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AVALIAÇÃO DO POTENCIAL INSETICIDA DE LECTINAS DE SEMENTES DE *Moringa oleifera* CONTRA LARVAS DE *Aedes aegypti* RESISTENTES E SUSCEPTÍVEIS A ORGANOFOSFATO E ADULTOS DE*Sitophilus zeamais*

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RESUMO

Algumas espécies de insetos podem atuar como vetores de doenças ou pragas agrícolas. O mosquito *Aedes aegypti* é o vetor da dengue e o besouro *Sitophilus zeamais* (gorgulho do milho) ataca diversos tipos de grãos armazenados. O surgimento de populações de insetos resistentes em decorrência da utilização de inseticidas sintéticos tem estimulado a busca por inseticidas naturais, incluindo as lectinas (proteínas que reconhecem especificamente carboidratos). Sementes de *Moringa oleifera* contêm as lectinas inseticidas cMoL (do inglês *coagulant M. oleifera lectin*) e WSMoL (do inglês *water-soluble M. oleifera lectin*). Para o isolamento de WSMoL, o extratoem água destilada de sementes de *M. oleifera*(EA) foi obtido e tratado com sulfato de amônio (60% de saturação) obtendo-se uma fração rica em lectina (FL), a qual foi aplicada em coluna de quitina. Já para o isolamento de cMoL, o extrato em NaCl 0,15 M foi obtido pela homogeneização das sementes em NaCl 0,15 M tratado com sulfato de amônio (60% de saturação) e cromatografado em coluna de gel de guar. Em seguida, o efeito das lectinasna sobrevivência e nas atividades de enzimas detoxificantes e digestivas de larvas de *A. aegypti* no quarto estágio das linhagens Rockefeller (susceptível ao organofosfato temefós) e Rec-R (resistente ao organofosfato temefós)foi avaliado. Investigou-se também os efeitos de EA, FL e WSMoLna sobrevivência e na atividade da tripsina de *S. zeamais*. WSMoL (0,197 mg/ml) matou as larvas Rockefeller (51,6%±2,8), enquanto cMoL não interferiu na sobrevivência dessas larvas. WSMoL e cMoL não apresentaram atividade larvicida contra Rec-R. WSMoL estimulou as atividades de proteases, tripsina e α-amilase das larvas Rockefeller, enquanto cMoL inibiu essas enzimas. WSMoL não interferiu na atividade de tripsina de larvas Rec-R, mas inibiu as atividades de protease e α-amilase. Dentre as atividades de enzimas digestivas de Rec-R, cMoLapenas inibiu a atividade de tripsina. cMoL inibiu fortemente a atividade de superóxido dismutase das larvas Rockefeller e Rec-R e WSMoL inibiu a atividade de β-esterase de larvas Rockefeller. As lectinas afetaramlevemente as atividades de α-esterase de ambas as linhagens. EA (58 à 145mg/g) apresentou toxicidade aguda para *S. zeamais* (taxas de mortalidade variando de 21,7 a 50%), enquanto FL não matou os insetos. WSMoL causou apenas baixa mortalidade (12,0%±2,7) na concentração de 60 mg/g (mg de lectina por g de farinha de trigo). A ingestão de EA reduziu a taxa de consumo relativo, sendo observado efeito deterrente moderado a forte. FL e WSMoL reduziram a taxa de ganho relativo de biomassa e a eficiência na conversão do alimento ingerido, mas não exerceram ação deterrente. Apenas WSMoLaumentou a atividade de enzimas tripsina do intestino de *S. zeamais*. A presente tese apresenta ainda um capítulo contendo uma revisão bibliográfica que contempla características da biologia e ecologia de insetos praga de grãos armazenados, bem como informações sobre danos causados por eles na agricultura. As principais estratégias de controle e o uso de inseticidas naturais como alternativa de combate a estas pragas são também discutidos. Em conclusão: (1) WSMoL, embora seja capaz de matar larvas de *A. aegypti* susceptíveis a organofosfato (Rockefeller), não promoveu mortalidade de larvas resistentes (Rec-R); (2) os efeitos opostos de WSMoL na sobrevivência de larvas Rockefeller e Rec-R podem indicar que estas populações são fisiologicamente distintas em outros aspectos além da resistência a temefós; (3) os efeitos distintos de WSMoL e cMoL sobre as enzimas digestivas de larvas Rockefeller e Rec-R indicam a expressão de diferentes formas de enzimas entre essas linhagens; (4) o mecanismo de atividade larvicida de WSMoL para larvas Rockefeller pode envolver a estimulação de enzimas digestivas (proteases, tripsina e α-amilase) e inibição da atividade de β-esterase; (5) cMoL pode ser avaliada, no futuro, como um agente sinérgico para aumentar a susceptibilidade de larvas de *A. aegypti* com atividade aumentada de superóxido dismutase; (6) a toxicidade de EA para *S. zeamais* adultos pode resultar do seu efeito deterrente de alimentação; e (7) os danos na fisiologia nutricional

de *S. zeamais* causados por WSMoL provavelmente envolvem um desequilíbrio do processo de digestão devido ao aumento da atividade de enzimas tripsina.

Palavras-chave: mosquito da dengue; gorgulho do milho; atividade larvicida; atividade deterrente; lectina.

ABSTRACT

Some insect species can act as disease vectors or agricultural pests. The *Aedes aegypti* mosquito is vector of dengue fever and the beetle *Sitophilus zeamais* (maize weevil) attacks several types of stored grains. The emergence of resistant insect populations due to the use of synthetic insecticides has stimulated the search for natural insecticides, including the lectins (proteins that specifically recognize carbohydrates). *Moringa oleifera* seeds contain the insecticidal lectins cMoL (coagulant *M. oleifera*lectin) and WSMoL (water-soluble *M. oleifera* lectin). In this work, the aqueous extract from *Moringa oleifera* seeds (AE) was obtained and treated with ammonium sulfate (60% saturation) for obtaining a lectin-rich fraction (LF), which was loaded onto a chitin column for isolation of WSMoL. The saline extract was obtained by homogenization of the seeds in 0.15 M NaCl and cMoL was isolated from it by treatment with ammonium sulphate (60%) and chromatography on guar gel column. Next, the effect of the lectins on survival and activities of detoxificant and digestive enzymes of*A. aegypti* fourthstage larvae (L_4) from Rockefeller and Rec-R (resistant to the organophosphate temephos) strains were evaluated. Were also evaluatedthe effects of AE, LF andWSMoL on survival and trypsin activity of *S. zeamais*. WSMoL (0.197 mg/ml) killed the Rockefeller larvae (51.6%±2.8) while cMoL did not affect the survival rate of them. WSMoL and cMoL did not show larvicidal activity against Rec-R. WSMoL stimulated the activities of proteases, trypsin and α-amylase from Rockefeller larvae, while cMoL inhibited these enzymes. WSMoL did not interfere with the activity of trypsin from Rec-R larvae but inhibited the activities of protease and α-amylase. Among the activities digestive enzymes from Rec-R, cMoL only inhibited the trypsin activity. cMoL strongly inhibited superoxide dismutase from Rockefeller and Rec-R larvae and WSMoL inhibited the activity of β-esterase from Rockefeller. The lectins slightly affected the activity of α-esterase of both larval strains. AE (58 à 145mg/g) was acutely toxic to *S. zeamais* (mortality rates ranging from 21.7 to 50%) while LF did not kill the insects. WSMoL caused only a slight mortality (12.0 ± 2.7) at 60 mg/g (mg of lectin per g of wheat flour). The intake of AE reduced the relative consumption rate, being observed a moderate to strong deterrent effect. LF and WSMoL decreased the relative biomass gain rates and the efficiency in conversion of ingested food, but did not exerted deterrent action. Only WSMoL interfered in trypsin-like activity from *S. zeamais* gut. This thesis also presents a chapter containing a literature review that includes characteristics of biology and ecology of stored-grain pests, as well as informations on damages caused by them in agriculture. The main strategies for control and the use of natural insecticides as an alternative to combat these pests are also discussed. In conclusion, (1) WSMoL, although is able to kill organophosphate-susceptible (Rockefeller) *A. aegypti* larvae, did not promote mortality of resistant (Rec-R) larvae; (2) the opposite effects of WSMoL on the survival of Rockefeller and Rec-R larvae may indicate that these populations are physiologically distinct in other aspects beyond the resistance to temephos; (3) the different effects of WSMoL and cMoL on digestive enzymes from Rockefeller and Rec-R larvae indicate expression of different enzyme forms between these strains; (4) the mechanism of larvicidal activity of WSMoL on Rockefeller larvae may involve stimulation of larval digestive enzymes (protease, trypsin, and α-amylase) and inhibition of β-esterase activity; (5) cMoL can be evaluated in the future as a synergist for increasing the susceptibility of *A. aegypti* larvae with increased SOD activity; (6) the toxicity of AE to *S. zeamais* adults can result from its feeding-deterrent activity; and (7) the damage to nutritional physiology of *S. zeamais* by WSMoL probably involves an imbalance of digestion process due to the increase in activity of trypsin-like enzymes.

Key words: dengue mosquito; maize weevil; larvicidal activity; deterrent activity; lectin.

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WSMoL (0.5 to 60 mg per g of wheat flour). The relative biomass gain rate indicates the amount of biomass in mg gained every day per mg of initial body weight. The efficiency in conversion of ingested food (%) indicates the amount of ingested food incorporated by insects as biomass. The relative consumption rate indicates the amount of food consumed in mg per mg of insect body weight per day. Each bar corresponds to the mean ± SD of four replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

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1. INTRODUÇÃO

Algumas espécies de insetos causam uma série de problemas para o homem, principalmente nas áreas médica e agrícola, atuando como vetores de inúmeras doenças ou como pragas de plantações e produtos armazenados (GALLO *et al*., 2002; NEVES *et al*., 2005).Na área médica, destaca-se o *Aedes aegypti*, mosquito pertencente à família Culicidae que apresenta grande importância epidemiológica por ser o transmissor do vírus da dengue, doença considerada um dos principais problemas de saúde pública no mundo. O *A. aegypti* apresenta comportamento antropofílico e possui a capacidade de adaptar-se a diferentes condições ambientais, estando bastante disseminado em países tropicais e subtropicais (TAUIL, 2002; HIRAGI *et al*., 2009).Na área agrícola, uma das principais pragas é o *Sitophilus zeamais*, conhecido como gorgulho do milho, pequeno coleóptero da família Curculionidae, que atua como uma praga primária interna de grãos armazenados, atacando grãos ainda sadios e apresentando em seu interior o desenvolvimento de seus estágios larvais.É encontrado em regiões tropicais e subtropicais, atacando arroz, trigo, milho, cevada, sorgo e outros grãos ainda no campoou armazenados (LOECK *et al*., 2002; LORINI *et al*., 2008; FAZOLIN *et al*., 2010).

O controle do*A. aegypti* e *S. zeamais*tem sido realizado pelo uso de inseticidas sintéticos, os quais, apesar de apresentarem resultados significativos, quando utilizados de forma não-planejada e intermitente, são tóxicos para organismos não-alvos e para o homem, bem como, favorecem o surgimento de populações resistentes (BRAGA & VALLE, 2007; ARAÚJO *et al*., 2011; WANG *et al*., 2012). Dessa forma, há um grande interesse de pesquisadores por métodos alternativos, com destaque para o controle biológico e para o controle químico utilizando agentes inseticidas de origem natural. As plantas sintetizam

diversos tipos de compostos que possuem potencial entomotóxico reconhecido (CLOYD, 2004; CORREIA& SALGADO, 2011).

As lectinas são proteínas de origem não-imunológica que se ligam reversivelmente a carboidratos, sendo encontradas em diversos organismos e isoladas principalmente de vegetais (MORIYAMA *et al*., 2003). As lectinassão capazes de promover aglutinação celular através de ligação com glicoconjugados de membrana e diferem quanto aestrutura, especificidade ao carboidrato ligante e atividades biológicas (PEUMANS *et al*., 1995). Estudos têm descrito os efeitos inseticidas das proteínas, e apontado possíveis mecanismos de ação. A toxicidade das lectinas para diversas ordens de insetos vem sendo relacionada com a ligação a glicoconjugados expostos nas células do epitélio intestinal; ligação à quitina presente na matriz peritrófica; interação com enzimas glicosiladas no trato digestivo, alterando a atividade catalítica e resistência à proteólise pelas enzimas digestivas (PEUMANS & VAN DAMME, 1995; SÁ *et al*., 2009; MICHIELS *et al*., 2010; NAPOLEÃO *et al*., 2012).

A *Moringa oleifera* é uma espécie nativa da África e Ásia, pertencente à família Moringaceae, sendo conhecida popularmente como lírio branco, quiabo-de-quina ou simplesmente moringa. Distribui-se em regiões tropicais e semi-áridas, tolerando condições climáticas inóspitas (ANWAR *et al*., 2007; SOUZA & LORENZI, 2008; MARACAJÁ *et al*., 2010). Seus tecidos apresentam múltiplas propriedades, tais como atividades antitumoral, antiinflamatória, antimicrobiana, antidiabética, antioxidante, diurética, anti-hipertensiva (ANWAR *et al*., 2007; GUPTA *et al*., 2005; COELHO *et al*., 2009). A partir de suas sementes, foram isoladas as lectinas cMoL (do inglês, coagulant *M. oleifera* lectin) e WSMoL (do inglês, water-soluble *M. oleifera* lectin) (COELHO *et al*., 2009; SANTOS *et al*., 2009). Ambas apresentaram ação inseticida: cMoL (1% p/p) promoveu mortalidade de pupas de *Anagasta kuehniella*e aumentou o tempo total de desenvolvimento (OLIVEIRA *et al*., 2011), enquanto WSMoL foi larvicida (LC_{50} = 0.197mg/mL) e ovicida (EC_{50} = 0.1 mg/mL)

contrapopulação de *A. aegypti*da linhagem Rockefeller(COELHO *et al*., 2009; SANTOS *et al.*, 2012).

O presente trabalho investigou: 1) o efeito de cMoLna sobrevivência de larvas de *A. aegypti*susceptíveis(linhagem Rockefeller) e resistentes (linhagem Rec-R) a organofosfato (temefós), bem como, o potencial inseticida de WSMoL contra as larvas Rec-R; 2) os efeitos de cMoL e WSMoL na atividade de enzimas digetivas (protease, tripsina e α-amilase) e detoxificadoras (superóxido dismutase, glutationa-S-transferases e esterases) de larvas de *A. aegypti* de ambas as linhagens; e 3) o potencial inseticida de WSMoLcontra*S. zeamais*, através de ensaio de toxicidade por ingestão. Foi também realizada uma revisão bibliográficasobrea biologia, ecologia e as formas de controle de besouros que atacam grãos armazenados, sendo as informações coletadas reunidas em um capítulo de livro.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. O mosquito *Aedes aegypti*

O *Aedes aegypti* (Linnaeus, 1762) pertence à ordem Diptera e à família Culicidae, sendo uma espécie originária da África. Distribui-se amplamente nas regiões tropicais e subtropicais do planeta, entre os paralelos 45° de latitude norte e 35° de latitude sul, sendo encontrado principalmente em regiões de baixa altitude. Apresenta alto poder de adaptação àscondições adversas encontradas em ambientes urbanos e poluídos (PONTES &RUFFINO-NETTO, 1994; BESERRA *et al*., 2009). Os adultos são considerados mosquitos de comportamento antropofílico e doméstico e apresentam hábito diurno, sendomais ativos principalmente no início da manhã e final da tarde. As fêmeas são hematófagas e põem seus ovos preferencialmente em águas limpas, incluindo recipientes artificiais encontrados no interior ou nas proximidades de habitações (ex. pneus, vasos, baldes, tanques e outros). Os ovos geralmente apresentam um alto grau de resistência à desidratação (HIRAGI *et al*., 2009; JANSEN & BEEBE, 2010).

A dinâmica vetorial do *A. aegypti* é afetada por alterações climáticas. O aumento e posterior redução na pluviosidade, por exemplo, pode ocasionar a formação de novos criadouros, contribuindo assim, para a elevação de sua distribuição geográfica e abundância sazonal. A elevação da temperatura, por sua vez, exerce influência direta sobre o *A. aegypti*, pois tende a aumentar a sua taxa metabólica e de crescimento, e consequentemente, sua capacidade de disseminação (KOVATS *et al*., 2001; ABRANTES & SILVEIRA, 2009).

O ciclo biológico do *A. aegypti*(Figura 1) compreende as fases de ovo, larva (quatro instares: L1, L2, L3 e L4), pupa e adulto, esta última correspondendo ao mosquito. Sua

duração média é de aproximadamente 10 dias entre a oviposição e início da fase adulta (CLEMONS *et al*., 2010). Os ovos possuem formato elíptico, alongado e fusiforme e apresentam-se inicialmente brancos, tornando-se negros e brilhantes após um tempo (CONSOLI & OLIVEIRA, 1994). Já as larvas são alongadas, vermiformes e esbranquiçadas. São exclusivamente aquáticas e alimentam-se de partículas orgânicas presentes na água (LOZOVEI, 2001). O primeiro ínstar (L1) surge após eclosão do ovo, os ínstares L2 e L3 são os intermediários e caracterizam-se por constante alimentação e,ao final do quarto e último ínstar (L4),a larva cessa sua alimentação preparando-se para a transformação em pupa (CONSOLI & OLIVEIRA, 1994; FORATTINI, 2002).A fase de puparepresenta a transição entre o ambiente aquático e o terrestre, e geralmente, por possuir um metabolismo lento, mantêm-se parada na superfície da água até transformar-se em adulto (FORATTINI, 2002). O adulto apresenta coloração escura e o tórax revestido por escamas escuras e branco-prateadas; o abdomem escurecido apresenta manchas anelares branco-prateadas e as pernas traseiras possuem faixas brancas semelhantes a listras (CLEMONS *et al*., 2010).

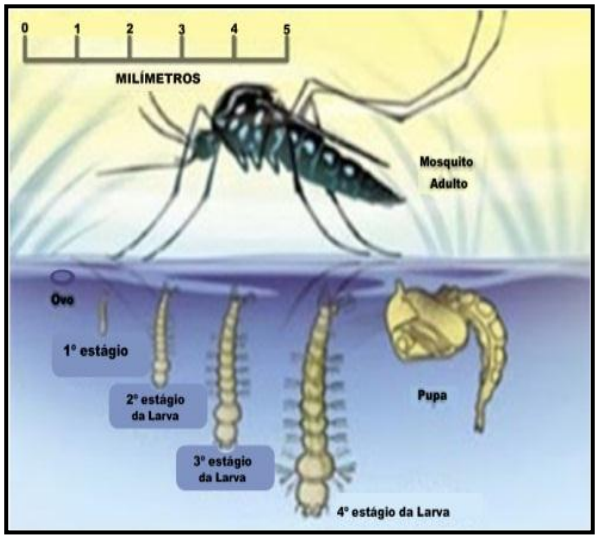

Figura 1. Ciclo biológico do*Aedes aegypti*
(Fonte:http://www.laboratorioriolab.com.br/?pg=controle)

2.1.1. Dengue

A dengue é uma arbovirose transmitida pelo*A. aegypti* que temcomo agente etiológico o vírus DEN. Este vírus pertencente ao gênero *Flavivirus*da família dos Flaviviridae, que apresenta cinco sorotipos: DEN 1, 2, 3, 4 e 5. A doença é considerada um sério problema de saúde pública, sendo disseminada em áreas tropicais e subtropicais e ocorre em mais de 100 países das Américas, África, Ásia, Ilhas do Pacífico e leste do Mediterrâneo (OMS, 2009; TIMERMAN *etal*., 2009; DIAS *et al*., 2010).

No Brasil, há registros de epidemias durante diferentes períodos. No ano de 2002, houve cerca de 700.000 casos notificados. (DIAS *et al*., 2010).Em anos mais recentes, houve aumento bastante expressivo, com destaque para 2010 e 2013, com 1.011.548 e 1.452.489 casos, respectivamente (BRASIL, 2014).

Os sintomas da dengue clássica são: febre, cefaléia, náuseas, vômitos e exantemas. Havendo evolução, a dengue clássica pode converter-se em dengue hemorrágica.O diagnóstico da dengue é realizado mediante manifestações clínicas e exames sanguíneos. Havendo a necessidade de confirmação faz-se o teste sorológico após 6 dias, através da detecção de anticorpos (SINGHI *et al*., 2007; DIAS *et al*., 2010).O tratamento na fase febril é sintomático, caracterizando-se pelo uso de medicação antitérmica e analgésica, bem como, hidratação oral e repouso. A cura surge, geralmente, após 8 dias de tratamento. No caso de dengue hemorrágica, recomenda-se internação com intensa hidratação via parenteral. (LUPI *et al*., 2007; SINGHI *et al*., 2007;OMS, 2009).

2.2. O besouro*Sitophilus zeamais*

O gorgulho do milho,*Sitophiluszeamais* (Coleoptera, Curculionidae) é um pequeno besouro, típico de áreas tropicais e subtropicais. Os adultos (Figura 2) são de coloração castanho-escuraeos élitros são densamente estriados com manchas avermelhadas e 3 mm de comprimento (GALLO *et al*., 2002; WAKEFIELD *et al*., 2005). Apresentam a cabeça projetada para frente na forma de rostro curvado, sendo mais curto e grosso no macho, enquanto na fêmea é longo e afilado (LORINI & SCHNEIDER, 1994; LOECK *et al*., 2002). Suas larvas são amarelo-claras e as pupas brancas (BOOTH *et al*., 1990). As fêmeas podem colocar em média 280 ovos e o ciclo de vida ocorre entre 4 e 5 semanas, em condições ótimas (28°C e 70% de umidade) (KEHINDE & ANGELA, 2004; MARSARO *et al*., 2005).

Figura 2. Morfologia externa de *Sitophilus zeamais*
(Fonte: http://bugguide.net/node/view/191577/bgpage)

O *S. zeamais* é uma praga primária, pois ataca os grãos inteiros e sadios a partir de perfurações feitas pelas fêmeas para depositar os ovos, com as larvas provenientes alimentando-se do tecido de reserva do grão. As fêmeas perfuram individualmente o grão liberando os ovos dentro de pequenos orifícios, e em seguida, as glândulas associadas ao aparelho ovipositor secretam uma substância gelatinosa que fecha a abertura. Após a eclosão no endosperma, as larvas alimentam-se e passam por quatro instares larvais e, posteriormente, transformam-se em pupa, até a emergência do adulto (COTTON & WILBUR, 1974; EVANS, 1981; LOECK *et al*., 2002; FAZOLIN *et al*., 2010).

Espécies do gênero *Sitophilus* são capazes de infestar grãos de culturas importantes mundialmente como, por exemplo, arroz, trigo, milho, aveia, cevada, sorgo e alimentos processados como macarrão e biscoitos (GALLO *et al*., 2002; LOECK *et al*., 2002; LORINI *et al*., 2008; FAZOLIN *et al*., 2010).Além de grãos armazenados, esta praga também pode atacar os grãos ainda em campo. Neste último caso, tendo como hospedeiro o milho, os indivíduos podem viver até 140 dias, sendo o período de oviposição de 104 dias, período de incubação entre 3 e 6 dias, e 34 dias a duração da fase de ovo até a emergência do adulto (LORINI & SCHNEIDER, 1994).

As perdas ocasionadas por *S. zeamais* no armazenamento de grãos são muitas vezes mais graves do que as que ocorrem na cultura em campo, pois são definitivas e irrecuperáveis. Miranda *et al* (1995) afirmam que a infestação desta praga pode gerar perdas em até 30% de grãos armazenados em fazendas. Estes danos podem ser quantitativos e qualitativos(GALLO *et al*., 2002; LORINI, 2008). Demissie *et al.* (2008) ressaltam que larvas do gorgulho podem ocasionar completa perda dos grãos de milho.

Fontes *et al.* (2003) verificaram a alta capacidade desse inseto em danificar grãos de variedades de arroz sem casca (Caiapó sequeiro e Paranaíba sequeiro) e com casca (Diamante irrigado). Picanço *et al.* (2003) atribuíram uma perda de 29,62% na produtividade de 49 cultivares de milho safrinha ao ataque de *S. zeamais* aos grãos. Caneppele*et al.* (2003), ao infestarem 500 mg de milho híbrido (variedade OC-705) com a praga, constataram que o aumento da infestação propicia redução significativa nos fatores de qualidade, como por exemplo, baixo poder germinativo, aumento do teor de umidade, redução do valor nutritivo e desvalorização comercial. Antunes *et al.* (2011), avaliando características físico-químicas de grãos de milho atacados por *S. zeamais*, constataram reduções no peso de 2,2, 3,0 e 17% para 30, 60 e 120 dias de armazenamento, respectivamente. Observaram que após 120 dias, houve elevação na quantidade de grãos defeituosos (33,48%) e na produção de resíduos (13,49 g).

S. zeamais também tem sido descrito atacando culturas de videira, nas quais os adultos perfuram as bagas de uva, facilitando a proliferação de fungos (*Aspergillus carbonarius*e *Penicillium sp*.) e, desta forma, contribuindo para a podridão do fruto e depreciação da qualidade de seus produtos finais, como por exemplo, o vinho. De acordo com relatos de agricultores do Rio Grande do Sul, nas safras de 2001 e 2002 foram constatadas perfurações em 80% das bagas nos parrerais desta região (BOTTON, 2005; ROUSSEAU, 2005).

2.3. Uso de produtos naturais no controle de *A. aegypti* e *S. zeamais*

2.3.1. Controle de *A. aegypti*

O controle do *A. aegypti* ainda é a estratégia prioritária no combate a dengue, visto que, há uma grande dificuldade em fabricar uma vacina que apresente respostas imunológicas eficazes para os quatro sorotipos do agente viral (SIMMONS *et al*., 2012).

O controle vetorial compreende ações preventivas simples, eficazes e integradas. Dentre elas, as medidas de controle mecânico e químico são as mais adotadas. As primeiras consistem na remoção e esvaziamento de recipientes, evitando o acúmulo de água, e consequentemente, a proliferação do vetor (SILVA*et al*., 2008, SVS/MS, 2009). Já o controle químico, implementado a partir da década de 80, caracteriza-se pelo uso predominante de inseticidas sintéticos das classes dos carbamatos, piretróides e organofosforados (BRAGA & VALLE, 2007). O temefós, pertencente a última classe citada, vem sendo amplamente estudado, visto que há registros de populações resistentes em várias localidades do Brasil, como em São Paulo (MACORIS *et al*., 2003), Rio de Janeiro (LIMA *et al*., 2003), Distrito Federal (CARVALHO *et al*., 2004). Tais compostos apresentam algumas desvantagens como

o alto custo, instabilidade química no meio (PALCHICK, 1996), e quando usados de forma contínua e não planejada, podem ocasionar contaminação ambiental, eliminação de organismos não-alvos e aumentar a pressão de seleção, favorecendo o surgimento de populações resistentes (POLANCZYK *et al*., 2003; LUNA *et al*., 2004, GUIRADO & BICUDO, 2009, PROFHIRO *et al*., 2011).

As plantas, ao longo da evolução, desenvolveram mecanismos contra a ação de insetos, sendo capazes de sintetizar, a partir de diferentes vias metabólicas, compostos de defesa como metabólitos secundários e proteínas que agem como toxinas inseticidas (TAGLIARI *et al*., 2004, SOARES & MACHADO, 2007, BARROS *et al*., 2010). Essas substâncias, de origem vegetal, vêm despertando interesse de vários pesquisadores na busca por estratégias alternativas para o controle químico do *A. aegypti*.

Há registros que avaliam os efeitos de extratos brutos, óleos essenciais, frações e metabólitos secundários isolados de vegetais contra *A. aegypti*. Garcez *et al*. (2012) mencionam 20 famílias (Annonaceae, Apiaceae, Asteraceae, Caesalpinoideae, Cupressaceae, Erythroxylaceae, Lauraceae, Meliaceae, Monimiaceae, Moraceae, Phrymaceae, Simaroubaceae, Sterculiaceae, Targionaceae, Taxodiaceae, Zingiberaceae, Rutaceae, Piperaceae, Fabaceae e Boraginaceae) como principais fontes de metabólitos larvicidas contra esse vetor. Dentre eles, destacam-se compostos pertencentes às classes: amidas, quinonas, terpenóides, fenólicos, rotenóides, flavonóides entre outros.

Saponinas isoladas de *Cordia piauhiensis* (Boraginaceae) foram agentes larvicidas contra *A. aegypti*, apresentando CL_{50} variando de 18,6 a 27,9 µg/mL (SANTIAGO *et al*., 2005). Freitas *et al* (2009) identificaram limonóides larvicidas e adulticidas a partir do fracionamento do extrato metanólico do caule de *Spathelia excelsa* (Rutaceae). O aerossol contendo óleo essencial de *Piper aduncum* (Piperaceae) promoveu uma mortalidade de 80% em adultos de *A. aegypti* (MISNI *et al*., 2011). Marimuthu *et al.* (2012) ao investigarem o

efeito larvicida contra esse inseto de extrato bruto de folhas de*Delonix elata* (Fabaceae) obtiveram CL_{50} de 163,69 ppm.

Outros estudos revelam que proteínas isoladas de plantas, como lectinas e inibidores de enzimas, também são capazes de interferir na sobrevivência e no desenvolvimento do *A. aegypti*.Gupta *et al.* (2011) demonstraram forte atividade larvicida de inibidor de α-amilase isolado de *Macrotyloma uniflorum* (Fabaceae) contra L1, L2, L3 e L4, bem como a redução na atividade da α-amilase em larvas tratadas e efeito deterrente na oviposição das fêmeas. Pontual *et al.* (2014) descreveram que inibidor de tripsina isolado das flores de *M. oleifera* causou a mortalidade de larvas de *A. aegypti* recém-eclodidas (CL_{50} = 0,3 mg/ml), atrasou o desenvolvimento das larvas que sobreviverame foi capaz de matar bactérias que compõem a microbiota do intestino das larvas.

2.3.2. Controle de *S. zeamais*

O controle de insetos que atacam grãos armazenados envolve uma série de estratégias integradas para assegurar a qualidade e segurança destes produtos agrícolas em longo prazo.Essas medidas incluem limpeza e secagem dos grãos, manutenção da aeração, regulação da temperatura e, sobretudo, aplicação de inseticidas sintéticos de contato na superfície dos silos de armazenamento ou inseticidas voláteis que atuamporfumigação(LAZZARI *et al*, 2006; MENEZES, 2005; PEREIRA *et al*, 2008). Os principais inseticidas utilizados são malathion, deltametrina e fosfina, sendo a fumigação o método mais usado (ESTRELA *et al*., 2006; VINHA *et al*., 2011).Dentre estes, a fosfina é uma das mais utilizadas e age afetando o sistema nervoso simpático, metabolismo energético e estado redox das células dos insetos(NATH *et al*., 2011). No entanto, tais compostos podem acarretar a morte de insetos não-alvos, bem como, exercer uma pressão seletiva que favorece

o aparecimento de populações resistentes (PEREZ-MENDOZA, 1999; COLLINS *et al*., 2001).

Métodos alternativos para controle da população de *S. zeamais* vêm sendo estudados, dentre eles, a utilização de produtos naturais com atividade inseticida. O pó da folha de *Eucalyptus citriodora* (Myrtaceae) foi repelente (96%) e o pó da folha de *Chenopodium ambrosioides*(Amaranthaceae)causou 100% de mortalidade de adultos de *S. zeamais* (PROCÓPIO *et al.*, 2003).Já os extratos de sementes de *Aframomummelegueta* (Zingiberaceae) e rizoma de *Zingiber officinale* (Zingiberaceae),quando aplicados sobre sementes de milho, apresentaram repelência contra o inseto (UKEH *et al*., 2010). Em outro estudo, essas mesmas preparações, promoveram redução significativa no número de indivíduos presentes em espigas de milho armazenadas por 12 semanas após a colheita (UKEH *et al*., 2012).

Alguns estudos também evidenciam a toxicidade de espécies de plantas pertencentes à família das Piperaceae contra *S. zeamais*. Por exemplo, óleos essenciais de *Piper hispidinervum* e *Piper aduncum* promoveram mortalidade acima de 70% quando aplicados por fumigação e por contato (aplicação tópica) (ESTRELA *et al*., 2006). Coitinho *et al.* (2010), ao avaliarem a persistência da atividade inseticida de *Piper marginatum*contra *S. zeamais*, constataram mortalidade de 53,1% após 120 dias de armazenamento.

Espécies da família das Zingiberaceae também apresentaram toxicidade para *S. zeamais*. O óleo essencial extraído do rizoma de *Alpinia conchigera* foiaplicado sobre diferentes fases de desenvolvimento do inseto, obtendo-se maior susceptibilidade de indivíduos adultos, com CL_{50} de 85µl/l (SUTHISUT *et al*., 2011). Já o óleo essencial de *Tagete patula*apresentou atividade inseticida na concentração de 10 µl, obtendo-se 94% de mortalidade dos insetos (RESTELLO *et al*., 2009). O metabólito secundário ar-turmerone, isolado a partir do rizoma de *Curcuma longa*, foi inseticida contra *S. zeamais*. Após 6 dias de

contato, esse composto (1%/mm) provocou a morte de 100% dosindivíduos adultos, e apresentou, ainda, atividade repelente em diferentes concentrações (10, 20, 30, 40 e 50µl) durante 45 dias de exposição (TAVARES *et al*., 2013).

A lectina de folhas de *Myracrodruon urundeuva* (MuLL) exerceu efeitos deletérios *S. zeamais* sobre adultos de *S. zeamais*, interferindo negativamente no ganho de biomassa crescimento, absorção de nutrientes e na atividade de diferentes enzimas digestivas (tripsina, α-amilase e protease) (NAPOLEÃO *et al*., 2013).

2.4. Lectinas

2.4.1. Aspectos gerais

As lectinas são proteínas que reconhecem e interagem reversível e seletivamente com carboidratos, através de ligações de hidrogênio e forças de Van der Waals entre as faces hidrofóbicas do açúcar e as cadeias laterais dos aminoácidos(FRANCO-FRAGUAS *et al*., 2003; CORREIA *et al*., 2008).O termo “lectina” é derivado do latim e remete às palavras “escolher” e “selecionar” devido ao fato de que a interação lectina-carboidrato ser tão específica quanto a interação que ocorre entre antígeno-anticorpo ou substrato-enzima (MINKO, 2004; SHARON & LIS, 2004). São encontradas em plantas,bactérias, fungos e animais.

Estudos realizados nos últimos anos revelam que lectinas de diferentes fontes, apresentam sequências primárias e estruturas tridimensionaissimilares, mas podendo apresentar-se distintas.A função ligadora de carboidratos tem sido atribuídaà existência de uma região denominada “sítio de ligação a carboidrato” ou “domínio de reconhecimento de carboidratos (DRC)” (SHARON & LIS, 2004). Baseando-se na estrutura geral e nas

características do DRC, Peumans e Van Damme (1995), classificaram as lectinas em: merolectinas, que possuem apenas um DRC, o qual se liga a açúcares simples, e não apresentam atividade aglutinante e catalítica; hololectinas (maioria), que possuem dois DRCs com elevada homologia e são capazes de aglutinar células ou precipitar gliconjugados; e quimerolectinas, possuindo ao menos um DRC, e outro domínio com atividade biológica distinta, como por exemplo, ação enzimática.

As lectinas podem ser detectadas numa amostra através de um ensaio de hemaglutinação (Figura 3) e isoladas por técnicas gerais de purificação de proteínas, tais como cromatografias de afinidade, troca iônica, interação hidrofóbica, e gel filtração, entre outras (LAM & NG, 2011).

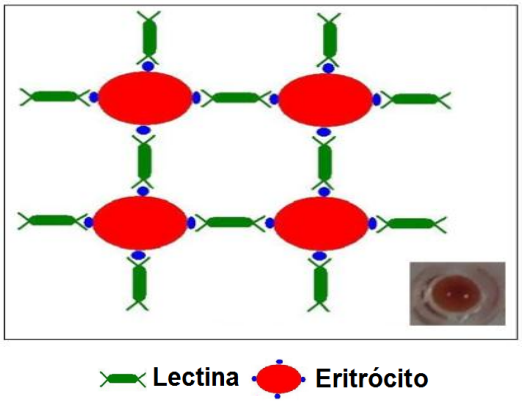

Figura 3. Ensaio de hemaglutinação
(Fonte: Paiva *et al*., 2011)

As lectinas são encontradas em diferentes partes das plantas, podendo ser isoladas de sementes (OLIVEIRA *et al*., 2011), folhas (NAPOLEÃO *et al*., 2011), cascas (SÁ *et al*., 2009) e raízes (SOUZA *et al*., 2011). Elas desempenham papel importante na defesa contra diferentes fitopatógenos como vírus, bactérias, fungos, nematóides e insetos pragas (RIPOLL *et al*., 2003; WONG *et al*., 2010). Diversas pesquisas vêm demonstrando o amplo potencial biotecnológico das lectinas vegetais, despertando cada vez mais o interesse por essas proteínas como ferramentas valiosas em diferentes áreas de estudo.Oliveira *et al.* (2008)

determinaram a atividade bactericida da lectina isolada de sementes de *Eugenia uniflora*contra *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus* sp., *Staphylococcus aureus*, *Corinebacterium bovis*, *Escherichia coli* e *Klebsiella* sp. As espécies de fungos *Fusarium oxysporum*, *Exserohilum turicicum* e *Colectrotrichum cassiicola* tiveram o crescimento *invitro*inibido quando tratados com diferentes concentrações (17,5 a 35 µg) da lectina isolada de rizoma de *Curcuma amarissima* (KHEEREE *et al*., 2010). Gomes *et al.* (2013) investigaram os efeitos antimicrobianos da lectina isolada de folhas de *Schinus terebinthifolius* e detectaram elevada ação basteriostática para *Salmonella enteritidis* (CMI: 0,45 µg/ mL) e bactericida para *Staphylococcus* aureus (CMB: 7,18 µg/mL). Além disto, a mesma lectina interferiu no crescimento (CMI: 6,5 mg/mL) e na sobrevivência (CMF: 26 ug/mL) de *Candida albicans*.

Swanson *et al.* (2010) e Fang *et al*. (2010) descreveram as atividades anti-viral (anti-HIV) e anti-tumoral de lectinas isoladas da banana (*Musa acuminata*)e do feijão (*Phaseolus vulgaris*). Assreuy *et al.* (2009) apresentaram importantes diferentes graus de efeitos vasodilatadores em ratos promovidos por lectinas isoladas de leguminosas do gênero *Canavalia*. Outro estudo, realizado por Nascimento *et al.*(2012), detectou relaxamento de anéis pré-contraídos da aorta de ratos, após interação com domínios da lectina de *Dioclea lasiocarpa*. Leite *et al.* (2012) isolaram das sementes de *Clitoria fairchildiana* uma lectina que apresentou efeito anti-inflamatório ao reduzir em 64% edema de pata induzido em camundongos. Esta mesma lectina mostrou-se antinociceptiva ao reduzir em 72% o número de contorções abdominais em camundongos.

As lectinas de origem vegetal são também investigadas para aplicação no diagnóstico de células neoplásicas e na cicatrização de ferimentos. Ensaios imunohistoquímicos realizados por Sobral *et al.* (2010) demonstraram que a marcação com lectinas de *Canavalia ensiformis*e*Ulex europaeus*apresentou correlação significativa com diferentes graus de

displasias. Emoutro estudo Neto *et al.* (2011) indicaram o potencial cicatrizanteda lectina isolada de sementes de *Bauhinia variegata*, a qual foi capaz de promover a remodelação do tecido conjuntivo quando aplicada topicamente em feridas, sugerindo-se, assim, uma ação estimulatória da lectina na divisão mitogênica e liberação de citocinas com consequente recrutamento de neutrófilos. As lectinas de origem vegetal também têm sido aplicadas em áreas como a terapia celular com células-tronco (IORDACHE *et al*., 2011) e imunologia (SOUSA *et al*., 2013), dentre outros.

Lectinas também são capazes de promover a estimulação mitogênica de células imunes (DONG *et al*., 2011), bem como, apresentar ação antiprotozoário (TEIXEIRA *et al*., 2006) e inseticida (PAIVA*et al*., 2011).

2.4.2. Atividade inseticida de lectinas

O potencial inseticida das lectinas vem sendo amplamente investigado contra espécies de diferentes ordens como Coleoptera, Diptera, Homoptera e Lepidoptera (LAM & NG, 2011). A atividade inseticida geralmente é avaliada através de bioensaios que incorporam a lectina em dietas artificiais oferecidas aos insetos, sendo possível investigar diferentes parâmetros, dentre eles, crescimento, desenvolvimento, fecundidade, inibição da alimentação, efeitos antimetabólicos emortalidade (VASCONCELOS & OLIVEIRA, 2004; LAWO & ROMEIS, 2008; COELHO *et al*., 2009).

Sá *et al.* (2009) e Napoleão *et al.* (2011) descrevaram a atividade larvicida contra L4 de lectinas isoladas de cerne, entrecasca e folha de *Myracrodruon urundeuva* (Anacardiaceae), com CL_{50} de 0,04, 0,125 e 0,202 mg/ml.

Tem sido demonstrado que as lectinas inseticidas geralmente são resistentes à digestão pelas proteases presentes no trato digestivo dos insetos, permanecendo ativas depois de

ingeridas (MACEDO *et al*., 2007; NAPOLEÃO *et al*., 2011). Estudos apontam possíveis alvos de ligação de lectinas, incluindo gliconjugados expostos nas células do epitélio intestinal, a quitina presente na matriz peritrófica, e enzimas glicosiladas presentes no lúmen intestinal. A ligação da lectina à matriz peritrófica pode resultar no rompimento da integridade e causar a descompartimentalização de enzimas digestivas (PEUMANS & VAN DAMME, 1995; FITCHES *et al*., 2001; SÁ *et al*., 2009; MICHIELS *etal*., 2010; PAIVA *et al.*, 2013).

Estudo do mecanismo de ação inseticida da lectina isolada de *Galanthus nivalis*(Amarilydaceae) sobre *Nilaparvata lugens* (Homoptera) demonstrou que a lectina não sofreu degradação proteolítica significativa quando ingerida pelos insetos e foi sugerido que ocorreu ligação específica desta proteína com glicoproteínas e/ou glicoconjugados presentes na superfície das células do epitélio intestinal (POWELL *et al*., 1998). Habibi *et al.* (2000), ao estudarem os efeitos da fitohemaglutinina (PHA) sobre o epitélio intestinal de *Lygushesperus* (Heteroptera), observaram através de imunofluorescência e microscopia eletrônica a ruptura e fechamento do lúmen deste órgão. Já a lectina de tubérculo de *Arum maculatum* (Araceae) ligou-se a glicoproteínas no intestino médio de *Lipaphis erysimi* (Homoptera) e *Aphiscraccivora* (Hemiptera) (MAJUMDER *et al*., 2005). Lectinaisolada de *Dioscorea batatas*(Discoreaceae) inibiu a emergência de adultos de *Helicoverpa armigera* (Lepidoptera) e técnica de imunomarcação revelou que ela ligou-se à membrana peritrófica destes insetos (OHIZUMI *et al*., 2009).

A modulação de atividade de enzimas digestivas por lectinas também vem sendo demonstrada. A alteração dessas atividades enzimáticas pode levar a um desequilíbrio metabólico e isto ocasionar a morte do inseto (MICHIELS *et al*., 2010). As lectinas de *G. nivalis* e a concanavalina A, lectina isolada de sementes de *Canavalia ensiformis* (Fabaceae),estimularam a atividade de aminopeptidase, tripsina e glucosidases no intestino de

larvas de *Lacanobia oleracea* (FITCHES & GATEHOUSE, 1998). A lectina isolada da folha de *Myracrodruon urundeuva* apresentou efeito inibitório sobre proteases e efeito estimulatório sobre α-amilase do intestino de larvas de *A. aegypti* (NAPOLEÃO *et al*., 2012). A atividade de α-amilase foi significativamente reduzida em larvas de 4° instar de *Spodoptera exigua* quando tratadascom a lectina de *G. nivalis*nas concentrações de 1% e 2%. Albuquerque *et al.* (2012), ao avaliarem os efeitos da lectina de rizoma de *Microgrammavacciniifolia*(Polypodiaceae) na sobrevivência de *Nasutitermes corniger*,determinaram CL_{50}(3 dias) de 0,130 e 0,085 mg/mL para operários e soldados, respectivamente. Essa lectina inibiu as atividades da tripsina e β-glicosidade do intestino de operários e estimulou as atividades deendoglucanase e fosfatase ácida. Com relação aos soldados, essa lectina inibiu endoglucanase e β-glicosidase e estimulou a atividade de fosfatases ácidas.

2.5. *Moringa oleifera*

M. oleifera (família das Moringaceae) é uma planta nativa da Índia, amplamente cultivada na Etiópia, Sudão, e também distribuída na África do Sul, Ásia Tropical, América Latina, Caribe, Flórida e nas Ilhas do Pacífico. É uma árvore perene (Figura 4), sobrevive por longos períodos em solos pobres e regiões pouco úmidas. Tem crescimento rápido e tem sido cultivada durante séculos devido ao seu uso na medicina tradicional e na indústria.

A  B 

Figura 4. *Moringa oleifera*: (A) árvore, (B) sementes
Fotos: (A) Maiara C. Moura. (B) Thiago H. Napoleão.

M. oleifera tem sido chamada de "árvore multi-uso", pois pode ser aplicada para diversos fins, como por exemplo: forragem animal (folhas e sementes), produção de biogás (folhas), agente de limpeza doméstica (folhas), fonte de corante azul (madeira), componente de fertilizantes (semente) e de adubo verde (folhas), complemento nutricional (suco das folhas), obtenção de goma (do tronco), fabricação de cordas (casca), fonte de taninos para curtimento (casca), aplicação no tratamento da água (sementes) e para fins ornamentais. Além disto, estudos em diferentes áreas da Biotecnologia, atribuem diversas propriedades biológicas às diferentes partes da *M. oleifera*, como atividades coagulante, inseticida, antitumoral, antiepilética, antiinflamatória, antimicrobiana, antidiabética, antioxidante, diurética e antihipertensiva (GUPTA *et al*., 2005; JED & FAHEY, 2005; ANWAR *et al*., 2007; COELHO *et al*., 2009).

2.5.1. Lectinas de *Moringa oleifera*

Três lectinas foram isoladas das sementes de *M. oleifera*:MoL (do inglês, *M. oleiferalectin*) (KATRE et al., 2008)cMoL (do inglês,*coagulant M. oleifera lectin*) e WSMoL (do inglês,*water-soluble M.oleifera lectin*). cMoL é extraída em solução salina (NaCl 0,15 M) e isolada através de cromatografia em coluna de gel de guar. Essa lectina apresenta atividade coagulante e é capaz de remover ácidos húmicos presentes na água (SANTOS *et al*., 2009; SANTOS *et al*., 2011). Oliveira *et al.* (2011) detectaram efeitos deletérios de cMoL sobre o desenvolvimento delarvas de *Anagastakuehniella*(Lepidoptera) e associaramtal fato à propriedade ligadora de quitina e à sua estabilidade frente à ação de enzimas. cMoL foi ainda capaz de matar as pupas dessa espécie. Na concentração de 1,5 mg/mL, ela foi ativa contra operários de cupins da espécie *N. corniger* (PAIVA *et al*., 2011).Luz *et al.* (2013)

descreveram que cMoL é uma proteína composto por 101 aminoácidos e com pI teórico 11,67, pertencente à classe de proteínas α/β quanto à sua estrutura terciária, sendo sua estrutura secundária composta por α-hélices (46%), folhas β (12%), voltas β (17%) e estruturas desordenadas (25%).

WSMoLé extraída em água destilada e foi isolada por cromatografia em coluna de quitina (COELHO*et al*., 2009). Essa lectina mostrou-se eficiente em coagular e matar bactérias presentes em corpos de água e não apresentou efeito mutagênico nem genotóxico nas concentrações de 0,0125 a 0,8 μg/μL,indicando que a utilização desta lectina, nesta faixa de concentração,para o tratamento de água para consumo humano é seguro (FERREIRA *et al*., 2011; ROLIM *et al*., 2011). Além disto, WSMoL apresentou atividade inseticida contra o quarto estágio larval (L4) de *A. aegypti* (CL_{50} = 0,197mg/mL), promovendo alterações morfológicas no trato digestivo (COELHO *et al*., 2009). WSMoL foi também capaz de matar ovos de *A. aegypti*, interferindo no desenvolvimento embrionário e na sobrevivência da larva ainda dentro do ovo, bem como apresentou efeito estimulante sobre a oviposição em condições de laboratório e de campo simulado (SANTOS *et al.*, 2012, 2014). Dessa forma, WSMoL é uma potencial candidata para uso no controle da população de *A. aegypti*em armadilhas de captura de ovos, uma vez que interfere na sobrevivência tanto dos ovos quando das larvas que vierem a eclodir.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar o potencial inseticida de lectinas de sementes de *Moringa oleifera* contra larvas de *Aedes aegypti* resistentes e susceptíveis a organofosfato e contra*Sitophilus zeamais* adultos

3.2. Objetivos específicos

- Obter extratos em água destilada e NaCl 0,15 M, e ainda, frações enriquecidas em lectinas a partir de sementes de *M. oleifera*.
- Isolar WSMoL e cMoL através de procedimentos previamentos estabelecidos.
- Avaliar a atividade larvicida de cMoL contra larvas (L_4) de *A. aegypti*de populações susceptível (Rockefeller) e resistente (Rec-R) a organofosfato (temefós).
- Avaliar a atividade larvicida de WSMoL contra larvas (L_4) de *A. aegypti* da população Rec-R.
- Determinar as atividades de enzimas digestivas (protease, tripsina e α-amilase) e detoxificantes (superóxido dismutase e α- e β-esterases) em extratos de larvas (L_4) de *A. aegypti* susceptíveis (Rockefeller) e resistentes (Rec-R) a organofosforados.
- Avaliar os efeitos de cMoL e WSMoL sobre as atividades das enzimas digestivas e detoxificantes.
- Reunir informações sobre a biologia, ecologia e estratégias de controle de insetos que atuam como pragas de grãos armazenados.

- Avaliar a toxicidade por ingestão de extrato aquoso, fração enriquecida em WSMoL e preparação de WSMoL isolada sobre*S. zeamais* adultos.
- Avaliar os efeitos de extrato aquoso, fração enriquecida em WSMoL e preparação de WSMoL isolada sobre parâmetros nutricionais de *S. zeamais* adultos.
- Determinar o efeito de extrato aquoso, fração enriquecida em WSMoL e preparação de WSMoL isolada sobre a atividade de tripsina do intestino de *S. zeamais* adultos.

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5. CAPÍTULO 1

Effect of *Moringa oleifera* lectins on survival and enzyme activities of *Aedes aegypti* larvae susceptible and resistant to organophosphate

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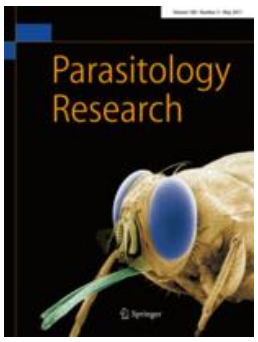

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Abstract The indiscriminate use of synthetic insecticides to control *Aedes aegypti* has led to emergence of resistant populations. *Moringa oleifera* seeds contain the lectins WSMoL and cMoL. WSMoL has larvicidal activity on fourth-stage of *A. aegypti* organophosphate-susceptible larvae (Rockefeller L_4). This study reports on the effects of cMoL on the survival of Rockefeller L_4 as well as of WSMoL and cMoL on L_4 from an organophosphate-resistant population (Rec-R). The effects of lectins on digestive (amylase, trypsin, and protease) and detoxifying (superoxide dismutase (SOD), α- and β-esterases) enzymes from larvae were also determined. cMoL (0.1–0.8 mg/ml) did not kill Rockefeller L_4 as well as WSMoL and cMoL (0.1–0.8 mg/ml) were not larvicidal for Rec-R L_4. WSMoL stimulated protease, trypsin-like, and α-amylase from Rockefeller L_4 while cMoL inhibited these enzymes. WSMoL had no effect on trypsin-like activity from Rec-R L_4 but inhibited protease and α-amylase. Among digestive enzymes of Rec-R L_4, cMoL inhibited only trypsin-like activity. cMoL inhibited SOD activities from Rockefeller and Rec-R L_4 in a higher level than WSMoL while β-esterase from Rockefeller L_4 was more inhibited by WSMoL. The lectins promoted low stimulation or inhibition of α-esterase activities from both populations. In conclusion, Rockefeller and Rec-R larvae were distinctly affected by *M. oleifera* lectins, and larvicidal mechanism of WSMoL on Rockefeller L_4 may involve deregulation of digestive enzymes. cMoL interfered mainly on SOD activity and thus it can be investigated as a synergistic agent for controlling populations whose resistance is linked to an increased detoxifying process mediated by this enzyme.

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Introduction

The reduction of *Aedes aegypti* populations is fundamental for dengue control (World Health Organization 2012). The continuous and indiscriminate use of synthetic insecticides has led to emergence of *A. aegypti* populations resistant to organochlorines (e.g., DDT), pyrethroids (e.g., permethrin and deltamethrin), and organophosphate (e.g., temephos) (Lima et al. 2003, 2011; Melo-Santos et al. 2010; Lumjuan et al. 2011; Polson et al. 2011; Somwang et al. 2011). The development of resistance towards temephos has been attributed to selection of individuals carrying genes that encode acetylcholinesterase forms insensitive to this pesticide as well as expressing forms of detoxifying enzymes (glutathione S-transferases, superoxide dismutase, and esterases) with increased activity (Lima et al. 2003; Braga and Valle 2007; Melo-Santos et al. 2010).

Natural insecticides have been investigated for larvicidal effect on *A. aegypti* resistant strains since they are usually

available at low cost and the larvae usually do not develop cross-resistance to them (Simas et al. 2007). Plant compounds with larvicidal activity have been investigated for inhibitory effect on acetylcholinesterase and detoxifying enzymes of insects aiming to identify agents that can be used as synergists for insecticides routinely used in control programs (Larson et al. 2010; Maheswaran and Ignacimuthu 2012).

Lectins, hemagglutinating proteins that recognize carbohydrates, have entomotoxic properties, and it has been suggested that the integrity and function of the peritrophic matrix can be altered by binding of lectin to chitin and *N*-acetylglucosamine residues (Albuquerque et al. 2012; Napoleão et al. 2012; Paiva et al. 2013). In the digestive tract of insects, lectins can also interact with glycan moiety of glycosylated enzymes as well as bind to sites other than the active site of both glycosylated and non-glycosylated enzymes (Paiva et al. 2013). In addition, lectins can cross the intestinal epithelial barrier and reach the hemolymph and other organs of the insect body (Fitches et al. 2001). Lectins from *Myracrodruon urundeuva* heartwood, bark, and leaf showed larvicidal activity on *A. aegypti* larvae (Sá et al. 2009; Napoleão et al. 2012).

Seeds of *Moringa oleifera* (Moringaceae) contain the water-soluble *M. oleifera* lectin (WSMoL) and the coagulant *M. oleifera* lectin (cMoL). WSMoL and cMoL have different structural characteristics such as monosaccharide specificity, molecular mass, and net charge (Santos et al. 2009; Rolim et al. 2011; Paiva et al. 2011; Luz et al. 2013). Coelho et al. (2009) reported that WSMoL showed larvicidal activity (LC_{50} of 0.197 mg/ml) against *A. aegypti* fourth-stage larvae (L_4) from an organophosphate-susceptible colony (Rockefeller strain). In addition, WSMoL showed ovicidal and oviposition-stimulant activities on *A. aegypti* eggs and females, respectively, from this same colony (Santos et al. 2012). cMoL showed insecticidal activity on *Anagasta kuehniella* when incorporated into artificial diet, resulting in reduction of weight gain, decrease in dietary utilization, and delay in development of larvae as well as reducing weight and survival of pupae (Oliveira et al. 2011).

This work evaluated WSMoL and cMoL effects on survival of L_4 from an organophosphate-resistant *A. aegypti* colony (Rec-R strain) as well as the effect of cMoL on Rockefeller L_4. The effects of WSMoL and cMoL on activities of digestive (protease, trypsin-like, and α-amylase) and detoxifying (superoxide dismutase as well as α- and β-esterases) enzymes from both Rockefeller and Rec-R L_4 are also described.

Materials and methods

Plant material

Seeds of *M. oleifera* (known as "moringa" in Portuguese, "árbol del ben" in Spanish, and horseradish tree or drumstick in English) were collected in Recife City, State of Pernambuco, northeastern Brazil. The authors possess the authorization for plant collection (number 38690–1) from the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of the Environment. The seeds were dried at 28 °C for 48 h, powdered using a blender, and stored at −20 °C. A voucher specimen is deposited under number 73,345 at the herbarium "Dárdano de Andrade Lima" (*Instituto Agronômico de Pernambuco*, Recife, Brazil).

A. aegypti larvae from Rockefeller and Rec-R strains

The colony of Rockefeller strain is maintained in the *Laboratório de Ecologia Química* from the *Departamento de Química Fundamental* of *Universidade Federal de Pernambuco* (Recife, Brazil). The organophosphate-resistant colony (Rec-R strain) was previously established under laboratory conditions (Melo-Santos et al. 2010) and is maintained in the *Departamento de Entomologia* from the *Centro de Pesquisas Aggeu Magalhães* of *Fundação Oswaldo Cruz* (Recife, Brazil). The larvae were hatched in distilled water at a temperature range of 25–27 °C and fed with cat food (Whiskas®). Early fourth-instar (L_4) larvae were used in bioassays.

Chemicals

α-Amylase from hog pancreas, azocasein, *N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA), bovine serum albumin, bovine trypsin, chitin powder from shrimp shells, 3,5-dinitrosalicylic acid (DNS), Fast Blue B, Folin–Ciocalteus's reagent, D(+)-glucose, glutaraldehyde, guar gum, α- and β-naphthyl acetate, potassium phosphate, sodium dodecyl sulphate (SDS), and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, acetone, ammonium sulphate, chloridric acid, monobasic and dibasic sodium phosphate, sodium carbonate, sodium chloride, and trichloroacetic acid were purchased from Vetec (Rio de Janeiro, Brazil). Ammonium persulfate, calcium chloride, copper sulphate, sodium acetate, soluble starch, *N*, *N*,*N*′,*N*′-tetramethylethylenediamine, and Triton X-100 were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

Isolation of WSMoL and cMoL

WSMoL was isolated according to the procedure described by Coelho et al. (2009). Powdered seeds (10 g) were homogenized with distilled water (100 ml) for 16 h at 4 °C using a magnetic stirrer (Fisatom, São Paulo, Brazil). Next, the homogenate was filtered through cotton gauze (Cremer, Blumenau, Brazil) and centrifuged at 3,000*g* for 15 min using a Sorvall® RC 6™ centrifuge (Thermo Scientific, MA, USA).

The supernatant (crude extract) was treated with 60 % saturation of ammonium sulphate (Green and Hughes 1955) for 4 h at 28 °C. The precipitated fraction was collected after centrifugation (3,000*g*, 15 min), dissolved in 0.15 M NaCl, and submitted to dialysis against 0.15 M NaCl (6 h at 4 °C) using a 3.5 kDa cut-off membrane (Sigma-Aldrich, USA). The dialyzed fraction (80 mg of proteins) was then loaded onto a chitin column (7.5×1.5 cm) previously equilibrated (flow rate of 20 ml/h) with 0.15 M NaCl. After extensive washing (until absorbance at 280 nm lower than 0.05), WSMoL was eluted with 1.0 M acetic acid and dialyzed against distilled water (6 h at 4 °C).

cMoL was isolated according to Santos et al. (2009). Ten grams of the seed powder were added to 100 ml of 0.15 M NaCl (100 ml) and the suspension was homogenized using a magnetic stirrer for 6 h at 28 °C. After filtration through cotton gauze and centrifugation (3,000*g* for 15 min at 4 °C), the crude extract was obtained. The proteins in this extract were precipitated using ammonium sulphate (60 % saturation) and the precipitate was collected and dialyzed against distilled water (4 h) and 0.15 M NaCl (4 h). The fraction was loaded (10 mg of protein) onto a guar gel column (7.5×1.5 cm), equilibrated (flow rate of 20 ml/h) with 0.15 M NaCl until that absorbance at 280 nm was lower than 0.05. cMoL was eluted with 1.0 M NaCl and dialyzed against 0.15 M NaCl (6 h at 4 °C).

Protein concentration

The protein concentration was estimated according to Lowry et al. (1951) using bovine serum albumin (31.25–500 μg/ml) as standard.

Hemagglutinating activity

The assay was conducted in microtiter plates (Kartell Spa, Italy) according to Paiva and Coelho (1992) using a suspension (2.5 % v/v) of rabbit erythrocytes treated with glutaraldehyde (Bing et al. 1967). Hemagglutinating activity was determined by mixing a twofold serial dilution of each sample (50 μl) with 0.15 M NaCl in microtiter plates. Next, the erythrocyte suspension (50 μl) was added to each well and the assay was incubated at 27 °C for 45 min. One hemagglutination unit was defined as the reciprocal value of the highest dilution of sample that promotes full agglutination of erythrocytes. The specific hemagglutinating activity was defined as the ratio between titer and protein concentration (unit per milligran).

Larvicidal assays

The larvicidal activity was evaluated according to the instructions of the World Health Organization (1981). Stock solutions of WSMoL or cMoL were used to provide a series of test solutions (0.1–0.8 mg/ml) obtained by dilution with distilled water. Each larvicidal assay had a final volume of 20 ml and contained 20–25 larvae in early L_4 stage. Distilled water or 0.15 M NaCl was used as negative controls. The mortality rate (in percent) was determined after 24 h of incubation at 28± 2 °C and 12–12 (light–dark) photoperiodism. Three independent experiments were run in triplicate.

A. aegypti L_4 extracts

Groups of 50 Rockefeller or Rec-R L_4 were collected and immobilized by cooling at 4 °C for 10 min. The gut of each larva was removed using an 8-mm-long, 0.3-mm needle (BD Ultra-Fine II from Becton, Dickinson and Company, NJ, USA) and immediately homogenized with 1 ml of acetate buffer (0.1 M sodium acetate at pH 5.5 containing 0.02 M $CaCl_2$ and 0.15 M NaCl) or Tris buffer (0.1 M Tris–HCl pH 8.0 containing 0.02 M $CaCl_2$ and 0.15 M NaCl) using a 2-ml tissue grinder. The homogenates were centrifuged at 9,000*g* at 4 °C for 15 min, and the supernatants (L_4 gut extracts) were collected (Napoleão et al. 2012). The extracts were evaluated for protein concentration as well as for activity of protease, trypsin-like, and α-amylase activities.

Extracts of whole larvae were prepared aiming to evaluate the activity of the detoxifying enzymes superoxide dismutase, α-esterase, and β-esterase. Groups of 50 Rockefeller L_4 or Rec-R L_4 were immobilized by placing them at 4 °C for 10 min. Next, they were homogenized in 2 ml tissue grinder with 1 ml of 0.05 M phosphate buffer pH 7.0 or 0.1 M phosphate potassium pH 7.2 containing 0.15 M NaCl. The whole body homogenates were centrifuged (9,000*g*, 4 °C, 15 min) and the supernatants (whole L_4 extracts) were evaluated for protein concentration and enzyme activities.

Protease activity

The protease activity was determined according to Azeez et al. (2007). A sample (70 μl; 150 μg of protein) of L_4 gut extracts in Tris buffer at pH 8.0 was mixed with 300 μl of 0.1 M sodium phosphate at pH 7.5 containing 50 μl of 0.6 % (w/v) azocasein. Next, 100 μl of 0.1 % (v/v) Triton X-100 were added and the mixture was incubated at 37 °C for 3 h. The reaction was stopped with 200 μl of 10 % (v/v) trichloroacetic acid and the assay was incubated at 4 °C for 30 min. The reaction mixture was then centrifuged (9,000 g for 10 min) and the absorbance (366 nm) of the supernatant was determined. One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 in absorbance.

Trypsin-like activity

The trypsin activity was determined by incubating (30 min, 37 °C) L_4 gut extract in Tris buffer (15 μl; 33 μg of protein)

with 8 mM BApNA (5 μl) in Tris–HCl 0.1 M pH 8.0 (160 μl). Trypsin activity was followed by measurement of absorbance at 405 nm (Kakade et al. 1969). One unit of trypsin activity was defined as the amount of enzyme that hydrolyzes 1 μmol of BApNA per minute. Control was performed by incubating (60 min, 37 °C) bovine trypsin (5 μg) with 8 mM BApNA (5 μl).

α-Amylase activity

The assay was carried out based on the method described by Bernfeld (1955). The L_4 gut extract in acetate buffer (100 μl; 212 μg of protein) was incubated at 50 °C for 10 min with 400 μl of a 1 % (w/v) soluble starch solution prepared in acetate buffer. The reaction was stopped by adding 500 μl of DNS. Next, the assays were heated at 100 °C in boiling water for 6 min, immediately cooled on ice for 15 min, and evaluated for absorbance at 540 nm. The amount of reducing sugars was determined using a standard curve of the reaction of different glucose concentrations with DNS (Y=1.2365X–0.06, where Y is the absorbance at 540 nm and X is the glucose concentration in milligrams per milliliter). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of glucose per minute. As positive control, the same procedure was carried out with 1.0 mg/ml α-amylase from hog pancreas. Reaction blanks were performed without starch.

Superoxide dismutase activity

The SOD activity was determined using SOD determination kit purchased from Sigma-Aldrich (USA). In the presence of superoxide anion, the water-soluble tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is reduced to a water-soluble formazan dye which shows a maximum absorbance at 440 nm. Since the amount of superoxide anion is linearly proportional to absorbance, the SOD activity can be quantified following the decrease in the color development at 440 nm (inhibition of WST-1 reduction).

The assay used the enzyme xanthine oxidase for generation of superoxide anion by oxidation of xanthine. The whole L_4 extract (20 μl; 48 μg of protein) in 0.05 M phosphate buffer pH 7.0 was added to a solution (20 μl) containing xanthine oxidase and its substrate, followed by addition of WST-1 solution (200 μl). The assay was incubated for 3 min at 28 °C, and, thus, the absorbance at 440 nm was determined. One unit of SOD activity was defined as the amount of enzyme required to inhibit the increase of absorbance at 440 nm in 50 %.

α- and β-esterase activities

For the determination of α- and β-esterase activities, whole L_4 extracts in 0.1 M phosphate potassium pH 7.2 were used. The extract (30 μl; 168 μg of protein) was mixed with 500 μl of a solution containing 0.3 mM α- or β-naphthyl acetate in 0.1 M phosphate potassium at pH 7.2 containing 1 % acetone. The reaction mixture was incubated for 20 min at 30 °C. Then, 0.1 ml of a mixture containing 0.3 % Fast Blue B and 3.3 % SDS was added. After centrifugation (3,000*g*, 28 °C), the supernatant absorbance at 590 nm was recorded. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of α- or β-naphthol per minute.

Effects of WSMoL and cMoL on the activity of digestive and detoxifying enzymes from Rockefeller and Rec-R L_4

The effect of lectins on the protease activity from larvae was evaluated by incubating (30 min at 37 °C) L_4 gut extracts in Tris buffer (150 μg of protein) with lectin (20–300 μg) before the determination of the protease activity as described above. The control assay was performed by submitting preparations containing only lectin (20–300 μg) to the same reaction steps. The activity of trypsin from L_4 gut extracts in Tris buffer (40 μg of protein) was determined after previous incubation (30 min, 37 °C) of them with WSMoL or cMoL (5–120 μg) in Tris buffer pH 8.0. Next, 8 mM BApNA (5 μl) were added and assay was incubated for 60 min at 37 °C.

The effect of lectins on the α-amylase activity was evaluated by incubating (30 min at 27 °C) L_4 gut extract (212 μg of protein) in acetate buffer with WSMoL or cMoL (15–200 μg) before determination of the α-amylase activity. The control assay was performed by submitting WSMoL (15–200 μg) in acetate buffer to the same reaction steps.

To determine the effect of lectins on the SOD activity, the whole L_4 extract (20 μl; 48 μg of protein) was incubated with WSMoL or cMoL (5–20 μg of protein) for 15 min at 28 °C. Next, the solution (20 μL) containing xanthine oxidase and its substrate was added to the mixture, followed by addition of WST-1 solution (20 μl). The assay was incubated for 3 min at 28 °C and then the absorbance at 440 nm was determined. The control assay was performed by incubating the lectin (5–20 μg) with xanthine oxidase solution in absence of whole L4 extract to assure that the lectins did not interfere in xanthine oxidase activity.

The effect of WSMoL and cMoL on α- and β-esterase activities was evaluated by incubating (30 min at 27 °C) whole L_4 extracts with WSMoL or cMoL (20–50 μg) before determination of the enzyme activities. The control assay was performed by submitting the lectin (15–200 μg) to the same reaction steps in absence of larval extract.

Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA) and data were expressed as a mean

of replicates±SD. Significant differences between treatment groups were analyzed by Student's *t* test (significance at $p<0.05$) using Origin 6.0 program. The lethal concentrations required to kill 16 % (LC_{16}), 50 % (LC_{50}), and 84 % (LC_{84}) of larvae in 24 h were calculated by probit analysis with a reliability interval of 95 % using the computer software StatPlus® 2006 (AnalystSoft, Canada).

Results

The larvicidal activity of WSMoL on Rockefeller L_4 was evaluated again in this work using the lectin at the LC_{50} (0.197 mg/ml) previously reported by Coelho et al. (2009) and similar mortality (51.6 %±2.8) was detected. Differently, cMoL did not cause mortality of Rockefeller L_4. Mortality of Rec-R was not promoted by both WSMoL and cMoL.

Protease activity was determined measuring the increase in absorbance at 366 nm resulting from the release of trichloroacetic acid-soluble peptides derived from hydrolysis of azocasein. Extract from gut of Rec-R L_4 showed protease activity (309.4 U/mg) higher than gut extract from Rockefeller L_4 (73.3 U/mg), indicating that organophosphate-resistant L_4 expressed a large amount of these enzymes and/or a set of proteases different from that of Rockefeller larvae. When WSMoL and cMoL were incubated with gut extracts from Rockefeller and Rec-R L_4 in absence of azocasein, no increase in absorbance at 366 nm was detected, revealing that the lectins were resistant to proteolysis by extracts.

WSMoL promoted distinct effect on protease activity from susceptible and resistant L_4, increasing activity from Rockefeller L_4 (Fig. 1a) but reducing activity from Rec-R larvae (Fig. 2a). cMoL promoted slight reduction (maximum of 30 %) of protease activity from Rockefeller L_4 (Fig. 1b), while it did not affect significantly the activity from Rec-R L_4 (Fig. 2b).

Trypsin-like activity from Rec-R L_4 (2.1 mU/mg), detected by the generation of ρ-nitroaniline resulting from the hydrolysis of BApNA, was also higher than that from Rockefeller L_4 (1.53 mU/mg). WSMoL promoted increase of trypsin-like activity from Rockefeller L_4 (Fig. 1c) but had no significant effect ($p>0.05$) on this enzyme activity from Rec-R larvae (Fig. 2c). cMoL strongly inhibited this enzyme activity from Rockefeller L_4 (Fig. 1d), and the activity from Rec-R L_4 was also reduced in the presence of this lectin (Fig. 2d).

Gut extracts from Rockefeller and Rec-R L_4 were able to hydrolyze starch, showing α-amylase activities of 1.3 and 1.56 U/mg, respectively. In presence of WSMoL, the amylase activity from organophosphate-susceptible larvae was stimulated (Fig. 1e) while that from Rec-R L_4 was inhibited (Fig. 2e). cMoL slightly inhibited (maximum of 34 %) amylase activity from Rockefeller L_4 (Fig. 1f) but had no effect on activity from Rec-R L_4 (Fig. 2f).

The SOD activity was significantly ($p<0.05$) higher in Rec-R (29.1 U/mg) than in Rockefeller larvae (21.7 U/mg). WSMoL interfered slightly in SOD activity from larvae of both populations (Fig. 3a, b), while cMoL showed a high inhibitory effect on this enzyme from Rockefeller L_4 (Fig. 3a) and neutralized the activity from Rec-R L_4 (Fig. 3b).

Rockefeller and Rec-R L_4 showed a higher level of α-esterase (21.6 and 36.0 U/mg, respectively) than β-esterase (7.02 and 12.0 U/mg, respectively). Also, Rec-R larvae showed esterase activities higher than Rockefeller L_4. WSMoL stimulated α-esterase activity from Rockefeller larvae (Fig. 3c) but did not promote significant changes in this activity from Rec-R larvae (Fig. 3d). cMoL did not affect the α-esterase activity from Rockefeller L_4 (Fig. 3c) but promoted increase of this activity from Rec-R L_4 (Fig. 3d). The β-esterase activity from Rockefeller larvae was inhibited by WSMoL, while cMoL did not promote significant alterations (Fig. 3e). WSMoL did not affect β-esterase activity from Rec-R L_4, and cMoL slightly reduced this activity (Fig. 3f).

Discussion

The indiscriminate use of the organophosphate temephos for controlling *A. aegypti* has induced the development of resistant populations. In this sense, it is increasing the search for larvicidal agents effective against both organophosphate-susceptible and resistant populations of *A. aegypti*.

The effects of *M. oleifera* lectins (WSMoL and cMoL) on organophosphate-resistant and susceptible larvae were evaluated aiming to provide a comparison between the effects of lectins on larvae from different populations. WSMoL was able to promote mortality of Rockefeller L_4 but not of Rec-R larvae. The distinct effects of this lectin on Rec-R and Rockefeller strains, which are already known by differing in regard to susceptibility to an organophosphate, indicate that they are also physiologically distinct in other aspects.

It has been reported that lectins may affect insect metabolism by inhibiting or stimulating the activity of digestive enzymes at insect midgut (Macedo et al. 2007; Napoleão et al. 2012). In order to investigate other possible alterations in larvae physiology beyond mortality, the effects of WSMoL and cMoL on protease, trypsin-like, and α-amylase activities from gut of Rockefeller and Rec-R L_4 were evaluated.

WSMoL and cMoL were resistant to proteolysis by extracts from larvae gut. An appropriate level of resistance against proteolysis in the insect gut is usually a prerequisite for lectins to exert their toxic effects (Macedo et al. 2007; Napoleão et al. 2012). WSMoL distinctly affected protease and α-amylase activities from Rockefeller and Rec-R L_4, corroborating with the assumption of different sets of enzymes being expressed by these strains.

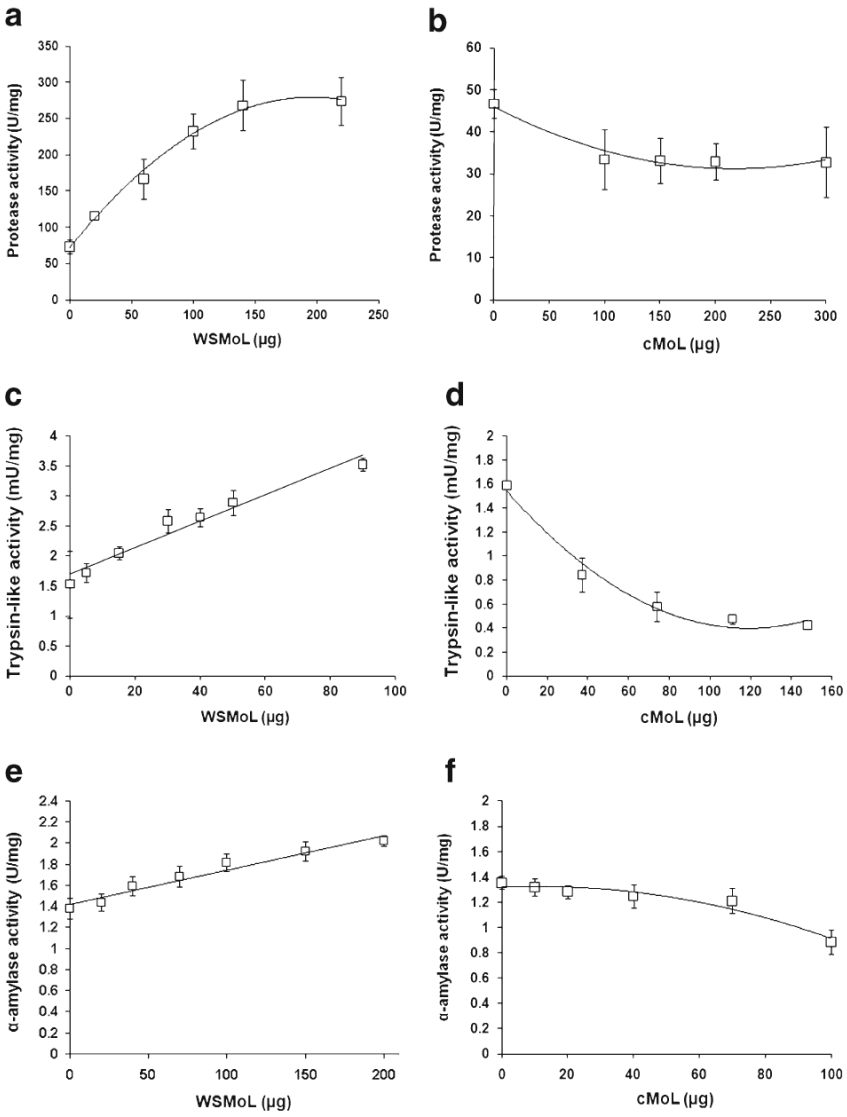

Fig. 1 Effect of WSMoL and cMoL on protease (**a**, **b**), trypsin-like (**c**, **d**), and α-amylase (**e**, **f**) activities from *A. aegypti* Rockefeller L_4

Macedo et al. (2007) reported that lectins can block enzyme activity by binding to protein or sugar moieties while stimulatory effect on enzyme activities may occur by increasing affinity of the enzyme to its substrate. The inhibition or stimulation of the activity of digestive enzymes in insects exposed to plant entomotoxic proteins may result in metabolic imbalance, impairment of growth, and induction of mortality (Macedo et al. 2007; Babu and Subrahmanyam 2010; Napoleão et al. 2012). Similar to WSMoL, the larvicidal lectin from *Myracrodruon urundeuva* leaf showed an in vitro stimulatory effect on α-amylase activity from *A. aegypti* L_4 (Napoleão et al. 2012).

The results raise the possibility that Rockefeller L_4 mortality may be linked to stimulation of digestive enzyme activities by WSMoL. Interestingly, WSMoL showed opposite effects (inhibition) on digestive enzymes from Rec-R larvae, which were not killed by this lectin. It is possible that WSMoL induces excessive proteolysis at the gut lumen of Rockefeller L_4, leading to degradation of important proteins and then resulting in the morphological damages at gut level reported by Coelho et al. (2009). cMoL showed a pronounced effect only on trypsin-like activity from Rockefeller larvae, but it seems that its inhibitory activity against this enzyme was not sufficient to impair larvae survival. The absence of very

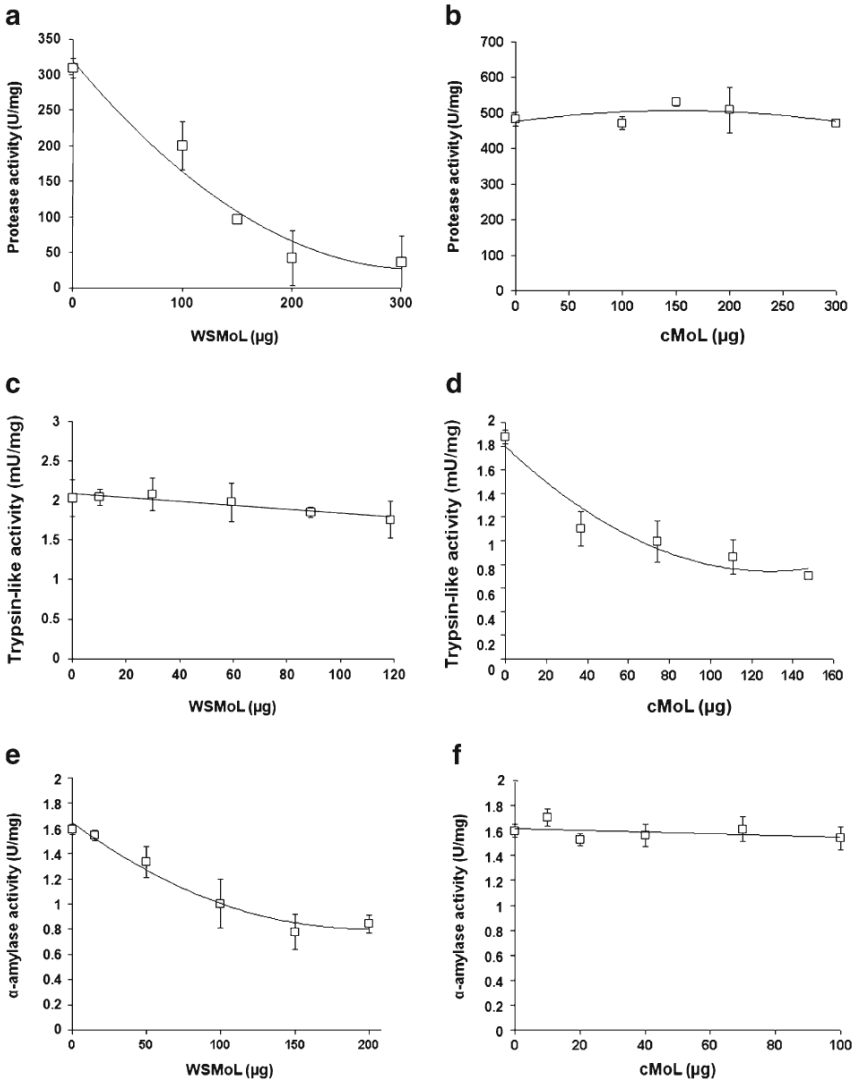

Fig. 2 Effect of WSMoL and cMoL on protease (**a**, **b**), trypsin-like (**c**, **d**), and α-amylase (**e**, **f**) activities from *A. aegypti* Rec-R L_4

significant changes promoted by cMoL on the other tested digestive enzyme activities from both populations is probably due to molecular characteristics of this lectin and may be a reason for its ineffectiveness on *A. aegypti* larvae mortality.

Detoxifying enzymes are involved in the development of insect resistance (Müller et al. 2007; Guirado and Bicudo 2009; Jagadeshwaran and Vijayan 2009; Lumjuan et al. 2011). The identification of compounds with an inhibitory effect on this group of enzymes may help to increase or restore the susceptibility of the insect to the insecticides used routinely, overpassing the metabolic resistance (Larson et al. 2010). The secondary metabolite α-mangostin from *Garcinia mangostana* decreased the LC_{50} of temephos on Rockefeller *A. aegypti* larvae because it was able to inhibit the activity of detoxifying esterases (Larson et al. 2010). In this sense, this work determined whether WSMoL and cMoL could interfere with the activity of detoxifying enzymes from Rockefeller and Rec-R L_4 and therefore if they would have potential as synergists for insecticides routinely used in control programs.

SOD activity of *A. aegypti* larvae has been detected in the anal gills of mosquito larvae, and this enzyme plays some important role in providing resistance against harmful oxygen derivatives (Nivsarkar et al. 1991). It has been showed that enzyme level increases with the maturation of the larvae from instar 1 to 4 and that elevated levels of SOD might increase pyrethroid resistance (Nivsarkar et al. 1991; Müller et al.

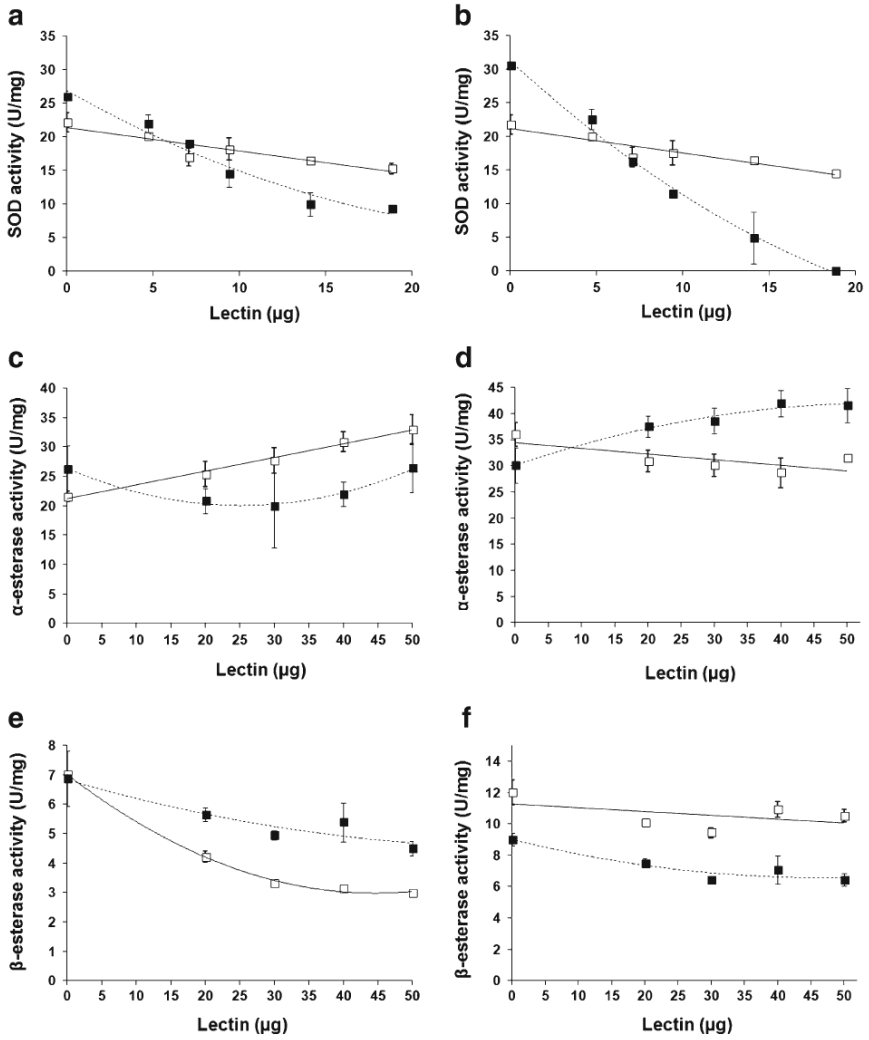

Fig. 3 Effects of WSMoL (*white square*) and cMoL (*black square*) on SOD (**a**, **b**), α-esterase (**c**, **d**), and β-esterase (**e**, **f**) activities from *A. aegypti* Rockefeller (**a**, **c**, **e**) and Rec-R (**b**, **d**, **f**) L_4

2007). The high inhibition of the SOD activity by cMoL, although it can lead to an oxidative stress due to impairment of superoxide radical detoxification, did not result in larval mortality. This result suggests that cMoL can be investigated as a synergistic agent for controlling populations whose resistance is linked to an increased detoxifying process mediated by SOD.

Rec-R larvae showed esterase activities higher than Rockefeller L_4. This fact is probably associated with their resistance to temephos since increased levels of these enzymes have been reported for larvae resistant to this pesticide (Flores et al. 2005; Melo-Santos et al. 2010; Polson et al. 2011). Although WSMoL and cMoL promoted reduction in β-esterase activity from Rec-R L_4, the inhibitory effect was slight and probably is not sufficient to promote an overpass of larval resistance to insecticide. In addition, the lectins showed no effect on α-esterase, which was the most active enzyme form detected.

In conclusion, the results showed that (1) WSMoL, although is able to kill organophosphate-susceptible (Rockefeller) larvae of *A. aegypti*, did not promote mortality of organophosphate-resistant (Rec-R) larvae; (2) the opposite effects of WSMoL on the survival of Rockefeller and Rec-R larvae indicate that these populations are physiologically distinct in other aspects beyond the resistance to temephos; (3) the different effects of WSMoL and cMoL on digestive enzymes from Rockefeller and Rec-R larvae indicate expression of different enzyme forms between these strains; (4) the mechanism of larvicidal activity of WSMoL on Rockefeller larvae may involve stimulation of larval digestive enzymes

(protease, trypsin, and α-amylase) and inhibition of β-esterase activity; and (5) cMoL can be evaluated in the future as a synergist for increasing the susceptibility of larvae with increased SOD activity.

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6. CAPÍTULO 2

Biology, Ecology and Strategies for Control of Stored-Grain Beetles: A Review

CAPÍTULO INCLUÍDO NO LIVRO

"*Beetles: Biodiversity, Ecology and Role in the Environment*"

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Chapter

BIOLOGY, ECOLOGY AND STRATEGIES FOR CONTROL OF STORED-GRAIN BEETLES: A REVIEW

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ABSTRACT

Beetle species belonging to the coleopteran families Bruchidae, Curculionidae, Laemophoeidae, Silvanidae and Tenebrionidae, as well as beetle-like insects from the psocopteran family Liposcelidae, are responsible for serious damages to agricultural products and resources. These beetles can be primary and/or secondary pests, feeding on integral and healthy grains or attacking those already damaged. The affected grains lose weight and germination power, are decreased in nutritive value and vigor, and are impaired by hygiene and sanitary conditions. This chapter summarizes information on biological and ecological aspects of stored product pests such as life cycle, fecundity, longevity, growth rate, voracity, natural habitats and hosts, and infestation focuses of beetles. These aspects are important for the development and choice of control measures. Classic strategies include mechanical methods, biological control, and the use of insecticide formulations such as powders, emulsions, aerosols, and microcapsules. Fumigation with phosphine has been the main strategy for control but this insecticide is highly volatile and toxic and increasingly there are reports of insect resistance to it. In this sense, natural insecticides, such as plant extracts, secondary metabolites, essential oils and lectins, have been investigated for insecticidal activity on destructive beetles. In summary, this chapter provides a brief and updated view of the state of the art on beetles that act as stored grain pests.

1. DAMAGES TO AGRICULTURE CAUSED BYSTORED-GRAIN PESTS

Grains such as sorghum, maize, rice and wheat are the main components of basic food in many countries. Since the harvest of grains occurs seasonally while the market demand is relatively unceasing, the storage step has a critical role in the global economy (FAO, 1994; Bilia et al., 2004). In this sense, the deterioration of stored products is a problem for agriculture, mainly in tropical regions, where the seeds are

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often subjected to high temperatures and humidity. In addition to physical and chemical factors, the stored grains can also be damaged by biological agents such as insect pests (Tavares and Vendramim, 2005; Silveira et al., 2006; Alencar et al., 2011). The insects belonging to the orders Coleoptera (beetles) and Psocoptera (booklice and barklice, also referred as beetle-like) cause most of the damage to stored grains. Figure 1 shows representative drawings of important species of the coleopteran families such as Bruchidae, Curculionidae, Laemophoeidae, Silvanidae and Tenebrionidae, and the psocopteran family, Liposcelidae.

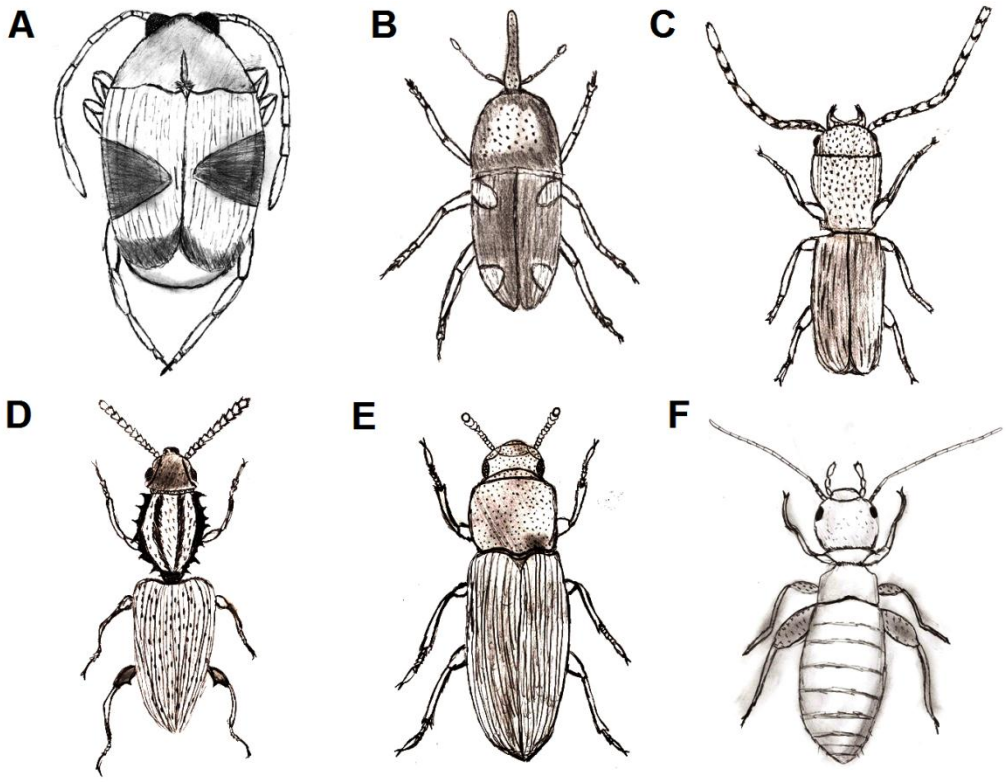

Figure 1. Representative drawings of stored-grain pests belonging to the respective families and species: (A)Bruchidae, *Callosobruchus maculatus*; (B) Curculionidae, *Sitophilus zeamais*; (C) Laemophoeidae, *Cryptolestes ferrugineus*; (D) Silvanidae, *Oryzaephilus surinamensis*; (E) Tenebrionidae, *Tribolium castaneum*; (F) Liposcelidae, *Liposceles* sp.

The insects that attack grains can be considered primary pests, which attack healthy grains throughout their development, and secondary pests, which are only able to attack grains that had been previously damaged (Silveira et al., 2006). The density of the insect population, the exposure time and the coexistence of primary and secondary pests dramatically increase the deterioration (Antunes et al., 2011; Tefera et al., 2011; Copatti et al., 2013). Alencar et al. (2011) assessed the effects of *Sitophilus zeamais* and *Tribolium castaneum* on maize and verified that the coexistence of these species during infestation promoted damage 20 times higher than that detected when these species infested the seeds separately. Similarly, Copatti et al. (2013) found more significant losses (91.48%) in rice grains concurrently attacked by *S. zeamais* and *Laemophloeus minutes*.

In general, insect pests usually feed on the endosperm because of its high nutritional content (Faroni, 1992; Nawrocka et al., 2010) and the most prevalent characteristics of damaged grains include reduction of dry matter, nutrient content and germination power. Caneppele et al. (2003) detected a loss of dry matter around 0.36% per day in maize grains infested during 150 days by *S. zeamais*. Mutungi et al. (2014) evaluated the effects of *Callosobruchus maculatus* on maize after six months of storage and found that 95% of the grains had their germination power reduced by more than 50%. This same pest also reduced the content of lipids and carbohydrates of beans by over 50% (Akintunde, 2012). Wheat grains infested by

Liposcelis bostrychophila showed weight reduction of approximately 15% after 90 days of exposure (Kucerova, 2002).

The affected grains also have impaired hygiene and sanitary conditions. The water generated by the respiratory metabolism of insects leads to increased moisture in the storage environment, which leads to the multiplication of other deteriorating agents such as fungi (FAO, 1985; Puzzi, 1986; Jayas and White, 2003) and fungi that develop in storage conditions can produce mycotoxins that are harmful to the human health (Moreno-Martinez et al., 2011; Smith et al., 2012; Suleiman et al., 2013). Ahmedani et al. (2011) observed a strong correlation between the increase of moisture content and weight loss of wheat infested by the beetle *Trogoderma granarium*.

2. Biology and Ecology of Beetles and Beetle-Like Insects That Attack Stored Grains

2.1. Bruchidae (Coleoptera)

The insects from Bruchidae family (approximately 1,300 species) are cosmopolitan and develop in the seeds of several plants, mainly leguminous, cultivated in almost all parts of the world. Some authors classify this family as a subfamily of the Crysomelidae family called Bruchinae. The bruchids are usually classified in two groups: the species that lay the eggs in the fruits of host plants and whose larvae feed on the seeds; and the species that lay the eggs directly on the seeds and thus act as stored-grain pests (Lima, 1952; Gallo et al., 2002; Buzzi, 2010).

Adult bruchids are usually less than 10 mm in length and the body has an oval shape (Figure 1A). Some of the characteristics of Bruchidae family were described by Lima (1952), Athié and de Paula (2002), Gallo et al. (2002), Kingsolver (2004) and Buzzi (2010) are: the head is free with a short and flat rostrum as well as serrated or pectinate antennae with 11 segments; the elytra are striated and do not cover all the abdomen, leading the last tergum (called pygidium) exposed; and the posterior legs are more robust than the others. The larvae are white or yellow and have a robust body, with a tiny and curved head that can retract into the thorax. After oviposition, the newly-hatched larvae penetrate into the grains and build a chamber consuming the entire cotyledon. The larva passes through four molts and remains feeding until the end of the last instar, after which it enters the pupa stage. The pupation may occur completely inside the larval chamber, may start within and complete out of the chamber, or may occur completely out of the seed where the larva developed.

Some of the most relevant pests from Bruchidae are *Acanthoscelides obtectus*, *Bruchus pisorum*, *Callosobruchus maculatus*, *Callosobruchus phaseoli* and *Zabrotes subfasciatus* (Athié and de Paula, 2002; Gallo et al., 2002). *A. obtectus* is a primary pest and the eggs may be laid on the pods in the field or directly on the stored seeds. The optimal conditions for development of larvae are ca. 30°C and 70% relative humidity. Although the period of adult life is short, this species has great infestation ability because the adults are good fliers and the life cycle is quickly completed (approximately 23 days) (Lorini, 2010). *C. maculatus* is native from Africa but distributed along all the tropics and subtropics. The most important hosts of *C. maculatus* are the beans from the *Vigna* genus and this beetle has a high ability for cross-infestation, attacking the tillage and the storage environment (Athié and de Paula, 2002; Gallo et al., 2002). An interesting study conducted by Cope and Fox (2003) revealed that the *C. maculatus* females distributed the eggs during oviposition according to the size of the seeds, aiming to optimize the use of resources. The oviposition of this species is stimulated by the alkanes present on the surface wax that cover the attacked seeds (Parr et al., 1998; Adhikary et al., 2014).

2.2. Curculionidae (Coleoptera)

The family Curculionidae represents the more numerous in the Animal Kingdom, with approximately 50,000 species described (Gallo et al., 2002; Buzzi, 2012) and about 30 species of this family are pests of

stored products around the world. The curculionids attack mainly fruits and seeds and a few subfamilies and the genus feed on dead vegetable materials. The most economically important species are *Sitophilus zeamais* (Figure 1B), *Sitophilus oryzae* and *Sitophilus granarium*, which can be found in storages of wheat, oat, rye, rice and maize and are also able to deteriorate beans, nuts, birdseeds, sunflower seeds and processed foods such as pasta.

According to Lima (1952), Athié and de Paula (2002), Gallo et al. (2002) and Buzzi (2010), the adults have a fairly elongated rostrum, straight or bent, which contains the chewing mouthparts at the end. The antennae are geniculate-capitate or geniculate-clavate with the scape inside a groove. The elytra are associated with the pro-thorax, usually cover the entire abdomen and can be glabrous, hairy and scaled. The scales may confer metallic coloration (green, blue, violet and golden). The posterior wings can be well-developed, rudimentary, obsolete or absent. The curculioniform larvae, which are apodal, robust, slightly bent and with a darker head, develop within the fruits, stems and seeds, consuming all the content present in these tissues. The pupae have a whitish color.

S. zeamais is found in all the warm and tropical regions. They are one of the major pests in stored grains in Brazil. This species possess a large number of hosts, including maize, wheat, sorghum and rice. It may also develop in processed cereals and food (Athié and de Paula, 2002; Gallo et al., 2002). *S. zeamais* have also been found attacking fruits such as apples, peaches and grapes (Botton et al., 2005). A remarkable characteristic of *S. zeamais* is the presence of reddish spots on the elytra and the curved rostrum present in the head is shorter and thicker in males. The adults are able to fly, quickly infesting the grains in the field and storage and are able to easily penetrate in the grains, and have a high potential for cross-infestation (Antunes and Dionello, 2010).

Danho et al. (2002) reported that the proportion of grains infected by *S. zeamais* is greater with decreasing in the amount of grains available. The females can live up to 140 days, 104 of these corresponding to oviposition period, and the average number of eggs per female is 282 (Botton et al., 2005). The females seal the hole made in the grain with a protein-rich secretion so that it is not possible to view the place of oviposition**.**

2.3. Laemophoeidae (Coleoptera)

The genus (*Cryptolestes*) is unique in the family Laemophoeidae that has importance as stored-grain pest. The *Cryptolestes* species feed on cereals, oleaginous seeds, nuts and dry fruits, and their presence is an indicator of very inadequate conditions in storage since these beetles develop in places already infested by other insects and fungi. The adults (Figure 1C) have a reddish-brown color, about 1.5–3.0 mm in length with a dorsoventral flattening. The antennae are filiform with 11 segments. The larvae penetrate grains with damaged or imperfect coats but the breaks only need to be microscopic to allow the entering of the larva. The eggs are elongated and more tapered at one of the ends. Each female is capable of laying 200 to 500 eggs and the oviposition occurs in the debris of plant material (Rillet, 1949; Athié and de Paula, 2002).

The species *Cryptolestes ferrugineus* is distributed at tropical, subtropical and temperate regions. It usually appears after an infestation by *Sitophilus* or *Rhyzopertha* and is able to develop in the grains of rye, wheat, maize, rice, oats, barley, corn, sunflower, flax, and soybeans (Rillet, 1949). *C. ferrugineus* also feeds on several types of fungi (mycophagous habit) found in storage. During cold seasons, they tend to move to the inner region of the grain mass (warmer region), and are present in the peripheral regions in warmer seasons (Athié & Cesar de Paula, 2002).

2.4. Silvanidae (Coleoptera)

The Silvanidae family includes about 500 species. The body of these insects is narrowed, brownish and densely punctured, with variable dimensions (1–15 mm in length), and the dorsoventral region is flattened (Figure 1D). Their antennae are clavate with 11 segments and their elytra entirely cover the abdomen.

Several species are mycophagous and some are important pests of grain products (Athié and de Paula, 2002).

The species *Oryzaephilus surinamensis* (sawtoothed grain beetle) has a cosmopolitan distribution and is found infesting cereals, flour, spices, dry fruits, pasta, chocolate and even jerky beef. These beetles did not develop well in oleaginous seeds. It is a major pest of stored barley and is classified as a secondary pest because it is only able to cause scratches or scars on healthy and whole grains; however it quickly develops in broken grains. The adults have limited flight ability and thus the infestations are usually resulting from residual populations present in the storage or previous contamination of grains (Athié and de Paula, 2002; Gallo et al., 2002; Beckel et al., 2002, 2007). Each female lays 37 eggs on average (Beckel et al., 2007).

2.5. Tenebrionidae (Coleoptera)

The family Tenebrionidae (darkling beetles) is present in tropical and temperate regions. About 15,000 species are described, commonly xerophiles with nocturnal habits. About 80 species are reported to act as stored-grain pests and are found attacking cereals and flours. The most important species that act as pests belong to the genus *Tribolium*, *Gnatocerus*, *Alphitobius*, *Tenebrio*, and *Latheticus*; all these beetles possess thoracic and abdominal glands of defense that secrete benzoquinones and hydroquinones. In addition to storage pests, in this family other crop pests are present such as mycetophagous, coprophagous, predators, myrmecophilous and polyphagous species (Lima, 1952; Athié and de Paula, 2002; Buzzi, 2010).

The adults have a variable coloration (black, brown, reddish-brown, cinereous) and body size (3–10 mm in length, with species that can reach 18 mm). The head is very small and narrower than the prothorax (Figure 1E). The antennae are filiform, moniliform or serrated (more common) with 11 segments. The exoskeleton is remarkably thick and stiff, shiny and glabrous. The elytra cover the entire abdomen and the wings are often stunted. The legs are ambulatorial (cursorial) or less frequently fossorial (adapted for digging). The larvae are elateriform (worm-like) with sclerotic, glabrous and shiny integument and have short legs (Lima, 1952; Athié and de Paula, 2002; Gallo et al., 2002; Buzzi, 2010).

Tenebrio mollitor (mealworm) is considered a pest because the larvae are able to feed on stored grains (Siemianowska et al., 2013). The species *Tribolium castaneum* is supposed to have an Indian origin and is distributed in tropical and subtropical regions being very tolerant to arid conditions. It attacks all kinds of ground cereals and is an important secondary pest that infests cereals, coffee, cocoa, soybeans, dried fruits, nuts, cotton seed and also stored milk powder. Occasionally, *T. castaneum* may attack stored peas and beans (Athié and de Paula, 2002).

2.6. Liposcelidae (Psocoptera)

The insects belonging to this family are known as booklice or barklice. Although they comprise a taxonomically distinct group, sometimes these insects are referred as “beetle-like”. They often live under dead bark, leaves and grass, in the nests of birds and mammals, on shelves, inside cracks of steps and furniture, and dusty places, among other locations. It grows easily in environments with relatively high temperature and humidity. Liposcelidae species can feed on fungi, algae, lichens, and pollen as well as on eggs and fragments of dead insects. Only a few species behave as pests of grain, for example, *Liposcelis bostrychophila*, which is able to damage rice. They are easily attracted by the presence of flour as well as moldy or wet food. Since these beetles are apterous the auto-dispersion is limited (Turner, 1998; Athié and de Paula, 2002; Buzzi, 2010; Chin et al., 2010).

The short (1–10 mm in length) body of these insects is tiny, flat and fragile (Figure 1F), with a yellowish or pale gray color and a semi-transparent appearance. The head is relatively large with a long and filiform (thread-like) antenna with 15 segments. The thorax is small and the abdomen is bigger than the rest of the body. Although the egg is approximately 1/3 of the female size, about 3-4 eggs are laid daily per female, resulting in a mean of 100 eggs in three weeks in the summer season. The metamorphosis is

incomplete, being the nymphal stages (4 for females and 3 for males) and adult forms are very similar only differing in size and color (Turner, 2002; Athié and Cesar de Paula, 2002; Buzzi, 2010).

3. CURRENT STRATEGIES FOR CONTROLOF STORED-GRAIN BEETLES

According to Gallo et al. (2002), three main techniques are usually applied to control pests in stored grains: fumigation, pulverizing and spraying. Other methods listed by Bond (1984) are sanitation, refrigeration, aeration, heating, drying, gamma radiation, microwaves, and infra-red radiation, as well as the use of insect growth regulators, predators, and pathogens. It also includes the use of insect resistant packaging.

Fumigation is the method most often used and can be applied to bulk or bagged products. It consists of the use of chemical compounds that are volatile at storage temperatures and toxic to the insect pests. However, only these characteristics are not enough to indicate the use of a substance. The compound should not be corrosive to containers or other materials in storage, should not react with the products originating irreversible residues and should not cause damage to the grains (Bond, 1984; Gallo et al., 2002). The fumigation technique is effective in eliminating insects in different stages of their life cycle.

The most used compounds in fumigation are methyl bromide and phosphine (aluminum phosphate and magnesium phosphate), which target the respiratory system of the insect. However, these substances are strongly toxic to non-target organisms, including humans (Gallo et al., 2002). Methyl bromide is acutely very toxic, mainly affecting the central nervous system, and intoxication may lead to death (Yang et al., 1995). Symptoms of methyl bromide poisoning are vomiting, headache, vertigo, imbalance while walking, slurred speech, and tremulousness of the upper limbs (Balogabal et al., 2011). Epidemiological evidence indicates that occupational exposure to methyl bromide is linked to incidence of human prostate cancer (Budnik et al., 2012). Phosphine is also toxic to non-target invertebrates and vertebrates disrupting the sympathetic nervous system, energy metabolism and the redox state of the cell (Nath et al., 2011). Exposure to high levels of phosphine causes acute respiratory problems, cough, headaches, dizziness, numbness, general fatigue and gastrointestinal disturbance. The chronic exposure results in anemia, bronchitis, gastrointestinal disorders, speech and motor disturbances, toothache, weakness, weight loss, mandible necrosis and spontaneous fractures (Takamiya, 2007).

The pulverizing method consists of mixing chemical powders with the grains and is recommended for treatment of small amounts of grains. Examples of chemicals used are bifenthrin, deltamethrin, fenitrothion, and pirimiphos (Gallo et al., 2002). The spraying method is achieved by micro pulverizations using an atomizer unit.

The excessive use of an insecticide inevitably results in a great selection pressure, which favors the proliferation of resistant individuals. Resistance of *S. zeamais* populations to the insecticides malathion, lindane, deltamethrin and phosphine has been described (Perez-Mendoza, 1999) and the development of strong resistance to phosphine by booklice has contributed to increase the importance of this species as a stored-grain pest (Collins et al., 2001). Opit et al. (2012) reported the detection in Oklahoma of a *Tribolium castaneum* population being 119-fold more resistant to phosphine in comparison with a laboratory susceptible strain, and highlighted that there is an increasing trend of phosphine resistance in the last 21 years. Figure 2 summarizes the steps involved in the emergence of a resistant insect population due to the prolonged, excessive and unplanned use of an insecticide.

4. NATURAL INSECTICIDES FOR CONTROLOF STORED-GRAIN BEETLES

The emergence of resistant populations and the risks that synthetic insecticides pose to human health and the environment have intensified the search for natural insecticides as an alternative in the control of agricultural pests. In general, natural products are environmentally safer because they are biodegradable and exhibit a greater selectivity than synthetic chemicals (Menezes, 2005; Duke et al., 2010; Kishore et al., 2011; Freitas et al., 2014).

Many parts of plants contain bioactive compounds that are involved in chemical defense against insect attack. A lot of naturally ant-insect substances have been extracted from plants in water and organic

solvents. The entomotoxicity of plant preparations is mainly characterized by neurotoxic actions, feeding inhibition, digestion impairment, developmental delay and alterations of reproductive and behavioral aspects (Kim et al., 2003; Menezes, 2005; Correa and Salgado, 2011).

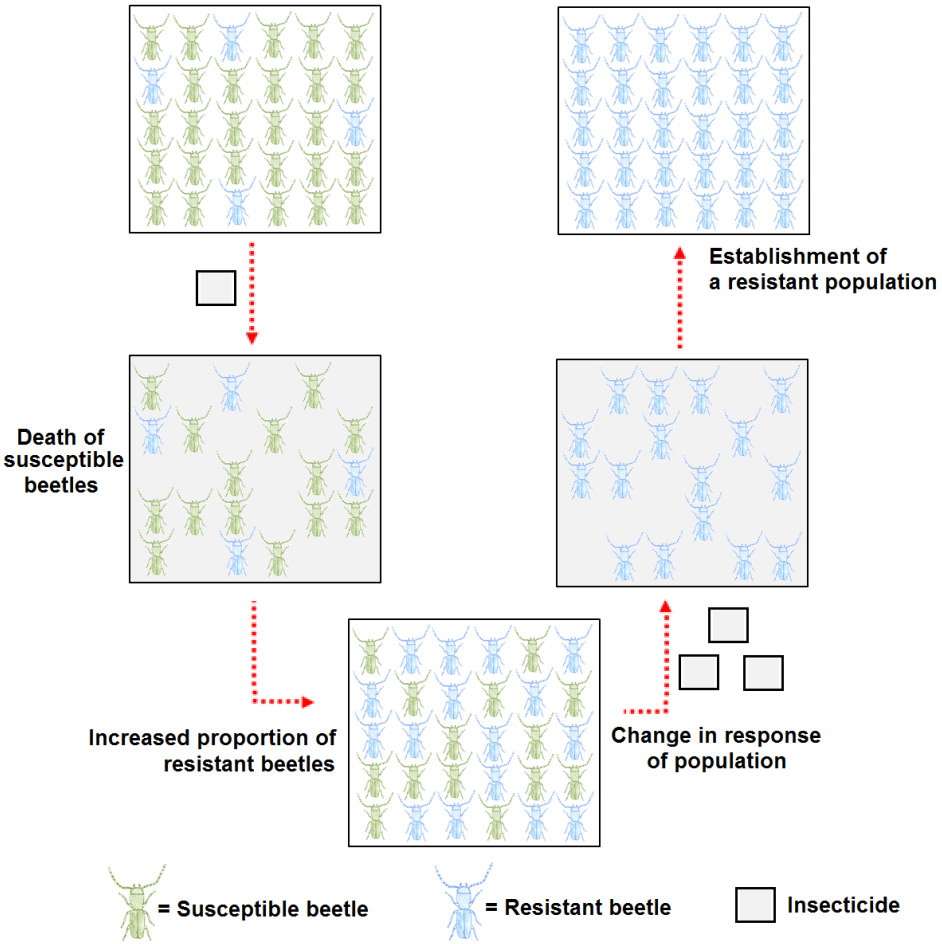

Figure 2.Establishment of an insect resistant population due to the excessive, intermittent and unplanned use of an insecticide.

Several plant extracts have shown insecticidal activity against stored-grain beetles, affecting survival and physiology. Hexane extracts from African nutmeg (7.5 and 10 mg/100mL) was strongly toxic to *Callosobruchus maculatus* adults promoting 100% mortality after 1 hour of exposure (Ogunsina et al., 2011). Treatment of *Sitophilus oryzae* with hexane extract from the *Capparis decidua* stem by 16 hours interfered on insect physiology reducing the levels of glycogen, protein, and amino acid as well as activity of enzymes phosphatases, transaminases, dehydrogenases and acetylcholinesterase involved in the physiology processes (Upadhyay, 2013). The powdered leaves of *Azadirachta indica* containing saponins and azadirachtin promoted a mortality of 50% of larvae and adults of *Tribolium castenum*, and also reduced more than 10% amylase activity (Sami, 2014).

Essential oils are complex mixtures of volatile secondary metabolites, mainly extracted from aromatic plants. They are able to affect survival, behavior and physiology of insect pests. Wang et al. (2011) showed that the essential oil from *Illicium fargesii* promoted mortality of *S. zeamais* through contact (LC_{50}=28.95 µg/adult) and by fumigation (LC_{50}=11.36 mg/L). Mossi et al. (2014) reported that the essential oil from *Ocotea odorifera* containing camphor (43%) and safrole (42%) as major constituents killed *S. zeamais* (DL_{50}=14.1µl cm^2) by contact after 24 h and was also a repellent agent. Food deterrence (74.52%) and reduction in oviposition rate (35.66%) were detected after treatment of *S. oryzae* with oil from *Aegles quinces* leaves (Mishra et al., 2014). Gusmão et al. (2013) determined that essential oils from *Eucalyptus citriodora*, *Eucalyptus staigeriana*, *Cymbopogon winterianus* and *Foeniculum vulgare* were toxic to

Callosobruchus maculatus by contact (LC_{50} ranging from 178.13 to 345.57 ppm) and fumigation (LC_{50} ranging from 2.58 to 7.85 μL/L of air), when used as repellent agents and reduced the oviposition rate as well as the emergence of adults in comparison with the control group.

In parallel, another progress in the search for alternative insecticides is the possible use of nanoparticles containing essential oils, since these systems are characterized by slow and persistent liberation of the oil in its active form. Gonzalez et al. (2014) determined that polyethylene glycol nanoparticles containing commercial essential oils (geranium and bergamot) have elevated residual toxicity against *Tribolium castaneum* and affected nutritional physiology reducing the relative growth rate, relative consumption rate and the efficiency of conversion of ingested food.

The deleterious effects of isolated secondary metabolites from different classes on insects are also reported. Sirinol, a compound isolated from garlic emulsion, showed a repellent effect on *T. castaneum* at a concentration of 10% while the metabolite allicin, also isolated from this plant, was toxic by fumigation to adults of *T. castaneum*, *Oryzaephilus surinamensis* and *Cryptolestes ferrugineus* with LC_{50} of 0.38, 0.51 and 0.51 mL/L in air, respectively (Jahromi et al., 2012; Lu et al., 2013). The ar-turmerone metabolite extracted from the rhizome of *Curcuma longa* affected the survival of *S. zeamais* at 1% (m/m), after six days of exposure by contact (Tavares et al., 2013).

Peptides and proteins isolated from plants also show insecticidal activity and thus have potential use in strategies for control of pests of stored products. Peptides purified from *Cicer arietinum* (chickpea) seeds were able to kill 83% and 100% of *S. oryzae* adults after 7 and 14 days of feeding (Mouhouche et al., 2009). Fields et al. (2010) demonstrated that peptide mixtures obtained from peas reduced the feeding and increased the mortality of *S. oryzae*. These authors also reported a synergistic effect between these peptides and insecticidal saponins and attributed this effect to the ability of saponins in impairing the hydrolysis of peptides by digestive enzymes at insect gut. Consequently, the increased time of contact between the peptides and insect gut probably allows the improvement of insecticidal action.

Zottich et al. (2014) revealed that the lipid transfer protein isolated from *Coffea canephora* seeds (Cc-LTP1, 0.5 %) inhibited the development of *C. maculatus* larvae, reduced the weight and the number of larvae, as well as decreased the oviposition rate. The same study showed that Cc-LTP1 inhibited the α-amylase activity of larval gut and was able to interact with the endoplasmic reticulum, mitochondria and microvilli of columnar cells from larvae.

Lectins are proteins whose structure contains carbohydrate-binding sites, which are able to interact with glycosylated molecules present in the lumen of insect gut, on the surface of epithelial cells or/and in the peritrophic matrix (Napoleão et al., 2012). The binding of lectins to glycosylated proteins at the midgut of insect larvae interferes with the nutrient uptake and the efficiency of diet utilization, resulting in a drop in mass gain. In this sense, lectins are insecticides that promote mortality or delay development of insects. The *N*-acetylglucosamine-specific lectin isolated from *Griffonia simplicifolia* leaf (GSII) at 1.0% (w/v) increased the WDST (within seed developmental time) of *C. maculatus* twice (Zhu et al., 1996) and the lectin isolated from *Myracrodruon urundeuva* leaves had a strong deterrent effect on adults of *S. zeamais*, and also reduced the activities of amylases, proteases, phosphatases, trypsin and endoglucanases (Napoleão et al., 2013). The lectin isolated from *Bauhinia monandra* leaf (BmoLL) was active on *C. maculatus* and *Z. subsfasciatus* larvae reducing the survival rate (LC_{50} of 0.5% and 0.3%, w/v, respectively) and decreasing body weight (Macedo et al., 2007). The authors demonstrated that the larvicidal effects of BmoLL probably involve resistance to proteolysis by larval enzymes and interaction with molecules at the membrane from midgut cells. *Talisia esculenta* seed lectin (TEL) also induced mortality of *C. maculatus* larvae (LC_{50}=1.0% w/v), was resistant to hydrolysis by cysteine proteases from larval midgut and bound proteins from the midgut of larvae (Macedo et al., 2004).

Products based on entomopathogenic fungi have also attracted the interest of researchers because they usually do not generate negative impacts to the environment and humans; they exhibit a residual effect that allows prolonged protection to stored grain, and the fungi applied are selective to the insect and do not develop in the grains (Alves et al., 2008; Michereff Filho et al., 2009). The fungi *Beauveria bassiana* caused mortality of *C. maculatus* (LC_{50}=3.17×10^6 conidia/mL) and *S. granarius* (LC_{50}= 6.08×10^7 conidia/mL) after 9 days (Shams et al., 2011). Nabaei et al. (2012) reported that the combination between diatomaceous earth and the entomopathogenic fungi *B. bassiana* or *Metharizium anisopliae* resulted in high

mortality rates of the *C. maculatus* adult with improved median lethal time. Pimentel and Ferreira (2012) reported on the insecticidal activity against *S. zeamais* of the products Metarril® (LC_{50}=181.9 ml/L for 3 days) and Boveril® (LC_{50}=2.1 g/L for 3 days). These products are formulations based on the entomopathogenic fungi *M. anisopliae* and *B. bassiana*, respectively. The fungi *Isaria fumosorosea* was able to induce the mortality of *S. oryzae* adults and it was demonstrated that the concentration was not a critical parameter that determined the speed of insect death (Kavallieratos et al., 2014). The insecticide spinosad, produced by fermentation of the soil actinobacteria, *Saccharopolyspora spinosa*, has been indicated as a promising insecticide for control of several species that attack stored grains such as *Cryptolestes ferrugineus*, *Cryptolestes pusillus*, *Liposcelis entomophila*, *Sitophilus oryzae*, *Sitophilus granarius*, *Tribolium castaneum* and *Tribolium confusum* (Chintzoglou et al., 2008; Vayias et al., 2010; Hertlein et al., 2011).

CONCLUSION

This chapter summarizes information on all families of stored-grain pests in relation to morphology, physiology, ecology, grains attacked and current strategies for control of insects. The finding of alternative insecticides will allow their use in rotation programs, which canlead to increasing efficiency in insect control as well as minimizing the development of resistance. The potential use as an insecticide of plant compounds (such as secondary metabolites, essential oils and peptides) and entomopathogenic fungi is described.

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7. CAPÍTULO 3

Evaluation of insecticidal activity of aqueous extract and water-soluble lectin from *Moringa oleifera* seeds against *Sitophilus zeamais* Motsch.

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ABSTRACT

The maize weevil*Sitophilus zeamais* hasa strong economic appeal since it causes deterioration of stored grains, processed cereals and industrial food.In this work, an aqueous extract (AE) from *Moringa oleifera* seeds was treated with ammonium sulfate for obtaining a lectin-rich fraction (LF), which was loaded onto a chitin column for isolation of the water-soluble *M. oleifera* lectin (WSMoL). AE, LF and WSMoL were investigated for effectson survival, feeding and nutritional parameters of *S. zeamais* adults. The assay consisted in maintain the insect in artificial diet corresponding to sample incorporated into wheat flour disks. Also, the effects of thesepreparations on trypsin-like activity from insect gut were investigated. AE (58–145 mg/g) was toxic to *S. zeamais* (mortality rates ranging from 21.7 to 50%) while LF (14.5–58 mg/g) did not kill the insects. WSMoL caused slight mortality (12.0 ± 2.7) only at 60 mg/g. The intake of AE reduced the relative consumption rate and a moderate to strong deterrent effect was observed.Both LFand WSMoL decreased the relative biomass gain rate and the efficiency in conversion of ingested food, but did not exerted deterrent action. Only WSMoL increasedin trypsin-like activity from *S. zeamais* gut.In conclusion, AE was toxic to *S. zeamais* probably due to its feeding-deterrent activity and WSMoL was damaging to the nutritional physiology of insects probably by promoting an imbalance of digestion process due to stimulation of trypsin-like activity.

KEYWORDS.*Moringa oleifera*; maize weevil; antinutritional effect; digestive enzymes; lectin.

Introduction

Cereal grains have a major relevance in the global economy since they are components of the basic diets for human populations worldwide. In general these grains are produced seasonally and the harvesting occurs once or at certain times of the year so that the storage of these products is a standard practice among major producers (FAO 1994). However, storage pests and pathogens can attack stored grains and cause quantitative and qualitative damages such as: weight loss; changes in color, smell and taste; loss of nutritional value; impairment of vigor; and contamination by mycotoxins (Gwinner et al. 1996; Gallo et al. 2002; Demissie et al. 2008; Lorini 2008).

The beetles of Curculionidae family, popularly known as grain weevils, are considered the major primary pests of cereals. More than 30 species have been recorded in stored food, and among them, the *Sitophilus zeamais* (maize weevil) is the major pest of corn that occurs in tropical regions, infecting healthy grains in the field and during postharvest storage (Semple et al. 1992; Abebe et al. 2009; Ukeh 2008).

The control of *S. zeamais* over the years has been performed through the use of synthetic insecticides. Nevertheless, these compounds are toxic to humans and non-target organisms and their continuous use can generate serious problems such as the emergence of resistant populations (Obeng-Ofori 2007; Sahaf et al. 2008; Mondal and Khalequzzaman 2010). Aiming to change this scenario and to contribute with integrated pest management programs, researchers have investigated plant preparations and isolated constituents for insecticidal activity against *S. zeamais*, such as: crude extracts (Ukeh et al. 2012; Thein et al. 2013), essential oils (Suthisut et al., 2011; Zoubiri and Baaliouamer 2012), isolated secondary metabolites (Yang et al. 2011; Tavares et al.2013), peptides (Mouhouche et al. 2009), and lectin (Napoleão et al. 2013).

Lectins are proteins that recognize and interact reversibly with carbohydrates and glycoconjugates. These proteins have shown deleterious effects against insects from several orders and at all development stages, interfering with growth, survival, nutrition, digestion and reproduction (Ohizumi et al. 2009; Oliveira et al. 2011; Napoleão et al. 2011. Santos et al. 2012; Hamshou et al. 2013; Kaur et al. 2013; Paiva et al. 2011).

Moringa oleifera (Moringaceae family) is a tree native from India and widely distributed in the tropics, resistant to drought and able to grow in poor soils. Its seeds contain a lectin called WSMoL (water-soluble *M. oleifera* lectin), which showed antibacterial activity (Ferreira et al. 2011) and was an insecticidal agent against *Aedes aegypti* eggs and larvae (Coelho et al. 2009; Santos et al. 2012). The larvicidal activity of WSMoL may be linked to disruption of gut structures as well as deregulation of the activity of digestive enzymes (Coelho et al. 2009; Agra-Neto et al. 2014).

This work reports the investigation of the effects of aqueous extract (AE) and a lectin-rich fraction (LF) from *M. oleifera* seeds as well as of WSMoL on feeding, survival, and nutritional parameters of *S. zeamais* adults. Also it was investigated the effects of WSMoL on protease and trypsin-like activities from insect gut.

Material and methods

Plant material

Moringa oleifera seeds were collected in Recife, Pernambuco, Brazil, powdered using a blender and stored at -20 ºC. The voucher specimen is deposited under number 73,345 at the herbarium *Dárdano de Andrade Lima* from the *Instituto Agronômico de Pernambuco*, Recife,

Brazil. Plant collection was authorized (number 38690-2) by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of Environment.

Moringa oleifera seed extract

Aqueous extract (AE) from *M. oleifera* seeds was obtained according to Coelho et al. (2009). Seed powder was homogenized for 16 h at 4°C with distilled water, in a proportion of 10% (w/v), using a magnetic stirrer. The mixture was filtered through gauze and centrifuged (3000 *g*, 15 min). The supernatant corresponded to the aqueous seed extract. Protein concentration was determined according to Lowry et al. (1951) using a standard curve of bovine serum albumin (31.25–500 µg/mL).

Isolation of WSMoL

WSMoL was isolated according to the procedure described by Coelho et al. (2009). The crude extract was treated with ammonium sulphate at 60% saturation (Green and Hughes, 1955) during 4 h at 28°C. Next, the precipitated proteins were collected by centrifugation (3000 *g*, 15 min) and dissolved in distilled water. After dialysis (3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (4 h), the lectin-rich fraction (LF) was obtained.

LF (10 mg of protein) was loaded onto chitin column (7.5 × 1.5 cm) equilibrated (flow rate of 20 ml/h) with 0.15 M NaCl. After washing with equilibrating solution, WSMoL was eluted from the column with 1.0 M acetic acid and dialyzed (3.5 kDa cut-off membrane) against distilled water (6 h at 4 °C) for eluent elimination.

Hemagglutinating activity

Hemagglutinating activity was determined in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho (1992). The assay was performed using a suspension (2.5%, v/v) of rabbit glutaraldehyde-treated erythrocytes (Bing*et al*. 1967) in 0.15 M NaCl. A two-fold serial dilution of sample (50 µL) in 0.15 M NaCl was performed in the microtiter plate and then the erythrocyte suspension (50 µl) was added to each well. The assay was incubated at 28°C for 45 min. One hemagglutination unit was defined as the reciprocal value of the highest dilution of sample that promotes full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between titer and protein concentration (mg/mL).

Insecticidal assay

S. zeamais adults were obtained from the breeding maintained at the *Departamento de Bioquímica* from the *Universidade Federal de Pernambuco* at 28±2°C in glass containers containing maize grains and closed with TNT-type fabric. Breeding is authorized (number 36301-2) by the ICMBio/Brazilian Ministry of Environment.

Insecticidal assay was performed according to an adaptation of Xie et al. (1996) method described by Napoleão et al. (2013). For each bioassay, 5 mL of a sample (AE, LF or WSMoL) solution in distilled water was added to 2.0 g of wheat flour and the mixture was stirred for 5 min in order to obtain a suspension. In the control treatment, 5 mL of distilled water was mixed with the wheat flour. Next, five aliquots of 200 µL of the suspension were placed in a Petri plate (90 x 100 mm), which was then incubated at 56°C for 16 h. After this period, 20 insects were transferred to the plate and the bioassay was maintained in dark at

28±2ºC. Each assay was performed in quadruplicate and the weight of flour disks and insects was determined before the starting of test and after 7 days. Mortality rates (%) were evaluated after 7 days of experiment. The final concentrations of AE, LF and WSMoL in the disks were 58.0–116.0, 10.5–41.0, and 0.5–60 mg/g (mg of protein per g of wheat flour), respectively.

Feeding-deterrence index (FDI) and nutritional parameters

FDI values were calculated as follows: FDI (%) = 100 × (*A* – *B*)/(*A*), where *A* is the mass of food ingested by insects in the control assay and *B* is the mass of food ingested by insects in the sample test (Isman et al., 1990). According to FDI, the samples were classified as: no-deterrent (FDI < 20%), weakly deterrent (50% > FDI ≥ 20%), moderately deterrent (70% > FDI ≥ 50%) or strongly deterrent (FDI ≥ 70%) (Liu et al., 2007).

The nutritional indices were calculated according to Xie et al. (1996) using the data obtained in the insecticidal assay after 7 days of experiment: (1) relative consumption rate = *C*/(*D* × days), where *C* is the mass (mg) of ingested food and *D* corresponds to the initial insect biomass (mg); (2) relative biomass gain rate = *E*/(*D* × days), where *E* corresponds to the biomass gained (mg) by the insects; (3) efficiency in conversion of ingested food = *E*/(*C* × 100).

Effect of extract, lectin-rich fraction and WSMoL on trypsin-like activity from gut of *S. zeamais* adults

Groups of 50 adults were collected and immobilized by placing them at -20 °C for 10 min. The gut of each insect was dissected by hand and immediately homogenized with 1 mL of Tris buffer (0.1 M Tris-HCl, pH 8.0, containing 0.02 M $CaCl_2$ and 0.15 M NaCl) by using a

3-mL tissue grinder (Pyrex®, Corning Inc., NY, USA). The homogenates were centrifuged at 9,000 × g at 4 °C for 15 min. The supernatants were collected, pooled (gut extract) and evaluated for protein concentration (Lowry et al., 1951).

Trypsin activity was determined by incubating (30 min, 37 ºC) gut extract in Tris buffer (50 µl; 5 µg of protein) with 8 mM*N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA, 5 µl) in Tris-HCl 0.1 M pH 8.0 (145 µl). Trypsin activity was followed by measurement of absorbance at 405 nm (Kakade et al. 1969). One unit of trypsin activity was defined as the amount of enzyme that hydrolyzes 1 µmol of BApNA per minute.

Theeffect of AE, LF and WSMoL on trypsin-like activitywas evaluated by incubating (30 min at 37 ºC) the gut extract in Tris buffer (50 µl; 5 µg of protein) with different volumes of sample before determination of trypsin-like activity as described above. Control assay was performed by submitting only the gut extract to the same reaction steps.

Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as the mean of replicates ± SD. Significant differences between treatment groups were analyzed using Tukey's test for multiple comparison (significance at $p < 0.05$) with Action 2.4.163.322 softwares.

Results

Incorporation of AE (specific hemagglutinating activity of 32) in diet at 58, 116 and 145 mg/g resulted in mortality of insects, ranging from 21,7 to 50%, after 7 days (Table 1). There was no interference on the relative biomass gain rate and efficiency in conversion of ingested food in regard to control but the relative consumption rates were lower than that determined for control treatment (Figure 1A). FDI values were 57.2±20.2%, 65.0±5.5%, and 70.8±2.8% for the treatments at 58.0, 116.0 and 145 mg/g, respectively, indicating a moderate to strong deterrent effect.

Mortality rates of *S. zeamais* adults that ingested LF (specific hemagglutinating activity of 114) were higher than 1.3 % after 7 days of experiment (Table 1). Differently from the extract, the incorporation of the fraction in diet caused biomass loss and non-ocorrence of conversion of ingested food in comparison with control (Figure 1B). Relative consumption rates wasaltered by LF at 14.5 mg/g(Figure 1B). The FDI values were 18.8±1.6 (14.5 mg/g), 16.3±5.8 (29 mg/g), 16.7±3.5 (43.5 mg/g), and 17.8±1.5 (58 mg/g), indicatingno-deterrent effect.

The ingestion of WSMoL (specific hemagglutinating activity of 2,240) only have impact on survival of *S. zeamais* adults at 60 mg/g showing a mortality rate of 12.0±2.7%(Table 1). In despite of this, WSMoL was damaging to physiology of *S. zeamais* adults since thevalues of relative biomass gain rate (Figure 2A) and efficiency in conversion of ingested food (Figure 2B) were negative in all treatments. There were no significant alterations in the relative consumption rate for all treatments in comparison with control (Figure 2C) and FDI values could not be determined since there was no significant differences ($p > 0.05$) between the amount of food ingested in treatments with lectin and in control.

Trypsin-like activity in extract from gut of *S. zeamais* adults was not affected by AE and LF while incubation with WSMoL resulted in increase of the enzyme activity in a dose-dependent manner (Figure 3).

Discussion

Crude extracts, essential oils, secondary metabolites and other biomolecules from plants have been indicated as alternatives for controlling *S. zeamais* by affecting survival and nutrition (Liu et al. 2007; Restello et al. 2009; Ootani et al. 2011; Almeida et al. 2013). The importance of *S. zeamais* as economic pest together with the presence of molecules with insecticidal activity in the seeds of *M. oleifera* stimulated the investigations described in this work.

Similarly to the results obtained with AE, the survival rate of *S. zeamais* adults was reduced after ingestion of artificial diet containing the leaf extract from *Myracroduon urundeuva* (LC_{50} = 72.4 mg/g) (Napoleão et al. 2013). The reduction in relative consumption rate in treatments with AE (58, 116 and 145 mg/g) and the strong feeding-deterrent effect may have contributed to the death of insects. Although the adults of *S. zeamais* have ingested a smaller amount of food, the efficiency in conversion of nutrients in biomass remained unchanged, showing that the digestibility of ingested food was not damaged and that the metabolism of insects was able to adjust their growth on the basis of food intake. In this sense, the deterrent action of the extract seems to be due a pre-ingestion effect.

Unlike, LF did not promote significant mortality of the insects, which suggests that active principles of the extract were not concentrated after treatment with ammonium sulfate. The data indicate that the compounds involved in the deterrent effect were eliminated and the absence of mortality is probable a consequence of this. However, although only LF was not

able to kill the insects within 7 days, it was damaging to the growth and to the conversion of food by the insects. These results indicate that LF still contains compounds with deleterious effects on *S. zeamais* adults.

Since WSMoL is well reported to be an insecticidal agent against *A. aegypti*, we investigate its effect on *S. zeamais*. Differently from *A. aegypti* larvae, WSMoL showed a week insecticidal activity against *S. zeamais* (rate mortality of 12.0±2.7% at 60mg/g). Similarly to WSMoL, the lectin isolated from *M. urundeuva* (MuLL) did not promote mortality of *S. zeamais* adults (Napoleão et al., 2013). Nevertheless, MuLL exerted a strong feeding-deterrent effect while WSMoL was not able to interfere with the relative consumption rate, showing no deterrent action. The effects of WSMoL were similar to those of LF, indicating that the lectin is the active principle of the fraction. Although a wide range of insecticidal lectins have already been published, to our knowledge the reports on the effects of these biomolecules on *S. zeamais* are restricted to this work with MuLL.

Other researches suggest the reduction in the growth and in feed consumption and conversion as a triggering factor of increased mortality of *S. zeamais*. These nutritional parameters were affected negatively by the treatment with eugenol, isoeugenol and methyleugenol resulting in mortality (LC_{50} = 30 mg/mg) of *S. zeamais* adults (Huang et al. 2002). Tavares et al. (2013) reported that the coumpound ar-turmerone isolated from *Curcuma longa* rhizome (Zingiberaceae) caused loss of biomass and mortality rate of adults of this species.

The imbalance in activity of digestive enzymes has been also reported as cause of mortality of insects, and thus we evaluated the effect of AE, LF and WSMoL on trypsin-like activity from gut of *S. zeamais*. AE did not affect the activity of this enzyme and, indeed, the values of efficiency in conversion of ingested food suggest that the digestibility was not affected. The deleterious effects of LF also seem not involve the interference with the activity

of trypsin-like enzymes. On the other hand, we detected an increase of activity of trypsin-like enzymes from *S. zeamais* gut after incubation with WSMoL. Similarly to detected here, WSMoL also increased the trypsin like activity from gut of *A. aegypti* Rockefeller larvae and the authors suggested that the excessive proteolytic activity may lead to imbalance of digestion and consequently result in the harmful effects of this lectin (Agra-Neto et al. 2014).

According to Macedo et al.(2007), lectins are able to interfere with the activity of enzymes by binding to the sugar moiety or to domains involved in catalysis in glycosylated or non-glycosylated enzymes, respectively. The activity of trypsin-like enzymes from the midgut of *Ephestia kuehniella* larvae was reduced after treatment with the *Annona coriacea* lectin (Coelho *et al.* 2007). Napoleão *et al*. (2013) reported that MuLL was also able to interfere with trypsin-like enzymes from *S. zeamais* gut, but unlike WSMoL, this lectin reduced the enzyme activity. Probably, themortality of insects is not related to changes in trypsin activity.

In conclusion, the extract from *M. oleifera* seeds induced mortality of *S. zeamais* adults and feeding-deterrent agents are probably linked to its toxicity. Purified WSMoL affected the nutritional physiology of the insects probably due to imbalance of digestion process but did not show deterrent effect and was not able to kill the insects in 7 days.

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Figure captions

Fig. 1. Nutritional parameters of *S. zeamais* adults reared on artificial diets containing (A) aqueous extract from *M. oleifera* seeds (58 to 145 mg of protein per g of wheat flour) or (B) lectin-rich fraction obtained after treatment of extract with ammonium sulphate (14.5 to 58 mg of protein per g of wheat flour). The relative biomass gain rate indicates the amount of biomass in mg gained every day per mg of initial body weight. The efficiency in conversion of ingested food (%) indicates the amount of ingested food incorporated by insects as biomass. The relative consumption rate indicates the amount of food consumed in mg per mg of insect body weight per day. Each bar corresponds to the mean ± SD of four replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

Fig. 2. Nutritional parameters of *S. zeamais* adults reared on artificial diets containing WSMoL (0.5 to 60 mg per g of wheat flour). The relative biomass gain rate indicates the amount of biomass in mg gained every day per mg of initial body weight. The efficiency in conversion of ingested food (%) indicates the amount of ingested food incorporated by insects as biomass. The relative consumption rate indicates the amount of food consumed in mg per mg of insect body weight per day. Each bar corresponds to the mean ± SD of four replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

Fig. 3. Effect of aqueous extract (AE), lectin-rich fraction (LF) and isolated WSMoL from *M. oleifera* seeds on trypsin-like activity from *S. zeamais* adults.

Table 1. Mortality rates of *S. zeamais* adults reared for 7 days on diets containing *M. oleifera* seed preparations and isolated WSMoL.

Sample concentration (mg/g of wheat flour)	Mortality rate (%) after 7 days
AE	
58	21.7 ± 7.0 a
116	50.0 ± 7.0 b
145	50.0 ± 0.0 b
Control	5.0 ± 0.0 d
LF	
14.5	2.5 ± 2.1 a
29	3.8 ± 2.0 a
43.5	2.5 ± 2.1 a
58	1.3 ± 2.5 a
Control	0.0 b
WSMoL	
0.5	4.0 ± 1.5 a
1.5	10.0 ± 6.1 a
2.5	6.0 ± 2.0a
15	5.0 ± 3.5 a
30	5.0 ± 0.0 a
60	12.0 ± 2.7 b
Control	4.0 ± 1.5 a

AE: aqueous extract. LF: lectin-rich fraction. Control treatments contained only wheat flour. Different letters indicate significant ($p<0.05$) differences between treatments.

Figure 1

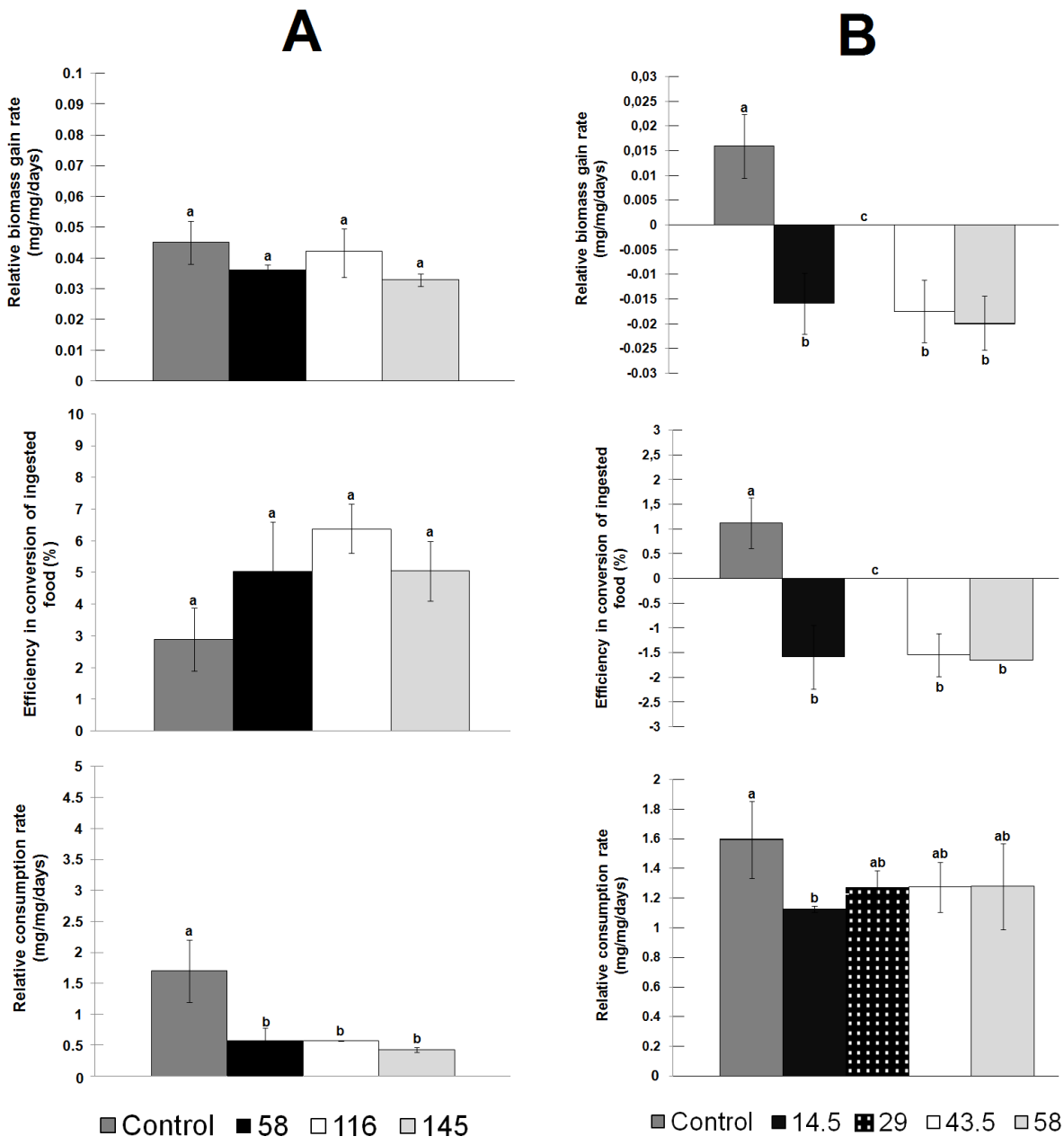

A
B
Relative biomass gain rate (mg/mg/days)
Efficiency in conversion of ingested food (%)
Relative consumption rate (mg/mg/days)
Control
58
116
145
14.5
29
43.5

Figure 2

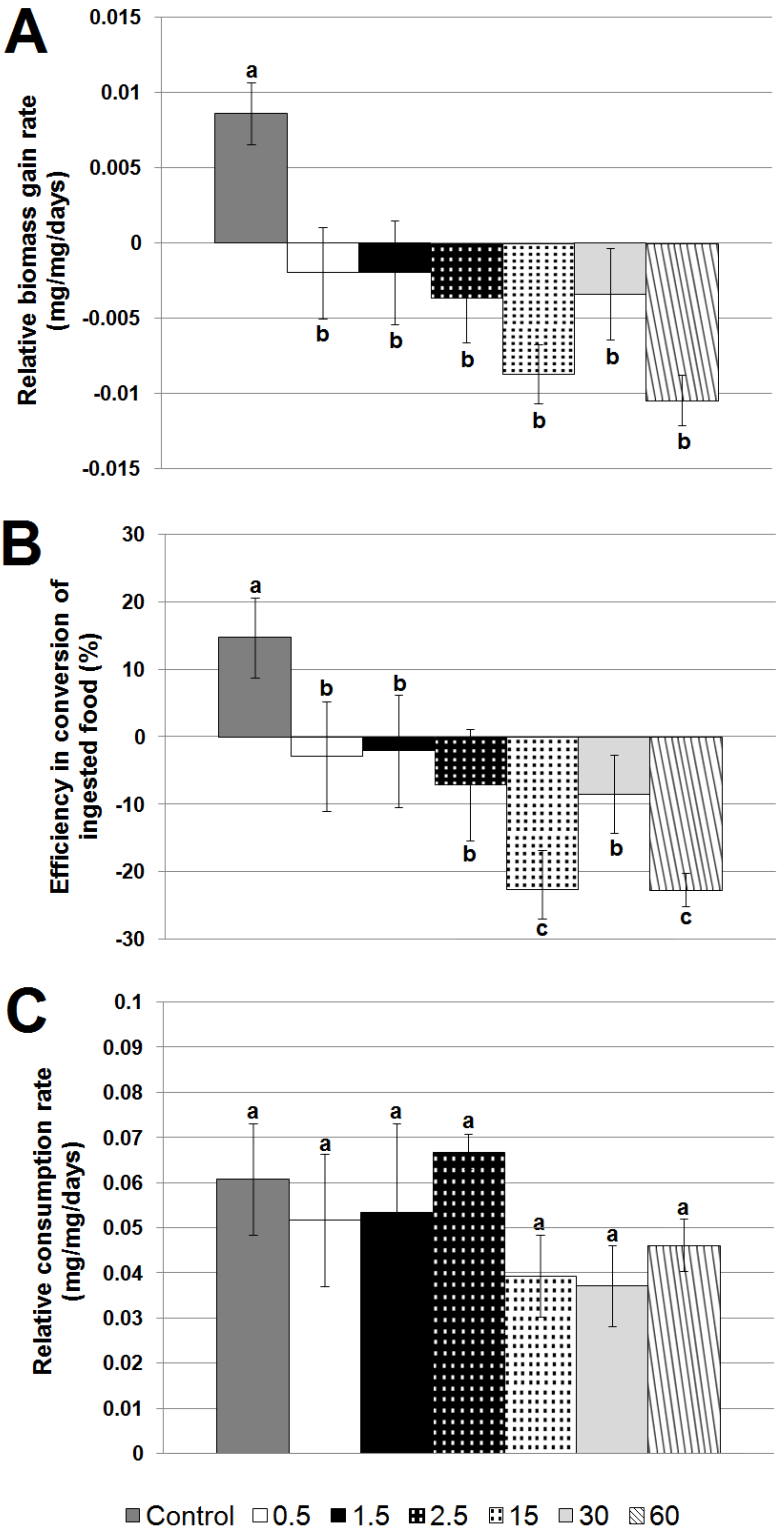
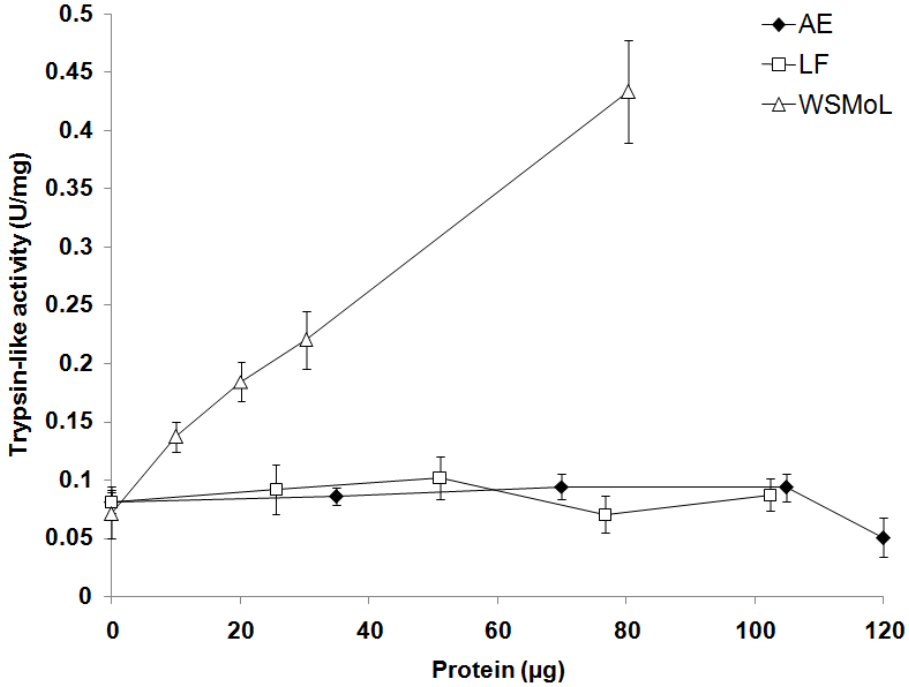

A
Relative biomass gain rate (mg/mg/days)
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
a
b
b
b
b
b
B
Efficiency in conversion of ingested food (%)
30
20
10
0
-10
-20
-30
a
b
b
b
c
b
c
C
Relative consumption rate (mg/mg/days)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
a
a
a
a
a
a
Control
0.5
1.5
2.5
15
30
60

Figure 3


AE
LF
WSMoL
Trypsin-like activity (U/mg)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
20
40
60
80
100
120
Protein (µg)

8. CONCLUSÕES

- ✓ WSMoL apresentou atividade larvicida somente contra L4 de *A. aegypti* susceptíveis a organofosfato (Rockefeller), enquanto cMoL não apresentou ação larvicida.

- ✓ O mecanismo de atividade larvicida de WSMoL para larvas Rockefeller pode envolver a estimulação de enzimas digestivas (proteases, tripsina e α-amilase) e inibição da atividade de β-esterase.

- ✓ cMoL tem potencial sinérgico para larvas de *A. aegypti* com atividade de SOD aumentada.

- ✓ O extrato aquoso (EA) de sementes foi tóxico para adultos de *S. zeamais,* provavelmente devido ao seu efeito deterrente de alimentação.

- ✓ WSMoL e Fração (LF) promoveram danos na fisiologia nutricional de *S. zeamais*.