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**Clérison Régis Perini**

# EFICIÊNCIA DE INSETICIDAS QUÍMICOS E IDENTIFICAÇÃO DE MECANISMOS MOLECULARES DE RESISTÊNCIA A PIRETROIDES EM *Chrysodeixis includens* (LEPIDOPTERA: NOCTUIDAE)

**Santa Maria, RS**
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Tese apresentada ao Curso de Pós-graduação em Agronomia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Agronomia**

Orientador: Prof. Dr. Jerson Vanderlei Carús Guedes

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**Aprovado em 7 de setembro de 2018:**

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**Janine Palma, Dra. (CCGL TEC)**

Santa Maria, RS
2018

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Dedico este trabalho de tese aos meus pais Nerci e Selmira e às minhas irmãs Carla e Gladis.

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“Todo mundo é um gênio. Mas se você julgar um peixe por sua habilidade de escalar uma árvore, viverá sua vida inteira acreditando que é estúpido.”

“Educação não é o aprendizado dos fatos, é sim o treinamento da mente para pensar.”

**Albert Einstein**

“Não são as respostas que movem o mundo, são as perguntas!”

**Autor desconhecido**

# RESUMO

## EFICIÊNCIA DE INSETICIDAS QUÍMICOS E IDENTIFICAÇÃO DE MECANISMOS MOLECULARES DE RESISTÊNCIA A PIRETROIDES EM *Chrysodeixis includens* (LEPIDOPTERA: NOCTUIDAE)

AUTOR: Clérison Régis Perini
ORIENTADOR: Jerson Vanderlei Carús Guedes

A lagarta falsa-medideira, *Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), é a mais importante praga da soja no Brasil devido à alta tolerância aos inseticidas e as falhas de controle em lavouras de soja. Neste sentido, foram realizadas pesquisas para avaliar o desempenho de inseticidas no controle de *C. includens* em soja em condições de campo durante três anos (2014, 2015 e 2016) e o sequenciamento de mRNA (RNA-seq) de duas populações brasileiras de *C. includens* que diferiram em suscetibilidade à λ-cialotrina piretroide. Na primeira pesquisa, o número de larvas pequenas e grandes de *C. includens* sobreviventes foi avaliado aos 3, 7 e 10 dias após a pulverização. Além disso, em 2016, foi comparada a eficácia de uma e duas aplicações de cada inseticida em um intervalo de sete dias. No segundo trabalho, o RNA total foi extraído das partes da cabeça e do tórax + abdômen de larvas de 3˚ instar de *C. includens* suscetíveis e resistentes e bibliotecas Illumina de extremidade dupla foram geradas usando o TruSeq® RNA Library Prep Kit. Os dados do sequenciamento foram utilizados para comparar os transcriptomas e avaliar mutações e a expressão diferencial de genes entre essas populações. Considerando os resultados dos ensaios de campo, a eficácia de controle da maioria dos inseticidas foi baixa para larvas pequenas e grandes de *C. includens* ao longo dos três anos de experimentos. Os inseticidas indoxacarbe e clorfenapir apresentaram consistentemente a maior redução de larvas de *C. includens*. A segunda aplicação de metoxifenozide, spinetoram, indoxacarb e flubendiamide + thiodicarb aumentou significativamente a eficácia de larvas grandes. A mistura de clorfluazuron + acefato reduziu a desfolha em 2016, mas não reduziu a densidade larval. Considerando o período de três anos, esses resultados demonstram que poucos inseticidas são eficazes para causar mortalidade de *C. includens* na soja, sugerindo uma investigação com bases moleculares da resistência a inseticidas. Alguns dos inseticidas precisaram de uma segunda aplicação para melhorar a eficácia ou reduzir a injúria nas folhas de soja. Com base nesses resultados, o produtor também deve levar em conta o custo desses inseticidas, pois os inseticidas mais eficazes, neste caso, são os mais caros. Por outro lado, com base na segunda pesquisa, nossos resultados revelaram vários mecanismos moleculares de resistência em *C. includens* responsáveis pela baixa suscetibilidade a piretroides. Com análise comparativa do transcrito do canal de sódio, que é alvo dos priretroides, entre as populações suscetível e resistente, MS vs. LAB, foram encontradas cinco mutações não sinônimas na região codificadora do gene na população resistente (N1013I, L1314V, Q1433H, F1608C e P1800S), especificamente nos domínios II, III e IV. Essas mutações podem alterar a conformação da proteína e insensibilizar a ligação dos piretroides no canal de sódio. Além disso, a superexpressão de transcritos relacionados a enzimas metabólicas incluindo do citocromo P450, glutationa S-transferase, esterases, e UDP-Glucosyltransferase sugere um intenso processo detoxificativo de inseticidas em *C. includens*. Algumas destas enzimas foram superexpressas na cabeça da população resistente, sugerindo que o processo detoxificativo inicia no aparelho bucal e continua através do aparelho digestivo das lagartas. Essa

superexpressão de genes detoxificantes na população MS pode estar sendo regulada por meio de uma via de sinalização de genes GPCR que foram superexpressos no tecido da cabeça. A fim de compensar a energia gasta no processo detoxificativo, larvas de *C. includens* apresentaram alta expressão de enzimas digestivas e de metabolismo energético como: tripsina, serina protease, lipase e quimotripsina. Além disso, verificou-se também que os genes da cutícula tiveram alta expressão nos tecidos do tórax + abdómen, o que representa uma potencial barreira aos inseticidas. Em resumo, nossas descobertas representam os primeiros *insights* sobre as dificuldades de controle e os mecanismos moleculares de resistência a inseticidas em *C. includens*. Assim, o manejo de *C. includens* na soja é desafiador e as táticas de controle devem ser combinadas em um manejo integrado de pragas e dentro de um manejo de resistência com o uso de inseticidas com diferences mecanismos de ação. O uso de inseticidas químicos, inseticidas biológicos e plantas geneticamente modificadas que expressam toxinas inseticidas devem ser utilizados no manejo para manter a eficiência da tática de controle e retardar ou evitar o aumento da frequência de indivíduos de *C. includens* resistentes.

**Palavras-chave:** Falsa-medideira. Controle químico. Transcriptoma. Mutação genética. Genes detoxificantes.

## ABSTRACT

### EFFICACY OF CHEMICAL INSECTICIDES AND IDENTIFICATION OF MOLECULAR MECHANISMS OF PYRETROID RESISTANCE IN *Chrysodeixis includens* (LEPIDOPTERA: NOCTUIDAE)

AUTHOR: Clérison Régis Perini
ADVISOR: Prof. Dr. Jerson Vanderlei Carús Guedes

Larvae of the soybean looper, *Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), is the most important soybean caterpillar pest in Brazil due to high tolerance to insecticides and control failures in soybean fields. In this sense, we conducted some researches to evaluate the performance of insecticides against *C. includens* on soybean in field conditions over three years (2014, 2015, and 2016) and a high-throughput RNA sequencing on two Brazilian populations of *C. includens*, LAB and MS, differing in susceptibility to pyrethroid λ-cyhalothrin. In the first research, the number of surviving small and large larvae of *C. includens* was evaluated at 3, 7, and 10 days after spray. Moreover, in 2016 we compared the efficacy of one and two applications of each insecticide within a seven-day interval. In the second research, RNA was extracted from head and thorax + abdomen parts of 3˚ instar larvae of *C. includens* susceptible and resistant and paired-end Illumina libraries were generated using the TruSeq® RNA Library Prep Kit. The Illumina data was used to compare the transcriptomes evaluating mutations and a global differential gene expression between these populations. Considering the results of the field trials the majority of insecticides showed low efficacy against larvae of *C. includens*, over three years of experiments. The insecticides indoxacarb and chlorfenapyr had consistently the highest reduction of larvae of *C. includens*. The second application of methoxyfenozide, spinetoram, indoxacarb, and flubendiamide+thiodicarb increased efficacy significantly against large larvae. The mixture of chlorfluazuron+acephate reduced defoliation in 2016 but did not effect larval density. Considering the three-year period, these findings demonstrate that few insecticides are effective to cause mortality of *C. includens* on soybean, suggesting further investigation of insecticide resistance. Some of the insecticides needed a second application to improve efficacy or to reduce the injury on soybean leaves. Based on these results, the grower also has to take into account the cost of these insecticides, because the most effective insecticides in this case are costlier. In the other hand, based on the second research, our results revealed several potential molecular mechanisms on *C. includens* responsible for its low susceptibility to pyrethroid insecticides. The comparison between sodium channel transcript, which is the target of pyrethroids, resistant vs. susceptible populations, MS vs. LAB, we found five nonsynonymous mutations within the coding region of the voltage gated sodium channel in the resistant population (N1013I, L1314V, Q1433H, F1608C, and P1800S), specifically in domains II, III, and IV. These mutations might alter the protein conformation and reduce sensitivity of connection between pyrethroids and sodium channel. Also, the high abundance of transcripts related to metabolic enzymes including cytochrome p450s, glutathione s-transferases, esterases, and UDP glycosyltransferases, suggests an intense detoxification process of pyrethroid in *C. includens*. Some of these enzymes were up-regulated in the head of the resistant population, suggesting that a detoxification process begins in the mouth parts and continues through the gut. This overexpression of detoxification genes in MS population might be enhanced via a signaling pathway of two overexpressed GPCR genes in the head. In order to compensate the spent energy in the detoxification

process, larvae of soybean looper showed high expression of some potential digestive and metabolic energy enzymes such as: trypsin, serine protease, lipase, and chymotrypsin. In addition, cuticle genes were found to be upregulated in the thorax + abdomen, which represents a potential barrier to insecticide penetrate in the resistant larvae. In summary, our findings represent the first insights into the molecular mechanisms underlying insecticide resistance in *C. includens*. Thus, the management of *C. includens* in soybean is challenging and the tactics have to be combined in an integrated pest management and insecticide resistance management.

**Key words:** Soybean looper. Chemical control. Transcriptome. Genetic mutation. Detoxification genes.

## LISTA DE FIGURAS

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**ARTIGO 2**

## LISTA DE TABELAS

**ARTIGO 1**



**ARTIGO 2**

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| ChinNaCh | Gene do canal de sódio de *Chrysodeixis includens* |
| CYP | Citocromo P450 monooxigenase |
| DAS | Days After Spray |
| DEG | Differentially Expressed Gene |
| EST | Esterase |
| FDR | False Discovery Rate |
| FPKM | Fragments per kilobase per million mapped reads |
| GO | Gene Ontology |
| GPCR | Receptor acoplado à proteína G |
| GPHH | Voltage-dependent L-type calcium channel, IQ-associated |
| GST | Glutationa S-transferase |
| ITR | Ion Transport Protein |
| LAB | População do Laboratório |
| MAPEG | Membrane-associated protein in glutathione metabolism |
| MIP | Manejo Integrado De Pragas |
| MRI | Manejo da Resistência de Inseticidas |
| mRNA | RNA mensageiro |
| MS | População do estado do Mato Grosso do Sul |
| Na-CG | Sodium channel gate |
| NaCh | Gene do canal de sódio |
| NaCYT | Cytoplasmic domain of voltage-gated $Na^+$ ion channel |
| NaTRA | Sodium ion transport-associated |
| NGS | Next-Generation Sequencing |
| ORF | Open Reading Frame |
| P450 | Citocromo P450 monooxigenase |
| refseq-PT | Database of reference sequence of Protein |
| RNA-seq | Sequenciamento do mRNA |
| SCBIs | Sodium channel blocker insecticides |
| SNP | Single Nucleotide Polymorphism |
| TM | Transmembrane Domains |
| TMHMM | Transmembrane helices based on a hidden Markov model |
| UDP | Uridina 5'-difosfo-glucuronosiltransferase |
| UGT | UDP-glicuronil transferase |

**SUMÁRIO**

# 1 APRESENTAÇÃO

A lagarta falsa-medideira, *Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), é atualmente a espécie mais importante dos lepidópteros que ocorrem na soja, sendo que os inseticidas químicos são sua principal forma de controle. Também, ocorrem outras lagartas importantes, como *Helicoverpa armigera* e *Spodoptera* spp., insetos sugadores, como os percevejos, e recentemente a mosca-branca, sendo que estes também exigem intervenções do controle químico sobre as populações. A intensidade de uso dessas aplicações de inseticidas químicos sobre o complexo de pragas da soja acaba por controlar ou selecionar populações de pragas cada vez menos suscetíveis aos inseticidas.

As falhas e as dificuldades de controle por inseticidas químicos, especialmente piretroides, reguladores de crescimento e diamidas, têm sido relatadas principalmente para a espécie *C. includens*. Embora haja estudos que reportem a eficácia satisfatória de inseticidas a *C. includens* no Brasil, acredita-se que estes resultados não podem ser mais aceitos para o manejo da praga. Em 2004, alguns inseticidas reguladores de crescimento (IGRs) tiveram eficácia de controle superior a 85%, de 7 a 30 dias após a pulverização (PINTO JUNIOR et al., 2011). Em 2006 e 2007, os inseticidas fenitrotion + esfenvalerato, metomil, tiodicarbe e clorpirifos foram considerados eficientes no controle de falsa-medideira (MARTINS; TOMQUELSKI, 2015).

No entanto, esses resultados aparentemente não condizem com a realidade atual no cultivo de soja no Brasil e a baixa eficácia dos inseticidas comumente aplicados pode resultar em elevadas injúrias nas folhas e a redução da produtividade da soja. Assim, a baixa eficácia dos inseticidas e as falhas de controle podem estar associadas à menor susceptibilidade ou resistência de *C. includens*, que são evidentes e/ou imprevisíveis, visto a pressão de seleção imposta com poucas táticas de manejo em uma área de soja de mais de 35 milhões de hectares como no Brasil.

Um dos grupos de inseticidas com intenso uso e vários casos de resistência já reportados são os piretroides (DONG et al., 2014). Os piretroides, que são usados para o controle de diversas pragas, como os percevejos, lagartas, mosca-branca e outras, acabam por pressionar indiretamente as populações de falsa-medideira. Não somente pelo motivo da pressão de seleção dos inseticidas do grupo químico dos piretroides, mas também pelas características evolutivas dos insetos que estão em constante adaptação no ambiente e que adquiriram a capacidade de resistir a esse grupo de pesticidas muito antes do inicio do seu uso na agricultura.

Os estudos de mapeamento de mecanismos moleculares que conferem menor suscetibilidade de insetos aos inseticidas são importantes para a implementação de estratégias de manejo integrado de pragas (MIP) e manejo da resistência de inseticidas (MRI). O uso do sequenciamento de alto desempenho, tal como RNA-seq, e outras técnicas de biologia molecular tem fornecido informações relevantes para novos entendimentos da biologia e da fisiologia de insetos, dos mecanismos de resistência à inseticidas e para aplicação de novas técnicas e estratégias de manejo de pragas. Os avanços nessa área conduzidos pelas novas tecnologias de sequenciamento de alto desempenho, como por exemplo, o sequenciamento de mRNA, torna possível fazer uma análise global da transcrição quantitativa de genes de detoxificação metabólica e mutações nas sequencias dos transcritos.

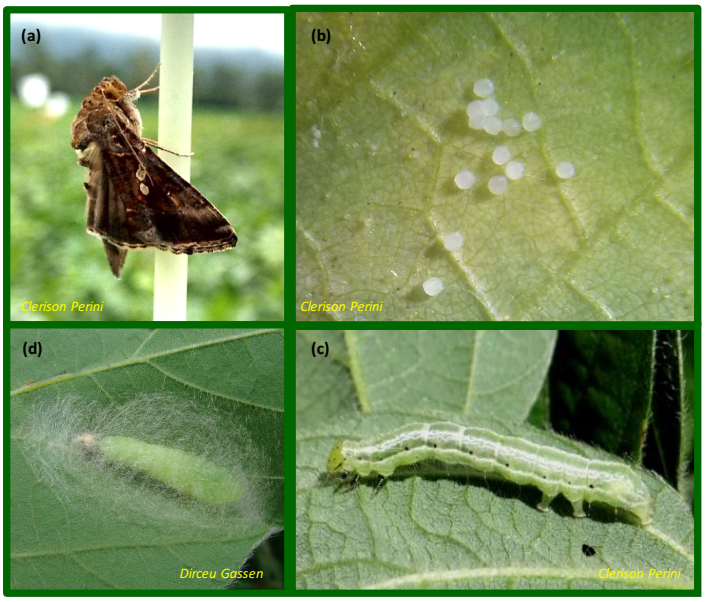
Portanto, a fim de abordar lacunas do conhecimento em *C. includens* por ser a lagarta a lagarta mais importante da soja no Brasil por ter alta tolerância aos inseticidas e ter a maior proporção entre as espécies de lagartas, foram realizados estudos de eficácia de inseticidas, que são amplamente usados no manejo de *C. includens* no Brasil, e o uso de técnicas inovadoras para a identificação de potenciais mecanismos de resistência de falsa-medideira aos piretroides. Potenciais mecanismos de resistência a inseticidas ainda não foram identificados em *C. includens*, ou em outras espécies do grupo das falsas-medideira. No entanto, a baixa eficácia de inseticidas piretroides no controle de *C. includens* em soja no Brasil sugerem que mecanismos genéticos estão influenciando essa baixa suscetibilidade. Assim, há a necessidade de monitorar a eficiência de inseticidas no controle de *C. includens* e buscar informações sobre os potenciais mecanismos de resistência para evitar ou retardar a seleção de insetos resistentes.

## 1.2 REFERENCIAL TEÓRICO

### 1.2.1 *Chrysodeixis includens* (Walker, 1857) (Lepidoptera: Noctuidae)

*Chrysodeixis includens* foi por muito tempo referida com o gênero *Pseudoplusia* e após a reavaliação por Goater et al. (2003) reclassificaram para o gênero *Chrysodeixis*, que é aceito e usado atualmente, sem alteração ou atualização nos caracteres de identificação. As espécies de Plusiinae são muito semelhantes na fase de larva e adulta e requerem um procedimento detalhado no momento da identificação seguindo Eichlin (1975). Logo após a oviposição por fêmeas de *C. includens*, os ovos apresentam coloração creme-clara passando para marrom-clara próximo da eclosão que perdura por aproximadamente 2,5 dias (PETERSON, 1964) (Figura 1). As larvas possuem coloração verde-clara com listras longitudinais brancas ao longo do corpo. Passam por cinco ínstares que duram em torno de 9 dias, sendo que 96-98% da alimentação ocorre a partir do terceiro e quarto ínstares (REID; GREENE, 1973). A fase de pupa de coloração verde-clara ocorre na parte abaxial das folhas de soja. Os adultos emergem após 7 a 9 dias com asas anteriores de coloração escura com duas manchas prateadas brilhantes no centro das asas, uma em forma de círculo e outra em forma de "U" (SOSA-GÓMEZ et al., 2010).

**Figure 1** - *Chrysodeixis includens* nas fases de adulto (a), ovo (b), lagarta (c) e pupa (d).



A ocorrência de *C. includens* aparentemente está restrita aos países das Américas do Sul, Central e do Norte (RATNASINGHAM; HEBERT, 2007) sendo esta a espécie mais abundante em soja no Brasil (GUEDES et al., 2015; LUZ et al., 2018) e uma das principais lagartas do algodão (BUSOLI et al., 2011). A maior ocorrência de *C. includens* no período reprodutivo da soja está atrelada com a preferencia de oviposição dos adultos no período de florescimento, sendo que as fêmeas depositam os ovos na parte abaxial das folhas e nos terços médio e inferior (JOST; PITRE, 2002). Consequentemente, as lagartas ocupam a parte mediana e inferior do dossel da cultura da soja (ZULIN; ÁVILA; SCHLICK-SOUZA, 2018). No período reprodutivo da soja, o dossel está fechado e dificulta o uso eficiente da tecnologia de aplicação para o controle dessa lagarta que está localizada nos terços inferiores das plantas de soja.

As lagartas falsas-medieiras eram consideradas como praga secundária nos anos 70 e 80 (HEINRICHS; SILVA, 1975; PRADO; CUNHA; SILVA, 1982; MORAES; LOECK; BELARMINO, 1991, a e b). Entretanto, as mudanças no manejo fitossanitário da soja verificado entre os anos de 1970 e 2010 está entre as principais causas da alteração na composição da lepidofauna em soja, sendo que neste período se faziam poucas pulverizações de inseticidas, fungicidas e herbicidas (GUEDES et al., 2015). As lagartas de *C. includens* eram mantidas sob baixa densidade populacional em resposta à elevada ocorrência de inimigos naturais (HEINRICHS; SILVA, 1975) e fungos entomopatogênicos, como *Nomuraea rileyi* (Farlow) Samson (doença branca) (SOSA-GÓMEZ; LASTRA; HUMBER, 2010).

As populações de *C. includens* ganharam maior importância com o aumento das aplicações de inseticidas, principalmente os não seletivos, como piretroides, por matar os inimigos naturais que controlavam essa praga (MOSCARDI et al., 2012). Além disso, houve recentemente a introdução dos inseticidas do grupo das diamidas (LIU et al., 2010) que devido ao uso intensivo em soja, que mesmo na dose recomendada, ajudou na redução de *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae) e aumento de *C. includens* (GUEDES, 2015, informação pessoal). Além disso, após a constatação e rápida disseminação da ferrugem-asiática da soja causada pelo fungo *Phakopsora pachyrhizi* (Sydow e P. Sydow) (YORINORI; LAZZAROTTO, 2004), as aplicações de fungicidas contribuíram com a redução de fungos entomopatogênicos que controlam *C. includens* (SOSA-GÓMEZ, 2012). Desse modo, as alterações no sistema de manejo fitossanitário da soja ao longo dos anos

acabaram por favorecer uma espécie e desfavorecer outras. O resultado disso está na predominância da lagarta com maior tolerância aos inseticidas, *C. includens*.

### 1.2.2 Modos de ação de inseticidas químicos

#### *1.2.2.1 Inseticidas que atuam no sistema nervoso dos insetos*

Os inseticidas que fazem parte desse grupo são os mais usados na agricultura atualmente, com cerca de 85% do valor total de vendas (SPARKS; NAUEN, 2015). Os inseticidas neurotóxicos atuam em alguma etapa da transmissão do impulso nervoso ao longo das células do sistema nervoso central ou nas células do neurônio motor, que possuem dendritos, corpo celular e axônio. Serão abordados os inseticidas que atuam na transmissão axônica do impulso nervoso, modulando ou bloqueando os canais de sódio ($Na^+$) dependentes de voltagem no sistema nervoso central.

Os inseticidas moduladores de canais de sódio agem nos canais de $Na^+$ dependentes de voltagem na membrana do axônio. A medida que a região intracelular passa do potencial negativo para o potencial positivo com a abertura do canal de sódio e influxo de íons $Na^+$ os piretroides podem se ligar a esse receptor em dois locais: sítio 1 - lipídio exposto na interface formada pelo linker entre as subunidades 4 e 5 do domínio II, a subunidade 5 do domínio II e a subunidade 6 do domínio III - IIL45–IIS5–IIIS6 - sítio 2 - triângulo formado entre IL45–IS5–IIS6). A ligação dos piretroides nesses locais evita e retarda o fechamento do canal de sódio causando um influxo contínuo de íons $Na^+$, despolarização da membrana e a consequente excitabilidade da transmissão sináptica levando o inseto a morte (DONG et al., 2014). Exemplo de inseticidas piretroides: cipermetrina, bifentrina e beta-ciflutrina.

Por outro lado, há os inseticidas bloqueadores do canal de sódio, como as oxadiazinas. O pró-inseticida indoxacarbe, que necessita ser bioativado para ter função de inseticidas, se ligam na proteína do canal de sódio, em local diferente dos piretroides, quando a célula está restaurando ou com o potencial já negativo. Ou seja, em repouso e o canal de sódio fechado. Assim, ocorre um bloqueio para o aumento do potencial elétrico de íons $Na^+$ para dentro da célula e a consequente morte do inseto por paralisia do impulso nervoso (WING et al., 1998).

*1.2.2.2 Inseticidas que atuam na respiração celular*

Os inseticidas com modo de ação na respiração celular atuam na região interna da membrana da mitocôndria inibindo a ATP sintase ou pelo desacoplamento da fosforilação oxidativa. O alimento ingerido pelos insetos é absorvido e convertido em uma forma de energia padrão na mitocôndria, a adenosina trifosfato, ou ATP, que é formada em uma reação chamada de fosforilação da adenosina difosfato (ADP), pela enzima ATP sintase, com a ligação de um grupo fosfato. Essa reação é possível com um gradiente de prótons ($H^+$) entre o espaço intermembranoso e a matriz mitocondrial que são usados pela ATP sintase para a geração da energia ATP (BASF, 2016).

O Clorfenapir faz parte dos inseticidas desacopladores da fosforilação oxidativa pela disrupção do gradiente de prótons (IRAC, 2018), sendo considerado um pró-inseticida. Clorfenapir é um ácido fraco que se liga a um próton no espaço intermembranoso que está rico em prótons ($H^+$) e o transporta através da membrana mitocondrial interna e o deposita na matriz mitocondrial. Após retorna através da membrana para se ligar a outro próton e repetir o ciclo. O resultado é a dissipação da energia armazenada no gradiente de prótons na forma de calor e a interrupção da síntese de ATP. Na ausência do gradiente de prótons, a ATP Sintase funciona em sentido inverso, hidrolisando rapidamente o ATP disponível na matriz mitocondrial, na tentativa de bombear prótons de volta para o espaço intermembranoso. Assim, sem a geração de ATP, o mesmo é rapidamente esgotado, levando à rápida paralisia e morte do inseto (HUNT; TREACY, 1998).

### 1.2.3 Mecanismos de resistência de insetos a inseticidas

A resistência de insetos a inseticidas pode ser compreendida como uma capacidade herdada de tolerar doses que são letais para outros indivíduos da mesma espécie. A resistência é afetada por fatores genéticos, bioecológicos e operacionais relacionados aos produtos químicos e com o modo de utilização desses (GEORGHIOU; TAYLOR, 1977; ROUSH; McKENZIE, 1987). Os fatores genéticos estão relacionados ao número de genes envolvidos

na resistência (GROETERS; TABASHNIK, 2000), à frequência inicial de alelos resistentes, ao padrão de herança desses e ao custo adaptativo da praga (ROUSH; McKENZIE, 1987).

O processo evolutivo dos insetos ao longo dos quase ~412 milhões de anos (MISOF et al., 2014), associado ao ambiente em que viveram, contribuiu para a evolução dos fatores genéticos que tornam os insetos resistentes a certos inseticidas. Os mecanismos de resistência a pesticidas podem ter evoluído nos insetos antes mesmo do uso de moléculas inseticidas (seleção da variabilidade genética permanente), ou após a introdução e utilização dessas com mutações *de novo* (HAWKINS et al., 2018). Assim, as pragas podem desenvolver ou terem desenvolvido mecanismos de resistência por comportamento, por redução da penetração cuticular, pelo aumento da expressão de enzimas metabólicas, por aumento da capacidade de excreção, ou também, por mutação no sítio de ação (MUTERO et al., 1994; JOUBEN; HECKEL, 2016).

Um dos fatores limitantes na identificação de mecanismos de resistência é a falta de informações quanto a sequência gênica e sua expressão. No caso de *C. includens*, não há informações disponíveis de genes do DNA nuclear que auxiliem para tal identificação e que são responsáveis por processos fisiológicos importantes, como por exemplo, o canal de sódio que regula o sistema nervoso dos insetos e é alvo de inseticidas. Além de genes alvos, a constelação de enzimas metabólicas (Citocromo P450, glutationa S-transferases, esterases, entre outras) também participam de processos importantes, como o de defesa contra xenobióticos.

#### *1.2.3.1 Insensibilidade do canal de sódio aos inseticidas piretroides e oxadiazinas*

O canal de sódio dependente de voltagem do sistema nervoso é um importante alvo de muitos inseticidas que são usados no manejo de pragas agrícolas, seja dos piretroides, DDT ou das oxadiazinas. A proteína do canal possui 4 domínios (I, II, III e IV) e cada domínio apresenta seis regiões trans-membrana (S1 a S6). As mutações no canal de sódio, associadas à resistência aos piretroides, ocorrem em diferentes posições ao longo da sequência de aminoácidos, dependendo das espécies de artrópodos. Atualmente, foram reportadas mais de 50 mutações não-sinônimas (*knock down resistance* - *kdr*) no canal de sódio de atrópodes, relacionadas a resistência e a menor suscetibilidade a inseticidas (DONG et al., 2014, LIEN et al., 2018). Assim, uma mutação pontual ou mais de uma, podem estar presentes

simultaneamente no gene, resultando em diferentes sensibilidades (níveis de resistência) para DDT e piretroides, ou em alguns casos, até eliminar por completo a sensibilidade (LEE et al., 1999; VAIS et al., 2000; BURTON et al., 2011; YOON et al., 2008).

Mais de 20 espécies de insetos apresentam a substituição de um único aminoácido, de leucina para fenilalanina, na posição 1014 da sequência de aminoácidos do gene do canal de sódio, o que resulta na resistência aos piretroides (DONG et al., 2014). A substituição de um único aminoácido altera a conformação da estrutura da proteína e leva a incapacidade da molécula inseticida se ligar no canal de sódio, conferindo a resistência e permitindo a sobrevivência da população. Duas substituições de aminoácidos em uma região conservada entre as espécies *H. armigera* e *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) foram encontradas entre os domínios III e IV, nas posições 1549 e 1553. Ambas resultaram da substituição de uma única base nitrogenada no códon, sendo a primeira de GAC para GTC e a segunda de GAA para GGA, o que resultou na substituição de um ácido aspartático por valina e ácido glutâmico por glicina, respectivamente (HEAD; McCAFFERY; CALLAGHAN, 1998). Outros estudos demonstraram múltiplas mutações em diferentes domínios e regiões de trans-membrana do canal de sódio associados a resistência aos piretroides. Populações de *H. virescens* tiveram mutações na posição 410 (IS6) e 1.014 (IIS6) (DONG et al., 2014). Populações de *H. zea* tiveram mutações na posição 421 (IS6), com a substituição de valina (GTG) por alanina (GCG) ou de valina por glicina (GGG), e na posição 1.029 (IIS6), com a substituição de leucina (CTT) por histidina (CAT) (HOPKINS, 2010). Assim, diferentes mecanismos de resistência aos piretroides com múltiplas mutações no canal de sódio podem ocorrer na mesma espécie.

Além dos piretroides, o inseticida indoxacarbe também tem como alvo o canal de sódio dos insetos, mesmo local em que foi identificada a substituição de um aminoácido levando a resistência aos piretroides. Em *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) a mutação foi identificada na posição 1.014 do domínio IIS6 do canal de sódio, a partir da troca no códon de CTT para TTT (GAO et al., 2014). No Brasil, o uso do inseticida indoxacarbe tem aumentado nos últimos anos, principalmente em aplicações visando o controle eficiente de populações de *C. includens* em soja. O elevado uso desse inseticida pode por em risco o manejo de *C. includens* em soja no Brasil, considerando os mecanismos de resistência podem ter evoluído previamente ao início do uso de inseticidas desse grupo químico.

*1.2.3.2 Enzimas oxirredutases do sistema metabólico que mediam resistência (P450, GST, EST e UGT)*

A resistência de artrópodes a inseticidas mediada por enzimas metabólicas compreende uma classe de resistência a compostos xenobióticos. Esses compostos sofrem redução da sua capacidade de interagir com uma molécula alvo, devido a uma transformação bioquímica resultante da ação das enzimas metabólicas.

As enzimas do citocromo P450 monooxigenases (P450s) e as redutases associadas ao P450 compreendem o único sistema metabólico que pode mediar resistência para todas as classes de inseticidas, devido a grande diversidade genética, ampla especificidade de substrato e versatilidade catalítica das P450s (FEYEREISEN, 2005). São enzimas metabólicas de fase I capazes de oxidar compostos endógenos e exógenos por oxidação ou outras reações relacionadas. Os modos mais comuns de resistência pelas P450 em insetos é via superexpressão de genes, seja por mutações, inserções ou deleções na região promotora *cis-acting* e/ou no locus *trans-regulatory* ou por mutações na região codificante que alteram a especificidade da ligação enzima/substrato. Os mecanismos pelos quais essas mutações, inserções ou deleções podem ocasionar superexpressão de P450s abrangem uma ampla gama de opções, seja com o bloqueio de elementos repressores existentes, com a introdução de elementos potenciadores, ou também a alteração da distância física entre os elementos reguladores e o local de início da transcrição (LI; SCHULER; BERENBAUM, 2007). Além desses, a duplicação dos genes sofrida por uma alteração genômica espontânea que aumenta o número de cópias do gene no genoma (fator principal da evolução das P450s nos organismos) e o *splicing* alternativo são também citados como mecanismos mediadores de superexpressão das P450 (FEYEREISEN, 2012) e que estão associados em insetos resistentes a inseticidas.

Por outro lado, as enzimas glutationa S-transferases (GSTs) podem mediar a detoxificação metabólica de maneira direta (fase I) ou pelo metabolismo de compostos secundários gerados por outras enzimas detoxificantes (fase II) (PAVLIDI; VONTAS; LEEUWEN, 2018). A reação das GSTs ocorre com a conjugação de glutationa (GSH), onde catalisam o ataque nucleofílico do grupo tiol da glutationa reduzida ($GS^-$) no centro eletrofílico dos produtos químicos (MANNERVIK, 1985). Isso neutraliza os locais nucleófilos altamente reativos da substância química e/ou aumenta sua solubilidade em água, facilitando sua excreção da célula. Assim, as GSTs podem catalisar a conjugação de GSH a inseticidas levando a produção de um conjugado solúvel menos tóxico, podem participar da

desintoxicação direta de DDT ao DDE não tóxico, utilizando glutationa como co-fator, podem ter atividade peroxidásica reduzindo os peróxidos tóxicos produzidos pelo estresse oxidativo da ingestão de inseticidas e podem conferir resistência através da ligação passiva não catalítica do inseticida (PAVLIDI; VONTAS; LEEUWEN, 2018). Esses são os possíveis mecanismos de resistência mediados por GSTs para organofosforados (HUANG et al., 1998), organoclorados (RANSON et al., 2001) e piretroides (VONTAS; SMALL; HEMINGWAY et al., 2001). Os mecanismos que as enzimas GSTs desenvolveram para agir em processos detoxificantes compreendem a duplicação de genes (VONTAS et al., 2002) e regulações nas regiões *cis* e *trans*-acting e região codificante dos genes (LI; SCHULER; BERENBAUM, 2007).

Outro grupo importante de enzimas metabólicas de inseticidas são as esterases (ESTs), resultando em resistência aos grupos químicos orgafosforados, carbamatos e piretroides (LI; SCHULER; BERENBAUM, 2007). Nessas enzimas, a duplicação de genes compreende ser um dos mecanismos de degradação e sequestro para esses três grupos químicos (FIELD; DEVONSHIRE, 1998), podendo sofrer processos de metilação no DNA na região promotora do gene que silenciam o gene ou a desmetilação que induz a transcrição (FIELD, 2000). Além desse, mutações nas regiões codificadoras do gene GSTs (CAMPBELL et al., 1998) ou regulação com aumento da expressão gênica (HEMINGWAY, 1998). Estas enzimas metabólicas são capazes de hidrolisar compostos que contêm ligações éster via adição de água na reação, formando um álcool e um metabólito ácido. Vale ressaltar que alguns inseticidas, como piretróides, carbamatos e organofosforados, possuem ligações éster e, consequentemente, são detoxificados por meio da hidrólise de enzimas esterase (MONTELLA; SCHAMA; VALLE, 2012).

Enzimas detoxificativas multifuncionais como a uridina 5'-difosfo-glucuronosiltransferase ou UDP-glucuronosiltransferase (UGT) foram relatadas por Pan et al. (2018) como metabolizantes de inseticidas em *P. xylostella* e *Aphis gossypii*. As UGTs catalisam a conjugação de uma série de pequenos compostos lipofílicos com açúcares para produzir glicosídeos, que são solúveis em água e podem ser eficientemente excretados (MACKENZIE et al., 1997). A conjugação de glicosídeos é uma das mais importantes vias metabólicas para a biotransformação de vários compostos lipofílicos exógenos e endógenos de xenobióticos e endobióticos (BOCK, 2003).

Aliado a expressão dessas e outras enzimas detoxificativas que conferem mecanismos de resistência aos inseticidas, estão um grupo importante de genes funcionais de transdução de sinal (*G protein coupled receptor* - GPCR). Em *Culex quinquefasciatus* (Diptera:

Culicidae) (Say, 1823) a transdução de sinal via GPCR sinaliza e desencadeia a superexpressão de uma série de outras enzimas em cascata (GPCR/Gαs/AC/cAMP/PKA) sendo altamente expressos nos tecidos da cabeça, mesêntero e túbulos do malpighi (LI; LIU, 2017). Os sinais extracelulares que são recebidos por membros de uma grande superfamília de receptores com sete regiões de membrana ativam as proteínas G que encaminham os sinais para várias vias de sinalização intracelular distintas. Essas vias interagem umas com as outras para formar redes de sinalização dentro da célula para as enzimas metabólicas, canais iônicos, transportadores e outros componentes da maquinaria celular. Essa cadeia controla uma ampla gama de processos celulares, incluindo transcrição, motilidade, contratilidade e secreção (NEVES; RAM; IYENGAR, 2002).

Assim, os mecanismos de resistência ou os mecanismos que conferem menor suscetibilidade de um inseto a uma ou diferentes classes de inseticidas podem ser inúmeras. A evolução das espécies nos ambientes diversos desenvolveu diferentes técnicas de os insetos suportar tais adversidades, que em muitos casos, foram anteriores a introdução de moléculas de inseticidas. Seja que esta evolução tenha ocorrido anterior ou até mesmo posterior ao início do uso dos inseticidas, como proposto por (HAWKINS et al., 2018), a evolução da resistência está diretamente ligada aos elementos transponíveis (LI; SCHULER; BERENBAUM, 2007). Como por exemplo, a inserção de um fragmento de 2.3-kb por transposons no gene da caderina conferiu resistência a toxina Bt em *Heliothis virescens* (GAHAN; GOULD; HECKEL, 2001) e a inserções na região regulatória do gene CYP6G1 em *D. melanogaster* foram associados com a superexpressão de CYP6G1 e resistência ao inseticida DDT (CATANIA et al., 2004). As alterações mediadas por transposon podem levar a mudanças massivas e rápidas na expressão de genes que conferem resistência. Essas respostas são potencialmente e altamente adaptáveis em face da introdução rápida de um agente de mortalidade no ambiente, ou seja, um inseticida.

### *1.2.3.3. Alterações na penetração cuticular*

O modo de contaminação dos insetos por inseticidas pode ser por ingestão, superfície do corpo e inalação. Após a entrada do produto no corpo do inseto, este deve exercer a sua função tóxica no sítio de ação. A redução da penetração pela cutícula é a principal barreira de entrada dos inseticidas nos insetos. Para os indivíduos que apresentam essa característica há

uma quantidade menor do pesticida que chega até o sítio de ação (BERNARDI; OMOTO, 2014). As espécies *H. zea* (ABD-ELGHAFAR; KNOWLES, 1996) e *C. virescens* (VINSON; LAW,1971) apresentam resistência por decréscimo e retardamento da penetração de piretroide (fenvalerate e cipermetrina) ao longo do tempo, como resultado de apresentarem conteúdos de proteínas e lipídios na cutícula e alto grau de esclerotização. Ou seja, há uma elevada expressão de genes da cutícula para a formação de uma camada espessa e pouco permeável por moléculas xenobióticas.

#### *1.2.3.4 Comportamento*

A resistência por comportamento foi relatada por alguns grupos de inseticidas, como os organoclorados, organofosforados, carbamatos e piretróides. Os insetos que utilizam esse mecanismo de resistência detectam ou reconhecem elementos xenobióticos e param de se alimentar, podendo sair da área pulverizada, mover-se para a parte inferior da folha ou para outra parte da planta ou até para outra área que não está tratada (BERNARDI; OMOTO, 2014). O sistema olfatório dos insetos é bem desenvolvido e pode contribuir para induzir a respostas adaptativas que favorecem a sobrevivência de indivíduos sem sequer entrar em contato com o inseticida. A identificação e análise de expressão de genes receptores do sistema olfatório têm sido identificados em alguns insetos, como por exemplo em *Apolygus lucorum* (Hemiptera: Miridae) (AN et al., 2016). Estudos de expressão gênica e bioensaios relacionados ao sistema olfatório dos insetos são importantes para compreender os mecanismos envolvidos na detecção dos inseticidas.

### 1.2.4 Genética molecular na pesquisa de mecanismos de resistência em insetos

O sequenciamento de genomas e transcriptomas de uma determinada praga agrícola, como descrito recentemente em *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) (CHIU et al., 2013), *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) e *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) (PEARCE et al., 2017) ou de insetos modelos como mosca-das-frutas *Drosophila melanogaster* (Meigen,

1830) (Diptera: Drosophilidae) (http://flybase.org) e *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) (http://silkworm.genomics.org.cn) tem contribuído para entender e melhorar o manejo de pragas. Nesse sentido, um dos potenciais usos dessas tecnologias é a identificação de potenciais mecanismos de resistência em insetos, ou seja, identificação de polimorfismo na sequência de bases nitrogenadas ou identificação de superexpressão de genes que codificam enzimas de desintoxicação (MURPHY et al., 2016). Um potencial uso em experimentos de análise global da regulação de genes é o uso de sequenciamento de mRNA (RNA-seq) (CONESA et al., 2016). Essa técnica pode auxiliar na descoberta de novos transcritos (MAHER et al., 2009), na anotação de genomas (GARBER et al., 2011), na análise de expressão de genes (PASTINEN, 2010), na detecção de fusão de genes (CARRARA et al., 2013), identificação de variações de *splicing* (CONESA et al., 2016), entre outros.

Cada molécula de inseticida se liga a uma proteína receptora no inseto que está envolvida no sistema fisiológico, e resulta na desregulação da função normal da proteína e a consequente morte do inseto (CASIDA; DURKIN, 2013). O sequenciamento dos genes que codificam a proteína receptora dos inseticidas permite a construção de marcadores moleculares para investigar a constituição genética de vários indivíduos de uma população simultaneamente. Assim, o uso de marcadores pode estar associado com bioensaios de inseticidas, para descobrir novas mutações com potencial causa de mecanismo de resistência (MURPHY et al., 2016).

Estudos de genômica, transcriptômica ou proteômica que reportam potenciais mecanismos de resistência aos inseticidas auxiliam imensamente no planejamento e aplicação de táticas de manejo de insetos resistentes de pragas agrícolas importantes, como *C. includens* em soja. O uso de técnicas para a identificação e desenho de marcadores moleculares a fim de amplificar e sequenciar os fragmentos do gene alvo dos inseticidas ou avaliar o nível de expressão de genes, em diferentes populações, permite a comparação entre indivíduos suscetíveis e resistentes. Além de identificar potenciais mutações não silenciosas ou não sinônimas que resultam em substituições de aminoácidos é possível verificar quantitativamente a produção de um determinado gene, que ambos mecanismos podem estar associados à menor suscetibilidade do inseto aos inseticidas.

## 1.3 OBJETIVOS

1. Avaliar a eficiência de inseticidas químicos, comumente utilizados no manejo de lagartas, na mortalidade de lagartas grandes e pequenas de *Chrysodeixis includens* na cultura da soja.

2. Comparar o número de aplicações, uma e duas, na mortalidade de lagartas grandes e pequenas e no percentual de desfolha da soja.

3. Fazer análise comparativa de transcriptomas de diferentes partes de lagartas suscetíveis e resistentes de *Chrysodeixis includens* a piretroide.

4. Investigar potenciais mecanismos de resistência em uma população de *Chrysodeixis includens* resistente ao piretroide λ-cialotrina.

# 2 ARTIGO 1

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**Field efficacy and time after spray of insecticides against larvae of** *Chrysodeixis includens* **(Lepidoptera: Noctuidae) in soybean (***Glycine max***) over three seasons in southern Brazil**

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**Abstract**

Larvae of the soybean looper, *Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), is the most important soybean caterpillar in Brazil due to its high tolerance to insecticides and control failures in field. We conducted field research to monitor the performance of several insecticides against *C. includens* on soybean over three years (2014, 2015, and 2016). The number of surviving small and large larvae of *C. includens* was evaluated at 3, 7, and 10 days after spray. Moreover, in 2016 we compared the efficacy of one and two applications of each insecticide within a seven-day interval. Majority of chemical insecticides showed low efficacy against larvae of *C. includens* over the three years of experiments. Except for insecticides indoxacarb and chlorfenapyr that had consistently high mortality upon small and large larvae of *C. includens*. The second application of spinetoram, chlorfenapyr, indoxacarb, and flubendiamide+thiodicarb increased efficacy significantly against large larvae. The mixture of chlorfluazuron+acephate reduced defoliation in 2016 but did not affect larval density. Treatment with chlorfenapyr resulted in reduced defoliation with both applications. Considering the three-year period, these findings demonstrate that few insecticides showed satisfactory performance to control larvae of *C. includens* on soybean, suggesting further investigation of insecticide resistance. Some of the insecticides needed a second application to improve efficacy or to reduce the injury on soybean leaves. Based on these results, the grower also has to take into account the cost of these insecticides, because the most effective insecticides in this case are more costly. Thus, the management of *C. includens* in soybean is challenging and the tactics have to be combined in an integrated pest management and insecticide resistance management.

**Keywords**: caterpillar, soybean pest, defoliation, insecticide application, pest management

**Highlights**

- Few active ingredients, such as chlorfenapyr and indoxacarb, showed high mortality of larvae of *C. includens* in soybean.
- Spinetoram, spinosad, chlorfenapyr and indoxacarb are effective against small larvae of *C. includens*.
- A second spray is required for spinetoram, indoxacarb, and fludendiamide + thiodicarb to increase mortality of *C. includens*.
- Chlorfenapyr does not require a second spray to increase larvae mortality and reduce defoliation caused by larvae of *C. includens*.
- Chlorfluazuron + acephate caused feeding inhibition effects on *C. includens*.

**Introduction**

*Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae) has significantly increased its predominance over the last decade in soybean due to successive changes in management practices and soybean cultivation (Guedes et al., 2010). These factors contributed to increase proportionality of *C. includens* on soybean in Brazil among other species, from 20% in the 80s and 90s (Moraes et al. 1991), to more than 70% nowadays (Guedes et al., 2015). This growth can also be related to areas where soybean and cotton crops are cultivated nearby (Burleigh, 1972), as it happens in the Midwestern Brazil. The area of soybean cultivation in Brazil has reached over 35 million hectares (Conab, 2018) distributed along seven months from north to south, indicating the immense food availability to be colonized by.

Currently, management tactics against soybean looper in Brazil have mainly relied on the use of chemical insecticides and transgenic soybean expressing the Cry1Ac insecticidal protein from *Bacillus thuringiensis* (Bt). There are currently no reports of *C. includens* resistance to *Bt* soybean (Bernardi et al., 2012; Yano et al., 2016) or chemical insecticides. In this regard,

the frequency of insecticide applications is often the most important issue for increasing the frequency of resistant insects if Insect Resistance Management plans are not used correctly (Onstad and Gassmann, 2014).

The highest *C. includens* population density occurs generally during the reproductive stage of soybean, when the canopy is closed (Czepak and Albernaz, 2014) and the larvae is larvae are predominately located in the lower half of the soybean canopy (Papa and Celoto, 2007). The positioning of *C. includens* in the lower half of the soybean canopy hinders control and requires an improved pesticide sprayer system or an insecticide with greater toxicity. Larvae of *C. includens* are more tolerant to the range of doses normally used to control other caterpillars and most insecticides recommended to control soybean caterpillars do not have satisfactory efficacy to control the soybean looper (Guedes et al., 2015). The tolerance of *C. includens* to insecticides was reported to be related to its capacity of enzymatic detoxification and excretion before the molecule binds to the target site (Dowd and Sparks, 1986).

Studies on insecticide efficacy against *C. includens* in Brazil were performed a long time ago. In 2004, insecticide growth regulators were tested and had control efficacy >85% from 7 to 30 days after spraying (Pinto Junior et al., 2011). In 2006 and 2007, the insecticides fenitrothion + esfenvalerate, methomyl, thiodicarb, and chlorpyrifos were considered efficient (Martins and Tomquelski, 2015). But, these results are outdated and no longer match the current reality of control of soybean looper. The low efficacy of insecticides can lead to leaf injury and yield loss of soybean, as reported to be a critical period when leaf injury occurs during the reproductive stages (Reichert and Costa, 2003). In order to address this critical knowledge gap, we performed three experiments during three years to determine the efficacy of insecticides that are currently widely used in the management of *C. includens* in Brazil.

**Material and methods**

Efficacy of soybean looper control was evaluated in field trials conducted over three years (from 2014 to 2016) in Santa Maria, Rio Grande do Sul state, Brazil. The soybean variety was BMX Potencia RR, which is widely used in Southern region. Soybean looper density was comparable among the growing seasons and field trials and the information of each experiment are detailed (Table 1).

Experiments were carried in completely randomized block designs with four replications and plot size of 4 × 6 m (24 $m^2$), randomly distributed with 0.5 m between each other on the soybean field in 2014 and 2015. In 2016 the treatments were arranged in a completely randomized block design in a 2 x 8 factorial design (plot area 24 $m^2$), corresponding to two time of spray and eight insecticide treatment, with four replications. The treatments were applied with a $CO_2$ pressurized backpack sprayer at a flow rate of 150 L $ha^{-1}$. All insecticides were obtained from each company that has the insecticide registered in Brazil (Table 2).

Larvae of *C. includens* collected during the experiment evaluations were identified at the Laboratory of Integrated Pest Management (LabMIP) at the Federal University of Santa Maria using the identification key of Eichlin (1975). The voucher specimens were deposited at LabMIP. Density of larvae of *C. includens* was evaluated with a vertical beat cloth method (Guedes et al., 2006). The number of small (<1.5 cm) and large (>1.5 cm) larvae sampled on a 1.0 $m^2$ area per plot at 3, 7 and 10 days after spraying (DAS) in 2013/2014 and 2014/2015 was recorded. In 2015/2016 the evaluations were performed at 3 and 7 days after the first (DAS1x) and the second spray (DAS2x) in all plots. In order to differentiate between treatments of one and two applications, we used the mean value of 3 and 7 DAS2x to analyze in a factor analysis where: factor 1 is insecticide (8 treatments) and factor 2 is time of spray (2 treatments). In 2015/2016, soybean defoliation was evaluated by the Stewart (2014) scale 7 days after the second spray and was also analyzed as factor analysis. Data were transformed into $\sqrt{x} + 0.5$, analyzed with ANOVA and means were separated using Scott-Knott grouping

test (P≤0.05). Control efficacy {E = [(control treatment – insecticide treatment)/control treatment] * 100} was calculated according to the Abbott equation (1925) with the treatments means. We assessed the mean efficacy of each insecticide among the three years in regards of an overlooking of the results.

**Results**

Experiment I - 2014

In this year the insecticides were applied at a high density of small *C. includens* larvae (13.0 larvae $m^2$). The number of small larvae was reduced significantly by chlorfenapyr, spinosad, and indoxacarb treatments at 3 DAS, ranging from 1.75 ± 1.0 to 3.50 ± 1.7 larvae $m^2$ (Table 3). These insecticides presented the lowest density at 7 DAS, comparing to other treatments, and there was no statistical difference between treatments (P > 0.05). These results highlight the fast effect of pesticides on small *C. includens* larvae, independently of the mode of action. Between 7 and 10 DAS the small larvae density was naturally reduced from 12.75 ± 1.9 larvae $m^2$ at 3 DAS to 2.25 ± 0.5 larvae $m^2$ at 10 DAS, probably due to larval development into larger categories. The number of large larvae was analyzed from 3 to 10 DAS. Only treatment with indoxacarb reduced significantly the number of large larvae at 3 DAS and the effect was maintained until 10 DAS. In comparison, chlorfenapyr significantly decreased the number of large larvae at 7 and 10 DAS, when in the last evaluation showed the lowest number of *C. includens* 0.75 ± 0.5 larvae $m^2$. The spinosad treatment, which was efficient to control small larvae, did not showed a consistent effect for large larvae in this year, with results ranging from 2.00 ± 2.2 larvae $m^2$ to 8.50 ± 1.3 larvae $m^2$. All other treatments increased the population density of large larvae almost at the same proportion as the untreated control, confirming the high difficulty to control this pest with chemical insecticides.

Experiment II – 2015

In 2015 we modified some treatments based on the results from 2014. The first evaluation of small larvae, at 3 DAS, did not show statistical differences between insecticides and untreated control, but spinetoram had the lowest number of small larvae per $m^2$ (Table 4). On the last two evaluations chlorfenapyr presented the lowest mean number of *C. includens* larvae per $m^2$ (0.38 ± 0.7 and 0.3 ± 0.4), but was not significantly different from other insecticides. All treatments were different from untreated control at 10 DAS, with less than one small larvae per $m^2$. The density of large larvae was high during all evaluations and chlorfenapyr presented the lowest mean number at 3, 7, and 10 DAS (2.63 ± 0.7, 2.38 ± 1.1, and 1.25 ± 0.8 larvae per $m^2$, respectively). Indoxacarb showed significant reduction of populations at 3 and 7 DAS, but at 10 DAS lost residual effect. Methoxyfenozide presented almost the same residual effect compared to chlorfenapyr. In this year, most treatments showed satisfactory field efficacy in controlling *C. includens*, which was related to significant reduction in number of small larvae. On the other hand, we could not detect the same effect for large larvae.

Experiment III – 2016

Data analyses of the number of large *C. includes* larvae and defoliation in 2016 detected factor interaction between insecticides and time of spray (P = 0.0233; P = 0.0000, respectively) (Supplemental information 1). But, there was no factor interaction for small larvae (P = 0.3652) and we present the results as normal analyses shown in Table 5. At 3 days after the first application (DAS1x) most treatments did not reduce the number of small or large larvae. The insecticides λ-cyhalothrin+chlorantraniliprole+diafenthiuron, chlorfenapyr,

and indoxacarb showed the lowest number of small larvae. For large larvae, chlorfenapyr and indoxacarb reduced significantly the population density at 3 DAS1x.

Treatment with the insecticide chlorfenapyr was the only one that significantly reduced small and large larvae along all evaluations, independently of the number of sprays. This observation supports that chlorfenapyr can preclude larval development from small to large stage with a long residual effect. Indoxacarb had a similar performance until 7 DAS1x, with a low infestation of small and large larvae. However, a second spray of indoxacarb was necessary to significantly diminish the number of small larvae from 4.50 ± 1.9 to 1.75 ± 1.3 and large larvae from 4.50 ± 1.7 to 0.50 ± 0.6, at 07 DAS2x. Also, the insecticide spinetoram had significant effects with the second spray reducing the number of large larvae from 6.00 ± 0.8 to 3.50 ± 0.6 at 03DAS2x and from 3.00 ± 0.8 to 2.00 ± 1.4 at 07DAS2x.

The data for the 2016 experiment was analyzed comparing one and two sprays for each insecticide considering the means of the two first (3 and 7 DAS1x) and the last two (03 and 07 DAS2x) evaluations. Thus, we plotted the control efficacy and defoliation percentage of large *C. includens* larvae to understand the interactions between insecticides and applications (Figure 1 and 2).

The second application at 07DAS1x resulted in reduced defoliation in some treatments comparing to just one spray, including methoxyfenozide, spinetoram, indoxacarb, and flubendiamide+thiodicarb. The highest efficacy and lowest defoliation was observed for indoxacarb and chlorfenapyr, considering that defoliation injury is caused mainly by large larvae of *C. includens*. Interestingly, even though chlorfluazuron+acephate was not efficient to control large larvae (Figure 1), defoliation in the soybean plots with this treatment was low (<10%, Figure 2).

The insecticide chlorfenapyr showed similarity between 1 and 2 applications, presenting the highest efficacy of *C. includens* and the lowest defoliation percentage. This result suggests

that the second spray of chlorfenapyr is not necessary at 7 days of interval to increase efficacy control of large larvae of *C. includens*.

**Discussion**

We tested diverse insecticides in the control of small and large larvae of *C. includes* in soybean under field conditions. Chlorfenapyr and indoxacarb showed reliable results among the three years of experiments, with high mortality of both sizes of *C. includens* caterpillars. Even more, besides the characteristics of the residual effect of all insecticides tested, we found significantly differences in mortality between one and two applications. Our results show that for large larvae of *C. includens* the second application is definitely necessary for some insecticides, including spinetoram, indoxacarb, and flubendiamide+thiodicarb.

Chlorfenapyr showed satisfactory results reducing the number of large larvae and having low defoliation percentage with one spray. The other insecticides tested, showed lower mortality of small and large larvae of *C. includens* and consequently lower efficacy, in all three years of experiments (Table 6). Interestingly, the highest efficacy of indoxacarb and chlorfenapyr was constant among the three years showing low standard deviation for small and large larvae. In contrast, methoxyfenozide showed high variation in mortality of small and large larvae. The other insecticides that performed low mortality resulted in low standard deviation, representing consistence of low efficacy.

This issue is common for *C. includens* larvae management in Brazil. The high tolerance of *C. includens* may be related to its capacity to detoxify insecticides as previously found (Dowd and Sparks, 1986) and recently noticed in a resistant population for pyrethroids in Brazil (Chapter II of this thesis). This feature of *C. includens* larvae might be related to the higher efficacy of chlorfenapyr and indoxacarb. Chlorfenapyr is a broad spectrum pro-insecticide activated by cytochrome P450, glutathione S-transferase, carboxylesterase, which activate the

pro-insecticide with oxidative removal of the N-ethoxymethyl group of chlorfenapyr to form a toxic compound that uncouples oxidative phosphorylation at the mitochondria (Hunt and Treacy, 1998; Feyereisen, 2012). Indoxacarb is bioactivated by enzymes that convert this compound to N-decarbomethoxyllated active metabolites, which are highly potent to block the voltage-gated sodium channel in the inactivated state (Wing et al., 1998).

Thus, in regards of these insecticide features, a combination of chlorfenapyr and indoxacarb should be considered and tested to control *C. includens*. It can result in a positive combination increasing efficacy and most important, help delaying insect resistance because the mode of action of chlorfenapyr and indoxacarb to be completely different. Chlorfenapyr and indoxacarb were also reported to have high efficacy controlling *Helicoverpa armigera* on soybean in Brazil (Perini et al., 2016). Furthermore, indoxacarb has a short period of residue due to its high photodegradation ratio ($DT_{50}$ = 4.5 days; FAO), compared to chlorfenapyr, which is less soluble in water and has less photolysis along five to eight days ($DT_{50}$ = 5-8 days; FAO).

One issue of *C. includens* related to its biology that type this species as difficult to control by several chemical insecticides that we tested, can be the continuous hatching of caterpillars and the residual effect of insecticides. The period from egg to 3$^{nd}$ instar larvae of *C. includes* takes about seven days (Moscardi et al., 2012) and these stages only scrape on the underside of leaves with little consumption (Bueno et al., 2007). The major consumption activity of *C. includens* (97%) is from 4º to 6º instar and its development takes about 8 days (Reid and Greene, 1973), and consequently the highest probability to get contaminated by insecticides. Thus, the insecticide needs a longer residual period (more than seven days) or additional applications after this interval in order to have the amount of insecticides available on soybean leaves when the greatest consumption of large larvae begins (after 3$^{rd}$ instar). It was observed for large larvae of *C. includens* at 7, 10 and 14 DAS in the chlorfenapyr treatment,

due to its long residual effect, even with just one application (Tables 3, 4, and 5). In the other hand, indoxacarb required a second application as we did in the experiment III - 2016, because its low residual effect as can be seen in experiments I and II and III with one spray.

Defoliation injury was avoided in treatments with spinetoram (2x), chlorfluazuron+acephate (1 and 2x), chlorfenapyr (1 and 2x) and indoxacarb (2x) (Figure 2). Interestingly, chlorfluazuron+acephate (1 and 2x) had low efficacy and but also low defoliation. We suggest that the larvae did not die in this treatment because the insecticide probably caused feeding inhibition. The type of injury of soybean looper larvae on soybean leaves, consuming only between veins, can result in water loss and reduction in photosynthetic efficacy, in addition of reduction of light interception and carbon assimilation as reported for cabbage looper with the same type of injury (Tang et al., 2006). Thus, low defoliation percentage helps to maintain the potential of soybean yield.

A relevant issue of soybean looper management in soybean is the cost of effective insecticides, wherein the most effective insecticides (indoxacarb and chlorfenapyr) are also the most expensive. Concerning the recommended range of application, the budget of these two insecticides to growers is between 2 and 4 times more costly than others. Thus, an usual decision is the grower to spray an insecticide with lower price first, leading to use those effective and expensive products just when the larvae density is very high leading to control failures. But, in regards our results, soybean growers should consider the tactics to control *C. includens* in an integrated pest management, independently of its cost. The consecutive use of just one strategy has a high risk to select resistant caterpillars and the tactics to control this pest on soybean should consider not only the efficient chemical insecticides (chlorfenapyr and indoxacarb), but also biological insecticides and varieties of transgenic soybean that express Bt toxins.

## Conclusions

Overall, few active ingredients have efficacy to control small and large larvae of *C. includens* in soybean. Among all tested insecticides, spinetoram, spinosad, chlorfenapyr and indoxacarb were consistently found to be effective against small larvae, while chlorfenapyr and indoxacarb were effective for small and large larvae. Effectiveness of chlorfluazuron + acephate mixtures may be related to feeding inhibition and subsequent lower defoliation, rather than killing larvae. Moreover, many insecticides need a second application to increase efficacy and to avoid defoliation, including: spinetoram, indoxacarb, and the association fludendiamide + thiodicarb. The exception was chlorfenapyr, which had similar defoliation percentage over one and two applications. Indoxacarb and chlorfenapyr are the most effective insecticides, besides being more costly. Thus, the management of *C. includens* in soybean have to consider the effectiveness of insecticides, the mode of action, the cost of insecticide application, and combination of tactics in an integrated pest management and insecticide resistance management.

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http://dx.doi.org/10.1002/(SICI)1520-6327(1998)37:1<91::AID-ARCH11>3.0.CO;2-5

Table 1 - Information of soybean and larval density for each experiment.

| Experiment | Year | Location | Spraying dates | Soybean growth stage at spray[1] | Larvae per $m^2$ | | Total |
|---|---|---|---|---|---|---|---|
| | | | | | Small[2] | Large | |
| I | 2014 | W 29° 42' 57.33'' S 53° 33' 49.55'' | 10 Feb 2014 | R5.1 | 13.00 | 4.50 | 17.50 |
| II | 2015 | W 29° 43' 19.05'' S 53° 33' 32.70'' | 21 Feb 2015 | R3 | 5.00 | 8.00 | 13.00 |
| III[3] | 2016 | W 29° 43' 51.65'' S 53° 32' 57.15'' | 03 Feb 2016 (1x) | R5.3 | 4.00 | 7.50 | 11.50 |
| | | | 10 Feb 2016 (2x) | R5.5 | 3.25 | 10.75 | 14.00 |

[1] Soybean growth stage (FEHR and CAVINESS, 1977)
[2] Small: < 1.5 cm; large: > 1.5 cm
[3] (1x) first spray; (2x) second spray at seven days of interval

Table 2 - Insecticides applied to control *C. includens* in soybean from 2014 to 2016.

| **Chemical name - 2014** | **Code** | **Trade names** | **Rate[1]** |
|---|---|---|---|
| 1. untreated control | UNT | - | - |
| 2. chlorantraniliprole | CHT | Premio® 200 SC | 10.0 |
| 3. flubendiamide | FLU | Belt® 480 SC | 33.6 |
| 4. indoxacarb | IND | Avatar® 150 CE | 60.0 |
| 5. chlorfenapyr | CLF | Pirate® 240 SC | 240.0 |
| 6. spinosad | SPD | Tracer® 480 SC | 33.6 |
| 7. chlorfluazuron + methomyl | CLZ + MEL | Atabron® 50 CE+Lannate® 215 SL | 25.0 + 215.0 |
| 8. methoxyfenozide | MET | Intrepid® 240 SC | 96.0 |
| 9. λ-cyhalothrin + chlorantraniliprole | LAC + CHT | Ampligo® 50+100 SC | 3.7 + 7.5 |
| 10. chlorpyrifos | CLP | Lorsban® 480 EC | 480.0 |
| **Chemical name - 2015** | | | |
| 1. untreated control | UNT | - | - |
| 2. λ-cyhalothrin + chlorantraniliprole + diafenthiuron | LAC + CHT + DAF | Ampligo® 50+100 SC+Polo® 500 SC | 6.0+12.0 + 75.0 |
| 3. methoxyfenozide | MET | Intrepid® 240 SC | 120.0 |
| 4. spinetoram | SPI | Exalt® 120 SC | 12.0 |
| 5. chlorfluazuron + acephate | CLZ + ACF | Atabron® 50 CE+Orthene® 750 PS | 25.0 + 750.0 |
| 6. chlorfenapyr | CLF | Pirate® 240 SC | 240.0 |
| 7. indoxacarb | IND | Avatar® 150 CE | 60.0 |

| | | | | |
|---|---|---|---|---|
| 8. diflubenzuron + chlorpyrifos | DIF + CLP | Dimax® 480 SC+Klorpan® 480 EC | 72.0 + 720.0 | |
| 9. flubendiamide + thiodicarb | FLU + TIO | Belt® 480 SC+Larvin® 800 WG | 38.4 + 200.0 | |

| **Chemical name - 2016** | | | | **Time** |
|---|---|---|---|---|
| 1. untreated control | UNT | - | - | - |
| 2. λ-cyhalothrin + chlorantraniliprole + diafenthiuron | LAC + CHT + DAF | Ampligo® 50+100 SC+Polo® 500 SC | 6.0+12.0 + 75.0 | 1x |
| | LAC + CHT + DAF | Ampligo® 50+100 SC+Polo® 500 SC | 6.0+12.0 + 75.0 | 2x |
| 3. methoxyfenozide | MET | Intrepid® 240 SC | 120.0 | 1x |
| | MET | Intrepid® 240 SC | 120.0 | 2x |
| 4. spinetoram | SPI | Exalt® 120 SC | 12.0 | 1x |
| | SPI | Exalt® 120 SC | 12.0 | 2x |
| 5. chlorfluazuron + acephate | CLZ + ACF | Atabron® 50 CE+Orthene® 750 PS | 25.0 + 750.0 | 1x |
| | CLZ + ACF | Atabron® 50 CE+Orthene® 750 PS | 25.0 + 750.0 | 2x |
| 6. chlorfenapyr | CLF | Pirate® 240 SC | 240.0 | 1x |
| | CLF | Pirate® 240 SC | 240.0 | 2x |
| 7. indoxacarb | IND | Avatar® 150 CE | 60.0 | 1x |
| | IND | Avatar® 150 CE | 60.0 | 2x |
| 8. diflubenzuron + chlorpyrifos | DIF + CLP | Dimax® 480 SC+Klorpan® 480 EC | 72.0 + 720.0 | 1x |
| | DIF + CLP | Dimax® 480 SC+Klorpan® 480 | 72.0 + 720.0 | 2x |

| | | | | |
|---|---|---|---|---|
| | | EC | | |
| | FLU + TIO | Belt® 480 SC+Larvin® 800 WG | 38.4 + 200.0 | 1x |
| 9. flubendiamide + thiodicarb | FLU + TIO | Belt® 480 SC+Larvin® 800 WG | 38.4 + 200.0 | 2x |

[1] Rate of active ingredient per hectare
[2] (1x) first spray; (2x) second spray after seven days from the first

Table 3 - Number of small and large larvae in the experiment I in 2014.

| Treatments | Small larvae | | | | | |
|---|---|---|---|---|---|---|
| | 3 DAS* | t | 7 DAS | t | 10 DAS | t |
| 1. untreated control | 12.75±1.9 | a | 4.00±1.8 | a | 2.25±0.5 | a |
| 2. chlorantraniliprole | 4.75±1.5 | c | 2.25±3.3 | a | 0.75±1.5 | b |
| 3. flubendiamide | 8.75±2.1 | b | 2.25±2.9 | a | 0.75±1.5 | b |
| 4. indoxacarb | 3.50±1.7 | d | 1.25±1.5 | a | 0.75±1.5 | b |
| 5. chlorfenapyr | 1.75±1.0 | d | 1.75±1.7 | a | 1.00±0.0 | a |
| 6. spinosad | 3.00±0.8 | d | 1.00±0.8 | a | 0.25±0.5 | b |
| 7. chlorfluazuron + methomyl | 8.00±1.8 | b | 2.00±1.6 | a | 1.75±1.7 | a |
| 8. methoxyfenozide | 11.25±2.8 | a | 2.75±1.7 | a | 0.75±1.5 | b |
| 9. λ-cyhalothrin + chlorantraniliprole | 10.75±1.7 | a | 3.00±0.8 | a | 2.00±0.8 | a |
| 10. chlorpyrifos | 5.75±1.0 | c | 2.75±1.3 | a | 0.50±1.0 | b |
| CV% | 12.1 | | 38.9 | | 37.2 | |
| Treatments | Large larvae | | | | | |
| | 3 DAS* | t | 7 DAS | t | 10 DAS | t |
| 1. untreated control | 4.50±2.1 | a | 15.25±2.6 | a | 13.00±2.2 | a |
| 2. chlorantraniliprole | 3.25±1.0 | a | 9.00±2.6 | b | 9.00±1.4 | b |
| 3. flubendiamide | 2.50±1.9 | a | 14.75±1.7 | a | 9.50±1.3 | b |
| 4. indoxacarb | 0.25±0.5 | b | 2.25±1.0 | d | 2.75±1.0 | d |
| 5. chlorfenapyr | 3.25±1.0 | a | 2.75±1.0 | d | 0.75±0.5 | e |
| 6. spinosad | 2.00±2.2 | a | 8.50±1.3 | b | 4.75±1.0 | c |
| 7. chlorfluazuron + methomyl | 2.25±1.0 | a | 5.75±2.7 | c | 10.00±3.6 | b |
| 8. methoxyfenozide | 3.25±1.5 | a | 10.25±2.9 | b | 15.50±1.7 | a |
| 9. λ-cyhalothrin + chlorantraniliprole | 3.25±0.5 | a | 18.50±1.7 | a | 12.00±1.8 | a |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10. chlorpyrifos | 3.50±1.3 | a | 6.25±1.5 | c | 5.00±1.4 | c |
| CV% | 23.3 | | 11.3 | | 10.5 | |

[1] Days after spray and the standard deviation for each mean value.
[2] Values followed by the same letter are not significantly different at $P \leq 0.05$ according to Scott-Knott test.

Table 4 - Number of small and large larvae of *C. includens* in experiment II in 2015.

| Treatments | Small larvae | | | | | |
|---|---|---|---|---|---|---|
| | 3 DAS[1] | t[2] | 7 DAS | t | 10 DAS | t |
| 1. untreated control | 4.25±1.6 | a | 3.25±1.0 | a | 3.6±1.3 | a |
| 2. λ-cyhalothrin + chlorantraniliprole + diafenthiuron | 2.50±2.3 | a | 2.50±1.5 | a | 0.8±0.8 | b |
| 3. methoxyfenozide | 2.50±2.3 | a | 1.25±1.0 | b | 0.9±0.8 | b |
| 4. spinetoram | 1.00±0.7 | a | 0.88±0.9 | b | 0.6±0.7 | b |
| 5. chlorfluazuron + acephate | 3.50±2.9 | a | 0.88±0.9 | b | 0.4±0.5 | b |
| 6. chlorfenapyr | 2.13±1.6 | a | 0.38±0.7 | b | 0.3±0.4 | b |
| 7. indoxacarb | 2.38±2.4 | a | 0.25±0.4 | b | 1.0±0.9 | b |
| 8. diflubenzuron + chlorpyrifos | 2.38±1.6 | a | 1.00±1.5 | b | 0.4±0.5 | b |
| 9. flubendiamide + thiodicarb | 3.13±1.6 | a | 2.00±1.1 | a | 0.9±1.1 | b |
| CV% | 34.7 | | 32.6 | | 32.2 | |
| Treatments | Large larvae | | | | | |
| | 3 DAS* | t | 7 DAS | t | 10 DAS | t |
| 1. untreated control | 7.25±1.9 | a | 7.50±1.2 | a | 7.00±1.5 | a |
| 2. λ-cyhalothrin + chlorantraniliprole + diafenthiuron | 8.38±1.8 | a | 6.50±1.6 | a | 4.75±1.9 | b |
| 3. methoxyfenozide | 5.63±1.0 | b | 2.75±1.1 | b | 2.50±1.1 | c |
| 4. spinetoram | 3.50±1.0 | c | 5.38±1.7 | a | 4.13±1.1 | b |
| 5. chlorfluazuron + acephate | 7.50±1.4 | a | 6.25±1.4 | a | 4.38±1.2 | b |
| 6. chlorfenapyr | 2.63±0.7 | c | 2.38±1.1 | b | 1.25±0.8 | d |
| 7. indoxacarb | 5.75±1.6 | b | 1.88±1.1 | b | 3.63±1.2 | b |
| 8. diflubenzuron + chlorpyrifos | 7.25±1.3 | a | 6.13±1.6 | a | 3.38±1.0 | b |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9. flubendiamide + thiodicarb | 5.88±1.4 | b | 5.88±1.5 | a | 5.63±1.8 | a |
| CV% | 12.3 | | 14.6 | | 15.7 | |

[1] Days after spray and the standard deviation for each mean value.
[2] Values followed by the same letter are not significantly different at $P \leq 0.05$ according to Scott-Knott test.

Table 5 - Number of small and large larvae of *C. includens* in experiment III in 2016.

| Treatments | | Small larvae | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 3 DAS1x | t | 7 DAS1x | t | 10 DAS1x | t | 14 DAS1x | t |
| 1. untreated control | | 3.75±1.0 | a | 3.25±1.3 | a | 2.25±1.0 | a | 3.00±1.4 | a |
| 2. λ-cyhalothrin + chlorantraniliprole + diafenthiuron | 1x | 1.50±1.0 | b | 3.25±0.5 | a | 2.50±0.6 | a | 4.25±1.3 | a |
| | 2x | 2.75±2.1 | b | 1.75±0.5 | b | 1.75±1.0 | a | 4.25±2.2 | a |
| 3. methoxyfenozide | 1x | 4.25±1.0 | a | 2.00±1.2 | b | 1.50±1.3 | a | 3.25±1.9 | a |
| | 2x | 3.50±0.6 | a | 4.00±0.8 | a | 2.25±1.3 | a | 3.25±1.3 | a |
| 4. spinetoram | 1x | 3.50±1.3 | a | 0.75±1.0 | c | 1.25±1.0 | a | 2.25±1.5 | b |
| | 2x | 3.25±1.5 | a | 0.50±1.0 | d | 0.75±1.0 | b | 1.75±1.5 | b |
| 5. chlorfluazuron+acephate | 1x | 4.00±0.8 | a | 1.75±1.0 | b | 1.00±1.2 | b | 3.25±2.1 | a |
| | 2x | 4.00±0.8 | a | 1.75±0.5 | b | 2.00±0.8 | a | 3.25±1.5 | a |
| 6. chlorfenapyr | 1x | 4.50±0.6 | a | 0.00±0.0 | d | 0.25±0.5 | b | 0.50±1.0 | b |
| | 2x | 2.00±0.8 | b | 0.00±0.0 | d | 0.00±0.0 | b | 1.25±1.0 | b |
| 7. indoxacarb | 1x | 2.00±0.8 | b | 1.25±1.0 | c | 0.00±0.0 | b | 4.50±1.9 | a |
| | 2x | 4.00±1.4 | a | 1.25±1.0 | c | 0.75±1.0 | b | 1.75±1.3 | b |
| 8. diflubenzuron+chlorpyrifos | 1x | 4.25±1.5 | a | 0.25±0.5 | d | 0.00±0.0 | b | 3.25±1.3 | a |
| | 2x | 5.50±1.7 | a | 1.25±1.0 | c | 0.25±0.5 | b | 1.50±1.0 | b |
| 9. flubendiamide+thiodicarb | 1x | 5.00±1.8 | a | 2.75±1.5 | a | 1.00±0.8 | b | 3.25±1.5 | a |
| | 2x | 6.00±2.4 | a | 1.25±1.0 | c | 1.25±1.0 | a | 2.00±1.6 | b |
| CV% | | 16.9 | | 23.4 | | 27.8 | | 25.4 | |

| Treatments | | Large larvae | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 3 DAS1x | t | 7 DAS1x | t | 10 DAS1x | t | 14 DAS1x | t |
| 1. untreated control | | 7.00±2.1 | a | 10.75±1.0 | a | 5.25±1.5 | b | 3.50±1.3 | a |
| 2. λ-cyhalothrin + | 1x | 6.50±1.3 | a | 11.00±2.4 | a | 7.75±1.7 | a | 3.50±1.3 | a |
| chlorantraniliprole + diafenthiuron | 2x | 6.50±0.6 | a | 9.00±2.4 | a | 8.00±2.2 | a | 3.25±0.5 | a |
| 3. methoxyfenozide | 1x | 6.25±1.5 | a | 10.25±1.0 | a | 6.25±1.0 | a | 3.25±1.0 | a |
| | 2x | 5.25±1.7 | a | 7.25±1.7 | b | 5.25±1.7 | b | 1.75±1.3 | b |
| 4. spinetoram | 1x | 7.00±1.4 | a | 7.25±1.0 | b | 6.00±0.8 | a | 3.00±0.8 | a |
| | 2x | 7.25±1.7 | a | 5.25±1.3 | c | 3.50±0.6 | b | 2.00±1.4 | b |
| 5. chlorfluazuron + | 1x | 8.25±1.0 | a | 8.75±1.0 | a | 4.50±1.7 | b | 3.50±1.3 | a |
| acephate | 2x | 6.00±1.4 | a | 6.25±2.2 | b | 3.75±1.0 | b | 1.75±0.5 | b |
| 6. chlorfenapyr | 1x | 1.50±1.3 | b | 4.25±1.3 | c | 2.25±1.3 | c | 0.50±0.6 | c |
| | 2x | 2.75±1.3 | b | 3.75±1.7 | c | 0.00±0.0 | d | 0.25±0.5 | c |
| 7. indoxacarb | 1x | 2.00±0.8 | b | 3.25±1.7 | c | 3.00±1.4 | c | 4.50±1.7 | a |
| | 2x | 2.25±1.7 | b | 4.00±1.4 | c | 1.25±1.9 | d | 0.50±0.6 | c |
| 8. diflubenzuron + | 1x | 6.25±1.3 | a | 6.50±1.9 | b | 4.00±2.3 | c | 0.25±0.5 | c |
| chlorpyrifos | 2x | 8.75±1.5 | a | 8.00±1.6 | b | 1.50±0.6 | a | 1.25±0.5 | b |
| 9. flubendiamide + | 1x | 6.00±1.6 | a | 7.25±1.0 | b | 5.75±1.7 | a | 2.00±1.4 | b |
| thiodicarb | 2x | 5.00±0.8 | a | 7.00±2.2 | b | 2.00±1.4 | c | 1.00±0.8 | b |
| CV% | | 13.8 | | 12.0 | | 17.4 | | 23.1 | |

1 Days after the first spray and the standard deviation for each mean value.

2 Values followed by the same letter are not significantly different at $P \leq 0.05$ according to Scott-Knott test.

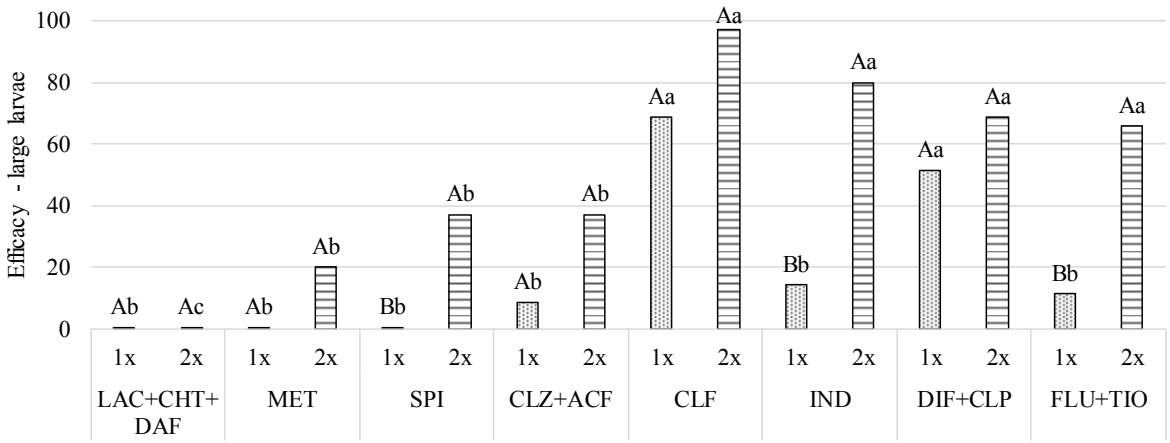



Figure 2 - Insecticide efficacy against large larvae of *C. includens* with one (1x) and two (2x) applications in 2016. Mean values followed by the same uppercase letters (between sprays 1 and 2 for each insecticide) and lowercase letters (between insecticides for each spray) do not differ significantly ($P \leq 0.05$). LAC+CHT+DAF = λ-cyhalothrin + chlorantraniliprole + diafenthiuron; MET = methoxyfenozide; SPI = spinetoram; CLZ+ACF = chlorfluazuron + acephate; CLF = chlorfenapyr; IND = indoxacarb; DIF+CLP = diflubenzuron + chlorpyrifos; FLU+TIO = flubendiamide + thiodicarb; UNT = untreated control.

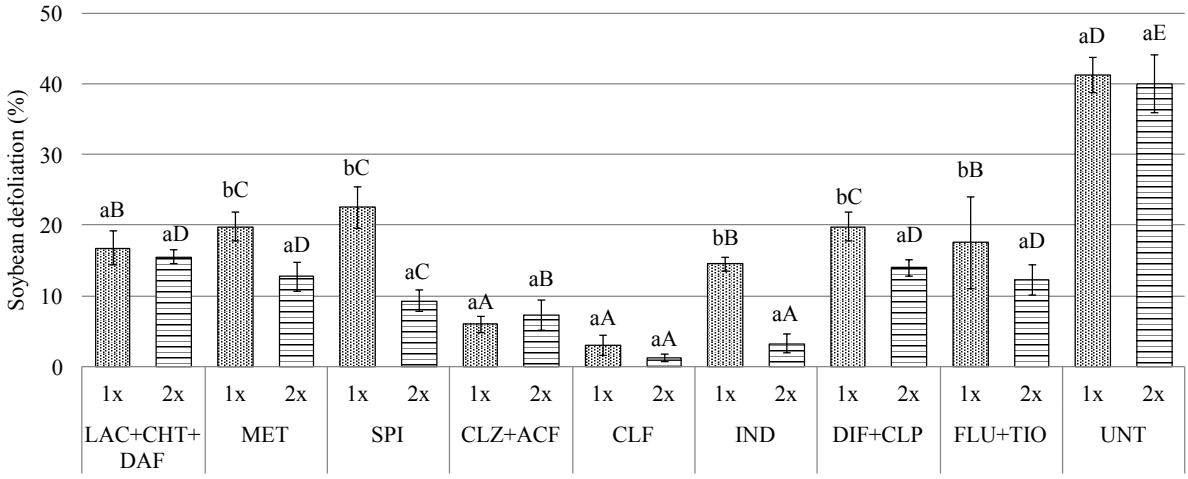



Figure 3 - Soybean defoliation percentage in 2016. Mean values followed by the same lowercase letters (between sprays 1 and 2 for each insecticide) and uppercase letters (between insecticides for each spray) do not differ significantly ($P \leq 0.05$). LAC+CHT+DAF = λ-cyhalothrin + chlorantraniliprole + diafenthiuron; MET = methoxyfenozide; SPI = spinetoram; CLZ+ACF = chlorfluazuron + acephate; CLF = chlorfenapyr; IND = indoxacarb; DIF+CLP = diflubenzuron + chlorpyrifos; FLU+TIO = flubendiamide + thiodicarb; UNT = untreated control.

Table 6 - Mean of control percentage for small and large *C. includens* larvae trials over three years: 2014, 2015, and 2016.

| | Small larvae | | | | |
|---|---|---|---|---|---|
| Treatments | 2014 | 2015 | 2016 | Mean | Standard deviation |
| 1. chlorantraniliprole* | 58 | - | - | 58 | - |
| 2. flubendiamide* | 47 | - | - | 47 | - |
| 3. indoxacarb | 69 | 70 | 54 | 64 | 9.0 |
| 4. chlorfenapyr | 66 | 77 | 50 | 64 | 13.6 |
| 5. spinosad* | 80 | - | - | 80 | - |
| 6. spinetoram | | 77 | 42 | 60 | 24.7 |
| 7. methoxyfenozide | 37 | 60 | 19 | 38 | 20.6 |
| 8. chlorpyrifos* | 55 | - | - | 55 | - |
| 9. diflubenzuron+chlorpyrifos | - | 68 | 46 | 57 | 15.6 |
| 10. lambda-cyhalothrin+chlorantraniliprole* | 17 | - | - | 17 | - |
| 11. lambda-cyhalothrin+chlorantraniliprole+ diafenthiuron | - | 48 | 30 | 39 | 12.7 |
| 12. chlorfluazuron+methomyl* | 36 | - | - | 36 | - |
| 13. chlorfluazuron+acephate | - | 60 | 23 | 42 | 26.2 |
| 14. flubendiamide+thiodicarb | - | 47 | 8 | 27 | 27.6 |
| | Large larvae | | | | |
| 1. chlorantraniliprole* | 33 | - | - | 33 | - |
| 2. flubendiamide* | 25 | - | - | 25 | - |
| 3. indoxacarb | 86 | 48 | 71 | 68 | 19.1 |
| 4. chlorfenapyr | 68 | 71 | 70 | 70 | 1.5 |

| | | | | | |
|---|---|---|---|---|---|
| 5. spinosad* | 54 | - | - | 54 | - |
| 6. spinetoram | - | 40 | 16 | 28 | 17.0 |
| 7. methoxyfenozide | 20 | 50 | 8 | 26 | 21.6 |
| 8. chlorpyrifos* | 48 | - | - | 48 | - |
| 9. diflubenzuron+chlorpyrifos | - | 23 | 25 | 24 | 1.4 |
| 10. lambda-cyhalothrin+chlorantraniliprole* | 12 | - | - | 12 | - |
| 11. lambda-cyhalothrin+chlorantraniliprole+ diafenthiuron | - | 15 | 4 | 9 | 7.8 |
| 12. chlorfluazuron+methomyl* | 45 | - | - | 45 | - |
| 13. chlorfluazuron+acephate | - | 18 | 9 | 14 | 6.4 |
| 14. flubendiamide+thiodicarb | - | 20 | 23 | 22 | 2.1 |

* Treatments used only in the first year.

**Table Supplemental 1:** ANOVA of the significant interaction (P value < 0.05) between insecticides and application time.

| **ANOVA - small larvae** | | | | | |
|---|---|---|---|---|---|
| **Factors** | **Degrees of freedom** | **Sum of squares** | **Mean square** | **Fc** | **Pr>Fc** |
| F1_APLICA | 1 | 0.107241 | 0.107241 | 1.498 | 0.2270 |
| F2_INSETIC | 7 | 4.613072 | 0.659010 | 9.205 | 0.0000 |
| F1_APLICA*F2_INSETIC | 7 | 0.562302 | 0.080329 | 1.122 | 0.3652 |
| Error | 48 | 3.436441 | 0.071593 | | |
| Total | 63 | 8.719056 | | | |
| CV(%) | 17.88 | | | | |
| General mean | 1.4962502 | Number of observations | | 64 | |
| Transformation | square root of Y + 0.5 | | | | |

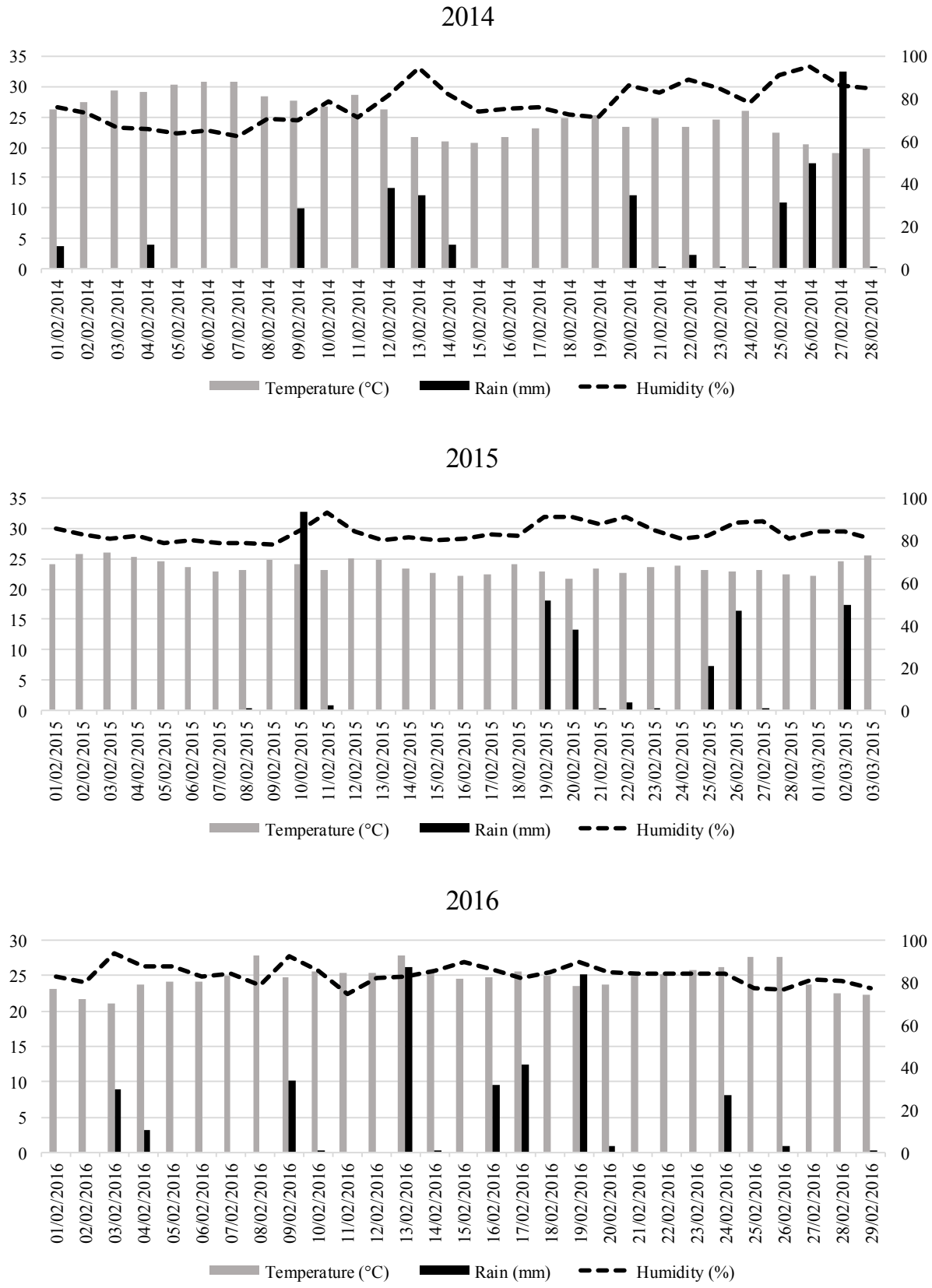
| **ANOVA - large larvae** | | | | | |
|---|---|---|---|---|---|
| **Factors** | **Degrees of freedom** | **Sum of squares** | **Mean square** | **Fc** | **Pr>Fc** |

| | | | | | |
|---|---|---|---|---|---|
| F1_APLICA | 1 | 3.008429 | 3.008429 | 44.576 | 0.0000 |
| F2_INSETIC | 7 | 10.582884 | 1.511841 | 22.401 | 0.0000 |
| F1_APLICA*F2_INSETIC | 7 | 1.228225 | 0.175461 | 2.600 | 0.0233 |
| Error | 48 | 3.239478 | 0.067489 | | |
| Total | 63 | 18.059016 | | | |
| CV(%) | 14.41 | | | | |
| General mean | 1.8025199 | Number of observations | | 64 | |
| Transformation | square root of Y + 0.5 | | | | |

**ANOVA - defoliation**

| **Factors** | **Degrees of freedom** | **Sum of squares** | **Mean square** | **Fc** | **Pr>Fc** |
|---|---|---|---|---|---|
| F1_APLICA | 1 | 435.125 | 435.125 | 64.774 | 0.0000 |
| F2_INSETIC | 8 | 7238.25 | 904.78125 | 134.688 | 0.0000 |
| F1_APLICA*F2_INSETIC | 8 | 400.75 | 50.09375 | 7.457 | 0.0233 |
| Error | 54 | 362.75 | 6.717593 | | |
| Total | 71 | 8436.875 | | | |
| CV(%) | 16.95 | | | | |
| General mean | 15.2916667 | Number of observations | | 72 | |
| Transformation | square root of Y + 0.5 | | | | |

**Figure Supplemental 1:** Temperature, humidity and rain during experiments.



# 3 ARTIGO 2

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**Transcriptional analyses of different *Chrysodeixis includens* larvae (Lepidoptera: Noctuidae) unveil potential molecular basis for field pyrethroid resistance in Brazil**

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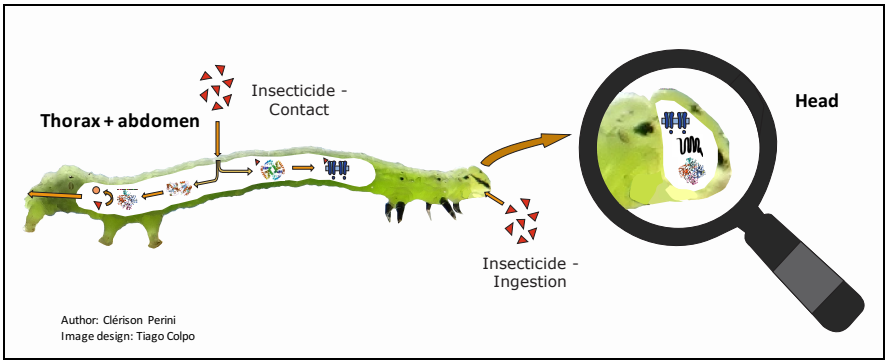
**Highlights**

- Five nonsynonymous mutations within the domains II, III, and IV of the voltage gated sodium channel were found in a resistant population of *C. inlcudens*.
- An intense detoxification process of metabolic enzymes (*Cyp*, *Gst*, *Est*, and *Ugt*) is upregulated in *C. includens*.
- GPCR genes upregulated in the head might be signaling a pathway to enhance the metabolic process.
- Detoxification process begins in the mouth parts of *C. includens* and continues through the gut.
- Energy enzymes (trypsin, serine protease, lipase, and chymotrypsin) overrepresented might be responsible to supply energy used in detoxification process.



**Abstract**

*Chrysodeixis includens*, also known as the soybean looper, is the most important caterpillar pest of soybean in Brazil due to its extensive distribution and high tolerance to insecticides. To investigate resistance mechanisms among individuals that tolerate high doses of insecticides, we performed high-throughput RNA sequencing on two Brazilian populations of *C. includens*, called by LAB and MS, differing in susceptibility to pyrethroid λ-cyhalothrin. RNA was extracted from the head and the thorax + abdomen parts and paired-end Illumina libraries were generated using the TruSeq® RNA Library Prep Kit. Sequence output was used to assemble a reference transcriptome and downstream analyses between LAB and MS. Assembly generated 102,249 contig sequences with an average of 1,005bp. After analyses, our results revealed several potential molecular mechanisms responsible for resistance to pyrethroids. First, based on comparison between the transcriptome of resistant vs. susceptible, MS vs. LAB, *C. includens*, we found five nonsynonymous mutations within the coding region of the voltage gated sodium channel in the resistant population (N1013I, L1314V, Q1433H, F1608C, and P1800S), specifically in domains II, III, and IV. These variants might alter the sensitivity of the sodium channel target site for pyrethroids. Second, overexpression of metabolic enzymes including *Cyp*, *Gst*, *Est*, and *Ugt* suggests an intense detoxification process in *C. includens*. Some of these enzymes were up-regulated in the head of the resistant population, suggesting a detoxification process that begins in the mouth parts and continues through the gut. Third, this overexpression of detoxification genes in MS population might be enhanced via a signaling pathway of two overexpressed GPCR genes in the head. Fourth, we found some potential digestive and metabolic energy enzymes that can be responsible to supply energy used in detoxification process such as: trypsin, serine protease, lipase, and chymotrypsin. Fifth, cuticle genes were found to be upregulated in the thorax + abdomen, which represents a potential barrier to insecticide penetrate in the resistant larvae. In summary, our findings represent the first insights into the molecular mechanisms underlying insecticide resistance in *C. includens*. The complexity of altered gene expression and nonsynonymous mutations can effectively be assessed with RNA-sequencing in order to implement integrated pest management strategies and insecticide resistance management.

**Key words**: soybean looper; differentially expressed genes; pyrethroid insecticide; sodium channel; nonsynonymous mutations; metabolic detoxification enzymes

## Introduction

*Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), commonly known as soybean looper, is widely distributed across the Americas with a high capacity to infest and feed on more than 70 species from 28 plant families (Herzog, 1980). In Brazil everywhere, from south to north, *C. includens* has a vast distribution availability to host plants for development, especially in summer when plants are being cultivated in a mosaic arrangement of plant species. This agricultural landscape supports broad migration of pests between regions. Polyphagous *C. includens* finds no impediments to simultaneous development on differing host species, all advantageous for egg-laying and larval nourishment (Moscardi et al., 2012). As a result of such wide distribution, *C. includens* has a low level of polymorphism over all populations, with high levels of gene flow and low genetic structure (Palma et al., 2016) that may implicate and be relevant to management strategies.

*C. includens* is the predominant Plusiinae species occurring mainly on soybean and cotton agro-ecosystems (Moscardi et al., 2012). In soybean, the predominance of *C. includens* has risen in recent years because of increased tolerance to insecticides (Guedes et al., 2015). Also, the low genetic structure of *C. includens*, indicating an exchange of genetic information among populations of different regions, and the low level of polymorphism and high level of gene flow (Palma et al., 2016), may influence such widespread occurrence and resistance-allele frequency in regards of selection intensity in all regions of Brazil. In spite of their abundance, the dispersal characteristics of male and female *C. includens* and their evolutionary process that makes it resistant to adverse conditions are not well understood.

The intensity of insecticide applications is often the most important factor driving the rate of resistance development, followed in importance by the genetic dominance, the dispersal of genotypes, and the fitness cost (Onstad, 2014). Genetic and molecular mechanisms that reveal how the insects can tolerate high doses of insecticide are significant for insecticide resistance management (IRM). Many insecticide molecules need a receptor protein to bind them and to cause functional disruption and insect death (Casida and Durkin, 2013). Either one single or more than one non-synonymous substitution in the nucleotide sequence of genes, that are expressed in transcripts, might result in a target site insensitivity, such as for pyrethroids (Dong et al., 2014), diamides (Troczka et al., 2012), oxadiazin (Gao et al., 2014), organophosphates and carbamates (Guo et al., 2017), benzoylureas (Douris et al., 2016), and *Bacillus thuringiensis* (Wu, 2014). Furthermore, metabolic detoxification of gene encoding esterases (EST), cytochrome P450 monooxygenases (P450s), and glutathione-S- transferases

(GSTs) is a common process for resistance to xenobiotics in insects (Li et al., 2007). Increased levels of these enzymes with sequence amplification or transcriptional enhancements, or with structural changes in an enzyme to provide metabolization and "break down" of insecticides, are results of internal spontaneous genomics alterations (Onstad, 2014).

Studies of resistance mechanisms reveal not only the risks of resistance but also provide information necessary for effective resistance management (Zhang et al., 2008). For this approach, RNA-seq is a powerful tool that provides the opportunity to evaluate global gene regulation and estimation of genetic differences between phenotypes (Fang and Cui, 2011; Williams et al., 2014). This technique greatly assists in the discovery of resistance mechanisms regarding the identification of single nucleotide polymorphism (SNPs), differential gene expression for metabolic resistance (Murphy et al., 2016), and identification of genes responding to different insecticide stress (Gao et al., 2017). Furthermore, RNA-seq data can provide transcript discovery (Maher et al., 2009) for constructing molecular markers in order to investigate mutations, differential gene expression, and splicing variants (Sacomoto et al., 2012; Yang et al., 2015; Murphy et al., 2016) related to resistance mechanisms.

Transcriptome analyses using high throughput sequencing and microarray have revealed that insecticide resistance mechanisms are often associated with multiple genes rather than with a single locus (Pedra et al., 2004; Puinean et al., 2010; Kalajdzic et al., 2012; Silva et al., 2012; David et al., 2014; Nascimento et al., 2015). Thus, the current study was performed using RNA sequencing based on a transcriptomic survey of two populations of *C. includens* differing in tolerance to pyrethroid insecticide in Brazil.

## Materials and methods

The workflow method has several steps, from experimental design, identification of suitable biological samples (and replicates), isolation of total RNA and library preparation, and sequencing, to downstream data analysis as are described in sections bellow.

### Sample collection and RNA extraction

A population of *C. includens* suspected of pyrethroid resistance (MS) was collected on a soybean field in the state of Mato Grosso do Sul, Brazil. A susceptible strain (LAB) which

had been reared for over 20 generations without any exposure to insecticides was taken from the laboratory LabMIP (Laboratório de Manejo Integrado de Pragas) at the Federal University of Santa Maria (UFSM). Both populations were reared with an artificial diet (Greene et al., 1976) in a room with controlled conditions of 25±2°C, 70±10% RH, and 14 hours of photophase. Bioassay experiments were performed and the resistance ratio of MS population was 38-fold for the pyrethroid Lambda-cyhalothrin (Stacke et al., 2017). In addition, a strain from a colony sourced originally from the University of Georgia, and reared for approximately 100 generations in a laboratory, was used as a reference for library and transcriptome (US strain).

Third instar larvae of each population were placed in 1.5 ml tubes with RNAlater™ stabilization solution and kept at -20ºC. Subsequently, total RNA was extracted from different body parts (head and thorax + abdomen) of pool 4 larvae, under 2 replicates, with TRI Reagent®, adapted from the manufacturer's protocol (Sigma-Aldrich, San Luis, MO, USA). To prevent potential contamination of the RNA samples with genomic DNA, RNA was treated with TURBO DNA-free™ Kit (Thermo Fisher Scientific, Vilnius, Lithuania). Total RNA quality and concentration were examined with a Nonodrop and an Experion™ RNA StdSens analysis kit (BioRad). All RNA samples were kept at -80ºC until library preparation.

**Library preparation and transcriptome sequencing**

Eight libraries were made from MS and LAB strains, using 2 replicates of head and thorax + abdomen. Because there is no information about genome or even transcriptome of *C. includens*, a US strain was used to build a reference transcriptome. Four libraries were created using the body parts of head and thorax + abdomen, and two replicates. TruSeq® RNA Library Prep Kit v2 (Illumina) was used to construct paired-end libraries from polyA RNA enrichment with an approximate average insert length of 300 bp. Size and concentration of inserts was analyzed with a High Sensitivity DNA Analysis Kit (Agilent) and a Qubit fluorometric quantitation (Invitrogen). Libraries were sequenced using a high-throughput sequencing technology with 150 bp paired-end Illumina HiSeq sequencing.

Raw Illumina RNA-seq reads were first analyzed with FastQC and trimmed (Trimmomatic - Version 0.36, released 03/21/2016) (Bolger et al., 2014) to remove low quality nucleotides ($Q < 20$), low-average quality of reads ($Q < 35$), adapters and over-represented sequences. Also, reads shorter than 25 bp were dropped. After trimming, FastQC was performed again to verify

the integrity of the remaining raw sequence reads. In accordance with the quality parameters chosen, reads were then ready to be used as input in the pipeline assembly.

**Transcriptome assembly and alignment**

The reference transcriptome from head and abdomen were *de novo* assembled using a *de Bruijn* graph-based assembler (Trinity v2.5.1, release 10/20/2017) (Haas et al., 2013). Assembly was performed using high-quality, cleaned, and filtered paired-end sequences with a fixed k-mer size of 25; minimum k-mer coverage was 3 and minimum isoform ratio was 0.05. The assembled reference transcriptome was then used to map trimmed RNA-seq reads of MS and LAB transcriptomes, and to identify splice junctions using the ultra high-throughput short read aligner Bowtie and TopHat (TopHat 2.1.0 release 6/29/2015) (Trapnell et al. 2009). Parameters for Tophat were as follows: segment length = 40; splice mismatch = 0; segment mismatches = 2; maximum insertion length = 1; and maximum deletion length = 1. Mapped sequences from TopHat were used for analyses of differential expression genes (DEG) and of sodium channel single nucleotide polymorphisms (SNPs).

**Transcriptome annotation of** *Chrysodeixis includens*

All contig sequences built on the reference transcriptome were annotated using a Blast2GO pipeline. A Blastx searches of transcripts was generated against a local database of refseq protein (refseq-PT) in the following order: *Bombyx mori* (Lepidoptera: Bombycidae) > *Drosophila melanogaster* (Diptera: Drosophilidae) > *Helicoverpa armigera* (Lepidoptera: Noctuidae) > *Danaus plexippus* (Lepidoptera: Nymphalidae). The parameters used were: word size of 3, 4 threads, HSP length cufoff of 33. Annotation of transcripts with Gene Ontology IDs (GO), which reveals the properties of biological process, molecular function, and cellular component, was accomplished using the following parameters: cutoff of 55 and blast filters with E-value-hit-filter of 1.0E-6. Also, GOs were annotated from blast descriptions with a minimum similarity of 85%. Statistics of the reference transcriptome of *C. includens* were kept to further investigate GO and annotation results.

**Sodium channel transcript and mutation discoveries**

A nucleotide BLAST was accomplished to find contigs sequences of sodium channel into the reference transcriptome using a query sequence of *Helicoverpa armigera* voltage-gated sodium channel mRNA (NCBI accession number KY247121.1). The reference transcriptome was first transformed into a BLAST database to be searchable with query sequences on a Megablast tool (BLASTn 2.7.1+) (Zhang et al. 2000). All output contig sequences with an E-value < 1E-5 were used to alignment and annotation. Each contig was searched on NCBI Open Reading Frame Finder (ORFfinder). These contig sequences were realigned with the query and predicted again on ORFfinder. The sodium channel predicted protein sequence was searched on NCBI database non-redundant protein sequences (nr) using Blastp (protein-protein BLAST).

Multiple sequence alignment was performed using Clustal W and a phylogenetic tree was generated by the Neighbor-Joining method using 1,000-fold bootstrap resampling. Cladogram was displayed graphically (MEGA7). A prediction of transmembrane regions of sodium channel protein was performed on TMHMM Server v. 2.0. The conserved structural domains were identified using the Conserved Domains Database (NCBI).

Single nucleotide polymorphisms (SNPs) analysis, regarding the high tolerance of MS population to pyrethroid, was performed using the IGV (version 2.4.4) (Thorvaldsdóttir et al., 2013) where each sodium channel contig of the reference transcriptome was loaded and the reads of the LAB and MS were aligned to find variant calls. Then, the nucleotide and protein sequence of the LAB strain was aligned with the MS strain mutant to search for non-synonymous mutations on sodium channel (CLC Sequence viewer 7).

**Differential gene expression analyses (DEG)**

The Cufflinks suite of tools was used following the 'classic' RNA-Seq workflow (Cufflinks 2.2.1 release 05/05/2014) (Trapnell et al. 2010; Roberts et al. 2011) to estimate the expression values of transcripts in FPKM (fragments per kilobase per million mapped reads). The workflow was accomplished as follows: Cufflinks with multi-read correction and upper-quartile normalization, Cuffcompare, and Cuffdiff with a minimum number of two-fragment alignments in a locus and with a false discovery rate (FDR) of 0.05, after a Benjamini-Hochberg correction for multiple-testing.

From Cuffdiff outputs four lists was created of all significant differentially expressed genes (q-value < 0.05) in head and thorax + abdomen: 1- transcripts expressed only in MS population, 2 - transcripts expressed only in LAB population, and transcripts expressed in

both populations, being 3 - upregulated, or 4 - downregulated. A Perl script was used to pull out these transcripts from the reference transcriptome followed by annotation using the Blast2GO pipeline. Transcripts involved in metabolic detoxification, receptor and metabolic energy were listed with gene description, GO IDs and terms, conserved domains, and the FPKMs between LAB and MS populations.

Visualization and plotting results of RNA-Seq DEG analyses were done by a multifaceted suite for streamlined analysis and visualization of massively parallel RNA differential expression data sequencing (CummeRbund version 2.0.0 released in 10/2/2012) in an R package (RStudio version 1.1.383). Analyses of dispersion, squared coefficient of variation ($CV^2$), distributions of FPKM, and pairwise comparisons were assessed for global statistics and quality control. An inference of different expression between populations and body parts required at least two-fold expression, FPKM $\geq 2$ and a q-value $< 0.05$.

**Results and discussions**

The assembled reference transcriptome from head and thorax + abdomen resulted in 102,249 contig sequences with an average of 1,005bp. This transcriptome was annotated and used to align and compare LAB and MS reads. Alignment summary of LAB and MS transcriptomes from the head and thorax + abdomen is presented in Table S1. The scores of gene ontologies gathered from functional analysis of proteins in InterPro and Blastx, and subsequent annotation related to molecular function, biological process, and cellular component are displayed in Figure S3.

**Sodium channel transcript and mutations discovery**

The nucleotide BLAST searched on the reference transcriptome database with a query sequence of *Helicoverpa armigera* voltage-gated sodium channel found six contig sequences with significant alignments (Table 1). Four sequences aligned with high identity percentage and E-value < 1E-5. These contig sequences were realigned and considered to annotate the sodium channel gene of *C. includens* (ChinNaCh). The length of the nucleotide sequence of *ChinNaCh* cDNA was 5,699 bp long, and resulted in a putative ORF of 5,322 nucleotides encoding 1,774 amino acids on sodium channel protein (Table 7). BLASTx and SmartBLAST

of both partial sequences showed similarity (E-value < 1E - 5) with other known insect proteins of the voltage-dependent sodium channel.

Table 7 - Contig sequences of *Chrysodeixis includens* with significant alignment for voltage-gated sodium channel and putative ORF of *ChinNaCh*.

| **Contig sequences** | **Length** | **Score** | **Bits** | **E-value** | **Identity (%)** | **Gaps** |
|---|---|---|---|---|---|---|
| TRINITY_DN6110_c0_g1_i1 | 1,134 | 1,738 | 941 | 0.0 | 94% | 0/1,130 |
| TRINITY_DN9553_c0_g1_i1 | 2,441 | 3,072 | 1,663 | 0.0 | 89% | 11/2,449 |
| TRINITY_DN9553_c0_g1_i2 | 1,798 | 1,884 | 1,020 | 0.0 | 90% | 2/1,480 |
| TRINITY_DN5867_c0_g1_i1 | 1,713 | 2,255 | 1,221 | 0.0 | 90% | 0/1,713 |
| TRINITY_DN30031_c0_g1_i1 | 600 | 534 | 289 | 7E-150 | 97% | 0/313 |
| TRINITY_DN9553_c0_g2_i1 | 759 | 172 | - | 6E-41 | - | - |
| **Putative ORF of voltage-gated sodium channel of** *Chrysodeixis includens* | | | | | | |
| **Sequence name** | **Total Length** | **Strand** | **Frame** | **Start / codon** | **Stop** | **Length (nt \| aa)** |
| *ChinNaCh* | 5,699 | + | 2 | 378 / CTG | >5,699 | 5,322 \| 1,774 |

Amino acid sequences of the voltage-gated sodium channel of *C. includens* were compared with other insects are shown in Table 8 and Figure 4. The evolutionary analysis involved 19 amino acid sequences from Lepidoptera, Coleoptera, Diptera, Blattodea, and Hemiptera orders. All positions containing gaps and missing data were eliminated, resulting in a total of 1,193 positions in the final dataset. These comparisons revealed that *C. includens* has the highest identity to the NaChs of Lepidoptera species and the *ChinNaCh* is more similar to *S. litura*, *H. armigera*, *S. exigua*, *H. zea*, and *H. virescens* (Table 8). Phylogenetic analysis of amino acid sequences of NaCh shows the well-segregated orders. NaCh of insects from Lepidoptera are most closely related to Coleoptera, Blattodea, and Hemiptera.

Table 8 - Pair-wise comparison among amino acid sequences of *ChinNaCh* and other voltage-gated sodium channels.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *C. includens* | - | | | | | | | | | | | | | | | | | | | | |
| 2 | *S. litura* | 0.010 | - | | | | | | | | | | | | | | | | | | | |
| 3 | *H. armigera* | 0.012 | 0.008 | - | | | | | | | | | | | | | | | | | | |
| 4 | *S. exigua* | 0.013 | 0.006 | 0.011 | - | | | | | | | | | | | | | | | | | |
| 5 | *H. zea* | 0.014 | 0.010 | 0.008 | 0.013 | - | | | | | | | | | | | | | | | | |
| 6 | *H. virescens* | 0.017 | 0.014 | 0.007 | 0.016 | 0.015 | - | | | | | | | | | | | | | | | |
| 7 | *B. mori* | 0.033 | 0.032 | 0.037 | 0.034 | 0.039 | 0.043 | - | | | | | | | | | | | | | | |
| 8 | *P. xylostella* | 0.054 | 0.053 | 0.059 | 0.057 | 0.060 | 0.066 | 0.054 | - | | | | | | | | | | | | | |
| 9 | *B. germanica* | 0.144 | 0.143 | 0.146 | 0.148 | 0.150 | 0.149 | 0.138 | 0.146 | - | | | | | | | | | | | | |
| 10 | *D. melanogaster* | 0.151 | 0.151 | 0.154 | 0.154 | 0.155 | 0.158 | 0.146 | 0.155 | 0.160 | - | | | | | | | | | | | |
| 11 | *A. glabripennis* | 0.153 | 0.150 | 0.154 | 0.155 | 0.157 | 0.158 | 0.145 | 0.160 | 0.148 | 0.158 | - | | | | | | | | | | |
| 12 | *L. decemlineata* | 0.153 | 0.152 | 0.158 | 0.157 | 0.161 | 0.161 | 0.142 | 0.166 | 0.154 | 0.166 | 0.047 | - | | | | | | | | | |
| 13 | *N. lugens* | 0.154 | 0.152 | 0.157 | 0.156 | 0.160 | 0.160 | 0.152 | 0.154 | 0.114 | 0.158 | 0.152 | 0.160 | - | | | | | | | | |
| 14 | *C. capitata* | 0.156 | 0.154 | 0.158 | 0.159 | 0.160 | 0.161 | 0.148 | 0.161 | 0.153 | 0.034 | 0.156 | 0.165 | 0.152 | - | | | | | | | |
| 15 | *C. lectularius* | 0.160 | 0.161 | 0.163 | 0.165 | 0.166 | 0.166 | 0.160 | 0.171 | 0.143 | 0.163 | 0.159 | 0.155 | 0.107 | 0.160 | - | | | | | | |
| 16 | *T. castaneum* | 0.162 | 0.163 | 0.166 | 0.166 | 0.169 | 0.171 | 0.157 | 0.174 | 0.159 | 0.172 | 0.086 | 0.089 | 0.162 | 0.171 | 0.170 | - | | | | | |
| 17 | *H. halys* | 0.176 | 0.175 | 0.178 | 0.179 | 0.181 | 0.181 | 0.172 | 0.180 | 0.134 | 0.174 | 0.154 | 0.149 | 0.110 | 0.170 | 0.069 | 0.161 | - | | | | |
| 18 | *M. domestica* | 0.178 | 0.176 | 0.181 | 0.181 | 0.182 | 0.184 | 0.172 | 0.184 | 0.178 | 0.060 | 0.179 | 0.176 | 0.178 | 0.066 | 0.177 | 0.188 | 0.188 | - | | | |

These values of distance estimation were done by Poisson model. Lower numbers mean less difference between sequences.

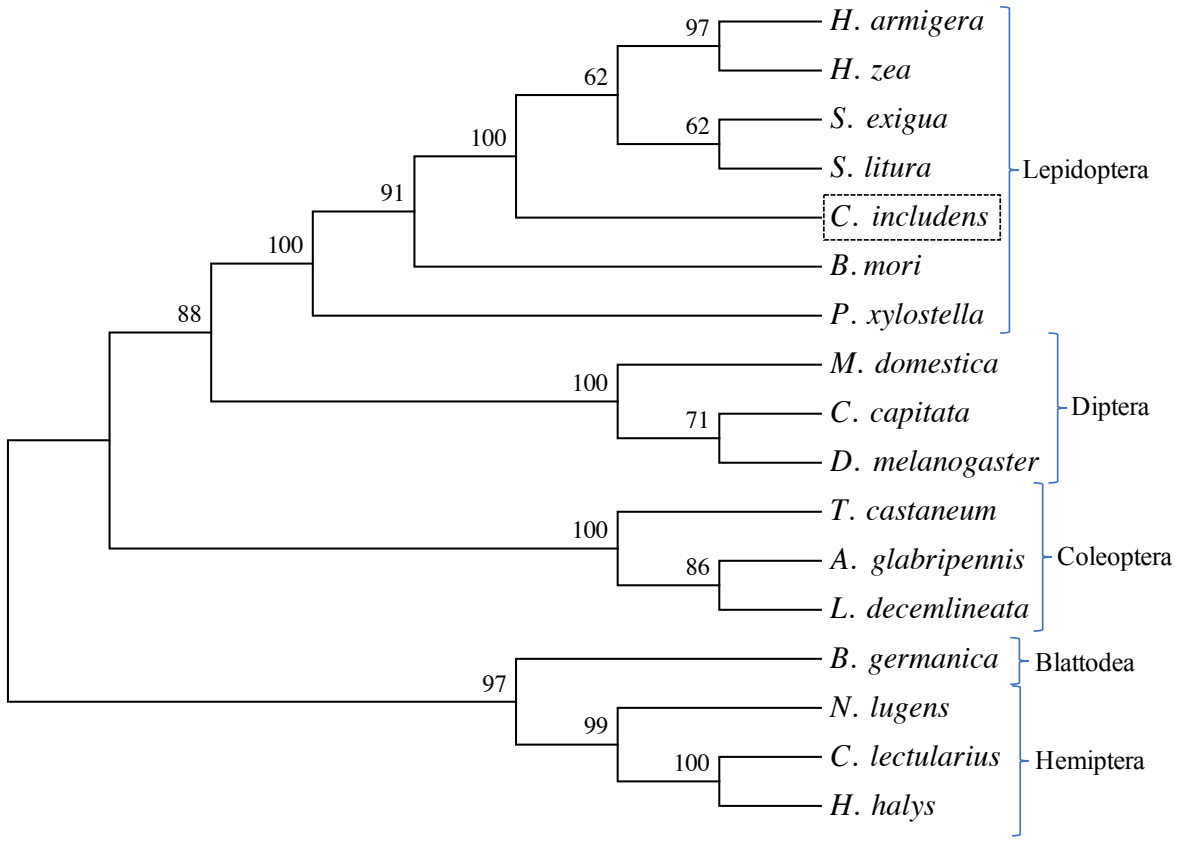



Figure 4 - Phylogenetic analysis of *ChinNaCh* with known orthologous genes. Sodium channel sequences were obtained from the following GenBank entries: *T. castaneum* (EFA11577), *L. decemlineata* (XP023023069), *A. glabripennis* (XP018568941), *M. domestica* (ARX79626), *D. melanogaster* (AAB59195), *C. capitata* (XP020717222), *H. halys* (XP024214489), *C. lectularius* (NP001303632), *N. lugens* (XP022195780), *B. germanica* (BBD13274), *H. armigera* (ARK07244), *P. xylostella* (AJR27944), *S. litura* (XP022824852), *S. exigua* (AON96178), *B. mori* (ACJ09096), and *H. zea* (ADF80418). The bootstrap values are shown next to each branch with a cutoff value of 50, and the genetic distance was drawn to scale.

The conserved structural domains and the transmembrane regions of *ChinNaCh* were predicted and they displayed five categories of conserved domains (Figure 5). These motifs include (1) **ITR** - four homologous domains [I - 74 to 358 aa, II - 597 to 808 aa, III - 1078 to 1347 aa, and IV - 1397 to 1647 aa] of the ion transport protein of sodium channel sub-family, which has six transmembrane helices; (2) **NaCYT** - cytoplasmic domain towards the start of

voltage-dependent $Na^+$ ion channel [384-455 aa]; (3) **Na-TRA** - region of sodium ion transport-associated that is found exclusively in eukaryotic organisms [834-1074 aa]; (4) **Na-CG** - inactivation gate of the voltage-gated $Na^+$ ion channel alpha subunits responsible for fast inactivation of the channel and essential for proper physiological function [1337-1389 aa]; and (5) **GPHH** - sequence motif found in this short domain on voltage-dependent L-type calcium channel [1650-1706 aa].

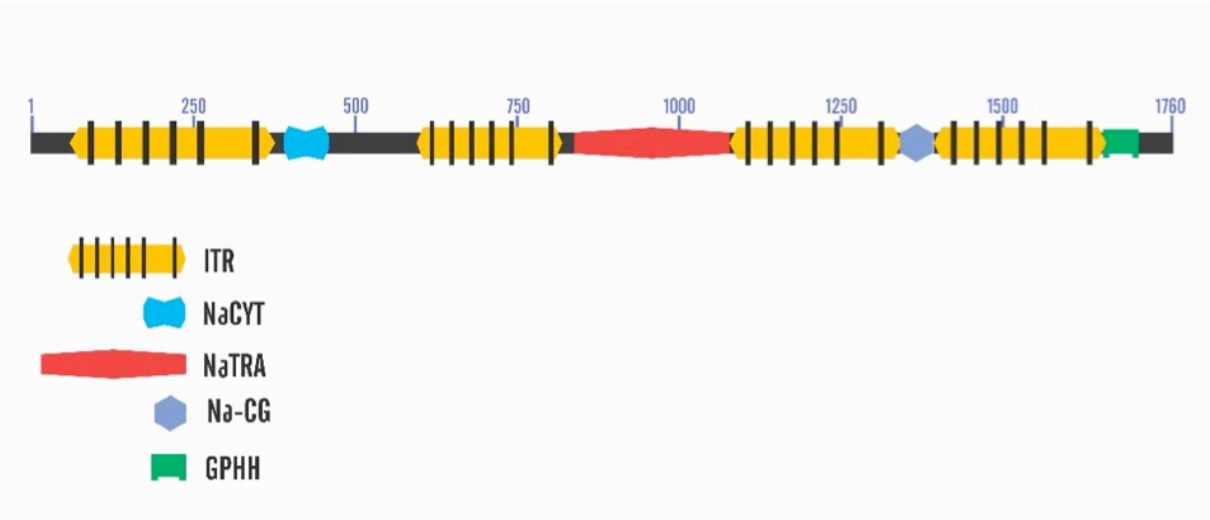



Figure 5 - Representation of conserved domains on voltage-gated $Na^+$ ion channel of *C. includens* shows the locations of predicted transmembrane domains (TM) and conserved structural domains: ITR (ion transport protein that is repeated four times and has six transmembrane helices), NaCYT (cytoplasmic domain of voltage-gated $Na^+$ ion channel), NaTRA (sodium ion transport-associated), Na-CG (sodium channel gate), and GPHH (voltage-dependent L-type calcium channel, IQ-associated).

We analyzed sodium channel contig sequences for nucleotide substitutions with IGV tools on both replicates of LAB and MS populations of head and thorax + abdomen. An ORF of *C. includens* sodium channel for LAB and MS population including all nucleotide substitutions and protein sequences was created to explore synonymous and non-synonymous mutations that can explain the resistance to Lambda-cyhalothrin in MS populations. Nucleotide and protein sequences of *ChinNaCh* was aligned with a sodium channel sequence of *H. armigera* (KY247120.1) (Figures S1 and S2). Five sites were found with non-synonymous mutations on *C. includens* sodium channel along the domains II, III, and IV (Figure 6). According to the prediction of transmembrane regions and linkers inside and outside of membranes, the first site is present in the transmembrane subunit IIS6 with a substitution of an asparagine to

isoleucine (N1013I). This region is potential for kdr mutations because it is a site where pyrethroids bind on the voltage-gated sodium ion channel, enhancing activation and prolonging open channel.

The second substitution is on the domain III in a linker between subunit S1 and S2, where a leucine is switched for a valine (L1314V) on the MS population. A third substitution occurs in the transmembrane region IIIS4 (Q1433H) from a glutamine to histidine. These two substitutions occurring in *C. includens* on domain III were not reported yet were associated with kdr to DDT or pyrethroids. But, there are many pyrethroid-sensing residues for site 1 on the transmembrane region IIIS6, where α-subunits S1 to S4 are a voltage-sensing module, while S5, S6, and the P-region connecting S5 and S6 form the pore module (DONG et al., 2014). Thus, any amino acid substitution in this region could lead to reduced sensitivity to insecticides that act on sodium channel, or alter the sensitivity and movement to activate and inactivate the voltage-gated sodium channel in *C. includens*.

Two site mutations were found on domain four, one at the S1 voltage-sensing region (F1608C) altering a phenylalanine (TTC) to a cysteine (TGC) and one mutation on the external membrane-reentrant loop connecting S5 and S6 segments, where a proline is altered (CCG) to a serine (TCG) (P1800S). Arthropod species have presented some mutation sites along this domain related to kdr mutations to pyrethroids (Du et al., 2013) and indoxacarb (Wang et al., 2016). Although these mutations across domain IV are positioned in the voltage-sensing regions and at the external linker between S5 and S6 subunits, the sodium channel of *C. includens* may account for a reduced sensitivity to Lambda-cyhalothrin.

For the transmembrane domain IIS6, several substitutions have already been reported which confer knockdown resistance in arthropod pests in positions 1010, 1011, 1013, 1014, 1020, and 1024 (Dong et al., 2014). Segments S4 of all domains move S5 and S6 subunits in response to membrane depolarization, which is responsible for opening and closing the ion channel, activating and inactivating the sodium channel (Dong et al., 2014). Silver et al. (2010) have mentioned a distinct mode of action from DDT and pyrethroids binding to sodium channels in the slow-inactivated state and blocking the channel for sodium channel blocker insecticides (SCBIs). Thus, further investigation should be done on the involvement of these two substitutions, and also to indoxacarb.

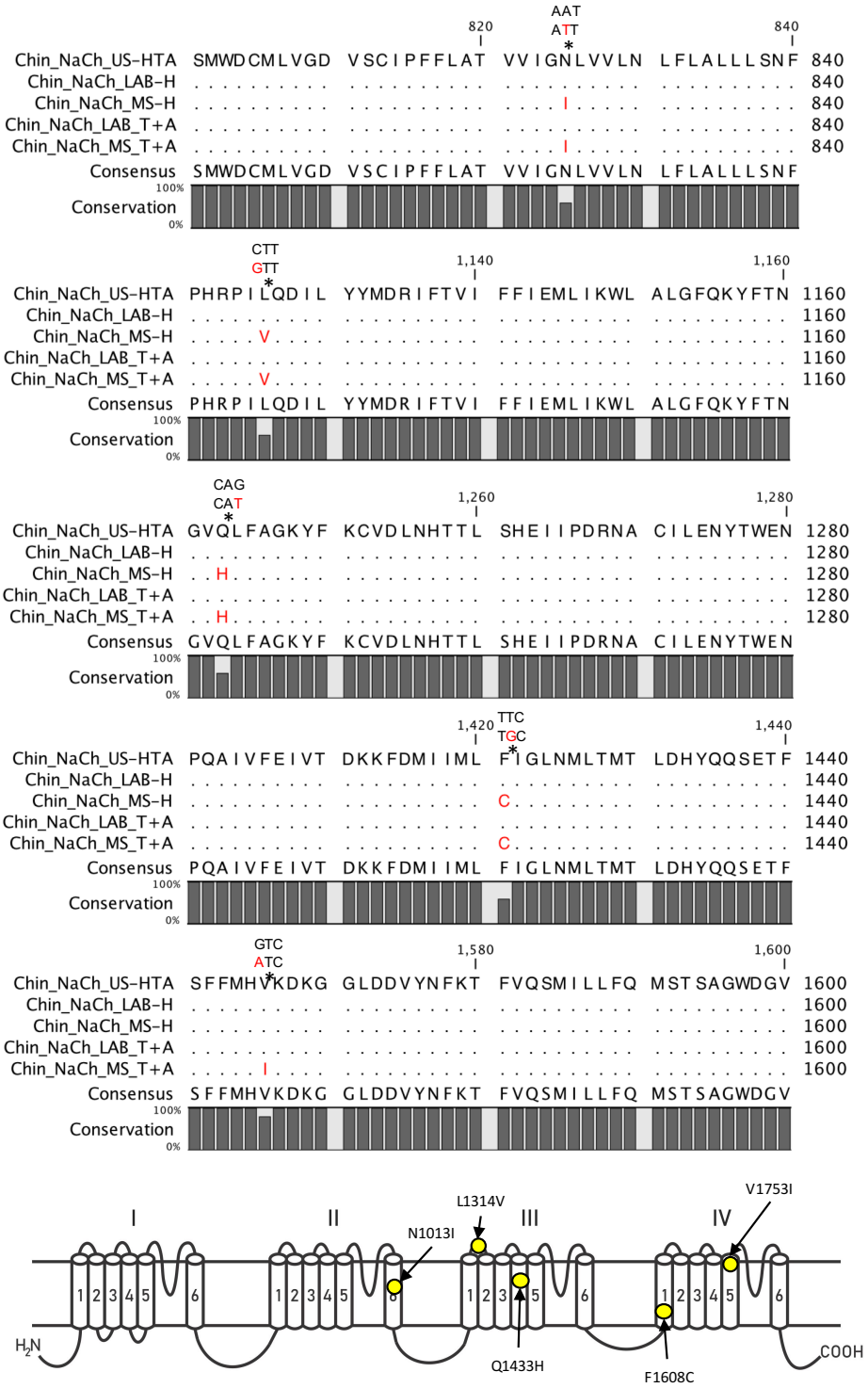



Figure 6 - Alignment of the *ChinNaCh* protein sequences of LAB and MS populations showing nonsynonymous mutation and its position in the transmembrane helices or linkers that may be conferring reduced target-site sensitivity to pyrethroid. Codons (*) showing the nucleotide substituted in red that changed the final amino acid. TMHMM Server v. 2.0 predictor of transmembrane regions was used to identify the position of each mutation. HTA - head, thorax, and abdomen; H - head; T+A - thorax + abdomen.

Even more, were found 10 synonymous mutations that did not alter amino acid from three-base-pair codon, where G change to C (1678), C changed to T (3839, 4229, 4709, 4730, 4817), G changed to A (4541) in thorax+abdomen part, A changed to G (4550), T changed to A in head part (4862), and G changed to A (5153). These mutations are called as silent substitutions. But, they are no-silent at all, because they can affect transcription, splicing, mRNA transport, and translation, and consequently, can alter the phenotype of individuals (Goymer, 2007). It sugest that synonymous mutations in the sodium channel gene of *C. includens* might cause some effect on larvae phenotype.

The voltage-gated sodium channel of agricultural arthropod pests and vectors of human diseases was already reported to have more than 50 non-synonymous mutations that are associated with knockdown resistance (kdr) to DDT and pyrethroids (Dong et al., 2014). Very recently two other novel mutations were found on sodium channel conferring kdr to DDT and pyrethroids in *Aedes aegypti* (Lien et al., 2018). Thus, one of these kdr mutations alone, or more than one, can be arranged simultaneously on sodium channels and can lead to different fold reduction in the sensitivity (levels of resistance) to DDT and pyrethroids, or in some cases can completely eliminate the sensitivity (Lee et al., 1999; Vais et al., 2000; Burton et al., 2011; Yoon et al., 2008; Tan et al., 2002; Liu et al., 2002).

**Differential gene expression analyzes (DEG)**

We used a high throughput RNA sequencing of different larvae body parts to perform pairwise comparisons between LAB and MS populations. We aimed unveil other molecular mechanisms that are implying in the decreased susceptibility of *C. includens* to the pyrethroid Lambda-cyhalothrin. The head presented paired-end reads from 135 million to 21.2 million, of which 4.4 million to 8.0 million had aligned pairs (Table S1). The thorax + abdomen presented paired-end reads from 17.8 million to 25.0 million, of which 4.7 million to 9.5 million had aligned pairs.

Estimated gene-level expression values in the generic FPKM done in the Cufflinks pipeline were more highly correlated in thorax + abdomen than in head (Figure 7). Also, the individual pairwise comparisons by scatter plots show a positive linear relationship between LAB and MS population transcripts for head and thorax + abdomen. The lower standard error values between LAB and MS populations indicate a high confidence of expression analysis. A normalized measure of cross-replicate variability was assessed with a squared coefficient of

variance ($CV^2$) and suggests lower variability among the head expressed genes than in the thorax + abdomen expressed genes (Figure S4). Further statistical analyses of distribution of FPKM scores across replicates and dispersion of mean counts between LAB and MS populations are available (Figure S5 and S6).

According to differential expressed analyses of Cuffdif with P value correction using Benjamini-Hochberg correction and fold change ≥ 2, we found some transcripts significantly differentially expressed ($P<0.05$) in head and thorax + abdomen between transcriptomes of LAB and MS populations (Figure 8). Comparisons of differentially expressed genes in each population and between body parts were made using Venn diagram plots regarding the Cuffdif output (Figure 9, Tables S2-9). There were 518 and 577 contigs expressed only in the MS population in the head and thorax + abdomen, respectively. In contrast, there were 147 contigs expressed in head and 468 contigs expressed only in thorax + abdomen, exclusively in the LAB population. Curiously, there were just a few contigs sharing expression in head between LAB and MS populations, of which 10 contigs were found to be upregulated and 8 contigs were downregulated. By contrast, in thorax + abdomen there were 790 mutual transcripts between LAB and MS populations, of which 492 were upregulated and 298 were downregulated. Thus, we performed further analyses to investigate the functionality of these contigs related to biological process, molecular function, and cellular components in the most tolerant population to pyrethroid insecticide.

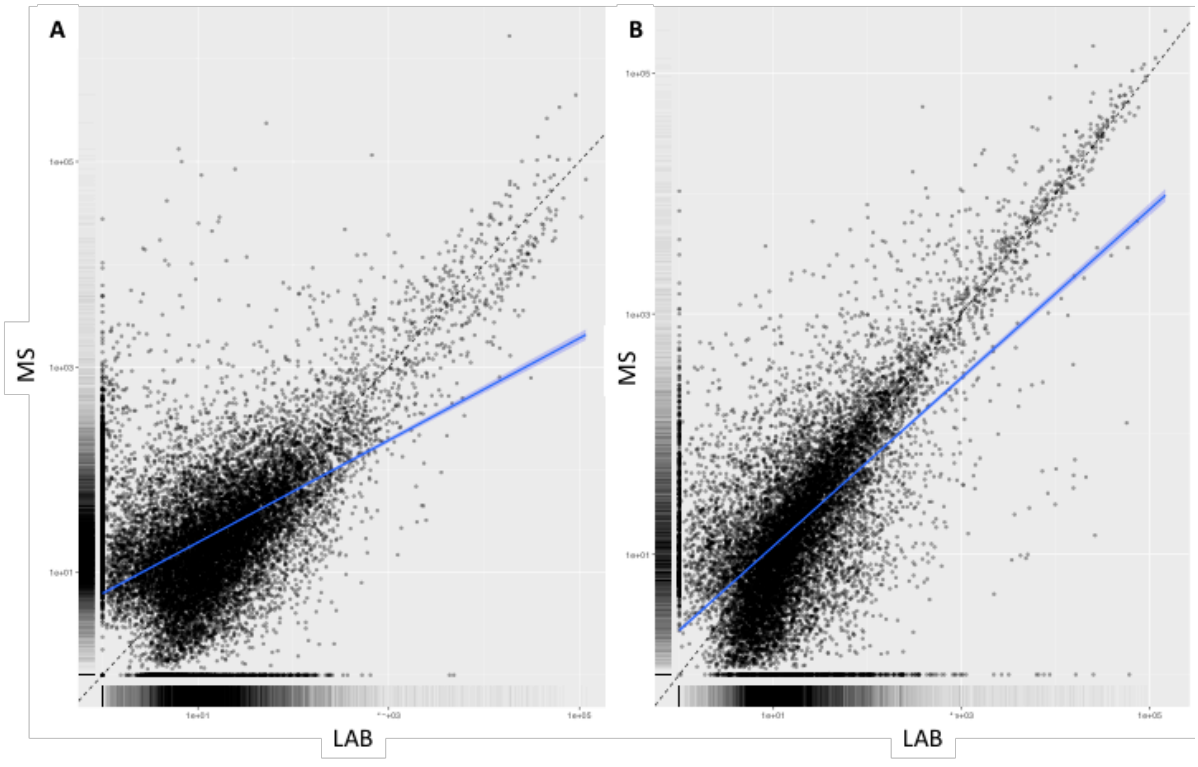


Figure 7 - Scatter plots of log10 (FPKM) showing comparisons of gene expression levels between LAB and MS populations of *C. includens* for (A) head and (B) thorax + abdomen. Blue line showing the fitted model and standard error of the fit from Poison method.

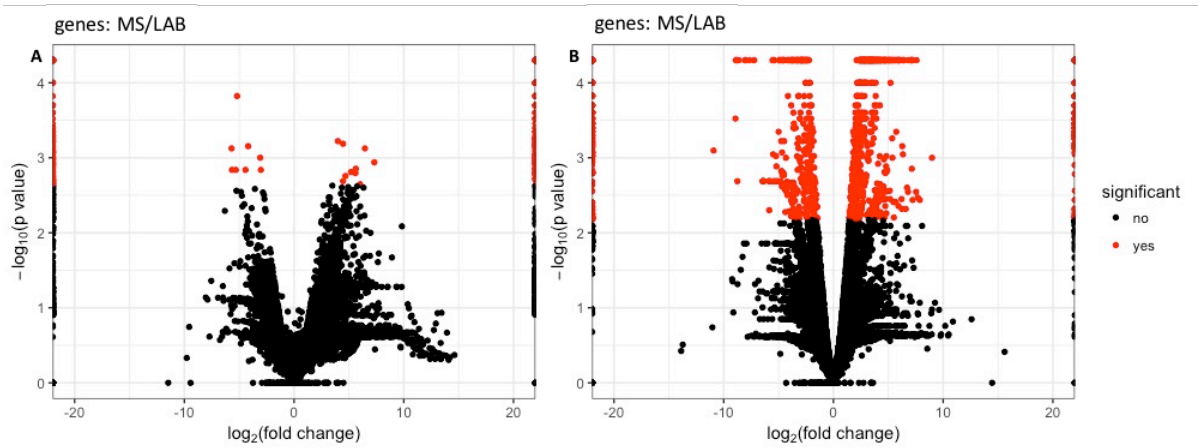



Figure 8 - Volcano plot of fold change vs. p value significance for individual pairwise comparisons between LAB and MS populations of *C. includens* for (A) head and (B) thorax + abdomen. Red dots show all significant differentially expressed contigs (q < 0.05).

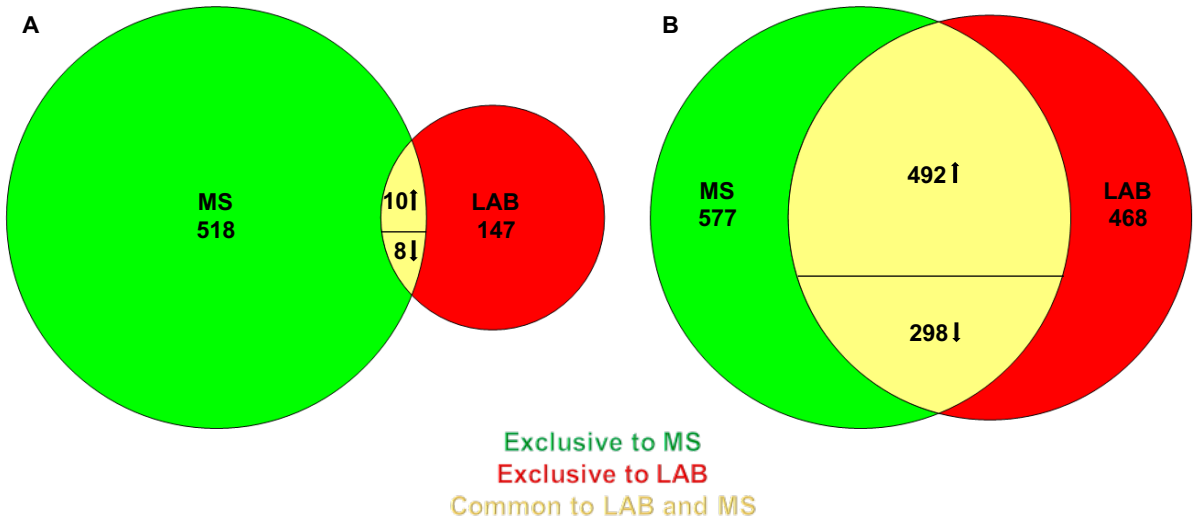



Figure 9 - Venn diagrams depicting commons and exclusives contigs among differentially expressed genes between *C. includens* LAB and MS populations in the head (A) and thorax + abdomen (B). Common contigs are separated into upregulated (↑) and downregulated (↓). Color representation is as follows: red denotes exclusive to LAB population; green denotes exclusive to MS population; and yellow denotes common to LAB and MS populations.

GO terms of differentially expressed transcripts of head and thorax + abdomen were used to functionally classify predicted proteins grouped into three primary divisions of biological process (Figures 10 and 11), molecular function, and cellular component (Figures S7-10). Among the four divisions on the Venn diagrams, there were more GO classifications involved in the metabolic and oxidation-reduction process of transcripts in upregulated and only MS (Figure 10). It can explain, in part, the low susceptibility to pyrethroid that also has the gene ontology of drug metabolic process in head. The thorax + abdomen presented higher scores of oxidation-reduction process and drug metabolic process for upregulated and only MS divisions that can be involved in metabolizing chemical compounds (Figure 11). Also, upregulated transcripts related to proteolysis showed a score higher than 60, suggesting a more organized energy metabolism in the MS population.

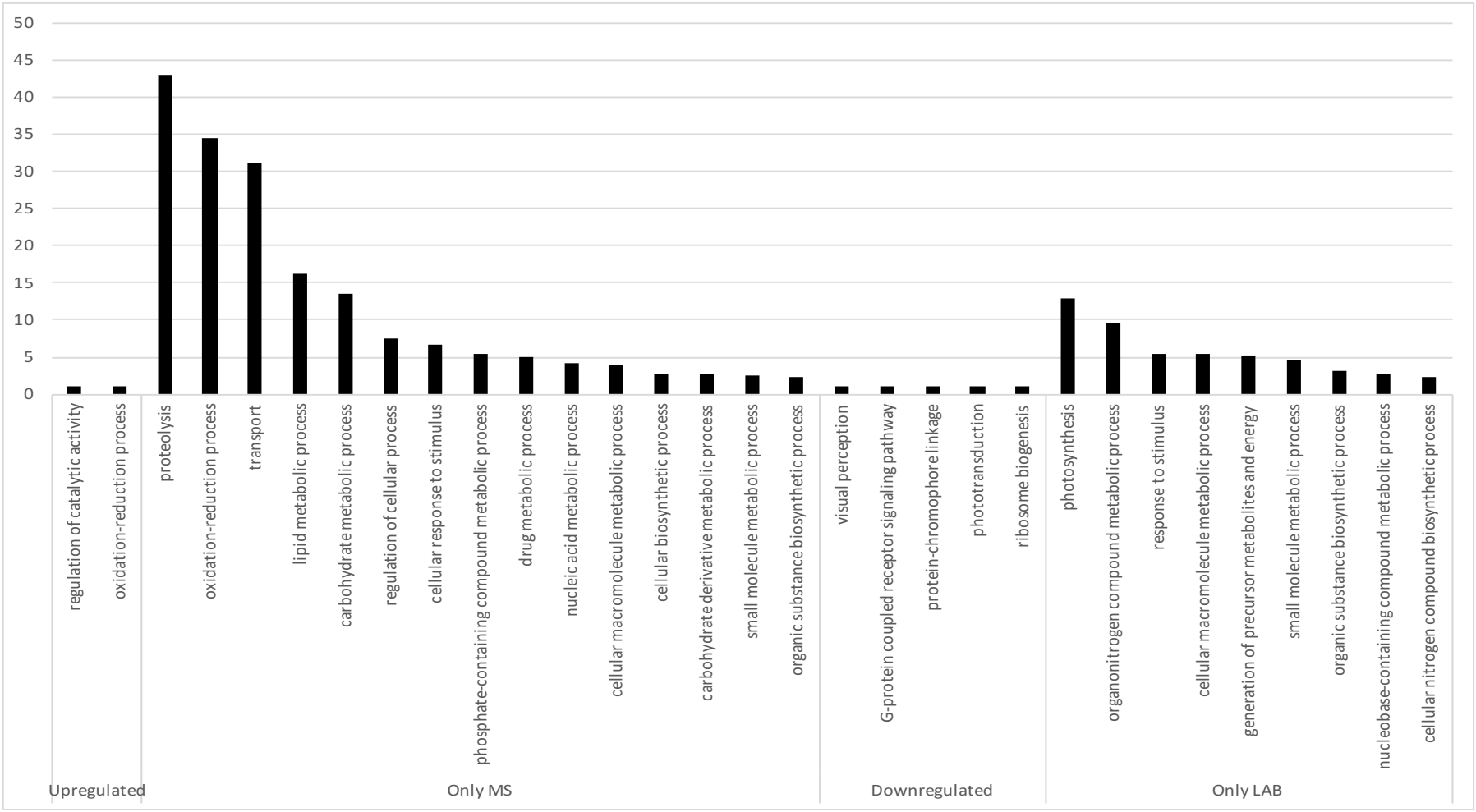



Figure 10 - Score of Gene Ontology (GO) classifications of *C. includens* head unigenes that were differentially expressed, according to their involvement in biological process. $score = \sum_{GOs} seq \times \alpha^{dist}$ (*seq* = number of different sequences annotated at a child GO term, $\alpha$ = factor alpha, *dist* = distance between the term and the term of direct annotation).

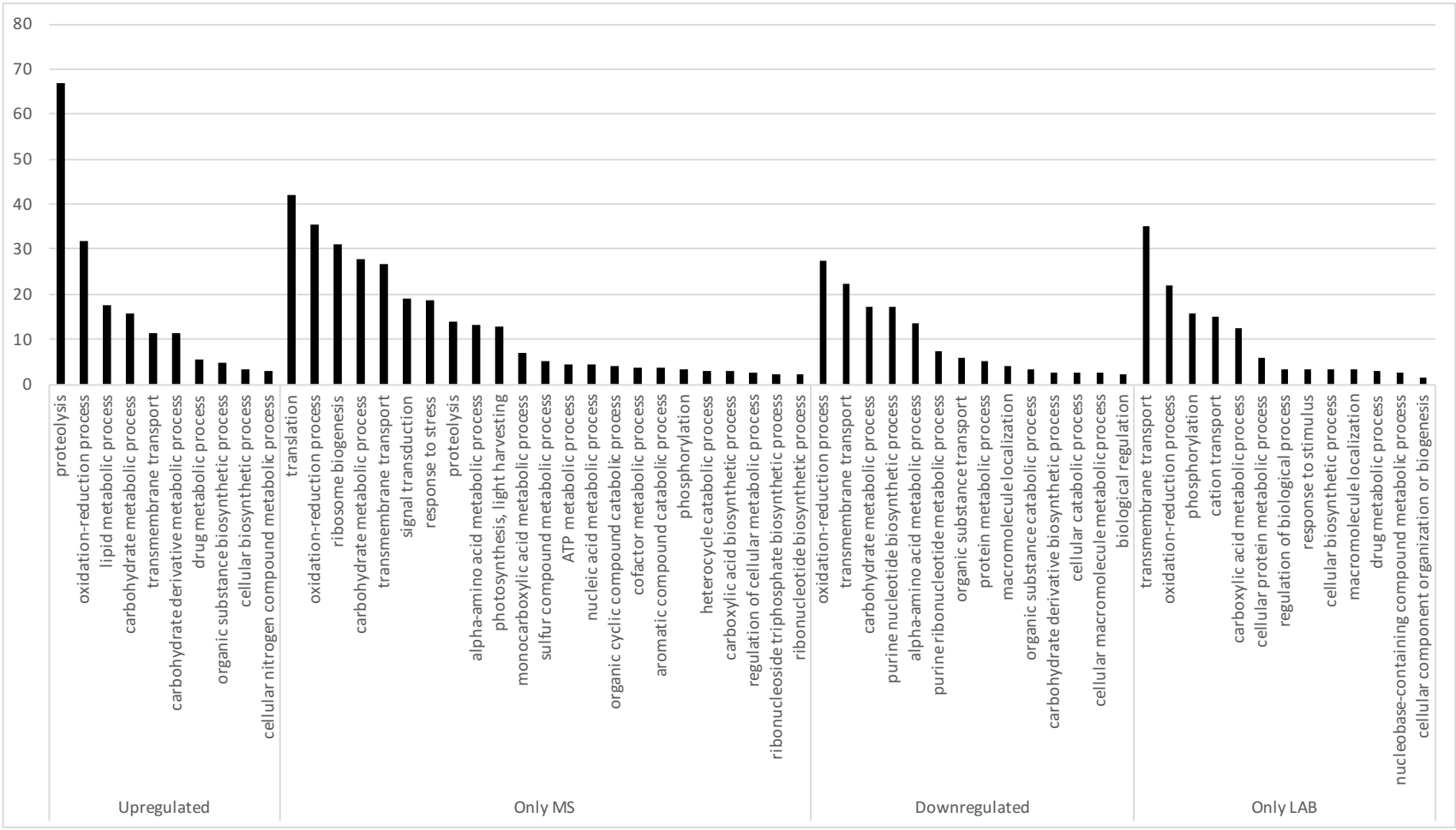



Figure 11 - Score of Gene Ontology (GO) classifications of *C. includens* thorax + abdomen unigenes that were differentially expressed, according to their involvement in biological process. $score = \sum_{GOs} seq \times \alpha^{dist}$ (*seq* = number of different sequences annotated at a child GO term, $\alpha$ = factor alpha, *dist* = distance between the term and the term of direct annotation).

In the head of *C. includens,* a greater number of detoxification transcripts (*Cyp*, *Gst*, *Est*, and *Ugt*) were found significantly upregulated or expressed only in the MS population (Figure 12; Tables S3, S5 and S10). The cytochrome P450 9e2-like, which belongs to the CYP9 family genes, showed the highest overexpression value with fold change of 7.3 (q value = 0.02925). Other P450 family genes were expressed in the MS population and not expressed in the LAB, of which six transcripts belong to CYP6 and one transcript belongs to each CYP4, CYP304, and CYP307 cytochrome P450 family genes. Conserved protein domains of P450 transcripts were analyzed (NCBI-Blastx) and the P450 superfamily domains were identified associated with oxidative degradation, metabolite biosynthesis, transport and catabolism, cyclodipeptide synthase-associated, and defense mechanisms (Table S10).

Seven transcripts of esterase E4-like and FE4-like were expressed only in MS population, having conserved domains of esterase enzymes and/or the gene ontology of hydrolase activity comprising catalysis of the hydrolysis of various bonds (e.g. C-O, C-N, C-C) (AmiGO). Two *Gst* enzymes were differentially expressed in the head with the same gene ontology term of transferase activity (GO:0016740) and conserved domain of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). Indeed, this implies that detoxification enzymes (*Cyp*, *Gst*, *Est*, and *Ugt*) might be being expressed in response to a signaling pathway receptor GPCR 64-like, since we found two transcripts differentially expressed in the head, where detoxification of chemicals begins. This broad detoxification transcripts expressed head body part of *C. includens*, possibly from the salivary glands as reported in other insect species (Rivera-Vega et al., 2017), indicates the first step of xenobiotics metabolism with contamination via ingestion.

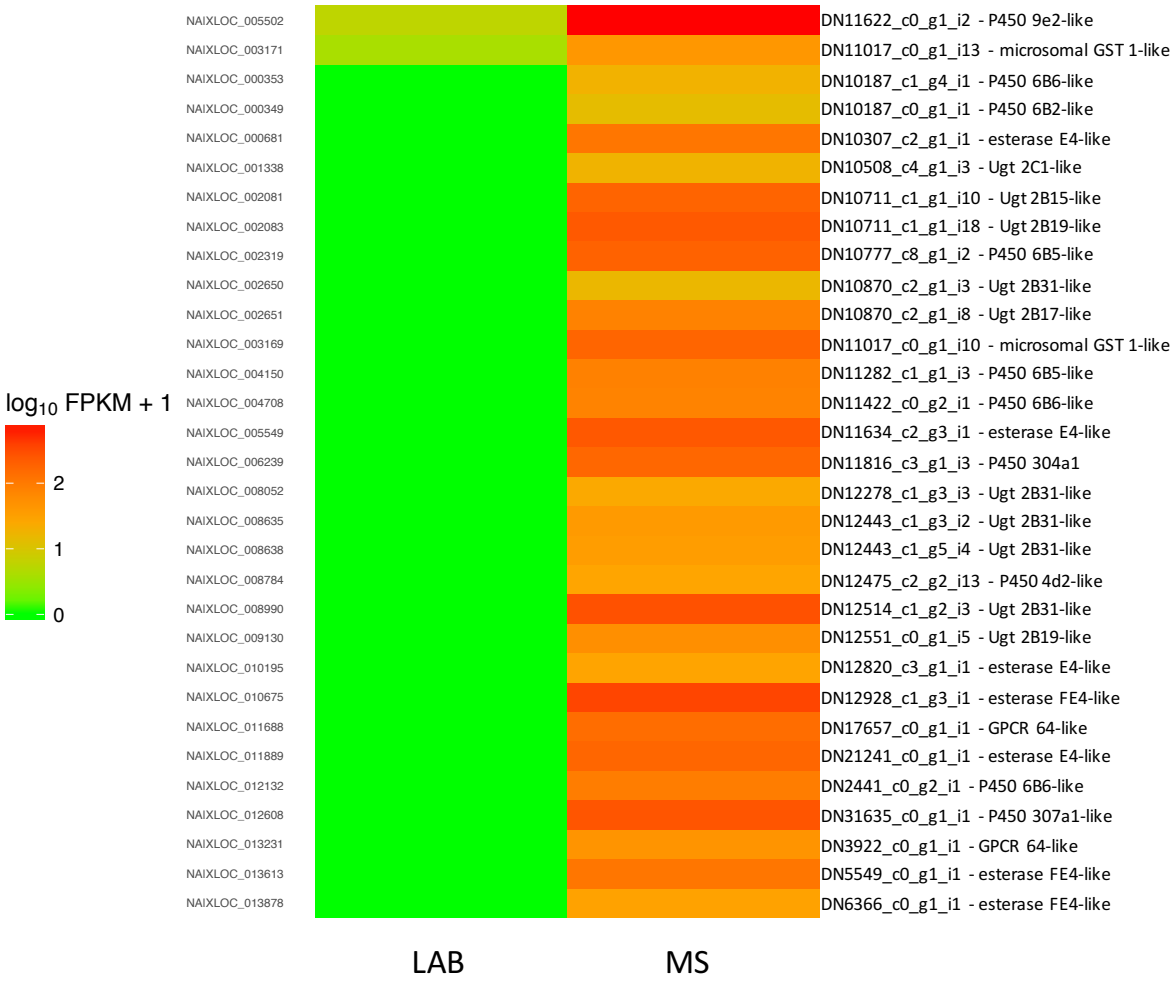



Figure 12 - Comparative distribution of FPKM expression values (fold change ≥ 2 and q < 0.05) of transcripts associated with detoxification metabolism and signaling pathway in the head between LAB and MS populations of *C. includens*. Scale of colors ranging from the lowest (green) to the highest (red) FPKM values.

Differentially expressed transcripts related to the function of detoxification by *Cyp*, *Gst*, *Est*, and *Ugt* were highly abundant in the thorax + abdomen (Figure 13). Of these, 12 transcripts of P450, 7 transcripts of GST, 9 transcripts of EST enzymes, and 5 transcripts of UGT were differentially expressed in MS population of *C. includens* (Figure 11, Table S11). Enzymes encoding cytochrome P450 monooxygenase belong to CYP6, CYP4, CYP9, and CYP 307 family genes, which were highly over-represented in MS population of *C. includens*. The overexpression of CYP enzymes via upregulation in resistant insects are now known to be controlled via mutations in *trans*-regulatory loci, via indels or mutations in *cis*-acting

elements, via coding sequence changes (Li et al., 2007), and CYP copy number variation or even amplification (Puinean et al., 2010; Feyereisen, 2012). Thus, further studies should be conducted to investigate the via of upregulation in these CYP enzymes within *C. includens* in order to understand and apply genome editing techniques for IRM.

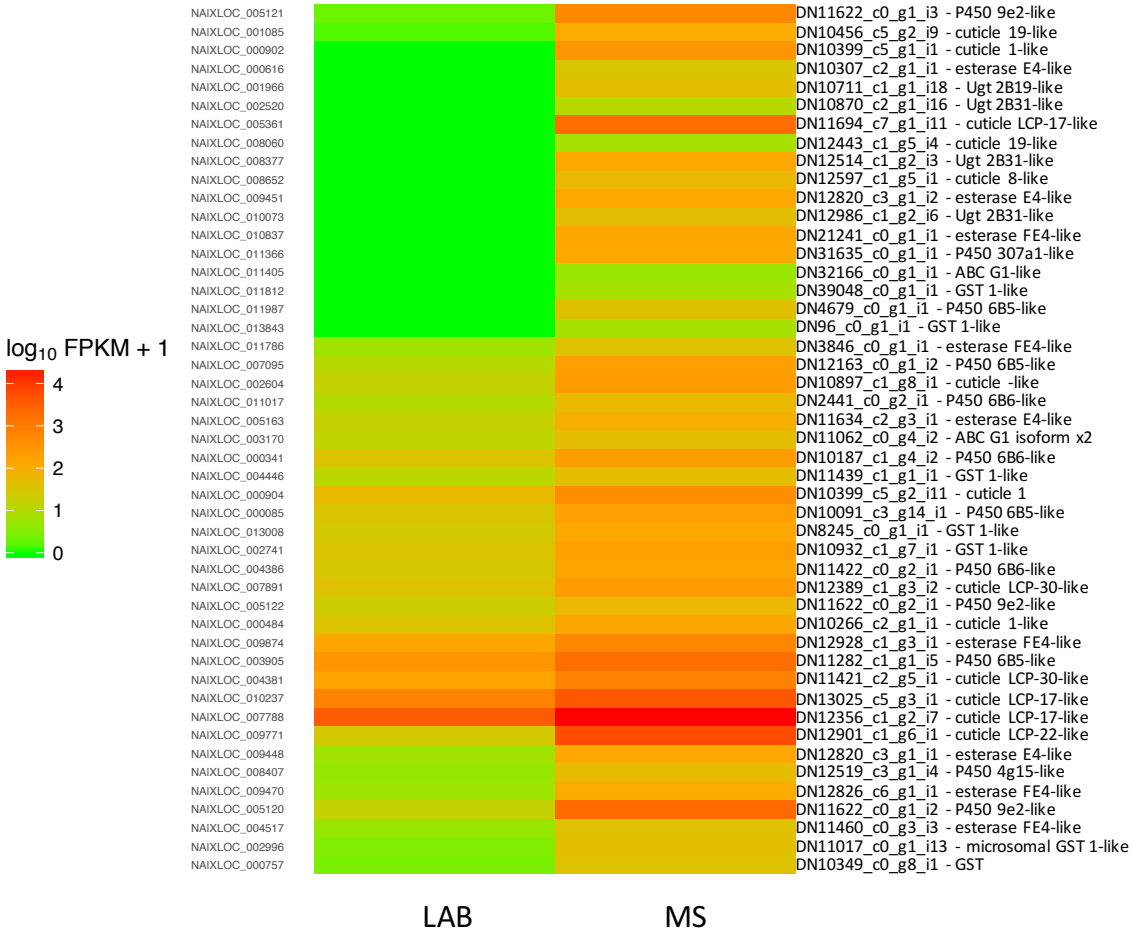



Figure 13 - Comparative distribution of FPKM expression values (fold change ≥ 2 and P < 0.05) of transcripts associated with detoxification metabolism and signaling pathway in the thorax + abdomen between LAB and MS populations of *C. includens*. Scale of colors ranging from the lowest (green) to the highest (red) FPKM values.

A greater number of detoxification *Cyp* genes encoding enzymes involved in phase I detoxification were markedly active in the head and thorax + abdomen of MS population. A CYP9 family transcript (DN11622_c0_g1_i2) was highly expressed (fold change > 7, q value < 0.05) in both body parts analyzed, head and thorax + abdomen. Indeed, another transcript of

CYP9 was greatly over-represented in thorax + abdomen (DN11622_c0_g1_i3) and not found in the head. Analyzing the OFR of these two transcripts, it was concluded that they have slight differences in the nucleotide sequence (36 synonymous substitutions), but the amino acid sequence is not altered. These results suggest that the low susceptibility of MS population to Lambda-cyhalothrin pyrethroid could be by the Cyp9e2-like gene expressed at different isoforms in the head and thorax + abdomen.

Overexpression of gene-encoding enzymes of *Cyp* families 4, 6, 9, 304, and 307 have already been reported to implicate insecticide resistance (Li et al., 2007). Furthermore, in regard to its expression level, the P450 enzymes can be upregulated in the lack of insecticide stress or in response to it (Nascimento et al., 2015; Mishra et al., 2015). The catalytic versatility of P450 enzymes to oxidize endogenous and exogenous compounds, plus the wide genetic diversity associated with a broad substrate of specificity, explain how these enzymes can be the metabolic system in arthropods that conditions resistance for all classes of insecticides (Feyereisen, 2005). As reported in brown planthopper, the insect evolution conditioned a duplicated *Cyp* gene in resistant strains with and without the gain of function mutations in substrate recognition sites (Zimmer et al., 2018). Thus, it suggests that the conditions wherein the insect evolution happens, and the versatility of genes to evolve, are the keys to insecticide resistance.

The microsomal glutathione S-transferase 1-like transcript (DN11017_c0_g1_i13) was found differentially expressed in the head and thorax + abdomen. This suggests that *C. includens* may have developed an extra body part to express this gene and not only in the gut. Regarding the results of Blastx of conserved domains, microsomal GST has a membrane-associated protein in glutathione metabolism (MAPEG), instead of specific domains of GST such as the N-terminal, C-terminal, and GST-DHAR1 (Tables S10 and S11). The GO term GO:0016740 is described as having catalysis for the transfer of a group, *e.g.* a methyl group, glycosyl group, acyl group, phosphorus-containing or other groups, from one compound to another.

GST enzymes involved in the phase II metabolism are known to mediate resistance to organophosphate, organochlorines, and pyrethroids conjugating reduced glutathione to the electrophilic centers of exogenous and endogenous compounds (LI et al., 2007). In the thorax + abdomen four transcripts of GSTs were found over-represented and two were expressed only in the MS population. Since it is known that genes having GST domains do not necessarily have GST activity (Snyder; Maddison, 1997), the GO terms and conserved

domains analyzed indicate that these enzymes are involved in detoxification via catalysis. Such catalysis occurs, apparently, from the conjugation of glutathione with a wide range of endogenous and xenobiotic alkylating agents, including drugs and environmental toxins. Indeed, GSTs are also involved in transport of endogenous lipophilic compounds, xenobiotic binding, and sequestration as possible resistance to organophosphate (Huang et al., 1998), organochlorines (Ranson et al., 2001), and pyrethroids (Vontas et al., 2001). Thus, *C. includens* might have developed a mechanism for GST enzymes to conjugate and sequester xenobiotic compounds such as insecticides. As reported, the overrepresentation of GST metabolic enzymes in insects can be regulated via gene amplification or overexpression (Li et al., 2007).

Some esterase enzymes were found being expressed in both transcriptome body parts of *C. includens*. Two esterase E4 and one FE4 were overexpressed in thorax + abdomen and found only in the MS population in the head. Two other E4 transcripts were found only in the MS population of *C. includens* (Figures 10 and 11; Tables S10 and S11). These metabolic enzymes are capable of hydrolyzing compounds that contain ester bonds via addition of water in the reaction, forming an alcohol and an acid metabolite. It is noteworthy that some insecticides, such as pyrethroids, carbamates, and organophosphates, have ester bonds and consequently are detoxified through hydrolysis of esterase enzymes (Montella et al., 2012). Overexpression via gene amplification of E4 or its paralog FE4 is responsible for enhancement of degradation and for sequestering organophosphates, carbamates, and pyrethroids in *M. persicae* (Field and Devonshire, 1998).

Recently discovered as multifunctional detoxification enzymes (Li et al., 2017; Pan et al., 2018), uridine diphosphate-glucuronosyltransferases (UGTs) in this experiment was found to be differentially expressed only in the MS population (Figures 12 and 13; Table S10). This class of enzymes was reported by Li and Pan to commonly metabolize insecticides, in *P. xylostella* and *Aphis gossypii.* Besides the metabolic detoxification process found in *C. includens* during our research, the transcriptome approach showed many larval cuticle transcripts to be over-represented or found only in the MS population. Regarding the most common mode of contamination from pyrethroids, which was mainly by contact of insect bodies with spray, the larval cuticle transcript genes seem essential for protection against insecticide contamination. Gene ontology and conserved domains analyses confirm that these transcripts are involved in the structural constituent of cuticle contributing to structural

integrity (Table S11). The reduced penetration of insecticides through the cuticle has already been reported for other insect species, such as for *H. armigera* (Gunning et al., 1991).

All detoxification enzymes found in our study are related as important mechanisms for resistance to synthetic and natural xenobiotic compounds, such as insecticides and secondary plant metabolites (Li et al., 2007). Our findings about overexpression transcripts in the head that encodes detoxification enzymes suggest a metabolic process involving insecticides that is initiated in the mouthparts of *C. includens*, before the insecticide molecule reaches the midgut or target site. Salivary secretions in insects contain enzymes involved in digestion, detoxification, host defense responses, lubrication of mouthparts, and immunity (Ribeiro, 1995; Gullan and Cranston, 2014). Rivera-Vega et al. (2017) proposed that detoxification enzymes released in saliva have utility for detoxification of toxins on the leaf surface during insect chewing.

Transcripts and proteins of detoxification enzymes were identified in salivary glands, including: cytochrome oxidase, glutathione S-transferase, esterases, and serine proteases in aphids *Acyrthosiphon pisum* (Carolan et al., 2011), *Diuraphis noxia* (Nicholson et al., 2012), *Megoura viciae* (Vandermoten et al., 2014), and *Macrosiphum euphorbiae* (Chaudhary et al., 2015), and *Sitobion avenae* (Zhang et al., 2017); glutathione S-transferases, esterases, cytochrome P450, and serine proteases in *Lygus lineolaris* (Zhu et al., 2016); cytochrome P450 oxidases and GSTs in hoppers *Empoasca fabae* (Delay et al., 2012), *Nilaparvata lugens* (Ji et al., 2013), and *Nephotettix cincticeps* (Matsumoto et al., 2014). Transcripts and proteins potentially involved in detoxification of Lepidoptera species were also reported in *Bombyx mori*, *Helicoverpa armigera*, *Helicoverpa zea*, *Manduca sexta*, *Spodoptera exigua*, *Vanessa cardui*, and *Vanessa gonerilla* in a review of genomics of Lepidoptera saliva (Rivera-Vega et al., 2017). Thus, this mechanism developed in insects to detoxify xenobiotic compound in mouth parts is a remarkable manner to survive and evolve under adverse conditions.

Two G-protein coupled receptor (GPCR) transcripts was annotated as G-coupled receptor 64-like in the head with GO IDs of transmembrane signaling receptor activity, signal transduction, and membrane. These GPCR transcripts were upregulated in the head of MS population and was not found expressed in the LAB population (FPKM = 0). GPCR genes are involved in many physiological pathways (Caers et al., 2012) and recently they were reported regulating a signaling pathway of expression for P450 insecticide resistance genes (Li et al., 2014). Previous study in *Musca domestica* suggests that regulation of P450 resistance gene is

driven by the signaling transduction cascades controlled by GPCRs, protein kinase/phosphates, and proteases (Li et al., 2013). In other recent study in *Culex quinquefasciatus* the GPCR signaling pathway was most overexpressed in the brain of resistant strains and the P450 genes were highly expressed in the brain, midgut and malpighian tubules (LI and LIU, 2017). As we found a wide number of detoxification enzyme transcripts (P450, GST, and EST) upregulated in the *C. includens* MS population, we suggest that there is a signaling pathway regulated by the G-coupled receptor 64-like which is over-represented in the head of soybean looper.

The transcriptome approach of a *C. includens* population having low susceptibility to pyrethroid-exhibited, massive overexpression of insecticide detoxification enzymes, cuticle proteins, and signaling receptor. To support the production of all proteins involved in resistance mechanisms in this population we found an energetic metabolic process well developed in *C. includens*. Many transcripts annotated as trypsin, serine protease, lipase, and chymotrypsins were significantly upregulated thorax + abdomen (Table S12).

The energy used for detoxification process might be channeled and disturbed from the normal energy used for development and reproduction (Hou et al., 2014). But, the insects have found a way to mitigate fitness cost associated with insecticide resistance using the energy and digestive enzymes (Araújo et al., 2008; Philippou et al., 2010). Once the insecticide or other xenobiotic access the insect body and the site of action, via oral, tegument contact or via spiracle, several changes are expected. Besides that, xenobiotic compounds can regulate and activate detoxification enzymes, they also can perform some changes in order to control and trigger digestive enzymes in an undetermined manner (Nath, 2000). Thus, these findings suggest a metabolic energy process in *C. includens* regulated with digestive enzymes to degrade proteins in order to compensate the energy spent to overexpress detoxification enzymes (*Cyp*, *Gst*, *Est*, and *Ugt*), cuticle proteins and receptors (GPCR).

Our results implicate greatly for adoption of suitable strategies on IRM of *C. includens* in Brazilian crops, since it has not been done yet. Soybean looper increased its importance on soybean in Brazil mainly due to population survivals to insecticide applications and became the predominant pest in soybean over the past few years (Guedes et al., 2015). This management practice, accounted with others, has selected the most tolerant Lepidoptera species to insecticides, which implications on the fitness cost were probably absent. The fitness depends on survival and reproduction of species, and all of the life history

characteristics that in turn effect survival and reproduction, which determines the rate of resistance-allele frequency (Onstad, 2014).

Even more, the low genetic structure of *C. includens* in Brazil indicates the exchange of genetic information among populations of different regions (Palma et al., 2016). These characteristics accounted with a wide area of soybean from north to south Brazil and the high survivorship of *C. includens* to insecticides may influence for a rapid resistance process. Caprio and Tabashnik (1992) suggested that low level of polymorphism between populations under constant insecticide application may develop resistance under high level of gene flow, even when the resistant allele has a low initial frequency.

Thus, the management of *C. includens* on soybean should be held in strategies of IPM and IRM, specially in regards of the use of distinct mode of actions. In summary, pyramiding of *Bt* proteins in plants, formulated products with *Bt*, baculoviruses, and insecticides with low risk to resistance are the best options at the moment.

## Conclusions

In conclusion, our findings represent the first insights of a transcriptome approach of *C. includens* regarding its low susceptibility to pyrethroid insecticide in Brazil. We found several molecular mechanisms that are driven its low susceptibility. Primary, sodium channel nonsynonymous mutations can characterize the insensitivity of the target site for pyrethroids. Second, the overexpression of metabolic enzymes of *Cyp*, *Gst*, *Est*, and *Ugt* suggest a wide detoxification process in the mouth parts and in the gut of soybean looper. This high expression pattern of detoxification enzymes might be enhanced and supported via signaling GPCR genes and the intense production of digestive and metabolic energy enzymes. These complex genes expression and nonsynonymous mutations assessed with the high throughput RNA sequencing can effectively to signal for the risk of insecticide resistance and lead time to implement strategies of IRM.

## Acknowledgements

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**Supplemental information**

**Table S1.** Alignment summary of *C. includens* LAB and MS population reads.

**Table S2.** Transcripts significantly differentially expressed exclusive in the LAB population in the head, with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S3.** Transcripts significantly differentially expressed exclusive in the MS population in the head, with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S4.** Significantly downregulated shared transcripts in LAB and MS populations in the head with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S5.** Significantly upregulated shared transcripts in LAB and MS populations in the head with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S6.** Transcripts significantly differentially expressed exclusive in the LAB population in thorax + abdomen, with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S7.** Transcripts significantly differentially expressed exclusive in the MS population in thorax + abdomen, with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S8.** Significantly downregulated shared transcripts in LAB and MS populations in thorax + abdomen with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S9.** Significantly upregulated shared transcripts in LAB and MS populations in thorax + abdomen with corrected P value (Benjamini-Hochberg correction) < 0.05.

**Table S10.** Significantly upregulated detoxification enzymes and receptor transcripts in the head, with corrected P value (Benjamini-Hochberg correction) < 0.05 and fold changes ≥ 2.

**Table S11.** Significantly upregulated detoxification enzymes and cuticle transcripts in the thorax + abdomen, with corrected P value (Benjamini-Hochberg correction) < 0.05 and fold changes ≥ 2.

**Table S12.** Significantly upregulated digestive and metabolic energy enzymes in the thorax + abdomen, with corrected P value (Benjamini-Hochberg correction) <0.05 and fold changes ≥ 2.

**Figure S1.** Nucleotide alignment of sodium channel sequence of *Chrysodeixis includens* Lab and MS Strain and *Helicoverpa armigera*.

**Figure S2.** Protein alignment of sodium channel sequence of *Chrysodeixis includens* Lab and MS Strain and *Helicoverpa armigera*.

**Figure S3.** Score of Gene Ontology (GO) classifications of the reference transcriptome of *C. includens* head and thorax + abdomen, according to their involvement in biological process, molecular function, and cellular component.

**Figure S4.** Squared coefficient of Variation ($CV^2$) plot to assess cross-replicate variability between *C. includens* LAB and MS population. Head (A) and thorax + abdomen (B).

**Figure S5.** Distributions of FPKM scores across replicates of each LAB and MS population samples of *C. includens*. Head (A) and thorax + abdomen (B).

**Figure S6.** Dispersion of mean counts between LAB and MS populations. Head (A) and thorax + abdomen (B).

**Figure S7.** Score of Gene Ontology (GO) classifications of *C. includens* head unigenes that were significantly differential expressed, according to their involvement in molecular function.

**Figure S8.** Score of Gene Ontology (GO) classifications of *C. includens* head unigenes that were significantly differential expressed, according to their involvement in cellular component.

**Figure S9.** Score of Gene Ontology (GO) classifications of *C. includens* thorax + abdomen unigenes that were significantly differential expressed, according to their involvement in molecular function.

**Figure S10.** Score of Gene Ontology (GO) classifications of *C. includens* thorax + abdomen unigenes that were significantly differential expressed, according to their involvement in cellular component.

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# 4 DISCUSSÃO

*Chrysodeixis includens* aumentou sua importância no Brasil e atualmente é o principal lepidóptero praga em lavouras de soja convencionais. Entre os fatores que levaram ao sucesso de *C. includens* como praga sobre a soja destaca-se (I) a maior tolerância a inseticidas (GUEDES et al., 2015) devido à alta expressão de enzimas metabólicas no aparelho bucal e digestivo das lagartas, encontrado no segundo trabalho, e (II) sua maior ocorrência nos estádios reprodutivos da soja (ZULIN; ÁVILA; SCHLICK-SOUZA, 2018), isto é, quando o dossel da soja está fechado e as lagartas se posicionam nos terços inferiores do dossel, resultando em maior dificuldade de controle (GUEDES et al., 2015). Desse modo, quando há ocorrência de altos níveis de infestação (> 15 lagartas.m$^{-2}$) de *C. includens* em soja, são necessárias aplicações de inseticidas mais caros, doses mais elevadas e, às vezes, mais de uma pulverização, com o risco de controle insatisfatório desta praga.

Atualmente, o manejo de lagartas em soja é realizado com inseticidas químicos e soja geneticamente modificada, expressando a toxina Cry1Ac e de forma simulada, no primeiro capítulo desta tese, a maioria dos inseticidas químicos testados não apresentou eficiência satisfatória. Os resultados de controle realizados ao longo de três anos em campo, mostram que apenas dois inseticidas, o clorfenapir e o indoxacarbe, apresentam resultados confiáveis de controle. Salvo as características físico-químicas de cada inseticida, o indoxacarbe apresentou menor período residual, exigindo uma segunda aplicação dentro de um intervalo de 7 dias para aumentar a taxa de mortalidade de falsa medideira em soja.

Tanto clorfenapir quanto indoxacarbe são definidos como pró-inseticidas e são ativados por enzimas que participam do processo metabólico do citocromo P450, glutationa S-transferase, carboxilesterase. Em clorfenapir essas enzimas removem o grupo N-etoximetil da molécula e um composto tóxico é formado, o que desregula a fosforilação oxidativa na mitocôndria (HUNT; TREACY, 1998; FEYEREISEN, 2012). O inseticida indoxacarbe também é bioativado por enzimas e transformado em metabólitos ativos N-descarbometoxilados que bloqueiam fortemente o canal de sódio no estado inativado (WING et al., 1998).

Curiosamente, indoxacarbe e clorfenapir, que são pró-inseticidas bioativados por enzimas, são os inseticidas mais eficazes. Além disso, o tratamento composto com piretroide

e diamida (λ-cialotrina + clorantraniliprole) apresentou a menor eficácia, independentemente do uso de uma ou duas aplicações. Em consequência, os resultados conduziram a uma investigação nossa investigação adicional sobre os mecanismos moleculares de resistência envolvidos em *C. includens*. Ficou demonstrado que enzimas do processo metabólico foram altamente expressas em lagartas de 3˚ instar de uma população de *C. includens*, como mostrado no segundo capítulo desta tese e explicam a alta eficácia do indoxacarbe e do clorfenapir e a baixa eficácia do tratamento com piretroide.

Os resultados das análises comparativas dos transcriptomas entre uma população suscetível do laboratório e outra população do estado de Mato Grosso do Sul (MS) que apresentou uma alta taxa de resistência ao piretroide lambda-cialotrina, mostram vários mecanismos moleculares associados à perda de suscetibilidade da população do MS. As mutações não-sinônimas nos domínios II, III e IV do canal de sódio, que podem alterar a sensibilidade do sítio de ligação dos piretroides no canal de sódio; a alta expressão de enzimas metabólicas de P450s, GSTs, ESTs e UGTs; e alta expressão do gene de sinalização GPCR sugere que *C. includens* tem um processo metabólico intenso para a desintoxicação de piretroides, e muito provavelmente para inseticidas de outros grupos químicos. Assim, a complexidade da expressão gênica alterada e de mutações não-sinônimas pode ser efetivamente avaliada com a técnica de RNA-seq, a fim de implementar estratégias para o manejo integrado de pragas e manejo da resistência a inseticidas, tais como, o manejo por moderação e por ataque múltiplo.

Com esses resultados, o controle químico de *C. includens* deve ser reavaliado e ser parte de um programa de manejo integrado, com outras táticas, como controle biológico por vírus, fungos, plantas de soja Bt ou mesmo produtos formulados com toxinas de Bt. Os dois inseticidas (indoxacarbe e clorfenapir), com elevada eficiência de controle, apresentam o maior custo de aplicação, variando de 2 a 3 vezes mais que os demais inseticidas testados. De outro lado, o custo desses inseticidas é similar ao do uso das variedades de soja Bt, que têm alta toxicidade para *C. includens*. Ou seja, o controle satisfatório de *C. includens* em soja é relativamente elevado, seja com o uso de inseticidas químicos ou com plantas Bt, deixando o produtor sem muitas opções.

Por fim, cabe discutir a sustentabilidade dos atuais métodos de controle da principal lagarta da soja estão em risco devido escassez de alternativas eficazes disponíveis para seu controle e ao intenso uso sobre uma área de soja de mais de 55 milhões de hectares na região

sul da América do Sul. O risco do surgimento da resistência para esses dois inseticidas tem uma menor probabilidade de ocorrer dado seu menor uso pelo custo elevado, comparado a outros inseticidas, como os piretroides, carbamatos, IGRs e as diamidas. Por outro lado, os piretroides são bastante usados, principalmente para controlar percevejos e outras pragas em soja, durante os estágios reprodutivos. Estas aplicações certamente estão atingindo populações de *C. includens* que ocorrem com maior intensidade após floração da soja. Possivelmente, a intensidade de pulverizações de piretroides está selecionando *C. includens*, mesmo que essa não seja a praga alvo desse grupo de inseticidas.

Futuras pesquisas sobre *C. includens* que envolvem mecanismos moleculares de resistência a inseticidas devem considerar:

1. Investigar mecanismos de regulação de expressão gênica (mutações nos locos trans-regulatórios, nos elementos cis-regulatórios, entre outros) das enzimas do processo metabólico;

2. Investigar níveis de resistência, caracteres de herdabilidade e o comportamento de diferentes fases de *C. includens*, incluindo as fases de larvas e adultos;

3. Investigar as respostas moleculares de populações, com perda de suscetibilidade, expostas a diferentes culturas hospedeiras e inseticidas;

4. Elaborar marcadores moleculares para ensaios de genotipagem de SNPs de alto rendimento em populações do campo;

5. Investigar potenciais genes alvos em *C. includens* para novos inseticidas e com aplicação de RNAi;

6. Usar tecnologias de edição de genoma (CRISPR, ZFNs, TALENs ou outros) com possiblidade de reverter essas mutações não-sinônimas no canal de sódio.

# 5 CONCLUSÃO

Os experimentos de campo demonstraram que apenas alguns inseticidas controlam larvas pequenas e grandes de *C. includens*. Os inseticidas spinetoram, o espinosad, o clorfenapir e o indoxacarbe foram efetivos no controle de larvas pequenas, enquanto que clorfenapir e indoxacarbe foram efetivos para larvas grandes. Muitos inseticidas precisam de uma segunda aplicação para aumentar a eficácia e evitar injúria de desfolha em soja, como o spinetoram, indoxacarbe e flubendiamide + tiodicarbe. A exceção foi o clorfenapir, que mostrou eficiência satisfatória semelhante entre uma e duas aplicações. Além disso, o tratamento composto com o piretroide λ-cialotrina apresentou uma eficácia insatisfatória.

Os resultados da análise comparativa dos transcriptomas dos tecidos da cabeça e do tórax + abdômen revelaram a expressão de vários mecanismos moleculares responsáveis pela baixa suscetibilidade de *C. includens* aos inseticidas piretroides. Com base na comparação entre o transcriptoma de *C. includens* resistente versus suscetível, encontramos cinco mutações não-sinônimas na região codificadora do canal de sódio na população do MS, especificamente nos domínios II, III e IV. Também, a superexpressão de enzimas metabólicas incluindo P450s, GSTs, ESTs e UGTs sugere um intenso processo de desintoxicação em *C. includens*, que possui a expressão sinalizada pela proteína receptora GPCR. Algumas dessas enzimas tiveram alta expressão nos tecidos da cabeça da população resistente, indicando que o processo de desintoxicação começa no aparelho bucal e continua no aparelho digestivo. Para suprir a energia despendida na expressão gênica de enzimas detoxificantes, *C. includens* possui um alto metabolismo energético com alta expressão de tripsina, serina protease, lipase e quimotripsina.

Os resultados desses experimentos representam informações relevantes sobre a eficiência de inseticidas no controle de lagartas grandes e pequenas de *C. includens* e são os primeiros *insights* sobre os mecanismos moleculares de resistência a inseticidas piretroides em *C. includens*. Portanto, o manejo de *C. includens* na soja deve considerar a eficácia dos inseticidas, o modo de ação, o custo da aplicação de inseticidas e combinar as táticas em um manejo integrado de pragas e no manejo da resistência a inseticidas.

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