASKOWSKI JR e QASIM, 2000). Pelo fato do complexo recém formados ser bioenergeticamente estável, a ligação com o centro ativo, em que a arginina 17 da SKTI interage com sítio S1 da enzima de forma mais estável por meio de interações hidrofóbicas, ligações de hidrogênio e outras ligações fracas (LASKOWSKI e KATO 1980, NAPOLEÃO, 2019). A ação direta dos IPs tem como primeiro desfecho um prejuízo na digestão e absorção das proteínas no intestino médio dos insetos, afetando o crescimento e o desenvolvimento larval ocasionado pela carência de aminoácidos essenciais à dieta dos insetos (WAR et al., 2018; AKBAR et al., 2018). Experimentos anteriores já comprovavam resultados similares na presença de IP, quanto ao comprometimento no desenvolvimento de insetos, mesmo com a adição de aminoácidos essenciais livre a dieta (WESTFALL et al., 1948).

Apesar de diversas pesquisas mostrarem a potencial eficácia dos IPs contra diversas classes de insetos, estes inibidores são desafiados pelas resistências dos insetos adquirida após determinado tempo de uso, podendo comprometer os programas de manejo de pragas, pois se adaptam a ingestão dos IP (COURA et al., 2022). Por outro lado, inibidores de proteases sintéticos tem sido uma alternativa para o uso mais efetivo de IPs para o controle de insetos pragas mastigadoras (SILVA-JÚNIOR et al., 2021; BARROS et al., 2022). Peptídeos miméticos de tamanho reduzido tem sido desenhado racionalmente por modelagem molecular a partir de inibidores de protease naturalmente produzidos pelas folhas da soja (SKTI - *Soybean*

Kunitz trypsin inhibitor), visando uma maior capacidade de inibição proteolítica, bem como uma redução do custo de produção biotecnológica. Dois tripeptídeos denominados GORE-1 e GORE-2 tem sido avaliado como promissores para a aplicação no controle de insetos mastigadores. Quando adicionados nas dietas das lagartas, promovem maiores reduções na atividade de proteases e na sobrevivência de *A. gemmatalis*, comparado ao PI SKTI. Em especial, o inibidor GORE-2 foi selecionado para conduzir o experimento, uma vez que este possui uma arginina em sua estrutura, que mimetiza a interação entre as enzimas trípiticas do inseto e SKTI, o qual é responsável por 80% do conteúdo de inibição em insetos (BARROS et al., 2022).

As respostas fisiológicas obtidas nos intestinos das lagartas alimentadas com os IPs GORE-1, GORE-2 e SKTI são distintas. Portanto, este trabalho objetivou verificar diferenças nas cascatas de regulação da expressão gênica e de defesa molecular em intestinos de *A. gemmatalis* alimentadas com os PIs GORE-2 e SKTI, uma vez que ambos SKTI e GORE-2 possuem o aminoácido Arginina se ligando ao sítio S_1 do centro ativo da tripsina (SCHECHTER & BERGER, 1967; BARROS et al., 2022). Perfis de expressão gênica, gerados por análises transcritômicas por RNASeq, foram comparados com alterações fisiológicas e histológicas e os seus impactos na redução da taxa de sobrevivência, observadas para os inibidores GORE-2 e SKTI.

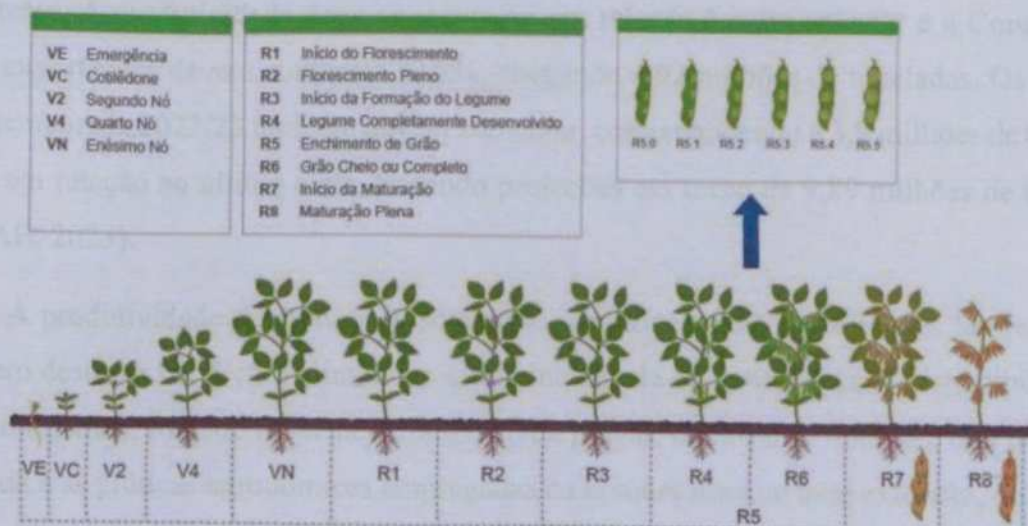
2. REVISÃO DE LITERATURA

2.1 Cultura de soja

A soja, *Glycine max* (L.) Merrill é uma das culturas mais importantes do mundo devido as suas propriedades nutricionais tanto humana quanto animal e seu potencial biotecnológico, agregando um grande valor econômico (AINSWORTH et al., 2012; KOFSKY et al., 2018). A soja é uma planta herbácea, da classe Rosideae, ordem Fabales, família Fabaceae, subfamília Papilionoideae, tribo Phaseoleae, gênero Glycine L., espécie max., classificada como uma leguminosa. Possui características que são diretamente associadas às condições ambientais, como altura, variando entre 30 e 200 cm e ciclo de vida entre 75 e 200 dias (MULLER et al., 1981).

As principais variedades da cultivar de interesse comercial apresentam caule híspido, pouco ramificado e raiz com eixo principal e muitas ramificações. Possuem folhas inicialmente unifoliar e após certo desenvolvimento, surgem as trifolioladas, que permanecem até a senescência (SEDIYAMA et al., 1985). Têm flores de fecundação autógama, de cor branca, púrpura ou intermediária. As sementes desenvolvem-se em vagens, as quais são levemente arqueadas, e à medida que amadurecem, transitam da cor verde para amareladas, marrom ou cinza, e que podem conter de uma a cinco sementes de característica lisa, elípticas ou globosas, de tegumento amarelo pálido, com hilo preto, marrom ou amarelo (NEUMAIER et al., 2000). A figura 1 representa o desenvolvimento de uma cultivar de soja.

Figura 1: Escala fenológica da soja. Disponível em: Caviness (1977).



A soja é amplamente utilizada como fonte de proteína vegetal para a alimentação humana e animal, uma vez que 34-40% de sua massa é composta por proteína (PEREIRA et al., 2021; FAO, 2020). Além disso, alguns de seus subprodutos, como o farelo de soja são usados extensivamente na agroindústria para a produção de rações, o seu conteúdo lipídico tem emprego comercial para produção de óleos e gordura hidrogenada, somado a isso, outros derivados são utilizados na produção de tintas, solventes, biocombustível e lubrificantes. Nos últimos anos tem ganhado destaque a produção de biodiesel a partir subprodutos descartados pela indústria (PIPOLO et al., 2015).

Historicamente, a domesticação da soja ocorreu a aproximadamente 5 mil anos atrás, na China e desde então, foi se tornando um alimento cada vez mais comum na alimentação humana e animal. No Brasil, sua introdução foi realizada em 1882, no estado da Bahia (BLACK et al., 2000), porém só em 1914 houve uma melhor adaptação ao clima e fotoperíodo do Rio Grande do Sul. Dessa data em diante, se iniciou a expansão agrícola para o Centro-Oeste, e a partir da década de 1970, adquiriu maior relevância de produção dentro do Brasil, passando de 2 para 20% da produção nacional em menos de 10 anos, figurando-se entre os maiores produtores mundiais (FREITAS, 2011).

A CONAB – Companhia Nacional de Abastecimento (2023) prevê um cenário recorde para a produção de soja no Brasil para safra 2022/23, mantendo-se como destaque mundial na produção deste grão, com uma projeção superior a 150 milhões de toneladas, com uma elevação 21% em comparação com a safra 2021/2022, superando os Estados Unidos em mais de 30 milhões de toneladas. A área destinada à cultura deve crescer 3,54%, chegando a 42,4 milhões de hectares. A produtividade deve se recuperar em relação à safra anterior e a Conab estima que as exportações devem aumentar 22,2%, chegando a 92 milhões de toneladas. Os estoques para a temporada 2022/23 também devem aumentar, correspondendo a 3,9 milhões de toneladas a mais em relação ao último ciclo, traçando projeções em torno de 9,89 milhões de toneladas (CONAB, 2023).

A produtividade da cultura de soja pode ser influenciada por diversos fatores, que se estendem desde as condições climáticas, como incidência de luminosidade, disponibilidade de água e nutrientes, somado ao manejo integrado de pragas, controle de doenças, tipo de cultivar escolhida e as práticas agronômicas empregadas na lavoura durante toda extensão, do cultivo à colheita (SANTOS et al., 2017; TEJO et al., 2019). As condições climáticas é um dos fatores

determinantes para uma boa produtividade, pois a mesma é frequentemente limitada por condições adversas, como a escassez hídrica, alagamento, alta concentração salina, entre outras de caráter abiótico (GAVA et al., 2015).

Outra vertente limitante à produtividade de soja são fatores de estresse bióticos, induzido por ataque de patógenos, representado por doenças de origem fúngica, bactérias, nematoides, vírus e insetos (SHANKER et al., 2016). Mais de 40 doenças já foram observadas e identificadas no Brasil e esse número continua aumentando, em função da expansão territorial da soja, iniciada com a revolução verde nas décadas de 1960 e 1970 para novas áreas, se mantendo até os dias atuais (EMBRAPA, 2021), combinada ao mau uso de agroquímicos, o que favorece a resistência de diversas pragas (BEL et al, 2017).

2.2 Inseto-praga da soja *Anticarsia gemmatalis*

A cultura de soja é constantemente alvo de pragas que reduzem sua produtividade e ocasiona a perda da qualidade dos grãos, que ocorre desde a fase vegetativa até estágios da planta desenvolvida, com danos locais a folha podendo se estender ao pecíolo e hastes (MACHADO et al., 2022). Os dois grupos de insetos que mais causam prejuízos à soja, são os percevejos sugadores pertencentes a família Pentatomidae e o outro grupo, corresponde as lagartas desfolhadoras pertencente a ordem Lepdoptera, cujas principais incluem *Helicoverpa armigera* (Hubner, 1805), *Anticarsia gemmatalis* (HUBNER, 1818) e *Chrysodeixis includens* (PANIZZI et al., 2012; MOSCARDI et al., 2012; SPECHT et al., 2013).

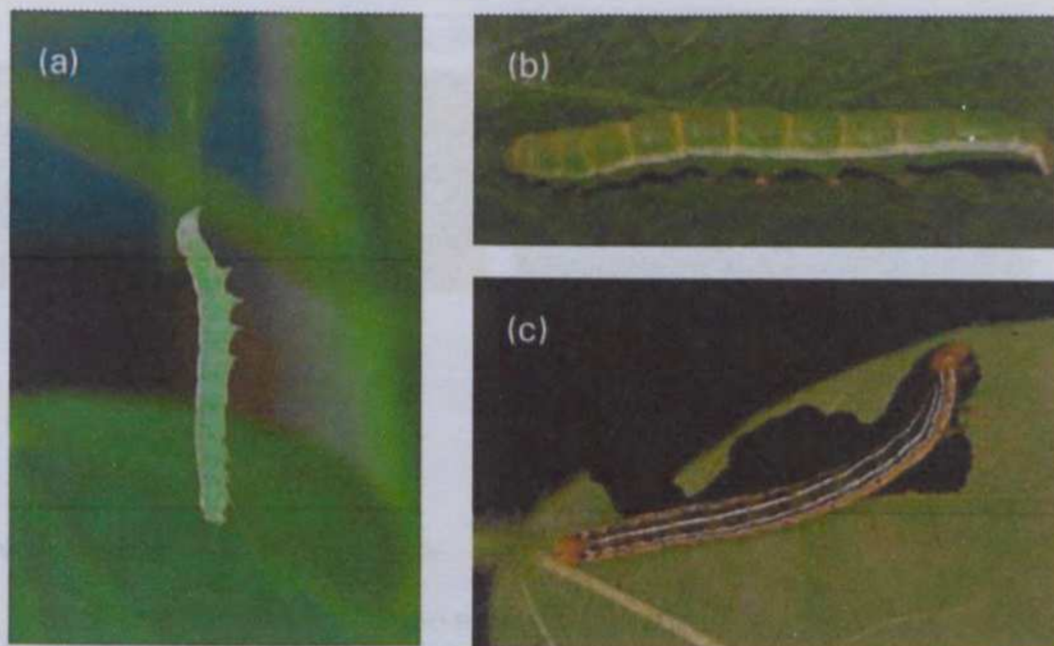
A *Anticarsia gemmatalis* também conhecida como lagarta-da-soja é considerada uma das principais pragas que ocasiona o desfolhamento da cultura de soja, caracterizada pela alta densidade populacional, são frequentemente encontradas em todas as localidades onde há cultivo de soja, com um potencial de desfolhamento podendo chegar a próximos dos 100% (HOFFMANN-CAMPO et al., 2000).

Os insetos adultos são mariposas, que possuem aproximadamente 5 centímetros de envergadura e se apresentam tipicamente nas colorações bege, marrom ou cinza, geralmente tem uma lista transversal escura ao longo das asas e na face interna possuem círculos brancos nas proximidades externas (Figura 2) (FUGI, 2003).

Figura 2: *Anticarsia gemmatalis* Hübner,1818 (Lepidoptera: Noctuidae): inseto adulto na forma de mariposa. Disponível em: Sosa-Gómez et al., (2014).

Todo processo reprodutivo de *A. gemmatalis* ocorre no fim da tarde ou período noturno, onde as mariposas gêmeas depositam seus ovos, preferencialmente na parte interna da folha e em menor frequência nas demais estruturas. esses ovos possuem estrutura arredondada, com tamanho variando de 1 a 2 mm e coloração branca, alterando para cor avermelhada próximo do período de eclosão (BARBARA, 2000). Os ovos depositados eclodem em três ou quatro dias, dando origem a lagartas que medem de 3 a 9 mm nos primeiros estágios de desenvolvimento, chegando a 1,5 cm nos instares superiores, assumindo coloração verde ou escuras com linhas longitudinais características no dorso (3 linhas) e na região abdominal (4 pares). Sua locomoção é do tipo mede palmo e fase larval de 12-15 dias, podendo chegar até 25 dias (Figura 3) (MOSCARDI et al., 2012; LIMA, 2023).

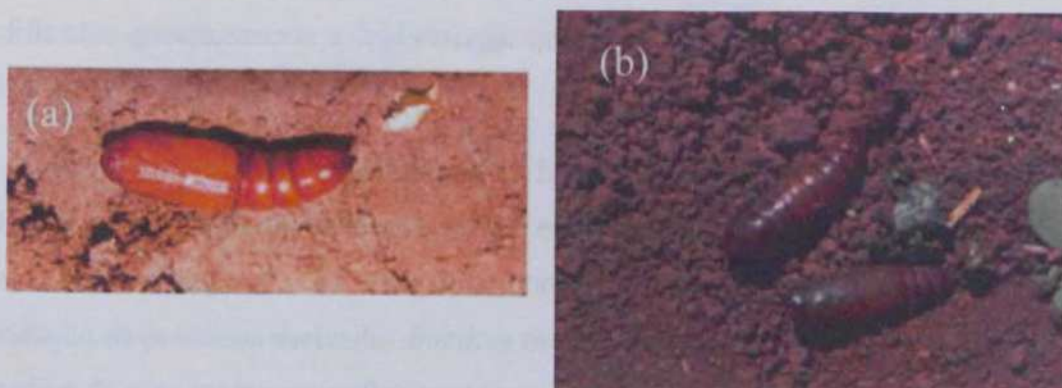
Figura 3: Lagarta da soja *Anticarsia gemmatalis* pequena (a), grande (b) e escura (c). Disponível em Moscardi et al., (2012).



Após 6 instares de evolução, as lagartas apresentam sua maior voracidade quanto à sua capacidade de desfolhamento, uma vez que nos estágios iniciais, tem um poder de herbivoria pouco maior que 30%, caso não seja feito o manejo correto. Os instares cinco e seis, corresponde ao estágio evolutivo com maior capacidade de injúria, uma vez que além das folhas, também devoram o pecíolo e haste da planta (HOFFMANN-CAMPO, 2000; MOSCARDI et al., 2012).

Cerca de dois dias antes da transformação em pupa ocorre a redução do tamanho da lagarta que se prepara para a próxima etapa do ciclo evolutivo. As pupas inicialmente tem cor verde-claro e posteriormente assumem a tonalidade marrom e se localizam no solo em sua maioria (Figura 4). Esta fase tem durabilidade de aproximadamente 7 dias, emergindo um novo adulto mariposa, que vão dar início a um novo ciclo de deposição de ovos, nas quais sua quantidade pode variar de 400 a 1200 a depender das condições climáticas e humidade do ambiente (SOSA-GOMEZ et al., 2014; SILVA-JUNIOR, 2022).

Figura 4: *Anticarsia gemmatalis*, pupa clara (a) e pupa escura (b). Disponível em Mascardi et al., (2012) e Hoffmann-Campo et al., (2000).



2.3 Controle de *Anticarsia gemmatalis*

Dentre os produtos existentes no mercado, os inseticidas químicos são os mais usados no manejo da *Anticarsia gemmatalis*, como os piretróides, organofosforados e carbamatos utilizados, já bastante conhecidos e utilizados a décadas (TOMQUELSKI et al., 2015). Outros mais recentes, tem ganhado destaque, como as benzoiluréias e diamida do ácido ftálico (MARTINS et al., 2015). No entanto, estes produtos muitas vezes estão associados a um risco potencial à saúde humana e ao ecossistema, elevando a contaminação do solo e rios (SOSA-GOMEZ et al., 2012).

Neste sentido, o uso intensivo de pesticidas e inseticidas químicos tem contribuído para o aumento da resistência de diversas espécies, pois tem sido constatado a seleção de genótipos de diversos insetos resistentes a algum grupo de inseticidas, já que os pequenos ciclos de vida dos insetos é um fator determinante para selecionar os fenótipos mais resistentes e provocar mutações genéticas espontânea (SOSA-GOMÉZ, 2012). Além disso, tem-se observado um notório desequilíbrio na cadeia alimentar, por reduzir a quantidade de espécies que são predadores naturais de diversas pragas, como a lagartas, percevejos e outras insetos que atacam a cultura de soja (WALSH et al., 2022).

Desde a década de 1960, o Brasil começou a identificar um potencial risco no uso excessivo de inseticidas químicos. Deste momento em diante, começaram a surgir novos estudos de estratégias voltadas ao controle de pragas (GUEDES et al., 2012). Estas novas ações compuseram o que passou a ser chamado de Manejo Integrado de Pragas (MIP), que tinham como premissas parâmetros econômicos, ambientais e sociais, de forma que a interferência ao ecossistema fosse minimizada, compondo um conjunto de estratégias de origem cultural, como

a rotação de cultura e aeração do solo; mecânico, uso de barreiras e armadilhas; físicos, como calor, frio, umidade e luminosidade; genéticos, como o desenvolvimento de cultivares modificadas geneticamente e legislativos, que são quarentenários e erradicação (WAQUIL, 2002).

Além das estratégias descritas, o MIP passou a associar ferramentas de origem biológica, pois ao monitor as pragas seriam então inseridas na cultura predadores naturais dos insetos. Além disso, outras tecnologias de controle biológico passaram a ser pesquisadas como introdução de proteínas derivadas *Bacillus thuringiensis var. kurstaki* (Berliner), uma bactéria portadora de uma toxina específica para controle de lepidópteros (BUENO et al., 2012; SOSA-GOMEZ et al., 2012).

Outra alternativa biológica é o uso de baculovirus, que é um vírus com DNA que infectam principalmente artrópodes e que não causa danos em vertebrados. Ao ser digerido pela lagarta, o produto viral atua exclusivamente no intestino do inseto, causando infecção das células, inserindo seu material genético, reproduzindo e lisando-as posteriormente, liberando novos vírus que infectam outras células, de forma a comprometer a hemolinfa e o sistema traqueal do inseto, levando-o a morte (ANDRADE et al., 2004). Uma terceira linha de bioinseticidas são os inibidores de protease, que age como um fator anti-metabólico por reduzir a biodisponibilidade de aminoácidos, afetando diretamente a síntese proteica limitando o desenvolvimento da mesma (MERIÑO-CABRERA et al., 2019; MENDONÇA et al., 2020).

2.4 Proteases

Proteases são enzimas que possuem a capacidade de catalisar a hidrólise de proteínas ou peptídeos em subunidades de peptídeos menores ou aminoácidos, possibilitam assim a sua absorção. Estas enzimas atuam em sítios específicos ocasionando a hidrólise da ligação, devido a uma redução da energia de ativação (LÓPEZ-OTÍN, 2006). Dessa forma, as proteases estão envolvidas numa série de processos regulatórios e estrutural, como o processamento, localização e destino de outras proteínas, ativação de proteínas bioativas, transdução e regulação de cascatas de sinalização, reciclagem de proteínas (*turnover*) e inativação das mesmas estão entre as principais atividade dessas enzimas (TURK, 2006).

Outras funções como interferência nos processos de replicação e transcrição do DNA, proliferação celular e diferenciação, morfogênese e remodelação tecidual, choque térmico e

respostas de proteínas desdobradas, imunidade, senescência, necrose e apoptose também foram descritas como influente pelas proteases (LÓPEZ-OTÍN e BOND, 2008). Por fim, as proteases tem desempenhado um papel promissor quanto ao uso biotecnológico, uma vez que são utilizadas como agente bioquímico na manufatura de diversos produtos (SAEKI et al., 2007).

As proteases foram inicialmente descritas como endopeptidases, ou seja, cuja função seria hidrolisar ligações peptídicas entre resíduos de aminoácidos no interior da cadeia proteica, já o outro grupo, corresponde às exopeptidases, as quais podem catalisar as extremidades amino-terminal (extremidade -NH2) ou carboxi-terminal (extremidade -COOH). Baseado na estrutura e mecanismo de ação, estes dois grupos se dividem em outras seis classes distintas, aspártil protease, glutâmil protease, metalo protease, cisteíno protease, treonil protease e serino protease (LÓPEZ-OTÍN e BOND, 2008). Estas nomenclaturas são derivadas dos resíduos de aminoácidos das proteínas o qual ocorre o ataque eletrofílico da enzima, posteriormente as classes de proteases ainda podem ser agrupadas em famílias de acordo com a sequência de aminoácido que as compõe, e afunilando ainda mais, podem se classificar em clans, de acordo com a similaridade da estrutura tridimensional (LÓPEZ-OTÍN e LYNN, 2007).

Os insetos fitófagos ao longo da evolução tiveram seus hábitos alimentares, fisiologia e seu metabolismo adaptado conforme a evolução do seu hospedeiro (TERRA et al., 1996). Na maior parte das vezes os alimentos são ingeridos e assim que triturados são deslocados ao trato gastrointestinal, onde degradam as macromoléculas (carboidratos, proteínas e lipídios) nas suas respectivas subunidades, por meio de enzimas específicas para cada tipo de substrato, situado em maior parte na porção média do intestino (SILVA et al., 2012). No geral, o intestino dos insetos é dividido em três partes, na porção medial (intestino médio) é onde ocorre o processo de liberação de enzimas, logo a digestão química e absorção (ZHU-SALZMAN e RENSEN, 2015).

Nos organismos fitófagos, a digestão das proteínas foi descrita inicialmente por Jongsma e Cols (1995), associando o uso de inibidores à dieta, em que a atividade proteolítica das enzimas ocorre principalmente pelas classes das serino, aspartil, cisteíno e metalo-proteases. Porém em alguns grupos de insetos como as leptopteras, as serino-proteases podem chegar a 95% da concentração de enzimas digestivas, cujas respectivos exemplares são as tripsinas e quimotripsinas. Estas enzimas estão diretamente envolvidas numa série de processos voltados ao desenvolvimento, sinalização e sistema imune (TERRA e FERREIRA, 1994).

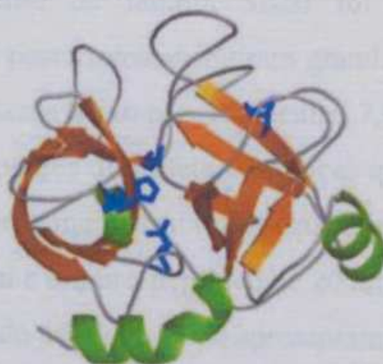
2.5 Inibidores de proteases

Os inibidores de protease (IP) naturais são moléculas cuja função é interromper a atividade de uma protease, sendo comumente de composição proteica, exceto alguns microrganismos que produzem IP não-peptídicos. Estes IPs compõem dois grupos, inibidores de baixo peso molecular, representado por aqueles que atuam no processo de germinação de sementes e o outro grupo podendo conter mais de uma cadeia peptídica em sua estrutura quaternária (POWERS et al., 2002). Os IPs ainda podem ser divididos em classes quanto aos grupos de proteases que inibem, sendo denominadas como: inibidores de serino, cisteíno, aspártil, glutâmil, treonino e metaloproteases. Estas moléculas podem ter uma menor afinidade por uma segunda protease a depender da constante de ionização (Ki), como o inibidor *kunitz* que possui uma forte afinidade por serino proteases, na ordem de 10^-9 a 10^-7 (DELFIN et al., 1996; SILVA-LOPEZ 2009).

No reino vegetal os IP ganharam notoriedade na década de 1940, com a descoberta de inibidores de alfa-amilase e posteriormente o inibidor *kunitz*, no qual foi identificado como um inibidor de serino protease, capaz de inibir tripsina (SILVA et al., 2000). Os inibidores do tipo *kunitz* foram identificados inicialmente nas leguminosas: *Mimosoideae, Caesalpinoideae, Papilionoideae* e também na família *Solanaceae*. Em 1973 conseguiram obter o sequenciamento completo da cadeia de aminoácidos do inibidor *kunitz* de soja, havendo alta atividade afinidade catalítica por tripsinas e um considerável termoestabilidade, devido ao alto peso molecular confirmado nestes inibidores (BATISTA et al., 1996; KOYDE e IKENAKA, 1973).

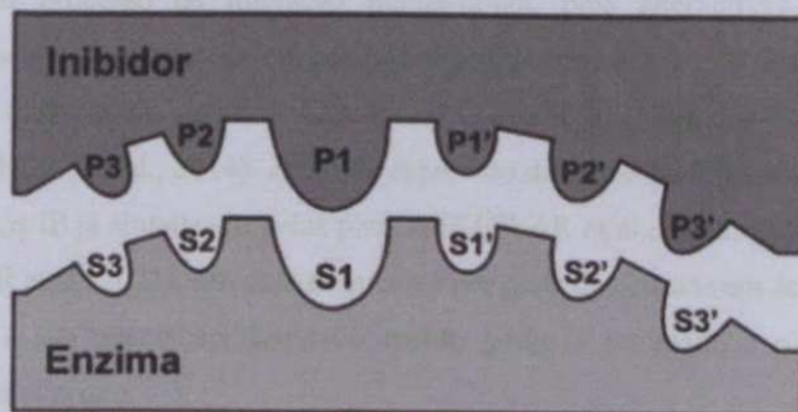
As serino-proteases são a família mais presente nos organismos como um todo, sua abundancia despertou interesse em pesquisas com potencial biotecnológico, sendo a classe de enzimas mais bem descritas e estudada, como observa-se na figura 5. Elas têm papel fundamental numa série de atividades fisiológicas e metabólicas. As serino-proteases tem peso molecular variado, podendo ser de único domínio ou multidomínos, a Tripsina por exemplo possui 229 resíduos de aminoácidos e massa 24000 Da resíduos de aminoácidos, pois cada enzima alvo possui estrutura de enovelamento e sequencias diferente (SILVA JÚNIOR, 2018).

Figura 5: Estrutura comum de uma serino-proteases. Os três resíduos catalíticos Asp102 Ser195-His57 estão designados de cor azul. Tripsina PDB1FY8. Na tripsina (A) mostramos também representado em azul o Asp189 (mais a direita) que confere uma maior especificidade. Fonte: disponível em Ribeiro (2009).



Esta classe de inibidores também pode apresentar variados mecanismos de ação, como ligação reversível (não forma ligações covalente), presente principalmente nas sementes em seu estágio de desenvolvimento ou irreversível (forma ligações covalente), as quais são induzidos principalmente por estímulos externo de herbivoria, além dessas, ainda há ligações fracas envolvidas como Van der Walls e interações hidrofóbicas (KROWARSCH et l., 2003; SILVA-LOPEZ, 2009).

Figura 6: Representação de um modelo de inibição entre protease (cinza claro) e inibidor (cinza escuro), representando os aminoácidos catalíticos (S) que sofreram, hidrólise nos respectivos resíduos de aminoácido. Disponível em: Schechter e Berg, (1967).



As duas famílias de inibidores de serino-proteases mais bem descritas são os inibidores de tripsina de soja Bowman-Birk (SBBI) e o do tipo Kunitz (SKTI). Estes inibidores representam uma parte considerável do conteúdo proteico de uma semente (6%), porém estes

dois IP não correspondem a quantidades equivalentes nas sementes, com uma maior prevalência do tipo SKTI frente ao SBBI (JOHNTON et al., 1993; BRANDON et al., 2002).

O inibidor serino-protease da família SBBI foi descoberto primeiramente em experimentos com soja, estudos posteriores atribuíram grande potencial biotecnológico a este inibidor. O IP de soja SBBI apresenta baixo peso molecular, 7,9 kDa e 71 aminoácidos, bastante estável devido a presença de 7 pontes dissulfeto com alças que inibem o ataque de proteases (COLARES et al., 2017). Esta proteína possui uma sequência sinalizadora de 19 aminoácidos a partir da porção amino-terminal e um pró-peptídeo de 20 aminoácidos ainda com função não identificada, os demais resíduos do peptídeo ainda apresentam dois domínios capaz de interagir com duas proteases distintas, um ligado tripsina e outro com quimotripsina (JOHNSTON et al., 1993; VOGTENTANZ et al., 2007).

Os inibidores da família SKTI também são proteicos, compostos por uma ou duas subunidades proteicas e com peso molecular superior a classe SBBI, variando de 18 a 20 kDa e cerca de 180 resíduos de aminoácidos com quatro resíduos de cisteína (ligações covalentes) que aumenta a força estrutural (BARROS et al., 2008). Estas propriedades moleculares conferem ao grupo dos inibidores *Kunitz* elevado termo estabilidade da sua estrutura enovelada, que é composta por folhas beta antiparalelas conectadas por *loops,* possuindo apenas um centro reativo contendo um resíduo de arginina 17 (SILVA-JÚNIOR, 2022; BHATTACHARYYA et al., 2006).

Os IP naturais produzidos pelas plantas em defesa à herbivoria estão em coevolução ocasionada pelo processo de interação planta-praga, pois alternativas metabólicas são desenvolvidas pelos insetos em defesa aos inibidores de protease, como a super-expressão de novas enzimas, aumentando assim a oferta de proteases no intestino dos insetos (NASERI et al., 2010; BAGHERY et al., 2014). Além da expressão de novas isoformas de enzima que são inertes à ação dos IP já sintetizado pelas plantas (KUWAR et al., 2015; COURA et al., 2021; SILVA-JÚNIOR et al., 2021). Em caso de sucesso por parte dos insetos em detrimento do efeito dos IP quanto a sua adaptação, seu crescimento tardio é retomado e o desenvolvimento completo poderá ocorrer.

No entanto, alguns estudos têm demonstrado que os danos causados aos insetos pelos IP vão além dos prejuízos da digestão proteica e alteração de expressão de proteases, como tripsinas e quimotripsinas, causando estresse oxidativo, alteração do padrão de resposta do

sistema imune, modulação dos componentes da matriz peritrófica dos insetos, seja por efeito de IP natural ou sintético (PAIXÃO et al., 2013; TAPROK et al., 2010).

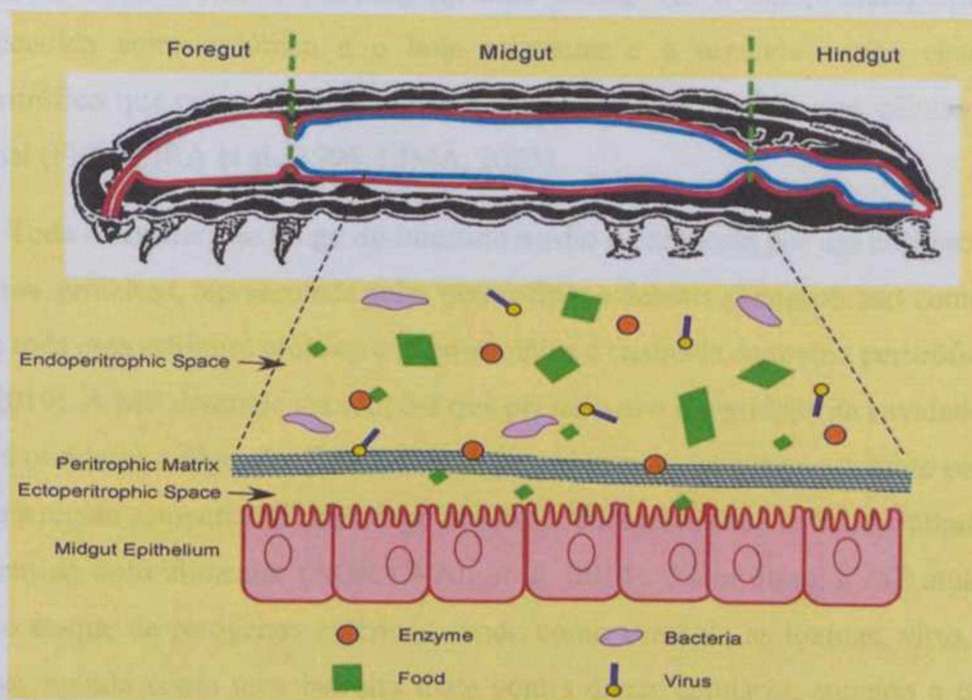
Os inibidores sintéticos de proteases são estruturas químicas ou proteica que mimetizam as interações entre protease e inibidor, o principal exemplar é a benzamidina, a qual é uma amidina aromática que é um inibidor competitivo de tripsina e tem características estruturais similares aos aminoácidos lisina e arginina (PILON et al., 2015). Este inibidor químico quando presente no meio reacional contendo tripsina, interage com o sítio ativo S1 de forma específica estabilizado por interações hidrofóbicas entre o grupo amidina e a porção carboxílica do aminoácido ácido aspártico presente no bolsão fundo do sítio S1 (PILON et al., 2004).

O derivado bis-benzamidina, o berenil, é composto por dois anéis da benzamidina, unidos por uma ligação triazeno no carbono C4 de cada anel, tal ligação pode ser rompida gerando dois produtos passíveis de inibir proteases do meio, com constante de inibição menor do que da benzamidina (OLIVEIRA et al., 1993; COSTA, 2021). Em experimentos, as lagartas quando desafiadas com diferentes concentrações de berenil, foi observado que o aumento da quantidade do inibidor na dieta resultou em efeitos adversos sobre diversos aspectos do ciclo de vida das lagartas. Dentre os parâmetros afetados, destacam-se um aumento da duração das fases larval e do ciclo de vida, o peso pupal e adultos, bem como o tamanho corporal e das asas. (MOREIRA, 2007).

2.6 Estrutura da matriz peritrófica

O trato gastrointestinal dos insetos é normalmente dividido em três partes morfológicas distintas: intestino anterior (estomadeu), intestino médio (mesentério) e intestino posterior (procteu), como mostrado na Figura 7, em que a passagem do alimento é controlado por sfincters. Nos insetos mastigadores, a preferência energética é extraída de polímeros de glicose, como a celulose, amido e hemicelulose, além de proteínas, que são degradadas por proteases intestinais, ocorrendo principalmente no intestino médio, local onde há a liberação de uma diversidade de enzimas digestivas (PINTO, 2019).

Figura 7: Representação da estrutura do intestino de um inseto mastigador. Na parte superior as três porções do intestino, anterior (*foregut*), média (*midgut*) e posterior (*hindgut*). Na parte central, compostos que situam e transitam pelo intestino médio e mais abaixo, a estrutura da membrana peritrófica e as células do intestino. Disponível em Pilon, (2004).



O intestino anterior desempenha a função de digestão mecânica, triturando o alimento, além de atuar como uma pré-digestão, enriquecendo o bolo alimentar com secreção das glândulas salivares. Na outra extremidade, o intestino posterior desempenha função de homeostase (reabsorção de água e sais minerais), além do processo de excreção final dos dejetos (NUÑES et al., 2023). A principal estrutura de proteção dessas duas porções intestinais é o revestimento da cutícula, uma camada proteica resistente e impermeável, conferindo proteção mecânica em toda sua extensão (ERLANDSON et al., 2019).

O mesentério, localizado entre as porções inicial e final do intestino, é onde ocorre a maior parte do processo de digestão química. Nesse compartimento, são secretadas enzimas digestivas que degradam os macronutrientes em suas subunidades. Posteriormente, também é realizado o processo de absorção (WIGGLESWORTH, 1972). O intestino médio é alvo de bastante estudos científicos, uma vez que é o principal local para a digestão proteica, convertendo-as em aminoácidos, que são absorvidos pelas células do mesentério (SILVA et al., 2012).

O sucesso do processo digestivo realizado pelo intestino médio é garantido por estruturas que desempenham papel de proteção, que envolve a síntese de um compartimento

estrutural denominado matriz peritrófica (MP), uma membra glico-proteica que reveste todo o compartimento do mesentério (HEGEDUS et al., 2019). A membrana peritrófica, formada no mesentério, divide o lúmen intestinal em duas partes, uma o espaço endoperitrófico, região compreendida entre o lúmen e o bolo alimentar e a segunda região chamada espaço ectoperitrófico que compreende a região da membrana peritrófica e as células do endotélio intestinal (FERREIRA et al., 1994; LIMA, 2023).

Toda a estrutura ao longo do intestino médio é composta por um polissacarídeo a base de quitina, proteínas, representada pelas peritrofinas e demais glicoproteínas como as mucinas, em que toda essa estrutura proteica e glico-protéica é chamada de matriz peritrófica (MP) (LIU et al., 2019). A MP desempenha funções que preservam a integridade da cavidade do intestino médio e otimizam a digestão. Esta estrutura possui características que confere permeabilidade seletiva à região ectoperitrófica, protege as células endoteliais contra a força abrasiva durante a passagem do bolo alimentar (AGRAWAL et al, 2014). Além disso, a MP atua na proteção contra o ataque de patógenos entéricos, tendo como exemplo as toxinas, vírus, bactérias ou parasitas, agindo como uma barreira inata contra danos celulares, somado a capacidade de compartimentalizar a secreção de enzimas digestivas, o que garante uma maior eficiência da digestão e absorção dos micro-nutrientes (PINTO, 2019).

A polissacarídeo quitina é o que corresponde a maior porção em volume da MP, sintetizado à partir da molécula precursora trealose, que formam este polissacarídeo. Somado a isso, há ação da enzima quitina sintase e posteriormente acetilação de parte da estrutura por meio da enzima quitina deacetilase (AMORIM, 2007). O polímero formado é então embebido na estrutura da MP por proteínas cuticulares ou proteínas da membrana peritrófica, o que garante a manutenção da estrutura da MP conferindo uma maior resistência durante a passagem do alimento. Neste processo, ocorre degradação de parte da estrutura da quitina por ação das enzimas quitinases e n-glicosaminidade forçando a reciclagem e nova síntese do polímero (ZHU et al., 2016; LIU et al., 2019).

O muco é uma estrutura produzido principalmente por proteínas mucinas, associadas à intervalos de glicosilação, formando assim, os proteoglicanos, que são secretados no lúmen do intestino médio dos insetos, o qual forma um filme protetor da MP (DIAS et al., 2017). Sua estrutura proteica predomina resíduos de aminoácidos serina, tirosina e prolina ligados em *tandem* (sequencias repetidas), que em grande parte são glicosilados, proporcionando proteção ao intestino contra fragmentos abrasivos dos alimentos e principalmente, proteção contra compostos acídicos e suas próprias enzimas digestivas compartimentalizadas no intestino

médio (HEGEDUS et al., 2009). De acordo com a função desempenhada, podem ser secretadas em duas classes distintas, a primeira compreende as formadoras de gel e não formadoras de gel e a outra, são mucinas aderidas à membrana, onde suas principais diferenças situam na sua estrutura proteica associada a membrana plasmática (DIAS et al., 2018; BOLOGNESI et al., 2008).

As proteínas correspondem a uma massa de 21 a 55% da composição da MP e as peritrofinas são o principal componente proteico, as quais são proteínas globulares, geralmente com tamanhos entre 10 e 20 kilodaltons (kDa) (BOLOGNESI, 2002). Estas estão associadas à quitina através da peritrofina-A. Estas proteínas são caracterizadas por conterem domínios com estruturas dobradas em formato de "dobradiça" (tripsina-*like*), que são essenciais para a sua atividade. Esses domínios permitem que as peritrofinas se liguem e inativem as enzimas digestivas presentes no intestino do inseto após o processo de digestão (BOLOGNESI, 2001; TELLAM et al., 1999).

As peritrofinas são secretadas pelas células epiteliais do intestino médio dos insetos e são liberadas no lúmen intestinal, onde exercem suas funções de inibição enzimática e proteção contra toxinas e patógenos. A estrutura dessas proteínas é altamente estável e resiste a condições adversas do trato digestório, garantindo sua efetividade na proteção e regulação da digestão (JASRAPURIA et al., 2012). Estas proteínas também são responsáveis pela organização das fibras de quitina, permitindo a formação da membrana peritrófica nos insetos. As peritrofinas estão ligadas a uma ou três interações com a quitina, estrutura essencial para demais processos, que vão além da proteção do tecido intestinal, atuando em processos fisiológicos de desenvolvimento, movimentação e sustentação da integridade da cutícula (TETRAU et al., 2015).

Os inibidores de protease, no combate à pragas agrícolas, têm se mostrado eficiente não somente como um agente antinutricional, mas também por danificar as estruturas que compõem a matriz peritrófica. De acordo com pesquisas desenvolvidas por Wang e Granado (2006), constataram a desintegração da MP pela ingestão de cisteíno proteases adicionadas a dieta *Spodoptera frugiperda,* devido à sua atividade proteolítica. Neste sentido, o dano causado em parte da estrutura da MP do inseto o torna mais suscetível ao ataque de patógenos, atraso no desenvolvimento, autofagia, podendo levar os mesmos a morte (WANG e GRANADOS, 2001; MOHAN et al., 2006).

2.7 Evolução das tecnologias de sequenciamento no estudo da Biologia Molecular

Nos últimos anos foi notado um expressivo aumento, evolução e facilidades de acesso às novas tecnologias de sequenciamento. Isto se tornou possível devido a uma maior capacidade de transferência, processamento de dados e redução dos custos evolvidos em todo processo, o que elevou exponencialmente o volume de dados gerados e o tamanho dos bancos de dados genômicos (ARDUI et al., 2018; WANG et al., 2019). Essa rápida evolução possibilitou uma melhor utilização das ferramentas de análise, estrutura e organização dos transcritos, além da melhor compreensão dos resultados de expressão e regulação gênica (LOWE et al., 2017).

As primeiras tecnologias desenvolvidas para o sequenciamento de DNA, foram nomeadas como sequenciamento de primeira geração, desenvolvida pelos cientistas Sanger e Coulson em 1975. Esse método se baseava na incorporação seletiva de dideoxinucleotídeos marcados que tinham a função de interromper o processo de polimerização, gerando diversos fragmentos de diferentes tamanhos, que posteriormente eram separados por eletroforese e sequenciados.

Pouco mais de um ano depois foi desenvolvida a primeira tecnologia de sequenciamento, Maxam e Gilbert (1977) desenvolveram uma outra técnica que envolvia a modificação química parcial do DNA em bases nucleotídicas específicas e a sua subsequente clivagem na estrutura do DNA próxima aos nucleotídeos modificados. Ao contrário da abordagem de Sanger, que exigia a clonagem do DNA de fita simples, o método de Maxam-Gilbert tinha a vantagem de poder utilizar diretamente o DNA purificado (SACCONE e PESOLE, 2003).

No entanto, o método de terminação de cadeia de Sanger provou ser mais escalável com o aprimoramento da técnica de terminação de cadeia e foi amplamente adotado nas três décadas seguintes, sendo considerado o padrão ouro, devido a uma pequena taxa de incorporação de nucleotídeos errôneos (SHUSTER, 2008). Embora o método de Sanger pudesse sequenciar fragmentos de DNA de até 1 kb com alta precisão bruta de leitura, sua aplicação era limitada devido ao baixo rendimento e ao alto custo (SCHLOSS, 2008).

A segunda fase das tecnologias de sequenciamento, também conhecida como (NGS – *Next Generation sequencing*), teve sua origem em meados dos anos 2000 com o objetivo de viabilizar as novas demandas que estavam surgindo, uma vez que centenas de milhares de fragmentos curtos de nucleotídeos (*reads*) eram gerados (BEJAHTI e TARPEY, 2013). Esses pequenos fragmentos eram aderidos a adaptadores, situados em uma matriz sólida e

sequenciados por meio de múltiplos ciclos de "lavagem e varredura" em que vão adicionando os nucleotídeos aos crescentes fragmentos (MOORTHIE et al., 2011; GOODWIN et al., 2016). Essa técnica é amplamente reproduzida nas plataformas Illumina HiSeq, que são capazes de gerar leituras na casa de bilhões e 1500 Gb por execução. Além disso, essa abordagem proporciona alta precisão de leitura a um custo significativamente inferior em relação às tecnologias de primeira geração (GOODWIN et al., 2016).

Porém, ainda havia algumas limitações recorrentes das tecnologias NGS, uma vez que os fragmentos gerados ultrapassavam 100 nucleotídeos (nt), a probabilidade de incorporação de um nt se elevava exponencialmente durante o processo, tal erro se amplificava também na reprodutibilidade via PCR (*polimerase chain reaction*), devido aos erros já presente nas *reads* geradas anteriormente (WHITEFORD et al., 2009). De fato, a NGS foi capaz de ampliar a aplicabilidade das técnicas de sequenciamento, encurtando imensamente o tempo de montagem de genomas, sequenciamento de RNA, identificação de polimorfismos e variações estruturais, possibilitando uma infinidade de estratégias e ferramentas de análise de dados (WADAPURKAR e VYAS, 2018).

Poucos anos após a adesão e o amplo uso das plataformas NGS, surgiram novas tecnologias de Sequenciamento de Terceira Geração (TGS - *Third-generation sequencing*), em que as principais características que fundamentavam esse novo modelo, eram as possibilidades de um sequenciamento ininterrupto de apenas uma única molécula de DNA ou RNA e o sequenciamento em tempo real, permitindo análises muito mais rápidas, visto que era uma das limitações dos modelos NGS (PUSHKAREV et al., 2009). A capacidade do TGS de sequenciar DNA ou RNA sem qualquer amplificação prévia do molde constitui um grande avanço, principalmente porque ameniza os vieses introduzidos pela PCR durante a construção da biblioteca (ATHANASOPOULOU et al., 2021).

Essas características tornaram a nova tecnologia TGS preferencial para casos como, montagem *de novo* de um genoma, anotações aprimoradas, caracterização epigenômica, viabilizando sequenciar regiões repetitivas do genoma ou altamente variáveis, que antes era impossibilitado, devido as leituras curtas geradas pelos modelos NGS. Uma vez que esta técnica TGS não utiliza ciclos de "varredura e lavagem", passou a ser viável o sequenciamento de longos fragmentos, de forma rápida e eficaz (SEARLI et al., 2023).

Dois principais modelos de maior relevância das TGS são o sequenciamento *Single Molecule Real-Time* (SMRT), pioneiro da Pacific Biosciences e o sequenciamento *Nanopore*

de Oxford, uma abordagem para o *single-molecule sequencing* (SMS), que tem comprimento de leitura, taxa de erro e taxa de transferência semelhante ao PacBio, porém disponível de forma portátil, barato e em tempo real, chamado MinION, que pode ser conectado diretamente a um computador e permitindo o seu deslocamento aos campos de experimentos (ZHENG et al., 2016; KINGAN et al., 2019). A representação dos dispositivos Oxford Nanopore e PacBio, respectivamente na Figura 8 A e B.

Figura 8: A) dispositivo de sequenciamento Oxford Nanopore MiniON. Poros proteicos acoplados a uma helicase incorporados a uma membrana. B) Sequenciamento SMTR, uso de polimerases guiadas em onda de modo zero, nucleotídeos marcados geram sinais característicos que são registrados. Disponível em: Searli (2023).

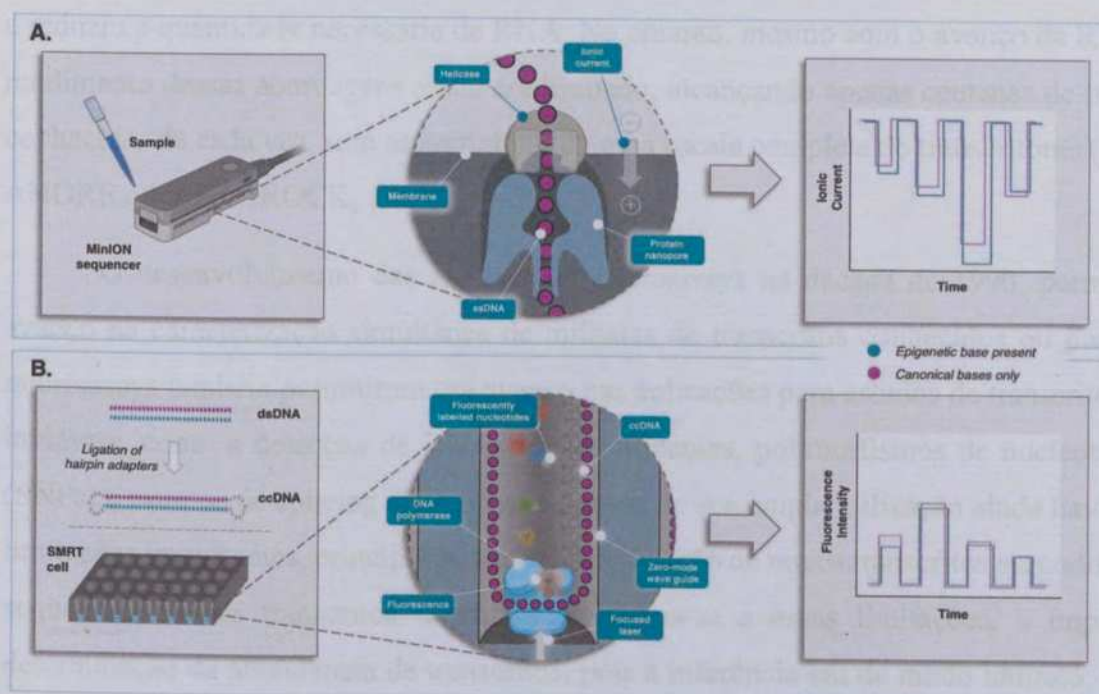
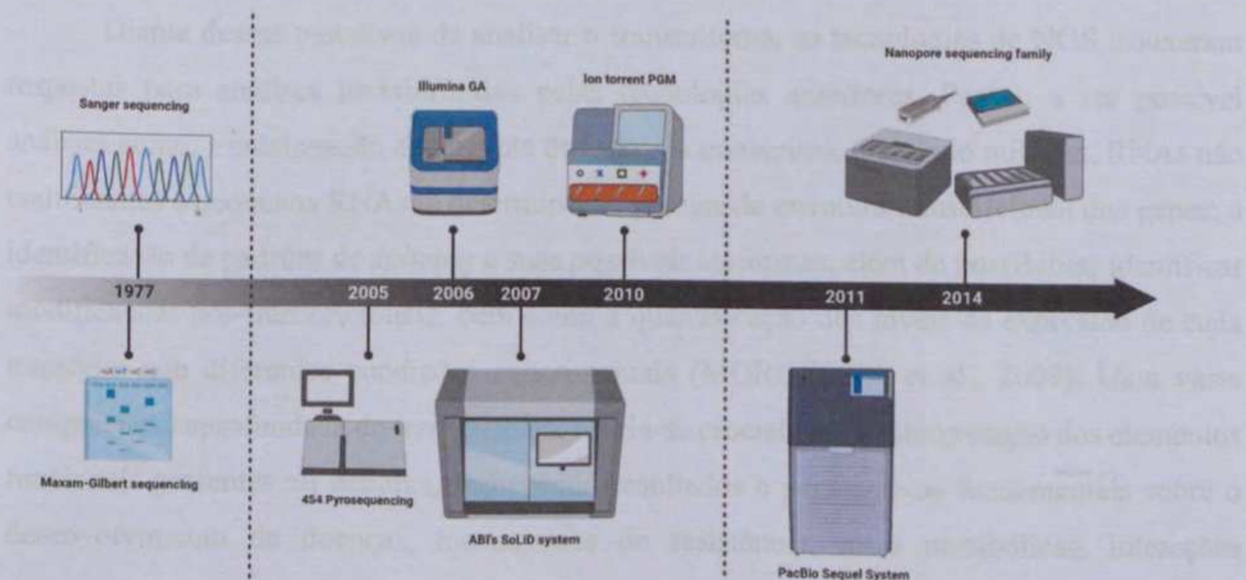


Figura 9: Evolução histórica das tecnologias de sequenciamento para biologia molecular. Disponível em: Athanasopoulou, (2022).



Historicamente, as primeiras tentativas de entender os transcritomas, partiram de experimentos que visavam examinar o conteúdo do RNA total das células de diferentes organismos, tecidos ou estados de doença para identificar os transcritos de interesse. Inicialmente, utilizaram a técnica de *Northern blot*, que fornecia resultados baixo rendimento, exigia o uso de radioatividade e carecia de grandes concentrações de RNA. Por causa dessas limitações, o *Northern blotting* só era capaz de detectar alguns transcritos conhecidos por vez em amostras com RNA abundante (ALWINE et al., 1977). A fim de superar essas restrições, novas metodologias foram desenvolvidas, como o PCR quantitativo de transcrição reversa (RT-qPCR). Essa técnica facilitou a detecção dos transcritos, aumentou o rendimento experimental e reduziu a quantidade necessária de RNA. No entanto, mesmo com o avanço da RT-qPCR, o rendimento dessas abordagens ainda era limitado, alcançando apenas centenas de transcrições conhecidas de cada vez, sem se aproximar de uma escala completa do transcritoma (BECKER-ANDRE e HAHLBROCK, 1989).

O desenvolvimento das análises de microarrays na década de 1990, permitiram um avanço na caracterização simultânea de milhares de transcritos conhecidos ou possíveis. Os microarrays também permitiram um avanço nas aplicações para estudos de transcritomas antes inviáveis, como a detecção de RNAs não codificantes, polimorfismos de nucleotídeo único (SNPs) e eventos de splicing alternativos. Apesar de sua ampla utilização ainda havia algumas limitações importantes, principalmente na identificação de novos transcritos e a codificação das sequencias desses transcritos identificados. Soma-se a essas limitações, a imprecisão na determinação da abundância de transcritos, pois a inferência era de modo indireto, resultando em dados ruidosos que afetam a reprodutibilidade e comparações entre amostras (SCHENA et al., 1995).

Diante dessas tentativas de analisar o transcritoma, as tecnologias de NGS trouxeram respostas para análises inviabilizadas pelas tecnologias anteriores. Passou a ser possível análises como: a catalogação abrangente de todos os transcritos, incluindo mRNAs, RNAs não codificantes e pequenos RNAs; a determinação precisa da estrutura transcricional dos genes; a identificação de padrões de *splicing* e suas possíveis isoformas, além de possibilitar identificar modificações pós-transcricionais; bem como a quantificação dos níveis de expressão de cada transcrito sob diferentes condições experimentais (MOROZOVA et al., 2009). Uma vasta compreensão aprofundada do transcritoma, revela-se crucial para a interpretação dos elementos funcionais presentes no genoma, fornecendo resultados e perspectivas fundamentais sobre o desenvolvimento de doenças, mecanismos de resistência, rotas metabólicas, interações

hospedeiro-patógeno, entre outros fenômenos de importância biológica (WANG et al., 2009; WESTERMANN et al., 2012).

As novas tecnologias desenvolvidas nos anos 2000 em diante, marcadas pelo sequenciamento paralelo em massa, mais conhecido como Sequenciamento de Próxima Geração (*Next-Generation Sequencing*, NGS), que tem se mostrado como uma ferramenta poderosa para a análise abrangente dos perfis transcritômicos, denominadas como RNA-Seq. Esta metodologia possibilitou identificação precisa da estrutura transcricional dos genes, descoberta de novos genes e isoformas expressas (originadas por splicing alternativo ou outras modificações pós-traducionais) e quantificação das mudanças nos níveis de expressão de cada transcrito em diferentes condições experimentais. Para a execução dessa abordagem, é essencial a extração de RNA de um tecido e a construção de uma biblioteca de cDNAs (HRDLICKOVA et al., 2017; MARGUERAT et al., 2010).

Nesse contexto, o RNA é submetido a processos de fragmentação e conversão em cDNA por meio de uma transcriptase reversa, com a adição de adaptadores em uma ou ambas as extremidades para preservar a direcionalidade do transcrito. Cada molécula de cDNA, amplificada ou não, é subsequente sequenciada utilizando métodos de alto rendimento, com o objetivo de obter sequências curtas a partir de um único lado (*single-end*) ou de ambos os lados (*paired-end*). A extensão dos *reads* pode alcançar até 10 kb, a depender da tecnologia de sequenciamento adotada (MARTIN & WANG, 2011; QUAIL et al., 2012; WANG et al., 2009).

O RNA-Seq permite inferir a respeito da expressão gênica devido à sua capacidade de fornecer uma representação quantitativa e abrangente dos transcritos de RNA presentes em uma amostra biológica (MARGUERAT et al., 2010). Isso é possível por meio de várias etapas e características fundamentais da técnica, como o mapeamento das *reads* (DNA complementar – cDNA) para o genoma de referência, o que permite identificar as regiões as quais os transcritos se originaram (LI et al., 2015). Os níveis de expressão de cada gene ou isoforma são estimados com base no mapeamento realizado e que também são normalizados. E por meio de métodos estatísticos, como os paramétricos, admitindo-se um gene mapeado em uma distribuição específica como a de poison ou binomial-negativa, os genes diferencialmente expressos (DEGs) são identificados. E por fim, esses resultados obtidos se associam à relevância do contexto biológico (COSTA-SILVA et al., 2017).

3. REFERÊNCIAS

A JONGSMA, Maarten; BEEKWILDER, Jules. Co-evolution of insect proteases and plant protease inhibitors. **Current protein and peptide Science**, v. 12, n. 5, p. 437-447, 2011.

AGOSTINI, L., OTUKA, A. K., SILVA, E. A., BAGGIO, M. V., de LAURENTIS, V. L., DUARTE, R. T., ... & POLANCZYK, R. A.. Compatibilidade de produtos à base de Bacillus thuringiensis (Berliner, 1911) com glifosato em diferentes dosagens, utilizado em soja (Glycine max (L.) Merrill). **Ciência et Praxis**, v. 6, n. 11, p. 37-40, 2013.

AGOSTINI, L., Otuka, A. K., Silva, E. A., Baggio, M. V., Laurentis, V. L., Duarte, R. T., Agostini, T. T., & Polanczyk, R. A. (2013). Compatibilidade de produtos à base de Bacillus thuringiensis (Berliner, 1911) com glifosato em diferentes dosagens, utilizado em soja (Glycine max (L.) Merrill). Ciência et Praxis, 6(11), 37-40.

AINSWORTH, Elizabeth A. et al. Accelerating yield potential in soybean: potential targets for biotechnological improvement. **Plant, cell & environment**, v. 35, n. 1, p. 38-52, 2012.

AKBAR, S. M. D. Jaba, J., Regode, V., Kumar, G. S., & Sharma, H. C. Plant protease inhibitors and their interactions with insect gut proteinases. In: **The biology of plant-insect interactions**. CRC Press, 2018. p. 1-47.

ALWINE, James C.; KEMP, David J.; STARK, George R. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. **Proceedings of the National Academy of Sciences**, v. 74, n. 12, p. 5350-5354, 1977.

AMORIM, Ticiana Maria Lúcio de. Avaliação da ação bioinseticida de SBTI e vicilina de Erythrina velutina em enzimas digestivas e membrana peritrófica de larvas de Plodia interpunctella (Lepidoptera: Pyralidae). Dissertação de Mestrado. **Universidade Federal do Rio Grande do Norte**. 2007.

ARAÚJO, Nadine Monteiro Salgueiro. ClTI, um inibidor de tripsina tipo KUNITZ purificado a partir de sementes de Cassia leiandra BENTH., exerce efeito candidicida, induzindo estresse oxidativo e apoptose celular em C. albicans. 2019.

ARDUI, Simon; AMEUR, A., VERMEESCH, J. R., & HESTAND, M. S.. Single molecule real-time (SMRT) sequencing comes of age: applications and utilities for medical diagnostics. **Nucleic acids research**, v. 46, n. 5, p. 2159-2168, 2018.

ATHANASOPOULOU, K., Boti, M. A., Adamopoulos, P. G., Skourou, P. C., & Scorilas, A. (2022). Third-Generation Sequencing: The Spearhead towards the Radical Transformation of Modern Genomics. **Life**, 12(1), 30.

ATHANASOPOULOU, Konstantina et al. Third-generation sequencing: the spearhead towards the radical transformation of modern genomics. **Life**, v. 12, n. 1, p. 30, 2022.

BARROS, Josie Gomes de Almeida, J. G. D. A., MORAES, R. M. A. D., PIOVESAN, N. D., BARROS, E. G. D., & MOREIRA, M. A.. Efeito do inibidor de protease Kunitz sobre níveis de lipoxigenases em sementes de soja. **Ciência e Agrotecnologia**, v. 32, p. 1126-1132, 2008.

BATISTA, I.F.C., OLIVA, M.L.V., ARAUJO, M.S., SAMPAIO, M.U., RICHARDSON, M., FRITZ, H., SAMPAIO, C.A.M. Primary structure of a Kunitz-type trypsin inhibitor from enterolobium contortisiliquum seeds. **Phytochemistry, Oxford**, v.41, n.4, p.1017-1022, 1996.

BECKER-ANDRE, Michael; HAHLBROCK, Klaus. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a P CR aided t ranscipt t itration assay (PATTY). **Nucleic acids research**, v. 17, n. 22, p. 9437-9446, 1989.

BEHJATI, Sam; TARPEY, Patrick S. What is next generation sequencing?. **Archives of Disease in Childhood-Education and Practice**, v. 98, n. 6, p. 236-238, 2013.

BEL, Yolanda; SHEETS, Joel J.; TAN, Sek Yee; NARVA, Kenneth E.; ESCRICHE, Baltasar. Toxicity and binding studies of Bacillus thuringiensis Cry1Ac, Cry1F, Cry1C, and Cry2A proteins in the soybean pests Anticarsia gemmatalis and Chrysodeixis (Pseudoplusia) includens. **Applied and Environmental Microbiology**, [S. l.], v. 83, n. 11, 2017.

BERNARDI, O., MALVESTITI, G. S., Dourado, P. M., OLIVEIRA, W. S., MARTINELLI, S., BERGER, G. U., ... & OMOTO, C.. Assessment of the high-dose concept and level of control provided by MON 87701× MON 89788 soybean against Anticarsia gemmatalis and Pseudoplusia includens (Lepidoptera: Noctuidae) in Brazil. **Pest management science**, v. 68, n. 7, p. 1083-1091, 2012.

BHATTACHARYYA, Arindam et al. A Kunitz proteinase inhibitor from Archidendron ellipticum seeds: purification, characterization, and kinetic properties. **Phytochemistry**, v. 67, n. 3, p. 232-241, 2006.

BLACK, R. J. Complexo soja: fundamentos, situação atual e perspectiva. In: câmara, G. M. S. (Ed.). Soja: tecnologia de produção II. Piracicaba: **ESALQ**, p.1-18, 2000.

BOLOGNESI, R., Terra, W.R. and Ferreira, C. (2008) Peritrophic membrane role in enhancing digestive efficiency. Theoretical and experimental models. **J Insect Physiol** 54: 1413–1422.

BOLOGNESI, Renata. Síntese, degradação e funções da membrana peritrófica dos insetos. Tese de Doutorado. **Universidade de São Paulo**. 2002.

BOLOGNESI, Renata. A membrana peritrófica de Spodoptera frugiperda: secreção de peritrofinas e papel na imobilização e na reciclagem de enzimas digestivas. Tese de Doutorado. **Universidade de São Paulo**. 2001.

CAMPOS, E. V. R., PROENÇA, P. L. F., OLIVEIRA, J. L., BAKSHI, M., ABHILASH, P. C., & FRACETO, L. F. Use of botanical insecticides for sustainable agriculture: Future perspectives. **Ecological Indicators**, v. 105, p. 483-495, 2019.

COLARES, Lara Franca. Detecção de inibidores de proteases em sementes de Punica granatum. **Química Nova**, v. 40, p. 270-274, 2017.

CONAB - Safra 2022/23: Produção de grãos pode chegar a 308 milhões de t impulsionada pela boa rentabilidade de milho, soja e algodão. Acesso em 11 de julho de 2023.

COSTA, Lara Farias Lustosa. Cinética de inibição das proteases digestivas de anticarsia gemmatalis. **Biologia: ensino, pesquisa e extensão-uma abordagem do conhecimento científico nas diferentes esferas do saber**, v. 1, n. 1, p. 79-95, 2021.

COSTA-SILVA, Juliana; DOMINGUES, Douglas; LOPES, Fabricio Martins. RNA-Seq differential expression analysis: An extended review and a software tool. **PloS one**, v. 12, n. 12, p. e0190152, 2017.

COURA, R. R., DA SILVA JUNIOR, N. R., MERIÑO-CABRERA, Y., DÍEZ, J. D. R., BARROS, R. A., BARBOSA, S. L., ... & DE ALMEIDA OLIVEIRA, M. G.. Extensive reprogramming of protein isoforms and histopathological alterations in the midgut of Anticarsia gemmatalis fed with protease inhibitors. **Annals of Applied Biology**, v. 180, n. 3, p. 383-397, 2022.

DELFÍN, J.; MARTÍNEZ, I.;ANTUCH, W.; MOREIRA, V.; GONZALÉZ, Y.; RODRÍGUEZ, R.; MÁRQUEZ, M.; SAROYÁN, A.; LARIONOVA, N.; DÍAZ, DIAS, R. O. CARDOSO, C., PIMENTEL, A. C., DAMASCENO, T. F., FERREIRA, C., & TERRA, W. R. l. The roles of mucus-forming mucins, peritrophins and peritrophins with mucin domains in the insect midgut. **Insect molecular biology**, v. 27, n. 1, p. 46-60, 2018.

DIAS, R. O. et al. The roles of mucus-forming mucins, peritrophins and peritrophins with mucin domains in the insect midgut. **Insect molecular biology**, v. 27, n. 1, p. 46-60, 2018.

ERLANDSON, Martin A.; TOPRAK, Umut; HEGEDUS, Dwayne D. Role of the peritrophic matrix in insect-pathogen interactions. **Journal of insect physiology**, v. 117, p. 103894, 2019.

FAO. (2020). FAOSTAT: Crops. Retrieved from http://www.fao.org/faostat/en/#data/QC.

FREIRES, Samya Thalyta dos Santos. Utilização de inseticidas naturais na agricultura: uma revisão. 2022.

FREITAS, Márcio. A cultura da soja no Brasil: o crescimento da produção brasileira e o surgimento de uma nova fronteira agrícola. **Enciclopédia Biosfera**, v. 7, n. 12, 2011.

GAVA, Ricardo et al. O estresse hídrico em diferentes fases da cultura da soja. **Revista Brasileira de Agricultura Irrigada-RBAI**, v. 9, n. 6, p. 349-359, 2015.

GOODWIN, Sara; MCPHERSON, John D.; MCCOMBIE, W. Richard. Coming of age: ten years of next-generation sequencing technologies. **Nature Reviews Genetics**, v. 17, n. 6, p. 333-351, 2016.

GUO H, ZHANG Y, TONG J, Ge P, WANG Q, ZHAO Z, ZHU-SALZMAN K, HOGENHOUT SA, Ge F, SUN Y An aphid-secreted salivary protease activates plant defense in phloem. **Current Biology**, v. 30, n. 24, p. 4826-4836. e7, 2020.

HABIB, Huma; FAZILI, Khalid Majid. Plant protease inhibitors: a defense strategy in plants. **Biotechnol Mol Biol Rev**, v. 2, n. 3, p. 68-85, 2007.

HEGEDUS, D.; ERLANDSON, M.; GILLOTT, C.; TOPRAK, U. New insights into peritrophic matrix synthesis, architecture, and function. **Annual Review of Entomology**, Stanford v. 54, p. 285-302, 2009.

HEGEDUS, Dwayne D.; TOPRAK, Umut; ERLANDSON, Martin. Peritrophic matrix formation. **Journal of Insect Physiology**, v. 117, p. 103898, 2019.

HRDLICKOVA, Radmila; TOLOUE, Masoud; TIAN, Bin. RNA-Seq methods for transcriptome analysis. **Wiley Interdisciplinary Reviews: RNA**, v. 8, n. 1, p. e1364, 2017.

https://www.conab.gov.br/ultimas-noticias/4731-safra-2022-23-producao-de-graos-pode-chegar-a-308-milhoes-de-toneladas-impulsionada-pela-boa-rentabilidade-de-milho-soja-e-algodao. Acesso em 05 de junho de 2023.

J.; PADRÓN, G.; CHÁVEZ, M. Purification, characterization and immobilization of proteinase inhibitors from Stichodactyla helianthus. **Toxicon**, v.34, p.1367-76, 1996.

JASRAPURIA, Sinu et al. Genes encoding proteins with peritrophin A-type chitin-binding domains in Tribolium castaneum are grouped into three distinct families based on phylogeny, expression and function. **Insect biochemistry and molecular biology**, v. 40, n. 3, p. 214-227, 2010.

JOHNSTON, K. A.; GATEHOUSE, J. A.; ANSTEE, J. H. Effects of soybean protease inhibitors on the growth and development of larval Helicoverpa armigera. **Journal of insect Physiology**, v. 39, n. 8, p. 657-664, 1993.

JONGSMA, Maarten A.; BAKKER, P. L., PETERS, J., BOSCH, D., & STIEKEMA, W. J. Adaptation of Spodoptera exigua larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. **Proceedings of the National Academy of Sciences**, v. 92, n. 17, p. 8041-8045, 1995.

KESSLER, André; BALDWIN, Ian T. Plant responses to insect herbivory: the emerging molecular analysis. **Annual review of plant biology**, v. 53, n. 1, p. 299-328, 2002.

KINGAN, S.B.; URBAN, J.; LAMBERT, C.C.; BAYBAYAN, P.; CHILDERS, A.K.; COATES, B.; SCHEFFLER, B.; HACKETT, K.; KORLACH, J.; GEIB, S.M. A high-quality genome assembly from a single, field-collected spotted lanternfly (Lycorma delicatula) using the PacBio Sequel II system. GigaScience, 8, giz122, 2019.

KOFSKY, Janice; ZHANG, Hengyou; SONG, Bao-Hua. The untapped genetic reservoir: the past, current, and future applications of the wild soybean (Glycine soja). **Frontiers in Plant Science**, v. 9, p. 949, 2018.

KOIDE, T., IKENAKA, T. Studies on soybean trypsin inhibitors: 3. amino-acid sequence of the carboxyl-terminal region and the complete amino-acid sequence of soybean trypsin inhibitor (Kunitz). **European Journal of Biochemistry**, Berlin, v.32, n.3, p.417-431, 1973.

KOIWA, H; BRESSAN, R.A; HASEGAWA, P.M. Regulation of protease inhibitors and plant defense. **Trends in Plants Science**, v.2, n.10, 1997, p.379-384.

LASKOWSKI JR, Michael; KATO, Ikunoshin. Protein inhibitors of proteinases. **Annual review of biochemistry**, v. 49, n. 1, p. 593-626, 1980.

LASKOWSKI JR, Michael; QASIM, M. A. What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes?. Biochimica et Biophysica Acta **(BBA)-Protein Structure and Molecular Enzymology**, v. 1477, n. 1-2, p. 324-337, 2000.

LI, Peipei; YONGJUN; Piao, HO; Sun Shon & KEUN Ho Ryu. Comparing the normalization methods for the differential analysis of Illumina high-throughput RNA-Seq data. **BMC bioinformatics**, v. 16, n. 1, p. 1-9, 2015.

LIMA, Bárbara Soares Amoroso. Efeitos de inseticidas no aparelho digestivo de Anticarsia gemmatalis (Lepidoptera: Noctuidae) e seu predador Podisus nigrispinus (Heteroptera: Pentatomidae). 2023.

LIMA, Fabrício. Caracterização de resistência de cultivares de soja a Anticarsia gemmatalis (LEPIDOPTERA: EREBIDAE). 2023.

LIU, Xiaojian et al. Biosynthesis, modifications and degradation of chitin in the formation and turnover of peritrophic matrix in insects. **Journal of insect physiology**, v. 114, p. 109-115, 2019.

LÓPEZ-OTÍN, Carlos, and LYNN M. Matrisian. "Emerging roles of proteases in tumour suppression." **Nature reviews cancer 7**, no. 10 (2007): 800-808.

LÓPEZ-OTÍN, Carlos; BOND, Judith S. Proteases: multifunctional enzymes in life and disease. **Journal of Biological Chemistry**, v. 283, n. 45, p. 30433-30437, 2008.

LÓPEZ-OTÍN, Carlos; BOND, Judith S. Proteases: multifunctional enzymes in life and disease. **Journal of Biological Chemistry**, v. 283, n. 45, p. 30433-30437, 2008.

LOWE, Rohan, SHIRLEY, N., BLEACKLEY, M., DOLAN, S., & SHAFEE, T. Transcriptomics technologies. **PLoS computational biology**, v. 13, n. 5, p. e1005457, 2017.

MACEDO, M. L. R., GARCIA, V. A., MARIA DAS GRAÇAS, M. F., & RICHARDSON, M. Characterization of Kunitz trypsin inhibitor with a single disulfide bridge from seeds of Ingra laurina. **Phytochemistry**, v. 68, 2007.

MARGUERAT, Samuel; BÄHLER, Jürg. RNA-seq: from technology to biology. Cellular and molecular life sciences, v. 67, p. 569-579, 2010.

MARGUERAT, Samuel; BÄHLER, Jürg. RNA-seq: from technology to biology. **Cellular and molecular life sciences**, v. 67, p. 569-579, 2010.

MARIONI, John C, MASON, C. E., MANE, S. M., STEPHENS, M., & GILAD, Y.. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. **Genome research**, v. 18, n. 9, p. 1509-1517, 2008.

MARTINS, Gustavo Luís Mamoré; TOMQUELSKI, Germison Vital. Eficiência de inseticidas no controle de Chrysodeixis includens (Lepidoptera: Noctuidae) na cultura da soja. **Revista de Agricultura Neotropical**, v. 2, n. 4, p. 25-30, 2015.

MAXAM, Allan M.; GILBERT, Walter. A new method for sequencing DNA. **Proceedings of the National Academy of Sciences**, v. 74, n. 2, p. 560-564, 1977.

MENDONÇA, E. G., DE ALMEIDA BARROS, R., CORDEIRO, G., DA SILVA, C. R., CAMPOS, W. G., DE OLIVEIRA, J. A., & DE ALMEIDA OLIVEIRA, M. G.. Larval development and proteolytic activity of Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) exposed to different soybean protease inhibitors. **Archives of insect biochemistry and physiology**, 103(1), e21637 (2020).

MERIÑO-CABRERA, Y., Mendes, de Oliveira, Macedo, T. A., de Almeida, M. L. R., & Oliveira, M. G. (2019). Inhibition of digestive trypsins by plant Kunitz proteins reduces the viability of Spodoptera cosmioides larvae. **Annals of Applied Biology**, 1–14. https://doi.org/10.1111/aab.12539.

MOHAN, S. et al. Degradation of the S. frugiperda peritrophic matrix by an inducible maize cysteine protease. **Journal of Insect Physiology**, v. 52, n. 1, p. 21-28, 2006.

MOORTHIE, Sowmiya; MATTOCKS, Christopher J.; WRIGHT, Caroline F. Review of massively parallel DNA sequencing technologies. **The HUGO journal**, v. 5, p. 1-12, 2011.

MOREIRA, Lílian Fernandes. Efeito do inibidor de serino-proteases, berenil, sobre a eficiência alimentar, atividade proteolítica e desenvolvimento pós-embrionário de Anticarsia gemmatalis

(Lepidoptera: Noctuidae). Dissertação (Mestrado em Ciência entomológica; Tecnologia entomológica) - Universidade Federal de Viçosa, Viçosa. 2007.

MOROZOVA, Olena; HIRST, Martin; MARRA, Marco A. Applications of new sequencing technologies for transcriptome analysis. **Annual review of genomics and human genetics**, v. 10, p. 135-151, 2009.

MOURA, Amanda Maria Alencar de. Purificação e caracterização bioquímica de um inibidor de protease cisteínica presente em sementes de Terminalia catappa, com potencial antimicrobiano frente a Staphylococcus aureus. 2019.

MÜLLER, L. Taxonomia e morfologia. In: MIYASAKA, S.; MEDINA, J. C. A soja no Brasil. 1 ed. **Campinas: Instituto de Tecnologia de Alimentos**, p. 65-104, 1981.

NAPOLEÃO, Thiago H. Insect midgut structures and molecules as targets of plant-derived protease inhibitors and lectins. **Pest Management Science**, v. 75, n. 5, p. 1212-1222, 2019.

NÚÑEZ, isauro beltrán. Alimentação de insetos herbívoros: exigências nutricionais e digestão (ênfase em lepidoptera). **Biologia: contextualizando o conhecimento científico**, v. 1, n. 1, p. 19-37, 2023.

OLIVEIRA, C. M. AUAD, A. M., MENDES, S. M., & FRIZZAS, M. R. l. Crop losses and the economic impact of insect pests on Brazilian agriculture. Crop Protection, v. 56, p. 50-54, 2014.

PAIXAO, G. P., Lourenção, A. L., Silva, C. R., Eduardo G. Mendonça, E. G., Silva, P. L., Oliveira, J.A., Zanuncio, J. C., Oliveira, M. G. A. Biochemical responses of Anticarsia gemmatalis (Lepidoptera: Noctuidae) in soybean cultivars sprayed with the protease inhibitor berenil. **Journal of agricultural and food chemistry**, v. 61, n. 34, p. 8034– 8038, 2013.

PEZENTI, L. F., SOSA-GÓMEZ, D. R., DE SOUZA, R. F., VILAS-BOAS, L. A., GONÇALVES, K. B., DA SILVA, C. R. M., ... & DA ROSA, R PEZENTI, Larissa Forim. Transcriptional profiling analysis of susceptible and resistant strains of Anticarsia gemmatalis and their response to Bacillus thuringiensis. **Genomics**, v. 113, n. 4, p. 2264-2275, 2021.

PILON, Anderson Martins. Adaptação da lagarta de soja Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) ao inibidor de protease benzamidina. **Ceres**, v. 56, n. 6, 2015.

POWERS, J.C.; ASGIAN, J.L.; EKICI, O.; JAMES, K.E. Irreversible inhibitors of serine, cysteine and threonine proteases. **Chemical Reviews**, v.102, p.4639-4750, 2002.

PUSHKAREV, Dmitry, NORMA F. Neff, and STEPHEN R. Quake. "Single-molecule sequencing of an individual human genome." Nature biotechnology 27.9 p. 847-850, 2009.

QI, Rui-Feng; SONG, Zhan-Wu; CHI, Cheng-Wu. Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application. **Acta biochimica et biophysica Sinica**, v. 37, n. 5, p. 283-292, 2005.

SACCONE, Cecilia; PESOLE, Graziano. **Handbook of comparative genomics: principles and methodology**. John Wiley & Sons, 2003.

SAEKI, Katsuhisa et al. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. **Journal of bioscience and bioengineering**, v. 103, n. 6, p. 501-508, 2007.

SANGER, Fred; COULSON, Alan R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. **Journal of molecular biology**, v. 94, n. 3, p. 441-448, 1975.

SANTOS, Aryelen Caroliny et al. Fatores e técnicas de produção e sua influência na produtividade e qualidade da soja. **XI EEPA–XI Encontro de Engenharia de Produção Agroindustrial**, p. 1-10, 2017.

SCHENA, Mark; SHALON, D., DAVIS, R. W., & BROWN, P. O. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. **Science**, v. 270, n. 5235, p. 467-470, 1995.

SCHLOSS, Jeffery A. How to get genomes at one ten-thousandth the cost. **Nature biotechnology**, v. 26, n. 10, p. 1113-1115, 2008.

SCHUSTER, Stephan C. Next-generation sequencing transforms today's biology. **Nature methods**, v. 5, n. 1, p. 16-18, 2008.

SEARLE, Bethany; Müller, Markus; Carell, Thomas; Kellett, Andrew. Third-generation sequencing of epigenetic DNA. **Angewandte Chemie**, v. 135, n. 14, p. e202215704, 2023.

SEDIYAMA, T.; PEREIRA, M. G.; SEDIYAMA, C. S.; GOMES, J. L. L. Botânica, descrição da planta e cruzamentos artificial. In: Cultura da Soja – I parte. **Viçosa: UFV**, p. 5-6, 1985.

SHANKER, Arun; SHANKER, Chitra (Ed.). Abiotic and biotic stress in plants: recent advances and future perspectives. 2016.

SILVA JÚNIOR, Neilier Rodrigues. Perfil proteômico de hidrolases do intestino de lagarta da soja, Anticarsia gemmatalis, no mecanismo de interação planta-inseto. 2018.

SILVA, Carlos Peres; LEMOS, Francisco José Alves; DA SILVA, José Roberto. CAPÍTULO 5 Digestão em Insetos. _. **Tópicos avançados em entomologia molecular**. INCT-EM, p. 32, 2012

SILVA, Mara Reis; SILVA, Maria Aparecida Azevedo Pereira da. Fatores antinutricionais: inibidores de proteases e lectinas. **Revista de Nutrição**, v. 13, p. 3-9, 2000.

SILVA-JÚNIOR, N. R., CABRERA, Y. M., BARBOSA, S. L., BARROS, R. D. A., BARROS, E., VITAL, C. E., ... & OLIVEIRA, M. G. A. Intestinal proteases profiling from Anticarsia gemmatalis and their binding to inhibitors. **Archives of Insect Biochemistry and Physiology**, v. 107, n. 3, p. e21792, 2021.

SILVA-LOPEZ, Raquel Elisa da. Inibidores de Proteases Oriundas de Plantas: Uma Abordagem Útil para o Desenvolvimento de Novos Fármacos. 2009.

SOSA-GÓMEZ, Daniel Ricardo; OMOTO, Celso. Resistência a inseticidas e outros agentes de controle em artrópodes associados à cultura da soja. **Soja: manejo integrado de insetos e outros artrópodes-praga**, v. 1, p. 673-723, 2012.

SULTANA, M. S., MILLWOOD, R. J., MAZAREI, M., & STEWART, C. N.. Proteinase inhibitors in legume herbivore defense: from natural to genetically engineered protectants. **Plant Cell Reports**, p. 1-13, 2022.

SWATHI, Marri; LOKYA, Vadthy; GUJJARLAPUDI, Mariyamma; VERMA, Shreya; KISKU, Pallabi; KUMAR, Nukapangu Sravan; PADMASREE, Kollipara. Proteinase Inhibitors. **Molecular Approaches for Sustainable Insect Pest Management**, p. 209-252, 2021.

TEJO, Débora Perdigão; FERNANDES, Carlos Henrique dos Santos; BURATTO, J. S. Soja: fenologia, morfologia e fatores que interferem na produtividade. **Revista Científica Eletrônica de Agronomia da FAEF**, v. 35, n. 1, p. 1-9, 2019.

TERRA, Walter Ribeiro, C. FERREIRA, and J. E. BAKER. "Compartmentalization of digestion." Biology of the insect midgut (1996): 206-235.

TETREAU, Guillaume et al. Analysis of chitin-binding proteins from Manduca sexta provides new insights into evolution of peritrophin A-type chitin-binding domains in insects. **Insect biochemistry and molecular biology**, v. 62, p. 127-141, 2015.

TOMQUELSKI, G.V.; MARTINS, G. L. M.; DIAS, T. S. Características e manejo de pragas da cultura da soja. Pesquisa, Tecnologia e Produtividade, Chapadão do Sul-MS, v. 2, n. 9, p. 61-82, 2015.

TONNANG, Henri EZ; SOKAME, B. M., ABDEL-RAHMAN, E. M., & DUBOIS, T.. Measuring and modelling crop yield losses due to invasive insect pests under climate change. **Current Opinion in Insect Science**, v. 50, p. 100873, 2022.

TURCO, Boris. "Targeting proteases: successes, failures and future prospects." **Nature reviews Drug discovery 5**, no. 9 (2006): 785-799.

TURK, Boris. Targeting proteases: successes, failures and future prospects. Nature reviews Drug discovery, v. 5, n. 9, p. 785-799, 2006.

VOGTENTANZ, Gudrun et al. A Bacillus subtilis fusion protein system to produce soybean Bowman–Birk protease inhibitor. Protein Expression and Purification, v. 55, n. 1, p. 40-52, 2007.

WADAPURKAR, Rucha M.; VYAS, Renu. Computational analysis of next generation sequencing data and its applications in clinical oncology. **Informatics in Medicine Unlocked**, v. 11, p. 75-82, 2018.

WANG, Zhong; GERSTEIN, Mark; SNYDER, Michael. RNA-Seq: a revolutionary tool for transcriptomics. **Nature reviews genetics**, v. 10, n. 1, p. 57-63, 2009.

WANG, Zhong; GERSTEIN, Mark; SNYDER, Michael. RNA-Seq: a revolutionary tool for transcriptomics. **Nature reviews genetics**, v. 10, n. 1, p. 57-63, 2009.

WAQUIL, José M. et al. Manejo Integrado de pragas: revisão histórica e perspectivas. In: Congresso Nacional de Milho e Sorgo [resumos expandidos]. Sete Lagoas: **ABMS: Embrapa Milho e Sorgo**. 2002.

WAR, A. R., TAGGAR, G. K., HUSSAIN, B., TAGGAR, M. S., NAIR, R. M., & SHARMA, H. C.. Plant defence against herbivory and insect adaptations. **AoB plants**, v. 10, n. 4, p. ply037, 2018.

WESTERMANN, Alexander J.; GORSKI, Stanislaw A.; VOGEL, Jörg. Dual RNA-seq of pathogen and host. **Nature Reviews Microbiology**, v. 10, n. 9, p. 618-630, 2012.

WESTFALL, R. J.; BOSSHARDT, D. K.; BARNES, Rw H. Influence of crude trypsin inhibitor on utilization of hydrolyzed protein. **Proceedings of the Society for Experimental Biology and Medicine**, v. 68, n. 3, p. 498-500, 1948.

WHITEFORD, Nava et al. Swift: primary data analysis for the Illumina Solexa sequencing platform. **Bioinformatics**, v. 25, n. 17, p. 2194-2199, 2009.

ZHENG, G.X.Y.; LAU, B.T.; SCHNALL-LEVIN, M.; JAROSZ, M.; BELL, J.M.; HINDSON, C.M.; KYRIAZOPOULOU-PANAGIOTOPOULOU, S.; MASQUELIER, D.A.; MERRILL, L.; TERRY, J.M.. Haplotyping germline and cancer genomes with high-throughput linked-read sequencing. Nat. Biotechnol., 34, p. 303–311, 2016.

ZHU-SALZMAN, Keyan, and RENSEN Zeng. "Insect response to plant defensive protease inhibitors." Annual review of entomology 60 (2015): 233-252.

CAPÍTULO I: Differential gene expression reprograming in *Anticarsia gemmatalis* midgut triggered by a SKTI-derivative tripeptide protease inhibitor compared to natural SKTI protein

ABSTRACT

Anticarsia gemmatalis is among the major insect pest that causes defoliation in soybean crops. Alternative strategies have been evaluated to reduce insect attacks, using protease inhibitors (PI) that act as an anti-nutritional factor. A tripeptide GORE-2 was designed from soybean SKTI PI with higher protease inhibition capabilities, reducing caterpillar survival. Thus, in the work, we evaluated the gene expression reprogramming by RNAseq to verify the cascades involved in the response to these different PIs. SKTI and GORE-2 triggered an extensive gene expression reprogramming after 24 hours. In general, the responses were similar for both PIs with changes in the expression of the genes encoding proteases and defense proteins and involved mainly in matrix peritrophic protection and reconstitution. However, these responses were more pronounced for SKTI, indicating a signaling cascade more efficient to trigger the insect defense mechanisms. These differences justified a more effective action of the GORE-2 in proteolysis inhibition and reduction of caterpillar survival, indicating that the signaling may be due the amino acid starvation but also by a perception general system developed to detect deterrent molecules from soybean. As GORE-2 is a protease inhibitor mimetic tripeptide, the signaling cascade appeared to be minus efficient. In fact, the gene expression for detoxifying and stress oxidative processes was higher for GORE-2. Thus, an additional advantage of the use of mimetic or synthetic protease inhibitors.

Keywords: *Anticarsia gemmatalis*; soybean; plant-pest interaction; transcriptome, resistance; intestine; peritrophic matrix.

1. INTRODUCTION

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) is a pest insect that causes the defoliation of several crops, mainly soybean, leading to significant economic losses (Walker et al., 2000; Fernandes et al., 2018). Chemical insecticides are a relevant tool facing this challenge and are effective against several pests, mainly caterpillars (Fernandes et al., 2018; Bel et al., 2019). However, the use of synthetic chemical insecticides on a large scale has caused the resistance of many insect pests (Agostini et al., 2013), requiring alternatives to reduce human health toxicity and cause less environmental contamination (Campos et al., 2019).

New strategies to reduce pest attacks have been developed, and bioinsecticides have shown promise, acting on limiting the caterpillar's development, as well as the low toxicity compared to already known insecticides (Meriño-Cabrera et al., 2018; da Silva Júnior et al., 2020). Examples of bioinsecticides include BT toxin derived from microorganisms *Bacillus thuringiensis* (Berliner, 1911) (Agostini et al., 2013; Pezenti et al., 2021) and protease inhibitors (PI).

PIs are proteins or peptides with insecticidal activity and can be naturally produced by plants or synthesized. PIs make up one of the most abundant classes of pesticides against insect herbivory (Zhao et al., 2019), such as the SKTI protein – Soybean Kunitz Trypsin Inhibitor. Its expression is induced in soybean plants after mechanical damage, produced by the chewing apparatus of phytophagous insects (Jongsma et al., 2011; Kuwar et al., 2015). The mechanical shocks produced by the caterpillar's chewing apparatus trigger a series of responses at the cellular level in plants, activating the lipoxygenase pathway (Fortunato et al., 2007) and increasing the translation of PI proteins. SKTI acts as a protease inhibitor when in contact with the caterpillar intestinal tract, as it inhibits specific active sites of digestive enzymes and reduces the availability of essential amino acids, which are necessary for insect development and several of its physiological activities (Kuwar et al. al., 2015; Mendonça et al., 2020).

Spraying PI-containing products on a large scale in the field is still a challenge since these molecules have low stability when dispersed in the environment, increasing the production cost of these bioinsecticides due to their large molecular size (Barros et al., 2022). Added to this are the evolutionary pressures in a plant-pest relationship, where the insect develops mechanisms to overcome this delay in development and against other plant defense mechanisms. To minimize the PIs effect added to a diet, the caterpillars induced high expression proteases (Shakeel et al., 2020) and produced new isoforms to avoid the action of inhibitors

(Coura et al., 2021). In this way, the caterpillars adapted to the new nutrient availability (Coura et al., 2021; Silva-Júnior et al., 2021).

Studies about how PIs mechanisms act along the caterpillar gut have focused mainly on the midgut, where the release of serine protease enzymes that correspond to about 95 % of digestive enzymes occurs (Taprok et al., 2015; Ponnuvel et al., 2015; Ponnuvel et al. al., 2012; Silva-Júnior et al., 2021). Other works have also explored the responses of the caterpillars' immune system since an up-regulated expression of oxidoreductase enzymes has been observed and protect structures on intestinal insect cells such as chitin, peritrophic membrane, cuticle and mucins (Taprok et al., 2010; Li et al., 2009; Liu et al., 2004).

Our research group has focused on studying new synthetic biological molecules, smaller and more stable, such as the tripeptide GORE-2 (Val-Leu-Arg),, that have two hydrophobic amino acids (valine and leucine) and another one hydrophilic (arginine), developed to cause more significant cellular damage to the caterpillar intestine without causing an exacerbated response to the immune system (Silva-Júnior et al., 2021). GORE-2 has a higher binding affinity for trypsin-like catalytic sites and acts as a competitor of proteases (Barros et al., 2022). In this work, we investigated the action mechanism of natural (SKTI) and synthetic (GORE-2) protease inhibitors on the physiology of the *A. gemmatalis* by transcriptome sequencing of caterpillars fed with PIs and identifying genes and metabolic pathways differential expressed.

2. METHODOLOGY

2.1. *Anticarsia gemmatalis* Soybean caterpillar

A. gemmatalis eggs have maintained at 25 °C ± 2 °C temperature and 70 % ± 10 % relative humidity in the Insect Laboratory of the Department of Biochemistry and Molecular Biology at UFV. The soybean caterpillar has a biological cycle average of four to five weeks. Cages measuring 50 x 50 cm were screened and internally coated with sheets of A4 bond paper. The pupae were placed in Petri dishes (150 x 20 mm) inside the cages until they emerge. The feeding of moths has constituted in a nutrient solution composed of honey (20.0 g), beer (350 mL), sucrose (50 g), ascorbic acid (1.05 g), nipagin (1.05 g), and water (650 mL), soaked in a cotton swab placed at the bottom of the cage, on a petri dish. After oviposition, the paper sheets containing *A. gemmatalis* eggs were cut into strips 2.5 cm wide and 10 cm long and placed in

a container (500 mL) with a circular hole in the lid of approximately 2 cm and with an organza fabric attached. The recipients were transferred to an acclimatized chamber at 25 °C temperature, with a 60 % ± 10 % relative humidity and a 14 h photoperiod. The caterpillar feeding started after the eggs hatched with an artificial diet, placing a diet cube in the recipients.

The fifth instar larvae were separated in cages 3 x 5 cm and fed ad libitum on a diet (control) and diets supplemented with sublethal PIs concentration: 0.12% (w/w) of serine-protease Kunitz (SKTI) (treatment 1) and 0,12% (w/w) of GORE-2 (treatment 2), as described by Chougule et al. (2008). After 24 h, the caterpillars were collected from each treatment and control. The intestines were removed and stored at -80 °C.

2.2. Preparation of the artificial diet

The artificial diet consisted of cooked Mulato beans, brewer's yeast, wheat germ, soy protein, casein, agar, and water. Agar and water were autoclaved for 20 min at 1.5 kgF cm^{-2} pressure, and the ingredients were mixed in an industrial blender. Then ascorbic acid (6.0 g), nipagin (methylparaben) (5.0 g), 40% formaldehyde (6.0 mL), and 23 mL of a vitamin solution composed of niacinamide (1.0 mg), calcium pantothenate (1.0 mg), thiamine (0.25 mg), riboflavin (0.5 mg), pyridoxine (0.25 mg), folic acid (0.25 mg), biotin (0.02 mg), inositol (20 mg) and water (1.0 L) to form a homogeneous paste, transferred while still hot to plastic containers with lids. The control group was fed a diet without protease inhibitors, and the other two groups were fed a plus diet with protease inhibitors GORE-2 and SKTI. The diets cooled in a germicidal chamber under ultraviolet light were kept at 4 °C, before being administered to the caterpillars.

2.3. RNA extraction and transcriptome sequencing

Was made a three technical and three biological pool replicates of the caterpillar intestine have split into two control, three SKTI, and three GORE-2 replicates. The total RNA extraction was performed using the Trizol reagent (Invitrogen), according to the manufacturer's recommendations. Three units of DNase free of RNAse (Invitrogen) treated the extracted RNA to eliminate contamination by DNA. RNA was quantified in a NanoDrop spectrophotometer (Thermo Scientific) 260/280 nm and analyzed with 1.5 % (w/v) denaturing agarose gel, stained

with 0.1 μg mL-1 ethidium bromide. The construction of individual cDNA libraries used the Illumina® TruSeq Stranded mRNA Kit according to the instructions provided by the manufacturer. Sequencing was performed on the Illumina® Hiseq 2500 by the Beijing Genomics Institute (BGI), producing paired-end reads of 100 nucleotides.

2.4. Quality analysis and assembly

The quality control of sequencing data used the reports of FastQC version 0.11.7 (Andrews, 2010) combined using MultiQC version 1.12 (Ewels et al., 2016). Sequences' adapters removal used the "auto-detection" setting of TrimGalore version 0.6.7 (Krueger et al., 2021). Then, the reads were trimmed for quality and filtered for length using the Trimmomatic version 0.39 (Bolger et al., 2014) with the following parameters: HEADCROP:15; LEADING:3; TRAILING:3; SLIDINGWINDOW:4:20; MINLEN: 50. Trimmed sequences were additionally processed by Rcorrector version 1.0.4 (Song & Florea, 2015), using the default settings and applying a sequence correction method based on k-mers sequences analysis.

Ab initio assembly of the transcriptome assembly used Trinity version 2.8.5 (Grabherr et al., 2011), selecting the default settings and filtering the transcripts to a minimum length of 500 nucleotides. The script "TrinityStats.pl" calculated descriptive statistics of the assembly. The transcriptome also was evaluated by mapping the reads to the transcript sequences using Bowtie2 tools version 2.2.6 (Langmead et al., 2012), selecting default settings "--end-to-end" and "--sensitive". After assembly, the cd-hit-est tool of CD-HIT version 4.8.1 (Fu et al., 2012) clustered the transcripts into non-redundant Unigene sequences, selecting a minimum global identity of 98 %.

2.5. ORF prediction

Unigenes sequences were analyzed to identify open reading frames (ORFs) using TransDecoder.LongOrfs version 5.5.0 (Haas et al., 2013) and considering only ORFs with a minimum length of 300 nucleotides. The protein sequences encoded by the predicted ORFs were compared with proteins available in the UniProt Knowledgebase database (UniProtKB, http://www.uniprot.org) (UniProt Consortium, 2014) using the BLASTp tool of BLAST version. 2.11.0 (Altschul et al., 1990). Protein sequences also were identified with the support

of Hidden Markov Models (HMMs) of domain families from the Pfam database (http://pfam.xfam.org/) (Finn et al., 2014) using HMMER version 3.1b2 (Eddy and Wheeler, 2015). Only alignments that showed an E-value score $\leq 1\times10^{-10}$ were considered significant. The BLASTp alignments also were evaluated for sequence similarity, selecting those showing the product coverage × similarity ≥ 40 %. The TransDecoder.predict tool predicted the proteins encoded by the Unigenes by consolidating the results of ORFs prediction with the results of similarity searches using BLASTp and HMMER.

2.6. Differentially expressed transcript analysis

The transcript abundance was quantified by Kallisto version 0.44.0 (Bray et al., 2016), performing a pseudo alignment of the reads against the Unigenes sequences. The differential expression analysis between contrasts was done by DESeq2 version 3.15 (Love, Hubner, Anders, 2014), with the following parameters: adjusted p-value < 0.01 and $|\log2 \text{ fold change}| \geq 2$ ($|FC| \geq 4$). And with the following contrasts: GORE-2 (2 replicates) × control (2 rep.); SKTI (2 rep.) × control (2 rep.); and GORE-2 (3 rep.) × SKTI (3 rep.).

2.7. Functional annotation

The functional annotation of the Unigenes sequences was done by the TRAPID tool version 2.0 (Van Bel et al., 2013), selecting the EggNOG database version 4.5.1 (Cepas et al., 2016, 2017) and considering an E-value score $\leq 1\times10^{-10}$ for gene family attribution, identification of functional domains, and gene ontology terms (GO) attribution. Unigenes identified as differentially expressed also were annotated using the BLAST2GO software (Conesa et al., 2005), performing an automatic annotation against Arthropoda protein sequences (NCBI Taxonomy ID 6656) available in the NCBI RefSeq Non-Redundant proteins (nr) database, and considering an E-value $\leq 1\times10^{-10}$ to select significant alignments. The metabolic pathway enrichment of differentially expressed Unigenes was evaluated by the KOBAS tool (Xie et al., 2011), using the hypergeometric statistical test (adjusted p-value < 0.05) and selecting as reference the genome annotations of *Helicoverpa armigera* and *Bombyx mori*.

3. RESULTS

3.1. Data analysis sequencing

The *A. gemmatalis* sequencing of midgut libraries generated 46 to 48 million reads with 100 nucleotides and an average GC content of 48 %. After removing the adapters and trimming the reads, the sequencing data contained 43 to 44 million reads with sizes ranging from 50 to 85 nucleotides (nt).

3.2. Reference transcriptome assembly

The reference transcriptome assembly identified 51,825 transcripts. The mean length of the transcripts obtained was 2,243.6 nt, and the N50 was 3,246 nt (**Table 1**). The validation of transcriptome by reads mapping showed that 86 % of aligned reads in the transcripts and 14.31 % did not align. Furthermore, 20.33 % of aligned uniquely, and 65.36 % had multiple alignments. The sequences of transcripts hadgrouped in 42,372 Unigenes, which have an average of 1,467 nt size and N50 of 2,894 nt.

Table 1: *A. gemmatalis* reference assembly statistics

Score:
Total trinity 'genes': 20,192
Total trinity transcripts: 51,825
Percent GC: 40.41

Statistics based on all transcripts:
Contig N50: 3,246
Median contig length: 1,617
Average contig: 2,243.60
Total assembled bases: 116,274,451

Statistics based on longest isoform per ''gene'':
Contig N50: 2,891
Median contig length: 1,204
Average contig: 1906,23
Total assembled bases: 38,490,674

3.3. Functional annotation

The set of Unigene sequences contains 51,802 ORFs. Among the proteins encoded by the predicted ORFs, 25,906 (50.01%) proteins significantly aligned with the available proteins in the Uniprot database. Among the sequences with significant alignments, 36 % aligned with sequences from *Bombyx mori,* 34 % with *Danaus plexippus*, 25 % with *Heliconius melpomene*, and 5 % with other species (**Fig. 1**). Protein sequences from the species *Helicoverpa armigera* and *Bombyx mori* represented 68% of the significant alignments against the EggNog database. Comparison against the SwissProt database found 22,040 (42.55%) significant alignments. The EggNog database identified several similar alignments to UniProt, but the protein sequences of the *Bombyx mori* and *Danaus plexippus* species corresponded with 70% of significant alignments. Analysis in the EggNog database also made it possible to assign 13,731 Gene Ontology (GO) terms to the protein sequences and classify them into 13,223 KEGG Orthology (KO) groups. The KO1312 group, which corresponds to the trypsin family, was the most frequent, with 145 proteins identified. The search for functional domains in the Pfam database identified 32,673 domains in the protein sequences.

The gene families identified in the transcriptome encompass all treatments and their respective replicates. Those with the higher frequency identification were the trypsin family with 102 different annotated Unigenes, associated with the metabolism and amino acids transport function; the serpin B family with 71 annotated Unigenes, associated with defense and inhibition mechanisms; and the cytochrome p450 family with 56 unigenes, associated with transport, biosynthesis, and catabolism of secondary metabolites. Three gene families with 36 (serine endopeptidases), 35 (trypsins), and 32 (serine proteases) Unigenes were associated with transport activity and amino acid metabolism function.

We can highlight the higher frequency of transcripts coding for digestive functions such as proteases and lipases (catalytic activity). A high number of expressed genes were involved in immunity, for example, 68 contigs encoding serpins, indicating a role of the midgut in insect immunity. As expected, these results confirmed the predicted role of gut enzymes in digestion, being: the most dominant enzymes being trypsin-like proteases (145 entries), serine endopeptidase (68 entries) and lipases (68 entries).

We also observed the transcription of a large number of genes coding for modification enzymes (Glucoronyltransferaes, cytochrome P450 family, glutathione transferases and glucosidases) involved in the detoxification and defense process (**Fig. 2**), indicating the

induction of genes encoding for enzymes involved in xenobiotic metabolism. Detoxification enzymes are induced in insects in response to plant allelochemicals. Glutathione S-transferase isozymes possess peroxidase activity and, together with independent mechanisms ascorbate peroxidase (APOX), metabolize peroxides in insect midgut tissues (Yepiskoposyan et al., 2006), considered to be one of the major modalities for xenobiotic detoxification. We also detected 36 entries for beta-glucosidases which may be involved in the disgestive and defense processes.

Figure 1. A) Similarity frequency distribution between different species, calculated from the EggNog database by the TrapID tool. B) Functional annotation distribution for similarity using different tools and databases from a total number of identified Unigenes.

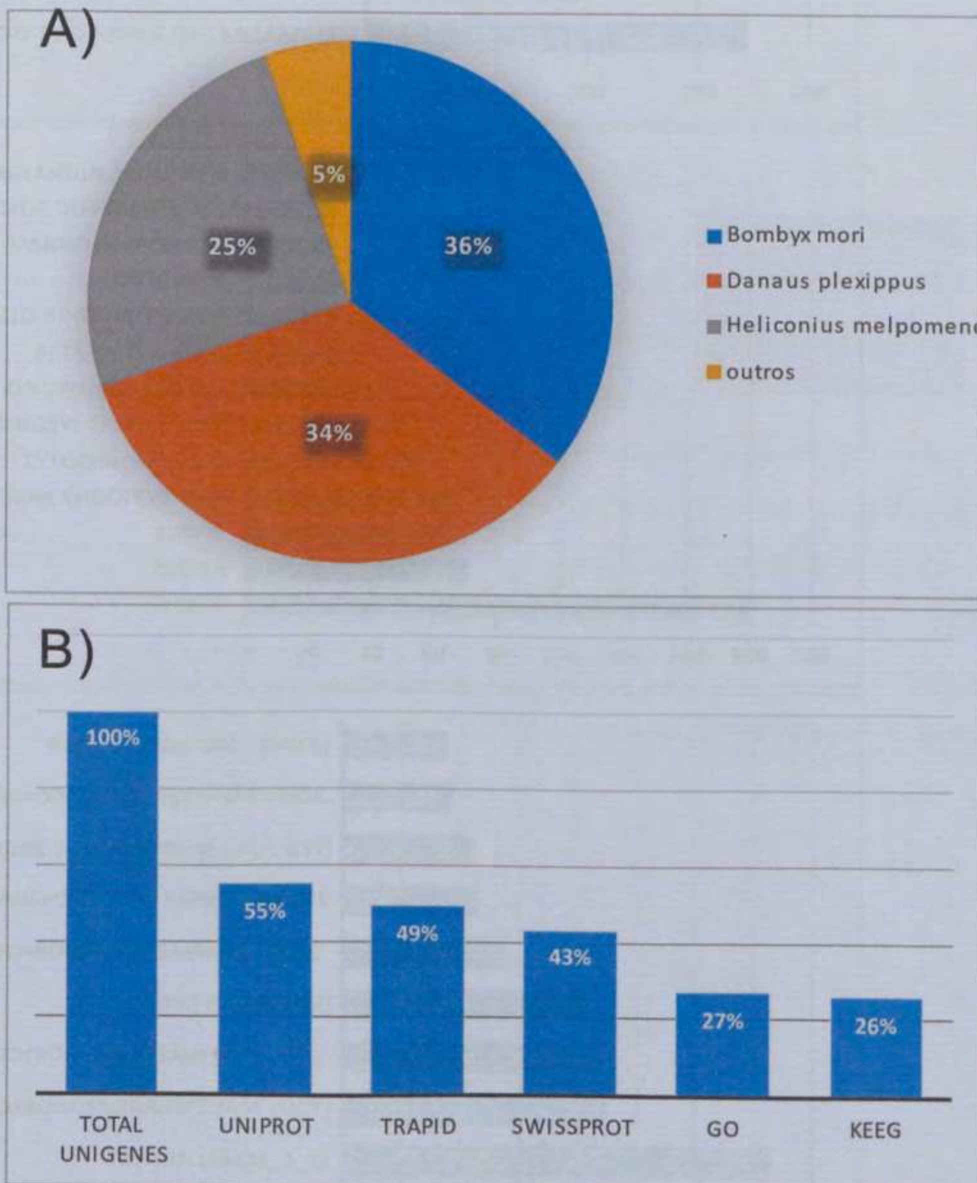
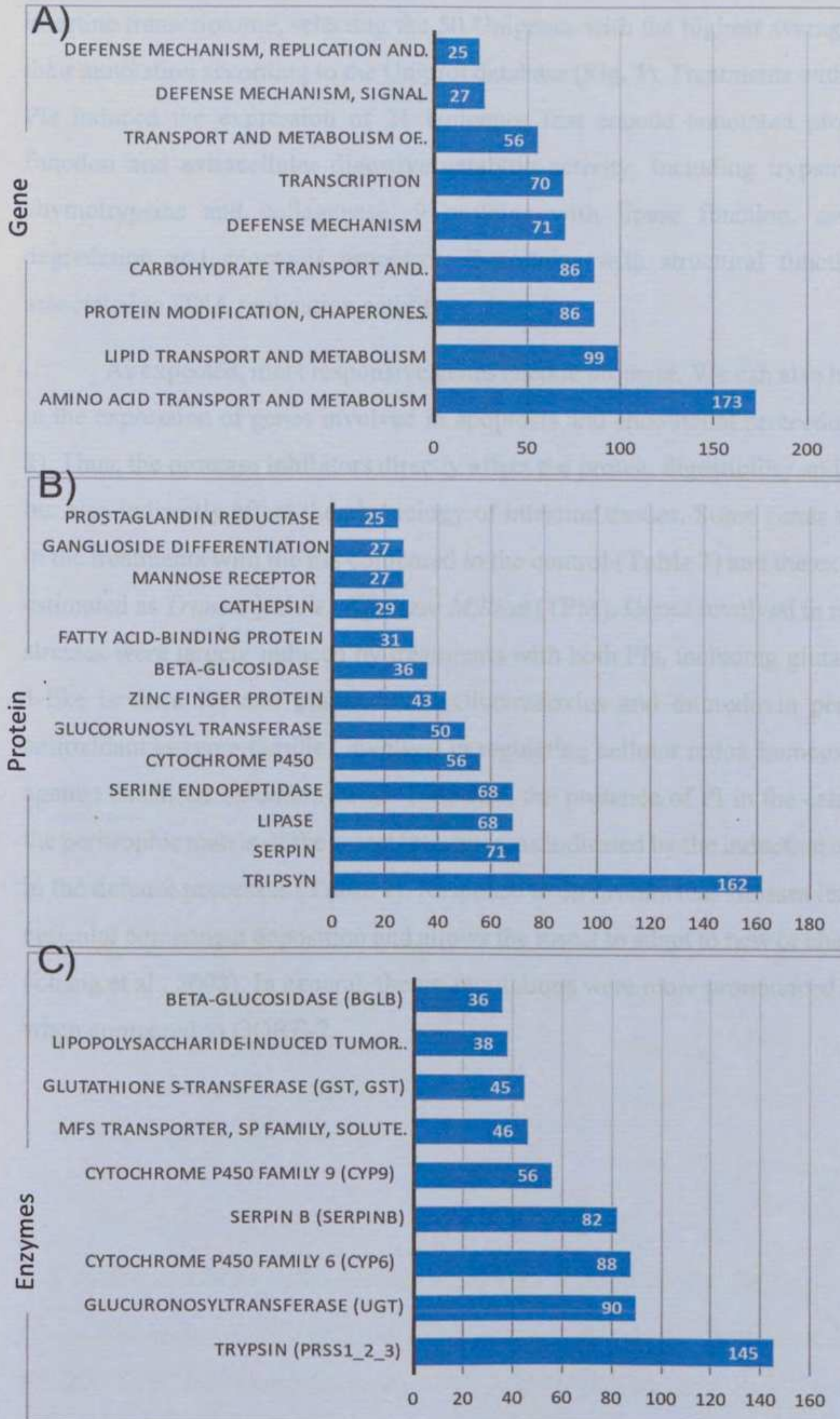


Figure 2. Distribution of frequency counts identified in the total (de Novo) transcriptome assembly approach A) Major gene families identified from the EggNog database using the TRAPID tool. B) The main annotations of proteins from the EggNog database by the TRAPID tool. C) The main enzymes annotated by the KAAS tool using the KEEG database as identification.



3.4. GORE-2 and SKTI inhibitors alter main protease expression

The average transcript counts per million (TPM) observed in the treatments with GORE-2 and with SKTI were used to evaluate the effect of these PIs on *A. gemmatalis* caterpillars intestine transcriptome, selecting the 50 Unigenes with the highest average TPM and that had their annotation according to the Uniprot database (**Fig. 3**). Treatments with GORE-2 and SKTI PIs induced the expression of 21 Unigenes that encode annotated proteins with protease function and extracellular digestive catalytic activity, including trypsins, serine proteases, chymotrypsins and collagenase, 9 proteins with lipase function, associated with lipid degradation and apoptosis processes; 7 proteins with structural function; and 4 proteins associated to DNA replication activity.

As expected, most responsive genes encode protease. We can also highlight the changes in the expression of genes involved in apoptosis and endothelial protection (**Fig. 3** and **Table 2**). Thus, the protease inhibitors directly affect the protein digestibility and protease expression but also indirectly affect the physiology of intestine tissues. Some genes were highly induced in the treatments with the PIs compared to the control (**Table 2**) and the expression levels were estimated as *Transcripts Per Kilobase Million* (TPM). Genes involved in response to oxidative stresses were largely induced by treatments with both PIs, including glutathione S-transferase 1-like isoform X1 and glutaredoxin. Glutaredoxins and thioredoxin peroxidases are major antioxidant enzyme families involved in regulating cellular redox homeostasis and in defense against enhanced oxidative stress. Likewise, the presence of PI in the caterpillar diet affected the peritrophic matrix of the insect intestines, as indicated by the induction of the genes involved in the defense processes (**Table 2**). Response to environmental stresses leads to an increase in cuticular component deposition and allows the insect to adapt to new or changing environments (Zhang et al., 2008). In general, the up-regulations were more pronounced in response to SKTI when compared to GORE-2.

Figure 3. Annotated Unigenes description with the highest normalized average expression rate in transcripts per million (TPM), obtained by the Kallisto and DESeq2 toolset, observing the effect of both treatments with protease inhibitors (GORE-2 and SKTI).

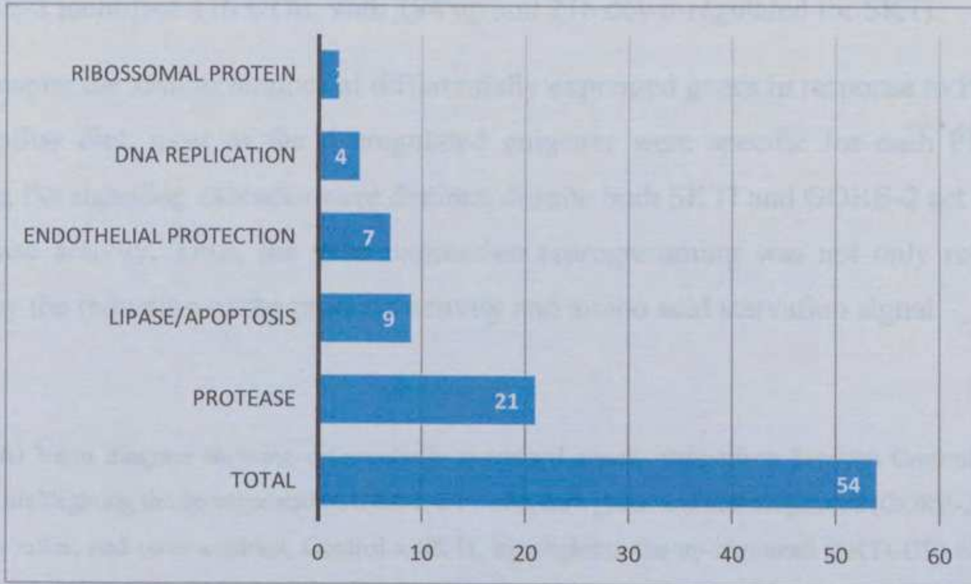


Table 2: Genes overexpression protection of *A. gemmatalis* peritrophic matrix related when fed with protease inhibitors (GORE-2 or SKTI) compared to the control.

target_id	TPM average SKTI	TPM average GORE-2	TPM average SKTI/GORE-2	function
TRINITY_DN360_c0_g3_i4	5965,37	11767,86667	8866,62	Putative cuticle protein CPH45
TRINITY_DN360_c0_g3_i2	1202,852667	2084,717333	1643,79	Putative cuticle protein CPH45
TRINITY_DN2772_c0_g1_i5	2064,003333	2478,556333	2271,28	Peritrophin type-A domain protein 2
TRINITY_DN2843_c0_g1_i1	1824,379333	1423,568	1623,97	Insect intestinal mucin
TRINITY_DN4963_c0_g2_i1	1530,629	1330,46	1430,54	glutathione S-transferase 1-like isoform X1
TRINITY_DN3649_c0_g1_i3	2493,056667	1622,8464	2057,95	Glutaredoxin
TRINITY_DN840_c0_g3_i1	5209,747333	795,9056667	3002,83	Chitin-binding protein
TRINITY_DN2469_c0_g1_i1	3650,155	702,9383333	2176,55	Chitin deacetylase 1

TPM : *Transcripts Per Kilobase Million*

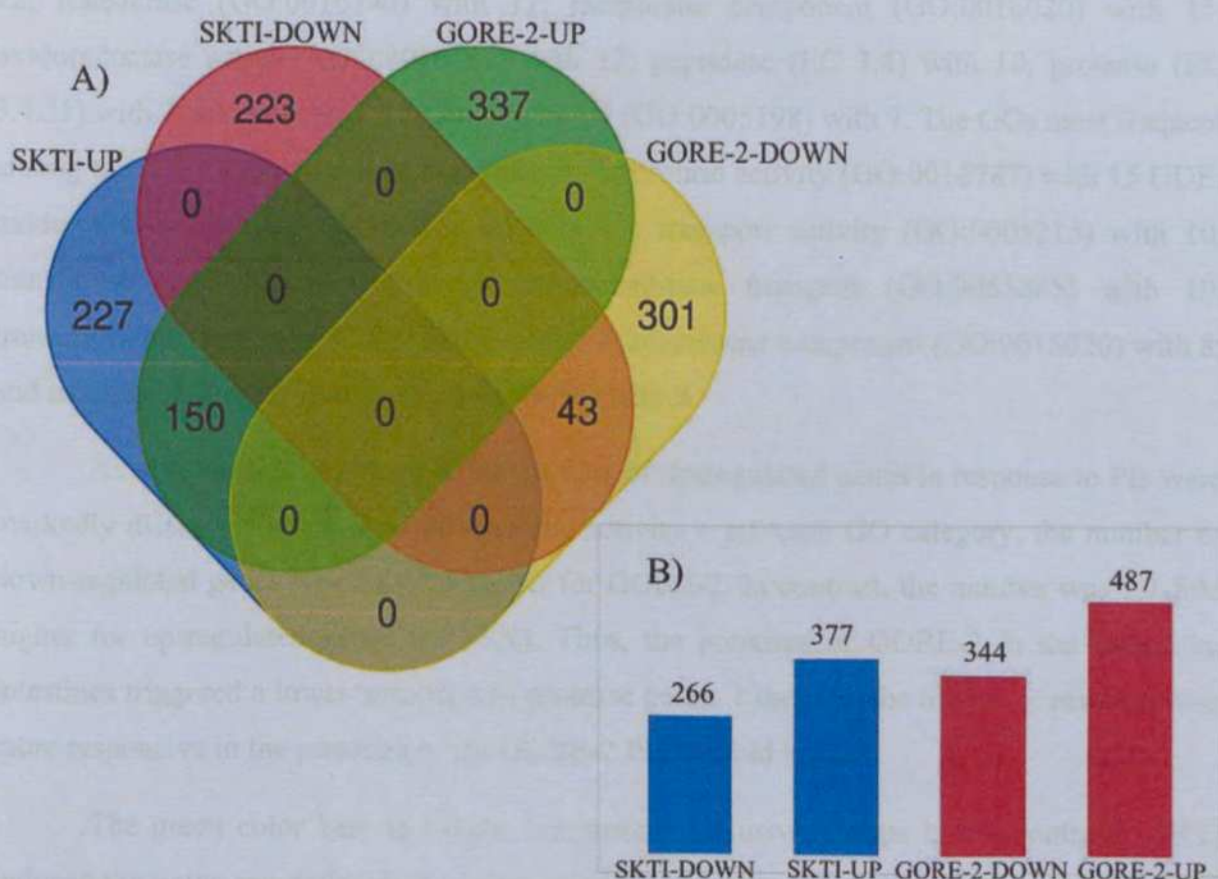
3.5. Differentially expressed transcripts were distinct for PIs SKTI and GORE-2

Comparison between SKTI and control identified 643 Unigenes differentially expressed (UDE), with 377 up-regulated and 266 down-regulated (**Fig. 4**). Overall, gene expression reprogramming was more pronounced in the presence of the tri-peptide GORE-2 than soybean

native protein inhibitor SKTI. The comparison between GORE-2 and control identified 831 UDE, with 487 up-regulated and 344 down-regulated. Likewise, the comparison between SKTI and GORE-2 identified 410 UDE, with 194 up and 216 down-regulated for SKTI.

Despite the similar number of differentially expressed genes in response to PI added to the caterpillar diet, most of the dysregulated unigenes were specific for each PI (**Fig. 4**). Therefore, the signaling cascades were distinct, despite both SKTI and GORE-2 act directly in the protease activity. Thus, the gene expression reprogramming was not only regulated or induced by the reduction of the protease activity and amino acid starvation signal.

Figure 4. A) Venn diagram showing differentially expressed genes, comparison between Control x GORE-2 treatments, highlighting the up-regulated (GORE-2-UP) with dark green and downregulated (GORE-2-DW) genes with yellow color, and other contrast, Control x SKTI, highlighting the up-regulated (SKTI-UP) with blue and downregulated (SKTI-DW) with pink color. B) The bar graph on the side shows the gene count of the respective contrasts Control x GORE-2 with red and Control x SKTI with blue color subdivided into up-regulated and down-regulated.



In the GORE-2 × Control contrast, 414 up-regulated and 425 down-regulated UDE were assigned to gene ontology (GO) terms. The most frequent GOs (**Fig. 5A and 5C**) among the up-regulated UDE are related to hydrolase activity (GO:0016787) with 27 UDE; transmembrane transport (GO:0055085) with 23; transport activity (GO:0005215) with 23; transferase (GO:0016740) with 20; oxidoreductase activity (GO:0016491) with 20 UDE; membrane component (GO:0016020) with 14; transcriptional regulation (GO:0005634) with 14; and catalytic activity - protease (GO:0140096) with 13. The most frequent GOs among the down-regulated UDE are related to hydrolase activity (GO:0016787) with 31; catalytic activity - protease (GO:0140096) with 21; transmembrane transport (GO:0055085) with 19; transport activity (GO:0005215) with 18; oxidoreductase activity (GO:0016491) with 17; transferase (GO:0016740) with 16; protein modifying process (GO:0036211) with 12; and lipid metabolism process (GO:0006629) with 11.

The analysis of SKTI × Control attributed GO terms to 333 UDE up-regulated and 272 down-regulated. The most frequent GOs (**Fig. 5B** and **5D**) among the up-regulated ones are related to hydrolase activity (GO:0016787) with 32 UDE; catalytic activity (GO:0140096) with 22; transferase (GO:0016740) with 17; membrane component (GO:0016020) with 15; oxidoreductase activity (GO:0016491) with 12; peptidase (EC 3.4) with 10; protease (EC 3.4.21) with 7; and structural molecular activity (GO:0005198) with 7. The GOs most frequent among the down-regulated ones are related to hydrolase activity (GO:0016787) with 15 UDE; oxidoreductase activity (GO:0016491) with 11; transport activity (GO:0005215) with 10; transferase (GO:0016740) with 10; transmembrane transport (GO:0055085) with 10; transcriptional regulation (GO:0005634) with 8; membrane component (GO:0016020) with 8; and catalytic activity - protease (GO:0140096) with 8.

As also verified in Figure 4, the profiles of dysregulated genes in response to PIs were markedly distinct (**Fig. 5**). For the catalytic activity – protease GO category, the number of down-regulated genes was 2.6-fold higher for GORE-2. In contrast, the number was 1.7-fold higher for up-regulated genes for SKTI. Thus, the presence of GORE-2 in the caterpillar intestines triggered a lower induction of protease genes. Likewise, the oxidative response was more responsive in the presence of the GORE-2 PI (1.7 fold higher).

The green color bars in Figure 5 represent exclusive groups in the contrasts. SKTI induced the down-regulation of 8 contigs involved in transcriptional regulation while GORE-2, in contrast, induced the up-regulation of 14 contigs. Thus, this result is additional evidence for distinct cascades triggered by SKTI and Gore 2 PIs. Likewise, 17 entries classified as

peptidases were up-regulated only for SKTI contrast. Furthermore, when comparing GO for SKTI-UP and GORE-2-UP, we observed contigs classified as RNA binding, splicing and proteasome process only in response to GORE-2 (**Fig. 5**).

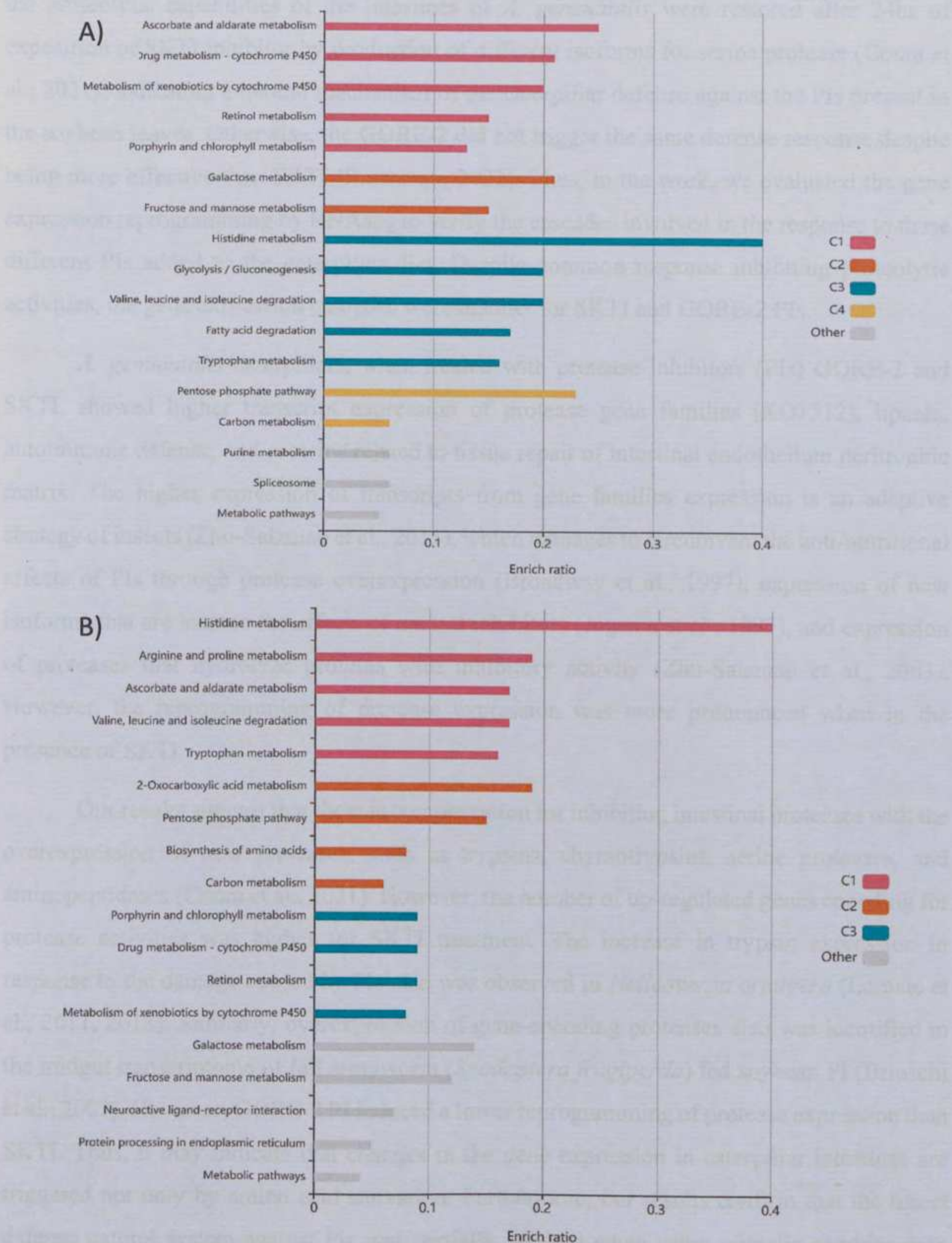
The metabolic pathways enrichment analysis identified four clusters of up-regulated pathways for the contrast GORE-2 x Control (**Fig. 6A**): autoimmune defense process, carbohydrate metabolism, amino acid and lipid metabolism, and metabolic processes. Three pathway clusters were up-regulated for the contrast SKTI x Control (**Fig. 6B**): amino acid metabolism, diverse metabolic processes, and autoimmune defense process. None down-regulated pathway had enriched for both treatments, GORE-2 and SKTI.

Despite some metabolic pathways showing enrichment for both PIs, the enrichment levels were distinct (**Fig. 6**). The levels were higher for pathways related to drug and xenobiotic by Cytochrome P450 metabolism when the caterpillars were fed with protease inhibitor GORE-2 (**Fig. 6B**). Thus, the presence of PIs in the intestines triggered metabolic response involved in biosynthesis of amino acid as expected, but also general processes of defense. These responses were more pronounced for treatment with GORE-2.

Furthermore, we observed higher enrichment for ascorbate and aldarate metabolism when the caterpillars were fed with the PI GORE-2, which is indicative high level of oxidative stress in the intestines. Ascorbic acid (AsA) is an essential member of antioxidants and also has functions in other enzymatic reactions and cellular processes, such as growth and mitosis.

Figure 5. The most frequent Representation and exclusive gene groups were classified according to GO (gene ontology). A) Main GO assigned to GORE-2-DW treatment, with green color bars representing 'exclusive groups in contrast to SKTI-DW. B) Main GO attributed to SKTI-DW treatment with green color bars represent exclusive groups in contrast to GORE-2-DW C) Main GO assigned to GORE-2-UP treatment, green color bars represent exclusive groups in contrast to SKTI-UP. D) Main GO attributed to the SKTI-UP treatment, with green color bars representing exclusive groups in contrast to GORE-2-UP.

Figure 6. Metabolic pathways enriched from differential expression. A) Contrast between Control x GORE-2 group. 4 groups are represented by genes associated with the same pathway functions. First pathway C1 (pink), second C2 (orange), third C3 (blue), fourth C4 (yellow), and a fifth group that did not have common functions. B) Contrast between Control x SKTI group have 4 groups are represented with genes associated with same pathway functions. First C1 (pink), second C2 (orange), third C3 (blue), and the fourth group that did not have common functions.



4. DISCUSSION

Tripeptídes showing protease inhibitory activity were designed from the natural PI SKTI produced by soybean plan. The tripeptide GORE-2 has been more effective in reducing protease activities and caterpillar survival when compared to the SKTI (Barros et., 2022). Furthermore, the proteolytic capabilities of the intestines of *A. gemmatalis* were restored after 24hs of exposition of SKTI inhibitor by production of different isoforms for serine protease (Coura et al., 2021), indicating a natural mechanism of the caterpillar defense against the PIs present in the soybean leaves. Otherwise, the GORE-2 did not trigger the same defense response despite being more effective than SKTI (Barros et., 2022). Thus, in the work, we evaluated the gene expression reprogramming by RNAseq to verify the cascades involved in the response to these different PIs added to the caterpillar diet. Despite common response inhibiting proteolytic activities, the gene expression cascades were distinct for SKTI and GORE-2 PIs.

A. gemmatalis caterpillars, when treated with protease inhibitors (PIs) GORE-2 and SKTI, showed higher transcript expression of protease gene families (KO1312), lipases, autoimmune defense, and proteins related to tissue repair of intestinal endothelium peritrophic matrix. The higher expression of transcripts from gene families expression is an adaptive strategy of insects (Zhu-Salzman et al., 2015), which manages to circumvent the anti-nutritional effects of PIs through protease overexpression (Broadway et al., 1997), expression of new isoforms that are inert to the effects of natural inhibitors (Jogsma et al., 1995), and expression of proteases that hydrolyze proteins with inhibitory activity (Zhu-Salzman et al., 2003). However, the reprogramming of protease expression was more pronounced when in the presence of SKTI.

Our results suggest that there is compensation for inhibiting intestinal proteases with the overexpression of new proteases, such as trypsins, chymotrypsins, serine proteases, and aminopeptidases (Coura et al., 2021). However, the number of up-regulated genes encoding for protease activities was higher for SKTI treatment. The increase in trypsin expression in response to the damage caused by PIs also was observed in *Helicoverpa armigera* (Lomate et al., 2011, 2018). Similarly, overexpression of gene-encoding proteases also was identified in the midgut transcriptome of fall armyworm (*Spodoptera frugiperda*) fed soybean PI (Brioschi et al., 2007). However, GORE-2 PI induced a lower reprogramming of protease expression than SKTI. Thus, it may indicate that changes in the gene expression in caterpillar intestines are triggered not only by amino acid starvation. Furthermore, our results confirm that the insect defense natural system against PIs was partially induced when using mimetic peptides with

reduced length, as also verified by (Barros et al., 2022). Differences in the protease inhibitory capabilities were justified by a lower induction of the expression of different protease isoforms in the presence of GORE-2 than SKTI. Thus, the intestines of *A. gemmatalis* evolved to induce an effective system of perception signal, leading to changes in the protease expression. This system was partially induced by the presence of GORE-2 inhibitor.

RNASeq has been widely used to identify and elucidate mechanisms by which phytophagous insects respond to PIs effects (Sousa et al., 2016; Lin et al., 2017; Lomate et al., 2018) or the use from *Bacillus thuringiensis* toxin derived (Sosa-Goméz et al., 2012; Dhania et al., Pezenti et al., 2021). This tool presents data with a high degree of reproducibility, leading to a precise quantification of expression levels (Schurch et al., 2016). Among the highly expressed transcripts, we found 7 Unigenes that encode proteins related to tissue intestinal endothelium repair. One Unigene encodes the peritrophin protein secreted by intestinal cells, and that composes the peritrophic matrix, which is responsible for the protection, compartmentalization, and permeability of insect gut cells (Terra et al., 2001). Another Unigene encodes the chitin deacetylase, which also plays a relevant function in chitin metabolism, which protects the intestine against mechanical damage, infections, and pathogen attacks (Alvarenga et al., 2016). Unigenes-encoding cuticle proteins are also highly expressed and, associated with chitin, reinforce intestinal protection by forming a physical barrier adhered to the peritrophic matrix (Muthukrishnan et al., 2019; Daquila et al., 2019). The expression of Unigenes that encode mucin glycoproteins increased after treatment fed with PIs, and these proteins are secreted during digestion, protecting tissue integrity by preventing the microorganism's adhesion and the action of enzymes in the local tissue (Fang et al., 2009). Our results suggest that the treatment with PIs induced the reprogramming of unigene expression aiming to protect the *A. gemmatalis* caterpillars' intestinal tissue cells.

The Unigenes that encode peritrophin protein had their expression up-regulated in response to the action of protease inhibitors GORE-2 and SKTI, and this protein is responsible for the peritrophic membrane synthesis that composes the peritrophic matrix. This structure has other protein and glycans components and plays significant roles in the caterpillar intestine protection, in addition to containing digestive enzymes released into the midgut lumen, which also aids the chitin synthesis (Ryerse et al., 1994). A similar result was proposed by Liu et al. (2004) when investigating insect larvae *Diabrotica undecimpunctata*, which attacks corn, subjected to cysteine PIs, and also observed a higher protein expression such as peritrophins

and chitins. This observed response appears to be a mechanism of caterpillar defense and it leads to rapid peritrophic membrane recycling and promotes intestinal epithelium protection.

Chitin metabolism and cuticle synthesis are significant for insect digestion and their innate defense. These structures actively participate in mechanical and chemical digestion by releasing digestive enzymes (Terra et al., 2009). Studies involving PIs in the *Helicoverpa armigera* diet and inducing protease expression also observed that PIs have cross-protective structures and accumulate in the peritrophic membrane and intestinal epithelium surface (Lomate et al., 2018). Some studies also highlight the association of PIs with lecithins to amplify the cellular damage caused to the phytophages insect intestine (Napoleão et al., 2018). Li et al. (2009) observed that Drosophila flies (Diptera) had up-regulated genes related to chitin and cuticle metabolism in response to cellular damage caused in microvilli and the peritrophic matrix structural integrity. The work of our research group also confirms these observations, in which the immune system seeks to regenerate the structural protein synthesis through the gene overexpression associated with the production of chitin, peritrophin, and cuticle protein.

Other highly induced genes by PIs on SKTI and GORE-2 treatment, encode for mucins. Mucins are proteins structure rich in proline, threonine, and serine amino acids associated with a high glycosylation content and are part of the insect's peritrophic matrix. Mucins protect epithelial intestine cells from physical and chemical damage, lubricate the food passage, protect against dehydration, and prevent autophagy through digestive enzymes (Toprak et al., 2010). Studies involving different fed stages of *Mamestra configurata* larvae observed the variation in the expression of the mucin-associated protein, suggesting that the regulation of mucin synthesis, and the structure of the peritrophic matrix, is a reflection of metabolic stress (Toprak et al., 2010). In this regulation, the thickness of the peritrophic matrix and its associated proteins are altered by secreted digestive enzymes, such as serine proteases and trypsins, as the production of the excess enzymes can trigger autophagy and degrade mucins that are rich in serine (Taprok et al., 2010; Napoleão et al., 2018).

Several studies seek to elucidate the mechanisms by which protease inhibitors act in the insect digestive system and how they react to the anti-nutritional effect and cellular damage of the intestinal epithelium (Silva-Júnior et al., 2019; 2021). These studies observed that the immune system activation increases protein expression involved in chitin metabolism, peritrophic membrane synthesis, and cuticle modulation, where all structures are part of the protection of the digestive tract and components of the midgut peritrophic matrix, which is the main target of PI action (Nascimento et al., 2015; Mohan et al., 2006). The results presented in

this work suggest that PIs decrease the availability of essential amino acids and affect the peritrophic matrix.

Gene expression results also indicated that the presence of PIs in the caterpillar intestines promoted oxidative stresses and triggered biochemical defense mechanisms. Our results indicated that many enriched genes were involved in the ascorbate-glutathione. Environmental stress could lead to the accumulation of reactive oxygen species (ROS). The ascorbate-glutathione cycle appeared to play an important role in detoxifying ROS.

The enrichment levels were higher for pathways related to metabolism to drugs and xenobiotics by Cytochrome P450 when the caterpillars were fed with protease inhibitor GORE-2. Cytochrome P450 (CYP450), a superfamily of heme-thiolate proteins, is involved primarily in the metabolism of endogenous and exogenous substances, such as pheromones and insecticides. This result is in accordance with a higher reduction of the caterpillar survival by GORE-2 compared to SKTI (Barros et al., 2022; Silva-Júnior et al., 2022). Experiments on insecticide treatments in bees have shown that the immune response activates the CYP9E2 gene, which has a detoxification function. Expression of genes involved in immune response was also changed by PI treatment, with 82 entries classified as serpin proteins.

5. CONCLUSION

The presence of the PIs SKTI and GORE-2 in the caterpillar diet triggered extensive gene expression reprogramming after 24 hours. In general, the responses were similar for both PIs with changes in the expression of the genes-encoding proteases and genes related to the defense mechanisms of the intestinal tract and mainly protection and matrix peritrophic reconstitution. However, these responses were more pronounced for SKTI indicating a signaling cascade more efficient to trigger the insect defense mechanisms. These differences justified a more effective action of the GORE-2 in proteolysis inhibition and reduction of caterpillar survival, compared to SKTI. The results also indicate that the signaling may be due the amino acid starvation but also by a perception general system developed to detect deterrent molecules produced by soybean plants. As GORE-2 is a protease inhibitor mimetic tripeptide, this signaling cascade appeared to be minus efficient. Thus, an additional advantage of the use of mimetic or synthetic protease inhibitors, as also verified by Coura et al., 2021.

The effect of the synthetic (GORE-2) and natural (SKTI) inhibitors on the *A. gemmatalis* caterpillars' intestinal tissue is the overexpression of genes-encoding proteases and genes related to the defense mechanisms of the intestinal tract and mainly protection and matrix peritrophic reconstitution. These results contribute to a better understanding of the plant-pest interaction mechanism and to the development of new strategies that are more effective in combating many pests.

6. REFERENCES

A. Jongsma, M., & Beekwilder, J. (2011). Co-Evolution of Insect Proteases and Plant Protease Inhibitors. *Current Protein & Peptide Science*, *12*(5). https://doi.org/10.2174/138920311796391115

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3). https://doi.org/10.1016/S0022-2836(05)80360-2

Alvarenga, E. S. L., Mansur, J. F., Justi, S. A., Figueira-Mansur, J., dos Santos, V. M., Lopez, S. G., Masuda, H., Lara, F. A., Melo, A. C. A., & Moreira, M. F. (2016). Chitin is a component of the Rhodnius prolixus midgut. *Insect Biochemistry and Molecular Biology*, *69*. https://doi.org/10.1016/j.ibmb.2015.04.003

Andrews, S. (2010). FastQC - A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.*BabrahamBioinformatics*

Apweiler, R., Bateman, A., Martin, M. J., O'Donovan, C., Magrane, M., Alam-Faruque, Y., Alpi, E., Antunes, R., Arganiska, J., Casanova, E. B., Bely, B., Bingley, M., Bonilla, C., Britto, R., Bursteinas, B., Chan, W. M., Chavali, G., Cibrian-Uhalte, E., da Silva, A., … Zhang, J. (2014). Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Research*, *42*(D1). https://doi.org/10.1093/nar/gkt1140

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15). https://doi.org/10.1093/bioinformatics/btu170

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, *34*(5). https://doi.org/10.1038/nbt.3519

Brioschi, D., Nadalini, L. D., Bengtson, M. H., Sogayar, M. C., Moura, D. S., & Silva-Filho, M. C. (2007). General up regulation of Spodoptera frugiperda trypsins and chymotrypsins allows its adaptation to soybean proteinase inhibitor. *Insect Biochemistry and Molecular Biology*, *37*(12). https://doi.org/10.1016/j.ibmb.2007.07.016

Broadway, R. M. (1997). Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. *Journal of Insect Physiology*, *43*(9). https://doi.org/10.1016/S0022-1910(97)00028-0

Campos, E. V. R., Proença, P. L. F., Oliveira, J. L., Bakshi, M., Abhilash, P. C., & Fraceto, L. F. (2019). Use of botanical insecticides for sustainable agriculture: Future perspectives. *Ecological Indicators*, *105*. https://doi.org/10.1016/j.ecolind.2018.04.038

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*(18). https://doi.org/10.1093/bioinformatics/bti610

Coura, R. R., da Silva Junior, N. R., Meriño-Cabrera, Y., Díez, J. D. R., Barros, R. A., Barbosa, S. L., de Oliveira, J. V. A., da Rocha, G. C., Serrão, J. E., de Oliveira Ramos, H. J., & de Almeida Oliveira, M. G. (2022). Extensive reprogramming of protein isoforms and

histopathological alterations in the midgut of Anticarsia gemmatalis fed with protease inhibitors. *Annals of Applied Biology*, *180*(3). https://doi.org/10.1111/aab.12740

Daquila, D. V., bergamo, r. H. S., oliveira junior, f. D. D., varoni, m. V., nanya, s., & conte, h. (2019). Análise ultraestrutural do intestino médio em larvas da *Diatraea saccharalis fabricius*, 1794 (lepidoptera: crambidae) tratadas com *Bacillus thuringiensis* (bacillales: bacillaceae).

da Silva Fortunato, F., de Almeida Oliveira, M. G., Brumano, M. H. N., Silva, C. H. O., Guedes, R. N. C., & Moreira, M. A. (2007). Lipoxygenase-induced defense of soybean varieties to the attack of the velvetbean caterpillar (Anticarsia gemmatalis Hübner). *Journal of Pest Science*, *80*(4). https://doi.org/10.1007/s10340-007-0179-4

de Almeida Barros, R., Meriño-Cabrera, Y., Severiche Castro, J. G., Rodrigues da Silva Júnior, N., Schultz, H., de Andrade, R. J., Aguilar de Oliveira, J. v., de Oliveira Ramos, H. J., & de Almeida Oliveira, M. G. (2022). Inhibition constant and stability of tripeptide inhibitors of gut trypsin-like enzyme of the soybean pest Anticarsia gemmatalis. *Archives of Insect Biochemistry and Physiology*, *110*(2). https://doi.org/10.1002/arch.21887

Dhania, N. K., Chauhan, V. K., Chaitanya, R. K., & Dutta-Gupta, A. (2019). Midgut de novo transcriptome analysis and gene expression profiling of Achaea janata larvae exposed with Bacillus thuringiensis (Bt)-based biopesticide formulation. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, *30*. https://doi.org/10.1016/j.cbd.2019.02.005

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19). https://doi.org/10.1093/bioinformatics/btw354

Fang, S., Wang, L., Guo, W., Zhang, X., Peng, D., Luo, C., Yu, Z., & Sun, M. (2009). Bacillus thuringiensis bel protein enhances the toxicity of Cry1Ac protein to Helicoverpa armigera larvae by degrading insect intestinal mucin. *Applied and Environmental Microbiology*, *75*(16). https://doi.org/10.1128/AEM.00532-09

Felix Krueger. (2021). *TrimGalore*. Https://Github.Com/FelixKrueger/TrimGalore#readme

Fernandes, F. O., Abreu, J. Á. de, Christ, L. M., & Rosa, A. P. S. A. da. (2018). Insecticides Management Used in Soybean for the Control of Anticarsia gemmatalis (Hübner, 1818) (Lepidoptera: Eribidae). *Journal of Agricultural Science*, *10*(11). https://doi.org/10.5539/jas.v10n11p223

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L. L., Tate, J., & Punta, M. (2014). Pfam: The protein families database. In *Nucleic Acids Research* (Vol. 42, Issue D1). https://doi.org/10.1093/nar/gkt1223

Finn, R. D., Clements, J., Arndt, W., Miller, B. L., Wheeler, T. J., Schreiber, F., Bateman, A., & Eddy, S. R. (2015). HMMER web server: 2015 Update. *Nucleic Acids Research*, *43*(W1). https://doi.org/10.1093/nar/gkv397

Fu, L., Niu, B., Zhu, Z., Wu, S., & Li, W. (2012). CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics*, *28*(23). https://doi.org/10.1093/bioinformatics/bts565

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7). https://doi.org/10.1038/nbt.1883

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M., Macmanes, M. D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C. N., … Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, *8*(8). https://doi.org/10.1038/nprot.2013.084

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., Rattei, T., Mende, D. R., Sunagawa, S., Kuhn, M., Jensen, L. J., von Mering, C., & Bork, P. (2016). EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research*, *44*(D1). https://doi.org/10.1093/nar/gkv1248

Jongsma, M. A., Bakker, P. L., Peters, J., Bosch, D., & Stiekema, W. J. (1995). Adaptation of Spodoptera exigua larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(17). https://doi.org/10.1073/pnas.92.17.8041

Kuwar, S. S., Pauchet, Y., Vogel, H., & Heckel, D. G. (2015). Adaptive regulation of digestive serine proteases in the larval midgut of Helicoverpa armigera in response to a plant protease inhibitor. *Insect Biochemistry and Molecular Biology*, *59*. https://doi.org/10.1016/j.ibmb.2015.01.016

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4). https://doi.org/10.1038/nmeth.1923

Li, H. M., Sun, L., Mittapalli, O., Muir, W. M., Xie, J., Wu, J., Schemerhorn, B. J., Sun, W., Pittendrigh, B. R., & Murdock, L. L. (2009). Transcriptional signatures in response to wheat germ agglutinin and starvation in Drosophila melanogaster larval midgut. *Insect Molecular Biology*, *18*(1). https://doi.org/10.1111/j.1365-2583.2008.00844.x

Lin, H., Lin, X., Zhu, J., Yu, X. Q., Xia, X., Yao, F., Yang, G., & You, M. (2017). Characterization and expression profiling of serine protease inhibitors in the diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae). *BMC Genomics*, *18*(1). https://doi.org/10.1186/s12864-017-3583-z

Liu, Y., Salzman, R. A., Pankiw, T., & Zhu-Salzman, K. (2004). Transcriptional regulation in southern corn rootworm larvae challenged by soyacystatin N. *Insect Biochemistry and Molecular Biology*, *34*(10). https://doi.org/10.1016/j.ibmb.2004.06.010

Lomate, P. R., Dewangan, V., Mahajan, N. S., Kumar, Y., Kulkarni, A., Wang, L., Saxena, S., Gupta, V. S., & Giri, A. P. (2018). Integrated transcriptomic and proteomic analyses suggest the participation of endogenous protease inhibitors in the regulation of protease gene expression in helicoverpa armigera. *Molecular and Cellular Proteomics*, *17*(7). https://doi.org/10.1074/mcp.RA117.000533

Lomate, P. R., & Hivrale, V. K. (2011). Differential responses of midgut soluble aminopeptidases of Helicoverpa armigera to feeding on various host and non-host plant diets. *Arthropod-Plant Interactions*, *5*(4). https://doi.org/10.1007/s11829-011-9140-9

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12). https://doi.org/10.1186/s13059-014-0550-8

Mendonça, E. G., de Almeida Barros, R., Cordeiro, G., da Silva, C. R., Campos, W. G., de Oliveira, J. A., & de Almeida Oliveira, M. G. (2020). Larval development and proteolytic activity of Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) exposed to different soybean protease inhibitors. *Archives of Insect Biochemistry and Physiology*, *103*(1). https://doi.org/10.1002/arch.21637

Mohan, S., Ma, P. W. K., Pechan, T., Bassford, E. R., Williams, W. P., & Luthe, D. S. (2006). Degradation of the S. frugiperda peritrophic matrix by an inducible maize cysteine protease. *Journal of Insect Physiology*, *52*(1). https://doi.org/10.1016/j.jinsphys.2005.08.011

Muthukrishnan, S., Merzendorfer, H., Arakane, Y., & Yang, Q. (2019). Chitin organizing and modifying enzymes and proteins involved in remodeling of the insect cuticle. In *Advances in Experimental Medicine and Biology* (Vol. 1142). https://doi.org/10.1007/978-981-13-7318-3_5

Nascimento, A. R. B. do, Fresia, P., Cônsoli, F. L., & Omoto, C. (2015). Comparative transcriptome analysis of lufenuron-resistant and susceptible strains of Spodoptera frugiperda (Lepidoptera: Noctuidae). *BMC Genomics*, *16*(1). https://doi.org/10.1186/s12864-015-2183-z

Pardo-López, L., Soberón, M., & Bravo, A. (2013). Bacillus thuringiensis insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. In *FEMS Microbiology Reviews* (Vol. 37, Issue 1). https://doi.org/10.1111/j.1574-6976.2012.00341.x

Pezenti, L. F., Sosa-Gómez, D. R., de Souza, R. F., Vilas-Boas, L. A., Gonçalves, K. B., da Silva, C. R. M., Vilas-Bôas, G. T., Baranoski, A., Mantovani, M. S., & da Rosa, R. (2021). Transcriptional profiling analysis of susceptible and resistant strains of Anticarsia gemmatalis and their response to Bacillus thuringiensis. *Genomics*, *113*(4). https://doi.org/10.1016/j.ygeno.2021.05.012

Pilon, A. M., Campos, W. G., Silva, C. R., Cordeiro, G., Silva, C. R., & Oliveira, M. G. A. (2018). Protease inhibitory, insecticidal, and deterrent effects of the trypsin-inhibitor benzamidine on the velvet bean caterpillar in soybean. *Anais da Academia Brasileira de Ciências*, 90, 3475-3482. https://doi.org/10.1590/0001-3765201820180159

Resh, V. H. (2009). Encyclopedia of Insects. In *Encyclopedia of Insects*. https://doi.org/10.1016/B978-0-12-374144-8.X0001-X

Ryerse, J. S., Purcell, J. P., & Sammons, R. D. (1994). Structure and formation of the peritrophic membrane in the larva of the southern corn rootworm, Diabrotica undecimpunctata. *Tissue and Cell*, *26*(3). https://doi.org/10.1016/0040-8166(94)90026-4

Shakeel, M., & Zafar, J. (2020). Molecular identification, characterization, and expression analysis of a novel trypsin inhibitor-like cysteine-rich peptide from the cotton bollworm, Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). *Egyptian Journal of Biological Pest Control*, 30(1), 1-7. https://doi.org/10.1590/1519-6984.223579

Silva-Júnior, N. R., Cabrera, Y. M., Barbosa, S. L., Barros, R. de A., Barros, E., Vital, C. E., Ramos, H. J. O., & Oliveira, M. G. A. (2021). Intestinal proteases profiling from Anticarsia gemmatalis and their binding to inhibitors. *Archives of Insect Biochemistry and Physiology*, *107*(3). https://doi.org/10.1002/arch.21792

Song, L., & Florea, L. (2015). Rcorrector: Efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience*, *4*(1). https://doi.org/10.1186/s13742-015-0089-y

Sosa-Gómez, D. R., Corrêa-Ferreira, B. S., Hoffmann-Campo, C. B., Corso, I. C., Oliveira, L. J., Moscardi, F., ... & Roggia, S. (2014). Manual de identificação de insetos e outros invertebrados da cultura da soja. http://www.infoteca.cnptia.embrapa.br/infoteca/handle/doc/991685

Souza, T. P., Dias, R. O., Castelhano, E. C., Brandão, M. M., Moura, D. S., & Silva-Filho, M. C. (2016). Comparative analysis of expression profiling of the trypsin and chymotrypsin genes from Lepidoptera species with different levels of sensitivity to soybean peptidase inhibitors. *Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology*, *196–197*. https://doi.org/10.1016/j.cbpb.2016.02.007

Stevens, J. A., Dunse, K. M., Guarino, R. F., Barbeta, B. L., Evans, S. C., West, J. A., & Anderson, M. A. (2013). The impact of ingested potato type II inhibitors on the production of the major serine proteases in the gut of Helicoverpa armigera. *Insect Biochemistry and Molecular Biology*, *43*(2). https://doi.org/10.1016/j.ibmb.2012.11.006

Terra, W. R. (2001). The origin and functions of the insect peritrophic membrane and peritrophic gel. *Archives of Insect Biochemistry and Physiology*, *47*(2). https://doi.org/10.1002/arch.1036

Toprak, U., Baldwin, D., Erlandson, M., Gillottt, C., & Hegedus, D. D. (2010). Insect intestinal mucins and serine proteases associated with the peritrophic matrix from feeding, starved and moulting Mamestra configurata larvae. *Insect Molecular Biology*, *19*(2). https://doi.org/10.1111/j.1365-2583.2009.00966.x

van Bel, M., Proost, S., van Neste, C., Deforce, D., van de Peer, Y., & Vandepoele, K. (2013). TRAPID: An efficient online tool for the functional and comparative analysis of de novo RNA-Seq transcriptomes. *Genome Biology*, *14*(12). https://doi.org/10.1186/gb-2013-14-12-r134

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C. Y., & Wei, L. (2011). KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Research*, *39*(SUPPL. 2). https://doi.org/10.1093/nar/gkr483

Zhu-Salzman, K., Koiwa, H., Salzman, R. A., Shade, R. E., & Ahn, J. E. (2003). Cowpea bruchid Callosobruchus maculatus uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. *Insect Molecular Biology*, *12*(2). https://doi.org/10.1046/j.1365-2583.2003.00395.x

Zhu-Salzman, K., & Zeng, R. (2015). Insect response to plant defensive protease inhibitors. In *Annual Review of Entomology* (Vol. 60). https://doi.org/10.1146/annurev-ento-010814-020816

CAPÍTULO II: Protease inhibitor tripeptide designed from natural SKTI differentially promoted protease expression, oxidative stress response and histological alterations in the midgut of *Anticarsia gemmatalis*

ABSTRACT

The physiological effects of PIs are not limited to nutritional damage to the insect. They lead to biochemical responses that can range from redirecting protein synthesis to produce new proteases to increasing the production of enzymes involved in defense pathways. Thus, the effects of the natural soybean PI (SKTI) and the rationally designed peptide GORE-2 were evaluated at the level of gene expression changes caused by PIs and their relationship with oxidative stress and histological alterations of the intestine cells. The presence of PI in the diet of *A. gemmatalis* handled 1474 differentially expressed genes, of which 643 and 831 genes responded to SKTI and GORE-2, respectively. Among the dysregulated genes to the PIs, 62 code for proteases, being 38 genes responsive to SKTI and 43 to GORE-2. However, some dysregulated protease genes were exclusive for each PIs, indicating different regulatory cascades in response to SKTI and GORE-2. This difference justified a more efficient gene expression reprograming triggered by SKTI to restore the protease activity than GORE-2. The activities of the main oxidative metabolism catalase (CAT), peroxidase (POX), glutathione S-transferase (GST) and superoxide dismutase (SOD) increased in response to both PIs. However, the activity levels were higher in the presence of SKTI, indicating that the antioxidant defense system was more active in response to SKTI. The fact that *A. gemmatalis* responds more efficiently to SKTI shows a better adaptability to the natural soybean inhibitor when compared to the tripeptide, revealing a valuable potential for the GORE-2 as an insecticide, as the cellular machinery was less responsive to the recognition and overcoming of the negative effects of tripeptide. In this sense, the tripeptide GORE-2 presents itself as an efficient alternative in the control of *A. gemmatalis*.

Keywords: coevolution, plant-pest interaction, resistance

1. INTRODUCTION

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) is a key pest in soybean, responsible for severe defoliation in the crop and great economic losses (Lima et al., 2020; Walker et al., 2000). The control of this caterpillar has been carried out by organic insecticides (Fiaz et al., 2018; Levy et al., 2007), sometimes associated with integrated pest management (Bueno et al., 2011; Panizzi, 2013) and transgenic soybean varieties that express entomotoxic proteins from *Bacillus thuringiensis* (Bt) (Bravo et al., 2007; Paula et al., 2014). However, the excessive use of pesticides affects non-target animals, and compromises the water table and the food chain, with consequences for human health (Campos et al., 2019; S. Sousa et al., 2020). In addition, conventional insecticides have selected resistant populations (Coelho et al., 2020; Horowitz et al., 2020; Jahan et al., 2008), making the development of alternative pest control technologies increasingly urgent.

Faced with this problem, protease inhibitors (PI) have emerged as an alternative to non-persistent molecules in the environment and are safer for non-target organisms, including natural enemies of agricultural and human pests. PIs are proteins or metabolites capable of interacting with proteolytic enzymes, compromising the digestibility and protein utilization of the animal (Pontual et al., 2012). This reduction in catalytic activity affects the development and reproductive success of the insect (Franco et al., 2004). Despite causing damage to the insect, natural PIs of protein origin (e.g., SKTI - soybean Kunitz trypsin inhibitor) are large molecules that have cleavage sites for other proteases (Singh et al., 2020; Yang et al., 2009; Zhao et al., 2010). In addition, insects express isoforms insensitive to many natural PIs (Coura et al., 2021; Silva-Júnior et al., 2021). To overcome the insect's adaptive mechanisms, we rationally designed the tripeptide GORE-2 (Val-Leu-Arg) to effectively bind to the *A. gemmatalis* gut trypsin-like catalytic triad as a potent PI (de Almeida Barros et al., 2021).

The physiological effects of PIs are not limited to the nutritional damage to the insect. They lead to biochemical responses that can range from redirecting protein synthesis to producing new proteases (Coura et al., 2021) to increasing the production of enzymes involved in the body's detoxification pathways (Farahani et al., 2020). The evolutionary success of insects on Earth largely results from a complex and efficient defense system based on antioxidant enzymes. At very low levels, reactive oxygen species (ROS) play a vital role in cell signaling and defense (Lijun et al., 2005; Wang et al., 2006); however, higher levels are harmful to DNA and proteins. During oxidative stress, the insect protects itself against ROS by the

catalysis of antioxidant enzymes. Because of this, increased activity of antioxidant enzymes in insects is often associated with insecticide resistance (Jahan et al., 2008).

Enzymes involved in lepidopteran oxidative stress respond to biotic stressors such as parasites (Dubovskiy et al., 2008; Farahani et al., 2020) and resistant plants (Aucoin et al., 1991; War et al., 2011, 2012). These enzymes are also reported to respond to abiotic stressors such as excess UV radiation. (Arif Ali et al., 2017), temperature and starvation (A. Ali et al., 2017; Farahani et al., 2020), besides exposure to organophosphate insecticides (Shadnia et al., 2005; Q. Y. Yu et al., 2011). In this sense, the enzymes catalase (CAT), peroxidase (POX), glutathione S-transferase (GST), and superoxide dismutase (SOD) are the main biomarkers of oxidative stress in insects. (Arif Ali et al., 2017; Farahani et al., 2020; Jia et al., 2011; Meng et al., 2009; Perić-Mataruga et al., 2019).

Therefore, we evaluated the effects of natural soybean protease inhibitor (SKTI) and the tripeptide inhibitor GORE-2 on the modulation of the expression of genes involved in the digestive process and the insect's defense mechanism. These changes were related to the activities of antioxidant and detoxifying enzymes and histological alterations in the insect's gut tissues.

2. MATERIAL AND METHODS

2.1. Insects and preparation of the artificial diet containing PIs

A. gemmatalis caterpillars were obtained from the Insects Laboratory of the Department of Biochemistry and Molecular Biology of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil, and maintained under controlled conditions of 25 ± 2 °C temperature, 70 ± 10% relative humidity and 12 h photophase. After egg hatching, the caterpillars were fed on an artificial diet according to Greene et al. (1976). The fifth instar larvae were separated in cages 3 x 5 cm and fed ad libitum on a diet (control) and diets supplemented with sublethal PIs concentration: 0.12% (w/w) of serine-protease Kunitz (SKTI) (treatment 1) and 0,12% (w/w) of GORE-2 (treatment 2), as described by Chougule et al. (2008). After 24 h, the caterpillars were collected from each treatment and control. The intestines were removed and stored at -80 °C.

2.2. Protein extraction from the caterpillar intestines

The midguts of fifth instar caterpillars were dissected and placed in 1 mL of 10−3 M hydrochloric acid solution at 4 °C, always maintaining the ratio of five intestines per 1 mL solution, followed by maceration on TissueLyser II (Qiagen). The extract was centrifuged at 10,000 g for 30 min at 4 °C, and the pellets were discarded. The supernatants containing the soluble material were separated and stored at -20 °C.

2.3. RNA Extraction and Sequencing

The treatments comprising control caterpillars, those fed with GORE2, and those fed with SKTI, were composed of three biological and technical replicates each. Total RNA from each treatment was isolated from the midgut of *A. gemmatalis* using TRIzol reagent (Invitrogen), following the manufacturer's instructions. DNA contamination was eliminated from the RNA samples using RNase-free DNase I (New England Biolabs). The purity and quantification of total RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific) and a Qubit 2.0 Fluorometer (Life Technologies). RNA integrity was verified using the RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 system (Agilent Technologies). High-quality total RNA isolated from independent biological replicates for each *A. gemmatalis* population was individually utilized to construct cDNA libraries.

The construction of individual cDNA libraries used the Illumina® TruSeq Stranded mRNA Kit according to the instructions provided by the manufacturer. Sequencing was performed on the Illumina® Hiseq 2500 by the Beijing Genomics Institute (BGI), producing paired-end reads of 100 nucleotides. Data cleaning and filtering were performed by BGI Genomics using SOAPnuke version 2.1.4 (Chen et al., 2018), which involved adapter removal and discarding reads with 50% of bases having a phred score below 20.

2.4. Quality Analysis and Assembly

The quality of the sequencing data was assessed using the reports generated by FastQC software version 0.11.7 (https://github.com/s-andrews/FastQC), and these reports were aggregated using MultiQC software version 1.12 (https://github.com/ewels/MultiQC) (Ewels et al., 2016). Reads were processed to remove adapter sequences from the libraries using the "auto-detect" configuration of TrimGalore software version 0.6.7

(https://github.com/FelixKrueger/TrimGalore). Subsequently, sequences were trimmed and filtered using Trimmomatic software version 0.39 (https://github.com/usadellab/Trimmomatic) (Bolger; Lohse; Usadel, 2014) with the following parameters: HEADCROP:15; LEADING:3; TRAILING:3; SLIDINGWINDOW:4:20; MINLEN:50. The trimmed sequences underwent processing using Rcorrector software version 1.0.4 (https://github.com/mourisl/Rcorrector) (Song; Florea, 2015), employing default settings and applying a k-mer-based sequence correction method.

Ab initio transcriptome assembly was performed using Trinity software version 2.8.5 (https://github.com/trinityrnaseq/trinityrnaseq) (Grabherr et al., 2011), utilizing default settings and filtering transcripts with a minimum size of 500 nucleotides. Descriptive statistics of the assembly were obtained using the "TrinityStats.pl" script. The assembly quality was also evaluated through the alignment of reads to transcript sequences using Bowtie2 software v2.2.6 (https://github.com/BenLangmead/bowtie2) (Langmead, 2009), employing default settings "--end-to-end" and "--sensitive".

Following the assembly, transcripts were clustered into sets of non-redundant Unigenes using the cd-hit-est tool from CD-HIT software version 4.8.1 (http://weizhong-lab.ucsd.edu/cd-hit/) (Fu et al., 2012), with a minimum identity threshold of 98% for sequence clustering.

2.5. Prediction of ORFs

The sequences of Unigenes were analyzed to identify open reading frames (ORFs) using TransDecoder.LongOrfs tool version 5.5.0 (https://github.com/TransDecoder/TransDecoder/wiki) (HAAS et al., 2013), considering only ORFs with a minimum size of 300 nucleotides.

The protein sequences encoded by the predicted ORFs were compared with proteins available in the UniProt Knowledgebase database (UniProtKB, http://www.uniprot.org) (UniProt Consortium, 2014) using the BLASTp tool of BLAST version. 2.11.0 (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) (ALTSCHUL et al., 1990). Protein sequences were also compared with Hidden Markov Models (HMMs) of domain families from the Pfam database (http://pfam.xfam.org/) (Finn et al., 2014) using HMMER version 3.1b2 (https://github.com/EddyRivasLab/hmmer) (Finn et al., 2015). Alignments with an E-value ≤ 1x10-10 were considered significant. BLASTp alignments were also evaluated for sequence similarity, selecting those with a product coverage x similarity ≥ 40%.

2.6. Analysis of Differentially Expressed Transcripts

The quantification of transcript abundance was performed using Kallisto software version 0.44.0 (https://github.com/pachterlab/kallisto) (Bray et al., 2016), conducting pseudo-alignment of reads against Unigene sequences.

Differential gene expression analysis between the SKTI and GORE2 treatments was conducted using the DESeq2 package version 3.15 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) (Love; Huber; Anders, 2014). This analysis aimed to quantify the number of aligned sequences for each transcript based on the data obtained from Kallisto. The applied parameters were adjusted p-value < 0.01 and |log2 fold change| ≥ 1 (|FC| ≥ 2).

Genes identified as differentially expressed were subjected to analysis using BLAST2GO (http://www.blast2go.de) (Conesa et al., 2005), performing automated annotation against the NCBI's arthropod nr database with an E-value of 1x10-10. The enrichment of metabolic pathways associated with the differentially expressed genes was assessed using the KOBAS tool (http://kobas.cbi.pku.edu.cn/) (Xie et al., 2011), referencing the genomes of Helicoverpa armigera and Bombyx mori, utilizing a hypergeometric statistical test.

2.7. Functional annotation

The functional annotation of the Unigenes sequences was done by the TRAPID tool version 2.0 (Van Bel et al., 2013), selecting the EggNOG database version 4.5.1 (Cepas et al., 2016, 2017) and considering an E-value score ≤ 1×10-10 for gene family attribution, identification of functional domains, and gene ontology terms (GO) attribution. Unigenes identified as differentially expressed also were annotated using the BLAST2GO software (https://github.com/blast2go-apps) (Conesa et al., 2005), performing an automatic annotation against Arthropoda protein sequences (NCBI Taxonomy ID 6656) available in the NCBI RefSeq Non-Redundant proteins (nr) database, and considering an E-value ≤ 1×10-10 to select significant alignments. The metabolic pathway enrichment of differentially expressed Unigenes was evaluated by the KOBAS tool (https://github.com/xmao/kobas) (Xie et al., 2011), using the hypergeometric statistical test (adjusted p-value < 0.05) and selecting as reference the genome annotations of *Helicoverpa armigera* and *Bombyx mori*.

2.8. Antioxidant and detoxifying enzyme assay

Catalase activity (CAT) was determined according to the method of Aebi (1984). The reaction mixture contained 100 μL of sample in a total volume of 1.5 mL of 30 mM H2O2 and 50 mM phosphate buffer (pH 7.4). Absorbance readings were obtained at 240 nm in an automatic mode with one reading per second for 120 s (Molecular Devices, Mod. SpectraMax ABS Plus). Specific catalase activity values were expressed in mmol s-1 mg protein-1.

Peroxidase (POX) activity was determined according to the method of Shannon et al. (1966) with minor modifications. The reaction occurred with the addition of 0.1 mL of enzyme extract in 1.45 mL of 0.1 M sodium phosphate buffer (pH 6.5), 0.8 mM H2O2, and 5 mM Guaiacol. The absorbance was read at 470 nm for 120 s with one reading per second on the spectrophotometer (Molecular Devices, Mod. SpectraMax ABS Plus). The final enzyme activity was expressed in U per gram of protein. One enzymatic unit (U) was defined as a change in absorbance of 0.1 units per minute under test conditions.

Glutathione S-transferase (GST) activity was determined according to the method described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. 50 μL of enzyme extract was added to the reaction mixture, which contained 50 μL of CDNB, 150 μL of 50 mM reduced glutathione (GSH), and 2.5 mL of 100 mM sodium phosphate buffer (pH 6.5). The reaction mixture was carefully homogenized and the formation of the GSH-CDNB complex was monitored in a spectrophotometer (Molecular Devices, Mod. SpectraMax ABS Plus) at 340 nm for 120 s, with automatic reading every second. The activity was determined using the molar extinction coefficient $\varepsilon = 9.6$ mM-1 cm-1.

Superoxide dismutase (SOD) activity was determined using a commercial colorimetric kit (Ref. CS0009, Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommendations.

Protein concentrations were determined by the method of Bradford (1976). All enzymatic activities were obtained in triplicates.

2.9. Histological analyzes of the caterpillar midgut

For histological analysis, five *A. gemmatalis* caterpillars fed on PIs and control were collected, dissected and transferred to the Zamboni's fixative solution for 12 h at 4 °C (Fiaz et al., 2018). The midguts were dehydrated in a graded ethanol series (70%, 80%, 90% and 95%) and embedded in historesin (Leica) following the manufacturer's instructions. Sections three

μm thick were got with rotation microtome and stained with hematoxylin (8 min) and eosin (30 s) and analyzed with a light microscope.

2.10. Statistical analysis

Data analyzes of all enzyme assays described were performed using R software. Differences among experimental groups were calculated by one-way ANOVA. The value $p < 0.05$ was used as the standard of statistical significance for hypothesis testing.

3. RESULTS

3.1. Data analysis sequencing

The *A. gemmatalis* sequencing of midgut libraries generated 46 to 48 million reads with 100 nucleotides and an average GC content of 48 %. After removing the adapters and trimming the reads, the sequencing data contained 43 to 44 million reads with sizes ranging from 50 to 85 nt.

3.2. Reference transcriptome assembly

The reference transcriptome assembly identified 51,825 transcripts. The mean length of the transcripts obtained was 2,243.6 nt, and the N50 was 3,246 nt (**Table 1**). The validation of transcriptome by reads mapping showed that 86 % of aligned reads in the transcripts and 14.31 % did not align. Furthermore, 20.33 % aligned uniquely, and 65.36 % had multiple alignments. The sequences of transcripts were grouped in 42,372 unigenes, which have an average of 1,467 nt size and N50 of 2,894 nt.

Table S1: *A. gemmatalis* reference assembly statistics

Score:
Total trinity 'genes': 20,192
Total trinity transcripts: 51,825
Percent GC: 40.41
Statistics based on all transcripts:
Contig N50: 3,246
Median contig length: 1,617
Average contig: 2,243.60
Total assembled bases: 116,274,451
Statistics based on longest isoform per ''gene'':
Contig N50: 2,891
Median contig length: 1,204
Average contig: 1906,23
Total assembled bases: 38,490,674

3.3. Functional Annotation

A total of 42,372 Unigenes were identified as gene candidates, and 51,802 open reading frames (ORFs) were located. Out of these, a total of 25,906 proteins were annotated for similarity against the Uniprot protein database, achieving a 50% annotation rate. The species Bombyx mori and Danaus plexippus accounted for approximately 70% of the best hits in the EggNog database (**Figure 1**). Additionally, 13,731 Gene Ontology (GO) terms were assigned based on Gene Ontology terms, and 13,223 enzyme KO terms were assigned for the KEGG database. The most frequent enzymes among the transcripts belonged to the classes of hydrolases (E.C. 3), transferases (E.C. 2), and oxidoreductases (E.C. 1). The trypsin-like enzymes (KO1312) had 145 occurrences, being the most frequently encountered code (**Figure 2**). A total of 32,673 genes were annotated with protein domains according to the Pfam database.

Figure 1. Distribution of Functional Annotation for Similarity Across Different Species, calculated from the EggNog Database using the TRAPID Tool.

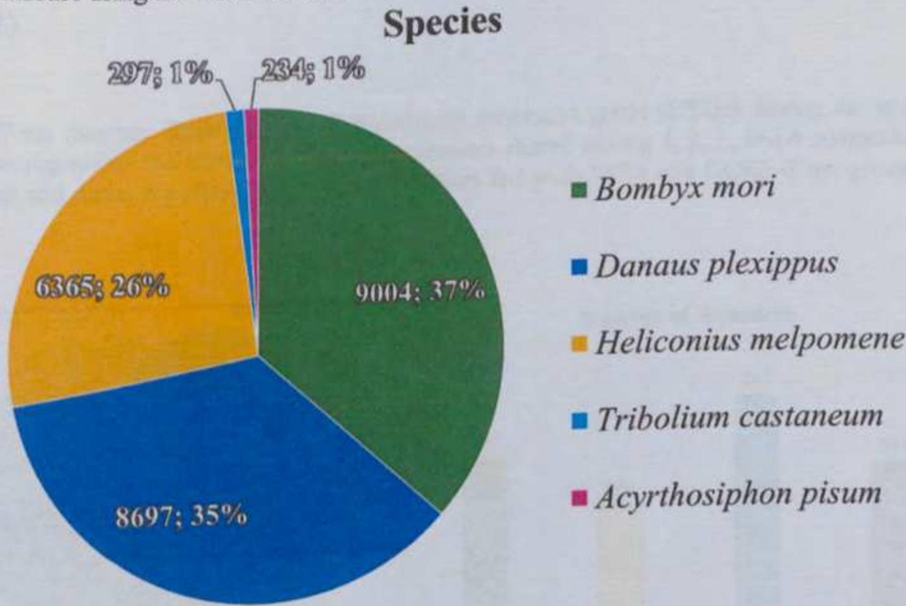
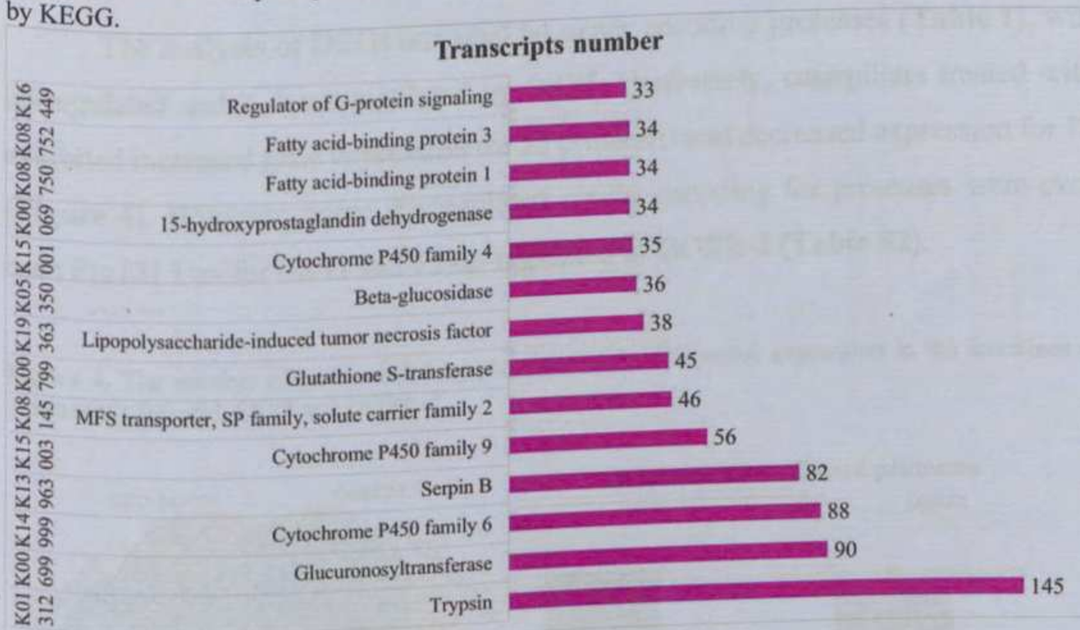


Figure 2. The most frequent protein domains represented in the transcriptome sequence of *A. gemmatalis* identified by KEGG.

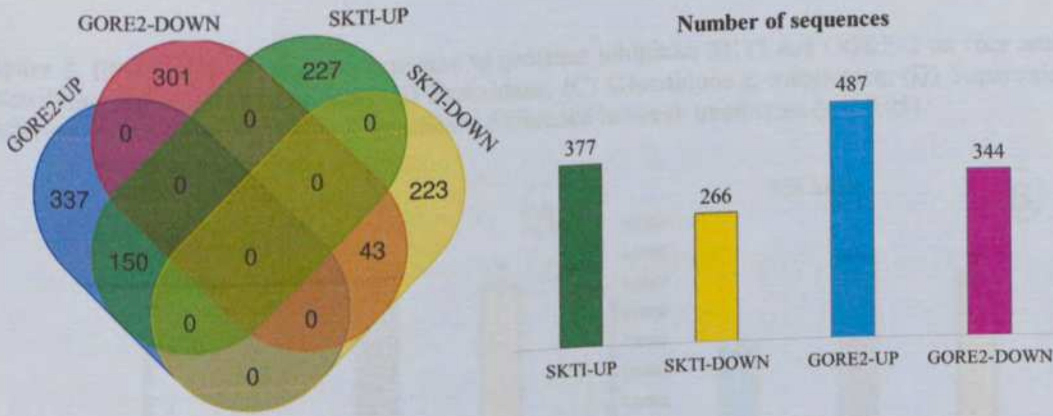


3.4. Analysis of Differentially Expressed Genes (DEGs)

In total, 1,474 differentially expressed genes (DEGs) were identified when caterpillars were fed SKTI and GORE-2, among which 266 genes were down-regulated and 377 genes were up-regulated in the SKTI-treated diet. Conversely, 487 genes were up-regulated, while 344 genes were down-regulated in the caterpillars treated with the tripeptide GORE-2. Overall, 643

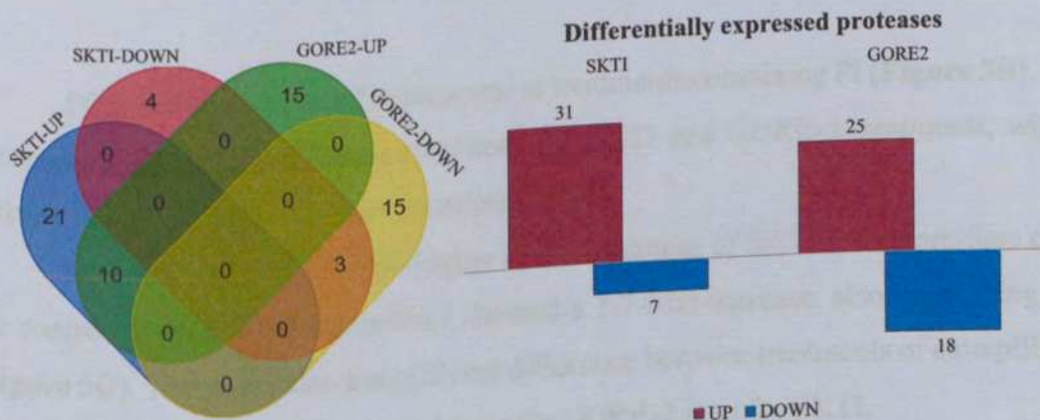
genes responded to the SKTI treatment, while GORE-2 modulated the expression of 831 genes (**Figure 3**).

Figure 3. Venn diagram illustrating the differentially expressed genes (DEGs) among the analyzed treatments. The diagram represents the numbers of specific elements shared among 1, 2, 3, and 4 comparisons. The absolute values of up and down-regulated genes for caterpillars fed with SKTI and GORE-2 are grouped in the column graph.



The analysis of DEGs unveiled 64 genes encoding proteases (**Table 1**), with 31 being up-regulated and 7 down-regulated by SKTI. Conversely, caterpillars treated with GORE-2 exhibited increased gene expression for 25 proteases and decreased expression for 18 proteases (**Figure 4**). However, some dysregulated contig encoding for proteases were exclusives for each PIs (21 Up- for SKTI and 15 up-regulated of GORE-2 (**Table S2**).

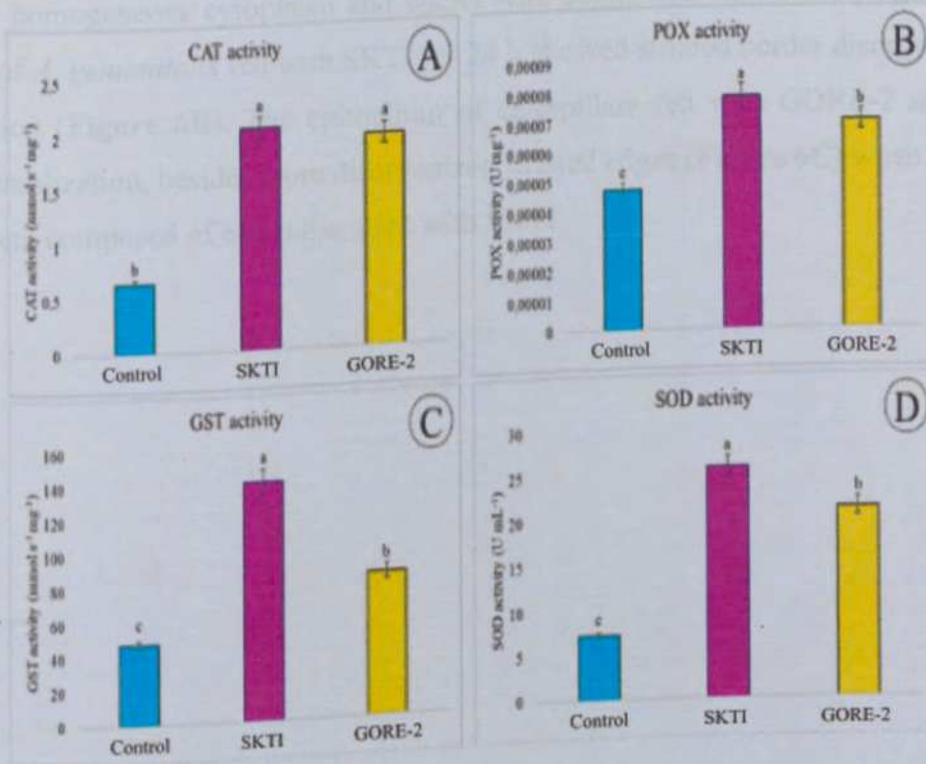
Figure 4. The number of genes encoding proteases with differential expression in the intestines of *Anticarsia gemmatalis* fed with SKTI and GORE-2.



3.5. Antioxidant Enzyme Activity

We observed evidence of oxidative stress in *A. gemmatalis* caterpillars when fed an artificial diet plus PI at sublethal concentrations for 24 h (**Figure 5**). CAT activity in the caterpillar gut increased significantly (4-fold) after 24 h of exposure to PI (**Figure 5A**). However, there was no difference in CAT activities between caterpillars fed SKTI and those fed GORE-2.

Figure 5. Effect of *A. gemmatalis* exposure to protease inhibitors SKTI and GORE-2 on four antioxidants and detoxifying enzymes: (A) Catalase; (B) Peroxidase; (C) Glutathione S-transferase; (D) Superoxide dismutase. Different letters on the bars show a significant difference between treatments ($p < 0.05$).



POX activity increased in response to treatments containing PI (**Figure 5B**). In addition, there was a significant difference between the SKTI and GORE-2 treatments, with the POX activity being higher for the diet containing SKTI.

GST activity was 2.9-fold higher in the intestines of SKTI-fed caterpillars compared to the control. GORE-2-fed caterpillars showed a 1.7-fold increase, also concerning the control (**Figure 5C**). There was also a significant difference between treatments of caterpillars exposed to PI, with GST activity being 40% lower for GORE-2 than for SKTI.

The PI-treated caterpillars also responded with increased SOD activity for both those fed SKTI and those treated with GORE-2 (**Figure 5D**). The increases in SOD activities for the caterpillars fed SKTI and GORE-2, about the control, were 3.5-fold and 2.9-fold, respectively.

As also indicated by RNAseq analysis, both PIs induced the expression of genes involved in oxidative metabolism (**Figure 2**). However, the effect of the PIst in the activities of the enzyme's oxidative metabolism was more pronounced for SKTI.

3.6. Histology

The caterpillars showed histological changes in the midgut when exposed to diets containing PI (**Figure 6**). The morphological organization of the control intestinal epithelium was a monolayer of goblet digestive cells with the presence of a well-defined striated border, besides to homogeneous cytoplasm and nuclei with condensed chromatin (**Figure 6A**). The intestines of *A. gemmatalis* fed with SKTI for 24 h showed striated border disorganization and vacuolization (**Figure 6B**). The epithelium of caterpillars fed with GORE-2 showed more intense vacuolization, besides more disorganized striated edges (**Figure 6C**) when compared to the treatment composed of caterpillars fed with SKTI.

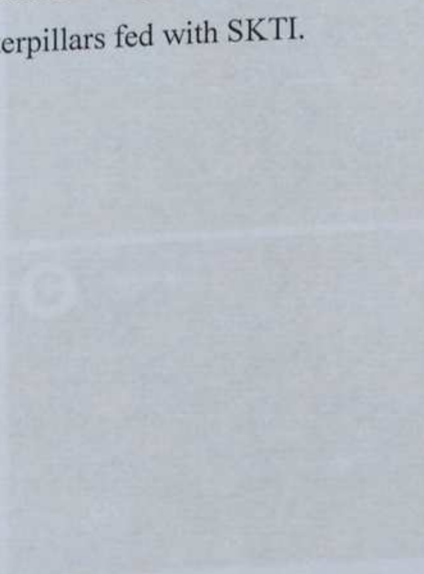
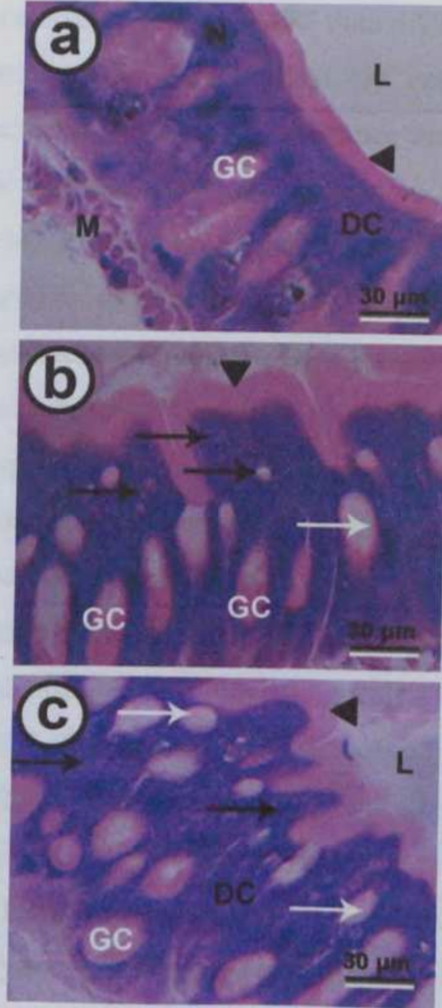


Figure 6. Light micrographs of the midgut of the Anticarsia gemmatalis caterpillar. [a] Control group epithelium showing the striated border (arrowhead) of digestive cells (DC) with homogeneous cytoplasm and nuclei (N) with decondensed chromatin, goblet cells (GC) with clear striated border, muscle layers (M) and lumen (L). [b] SKTI-fed caterpillar epithelium showing striated border (arrowhead) of digestive cells (DC) with cytoplasm showing vacuolization (black arrows) and goblet cells (GC) with disorganized striated border (white arrows). [c] Caterpillar epithelium fed on GORE-2 showing striated border (arrowhead) of digestive cells (DC) that present strongly vacuolated cytoplasm (black arrows), goblet cells (GC) with disorganized striated border (white arrows).



4. DISCUSSION

Previous studies have focused on the basic and immediate function of PIs related to the decrease in protein hydrolysis in the gut of insect pests. In fact, the tripeptides GORE-1 and GORE-2 have shown potential for the use of IPs as pesticides. However, the physiological effects caused by PIs go much further and seem to be more complex than a simple reduction of the proteolytic potential in the arthropod intestine. As expected, both PIs changed the expression of several genes encoding for protease. However, the protease profiles were distinct, indicating differential cascades induced gene expression. The overall physiology of the

intestine was also affected triggered directly or indirectly by the PIs presence. PIs were also responsible for the increase in the activity of antioxidant enzymes, showing oxidative stress. The soybean caterpillar was more efficient in the defense against SKTI, presenting a more intense and efficient defensive response when compared to the GORE-2, showing a better detoxification response to the natural PI of soybean. Furthermore, histological damage was more extensive in the gut of caterpillars fed GORE-2 than SKTI.

The RNAseq technology has been extensively employed in characterizing and identifying signaling genes and related to insect resistance mechanisms (Dhania et al., 2019; Nanoth Vellichirammal et al., 2015; XU et al., 2015) due to its high reproducibility and accuracy in quantifying varying gene expression rates (Conesa et al., 2016; Wang; Gerstein; Snyder, 2009). The transcriptome assembly successfully identified 42,372 unigenes, with 25,906 proteins annotated in the Gene Ontology (GO). This outcome surpassed previous studies conducted on other lepidopteran species (Gazara et al., 2017; Perera et al., 2015; Shao et al., 2020; Tang et al., 2017). The species with the highest similarity hits were *Bombyx mori*, *Danaus plexippus*, and *Heliconius melpomene*, accounting for 98% of the identified genes. Indeed, these three lepidopterans possess extensive deposited data.

The trypsin-like proteins were the most frequently represented in the transcriptome sequence based on KEGG identifications, with 145 annotated transcripts. This prevalence exceeds what has been found in other lepidopterans like *Spodoptera frugiperda* (Chen et al., 2022; Silva-Brandão et al., 2021) and *Pieris rapae* (Xiang et al., 2018). This substantial number underscores the significance of the serine endopeptidase sub-subclass within the Lepidoptera order as a target for efficient Integrated Pest Management (IPM) strategies against important agricultural pests. Glucuronosyltransferases were also highly represented in the RNAseq, with 90 transcripts. These enzymes are known to participate in the detoxification of plant allelochemicals in insects (Ahmad; Hopkins, 1993; Ahn; Vogel; Heckel, 2012; Krempl et al., 2016), and other studies link them to insecticide resistance due to their capacity to metabolize toxic xenobiotics (Bull; Whitten, 1972; Krempl et al., 2016). The third most represented enzyme family was Cytochrome P450, a hemoprotein of crucial importance for insect adaptation to plant defense systems, as well as insect growth and development (Nelson, 2011; Pottier et al., 2012; Zhou et al., 2010).

The tripeptide GORE-2 was responsible for the differential expression of a larger set of genes compared to SKTI. However, caterpillars challenged with SKTI exhibited a higher number of up-regulated genes encoding proteases. On the other hand, down-regulated genes for proteases were more abundant in response to GORE-2. The fact that caterpillars increased the

expression of proteases more intensely when challenged with the natural soybean inhibitor reinforces one of the most common counter-responses of generalist insects as adaptation mechanisms to cope with plant defenses (Bayés et al., 2006; Volpicella et al., 2003). Caterpillars fed diets rich in protease inhibitors (PIs) respond by increasing the abundance of inhibitor-insensitive isoforms (Coura et al., 2022; Jongsma et al., 1995). A study conducted on *Helicoverpa armigera* demonstrated that caterpillars exposed to SKTI in their diet exhibited an increase in mRNA coding for proteases (Bown; Wilkinson; Gatehouse, 2004).

The overall increase in the expression of serine proteases can be attributed to two approaches: universal or selective. In the universal approach, the insect positively modulates all genes encoding digestive enzymes, even in the presence of protease inhibitors (PIs) in its diet. In the selective approach, only the genes encoding PI-insensitive hydrolases would be maintained. This selective approach implies an as-yet-unknown feedback mechanism that informs which proteases should be preserved. This selective mechanism would explain why GORE2 doesn't strongly elicit the insect's signaling pathways. The insect produces a broad repertoire of proteases under both strategies, aiming to ensure the production of some isoforms insensitive to PIs. This approach might seem energetically costly for the insect, but it significantly enhances adaptation chances. Our results demonstrated a greater increase in the expression of genes encoding proteases not belonging to the trypsin-like family. Bown et al. (2004) also demonstrated that *Helicoverpa armigera* employed a selective approach to overcome the negative effects of PIs in its diet.

Both SKTI and GORE-2 increased the activity of antioxidant enzymes such as SOD, CAT, POX, and GST indicates oxidative stress, as these defensive enzymes work cooperatively to deal with the relatively high amounts of ROS within the cell (Foyer et al., 1994). Both these enzymes and ROS play an important role in homeostasis, immunity, and detoxification. The extensive literature suggests that ROS are continuously generated upon exposure to environmental stressors (Bagchi et al., 1995; Ledirac et al., 2005; Melchiorri et al., 1998). Insecticides are among the most important environmental factors and can also induce significant physiological changes and oxidative stress in insects (Müller, 2018; Q. Y. Yu et al., 2011).

CAT activity was higher in the gut of caterpillars fed with PIs when compared to the control. However, the CAT activities between caterpillars challenged with SKTI and GORE-2 showed no significant difference between them after 24 h of exposure. This may have occurred because of the large production of peroxides and superoxides, which cause oxidative stress. Very high levels of H_2O_2 limit the catalytic potential of CAT through the formation of excess

hydroxyl radicals. CAT is also sensitive to O_2•− and can be inhibited by high levels of this superoxide (Karthi et al., 2014).

When the increase in CAT activity is observed together with the increase in SOD activity, it is considered that this has occurred for the elimination of H_2O_2 (Munday & Winterbourn, 1989; Sies, 1991). This cooperative action was observed as SOD showed an increase in activity in the intestines of caterpillars exposed to PIs. The rapid induction of SOD activity may have led to the conversion of superoxide radicals to H_2O_2, and a higher intracellular concentration of H_2O_2 induces CAT and POX activity (Dmochowska-Ślęzak et al., 2015; Felton & Summers, 1995; Łukasik et al., 2009).

In addition, SOD showed higher activity in caterpillars fed with SKTI concerning the tripeptide GORE-2. The fact that the insect responded more efficiently to SKTI shows a valuable insecticidal potential for the GORE-2, as the cellular machinery was less responsive to recognizing and overcoming the negative effects of the tripeptide. SOD is the first antioxidant enzyme in the defense against ROS, and increased SOD activity is the main cellular response to superoxide anion production (Emre et al., 2013; Kayis et al., 2015). The enzyme catalyzes the dismutation of superoxide anion radicals into hydrogen peroxides and molecular oxygen, playing a crucial role in antioxidant defense against these ROS (Sankarapandi & Zweier, 1999). In this sense, circumventing the insect's defense system implies stimulating the metabolic pathways of detoxification of the physiological system as little as possible. The importance of key antioxidant enzymes has already been highlighted by studies involving synthetic insecticides that aimed to suppress SOD activity (Altuntas et al., 2002; Büyükgüzel, 2009; Sobeková et al., 2009).

Altuntaş (2015) demonstrated that plant metabolites increase SOD activity in lepidopterans to prevent oxidative stress, even at low doses. Other studies have also shown that SOD can rapidly eliminate pesticide-induced ROS and increase insecticide tolerance in Galleria mellonella, Oxya chinensis and Lymantria dispar (Büyükgüzel, 2009; H. Wu et al., 2017; Zeng et al., 2019). SOD activity was also induced under sublethal concentrations of chlorantraniliprole in *Agrotis ipsilon* (He et al., 2019; Li et al., 2021) and in *Bombyx mori* (Mao et al., 2020), being related to insecticide resistance.

The GST activity showed a behavior similar to SOD in the caterpillars treated with PI. There was a significant increase in enzymatic activity compared to control after 24 h of exposure. In addition, GST activity was higher in caterpillars fed with the natural soybean inhibitor compared to GORE-2. GST is an enzyme that catalyzes the conjugation of GSH with electrophilic metabolites to overcome allelochemical toxicity in lepidopteran species

(Büyükgüzel et al., 2010; Chen et al., 2019; Su et al., 2018). GST can detoxify various chemical classes of insecticides such as organophosphates, pyrethroids, carbamates and chlorinated hydrocarbons (Konanz & Nauen, 2004). Therefore, GST is often identified as a biomarker of resistance, as demonstrated for Lymantria dispar larvae (Lindroth et al., 1990).

Navarro-Roldán et al. (2020) reviewed 92 cases of detoxification mechanisms in lepidopterans and found that GST was involved in only 32% of cases. Previous studies have also shown that several plants allelochemicals act as GST activators or GST substrates in phytophagous insects (Piskorski et al., 2011; Thiboldeaux et al., 1998; S. J. Yu & Abo-Elghar, 2000). In this context, GST has become an important suppression target in the rational design of synthetic insecticides to minimize the effects of target insect response (Cossio-Bayugar et al., 2002; Papadopoulos et al., 2004; S. Wu et al., 2009). Therefore, a compound harmful to the insect that presents low GST activation is relevant in the study of more efficient insecticides.

POX exhibited similar behavior to the other enzymes involved in the cellular detoxification process. There was an increase in POX activity in the gut of caterpillars when exposed to PIs; however, insects fed a diet plus GORE-2 responded less intensely to oxidative stress when compared to those treated with SKTI. The POX results are consistent with the patterns observed for the other antioxidant enzymes evaluated. Another important implication of the lower POX activity in caterpillars fed GORE-2 is the susceptibility to greater cellular damage, observed in the insect intestines, as will be discussed later. POX-derived prostaglandins act in the signaling of the cellular immune system, which may compromise the tissue integrity of Lepidoptera such as Spodoptera exigua (Park et al., 2014).

Changes in the activities of CAT, POX, GST and SOD have shown important metabolic adaptations that imply the ability of insects to detoxify (Ding et al., 2005; Heng-Moss et al., 2004; Vontas et al., 2001). The up-regulation of many of these antioxidant enzymes in response to insecticides is responsible for the resistance of several insect species (Coleman et al., 2002; Hemingway & Karunaratne, 1998; Vontas et al., 2001). The adaptation of insect pests to PIs has attracted researchers to understand the biochemical responses involved and to design better approaches to the use of PIs as pesticides (Bolter & Jongsma, 1995; Brioschi et al., 2007).

The contrast established between both PIs studied was important because SKTI is a natural protein of *Glycine max*, the main food source of *A. gemmatalis*. Throughout the coevolution between the plant and the insect, the expression of PIs aimed to mitigate the herbivory (Jongsma & Bolter, 1997); on the other hand, the caterpillar developed strategies to overcome the plant's defense system. The versatile physiological responses exhibited by the insect facilitated its adaptation to the PI-based plant defense. This performance was evidenced

in our results when *A. gemmatalis* responded more efficiently to natural soybean PI (i.e., SKTI). This suggests that the difficulty in recruiting antioxidant enzymes in caterpillars fed GORE-2 makes the caterpillar more susceptible. Consequently, non-plant PIs can be explored as a more effective measure to avoid the specific co-evolutionary adaptations in the plant-pest interaction (Harsulkar et al., 1999). Part of the challenge faced in the development of efficient insecticides is based on the search for molecules that cause physiological damage and are neutralized with low efficiency by the defense system of the target organism.

Previous studies have reported a strong correlation between CAT gene expression and longevity in *Drosophila melanogaster* (Orr & Sohal, 1994). Decreased CAT activity or interruption of CAT gene expression led to death after hatching (Griswold et al., 1993; Orr & Sohal, 1994). POX was also responsible for increasing stress tolerance in insects (Mittler & Poulos, 2007). Several conventional insecticides, especially organophosphates, organochlorines and pyrethroids, are known to decrease the activities of enzymes such as SOD (Büyükgüzel, 2009), GST (Cossio-Bayugar et al., 2002; Papadopoulos et al., 2004; S. Wu et al., 2009) and CAT (Sowjanya Sree et al., 2010). Insects resistant to these insecticides show increased activity of antioxidant enzymes (Coleman et al., 2002; Ishaaya & Casida, 1974). Therefore, analyzing the inhibitory potential of a PI is very important when considering a rational design. But that's just the tip of the iceberg. It is important that IPs modulate the insect's defense system to minimize the emergence of resistant populations.

Besides to circumventing the insect's defense mechanisms, the GORE-2 caused more significant damage to the insect's midgut when compared to the SKTI. Similar changes in cell morphology, such as increased vacuolization in treatments containing PI, were pointed out by other studies that used conventional insecticides (Fiaz et al., 2018; Gonçalves et al., 2018). The increased secretory activity was also reported in the midgut of A. gemmatalis (Fiaz et al., 2018) and in Aedes aegypti larvae (Gaban et al., 2015) when treated with insecticides. This intense vacuolization was observed in A. gemmatalis treated with tebufenozide, an insecticide that mimics the hormone ecdysone (Beaulaton & Lockshin, 1977; Romanelli et al., 2014).

The presence of vacuoles in midgut cells is common in insects (Alves et al., 2010; Fernandes et al., 2015), but their increased number in the cytoplasm because of insecticides has been characterized as a precedent of autophagy (Hariri et al., 2000; D. E. Santos et al., 2015). The many cytoplasmic vacuoles observed, and the striated border disorganization in the midgut of *A. gemmatalis* are like the changes observed in Lepidoptera and Diptera when challenged with *B. thuringiensis* (Loeb et al., 2001; Mathavan et al., 1989; Rey et al., 1998), suggesting a detoxification response to the stressor and initiation of the cell death process (M. C. dos Santos

et al., 2015). Stresses such as the decrease in free amino acids caused by the immediate action of PI, oxidative stress, DNA damage caused by ROS and damaged cells can activate the autophagy pathway (Kroemer et al., 2010).

The morphological disorganizations in the midgut lumen of *A. gemmatalis* caterpillars fed with GORE-2 suggest a cytotoxic effect of this PI, which may cause apoptosis (Ihara et al., 1998). The elimination of cells by death would therefore be a response to damage to midgut epithelial cells after 24 h of PI ingestion. Apoptosis is fundamental to many important biological processes (Ottaviani & Malagoli, 2016) such as development, tissue homeostasis, DNA damage response (Amundson et al., 1998) and immune response (Creagh et al., 2003). Damage to the insect's digestive system by toxins that activate defensive responses also culminated in apoptosis in *Heliothis virescens* (Forcada et al., 1999; Martínez-Ramírez et al., 1999).

Antioxidant enzymes make up defenses that maintain ROS balance in normal cells. Reduction in CAT, POX, SOD, and GST activity can lead to ROS accumulation, resulting in cellular apoptosis (Jacobson, 1996; Wertz & Hanley, 1996). The production of ROS leads to the peroxidation of membrane lipids in the presence of oxygen (Slater, 1984) and the lipid peroxides formed are metabolized by GST in insects (Ahmad et al., 1991; Konno & Shishido, 1992). Therefore, the more intense histological damage observed in caterpillars fed with GORE-2 can be explained by the low response of the insect to this antioxidant enzyme. Cellular impact on the midgut has been reported for Alabama argillacea (M. E. C. Sousa et al., 2010) and *Plutella xylostella* (da Solidade Ribeiro et al., 2013) similarly after exposure to insecticides.

Therefore, the fact that the tripeptide triggers a lower response from antioxidant enzymes does not mean that this PI spares the insect. On the contrary, the GORE-2 causes more severe damage to the caterpillar by eliciting less of the insect's detoxification pathways.

To date, there are no commercially available transgenic plants expressing PIs, but the use of small peptides may have large-scale advantages. Besides the adaptive advantages circumvented by the tripeptide, its reduced size makes production economically viable for spraying and bio-energetically favorable for localized expression in genetically modified plants. This approach may represent a paradigm shift in the development of new environmentally friendly agrochemicals.

5. CONCLUSION

Our work evaluated the damage caused by two PIs under two levels: oxidative stress through key enzymes and from a gut morphological perspective. The presence of PI in the diet of *A. gemmatalis* caused oxidative stress and histological changes in the insect's midgut. In addition, GORE-2 generated less intense oxidative stress than SKTI, but more intense morphological damage. In this sense, the tripeptide GORE-2 presents itself as an efficient alternative in the control of this Lepidoptera.

6. REFERENCES

AEBI, Hugo. [13] Catalase in Vitro. Methods in Enzymology, [S. l.], v. 105, n. C, p. 121–126, 1984. DOI: 10.1016/S0076-6879(84)05016-3. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0076687984050163. Acesso em: 9 fev. 2022.

AHMAD, S. A.; HOPKINS, T. L. β-Glucosylation of plant phenolics by phenol β-glucosyltransferase in larval tissues of the tobacco hornworm, Manduca sexta (L.). Insect Biochemistry and Molecular Biology, [S. l.], v. 23, n. 5, p. 581–589, 1993. DOI: 10.1016/0965-1748(93)90031-M.

AHMAD, Sami; DUVAL, Dawn L.; WEINHOLD, Leanne C.; PARDINI, Ronald S. Cabbage looper antioxidant enzymes: Tissue specificity. Insect Biochemistry, [S. l.], v. 21, n. 5, p. 563–572, 1991. DOI: 10.1016/0020-1790(91)90111-Q.

AHN, Seung Joon; VOGEL, Heiko; HECKEL, David G. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. Insect Biochemistry and Molecular Biology, [S. l.], v. 42, n. 2, p. 133–147, 2012. DOI: 10.1016/j.ibmb.2011.11.006.

ALI, A.; RASHID, M. A.; HUANG, Q. Y.; WONG, C.; LEI, C. L. Response of antioxidant enzymes in Mythimna separata (Lepidoptera: Noctuidae) exposed to thermal stress. Bulletin of Entomological Research, [S. l.], v. 107, n. 3, p. 382–390, 2017. a. DOI: 10.1017/S0007485316001000. Disponível em: https://www.cambridge.org/core/journals/bulletin-of-entomological-research/article/abs/response-of-antioxidant-enzymes-in-mythimna-separata-lepidoptera-noctuidae-exposed-to-thermal-stress/66668A0318DBEBA1A7F26EE218E2CA0A. Acesso em: 11 mar. 2022.

ALI, Arif; RASHID, Muhammad Adnan; HUANG, Qiu Ying; LEI, Chao Liang. Influence of UV-A radiation on oxidative stress and antioxidant enzymes in Mythimna separata (Lepidoptera: Noctuidae). Environmental Science and Pollution Research, [S. l.], v. 24, n. 9, p. 8392–8398, 2017. b. DOI: 10.1007/s11356-017-8514-7. Disponível em: https://europepmc.org/article/med/28185177. Acesso em: 21 out. 2021.

ALTSCHUL, Stephen F.; GISH, Warren; MILLER, Webb; MYERS, Eugene W.; LIPMAN, David J. Basic local alignment search tool. Journal of Molecular Biology, [S. l.], v. 215, n. 3, p. 403–410, 1990. DOI: 10.1016/S0022-2836(05)80360-2.

ALTUNTAŞ, H. Determination of gibberellic acid (GA3)-induced oxidative stress in a model organism galleria mellonella l. (Lepidoptera: Pyralidae). Environmental Entomology, [S. l.], v. 44, n. 1, p. 100–105, 2015. DOI: 10.1093/ee/nvu020. Disponível em: https://academic.oup.com/ee/article/44/1/100/2464823. Acesso em: 8 abr. 2022.

ALTUNTAS, Irfan; DELIBAS, N.; SUTCU, R. The effects of organophosphate insecticide methidathion on lipid peroxidation and anti-oxidant enzymes in rat erythrocytes: Role of vitamins E and C. Human and Experimental Toxicology, [S. l.], v. 21, n. 12, p. 681–685, 2002. DOI: 10.1191/0960327102ht304oa. Disponível em: https://journals.sagepub.com/doi/abs/10.1191/0960327102ht304oa. Acesso em: 8 abr. 2022.

ALVES, Stênio Nunes; SERRÃO, José Eduardo; MELO, Alan Lane. Alterations in the fat body and midgut of Culex quinquefasciatus larvae following exposure to different insecticides. Micron, [S. l.], v. 41, n. 6, p. 592–597, 2010. DOI: 10.1016/j.micron.2010.04.004.

AMUNDSON, Sally A.; MYERS, Timothy G.; FORNACE, Albert J. Roles for p53 in growth arrest and apoptosis: Putting on the brakes after genotoxic stress. Oncogene, [S. l.], v. 17, n. 25, p. 3287–3299, 1998. DOI: 10.1038/sj.onc.1202576. Disponível em: https://www.nature.com/articles/1202576. Acesso em: 13 abr. 2022.

AUCOIN, Richard R.; PHILOGÈNE, Bernard J. R.; ARNASON, John T. Antioxidant enzymes as biochemical defenses against phototoxin-induced oxidative stress in three species of herbivorous lepidoptera. Archives of Insect Biochemistry and Physiology, [S. l.], v. 16, n. 2, p. 139–152, 1991. DOI: 10.1002/arch.940160206. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.940160206. Acesso em: 18 nov. 2021.

BAGCHI, D.; BAGCHI, M.; HASSOUN, E. A.; STOHS, S. J. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology, [S. l.], v. 104, n. 1–3, p. 129–140, 1995. DOI: 10.1016/0300-483X(95)03156-A.

BAYÉS, Alex; DE LA VEGA, Mónica Rodríguez; VENDRELL, Josep; AVILES, Francesc X.; JONGSMA, Maarten A.; BEEKWILDER, Jules. Response of the digestive system of Helicoverpa zea to ingestion of potato carboxypeptidase inhibitor and characterization of an uninhibited carboxypeptidase B. Insect Biochemistry and Molecular Biology, [S. l.], v. 36, n. 8, p. 654–664, 2006. DOI: 10.1016/j.ibmb.2006.05.010.

BEAULATON, Jacques; LOCKSHIN, Richard A. Ultrastructural study of the normal degeneration of the intersegmental muscles of Antheraea polyphemus and Manduca sexta (Insecta, lepidoptera) with particular reference to cellular autophagy. Journal of Morphology, [S. l.], v. 154, n. 1, p. 39–57, 1977. DOI: 10.1002/jmor.1051540104. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/jmor.1051540104. Acesso em: 13 abr. 2022.

BOLGER, Anthony M.; LOHSE, Marc; USADEL, Bjoern. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics, [S. l.], v. 30, n. 15, p. 2114–2120, 2014. DOI: 10.1093/bioinformatics/btu170. Disponível em: https://academic.oup.com/bioinformatics/article/30/15/2114/2390096. Acesso em: 7 jun. 2022.

BOLTER, Caroline J.; JONGSMA, Maarten A. Colorado potato beetles (leptinotarsa decemlineata) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. Journal of Insect Physiology, [S. l.], v. 41, n. 12, p. 1071–1078, 1995. DOI: 10.1016/0022-1910(95)00073-4.

BOWN, David P.; WILKINSON, Hillary S.; GATEHOUSE, John A. Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors. Physiological Entomology, [S. l.], v. 29, n. 3 SPEC. ISS., p. 278–290, 2004. DOI: 10.1111/j.0307-6962.2004.00402.x. Disponível em: http://doi.wiley.com/10.1111/j.0307-6962.2004.00402.x. Acesso em: 10 jun. 2022.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, [S. l.], v. 72, n. 1–2, p. 248–254, 1976. DOI: 10.1016/0003-2697(76)90527-3. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/0003269776905273. Acesso em: 25 mar. 2021.

BRAVO, Alejandra; GILL, Sarjeet S.; SOBERÓN, Mario. Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. Toxicon, [S. l.], v. 49, n. 4, p. 423–435, 2007. DOI: 10.1016/j.toxicon.2006.11.022.

BRAY, Nicolas L.; PIMENTEL, Harold; MELSTED, Páll; PACHTER, Lior. Near-optimal probabilistic RNA-seq quantification. Nature Biotechnology, [S. l.], v. 34, n. 5, p. 525–527, 2016. DOI: 10.1038/nbt.3519. Disponível em: https://www.nature.com/articles/nbt.3519. Acesso em: 7 jun. 2022.

BRIOSCHI, Daniela; NADALINI, Larissa D.; BENGTSON, Mario H.; SOGAYAR, Mari Cleide; MOURA, Daniel S.; SILVA-FILHO, Marcio C. General up regulation of Spodoptera

frugiperda trypsins and chymotrypsins allows its adaptation to soybean proteinase inhibitor. Insect Biochemistry and Molecular Biology, [S. l.], v. 37, n. 12, p. 1283–1290, 2007. DOI: 10.1016/j.ibmb.2007.07.016. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0965174807001919. Acesso em: 24 mar. 2022.

BUENO, Adeney de Freitas; BATISTELA, Marcelo José; BUENO, Regiane Cristina Oliveira de Freitas; FRANÇA-NETO, José de Barros; NAIME NISHIKAWA, Marcelo Akita; FILHO, Adeone Libério. Effects of integrated pest management, biological control and prophylactic use of insecticides on the management and sustainability of soybean. Crop Protection, [S. l.], v. 30, n. 7, p. 937–945, 2011. DOI: 10.1016/j.cropro.2011.02.021.

BULL, Don L.; WHITTEN, Chandler J. Factors Influencing Organophosphorus Insecticide Resistance in Tobacco Budworms. Journal of Agricultural and Food Chemistry, [S. l.], v. 20, n. 3, p. 561–564, 1972. DOI: 10.1021/jf60181a061. Disponível em: https://pubs.acs.org/doi/pdf/10.1021/jf60181a061. Acesso em: 10 jun. 2022.

BÜYÜKGÜZEL, Ender. Evidence of oxidative and antioxidative responses by Galleria mellonella larvae to malathion. Journal of Economic Entomology, [S. l.], v. 102, n. 1, p. 152–159, 2009. DOI: 10.1603/029.102.0122. Disponível em: https://academic.oup.com/jee/article/102/1/152/2199106. Acesso em: 25 mar. 2022.

BÜYÜKGÜZEL, Ender; HYRŠL, Pavel; BÜYÜKGÜZEL, Kemal. Eicosanoids mediate hemolymph oxidative and antioxidative response in larvae of Galleria mellonella L. Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, [S. l.], v. 156, n. 2, p. 176–183, 2010. DOI: 10.1016/j.cbpa.2010.01.020.

CAMPOS, Estefânia V. R.; PROENÇA, Patrícia L. F.; OLIVEIRA, Jhones L.; BAKSHI, Mansi; ABHILASH, P. C.; FRACETO, Leonardo F. Use of botanical insecticides for sustainable agriculture: Future perspectives. Ecological Indicators, [S. l.], v. 105, n. July 2017, p. 483–495, 2019. DOI: 10.1016/j.ecolind.2018.04.038. Disponível em: http://dx.doi.org/10.1016/j.ecolind.2018.04.038.

CHEN, Haoliang; XIE, Minghui; LIN, Lulu; ZHONG, Yongzhi; ZHANG, Feng; SU, Weihua. Transcriptome Analysis of Detoxification-Related Genes in Spodoptera frugiperda (Lepidoptera: Noctuidae). Journal of Insect Science, [S. l.], v. 22, n. 1, 2022. DOI: 10.1093/jisesa/ieab108. Disponível em: https://academic.oup.com/jinsectscience/article/22/1/11/6520543. Acesso em: 10 jun. 2022.

CHEN, Shi; ELZAKI, Mohammed Esmail Abdalla; DING, Chaohui; LI, Zheng fang; WANG, Jie; ZENG, Ren Sen; SONG, Yuan Yuan. Plant allelochemicals affect tolerance of polyphagous lepidopteran pest Helicoverpa armigera (Hübner) against insecticides. Pesticide Biochemistry and Physiology, [S. l.], v. 154, p. 32–38, 2019. DOI: 10.1016/j.pestbp.2018.12.009.

CHEN, Yuxin et al. SOAPnuke: A MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. GigaScience, [S. l.], v. 7, n. 1, p. 1–6, 2018. DOI: 10.1093/gigascience/gix120. Disponível em: https://academic.oup.com/gigascience/article/7/1/gix120/4689118. Acesso em: 7 jun. 2022.

CHOUGULE, Nanasaheb P.; DOYLE, Emily; FITCHES, Elaine; GATEHOUSE, John A. Biochemical characterization of midgut digestive proteases from Mamestra brassicae (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. Journal of Insect Physiology, [S. l.], v. 54, n. 3, p. 563–572, 2008. DOI: 10.1016/j.jinsphys.2007.12.005. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/18241882. Acesso em: 31 mar. 2021.

COELHO, M.; GODOY, A. F.; BAPTISTA, Y. A.; BENTIVENHA, J. P. F.; LOURENÇÃO, A. L.; BALDIN, E. L. L.; CATCHOT, A. L. Assessing soybean genotypes for resistance to helicoverpa armigera (Lepidoptera: Noctuidae). Journal of Economic Entomology, [S. l.], v. 113, n. 1, p. 471–481, 2020. DOI: 10.1093/jee/toz269. Disponível em: https://academic.oup.com/jee/article/113/1/471/5588651.

COLEMAN, Michael; VONTAS, John G.; HEMINGWAY, Janet. Molecular characterization of the amplified aldehyde oxidase from insecticide resistant Culex quinquefasciatus. European Journal of Biochemistry, [S. l.], v. 269, n. 3, p. 768–779, 2002. DOI: 10.1046/j.0014-2956.2001.02682.x. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1046/j.0014-2956.2001.02682.x. Acesso em: 25 mar. 2022.

CONESA, Ana et al. A survey of best practices for RNA-seq data analysis. Genome Biology, [S. l.], v. 17, n. 1, p. 1–19, 2016. DOI: 10.1186/s13059-016-0881-8. Disponível em: https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0881-8. Acesso em: 10 jun. 2022.

CONESA, Ana; GÖTZ, Stefan; GARCÍA-GÓMEZ, Juan Miguel; TEROL, Javier; TALÓN, Manuel; ROBLES, Montserrat. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, [S. l.], v. 21, n. 18, p. 3674–3676,

2005. DOI: 10.1093/bioinformatics/bti610. Disponível em: https://academic.oup.com/bioinformatics/article/21/18/3674/202517. Acesso em: 4 fev. 2022.

COSSIO-BAYUGAR, Raquel; BARHOUMI, Rola; BURGHARDT, Robert C.; WAGNER, G. Gale; HOLMAN, Patricia J. Basal cellular alterations of esterase, glutathione, glutathione S-transferase, intracellular calcium, and membrane potentials in coumaphos-resistant Boophilus microplus (Acari: Ixodidae) cell lines. Pesticide Biochemistry and Physiology, [S. l.], v. 72, n. 1, p. 1–9, 2002. DOI: 10.1006/pest.2001.2578.

COURA, Roberta Ribeiro et al. Extensive reprogramming of protein isoforms and histopathological alterations in the midgut of Anticarsia gemmatalis fed with protease inhibitors. Annals of Applied Biology, [S. l.], v. 180, n. 3, p. 383–397, 2022. DOI: 10.1111/aab.12740. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/aab.12740. Acesso em: 18 nov. 2021.

CREAGH, Emma M.; CONROY, Helen; MARTIN, Seamus J. Caspase-activation pathways in apoptosis and immunity. Immunological Reviews, [S. l.], v. 193, p. 10–21, 2003. DOI: 10.1034/j.1600-065X.2003.00048.x.

DA SILVA JÚNIOR, Neilier R.; VITAL, Camilo E.; DE ALMEIDA BARROS, Rafael; FAUSTINO, Verônica A.; MONTEIRO, Luana P.; BARROS, Edvaldo; DE OLIVEIRA, Eugênio E.; DE OLIVEIRA RAMOS, Humberto J.; DE ALMEIDA OLIVEIRA, Maria G. Intestinal proteolytic profile changes during larval development of Anticarsia gemmatalis caterpillars. Archives of Insect Biochemistry and Physiology, [S. l.], v. 103, n. 1, p. e21631, 2020. DOI: 10.1002/arch.21631. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.21631. Acesso em: 31 mar. 2021.

DA SOLIDADE RIBEIRO, Lílian Maria; WANDERLEY-TEIXEIRA, Valéria; DE SIQUEIRA, Herbert Álvaro Abreu; DE OLIVEIRA, Andresa Cristina Batista; LEMOS, Ana Janaína Jeanine Martins; TEIXEIRA, Álvaro Aguiar Coelho. Midgut histopathology of resistant and susceptible Plutella xylostella exposed to commercial formulations of Bacillus thuringiensis. Bulletin of Insectology, [S. l.], v. 66, n. 2, p. 162–168, 2013. Disponível em: www.bulletinofinsectology.org. Acesso em: 13 abr. 2022.

DE ALMEIDA BARROS, Rafael; MERIÑO-CABRERA, Yaremis; VITAL, Camilo E.; DA SILVA JÚNIOR, Neilier R.; DE OLIVEIRA, Cauê N.; LESSA BARBOSA, Samuel; MARQUES GONÇALVES ASSIS, João V.; RAMOS, Humberto J. O.; DE ALMEIDA

OLIVEIRA, Maria G. Small peptides inhibit gut trypsin-like proteases and impair Anticarsia gemmatalis (Lepidoptera: Noctuidae) survival and development. Pest Management Science, [S. l.], v. 77, n. 4, p. 1714–1723, 2021. DOI: 10.1002/ps.6191. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/ps.6191. Acesso em: 31 mar. 2021.

DHANIA, Narender K.; CHAUHAN, Vinod K.; CHAITANYA, R. K.; DUTTA-GUPTA, Aparna. Midgut de novo transcriptome analysis and gene expression profiling of Achaea janata larvae exposed with Bacillus thuringiensis (Bt)-based biopesticide formulation. Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics, [S. l.], v. 30, p. 81–90, 2019. DOI: 10.1016/j.cbd.2019.02.005.

DING, Yunchuan; HAWKES, Nicola; MEREDITH, Janet; EGGLESTON, Paul; HEMINGWAY, Janet; RANSON, Hilary. Characterization of the promoters of Epsilon glutathione transferases in the mosquito Anopheles gambiae and their response to oxidative stress. Biochemical Journal, [S. l.], v. 387, n. 3, p. 879–888, 2005. DOI: 10.1042/BJ20041850. Disponível em: /biochemj/article/387/3/879/44323/Characterization-of-the-promoters-of-Epsilon. Acesso em: 6 maio. 2022.

DMOCHOWSKA-ŚLĘZAK, Kamila; GIEJDASZ, Karol; FLISZKIEWICZ, Monika; ŻÓŁTOWSKA, Krystyna. Variations in antioxidant defense during the development of the solitary bee Osmia bicornis. Apidologie, [S. l.], v. 46, n. 4, p. 432–444, 2015. DOI: 10.1007/s13592-014-0333-y. Disponível em: https://link.springer.com/article/10.1007/s13592-014-0333-y. Acesso em: 8 abr. 2022.

DOS SANTOS, M. C.; RESENDE JUNQUEIRA, A. M.; MENDES DE SÁ, V. G.; ZANÚNCIO, J. C.; SERRÃO, José Eduardo. Effect of silicon on the morphology of the midgut and mandible of tomato leafminer tuta absoluta (Lepidoptera: Gelechiidae) larvae. 2015. Disponível em: https://www.isj.unimore.it/index.php/ISJ/article/view/356. Acesso em: 13 abr. 2022.

DUBOVSKIY, I. M.; MARTEMYANOV, V. V.; VORONTSOVA, Y. L.; RANTALA, M. J.; GRYZANOVA, E. V.; GLUPOV, V. V. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of Galleria mellonella L. larvae (Lepidoptera, Pyralidae). Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, [S. l.], v. 148, n. 1, p. 1–5, 2008. DOI: 10.1016/j.cbpc.2008.02.003.

EMRE, Iskender; KAYIS, Tamer; COSKUN, Mustafa; DURSUN, Osman; COGUN, Hikmet Yeter. Changes in antioxidative enzyme activity, glycogen, lipid, protein, and malondialdehyde content in cadmium-treated galleria mellonella larvae. Annals of the Entomological Society of America, [S. l.], v. 106, n. 3, p. 371–377, 2013. DOI: 10.1603/AN12137. Disponível em: https://academic.oup.com/aesa/article/106/3/371/90679. Acesso em: 8 abr. 2022.

EWELS, Philip; MAGNUSSON, Måns; LUNDIN, Sverker; KÄLLER, Max. MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics, [S. l.], v. 32, n. 19, p. 3047–3048, 2016. DOI: 10.1093/bioinformatics/btw354. Disponível em: https://academic.oup.com/bioinformatics/article/32/19/3047/2196507. Acesso em: 7 jun. 2022.

FARAHANI, Saeed; BANDANI, Ali R.; ALIZADEH, Houshang; GOLDANSAZ, Seyed Hossein; WHYARD, Steven. Differential expression of heat shock proteins and antioxidant enzymes in response to temperature, starvation, and parasitism in the Carob moth larvae, Ectomyelois ceratoniae (Lepidoptera: Pyralidae). PLoS ONE, [S. l.], v. 15, n. 1, p. e0228104, 2020. DOI: 10.1371/journal.pone.0228104. Disponível em: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0228104. Acesso em: 18 nov. 2021.

FELTON, Gary W.; SUMMERS, Clinton B. Antioxidant systems in insects. Archives of Insect Biochemistry and Physiology, [S. l.], v. 29, n. 2, p. 187–197, 1995. DOI: 10.1002/arch.940290208. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.940290208. Acesso em: 8 abr. 2022.

FERNANDES, K. M.; GONZAGA, W. G.; PASCINI, T. V.; MIRANDA, F. R.; TOMÉ, H. V. V.; SERRÃO, J. E.; MARTINS, G. F. Imidacloprid impairs the post-embryonic development of the midgut in the yellow fever mosquito Stegomyia aegypti (=Aedes aegypti). Medical and Veterinary Entomology, [S. l.], v. 29, n. 3, p. 245–254, 2015. DOI: 10.1111/mve.12122. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/mve.12122. Acesso em: 13 abr. 2022.

FIAZ, Muhammad; MARTÍNEZ, Luis Carlos; COSTA, Marilza da Silva; COSSOLIN, Jamile Fernanda Silva; PLATA-RUEDA, Angelica; GONÇALVES, Wagner Gonzaga; SANT'ANA, Antônio Euzébio Goulart; ZANUNCIO, José Cola; SERRÃO, José Eduardo. Squamocin induce histological and ultrastructural changes in the midgut cells of Anticarsia gemmatalis (Lepidoptera: Noctuidae). Ecotoxicology and Environmental Safety, [S. l.], v. 156, n. March,

p. 1–8, 2018. DOI: 10.1016/j.ecoenv.2018.02.080. Disponível em: https://doi.org/10.1016/j.ecoenv.2018.02.080. Acesso em: 31 mar. 2021.

FINN, Robert D. et al. Pfam: The protein families database. Nucleic Acids Research, [S. l.], v. 42, n. D1, p. D222–D230, 2014. DOI: 10.1093/nar/gkt1223. Disponível em: https://academic.oup.com/nar/article/42/D1/D222/1062431. Acesso em: 7 jun. 2022.

FINN, Robert D.; CLEMENTS, Jody; ARNDT, William; MILLER, Benjamin L.; WHEELER, Travis J.; SCHREIBER, Fabian; BATEMAN, Alex; EDDY, Sean R. HMMER web server: 2015 Update. Nucleic Acids Research, [S. l.], v. 43, n. W1, p. W30–W38, 2015. DOI: 10.1093/nar/gkv397. Disponível em: https://academic.oup.com/nar/article/43/W1/W30/2467897. Acesso em: 7 jun. 2022.

FORCADA, Carmen; ALCÁCER, Encarna; GARCERÁ, Ma Dolores; TATO, Agustín; MARTÍNEZ, Rafael. Resistance to Bacillus thuringiensis Cry1Ac toxin in three strains of Heliothis virescens: Proteolytic and SEM study of the larval midgut. Archives of Insect Biochemistry and Physiology, [S. l.], v. 42, n. 1, p. 51–63, 1999. DOI: 10.1002/(SICI)1520-6327(199909)42:1<51::AID-ARCH6>3.0.CO;2-6.

FOYER, C. H.; DESCOURVIÈRES, P.; KUNERT, K. J. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant, Cell & Environment, [S. l.], v. 17, n. 5, p. 507–523, 1994. DOI: 10.1111/j.1365-3040.1994.tb00146.x. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-3040.1994.tb00146.x. Acesso em: 8 abr. 2022.

FRANCO, Octávio L.; DIAS, Simoni C.; MAGALHÃES, Claudio P.; MONTEIRO, Ana C. S.; BLOCH, Carlos; MELO, Francislete R.; OLIVEIRA-NETO, Osmundo B.; MONNERAT, Rose G.; GROSSI-DE-SÁ, Maria Fátima. Effects of soybean Kunitz trypsin inhibitor on the cotton boll weevil (A nthonomus grandis). Phytochemistry, [S. l.], v. 65, n. 1, p. 81–89, 2004. DOI: 10.1016/j.phytochem.2003.09.010.

FU, Limin; NIU, Beifang; ZHU, Zhengwei; WU, Sitao; LI, Weizhong. CD-HIT: Accelerated for clustering the next-generation sequencing data. Bioinformatics, [S. l.], v. 28, n. 23, p. 3150–3152, 2012. DOI: 10.1093/bioinformatics/bts565. Disponível em: https://academic.oup.com/bioinformatics/article/28/23/3150/192160. Acesso em: 7 jun. 2022.

GABAN, Cleusa Rocha Garcia; ARRUDA, Eduardo José De; DOURADO, Doroty Mesquita; SILVA, Lilliam May Grespan E. Da; VILA NOVA, Paulo César Cavalcante; CABRINI, Isaías.

Morphological Changes in the Digestive System of Aedes aegypti L. Induced by [Cu(EDTA)]2- Complex Ions. Journal of Mosquito Research, [S. l.], 2015. DOI: 10.5376/jmr.2015.05.0021. Disponível em: https://www.researchgate.net/publication/308815082. Acesso em: 13 abr. 2022.

GAZARA, Rajesh K.; CARDOSO, Christiane; BELLIENY-RABELO, Daniel; FERREIRA, Clélia; TERRA, Walter R.; VENANCIO, Thiago M. De novo transcriptome sequencing and comparative analysis of midgut tissues of four non-model insects pertaining to Hemiptera, Coleoptera, Diptera and Lepidoptera. Gene, [S. l.], v. 627, p. 85–93, 2017. DOI: 10.1016/j.gene.2017.06.008. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0378111917304596. Acesso em: 10 jun. 2022.

GONÇALVES, Wagner Gonzaga; FERNANDES, Kenner Morais; SANTANA, Weyder Cristiano; MARTINS, Gustavo Ferreira; ZANUNCIO, José Cola; SERRÃO, José Eduardo. Post-embryonic development of the Malpighian tubules in Apis mellifera (Hymenoptera) workers: morphology, remodeling, apoptosis, and cell proliferation. Protoplasma, [S. l.], v. 255, n. 2, p. 585–599, 2018. DOI: 10.1007/s00709-017-1171-3. Disponível em: https://link.springer.com/article/10.1007/s00709-017-1171-3. Acesso em: 13 abr. 2022.

GRABHERR, Manfred G. et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology, [S. l.], v. 29, n. 7, p. 644–652, 2011. DOI: 10.1038/nbt.1883. Disponível em: https://www.nature.com/articles/nbt.1883. Acesso em: 31 mar. 2021.

GREENE, G. L.; LEPPLA, N. C.; DICKERSON, W. A. Velvetbean Caterpillar: A Rearing Procedure and Artificial Medium123. Journal of Economic Entomology, [S. l.], v. 69, n. 4, p. 487–488, 1976. DOI: 10.1093/jee/69.4.487. Disponível em: https://academic.oup.com/jee/article/69/4/487/2212175. Acesso em: 31 mar. 2021.

GRISWOLD, C. M.; MATTHEWS, A. L.; BEWLEY, K. E.; MAHAFFEY, J. W. Molecular characterization and rescue of acatalasemic mutants of Drosophila melanogaster. Genetics, [S. l.], v. 134, n. 3, p. 781–788, 1993. DOI: 10.1093/genetics/134.3.781. Disponível em: https://academic.oup.com/genetics/article/134/3/781/6009305. Acesso em: 8 abr. 2022.

HAAS, Brian J. et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols, [S. l.], v. 8, n. 8, p.

1494–1512, 2013. DOI: 10.1038/nprot.2013.084. Disponível em: https://www.nature.com/articles/nprot.2013.084. Acesso em: 7 jun. 2022.

HARIRI, Mehrdad; MILLANE, Ghania; GUIMOND, Marie Pierre; GUAY, Ginette; DENNIS, James W.; NABI, Ivan R. Biogenesis of multilamellar bodies via autophagy. Molecular Biology of the Cell, [S. l.], v. 11, n. 1, p. 255–268, 2000. DOI: 10.1091/mbc.11.1.255. Disponível em: https://www.molbiolcell.org/doi/full/10.1091/mbc.11.1.255. Acesso em: 13 abr. 2022.

HARSULKAR, Abhay M.; GIRI, Ashok P.; PATANKAR, Aparna G.; GUPTA, Vidya S.; SAINANI, Mohini N.; RANJEKAR, Prabhakar K.; DESHPANDE, Vasanti V. Successive use of non-host plant proteinase inhibitors required for effective inhibition of Helicoverpa armigera gut proteinases and larval growth. Plant Physiology, [S. l.], v. 121, n. 2, p. 497–506, 1999. DOI: 10.1104/pp.121.2.497. Disponível em: https://academic.oup.com/plphys/article/121/2/497/6096195. Acesso em: 24 mar. 2022.

HE, Falin; SUN, Shiang; TAN, Haili; SUN, Xiao; QIN, Chao; JI, Shoumin; LI, Xiangdong; ZHANG, Jiwang; JIANG, Xingyin. Chlorantraniliprole against the black cutworm Agrotis ipsilon (Lepidoptera: Noctuidae): From biochemical/physiological to demographic responses. Scientific Reports, [S. l.], v. 9, n. 1, 2019. DOI: 10.1038/s41598-019-46915-0.

HEMINGWAY, J.; KARUNARATNE, S. H. P. P. Mosquito carboxylesterases: A review of the molecular biology and biochemistry of a major insecticide resistance mechanism. Medical and Veterinary Entomology, [S. l.], v. 12, n. 1, p. 1–12, 1998. DOI: 10.1046/j.1365-2915.1998.00082.x. Disponível em: https://europepmc.org/article/med/9513933. Acesso em: 25 mar. 2022.

HENG-MOSS, Tiffany; SARATH, Gautam; BAXENDALE, Frederick; NOVAK, Dana; BOSE, Shauna; XINHI, N. I.; QUISENBERRY, Sharron. Characterization of oxidative enzyme changes in buffalograsses challenged by Blissus occiduus. Journal of Economic Entomology, [S. l.], v. 97, n. 3, p. 1086–1095, 2004. DOI: 10.1093/jee/97.3.1086. Disponível em: https://academic.oup.com/jee/article/97/3/1086/2218140. Acesso em: 6 maio. 2022.

HOROWITZ, A. Rami; GHANIM, Murad; RODITAKIS, Emmanouil; NAUEN, Ralf; ISHAAYA, Isaac. Insecticide resistance and its management in Bemisia tabaci species. Journal of Pest Science, [S. l.], v. 93, n. 3, p. 893–910, 2020. DOI: 10.1007/s10340-020-01210-0. Disponível em: https://link.springer.com/article/10.1007/s10340-020-01210-0. Acesso em: 24 nov. 2021.

HUERTA-CEPAS, Jaime et al. EGGNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. Nucleic Acids Research, [S. l.], v. 44, n. D1, p. D286–D293, 2016. DOI: 10.1093/nar/gkv1248. Disponível em: https://academic.oup.com/nar/article/44/D1/D286/2503059. Acesso em: 7 jun. 2022.

HUERTA-CEPAS, Jaime; FORSLUND, Kristoffer; COELHO, Luis Pedro; SZKLARCZYK, Damian; JENSEN, Lars Juhl; VON MERING, Christian; BORK, Peer. Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. Molecular Biology and Evolution, [S. l.], v. 34, n. 8, p. 2115–2122, 2017. DOI: 10.1093/molbev/msx148. Disponível em: https://academic.oup.com/mbe/article/34/8/2115/3782716. Acesso em: 7 jun. 2022.

IHARA, Tomomi; YAMAMOTO, Tsukiko; SUGAMATA, Masao; OKUMURA, Hiroki; UENO, Yoshio. The process of ultrastructural changes from nuclei to apoptotic body. Virchows Archiv, [S. l.], v. 433, n. 5, p. 443–447, 1998. DOI: 10.1007/s004280050272. Disponível em: https://link.springer.com/article/10.1007/s004280050272. Acesso em: 13 abr. 2022.

ISHAAYA, Isaac; CASIDA, John E. Dietary TH 6040 alters composition and enzyme activity of housefly larval cuticle. Pesticide Biochemistry and Physiology, [S. l.], v. 4, n. 4, p. 484–490, 1974. DOI: 10.1016/0048-3575(74)90073-X.

JACOBSON, Michael D. Reactive oxygen species and programmed cell death. Trends in Biochemical Sciences, [S. l.], v. 21, n. 3, p. 83–86, 1996. DOI: 10.1016/S0968-0004(96)20008-8.

JAHAN, Fatima N.; RAHMAN, Mohammad S.; HOSSAIN, Mahboob; RASHID, Mohammad A. Antimicrobial activity and toxicity of Quisqualis indica. Oriental Pharmacy and Experimental Medicine, [S. l.], v. 8, n. 1, p. 53–58, 2008. DOI: 10.3742/opem.2008.8.1.053. Disponível em: www.opem.orgOPEM. Acesso em: 16 nov. 2021.

JIA, Fu Xian; DOU, Wei; HU, Fei; WANG, Jin Jun. Effects of thermal stress on lipid peroxidation and antioxidant enzyme activities of oriental fruit fly, bactrocera dorsalis (Diptera: Tephritidae). Florida Entomologist, [S. l.], v. 94, n. 4, p. 956–963, 2011. DOI: 10.1653/024.094.0432. Disponível em: https://bioone.org/journals/florida-entomologist/volume-94/issue-4/024.094.0432/Effects-of-Thermal-Stress-on-Lipid-Peroxidation-and-Antioxidant-Enzyme/10.1653/024.094.0432.full. Acesso em: 11 mar. 2022.

JONGSMA, Maarten A.; BAKKER, Petra L.; PETERS, Jeroen; BOSCH, Dirk; STIEKEMA, Willem J. Adaptation of Spodoptera exigua larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proceedings of the National Academy of Sciences of the United States of America, [S. l.], v. 92, n. 17, p. 8041–8045, 1995. DOI: 10.1073/pnas.92.17.8041. Disponível em: https://www.pnas.org. Acesso em: 8 abr. 2022.

JONGSMA, Maarten A.; BOLTER, Caroline. The adaptation of insects to plant protease inhibitors. Journal of Insect Physiology, [S. l.], v. 43, n. 10, p. 885–895, 1997. DOI: 10.1016/S0022-1910(97)00040-1. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0022191097000401. Acesso em: 31 mar. 2021.

KARTHI, Sengodan; SANKARI, R.; SHIVAKUMAR, Muthugounder S. Ultraviolet-B light induced oxidative stress: Effects on antioxidant response of Spodoptera litura. Journal of Photochemistry and Photobiology B: Biology, [S. l.], v. 135, p. 1–6, 2014. DOI: 10.1016/j.jphotobiol.2014.04.008.

KATO, S.; NAITO, Z. Glutathione S-transferase. Nippon rinsho. Japanese journal of clinical medicine, [S. l.], v. 57 Suppl, n. 22, p. 451–453, 1999. DOI: 10.1016/s0021-9258(19)42083-8. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0021925819420838. Acesso em: 4 fev. 2022.

KAYIS, T.; COSKUN, M.; DURSUN, O.; EMRE, I. Alterations in Antioxidant Enzyme Activity, Lipid Peroxidation, and Ion Balance Induced by Dichlorvos in Galleria mellonella (Lepidoptera: Pyralidae). Annals of the Entomological Society of America, [S. l.], v. 108, n. 4, p. 570–574, 2015. DOI: 10.1093/aesa/sav038. Disponível em: https://academic.oup.com/aesa/article/108/4/570/72622. Acesso em: 9 fev. 2022.

KONANZ, Stefanie; NAUEN, Ralf. Purification and partial characterization of a glutathione S-transferase from the two-spotted spider mite, Tetranychus urticae. Pesticide Biochemistry and Physiology, [S. l.], v. 79, n. 2, p. 49–57, 2004. DOI: 10.1016/j.pestbp.2004.03.004.

KONNO, Yasuhiko; SHISHIDO, Takashi. Distribution of Glutathione S-transferase Activity in Insect Tissues. Applied Entomology and Zoology, [S. l.], v. 27, n. 3, p. 391–397, 1992. DOI: 10.1303/aez.27.391.

KREMPL, Corinna; SPORER, Theresa; REICHELT, Michael; AHN, Seung Joon; HEIDEL-FISCHER, Hanna; VOGEL, Heiko; HECKEL, David G.; JOUSSEN, Nicole. Potential detoxification of gossypol by UDP-glycosyltransferases in the two Heliothine moth species

Helicoverpa armigera and Heliothis virescens. Insect Biochemistry and Molecular Biology, [S. l.], v. 71, p. 49–57, 2016. DOI: 10.1016/j.ibmb.2016.02.005.

KROEMER, Guido; MARIÑO, Guillermo; LEVINE, Beth. Autophagy and the Integrated Stress Response. Molecular Cell, [S. l.], v. 40, n. 2, p. 280–293, 2010. DOI: 10.1016/j.molcel.2010.09.023.

LANGMEAD, Benjamin Thomas. Highly Scalable Short Read Alignment with the Burrows-Wheeler Transform and Cloud Computing. [s.l.] : University of Maryland, College Park, 2009. Disponível em: https://www.proquest.com/openview/3dbe4cf8c99e49308a6f6b5e7bd4934e/1?pq-origsite=gscholar&cbl=18750. Acesso em: 7 jun. 2022.

LEDIRAC, Nathalie; ANTHERIEU, Sebastian; D'UBY, Anne Dupuy; CARON, Jean Claude; RAHMANI, Roger. Effects of organochlorine insecticides on MAP kinase pathways in human HaCaT keratinocytes: Key role of reactive oxygen species. Toxicological Sciences, [S. l.], v. 86, n. 2, p. 444–452, 2005. DOI: 10.1093/toxsci/kfi192. Disponível em: https://academic.oup.com/toxsci/article/86/2/444/1653598. Acesso em: 2 fev. 2022.

LEVY, Sheila M.; FALLEIROS, Ângela M. F.; MOSCARDI, Flávio; GREGÓRIO, Elisa A. Susceptibility/resistance of Anticarsia gemmatalis larvae to its nucleopolyhedrovirus (AgMNPV): Structural study of the peritrophic membrane. Journal of Invertebrate Pathology, [S. l.], v. 96, n. 2, p. 183–186, 2007. DOI: 10.1016/j.jip.2007.04.007.

LI, Mao Ye; TU, Xiao Hui; CAO, Ye; LI, Shi Guang; LIU, Su. Characterisation of a copper/zinc superoxide dismutase from Pieris rapae and its role in protecting against oxidative stress induced by chlorantraniliprole. Pesticide Biochemistry and Physiology, [S. l.], v. 174, p. 104825, 2021. DOI: 10.1016/j.pestbp.2021.104825.

LIJUN, Li; XUEMEI, Liu; YAPING, Guo; ENBO, Ma. Activity of the enzymes of the antioxidative system in cadmium-treated Oxya chinensis (Orthoptera Acridoidae). Environmental Toxicology and Pharmacology, [S. l.], v. 20, n. 3, p. 412–416, 2005. DOI: 10.1016/j.etap.2005.04.001.

LIMA, Ana T.; BASTOS, Felipe A.; TEUBNER, Fernando Jakes; NETO, Renato R.; COOPER, Andrew; BARROSO, Gilberto F. Strengths and Weaknesses of a Hybrid Post-disaster Management Approach: the Doce River (Brazil) Mine-Tailing Dam Burst. Environmental Management, Department of Biochemistry, George S. Wise Faculty of Life

Sciences, Tel Aviv University, Ramat Aviv 69978, Israel. DOI - 10.1093/nar/gki370 SRC - Pubmed ID2 - 15980475 FG - 0, v. 65, n. 6, p. 711–724, 2020. DOI: 10.1007/s00267-020-01279-4. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-204X2008000700003&lng=en&tlng=en. Acesso em: 31 mar. 2021.

LINDROTH, Richard L.; ANSON, Blake D.; WEISBROD, Anne V. Effects of protein and juglone on gypsy moths: Growth performance and detoxification enzyme activity. Journal of Chemical Ecology, [S. l.], v. 16, n. 8, p. 2533–2547, 1990. DOI: 10.1007/BF01017476. Disponível em: https://link.springer.com/article/10.1007/BF01017476. Acesso em: 8 abr. 2022.

LOEB, Marcia J.; MARTIN, Phyllis A. W.; HAKIM, Raziel S.; GOTO, Shintaro; TAKEDA, Makio. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of Bacillus thuringiensis. Journal of Insect Physiology, [S. l.], v. 47, n. 6, p. 599–606, 2001. DOI: 10.1016/S0022-1910(00)00150-5.

LOVE, Michael I.; HUBER, Wolfgang; ANDERS, Simon. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology, [S. l.], v. 15, n. 12, 2014. DOI: 10.1186/s13059-014-0550-8.

ŁUKASIK, Iwona; GOŁAWSKA, Sylwia; WÓJCICKA, Agnieszka. Antioxidant defense mechanisms of cereal aphids based on ascorbate and ascorbate peroxidase. Biologia, [S. l.], v. 64, n. 5, p. 994–998, 2009. DOI: 10.2478/s11756-009-0164-x. Disponível em: https://link.springer.com/article/10.2478/s11756-009-0164-x. Acesso em: 8 abr. 2022.

MAO, Tingting et al. Induction of ER stress, antioxidant and detoxification response by sublethal doses of chlorantraniliprole in the silk gland of silkworm, Bombyx mori. Pesticide Biochemistry and Physiology, [S. l.], v. 170, p. 104685, 2020. DOI: 10.1016/j.pestbp.2020.104685.

MARTÍNEZ-RAMÍREZ, Amparo C.; GOULD, Fred; FERRÉ, Juan. Histopathological effects and growth reduction in a susceptible and a resistant strain of Heliothis virescens (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from Bacillus thuringiensis. Biocontrol Science and Technology, [S. l.], v. 9, n. 2, p. 239–246, 1999. DOI: 10.1080/09583159929811. Disponível em: https://www.tandfonline.com/doi/abs/10.1080/09583159929811. Acesso em: 13 abr. 2022.

MATHAVAN, S.; SUDHA, P. M.; PECHIMUTHU, S. M. Effect of Bacillus thuringiensis israelensis on the midgut cells of Bombyx mori larvae: A histopathological and histochemical

study. Journal of Invertebrate Pathology, [S. l.], v. 53, n. 2, p. 217–227, 1989. DOI: 10.1016/0022-2011(89)90010-4.

MELCHIORRI, D.; ORTIZ, G. G.; REITER, R. J.; SEWERYNEK, E.; DANIELS, W. M. U.; PABLOS, M. I.; NISTICÒ, G. Melatonin reduces paraquat-induced genotoxicity in mice. Toxicology Letters, [S. l.], v. 95, n. 2, p. 103–108, 1998. DOI: 10.1016/S0378-4274(98)00025-3.

MENG, Jian Yu; ZHANG, Chang Yu; ZHU, Fen; WANG, Xiao Ping; LEI, Chao Liang. Ultraviolet light-induced oxidative stress: Effects on antioxidant response of Helicoverpa armigera adults. Journal of Insect Physiology, [S. l.], v. 55, n. 6, p. 588–592, 2009. DOI: 10.1016/j.jinsphys.2009.03.003.

MITTLER, Ron; POULOS, Thomas L. Ascorbate Peroxidase. Antioxidants and Reactive Oxygen Species in Plants, [S. l.], p. 87–100, 2007. DOI: 10.1002/9780470988565.ch4.

MÜLLER, Caroline. Impacts of sublethal insecticide exposure on insects — Facts and knowledge gaps. Basic and Applied Ecology, [S. l.], v. 30, p. 1–10, 2018. DOI: 10.1016/j.baae.2018.05.001.

MUNDAY, Rex; WINTERBOURN, Christine C. Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. Biochemical Pharmacology, [S. l.], v. 38, n. 24, p. 4349–4352, 1989. DOI: 10.1016/0006-2952(89)90641-2.

NANOTH VELLICHIRAMMAL, Neetha; WANG, Haichuan; EYUN, Seong Il; MORIYAMA, Etsuko N.; COATES, Brad S.; MILLER, Nicholas J.; SIEGFRIED, Blair D. Transcriptional analysis of susceptible and resistant European corn borer strains and their response to Cry1F protoxin. BMC Genomics, [S. l.], v. 16, n. 1, p. 1–11, 2015. DOI: 10.1186/s12864-015-1751-6. Disponível em: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-015-1751-6. Acesso em: 10 jun. 2022.

NAVARRO-ROLDÁN, M. A.; BOSCH, D.; GEMENO, C.; SIEGWART, M. Enzymatic detoxification strategies for neurotoxic insecticides in adults of three tortricid pests. Bulletin of Entomological Research, [S. l.], v. 110, n. 1, p. 144–154, 2020. DOI: 10.1017/S0007485319000415. Disponível em: https://www.cambridge.org/core/journals/bulletin-of-entomological-

research/article/abs/enzymatic-detoxification-strategies-for-neurotoxic-insecticides-in-adults-of-three-tortricid-pests/A692780A3EE4C219163844CB09FD3C60. Acesso em: 21 out. 2021.

NELSON, David R. Progress in tracing the evolutionary paths of cytochrome P450. Biochimica et Biophysica Acta - Proteins and Proteomics, [S. l.], v. 1814, n. 1, p. 14–18, 2011. DOI: 10.1016/j.bbapap.2010.08.008.

ORR, William C.; SOHAL, Rajindar S. Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. Science, [S. l.], v. 263, n. 5150, p. 1128–1130, 1994. DOI: 10.1126/science.8108730. Disponível em: https://www.science.org/doi/abs/10.1126/science.8108730. Acesso em: 8 abr. 2022.

OTTAVIANI, Enzo; MALAGOLI, Davide. Cell Death Pathways in an Unconventional Invertebrate Model. Lessons in Immunity: From Single-cell Organisms to Mammals, [S. l.], p. 17–27, 2016. DOI: 10.1016/B978-0-12-803252-7.00002-3.

PANIZZI, A. R. History and Contemporary Perspectives of the Integrated Pest Management of Soybean in Brazil. Neotropical Entomology, [S. l.], v. 42, n. 2, p. 119–127, 2013. DOI: 10.1007/s13744-013-0111-y. Disponível em: https://link.springer.com/article/10.1007/s13744-013-0111-y. Acesso em: 30 set. 2021.

PAPADOPOULOS, Athanasios I.; POLEMITOU, Irene; LAIFI, Pshychoula; YIANGOU, Astero; TANANAKI, Chrysoula. Glutathione S-transferase in the insect Apis mellifera macedonica: Kinetic characteristics and effect of stress on the expression of GST isoenzymes in the adult worker bee. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, [S. l.], v. 139, n. 1–3, p. 93–97, 2004. DOI: 10.1016/j.cca.2004.09.010.

PARK, Jiyeong; STANLEY, David; KIM, Yonggyun. Roles of peroxinectin in PGE2-mediated cellular immunity in spodoptera exigua. PLoS ONE, [S. l.], v. 9, n. 9, p. e105717, 2014. DOI: 10.1371/journal.pone.0105717. Disponível em: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0105717. Acesso em: 4 maio. 2022.

PAULA, Débora Pires; ANDOW, David A.; TIMBÓ, Renata Velozo; SUJII, Edison R.; PIRES, Carmen S. S.; FONTES, Eliana M. G. Uptake and transfer of a Bt toxin by a lepidoptera to its eggs and effects on its offspring. PLoS ONE, [S. l.], v. 9, n. 4, p. e95422, 2014. DOI: 10.1371/journal.pone.0095422. Disponível em:

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0095422. Acesso em: 29 out. 2021.

PERERA, Omaththage P.; SHELBY, Kent S.; POPHAM, Holly J. R.; GOULD, Fred; ADANG, Michael J.; JURAT-FUENTES, Juan Luis. Generation of a transcriptome in a model lepidopteran pest, Heliothis virescens, using multiple sequencing strategies for profiling midgut gene expression. PLoS ONE, [S. l.], v. 10, n. 6, p. e0128563, 2015. DOI: 10.1371/journal.pone.0128563. Disponível em: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0128563. Acesso em: 10 jun. 2022.

PERIĆ-MATARUGA, Vesna; ILIJIN, Larisa; MRDAKOVIĆ, Marija; TODOROVIĆ, Dajana; PROKIĆ, Marko; MATIĆ, Dragana; VLAHOVIĆ, Milena. Parameters of oxidative stress, cholinesterase activity, Cd bioaccumulation in the brain and midgut of Lymantria dispar (Lepidoptera: Lymantriidae) caterpillars from unpolluted and polluted forests. Chemosphere, [S. l.], v. 218, p. 416–424, 2019. DOI: 10.1016/j.chemosphere.2018.11.112. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0045653518322197. Acesso em: 11 mar. 2022.

PISKORSKI, Rafal; INEICHEN, Simon; DORN, Silvia. Ability of the Oriental Fruit Moth Grapholita molesta (Lepidoptera: Tortricidae) to Detoxify Juglone, the Main Secondary Metabolite of the Non-host Plant Walnut. Journal of Chemical Ecology, [S. l.], v. 37, n. 10, p. 1110–1116, 2011. DOI: 10.1007/s10886-011-0015-4. Disponível em: https://link.springer.com/article/10.1007/s10886-011-0015-4. Acesso em: 8 abr. 2022.

PONTUAL, Emmanuel Viana; NAPOLEÃO, Thiago Henrique; DIAS DE ASSIS, Caio Rodrigo; DE SOUZA BEZERRA, Ranilson; XAVIER, Haroudo Satiro; NAVARRO, Daniela Maria do Amaral Ferraz; COELHO, Luana Cassandra Breitenbach Barroso; PAIVA, Patrícia Maria Guedes. Effect of moringa oleifera flower extract on larval trypsin and acethylcholinesterase activities in aedes aegypti. Archives of Insect Biochemistry and Physiology, [S. l.], v. 79, n. 3, p. 135–152, 2012. DOI: 10.1002/arch.21012. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.21012. Acesso em: 9 nov. 2021.

POTTIER, M. A.; BOZZOLAN, F.; CHERTEMPS, T.; JACQUIN-JOLY, E.; LALOUETTE, L.; SIAUSSAT, D.; MAÏBÈCHE-COISNE, M. Cytochrome P450s and cytochrome P450 reductase in the olfactory organ of the cotton leafworm Spodoptera littoralis. Insect Molecular Biology, [S. l.], v. 21, n. 6, p. 568–580, 2012. DOI: 10.1111/j.1365-2583.2012.01160.x.

Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2583.2012.01160.x. Acesso em: 10 jun. 2022.

REY, D.; LONG, A.; PAUTOU, M. P.; MEYRAN, J. C. Comparative histopathology of some Diptera and Crustacea of aquatic alpine ecosystems, after treatment with Bacillus thuringiensis var. israelensis. Entomologia Experimentalis et Applicata, [S. l.], v. 88, n. 3, p. 255–263, 1998. DOI: 10.1046/j.1570-7458.1998.00370.x. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1046/j.1570-7458.1998.00370.x. Acesso em: 6 maio. 2022.

ROMANELLI, Davide; CASATI, Barbara; FRANZETTI, Eleonora; TETTAMANTI, Gianluca. A Molecular View of Autophagy in Lepidoptera. BioMed Research International, [S. l.], v. 2014, 2014. DOI: 10.1155/2014/902315.

SANKARAPANDI, Sornampillai; ZWEIER, Jay L. Evidence against the generation of free hydroxyl radicals from the interaction of copper,zinc-superoxide dismutase and hydrogen peroxide. Journal of Biological Chemistry, [S. l.], v. 274, n. 49, p. 34576–34583, 1999. DOI: 10.1074/jbc.274.49.34576. Disponível em: http://www.jbc.org/article/S0021925819533634/fulltext. Acesso em: 8 abr. 2022.

SANTOS, Douglas Elias; AZEVEDO, Dihego Oliveira; CAMPOS, Lúcio Antônio Oliveira; ZANUNCIO, José Cola; SERRÃO, José Eduardo. Melipona quadrifasciata (Hymenoptera: Apidae) fat body persists through metamorphosis with a few apoptotic cells and an increased autophagy. Protoplasma, [S. l.], v. 252, n. 2, p. 619–627, 2015. DOI: 10.1007/s00709-014-0707-z. Disponível em: https://link.springer.com/article/10.1007/s00709-014-0707-z. Acesso em: 13 abr. 2022.

SHADNIA, Shahin; AZIZI, Ebrahim; HOSSEINI, Rohollah; KHOEI, Samideh; FOULADDEL, Shamileh; PAJOUMAND, Abdolkarim; JALALI, Nasser; ABDOLLAHI, Mohammad. Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. Human and Experimental Toxicology, [S. l.], v. 24, n. 9, p. 439–445, 2005. DOI: 10.1191/0960327105ht549oa. Disponível em: https://journals.sagepub.com/doi/10.1191/0960327105ht549oa. Acesso em: 18 nov. 2021.

SHANNON, Leland M.; KAY, Ernest; LEW, Jow Y. Peroxidase Isozymes from Horseradish Roots. Journal of Biological Chemistry, [S. l.], v. 241, n. 9, p. 2166–2172, 1966. DOI:

10.1016/s0021-9258(18)96680-9. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0021925818966809. Acesso em: 10 mar. 2022.

SHAO, Zuo Min; LI, Yi Jiangcheng; ZHANG, Xiao Rui; CHU, Jie; MA, Jia Hui; LIU, Zhi Xiang; WANG, Jun; SHENG, Sheng; WU, Fu An. Identification and functional study of chitin metabolism and detoxification-related genes in glyphodes pyloalis walker (Lepidoptera: Pyralidae) based on transcriptome analysis. International Journal of Molecular Sciences, [S. l.], v. 21, n. 5, p. 1904, 2020. DOI: 10.3390/ijms21051904. Disponível em: https://www.mdpi.com/1422-0067/21/5/1904. Acesso em: 10 jun. 2022.

SIES, Helmut. Oxidative stress: From basic research to clinical application. The American Journal of Medicine, [S. l.], v. 91, n. 3 SUPPL. 3, p. S31–S38, 1991. DOI: 10.1016/0002-9343(91)90281-2.

SILVA-BRANDÃO, Karina Lucas; MURAD, Natália Faraj; PERUCHI, Aline; MARTINS, Carlos Henrique Zanini; OMOTO, Celso; FIGUEIRA, Antonio; BRANDÃO, Marcelo Mendes; TRIGO, José Roberto. Transcriptome differential co-expression reveals distinct molecular response of fall-armyworm strains to DIMBOA. Pest Management Science, [S. l.], v. 77, n. 1, p. 518–526, 2021. DOI: 10.1002/ps.6051. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/ps.6051. Acesso em: 10 jun. 2022.

SILVA-JÚNIOR, Neilier R.; CABRERA, Yaremis M.; BARBOSA, Samuel L.; BARROS, Rafael de A.; BARROS, Edvaldo; VITAL, Camilo E.; RAMOS, Humberto J. O.; OLIVEIRA, Maria Goreti A. Intestinal proteases profiling from Anticarsia gemmatalis and their binding to inhibitors. Archives of Insect Biochemistry and Physiology, [S. l.], v. 107, n. 3, p. e21792, 2021. DOI: 10.1002/arch.21792. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.21792. Acesso em: 13 maio. 2021.

SINGH, Sujata; SINGH, Archana; KUMAR, Sumit; MITTAL, Pooja; SINGH, Indrakant K. Protease inhibitors: recent advancement in its usage as a potential biocontrol agent for insect pest management. Insect Science, Molecular Biology Research Laboratory, Department of Zoology, Deshbandhu College, University of Delhi, Kalkaji, New Delhi, India. DOI - 10.1111/1744-7917.12641 SRC - Pubmed ID2 - 30230264 FG - 0, v. 27, n. 2, p. 186–201, 2020. DOI: 10.1111/1744-7917.12641. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/1744-7917.12641. Acesso em: 27 maio. 2021.

SLATER, T. F. [33] Overview of Methods Used for Detecting Lipid Peroxidation. Methods in Enzymology, [S. l.], v. 105, n. C, p. 283–293, 1984. DOI: 10.1016/S0076-6879(84)05036-9. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/6427549. Acesso em: 8 abr. 2022.

SOBEKOVÁ, Anna; HOLOVSKÁ, Katarína; LENÁRTOVÁ, Viera; FLEŠÁROVÁ, Slávka; JAVORSKÝ, P. The another toxic effect if carbamate insecticides. Acta Biologica Hungarica, [S. l.], v. 60, n. 1, p. 45–54, 2009. DOI: 10.1556/ABiol.60.2009.1.5. Disponível em: https://akjournals.com/view/journals/018/60/1/article-p45.xml. Acesso em: 8 abr. 2022.

SONG, Li; FLOREA, Liliana. Rcorrector: Efficient and accurate error correction for Illumina RNA-seq reads. GigaScience, [S. l.], v. 4, n. 1, p. 48, 2015. DOI: 10.1186/s13742-015-0089-y. Disponível em: https://academic.oup.com/gigascience/article/4/1/s13742-015-0089-y/2707778. Acesso em: 7 jun. 2022.

SOUSA, Maria Esmeralda C.; SANTOS, Fábio A. B.; WANDERLEY-TEIXEIRA, Valéria; TEIXEIRA, Álvaro A. C.; DE SIQUEIRA, Herbert Álvaro A.; ALVES, Luiz C.; TORRES, Jorge B. Histopathology and ultrastructure of midgut of Alabama argillacea (Hübner) (Lepidoptera: Noctuidae) fed Bt-cotton. Journal of Insect Physiology, [S. l.], v. 56, n. 12, p. 1913–1919, 2010. DOI: 10.1016/j.jinsphys.2010.08.014. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0022191010002593. Acesso em: 13 abr. 2022.

SOUSA, S.; MAIA, M. L.; CORREIRA-SÁ, L.; FERNANDES, V. C.; DELERUE-MATOS, C.; CALHAU, C.; DOMINGUES, V. F. Chemistry and Toxicology Behind Insecticides and Herbicides. In: Controlled Release of Pesticides for Sustainable Agriculture. Cham: Springer International Publishing, 2020. p. 59–109. DOI: 10.1007/978-3-030-23396-9_3. Disponível em: http://link.springer.com/10.1007/978-3-030-23396-9_3.

SOWJANYA SREE, K. S.; SACHDEV, Bindiya; PADMAJA, V.; BHATNAGAR, Raj K. Electron spin resonance spectroscopic studies of free radical generation and tissue specific catalase gene expression in Spodoptera litura (Fab.) larvae treated with the mycotoxin, destruxin. Pesticide Biochemistry and Physiology, [S. l.], v. 97, n. 3, p. 168–176, 2010. DOI: 10.1016/j.pestbp.2010.01.002.

SRINIVASAN, Ajay; GIRI, Ashok P.; GUPTA, Vidya S. Structural and functional diversities in lepidopteran serine proteases. Cellular and Molecular Biology Letters, Plant Molecular Biology Group, Division of Biochemical Sciences, National Chemical Laboratory, Pune, India. DOI - 10.2478/s11658-006-0012-8 SRC - Pubmed ID2 - 16847755 FG - 0, v. 11, n. 1, p. 132–

154, 2006. DOI: 10.2478/s11658-006-0012-8. Disponível em: https://www.degruyter.com/document/doi/10.2478/s11658-006-0012-8/html. Acesso em: 27 maio. 2021.

SU, Qi; ZHOU, Zhixiong; ZHANG, Jianmin; SHI, Caihua; ZHANG, Guohui; JIN, Zhenyu; WANG, Wenkai; LI, Chuanren. Effect of plant secondary metabolites on common cutworm, Spodoptera litura (Lepidoptera: Noctuidae). Entomological Research, [S. l.], v. 48, n. 1, p. 18–26, 2018. DOI: 10.1111/1748-5967.12238. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1111/1748-5967.12238. Acesso em: 8 abr. 2022.

TANG, Pei An; WU, Hai Jing; XUE, Hao; JU, Xing Rong; SONG, Wei; ZHANG, Qi Lin; YUAN, Ming Long. Characterization of transcriptome in the Indian meal moth Plodia interpunctella (Lepidoptera: Pyralidae) and gene expression analysis during developmental stages. Gene, [S. l.], v. 622, p. 29–41, 2017. DOI: 10.1016/j.gene.2017.04.018. Disponível em: http://dx.doi.org/10.1016/j.gene.2017.04.018. Acesso em: 10 jun. 2022.

THIBOLDEAUX, R. L.; LINDROTH, R. L.; TRACY, J. W. Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in Saturniid moth larvae. Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology, [S. l.], v. 120, n. 3, p. 481–487, 1998. DOI: 10.1016/S0742-8413(98)10070-1.

VAN BEL, Michiel; PROOST, Sebastian; VAN NESTE, Christophe; DEFORCE, Dieter; VAN DE PEER, Yves; VANDEPOELE, Klaas. TRAPID: An efficient online tool for the functional and comparative analysis of de novo RNA-Seq transcriptomes. Genome Biology, [S. l.], v. 14, n. 12, p. 1–10, 2013. DOI: 10.1186/gb-2013-14-12-r134. Disponível em: https://genomebiology.biomedcentral.com/articles/10.1186/gb-2013-14-12-r134. Acesso em: 7 jun. 2022.

VOLPICELLA, Mariateresa; CECI, Luigi R.; CORDEWENER, Jan; AMERICA, Twan; GALLERANI, Raffaele; BODE, Wolfram; JONGSMA, Maarten A.; BEEKWILDER, Jules. Properties of purified gut trypsin from Helicoverpa zea, adapted to proteinase inhibitors. European Journal of Biochemistry, [S. l.], v. 270, n. 1, p. 10–19, 2003. DOI: 10.1046/j.1432-1033.2003.03368.x. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1046/j.1432-1033.2003.03368.x. Acesso em: 31 mar. 2021.

VONTAS, John G.; SMALL, Graham J.; HEMINGWAY, Janet. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. Biochemical

Journal, [S. l.], v. 357, n. 1, p. 65–72, 2001. DOI: 10.1042/bj3570065. Disponível em: /biochemj/article/357/1/65/39593/Glutathione-S-transferases-as-antioxidant-defence. Acesso em: 25 mar. 2022.

WALKER, David R.; ALL, John N.; MCPHERSON, Robert M.; BOERMA, H. Roger; PARROTT, Wayne A. Field evaluation of soybean engineered with a synthetic cry1Ac transgene for resistance to corn earworm, soybean looper, velvetbean caterpillar (Lepidoptera: Noetuidae), and lesser cornstalk borer (Lepidoptera: Pyralidae). Journal of Economic Entomology, Department of Crop and Soil Sciences, University of Georgia, Athens 30602-7272, USA. DOI - 10.1603/0022-0493-93.3.613 SRC - Pubmed ID2 - 10902306 FG - 0, v. 93, n. 3, p. 613–622, 2000. DOI: 10.1603/0022-0493-93.3.613. Disponível em: https://academic.oup.com/jee/article-lookup/doi/10.1603/0022-0493-93.3.613. Acesso em: 31 mar. 2021.

WANG, Gao-Ping; ZHANG, Qing-Wen; YE, Zhi-Hua; LUO, Li-Zhi. The role of nectar plants in severe outbreaks of armyworm Mythimna separata (Lepidoptera: Noctuidae) in China. Bulletin of entomological research, [S. l.], v. 96, n. 5, p. 445–55, 2006. DOI: 10.1079/BER2006433. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/17092356. Acesso em: 16 nov. 2021.

WANG, Zhong; GERSTEIN, Mark; SNYDER, Michael. RNA-Seq: A revolutionary tool for transcriptomics. Nature Reviews Genetics, [S. l.], v. 10, n. 1, p. 57–63, 2009. DOI: 10.1038/nrg2484. Disponível em: https://www.nature.com/articles/nrg2484. Acesso em: 10 jun. 2022.

WAR, Abdul Rashid; PAULRAJ, Michael Gabriel; WAR, Mohd Yousf; IGNACIMUTHU, Savarimuthu. Herbivore- and elicitor-induced resistance in groundnut to Asian armyworm, Spodoptera litura (Fab.) (lepidoptera: Noctuidae). Plant Signaling and Behavior, [S. l.], v. 6, n. 11, p. 1769–1777, 2011. DOI: 10.4161/psb.6.11.17323. Disponível em: https://www.tandfonline.com/doi/abs/10.4161/psb.6.11.17323. Acesso em: 18 nov. 2021.

WAR, Abdul Rashid; PAULRAJ, Michael Gabriel; WAR, Mohd Yousf; IGNACIMUTHU, Savarimuthu. Herbivore-induced resistance in different groundnut germplasm lines to Asian armyworm, Spodoptera litura (Fab.) (Lepidoptera: Noctuidae). Acta Physiologiae Plantarum, [S. l.], v. 34, n. 1, p. 343–352, 2012. DOI: 10.1007/s11738-011-0833-6. Disponível em: https://link.springer.com/article/10.1007/s11738-011-0833-6. Acesso em: 18 nov. 2021.

WERTZ, I.; HANLEY, M. Diverse molecular provocation of programmed cell death. Trends in Biochemical Sciences, [S. l.], v. 21, n. 10, p. 359–364, 1996. DOI: 10.1016/s0968-0004(96)40002-0. Disponível em: http://www.cell.com/article/S0968000496400020/fulltext. Acesso em: 7 abr. 2022.

WU, Haihua; ZHANG, Yiwei; SHI, Xuekai; ZHANG, Jianzhen; MA, Enbo. Overexpression of Mn-superoxide dismutase in Oxya chinensis mediates increased malathion tolerance. Chemosphere, [S. l.], v. 181, p. 352–359, 2017. DOI: 10.1016/j.chemosphere.2017.04.087. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/28456037. Acesso em: 11 abr. 2022.

WU, Shuang; DOU, Wei; WU, Jing Jing; WANG, Jin Jun. Purification and partial characterization of glutathione S-transferase from insecticide-resistant field populations of Liposcelis paeta Pearman (Psocoptera: Liposcelididae). Archives of Insect Biochemistry and Physiology, [S. l.], v. 70, n. 2, p. 136–150, 2009. DOI: 10.1002/arch.20285. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.20285. Acesso em: 25 mar. 2022.

XIANG, Mian; ZHANG, Xian; DENG, Yin; LI, Yangyang; YU, Jihua; ZHU, Jianquan; HUANG, Xinhe; ZHOU, Jiayu; LIAO, Hai. Comparative transcriptome analysis provides insights of anti-insect molecular mechanism of Cassia obtusifolia trypsin inhibitor against Pieris rapae. Archives of Insect Biochemistry and Physiology, [S. l.], v. 97, n. 1, p. e21427, 2018. DOI: 10.1002/arch.21427. Disponível em: https://onlinelibrary.wiley.com/doi/full/10.1002/arch.21427. Acesso em: 10 jun. 2022.

XIE, Chen et al. KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Research, [S. l.], v. 39, n. SUPPL. 2, p. W316–W322, 2011. DOI: 10.1093/nar/gkr483. Disponível em: https://academic.oup.com/nar/article/39/suppl_2/W316/2507217. Acesso em: 7 jun. 2022.

XU, Li Na; WANG, Yue Qin; WANG, Zhen Ying; HU, Ben Jin; LING, Ying Hui; HE, Kang Lai. Transcriptome differences between Cry1Ab resistant and susceptible strains of Asian corn borer. BMC Genomics, [S. l.], v. 16, n. 1, p. 1–15, 2015. DOI: 10.1186/s12864-015-1362-2. Disponível em: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-015-1362-2. Acesso em: 10 jun. 2022.

YANG, Limei; FANG, Zhiyuan; DICKE, Marcel; LOON, Joop J. A. va.; JONGSMA, Maarten A. The diamondback moth, Plutella xylostella, specifically inactivates Mustard Trypsin

Inhibitor 2 (MTI2) to overcome host plant defence. Insect Biochemistry and Molecular Biology, [S. l.], v. 39, n. 1, p. 55–61, 2009. DOI: 10.1016/j.ibmb.2008.09.012.

YAO, Jianxiu; BUSCHMAN, Lawrent L.; OPPERT, Brenda; KHAJURIA, Chitvan; ZHU, Kun Yan. Characterization of cDNAs Encoding Serine Proteases and Their Transcriptional Responses to Cry1Ab Protoxin in the Gut of Ostrinia nubilalis Larvae. PLoS ONE, [S. l.], v. 7, n. 8, p. 44090, 2012. DOI: 10.1371/journal.pone.0044090. Disponível em: www.plosone.org. Acesso em: 25 jun. 2021.

YU, Quan You; FANG, Shou Min; ZUO, Wei Dong; DAI, Fang Yin; ZHANG, Ze; LU, Cheng. Effect of organophosphate phoxim exposure on certain oxidative stress biomarkers in the silkworm. Journal of Economic Entomology, [S. l.], v. 104, n. 1, p. 101–106, 2011. DOI: 10.1603/EC10260. Disponível em: https://academic.oup.com/jee/article/104/1/101/2199561. Acesso em: 18 nov. 2021.

YU, S. J.; ABO-ELGHAR, G. E. Allelochemicals as inhibitors of glutathione S-transferases in the fall armyworm. Pesticide Biochemistry and Physiology, [S. l.], v. 68, n. 3, p. 173–183, 2000. DOI: 10.1006/pest.2000.2514.

ZENG, Jian Yong; BI, Bing; ZHANG, Fang Ming; CHENG, Gong; VUONG THI, Minh Dien; ZHANG, Guo Cai. Cu/ZnSOD always responded stronger and rapider than MnSOD in Lymantria dispar larvae under the avermectin stress. Pesticide Biochemistry and Physiology, [S. l.], v. 156, p. 72–79, 2019. DOI: 10.1016/j.pestbp.2019.02.010.

ZHAO, Ping; WANG, Gen Hong; DONG, Zhao Ming; DUAN, Jun; XU, Ping Zhen; CHENG, Ting Cai; XIANG, Zhong Huai; XIA, Qing You. Genome-wide identification and expression analysis of serine proteases and homologs in the silkworm Bombyx mori. BMC Genomics, [S. l.], v. 11, n. 1, p. 1–11, 2010. DOI: 10.1186/1471-2164-11-405. Disponível em: https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-11-405. Acesso em: 2 out. 2021.

ZHOU, Xiaojie; SHENG, Chengfa; LI, Mei; WAN, Hua; LIU, Dong; QIU, Xinghui. Expression responses of nine cytochrome P450 genes to xenobiotics in the cotton bollworm Helicoverpa armigera. Pesticide Biochemistry and Physiology, [S. l.], v. 97, n. 3, p. 209–213, 2010. DOI: 10.1016/j.pestbp.2010.02.003.

Table 1. Differentially expressed protease genes in the *A. gemmatalis* midgut in response to the SKTI and GORE-2 Pis.

Regulation	Description	SeqName	log2 FC	e-Value	p adj	GO IDs
SKTI-UP	serine protease 33	CL1225.Contig1_All	11,9750225	5,10E-156	1,49E-27	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease	CL1225.Contig3_All	10,70751206	3,40E-155	5,11E-35	F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin-like protease	CL1397.Contig1_All	5,418909696	4,98E-81	0,000514654	F:GO:0016787; F:GO:0140096
SKTI-UP	presequence protease, mitochondrial	CL157.Contig1_All	7,147367106	0.0	2,83E-17	F:GO:0016787; F:GO:0140096
SKTI-UP	protease homolog, mitochondrial-like isoform X4	CL1577.Contig6_All	2,448953637	0.0	1,41E-07	F:GO:0003677; C:GO:0005739; F:GO:0016787; P:GO:0030163; P:GO:0065003; F:GO:0140096; F:GO:0140657
SKTI-UP	trypsin, alkaline C-like	CL16.Contig2_All	2,907698393	8,13E-78	0,001461125	F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline C-like	CL2010.Contig1_All	3,968553061	6,02E-86	0,001017485	C:GO:0005576; F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline C	CL2410.Contig3_All	1,680681833	1,34E-103	0,001391058	C:GO:0005615; F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline C-like	CL2650.Contig2_All	3,813550071	2,55E-80	0,000220762	F:GO:0016787; F:GO:0140096
SKTI-UP	venom serine protease Bi-VSP-like isoform X2	CL3952.Contig1_All	8,430010761	0.0	0,000152995	C:GO:0005576; F:GO:0016787; F:GO:0140096
SKTI-UP	mitochondrial-processing peptidase subunit beta	CL5050.Contig1_All	3,439106163	0.0	0,000547926	F:GO:0016787; F:GO:0140096
SKTI-UP	collagenase-like	CL51.Contig11_All	2,599473031	2,19E-99	0,001383029	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease	CL51.Contig4_All	2,288541907	4,32E-100	0,001968344	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease	CL51.Contig5_All	3,76344362	1,48E-90	4,93E-25	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease	CL51.Contig6_All	2,3820552	1,13E-70	9,69E-07	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease	CL51.Contig9_All	1,431517724	2,46E-102	0,006790245	F:GO:0016787; F:GO:0140096
SKTI-UP	carboxypeptidase B-like	CL5209.Contig2_All	10,1788689	0.0	5,11E-45	F:GO:0016787; F:GO:0140096
SKTI-UP	carboxypeptidase B-like	CL5825.Contig3_All	7,010970591	0.0	1,09E-05	C:GO:0005576; F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline C-like	CL6108.Contig1_All	1,918163492	2,58E-92	0,001461125	F:GO:0016787; F:GO:0140096

SKTI-UP	uncharacterized peptidase C1-like protein F26E4.3	CL6268.Contig1_All	11,62414956	0.0	7,06E-07	F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline B-like	CL785.Contig1_All	4,022253309	2,14E-60	0,000685466	F:GO:0016787
SKTI-UP	trypsin-like serine protease	Unigene12150_All	3,638221909	6,66E-18	0,003194793	F:GO:0016787; F:GO:0140096
SKTI-UP	trypsin, alkaline C-like	Unigene12501_All	2,429827754	4,96E-90	0,003148514	F:GO:0016787; F:GO:0140096
SKTI-UP	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 isoform X1	Unigene2216_All	5,68935337	0.0	4,14E-07	F:GO:0016787; P:GO:0023052; F:GO:0098772; F:GO:0140096
SKTI-UP	trypsin, alkaline C-like	Unigene2276_All	3,146069419	7,19E-36	0,002415837	F:GO:0016787; F:GO:0140096
SKTI-UP	serine protease 59	Unigene2402_All	11,91139868	3,93E-90	1,23E-20	F:GO:0016787; F:GO:0140096
SKTI-UP	ATP-dependent zinc metalloprotease YME1 homolog isoform X2	Unigene2627_All	4,181955231	0.0	4,44E-17	F:GO:0016787; F:GO:0140096; F:GO:0140657
SKTI-UP	serine protease 36	Unigene7719_All	4,548315715	3,51E-117	2,41E-05	F:GO:0016787; F:GO:0140096
SKTI-DOWN	disintegrin and metalloproteinase domain-containing protein 10	CL3059.Contig1_All	-8,462010975	0.0	0,00196197	F:GO:0016787; P:GO:0023052; F:GO:0140096
SKTI-DOWN	trypsin-like protease	CL461.Contig1_All	-2,132838265	2,40E-104	0,0001261	C:GO:0005615; F:GO:0016787; F:GO:0140096
SKTI-DOWN	ubiquitin carboxyl-terminal hydrolase 32-like isoform X4	Unigene4082_All	-4,387297501	0.0	6,98E-05	F:GO:0016787; P:GO:0030163; P:GO:0036211; F:GO:0140096
GORE2 -UP	presequence protease, mitochondrial	CL157.Contig1_All	6,089406556	0.0	2,54E-11	F:GO:0016787; F:GO:0140096
GORE2 -UP	dipeptidyl peptidase 3 isoform X2	CL2216.Contig2_All	1,302839553	0.0	0,008377606	C:GO:0005737; F:GO:0016787; F:GO:0140096
GORE2 -UP	leukotriene A-4 hydrolase isoform X2	CL3113.Contig3_All	10,2044005	0.0	1,70E-20	F:GO:0016787; F:GO:0140096
GORE2 -UP	methionine aminopeptidase 1D, mitochondrial isoform X1	CL3143.Contig3_All	2,006182744	8,74E-169	0,008824794	F:GO:0016787; P:GO:0036211; F:GO:0140096
GORE2 -UP	proteasome subunit beta type-4	CL3921.Contig1_All	8,088182282	1,28E-170	1,21E-06	C:GO:0005634; C:GO:0005737; F:GO:0016787; P:GO:0030163; C:GO:0032991; F:GO:0140096
GORE2 -UP	mitochondrial-processing peptidase subunit beta	CL5050.Contig1_All	4,160957797	0.0	4,03E-06	F:GO:0016787; F:GO:0140096
GORE2 -UP	collagenase-like	CL51.Contig11_All	2,102057828	2,20E-99	0,000122163	F:GO:0016787; F:GO:0140096
GORE2 -UP	trypsin alpha-3-like	CL51.Contig3_All	1,66091933	1,16E-23	0,008761093	F:GO:0016787; F:GO:0140096
GORE2 -UP	serine protease	CL51.Contig4_All	1,932342636	4,32E-100	4,88E-11	F:GO:0016787; F:GO:0140096
GORE2 -UP	serine protease	CL51.Contig5_All	5,05220764	1,49E-90	7,26E-25	F:GO:0016787; F:GO:0140096
GORE2 -UP	serine protease	CL51.Contig6_All	1,577863011	1,14E-70	3,14E-16	F:GO:0016787; F:GO:0140096
GORE2 -UP	carboxypeptidase B-like	CL5209.Contig2_All	7,904137745	0.0	0,002140265	F:GO:0016787; F:GO:0140096
GORE2 -UP	sentrin-specific protease 6-like isoform X3	CL5231.Contig4_All	7,846312629	0.0	0,00101668	F:GO:0016787; F:GO:0140096
GORE2 -UP	dipeptidyl peptidase 9 isoform X2	CL6222.Contig1_All	9,017842177	0.0	0,000308924	F:GO:0016787; F:GO:0140096
GORE2 -UP	pyroglutamyl-peptidase 1	Unigene1265_All	7,550171361	2,21E-38	0,000547424	C:GO:0005829; F:GO:0016787;
GORE2 -UP	Golgi-specific brefeldin A-resistance guanine	Unigene2216_All	4,465360198	0.0	0,00682728	F:GO:0016787; P:GO:0023052;

	nucleotide exchange factor 1 isoform X1					
GORE2 -UP	ATP-dependent zinc metalloprotease YME1 homolog isoform X2	Unigene2627_All	3,478315673	0.0	1,83E-12	F:GO:0016787; F:GO:0140096; F:GO:0140657
GORE2 -UP	carboxypeptidase B-like	Unigene5732_All	5,175631623	3,89E-32	0,004095005	F:GO:0016787; F:GO:0140096
GORE2 -UP	carboxypeptidase B-like	Unigene6320_All	4,793606264	1,90E-153	2,34E-05	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	probable alpha-aspartyl dipeptidase	CL1552.Contig1_All	-9,701270345	8,12E-122	4,03E-06	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	membrane alanyl aminopeptidase-like	CL1713.Contig3_All	-26,87443177	0.0	3,30E-06	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	ubiquitin thioesterase otubain-like isoform X1	CL1733.Contig2_All	-2,540511301	1,37E-167	1,16E-06	C:GO:0005634; F:GO:0016787; P:GO:0036211; F:GO:0140096
GORE2 -DOWN	dipeptidyl peptidase 3 isoform X2	CL2216.Contig3_All	-1,490430532	0.0	4,00E-06	C:GO:0005737; F:GO:0016787; F:GO:0140096
GORE2 -DOWN	glutathione hydrolase 1 proenzyme-like isoform X2	CL2266.Contig1_All	-4,530341979	0.0	0,008444935	P:GO:0006575; P:GO:0006790; F:GO:0016787; F:GO:0140096
GORE2 -DOWN	zinc carboxypeptidase-like	CL2550.Contig3_All	-7,093489585	0.0	0,003747412	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	chymotrypsin-1 isoform X1	CL3591.Contig1_All	-14,75108151	5,01E-83	0,000422577	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	zinc metalloproteinase nas-4-like isoform X1	CL4422.Contig3_All	-7,572330767	5,08E-174	2,98E-12	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	aminopeptidase N	CL5022.Contig1_All	-12,26843116	0.0	0,001496461	C:GO:0005886; F:GO:0016787; F:GO:0140096
GORE2 -DOWN	ATP-dependent zinc metalloprotease YME1 homolog	CL5330.Contig2_All	-1,165401994	0.0	0,009093881	F:GO:0016787; F:GO:0140096; F:GO:0140657
GORE2 -DOWN	collagenase-like isoform X1	CL6019.Contig1_All	-2,317611091	9,52E-57	0,002938325	F:GO:0016787
GORE2 -DOWN	aminopeptidase N	CL906.Contig5_All	-23,75848631	0.0	7,22E-05	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	aminopeptidase N	Unigene1311_All	-13,08888841	0.0	0,000497904	F:GO:0016787; F:GO:0140096
GORE2 -DOWN	midgut aminopeptidase N4	Unigene6594_All	-11,46871248	0.0	0,002265247	F:GO:0016787; F:GO:0140096

CONSIDERAÇÕES FINAIS

Neste trabalho buscou-se compreender respostas a nível molecular ao analisar o transcriptoma de *Anticarsia gemmatalis* desafiadas com inibidores de protease e com isso, possibilitar encontrar genes específicos e rotas metabólicas que possibilitem descrever os mecanismos pelos quais desdobram as interações entre a lagarta e os respectivos inibidores de protease natural e sintético, avaliando as diferenças entre eles. Entretanto, para elucidar os impactos biológicos na sobrevivência e desenvolvimento dos insetos, é essencial empregar ferramentas que possam fornecer respostas mais específicas e detalhadas. Compreender a reação adaptativa dos insetos diante da pressão seletiva de inseticidas exige um conhecimento molecular mais aprofundado.

Diante dos resultados encontrados, é nítido que os inibidores de protease, descritos como fatores antinutricionais desencadearam um súbito aumento da quantidade e expressão de genes relacionado às proteases, como tripsinas e quimotripsinas. A tecnologia de sequenciamento de RNA, RNA-Seq possibilitou a identificação de um número muito maior de enzimas proteases quando se comparado ao proteoma realizado anteriormente desse mesmo perfil de experimento, confirmando que a família das proteases foram as mais abundantes no transcritoma. Ademais, foi possível realizar a montagem de um transcritoma de referência por meio de uma montagem *De novo*, com cerca de 55% dos genes anotados pela ferramenta BLAST (*Basic Local Alignment Search Tool*), servindo como modelo para demais estudos do próprio grupo de pesquisa.

Também foi avaliado o perfil de expressão de genes quando a lagarta foi tratada com dois diferentes inibidores de protease, SKTI e GORE-2. Foi observado que o inibidor sintético GORE2 promoveu uma maior quantidade de genes que foram considerados diferencialmente expressos, evidenciando uma ação de inibição das proteases mais eficiente, reduzindo a sobrevivência das lagartas. As alterações do perfil transcritomico de forma geral, mostram também um aumento na expressão de isoformas das proteases up-reguladas em ambos tratamentos, como uma tentativa fisiológica de driblar os efeitos negativos dos IP. Neste sentido, também foram observadas respostas da lagarta referentes a detoxificação, no intuito de proteger os efeitos danosos causado pelos IP à matriz peritrófica, elevando a expressão de genes relacionado a síntese de proteínas relacionadas a proteção do epitélio intestinal.

É evidente que os resultados obtidos mostram que os efeitos dos IP vão muito além da inibição de proteases intestinais. As análises histológicas revelam danos celulares notavelmente

mais extensos e intensos nas lagartas que se alimentam do tripeptídeo GORE-2 em comparação com os efeitos causados pelo inibidor natural presente na soja (SKTI). Além disso, observa-se que as lagartas tratadas com GORE-2 apresentam uma redução significativa na capacidade de desintoxicação fisiológica mediada por enzimas antioxidantes.

Ao longo da evolução, houve uma adaptação das proteases dos insetos herbívoros em relação ao mecanismo de defesa das plantas relacionado à produção de inibidores de protease, que a princípio atua como um fator antinutricional mas que foi desenvolvendo ferramentas para contornar a ação desses IP. O conhecimento abordado em torno dessa pesquisa, possibilitou desenvolver um produto biotecnológico, capaz de atuar como uma nova estratégia no manejo de pragas agrícolas.