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ASSOCIAÇÃO DE PIRETROIDES NANOENCAPSULADOS OU NÃO COM SINERGISTAS PARA O MANEJO DA RESISTÊNCIA DE *Chrysodeixis includens* (WALKER) E *Euschistus heros* (F.)

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Dissertação apresentada ao Programa de Pós-Graduação em Agronomia da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Agronomia**.

Orientador: Prof. Dr. Oderlei Bernardi

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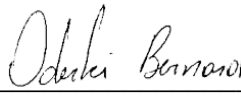

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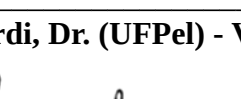

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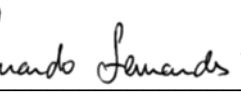

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DEDICATÓRIA

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"Se o dinheiro for a sua esperança de independência, você jamais a terá. A única segurança verdadeira consiste numa reserva de sabedoria, de experiência e de competência".
Henry Ford

RESUMO

ASSOCIAÇÃO DE PIRETROIDES NANOENCAPSULADOS OU NÃO COM SINERGISTAS PARA O MANEJO DA RESISTÊNCIA DE *Chrysodeixis includens* (WALKER) E *Euschistus heros* (F.)

AUTORA: Jéssica Santi Boff
ORIENTADOR: Oderlei Bernardi

O percevejo-marrom, *Euschistus heros* (F.) (Hemiptera: Pentatomidae), e a lagarta falsa-medideira, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), são os principais insetos-praga da soja na América do Sul. O controle químico é a principal estratégia para manejo dessas espécies, entretanto, uma baixa suscetibilidade a piretroides foi relatada no Brasil para ambas as espécies. No primeiro estudo, avaliou-se a adição dos sinergistas butóxido de piperonila (PBO) e maleato de dietila (DEM) para o manejo de *E. heros* e *C. includens* com resistência a λ-cialotrina e bifentrina. Os valores DL_{50} dos produtos técnicos e comerciais contendo λ-cialotrina e bifentrina foram menores para *E. heros* coletados a campo quando expostos ao PBO e DEM, em relação a insetos não tratados com os sinergistas, com razão de sinergismo de até 4,75 vezes. A mortalidade de *E. heros* também aumentou quando expostos a doses dos produtos comerciais contendo λ-cialotrina (de 4% para 44%) e bifentrina (de 44% para 88%) na presença dos sinergistas. Houve também uma maior suscetibilidade de *C. includens* coletada a campo a λ-cialotrina de grau técnico na presença de PBO; razão de sinergismo de 5,50 vezes. Uma maior letalidade de formulação com λ-cialotrina também foi verificado na presença de PBO, com mortalidade aumentando de 6% para 57%. No segundo estudo, desenvolveram-se e avaliaram-se nanoformulações de λ-cialotrina e bifentrina com os sinergistas PBO e DEM para o manejo da resistência de *E. heros* e *C. includens*. Nanoformulações de bifentrina e λ-cialotrina com PBO e DEM apresentaram boas características físico-químicas e estabilidade em função do tempo (>90 dias). Também foi observado que a morfologia esférica de todos os sistemas preparados e a eficiência de encapsulação em sistemas carreadores de lipídios nanoestruturados não se alteraram com a adição dos sinergistas. Em bioensaios toxicológicos, bifentrina nanoencapsulado com DEM aumentou a suscetibilidade de *E. heros* a este ingrediente ativo em até 3,50 vezes, conforme valores de CL_{90}. Uma maior suscetibilidade bifentrina e λ-cialotrina nanoencapsulada com PBO também foi detectada em *C. includens*, com os valores de CL_{90} indicando uma razão de sinergismo de até 2,16 vezes. Também detectou-se que bifentrina e λ-cialotrina nanoencapsulada com PBO e DEM causaram maior mortalidade de *E. heros* (68% e 72%) e *C. includens* (33% e 21%), em comparação aos produtos comerciais com bifentrina e λ-cialotrina. Os resultados indicam a possibilidade de uso dos sinergistas na reversão da resistência à λ-cialotrina e bifentrina em *E. heros* e *C. includens* e sugerem um efeito significativo de mecanismos metabólicos na desintoxicação de ambos os piretroides. Também foi possível desenvolver formulações de λ-cialotrina e bifentrina nanoencapsuladas com PBO e DEM, as quais demonstram potencial para restaurar a suscetibilidade de *E. heros* e *C. includens* a piretroides. Este é o primeiro estudo que relata o desenvolvimento e avaliação de inseticidas nanoencapsulados com sinergistas para o manejo da resistência de insetos.

Palavras-chave: Praga da soja. Inseticida. Nanoformulação. Controle.

ABSTRACT

ASSOCIATION OF PYRETHROIDS NANOENCAPSULATED OR NOT WITH SYNERGISTS FOR RESISTANCE MANAGEMENT OF *Chrysodeixis includens* (WALKER) AND *Euschistus heros* (F.)

AUTHOR: Jéssica Santi Boff
SUPERVISOR: Oderlei Bernardi

The brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae), and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), are the main pests of soybean in South America. Chemical control is the main strategy for controlling these insects-pest, however, low susceptibility to pyrethroids was reported in Brazil for both species. In the first study, it was evaluate the addition of synergists piperonyl butoxide (PBO) and diethyl maleate (DEM) to manage *E. heros* and *C. includens* with resistance to λ-cyhalothrin and bifenthrin. The LD_{50} of technical grade and commercial products containing λ-cyhalothrin and bifenthrin decreased against field-collected *E. heros* exposed to PBO and DEM relative to unexposed insects; synergistic ratios up to 4.75-fold. The mortality also increased when *E. heros* were exposed at fixed doses of commercial λ-cyhalothrin (from 4% to 44%) and bifenthrin (from 44% to 88%) in the presence of synergists. There was also a higher susceptibility of field-collected *C. includens* to technical grade λ-cyhalothrin when PBO was used; synergistic ratio of 5.50-fold. A high lethally of technical grade λ-cyhalothrin was also verified in the presence of PBO, with mortality increasing from 6% to 57%. In the second study, we developed and evaluated nanoencapsulated-based bifenthrin and λ-cyhalothrin with the synergistic compounds PBO and DEM for resistance management of *E. heros* and *C. includens*. Nanoformulations of bifenthrin and λ-cyhalothrin with PBO and DEM presented good physical-chemical characteristics and stability in function of the time (>90 days). It was also observed that the spherical morphology of all prepared systems and the encapsulation efficiency in nanostructured lipid carrier systems did not change with the addition of synergists. In toxicological bioassays, nanoencapsulated bifenthrin with DEM increased the susceptibility of *E. heros* to bifenthrin by 3.50-fold, as indicated by LC_{90} values. A high susceptibility to nanoencapsulated bifenthrin and λ-cyhalothrin with PBO was also detected in *C. includens*, reveling a synergistic ratio up to 2.16-fold according to LC_{90} values. We also found that nanoencapsulated bifenthrin and λ-cyhalothrin with PBO and DEM increased mortality of *E. heros* (68% and 72%) and *C. includens* (33% and 21%), compared to commercial products with bifenthrin and λ-cyhalothrin. Our results indicate the utility of synergists in reversing λ-cyhalothrin and bifenthrin resistance in *E. heros* and *C. includens* and suggest a significant role of metabolic mechanisms in the detoxification of both pyrethroids. We also reported that it is possible to develop nanoencapsulated-based formulations of bifenthrin and λ-cyhalothrin with the synergists PBO and DEM, which demonstrate the potential to restore the susceptibility of *E. heros* e *C. includens* to pyrethroids. This is the first study to report the development of nanoencapsulated insecticides with synergists for insect resistance management.

Keywords: Soybean pest. Insecticide. Nanoformulation. Control.

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1 INTRODUÇÃO

A cultura da soja é atacada por diversos insetos-praga durante todo o seu desenvolvimento. Dentre estes, se destacam a lagarta-falsa-medideira, *Chrysodeixis includens* (Walker, [1858]) (Lepidoptera: Noctuidae), e o percevejo-marrom, *Euschistus heros* (Fabricius, 1798) (Hemiptera: Pentatomidae) (STACKE et al., 2019; PANIZZI; LUCINI; ALDRICH, 2022). *Chrysodeixis includens* caracteriza-se por ser uma espécie desfolhadora e com capacidade de ocasionar danos econômicos significativos à soja (BUENO et al., 2010; MARTINS; TOMQUELSKI, 2015). Embora essa espécie, até meados da década de 1990, era considerada praga-secundária da soja (MORAES; LOECK; BELARMINO, 1991), atualmente tem sido um sério problema fitossanitário, devido aos seus surtos populacionais em todas as regiões produtoras de soja (BERNARDI et al., 2014; SOSA-GÓMEZ; OMOTO, 2012).

De forma similar, *E. heros* têm ampla ocorrência e distribuição no Brasil (DEGRANDE; VIVAN, 2008; PANIZZI; BUENO; SILVA, 2012; PANIZZI, 2015; MARQUES et al., 2019). Trata-se de um inseto sugador que ataca principalmente a cultura da soja, podendo ocasionar perdas significativas à produtividade (FIORIN et al., 2011; SOUZA et al., 2016, TIBOLA et al., 2021). *Euschistus heros* também ocasiona danos em milho e algodão (ROZA-GOMES et al., 2011; SORIA et al., 2017). As perdas geradas por *E. heros* em soja podem atingir até 30% (VIVAN; DEGRANDE, 2011), sendo decorrentes danos diretos, provocados pela alimentação nas vagens (aborto, deformação dos grãos ou redução do peso e qualidade da semente) e indiretos por provocarem distúrbios fisiológicos que retardam a maturação normal das plantas, além de serem porta de entrada de infecção dos grãos por patógenos (DEPIERI; PANIZZI, 2011, SILVA et al., 2012; GIACOMETTI et al., 2016; SOUZA et al., 2016).

O controle químico tem sido uma das principais estratégias para controle de *C. includens* e *E. heros* em soja (PANIZZI, 2013; BUENO et al., 2020). O controle de *C. includens* com inseticidas é limitado pela dificuldade do produto em atingir o alvo (lagartas), que fica localizado na região mediana do dossel da planta (GUEDES et al., 2012; MOSCARDI et al., 2012) ou por falhas de controle devido à evolução da resistência (STACKE et al., 2019; 2020). *Euschistus heros* tem sido manejado quase que exclusivamente com inseticidas, no entanto, algumas moléculas inseticidas (principalmente do grupo dos piretroides) tem apresentado reduzida eficácia (SOMAVILLA et al., 2020). De maneira geral, o uso frequente de inseticidas, tem favorecido a seleção de indivíduos resistentes em ambas as

espécies, dificultando o seu manejo (SOSA-GÓMEZ; CORSO; MORALES, 2001; TUELHER et al., 2018; SOMAVILLA et al., 2020; STACKE et al., 2020).

Ao ocorrer a evolução da resistência, os insetos desenvolvem mecanismos para dificultar que os inseticidas atinjam o sítio de ação, ou mesmo, se o atingir, não exerçam seu poder letal (GRESSEL, 2019; DAVID, 2021). Sendo assim, os insetos podem evoluir para resistência mediante mecanismos de redução na penetração cuticular, aumento na taxa de metabolismo do inseticida pela atividade de enzimas esterases, monooxigenases, oxidases ou glutathiona-S-transferases, ou por uma alteração no sítio de ação do produto (MUTERO et al., 1994). Por exemplo, *C. includens* tem a capacidade de desintoxicar e excretar determinados inseticidas antes mesmo da ativação do composto *in vivo* (MARTIN; BROWN, 1984). Essa capacidade está relacionada com algumas enzimas, como glutationa-transferases e monooxigenases que são as principais responsáveis pela baixa suscetibilidade de *C. includens* a inseticidas moduladores dos canais de sódio (piretroides) (DOWD; SPARKS, 1986; ROSE et al., 1990; THOMAS; BOETHEL, 1995; THOMAS et al., 1996; PERINI et al., 2021). De forma similar, em *E. heros* foi reportado que as enzimas α-esterase, β-esterase e glutationa S-transferases exercem a atividade de desintoxicação metabólica de piretroides em populações de baixa suscetibilidade a este modo de ação (SOSA-GÓMEZ et al., 2009; 2019).

Nesse contexto, o uso de sinergistas como butóxido de piperonila (PBO) pode ser útil para reduzir a atividade de enzimas oxidativas dependentes do citocromo P-450, pois o PBO inibe a ação do citrocomo P-450, reduzindo a incorporação do oxigênio na molécula inseticida, ou seja, essa é pouco degradada e maior quantidade de ingrediente ativo atinge o sítio de ação (MAMIDALA; JONES; MITTAPALLI, 2011; FEYEREISEN, 2014). Para GSH-transferases, o sinergista é o maleato de dietila (DEM) (CHELVANAYAGAM; PARKER; BOARD, 2000; ENAYATI; RANSON; HEMINGWAY, 2005; HILLIOU et al., 2021). Nesse sentido, o uso de sinergistas pode reduzir a quantidade de inseticida necessária para controle de insetos, pois eles retardam ou impedem a desintoxicação ou eliminação dos inseticidas (METCALF, 1967; CASIDA, 1970; GONZALEZ-MORALES; ROMERO, 2019; NAUEN et al., 2021). Portanto, entender o efeito dos sinergistas na toxicidade de inseticidas em *C. includens* e *E. heros* é importante para subsidiar o manejo destas espécies-praga.

O uso de nanotecnologia na agricultura tem demonstrado grande potencial na criação de novas formulações, bem como, para melhorar a eficiência e segurança dos pesticidas s (ZHAO et al., 2018). Estas formulações podem proporcionar maior proteção do ingrediente ativo, melhorar a estabilidade, absorção e eficácia de controle, minimiza os efeitos adversos em organismos não-alvo e reduzem a necessidade de pulverizações (BORGATTA et al.,

2018; OLIVEIRA et al., 2019a). Para a nanoformulação de pesticidas, umas das técnicas utilizadas têm sido via sistemas carreadores com nanopartículas lipídicas nanoestruturadas (SLN), as quais tem o potencial de evitar a rápida degradação, melhorar a estabilidade e eficácia dos pesticidas (OLIVEIRA et al. 2019b). Nesse contexto, o desenvolvimento de nanoformulações de inseticidas com sinergistas para o manejo da resistência de insetos-praga pode ser uma alternativa viável para prolongar a vida útil das moléculas. Diante disso, os objetivos deste estudo foram:

1) Avaliar a adição dos sinergistas PBO e DEM para o manejo de *E. heros* e *C. includens* com resistência a λ-cialotrina e bifentrina.
2) Desenvolver e avaliar nanoformulações de bifentrina e λ-cialotrina com PBO e DEM para o manejo da resistência de *E. heros* e *C. includens* a piretroides.

2 ARTIGO 1

The effect of synergistic compounds on the susceptibility of *Euschistus heros* (Hemiptera: Pentatomidae) and *Chrysodeixis includens* (Lepidoptera: Noctuidae) to pyrethroids

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Abstract — The Neotropical brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae), and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), are key pests of soybean in South America. Low susceptibility to pyrethroids has been reported for both species in Brazil. Here, we evaluate the addition of synergistic compounds piperonyl butoxide (PBO) and diethyl maleate (DEM) to manage *E. heros* and *C. includens* with resistance to λ-cyhalothrin and bifenthrin. The LD_{50} of technical grade and commercial products containing λ-cyhalothrin and bifenthrin decreased against field-collected *E. heros* exposed to PBO and DEM relative to unexposed insects; synergistic ratios up to 4.75-fold. The mortality also increased when *E. heros* were exposed to commercial formulations containing λ-cyhalothrin (from 4% to 44%) and bifenthrin (from 44% to 88%) in the presence of synergists. There was also a higher susceptibility of field-collected *C. includens* to technical grade λ-cyhalothrin when PBO was used; synergistic ratio of 5.50-fold. High lethally of technical grade λ-cyhalothrin was also verified in the presence of PBO, with mortality increasing from 6% to 57%. Our findings indicate the potential utility of synergists in reversing the resistance to λ-cyhalothrin and bifenthrin in *E. heros* and *C. includens* and suggest a significant role of metabolic mechanisms underlying the detoxification of both pyrethroids.

Keywords: Neotropical brown stink bug, soybean looper, insecticide, resistance management

Introduction

The Neotropical brown stink bug, *Euschistus heros* (F. 1798) (Hemiptera: Pentatomidae), and the soybean looper, *Chrysodeixis includens* (Walker [1858]) (Lepidoptera: Noctuidae), are two of the major pests of soybean in South America (Panizzi 2015, Horikoshi et al. 2021, Panizzi et al. 2022). The damage from *E. heros* is caused by the insertion of the stylet into the plants that causes leaf retention in vegetative stages or reduction in grain weights and quality when feeding on pods (Panizzi and Slansky Jr. 1985, Sosa-Gómez and Moscardi 1995, Souza et al. 2016). Conversely, *C. includens* causes defoliation that reduces the photosynthesis capacity, thus affecting yield (Bueno et al. 2010, Martins and Tomquelski 2015).

The use of insecticides is one of the main strategies for controlling *E. heros* and *C. includens* in soybean in South America (Panizzi 2013, Bueno et al. 2020, Ramos et al. 2020). Pyrethroids (voltage-gated sodium channel modulators) are one of the major modes of action (MoA) applied for managing these pests in soybean fields in Brazil, with more than 60 commercial products registered for use in soybean (Agrofit 2021). The repeated and longtime use of pyrethroids against *E. heros* and *C. includens* selected insects with reduced susceptibility to this MoA in the main regions of soybean production in Brazil (Tuelher et al. 2018, Stacke et al. 2019, Somavilla et al. 2020, Stacke et al. 2020, Perini et al. 2021).

The major mechanisms that mitigate pyrethroid actions in insects are linked with altered target-site sensitivity or detoxification processes (Enayati et al. 2005, Khot et al. 2008, Mamidala et al. 2011, Dang et al. 2017, Hilliou et al. 2021). For instance, the low susceptibility of *E. heros* to λ-cyhalothrin was associated with differential expression of α-esterase, β-esterase, glutathione-S-transferases, acetylcholinesterase, and 7-ethoxycoumarin-O-deethylase, (Sosa-Gómez et al. 2009, Sosa-Gómez et al. 2019) whereas the resistance to λ-

cyhalothrin by *C. includens* was linked with overexpression of cytochrome P-450 and glutathione S-transferase enzymes (Perini et al. 2021).

The resistance of insects to insecticides can be inhibited or reversed by synergists (Metcalf 1967, Hodgson 1983, Bernard and Philogene 1993, Feyereisen 2014, Gonzalez-Morales and Romero 2019, Nauen et al. 2021). From a practical viewpoint, understanding the mechanisms of detoxification by using synergistic agents can shed light on the activity of insecticides in insects. Previous studies stated that the synergist piperonyl butoxide (PBO), an inhibitor of detoxification enzymes from cytochrome P-450 monooxygenases, increased the susceptibility of *E. heros* to λ-cyhalothrin, β-cyfluthrin and thiamethoxam and diethyl maleate (DEM), an inhibitor of glutathione S-transferase and esterase enzymes, improved its susceptibility to acephate (Sosa-Gómez et al. 2019). For lepidopteran pests, the synergist PBO reduced the resistance of *Spodoptera litura* F., and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) to deltamethrin (Ishtiaq et al. 2012, Cheema et al. 2020) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to deltamethrin and fenvalerate (Durigan et al. 2017). It was also reported that the synergist DEM increased the susceptibility of *S. litura* to several pyrethroids (Kaur and Kang 2015).

On this basis, the objective of this study was to evaluate the addition of synergists PBO and DEM to manage *E. heros* and *C. includens* with resistance to λ-cyhalothrin and bifenthrin.

Material and Methods

Collecting insects and rearing. During the 2021 crop season, two populations of *E. heros* were collected in soybean fields in Restinga Sêca (29°44’00”S; 53°26’43”W) and Santiago (29°18’47”S; 54°38’30”W), RS, Brazil. At the same time, a single population of *C. includens*

was obtained from a non-Bt soybean area in Campo Mourão, PR, Brazil (23°53'44"S; 52°19'42"W). These populations were chosen because low susceptibility to λ-cyhalothrin (resistance ratio >12-fold) and bifenthrin (resistance ratio >40-fold) has already been reported (Stacke et al. 2019, Somavilla et al. 2020). After collection, insects were transported to the laboratory with *E. heros* maintained on aerated plastic containers (41 cm long × 29 cm wide × 13 cm height) with fresh green bean pods (*Phaseolus vulgaris* L.), dry soybean seeds and peanuts (*Arachis hypogaea* L.), whereas larvae of *C. includens* were transferred to 50-ml plastic cups containing an artificial diet proposed by Greene et al. (1976). In the bioassays, we also used a susceptible population of each species (hereafter susceptible), which had been maintained in the laboratory for more than 7 years without exposure to insecticides. All populations were placed in the laboratory in a room at 25 ± 2°C, 65 ± 5% RH, and a photoperiod of 14:10 h.

Insecticides. Technical grade λ-cyhalothrin (96.5% purity; Sumitomo Chemical Brasil Indústria Química S.A., Maracanaú, CE, Brazil) and bifenthrin (99.1% purity; Sigma-Aldrich, São Paulo, SP, Brazil) were used in topical bioassays. We also used the commercial products containing λ-cyhalothrin (Kaiso 250 CS, 250 g a.i./L, Sumitomo Chemical Brasil Indústria Química S.A., Maracanaú, CE, Brazil) and bifenthrin (Talstar 100 EC, 100 g a.i./L, FMC Química do Brasil Ltda, Campinas, SP, Brazil) in dip-test and ingestion bioassays. The lethality of previous active ingredients against both species was evaluated in the presence of two synergists: piperonyl butoxide (PBO, 88% purity; Sigma-Aldrich, São Paulo, SP, Brazil), and diethyl maleate (DEM, 97% purity; Sigma-Aldrich, São Paulo, SP, Brazil). Non-lethal doses of PBO and DEM for *E. heros* were based on Sosa-Gómez et al. (2019) and for *C. includens* obtained in preliminary topical bioassays as described below.

Topical bioassays. For the bioassays with *E. heros*, initially fresh green bean pods were washed in 1% chlorine solution and allowed to dry. Then, bean pods were cut into pieces ~5 cm in length. Test arenas were prepared by placing a sheet of filter paper in Petri dishes (100 × 15 mm) and the paper was moistened with distilled water (1 ml), and then 2 pieces of bean pod were placed into each arena. Adults of *E. heros* were exposed to synergists and insecticides using two methods: (i) PBO or DEM at a concentration of 300.00 ppm were applied topically (dorsum 2 µl) for each stink bug, and at 30 min post-exposure, each insect received λ-cyhalothrin or bifenthrin (2 µl) diluted in acetone (99.5% purity; Sigma Aldrich, São Paulo, SP, Brazil), and (ii) PBO or DEM at the same concentration above was added to the solution of insecticide + acetone and then applied topically to each stink bug (2 µl). For each insecticide and bioassay method, 4–8 concentrations were tested using a hand microapplicator (Burkard Manufacturing, Rickmansworth, UK). Each replicate was composed of 5 stink bugs in a total of 5–6 replicates/concentration. Mortality was assessed after 72 h.

Bioassays with *C. includens* were performed in 24-well acrylic plates (Costar®, São Paulo, SP, Brazil) containing an artificial diet (1 ml/well) proposed by Greene et al. (1976). Early L3 larvae of *C. includens* were exposed to insecticides and synergists while also using two procedures: (i) PBO or DEM applied to the larvae pronotum (1µl/larva) at concentrations of 100 and 1000 ppm, respectively, and 2 h post-exposure, each larva received λ-cyhalothrin or bifenthrin (1µl) diluted in acetone, or (ii) PBO or DEM added to the solution of insecticide + acetone at the same concentrations above and then applied topically (1 µl/larva). From 6–9 concentrations of each insecticide were tested by each bioassay method, with 4 replicates (plates) per concentration. Mortality was assessed after 48 h. Petri dishes (arenas) and plates were maintained in a room at 25 ± 2°C, 65 ± 5% RH, and a photoperiod 14:10 h.

In addition to previous bioassays, single doses of technical grade λ-cyhalothrin and bifenthrin that caused more than 80% mortality of the susceptible populations in dose-

response bioassays were defined (0.11 µg a.i./stink bug and 0.90 µg a.i./larva) and then applied in field populations with and without synergists, following the same procedures and experimental design previously described.

Dip-test bioassays. The commercial products containing λ-cyhalothrin and bifenthrin were diluted in distilled water to prepare the concentrations to be tested. Triton X-100 (Sigma-Aldrich, São Paulo, SP, Brazil) at 0.1% was added to each concentration to enable spreading on the diet or pods surface. Test arenas and plates were prepared as previously described. Insects were exposed to PBO or DEM applied 30 min. (*E. heros*) or 2 h (*C. includens*) before exposure to λ-cyhalothrin and bifenthrin, following the same procedures earlier described. Then, adults of *E. heros* were placed in Petri dishes (arenas) containing bean pods treated with 4–6 doses of λ-cyhalothrin or bifenthrin (pods were immersed for 5 s in the insecticide solution), whereas L3 larvae of *C. includens* were transferred to acrylic plates with the diet surface treated with 6–8 concentrations of λ-cyhalothrin (30 µl/well). Single doses of commercial formulations containing λ-cyhalothrin (0.12 µg a.i./ml and 0.51 µg a.i./cm^2 for *E. heros* and *C. includens*, respectively) and bifenthrin (2 µg a.i./ml) were chosen as earlier described and used against field populations in the presence of PBO and DEM, following the same bioassay methods described above. The number of replicates and mortality assessment was the same as for topical bioassays.

Statistical analysis. Concentration-mortality data were initially subjected to a goodness of fit test to measure how well data fit the assumptions of the Probit model using a χ^2 test. Then, mortality data were subjected to Probit analysis to estimate LD_{50} and respective confidence interval (95% CI) using the PROC PROBIT procedure in SAS® 9.1 (SAS Institute 2000). The LD_{50} values were pairwise compared, and significance was declared if 95% CIs did not

overlap (Dorai-Raj 2009, Savin et al. 1977). The synergistic ratios (SR) were calculated by dividing the LD_{50} of insects treated with insecticide by the LD_{50} of insects treated with synergist + insecticide. Mortality of insects exposed to single doses of λ-cyhalothrin and bifenthrin in the presence of PBO and DEM was corrected based on controls (untreated insects) (Abbott 1925). Corrected mortalities were then submitted to analysis of variance (ANOVA) and means were compared by the Scott-Knott test ($P < 0.05$) using the R software (R Development Core Team 2017).

Results

Synergist effects on *E. heros*. The estimated LD_{50} values of technical grade λ-cyhalothrin and bifenthrin against the susceptible population of *E. heros* pre-exposed to PBO and DEM or both synergists were added in the insecticide solution in topical bioassays did not differ due to overlapping of 95% CIs (Table 1). For this population, the LD_{50} values of technical grade λ-cyhalothrin and bifenthrin with and without synergists ranged from 0.04 to 0.05 µg a.i./stink bug, indicating synergistic ratios <1.33-fold. Contrary to this, the susceptibility to technical grade λ-cyhalothrin and bifenthrin by field populations of *E. heros* significantly increased when insects were pre-exposed to PBO and DEM or synergists were added to the insecticide solution in topical bioassays, as demonstrated by the non-overlap of 95% CIs of LD_{50} values (Table 1). Synergists reduced the LD_{50} values of λ-cyhalothrin from 0.17 to 0.05 µg a.i./stink bug and bifenthrin from 0.09 to 0.03 µg a.i./stink bug, showing a synergistic ratio up to 3.40-fold.

When insecticides and synergists were used in dip-test bioassays, the lethality of commercial insecticides based on λ-cyhalothrin and bifenthrin also increased against the populations of *E. heros* tested (Table 2). The LD_{50} values differed significantly (based on

non-overlap of 95% CIs), ranging from 44.39 to 75.53 µg a.i./ml for λ-cyhalothrin and from 58.79 to 105.72 µg a.i./ml for bifenthrin against the susceptible population pre-exposed to PBO and DEM, whereas it was 715.58 µg a.i./ml and 193.02 µg a.i./ml for unexposed insects, respectively. These results indicated synergistic ratios up to 16.12-fold. For the field populations, the LD_{50} values of λ-cyhalothrin also differed significantly, ranging from 443.53 to 853.83 µg a.i./ml for insects treated with PBO and DEM, whereas for untreated insects, the LD_{50} was 2106.81 µg a.i./ml (Table 2). For bifenthrin the LD_{50} values were 744.49 and 1323.86 µg a.i./ml for insects pre-exposed to PBO and DEM, respectively, while the value was >2700 µg a.i./ml for unexposed insects. These findings indicate that PBO and DEM increased the lethality of λ-cyhalothrin and bifenthrin up to 4.75- and 3.72-fold for the susceptible and field populations of *E. heros*, respectively.

At a fixed dose of technical grade λ-cyhalothrin, the mortality rate of *E. heros* did not differ for the susceptible (80% to 84%; $F = 0.49$; $df = 4, 16$; $P = 0.7434$) and field (68% to 72%; $F = 2.42$; $df = 4, 10$; $P = 0.0824$) populations when exposed to PBO and DEM in topical bioassays (Fig. 1A). The mortality rate was also similar for the susceptible population of *E. heros* exposed to a fixed dose of technical grade bifenthrin and both synergists, ranging from 83.3% to 93.3% ($F = 0.72$; $df = 4, 17$; $P = 0.5867$) (Fig. 1B). However, the field population of *E. heros* had a significantly higher susceptibility to bifenthrin when insects were pre-exposed to PBO and DEM or both synergists were added in the insecticide solution, with increased mortality of 36% ($F = 3.27$; $df = 4, 16$; $P = 0.0323$) (Fig. 1B). In dip-test bioassays using a fixed dose of the commercial λ-cyhalothrin, the mortality of the susceptible and field populations also increased when insects were pre-exposed to synergists ($F = 21.44$; $df = 2, 72$; $P < 0.0001$ for the susceptible and $F = 7.64$; $df = 2, 22$; $P = 0.0073$ for the field population) (Fig. 2A). The lethality of commercial insecticide containing λ-cyhalothrin in the presence of PBO and DEM increased in 72% and 52%, respectively, for the susceptible population,

whereas for the field population this increase was near to 40%. At the fixed dose of commercial insecticide bifenthrin, the mortality ratios did not vary for insects from the susceptible (F = 0.50; df = 2, 26; P = 0.6186) and field (F = 2.52; df = 2, 25; P = 0.1220) populations pre-exposed or unexposed to synergists (Fig. 2B). The mortality rates were 96% and 44% for the susceptible and field populations, respectively.

Synergist effects on *C. includens*. There were no significant differences in LD_{50} values of technical grade λ-cyhalothrin (based on the overlap of 95% CIs) against the susceptible population of *C. includens* pre-exposed to PBO and DEM or when synergists were added to the insecticide solution in topical bioassays (Table 3). The LD_{50} values ranged from 0.005 to 0.008 µg a.i./larva, suggesting synergist ratios <1.60-fold. For larvae from the field population, the LD_{50} values of technical grade λ-cyhalothrin ranged from 0.02 to 0.11 µg a.i./larva differing significantly only when PBO was added to the solution, as demonstrated by its non-overlap of 95% CIs. These results revealed a synergistic ratio of 5.50-fold with the presence of PBO (Table 3).

In ingestion bioassays, the susceptible population of *C. includens* had similar susceptibility to commercial insecticide containing λ-cyhalothrin (LD_{50} = 0.07 to 0.08 µg a.i./cm^2 of diet) independently of the presence or absence of synergists, as indicated by the overlapping of 95% CIs of LD_{50} values (Table 4). These results indicate synergistic ratios <1.14-fold. For the field population, the LD_{50} values of λ-cyhalothrin varied from 0.47 and 1.12 µg a.i./cm^2 for insects pre-exposed to PBO and DEM, respectively, while for unexposed insects was 1.17 µg a.i./cm^2 (Table 4). A significantly synergistic effect was only observed when larvae were pre-exposed to PBO, resulting in a synergistic ratio of 2.47-fold.

At the fixed dose of technical grade and commercial insecticide based on λ-cyhalothrin, the mortality rates of a susceptible population of *C. includens* exposed or unexposed to PBO and

DEM in topical (F = 0.34; df = 4, 51; P = 0.8452) and ingestion (F = 1.00; df = 2, 12; P = 0.4053) bioassays were similar (Fig. 3A and 3B). Mortality rates ranged from 63% to 73% and 95% to 98% in these bioassay methods, respectively. In contrast, the mortality of *C. includens* significantly increased in 13% and 46% when DEM and PBO, respectively, were used with technical grade λ-cyhalothrin (F = 15.38; df = 4, 13; P < 0.0001) (Fig. 3A). Similarly, the commercial insecticide λ-cyhalothrin had 15% and 48% higher lethality against *C. includens* from the field population when pre-exposed to PBO and DEM, respectively (F = 59.64; df = 2, 24; P < 0.0001) (Fig. 3B).

Discussion

An increased lethality of λ-cyhalothrin and bifenthrin was observed, mainly against field-collected *E. heros* when pre-exposed to PBO and DEM or when both synergists were added to the insecticide solutions. Results from this work also indicate a more pronounced lethality of λ-cyhalothrin against field-collected *C. includens* when larvae were pre-exposed to PBO. The low effects of synergists in the toxicity of pyrethroids against susceptible populations can be explained by it not altering expression of enzymes that detoxify insecticides, as they were not exposed to selection pressure for a long time. Previous studies also indicated that field populations of *E. heros* were most susceptible to λ-cyhalothrin + thiamethoxam, β-cyfluthrin, and thiamethoxam when pre-exposed to PBO, whereas the synergist DEM increased its susceptibility to acephate (Sosa-Gómez et al. 2019). According to our findings and others, it is evident that metabolic detoxification enzymes from cytochromes P-450, glutathione S-transferases and esterases had a significant role underlying the detoxification of pyrethroids by these pest species.

The effects of the synergistic compounds PBO and DEM in altering the susceptibility to insecticides by several other pest species were also previously reported. Studies stated that the resistance of *S. litura* to fenvalerate and *H. armigera* to deltamethrin and fenvalerate diminished when larvae were pre-exposed to PBO (Durigan et al. 2017, Cheema et al. 2020). The synergist PBO also restored the susceptibility of *H. armigera* and *S. frugiperda* to fenvalerate and zeta-cypermethrin (Young et al. 2005, Joußen and Heckel 2021). Similarly, the lethality of avermectin, methamidophos, fenvalerate, and fipronil were also more pronounced against larvae of *P. xylostella* pre-exposed to PBO (Wu et al. 2007). It was also reported that the synergist DEM abolished the resistance of *S. litura* to deltamethrin (Kaur and Kang 2015).

The lethality of λ-cyhalothrin and bifenthrin against field-collected *E. heros* was directly influenced by metabolic detoxification enzymes. According to our results, the major metabolic enzymes that detoxify pyrethroids in *E. heros* were P-450 monooxygenases and glutathione S-transferases. Previous studies also demonstrated that the low susceptibility of *E. heros* to other pyrethroids was associated with high expression of α-esterase, β-esterase, and glutathione S-transferases (Sosa-Gómez et al. 2009, Sosa-Gómez et al. 2019). Similar mechanisms of resistance were related to the metabolic detoxification of pyrethroids by bed bugs of the genus *Cimex* (Hemiptera: Cimicidae) (How and Lee 2011, Lilly et al. 2016).

The toxicity of λ-cyhalothrin also increased against field-collected *C. includens* in the presence of PBO. This finding indicates that detoxification enzymes from cytochrome P-450 monooxygenases are involved with the mechanism of resistance. Earlier studies using a *C. includens* strain resistant to λ-cyhalothrin also revealed that the detoxification of this insecticide was linked with the overexpression of P-450 and glutathione S-transferases enzymes (Perini et al. 2021). Similar mechanisms of resistance to pyrethroids were reported in

several other lepidopteran pests (Ishtiaq et al. 2012, Kaur and Kang 2015, Durigan et al. 2017, Cheema et al. 2020).

The restoration of susceptibility to insecticides by insects with the use of synergists can be explained by the reduction in the activity of detoxification enzymes (Metcalf 1967, Feyereisen 2014, Nauen et al. 2021). The synergist PBO acts in the active sites of some detoxification P-450 enzymes metabolizing it to an irreversible inhibitor complex between a carbene radical of the methylenedioxyphenyl group and the ferrous iron of the P-450. Because some P-450 enzymes are involved with the physiological process of detoxification, the effect of PBO reducing its activity allows insecticides to exert their toxic effects before metabolization (Correia and Ortiz de Montellano 2005, Mamidala et al. 2011, Feyereisen 2014). Similarly, the synergist DEM inhibits glutathione S-transferases that play a central role in the detoxification of xenobiotics. These enzymes can metabolize insecticides into water-soluble metabolites that are more readily excreted (Atkins et al. 1993, Enayati et al. 2005). The effects of DEM reducing the activity of glutathione S-transferases favor a greater amount of active ingredient reaching the target sites to exert its lethality.

The present study documents that the synergistic compounds PBO and DEM increased the lethality of λ-cyhalothrin and bifenthrin against field populations of *E. heros*, whereas PBO mainly increased its lethality against *C. includens*. These results indicate that the physiological detoxification process by enzymes from cytochrome P-450 monooxygenases and glutathione S-transferases are involved with the metabolic detoxification of λ-cyhalothrin and bifenthrin by *E. heros*, whereas P-450 monooxygenase enzymes are the major ones responsible for the reduced susceptibility of *C. includens* to previous pyrethroids. These findings provide new insights for the design of insecticides formulations containing synergistic compounds to reverse resistance to pyrethroid-based insecticides and prolong the

lifetime of chemistries for managing these notorious pest species in Brazil, where the resistance to pyrethroids is already reported.

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Table 1. Lethal doses of technical grade λ-cyhalothrin (insects from Restinga Sêca) and bifenthrin (insects from Santiago) combined with PBO or DEM against populations of *E. heros* in topical bioassays.

Treatment	*n*	Fit of probit lines			LD_{50}[b]	SR[c]
		Slope ± SE	χ^2 (*df*[a])	*P*		
— Susceptible *E. heros* —						
λ-cyhalothrin	175	2.58 ± 0.52	0.80 (4)	0.98	0.05 (0.03–0.06) a	–
PBO (30 min. before) + λ-cyhalothrin	200	2.74 ± 0.83	3.48 (5)	0.70	0.04 (0.02–0.06) a	1.25
DEM (30 min. before) + λ-cyhalothrin	200	2.31 ± 0.35	1.67 (5)	0.91	0.04 (0.03–0.05) a	1.25
λ-cyhalothrin and PBO (both in acetone)	240	2.43 ± 0.41	1.57 (5)	0.87	0.05 (0.03–0.06) a	1.00
λ-cyhalothrin and DEM (both in acetone)	270	2.26 ± 0.35	3.00 (6)	0.96	0.05 (0.03–0.07) a	1.00
Bifenthrin	175	2.43 ± 0.40	2.32 (4)	0.99	0.04 (0.02–0.05) a	–
PBO (30 min. before) + Bifenthrin	240	2.69 ± 0.45	2.80 (5)	0.91	0.04 (0.03–0.05) a	1.00
DEM (30 min. before) + Bifenthrin	210	2.77 ± 0.45	1.59 (4)	0.86	0.04 (0.03–0.04) a	1.00
Bifenthrin and PBO (both in acetone)	175	2.39 ± 0.50	3.68 (4)	0.79	0.03 (0.02–0.05) a	1.33
Bifenthrin and PBO (both in acetone)	175	3.22 ± 0.55	2.07 (4)	0.83	0.03 (0.03–0.04) a	1.33
— Field *E. heros* —						
λ-cyhalothrin	200	1.84 ± 0.34	1.21 (5)	0.94	0.17 (0.09–0.25) a	–
PBO (30 min. before) + λ-cyhalothrin	225	1.84 ± 0.29	2.02 (6)	0.91	0.07 (0.04–0.11) ab	2.43
DEM (30 min. before) + λ-cyhalothrin	175	1.43 ± 0.29	0.78 (4)	0.92	0.05 (0.02–0.09) b	3.40
λ-cyhalothrin and PBO (both in acetone)	200	1.51 ± 0.28	3.13 (6)	0.79	0.14 (0.07–0.21) ab	1.21
λ-cyhalothrin and DEM (both in acetone)	200	1.53 ± 0.29	3.43 (5)	0.67	0.06 (0.03–0.09) b	2.83
Bifenthrin	175	3.59 ± 0.61	2.16 (4)	0.70	0.09 (0.07–0.11) a	–
PBO (30 min. before) + Bifenthrin	125	2.62 ± 0.55	1.88 (2)	0.38	0.08 (0.05–0.13) ab	1.13
DEM (30 min. before) + Bifenthrin	125	2.24 ± 0.54	0.23 (2)	0.88	0.03 (0.02–0.05) b	3.00
Bifenthrin and PBO (both in acetone)	125	2.02 ± 0.44	0.02 (2)	0.98	0.07 (0.04–0.11) ab	1.29
Bifenthrin and PBO (both in acetone)	125	1.75 ± 0.33	1.97 (2)	0.37	0.04 (0.02–0.06) b	2.25

[a]Degrees of freedom.

[b]LD_{50}: dose of technical grade insecticide (µg a.i./stink bug) required to kill 50% of insects after 72 h. LD_{50} values designated by different letters within a column for each insecticide are significantly different from each other through non-overlap of 95% CIs.

[c]Synergistic Ratio (SR) = LD_{50} of insecticides without synergist/LD_{50} of synergist + insecticide.

Table 2. Lethal doses of commercial products containing λ-cyhalothrin (insects from Restinga Sêca) or bifenthrin (insects from Santiago) combined with PBO or DEM against populations of *E. heros* in dip-test bioassays.

Treatment	*n*	Fit of probit lines			LD_{50}[b]	SR[c]
		Slope ± SE	χ^2 (*df*[a])	*P*		
— Susceptible *E. heros* —						
λ-cyhalothrin	150	3.17 ± 0.76	1.18 (3)	0.75	715.78 (481.88–934.13) a	–
PBO (30 min. before) + λ-cyhalothrin	125	2.71 ± 0.63	1.27 (2)	0.52	44.39 (27.29–58.85) c	16.12
DEM (30 min. before) + λ-cyhalothrin	150	1.51 ± 0.35	0.07 (3)	0.99	75.53 (25.27–127.48) bc	9.47
Bifenthrin	175	2.66 ± 0.45	2.80 (4)	0.58	193.02 (135.81–250.83) a	–
PBO (30 min. before) + Bifenthrin	150	2.58 ± 0.48	0.86 (3)	0.83	58.79 (41.57–77.28) b	3.28
DEM (30 min. before) + Bifenthrin	150	1.43 ± 0.32	0.91 (3)	0.82	105.72 (44.19–168.24) ab	1.82
— Field *E. heros* —						
λ-cyhalothrin	175	2.94 ± 0.58	1.09 (4)	0.89	2106.81 (1545.21–2691.98) a	–
PBO (30 min. before) + λ-cyhalothrin	150	1.97 ± 0.41	2.42 (3)	0.48	443.56 (284.57–615.46) bc	4.75
DEM (30 min. before) + λ-cyhalothrin	125	4.41 ± 1.14	1.11 (2)	0.71	853.83 (599.83–1045.81) b	2.01
Bifenthrin	125	2.17 ± 0.43	0.45 (2)	0.57	2771.15 (1610.53–4128.27) a	–
PBO (30 min. before) + Bifenthrin	125	2.47 ± 0.51	1.43 (2)	0.48	744.49 (527.90–1019.78) b	3.72
DEM (30 min. before) + Bifenthrin	125	4.02 ± 0.90	0.36 (2)	0.83	1323.86 (947.25–1937.39) ab	2.09

[a]Degrees of freedom.

[b]LD_{50}: dose of commercial insecticides (µg a.i. /ml) required to kill 50% of insects after 72 h. LD_{50} values designated by different letters within a column for each insecticide are significantly different from each other through non-overlap of 95% CIs.

[c]Synergistic Ratio (SR) = LD_{50} of insecticides without synergist/LD_{50} of synergist + insecticide.

Table 3. Lethal doses of technical grade λ-cyhalothrin combined with PBO or DEM against the populations of *C. includens* in topical bioassays.

Treatment	*n*	Fit of probit lines			LD_{50}[b]	SR[c]
		Slope ± SE	χ^2 (*df*[a])	*P*		
— Susceptible *C. includens* —						
λ-cyhalothrin	700	1.68 ± 0.19	8.17 (4)	0.08	0.008 (0.005–0.013) a	–
PBO (2 h before) + λ-cyhalothrin	810	1.48 ± 0.10	6.02 (6)	0.41	0.006 (0.005–0.007) a	1.33
DEM (2 h before) + λ-cyhalothrin	970	1.67 ± 0.16	9.00 (4)	0.06	0.008 (0.005–0.011) a	1.00
λ-cyhalothrin and PBO (both in acetone)	700	1.44 ± 0.14	8.16 (4)	0.08	0.005 (0.003–0.007) a	1.60
λ-cyhalothrin and DEM (both in acetone)	700	1.28 ± 0.16	7.89 (4)	0.09	0.005 (0.003–0.008) a	1.60
— Field *C. includens* —						
λ-cyhalothrin	770	2.29 ± 0.40	9.91 (4)	0.41	0.11 (0.05–0.16) a	–
PBO (2 h before) + λ-cyhalothrin	700	1.48 ± 0.18	9.38 (4)	0.52	0.05 (0.03–0.07) a	2.20
DEM (2 h before) + λ-cyhalothrin	600	2.25 ± 0.20	3.45 (3)	0.32	0.06 (0.05–0.07) a	1.83
λ-cyhalothrin and PBO (both in acetone)	600	2.44 ± 0.36	7.88 (3)	0.48	0.02 (0.01–0.02) b	5.50
λ-cyhalothrin and DEM (both in acetone)	800	2.16 ± 0.20	4.14 (5)	0.52	0.07 (0.06–0.08) a	1.57

[a]Degrees of freedom.

[b]LD_{50}: dose of technical grade insecticide (µg a.i./larva) required to kill 50% of larvae after 48 h. LD_{50} designated by different letters within a column for each insecticide are significantly different from each other through non-overlap of 95% CIs.

[c]Synergistic Ratio (SR) = LD_{50} of insecticides without synergist/LD_{50} of synergist + insecticide.

Table 4. Lethal doses of commercial product containing λ-cyhalothrin combined with the synergists PBO or DEM against populations of *C. includens* in ingestion bioassays.

Treatment	*n*	Fit of probit lines			LD_{50}[b]	SR[c]
		Slope ± SE	χ^2 (*df*[a])	*P*		
— Susceptible *C. includens* —						
λ-cyhalothrin	700	3.27 ± 0.32	3.43 (4)	0.48	0.08 (0.07–0.09) a	–
PBO (2 h before) + λ-cyhalothrin	800	2.91 ± 0.28	4.28 (5)	0.50	0.07 (0.06–0.09) a	1.14
DEM (2 h before) + λ-cyhalothrin	700	2.46 ± 0.22	6.78 (4)	0.14	0.08 (0.07–0.09) a	1.00
— Field *C. includens* —						
λ-cyhalothrin	600	4.08 ± 0.33	0.97 (3)	0.25	1.17 (1.07–1.28) a	–
PBO (2 h before) + λ-cyhalothrin	600	2.22 ± 0.20	5.08 (3)	0.12	0.47 (0.32–0.61) b	2.47
DEM (2 h before) + λ-cyhalothrin	600	3.12 ± 0.27	5.53 (3)	0.13	1.12 (0.97–1.26) a	1.04

[a]Degrees of freedom.

[b]LD_{50}: dose of commercial insecticide (μg a.i./cm^2) required to kill 50% of larvae after 48 h. LD_{50} values designated by different letters within a column for each insecticide are significantly different from each other through non-overlap of 95% CIs.

[c]Synergistic Ratio (SR) = LD_{50} of insecticides without synergist/LD_{50} of synergist + insecticide.

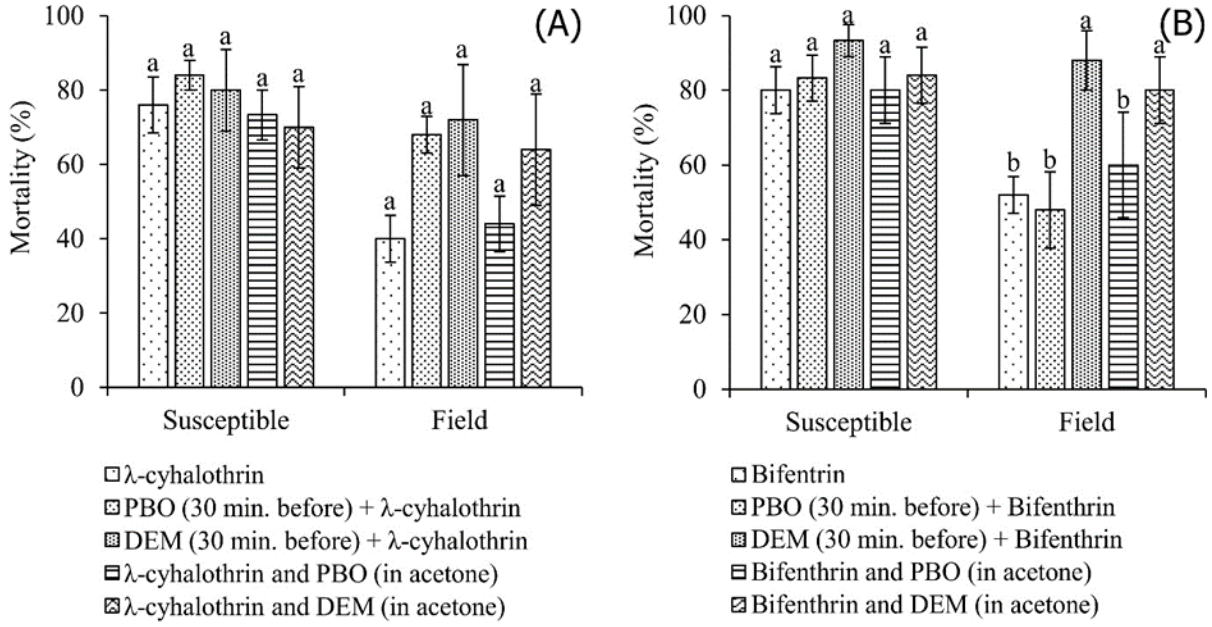

Figure 1. Mortality of the susceptible and field populations of *E. heros* exposed to technical grade λ-cyhalothrin (A) (insects from Restinga Sêca) and bifenthrin (B) (insects from Santiago) at a dose of 0.11 µg a.i./stink bug with and without PBO and DEM in topical bioassays. Group of bars (± SE) with the same letters do not differ significantly.

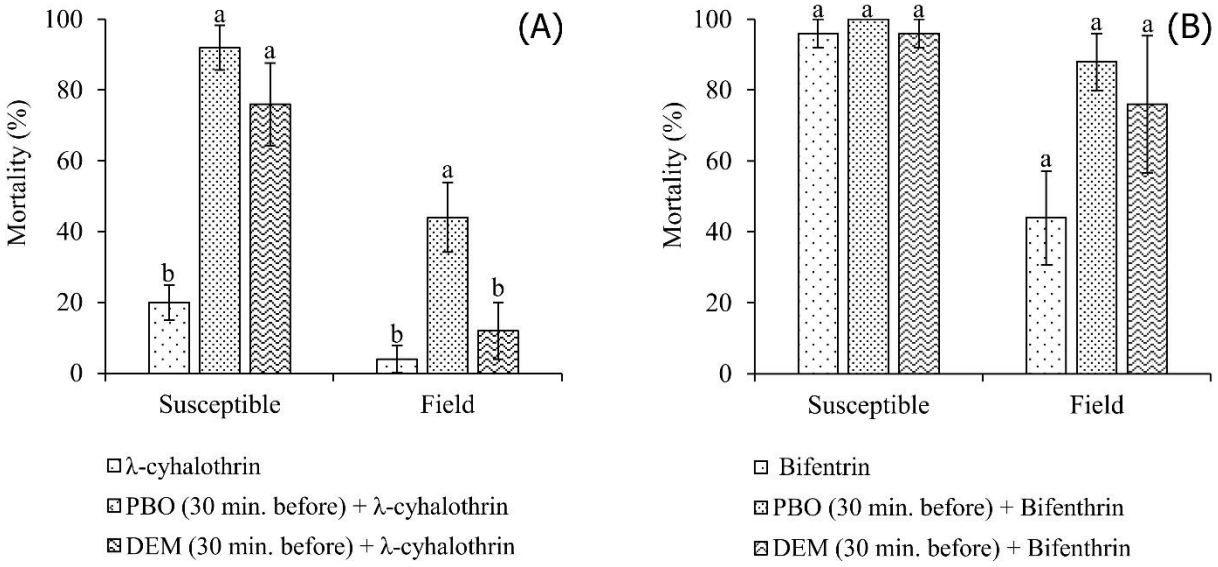

Figure 2. Mortality of the susceptible and field populations of *E. heros* exposed to the commercial products containing λ-cyhalothrin (0.12 µg a.i./ml) (A) (insects from Restinga Sêca) or bifenthrin (2 µg a.i./ml) (B) (insects from Santiago) with and without PBO and DEM in dip-test bioassays. Group of bars (± SE) with the same letters do not differ significantly.

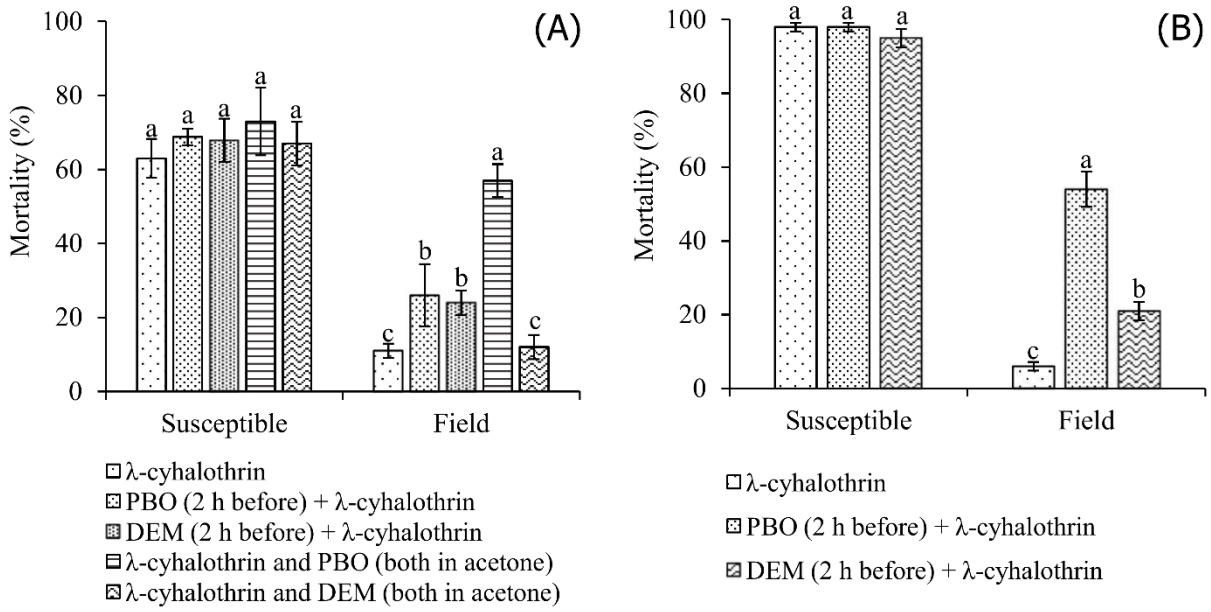

Figure 3. Mortality of *C. includens* populations exposed to technical grade λ-cyhalothrin (0.90 µg a.i./larva) in topical (A) and to the commercial insecticide containing λ-cyhalothrin (0.51 µg a.i./cm^2) in ingestion (B) bioassays with and without PBO and DEM. Group of bars (± SE) with the same letters do not differ significantly.

3 ARTIGO 2

Development and biological evaluation of nanoencapsulated-based pyrethroids in association with synergists for resistance management of soybean pests

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ABSTRACT

Chemical control is one of the main tactics used against Neotropical brown stink bug, *Euschistus heros* (F.), and the soybean looper, *Chrysodeixis includens* (Walker), in soybean fields in South America. Previous studies reported that both species have reduced susceptibility to bifenthrin (BFT) and λ-cyhalothrin (LAM) in Brazil. On this basis, we developed and evaluated nanoencapsulated-based BFT and LAM with the synergistic compounds piperonyl butoxide (PBO) and diethyl maleate (DEM) for the resistance

management of these species. Nanoformulations of BFT and LAM with PBO and DEM presented good physical-chemical characteristics and stability in function of the time (90 d). It was also observed that the spherical morphology of all prepared systems and the encapsulation efficiency in nanostructured lipid carrier systems did not change with the addition of synergists. In toxicological bioassays, nanoencapsulated BFT with DEM increased the susceptibility of *E. heros* to BFT by 3.50-fold, as indicated by LC_{90} values. A high susceptibility to nanoencapsulated LAM and BFT with PBO was also detected in *C. includens* according to LC_{90} values; synergistic ratio up to 2.16-fold. We also detected that nanoencapsulated BFT and LAM with PBO and DEM caused higher mortality of *E. heros* and *C. includens* than the same dose of commercial products containing BFT or LAM only. Our findings indicate that it is possible to develop nanoencapsulated-based formulations of BFT and LAM with the synergists PBO and DEM, and that these nanoformulations have the potential to improve control and restore the susceptibility of *E. heros* and *C. includens* to insecticides. This is the first study to report on the development and to evaluate nanoencapsulated insecticides with synergists for insect resistance management.

Keywords: Neotropical brown stink bug, soybean looper, nanoformulation, sodium channel modulators

1. Introduction

Chemical control is commonly used for managing the Neotropical brown stink bug, *Euschistus heros* (F. 1798) (Hemiptera: Pentatomidae) and the soybean looper, *Chrysodeixis includens* (Walker [1858]) (Lepidoptera: Noctuidae), in soybean fields in South America (Bueno et al., 2013; Panizzi, 2013; Marques et al., 2019; Panizzi et al., 2022). The repeated

use of insecticides with the same mode of action against *E. heros* and *C. includens* favors the selection of resistant populations in Brazilian soybean fields, as reported for the pyrethroids (sodium channel modulators) beta-cyfluthrin, bifenthrin and λ-cyhalothrin (Tuelher et al., 2018; Somavilla et al., 2020; Stacke et al., 2019, 2020; Perini et al., 2021).

The reduced susceptibility to pyrethroids in insects is linked with physiological mechanisms that cause alterations in target-site sensitivity or overexpression of detoxification enzymes (Khot et al., 2008; Mamidala et al., 2011; Dang et al., 2017). Previous studies indicated that the reduced susceptibility to pyrethroid in Brazilian populations of *E. heros* and *C. includens* was associated with the higher expression of detoxification enzymes (Sosa-Gómez et al., 2009, 2019; Perini et al., 2021).

Synergistic compounds can reduce metabolic resistance to insecticide by inhibiting detoxification enzymes responsible for its detoxification in insects (Nauen et al., 2021). For example, the synergist piperonyl butoxide (PBO) acts on cytochrome P450 content reducing the activity of these enzymes in detoxifying insecticides (Mamidala et al., 2011; Feyereisen, 2014). Similarly, the synergist diethyl maleate (DEM) inhibits glutathione S-transferases that play an important role in the detoxification of xenobiotics (Enayati et al., 2005; Hilliou et al., 2021). Previous studies indicated that the exposure of *E. heros* to PBO reduced its resistance ratio to pyrethroids and neonicotinoids, and that DEM improved the susceptibility to acephate, suggesting that these enzymes are involved with the detoxification of these insecticides (Sosa-Gómez et al., 2019). Similarly, in lepidopteran pests such as *Spodoptera litura* F., *Spodoptera exigua* (Hübner), and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) a high susceptibility to pyrethroids was verified in the presence of PBO (Ishtiaq et al., 2012; Kaur and Kang, 2015; Durigan et al., 2017; Cheema et al., 2020). It was also reported that the synergist DEM reduced the resistance of *S. litura* and *S. exigua* to pyrethroids (Kaur and Kang 2015; Hu et al., 2019).

From a practical viewpoint, the use of nanotechnology in agriculture has shown great potential to create new formulations with insecticides (Zhao et al., 2018). The main advantages of these systems are: (i) reduction in the amount of active ingredient needed for biological response; (ii) low environmental contamination; (iii) reduction in energy, labor costs, and number of applications, (iv) greater safety for applicators, and (v) reduced adverse effects on non-target insects (Grillo et al., 2016; Shah et al., 2016; Prasad et al., 2017; Oliveira et al., 2019a).

The development of new insecticide formulations containing synergistic compounds can improve the resistance management and the use of these chemicals in soybean pest management programs. For this, the use of nanostructured lipid carrier systems (SLNs) can contribute to delay the degradation molecules, improving its stability and efficiency (Oliveira et al., 2019b). Many benefits can be obtained by using SLNs, such as lower scale production costs, greater physicochemical stability, hydrophilic and/or hydrophobic encapsulation, and the use of natural products in the preparation of formulation (Naseri et al., 2015; Lingayat et al., 2017). Therefore, the development of nanoformulations of insecticides can maintain a stable concentration of the active ingredient improving its efficacy for pest management, even in small concentrations.

On this basis, we developed and evaluated nanoencapsulated-based bifenthrin and λ-cyhalothrin with the synergistic compounds PBO and DEM for the resistance management of *E. heros* and *C. includens* to pyrethroids in soybean fields.

2. Material and Methods

2.1 Chemicals

The insecticides used in the encapsulation process were technical grade bifenthrin (hereafter BFT) (99.1% purity; Sigma-Aldrich, São Paulo, SP, Brazil) and λ-cyhalothrin (hereafter LAM) (96.5% purity; Sumitomo Chemical Brasil Indústria Química S.A., Maracanaú, CE, Brazil). These insecticides were nanoencapsulated with the synergists PBO (88% purity; Sigma-Aldrich, São Paulo, SP, Brazil), an inhibitor of cytochrome P450s enzymes; and DEM (97% purity; Sigma-Aldrich, São Paulo, SP, Brazil), an inhibitor of glutathione S-transferases in insects. We also used formulated commercial products containing LAM (Kaiso 250 CS, 250 g ai/L, Sumitomo Chemical Brasil Indústria Química SA, Maracanaú, CE, Brazil) and BFT (Talstar 100 EC, 100 g ai/L, FMC Química do Brasil Ltda, Campinas, SP, Brazil) to compare with nanoencapsulated BFT and LAM with previous synergists.

2.2 Preparation of lipid carrier systems for insecticides and synergists

Nanostructured lipid carriers (NLC) containing chemical agents (insecticides and synergists) were prepared according to the solvent evaporation and emulsification method described by Vitorino et al. (2011), with slight modifications. For this, an aqueous solution of PVA (1.25%) was prepared under agitation (100 rpm). An organic solution containing chloroform (5 mL) and stearic acid (250 mg) was prepared, and the active compounds BFT (10 mg) or LAM (10 mg) and Myritol oil (100 mg) were dissolved in this phase. Subsequently, the organic phase was poured into the PVA solution and 10 mL of ethanol was added and subjected to emulsion in an UltraTurrax (14000 rpm) for 10 minutes. The organic solvent was removed by rotary evaporation. The final concentration of BFT and LAM in the nanoformulation was 10 mg/mL, the synergists were added to the formulations after their preparation, at concentrations of 0.1 mg/mL for PBO and 10 mg/mL for DEM. Figure 1

shows a schematic of the formulation preparation steps and the addition of synergistic compounds.

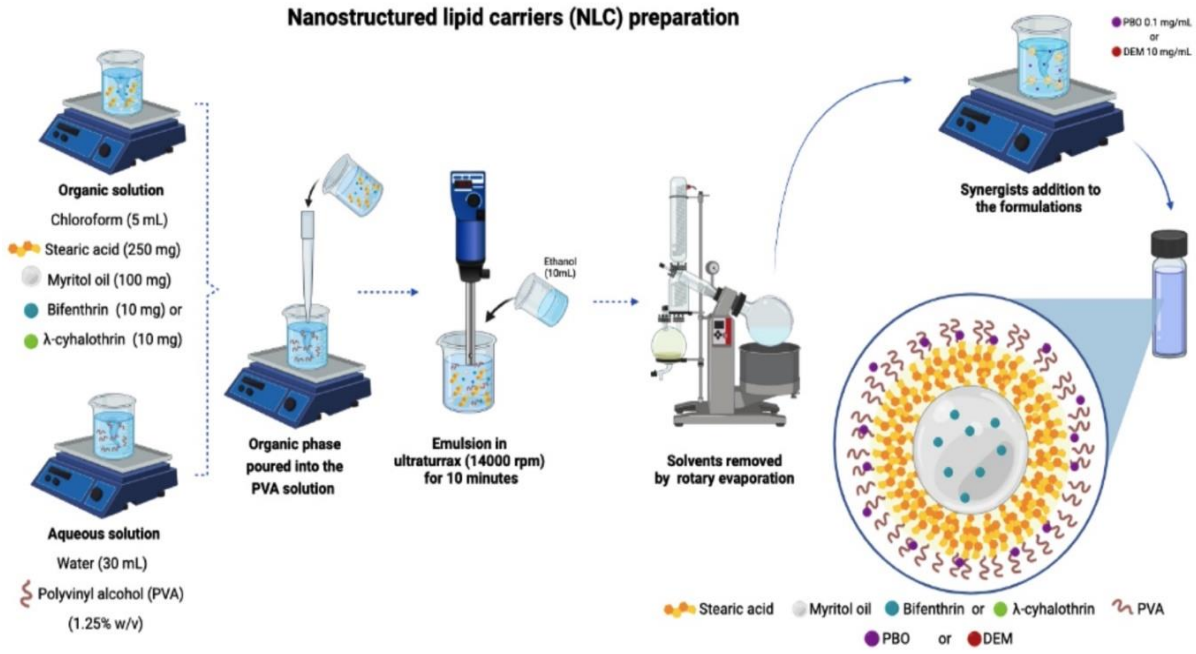

Figure 1. Schematic representation of the preparation steps of nanostructured lipid carriers (NLC) containing BFT and LAM and the synergists PBO and DEM. An illustration of the system produced is also presented, showing its structure and chemical composition.

2.3 Physicochemical characterization of NCL

Analysis of size distribution and polydispersity was performed using the dynamic light scattering (DLS) technique. The zeta potential was determined by the microelectrophoresis method. For both techniques a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) was used at a fixed angle of 90°C and 25°C, the samples were diluted about 1000-fold. In addition, the nanoparticle tracking analysis technique (NTA) was used to measure size distribution and nanoparticle concentration. For this, NanoSight LM 10 cell (green laser, 532 nm) and a sCMOS camera controlled by NanoSight v. 3.1 were used. The results were

expressed as the average of three determinations. The formulations were stored in amber bottles at room temperature and their stability was investigated as a function of time (after 0, 15, 30, 60, and 90 d).

The morphology of the nanoparticles was investigated by atomic force microscopy (AFM). For this, the nanoparticles were diluted (10,000 times) and deposited on silicon plates that were dried in a desiccator. The analyses were performed using an atomic force microscope Easy Scan 2 Basic BT02217 (Nanosurf, Switzerland), operated in contactless mode with the TapAl–G (BudgetSensors, Bulgaria) cantilevers and a scan rate of 90 Hz. The images (256 × 256 pixels, TIFF format) were captured in time mode and analyzed using Gwyddion software.

The insecticides quantification was carried out using high performance liquid chromatography (HPLC). Analysis of LAM employed a reverse phase Phenomenex Luna C18 column (250 mm × 4.6 mm; 5.0 μm) maintained at 30°C. The mobile phase was acetonitrile: water (80:20, v/v), at a flow rate of 1 mL/min. The injection volume was 100 μL and the detector wavelength was 212 nm. Analysis of BFT employed a reverse phase Phenomenex Luna C18 column (250 mm × 4.6 mm; 5.0 μm) maintained at 45°C. The mobile phase was acetonitrile: water (87:13, v/v), at a flow rate of 1 mL/min. The injection volume was 100 μL and the detector wavelength was 215 nm. All chromatographic analyses were performed in an UltiMate 3000 system (Thermo Scientific), operated by Chromeleon 7.2 software, which was used for the acquisition and analysis of the chromatograms. All analytical curves showed correlation coefficients (r2) higher than 0.99.

The ultrafiltration/centrifugation method was used to quantify insecticides encapsulated in NLC. The technique is based on the use of Microcon 10 kDa regenerated cellulose ultrafilters (Millipore), which allows the passage of only the non-encapsulated substances. Thus, the difference between the quantity initially added and the quantity not encapsulated gives the encapsulation efficiency (EE).

2.4 Insects

The *E. heros* population used in this study was collected in a soybean field in Restinga Sêca, Rio Grande do Sul, Brazil (29°44'00" S; 53°26'43" W), during the 2021 season. At the same time, a population of *C. includens* was obtained from a non-Bt soybean area in Chapadão do Sul, Mato Grosso do Sul, Brazil (18º37'25" S; 52º54'13" W). These populations were chosen due to their low susceptibility to pyrethroids, including BFT and LAM, with resistance ratios >31-fold in relation to a susceptible population (Stacke et al., 2019; Somavilla et al., 2020). After collection, stink bugs were transported to the laboratory in aerated plastic containers (41 cm long × 29 cm wide × 13 cm high) containing fresh green bean pods (*Phaseolus vulgaris* L.), dry soybean seeds, and peanuts in shell (*Arachis hypogaea* L.) for food. Larvae of *C. includens* were transported in 100 ml plastic pots containing an artificial diet (Greene et al., 1976) and, in laboratory, were transferred to 50 ml plastic cups containing the same artificial diet. All populations were kept at 25 ± 2ºC, relative humidity of 60% ± 10%, and photoperiod of 14:10 h.

2.5 Toxicological bioassays

For the bioassays with *E. heros*, initially the fresh green bean pods (*P. vulgaris* L.) were washed in a 1% chlorine solution and allowed to dry. Then, the bean pods were cut into pieces 5 cm long. The test arenas were prepared by placing a sheet of filter paper in Petri dishes (100 × 15 mm), the paper was moistened with 1 ml of distilled water and then 2 pieces of pod were placed in each test arena. Adults of *E. heros* were exposed to nanoencapsulated BFT and LAM with or without PBO or DEM diluted in distilled water. All solutions were agitated for

10 min and applied to the back (dorsum) of each stink bug at a volume of 2 µL/stink bug using a hand microapplicator (Burkard Manufacturing, Rickmansworth, UK). From 4 to 6 concentrations of each insecticide were tested, each replicate being composed of 5 stink bugs (25 stink bugs tested/concentration). The control treatment was composed by nanostructured lipid carriers without insecticides and synergists. Mortality was assessed after 72 h (stink bugs without movement were considered dead).

Bioassays with *C. includens* were performed in 24-well acrylic plates (Costar®, São Paulo, SP, Brazil) containing an artificial diet proposed by Greene et al. (1976) at a volume of 1 mL/well. The nanoencapsulated formulations were diluted in distilled water to prepare different concentrations to be tested. Triton X-100 (Sigma-Aldrich, São Paulo, SP, Brazil) at 0.1% was added to each concentration to allow it to spread on the diet surface. Then, early L3 larvae were transferred to acrylic plates with the diet surface treated with 4 concentrations of nanoencapsulated formulations of BFT and LAM with and without PBO or DEM (30 µL/well), in a total of 2 replicates (plates)/concentration (48 larvae tested/concentration). The control treatment was nanostructured lipid carriers without insecticides and synergists. The larval mortality was assessed after 48 h (larvae without movement were considered dead).

In addition to previous bioassays, single doses of nanoencapsulated BFT and LAM with and without PBO and DEM were compared to the same doses of commercial products containing BTF and LAM to measure control efficacy of field populations of *E. heros* (2 µg BFT/mL and 0.64 µg LAM/mL – 5 replicates of 5 stink bugs/dose) and *C. includens* (1.6 µg BFT/mL and 0.89 µg LAM/mL – 4 replicates of 24 larvae/dose) in ingestion bioassays as described above. However, thirty minutes (*E. heros*) and 2h (*C. includens*) before exposure to BFT and LAM, 2µL of PBO or DEM were applied in the dorsum of insects as previously described. The time before exposure to insecticides and dose of synergists was reported by Boff et al. (2022). The mortality assessment was the same as earlier defined for each species.

Concentration-mortality data in bioassays with *E. heros* and *C. includens* were subjected to Probit analysis to estimate LC_{50} and LC_{90} and respective 95% confidence intervals (CI) using the PROC PROBIT procedure in SAS® 9.1 (SAS Institute, 2000). The LC values were pairwise compared, being the significance declared if 95% CIs did not overlap (Savin et al., 1977; Robertson et al., 2007). The synergistic ratios (SR) were calculated by dividing LC_{50} and LC_{90} of insects treated with nanoencapsulated BFT and LAM by the respective values of BFT and LAM nanoencapsulated with PBO or DEM. Percent mortality of *E. heros* and *C. includens* exposed to single doses of nanoencapsulated and commercial BFT and LAM were corrected based on controls (untreated insects) and then compared by the Scott-Knott test ($P < 0.05$) using the R software (R Development Core Team, 2017).

3. Results and Discussion

3.1 Characterization and physicochemical stability of NLCs containing BFT, LAM, and synergists

To characterize as well as evaluate the physicochemical stability of the formulations, the following were measured: mean diameter (MD, nm), polydispersity index (PDI), zeta potential (ZP, mV), nanoparticle concentration (CT, particles/mL), and encapsulation efficiency (EE, %); the results are presented in Table 1.

The results presented in Table 1 and Fig. 2; show that the formulations prepared in the present study presented good physical-chemical characteristics and good stability during storage time (90 d). The average diameter results analyzed by two different techniques (DLS and NTA) showed that the formulations containing the actives BFT and LAM, in the absence and presence of synergists, had sizes from 200 to 350 nm. Changes in these values were

observed over the storage time, however no changes were observed in the stability of the formulations, as shown by the other parameters that will be discussed below. The mean diameter values for analyses performed by NTA were smaller compared to the DLS values. This is mainly due to the particularity of each technique, since NTA performs the individual analysis of each particle, while DLS performs a combined analysis, which favors an increase in the size, with the presence of larger particles (Zhdanov, 2020).

Table 1. Characterization of nanostructured lipid carriers containing the active compounds BFT and LAM (10 mg/ml) in the presence and absence of PBO (0.1 mg/ml) and DEM (10 mg/ml).

Formulations	MD (nm)		PDI	ZP (mV)	CT (x10^{12} particles/mL)	EE (%)
	DLS	NTA				
NP	306 ± 7	229 ± 8	0.12 ± 0.04	–18 ± 1.2	8.2 ± 0.2	–
NP_BFT	220 ± 3	215 ± 2	0.14 ± 0.02	–20 ± 0.7	8.4 ± 0.2	99.5 ± 0.2
NP_BFT+DEM	309 ± 5	290 ± 7	0.14 ± 0.08	–14 ± 0.7	7.6 ± 0.5	99.3 ± 0.2
NP_BFT+PBO	299 ± 4	298 ± 6	0.09 ± 0.02	–18 ± 0.6	7.2 ± 0.4	99.4 ± 0.5
NP_LAM	316 ± 3	238 ± 5	0.08 ± 0.05	–17 ± 0.3	5.7 ± 0.8	98.7 ± 0.3
NP_LAM+DEM	308 ± 4	224 ± 5	0.15 ± 0.01	–17 ± 0.5	5.0 ± 0.5	98.8 ± 0.3
NP_LAM+PBO	306 ± 1	241 ± 2	0.17 ± 0.05	–16 ± 0.8	4.8 ± 1.0	98.5 ± 0.4

MD - Mean diameter; PDI - Polydispersity index; ZP - zeta potential; CT - Nanoparticle concentration; EE - Encapsulation efficiency

The formulations were also subjected to microscopy analysis, as shown in Fig. 3. The spherical morphology of all prepared systems is observed, and the addition of synergists did not significantly change the size distribution of the nanoparticles, corroborating the DLS and NTA results (Fig. 2A and B). It is noteworthy that the mean diameter values for the AFM analysis showed particle sizes from 125 to 168 nm. This is also due to the particularity of the

AFM technique since the formulations are deposited on a support and dried for further analysis.

As mentioned, despite the change in the mean diameter values of the formulations over time, we can observe that the polydispersion index (Fig. 2C) of the formulations remained below 0.2, indicating the good homogeneity of the prepared formulations. Furthermore, the results for the zeta potential (Fig. 2D) show mean values of -20 mV, however, for these systems, a surfactant was also used, which plays a role in the steric stability of these formulations (Cacua et al., 2019). The concentration of nanoparticles in the formulations (Fig. 2E) was also a parameter that did not show major changes over time. These results reinforce the good physicochemical stability of the prepared nanoparticle formulations, which is extremely important for commercial applications that require long-term storage.

Finally, encapsulation efficiency was also investigated in the current study (Fig. 2F). Both active compounds (BFT and LAM) showed high encapsulation efficiency in nanostructured lipid carriers, and no significant changes were observed with the addition of synergists and as a function of storage time. The high encapsulation efficiency for these compounds is mainly due to their strong hydrophobic characteristic, which helps in the interaction with the lipid matrix of the nanocarriers. The BFT compound had higher EE values than LAM, which corroborates its water solubility values (<1ug/L for BFT and <5 ug/L for LAM) (PubChem 2021a, 2021b).

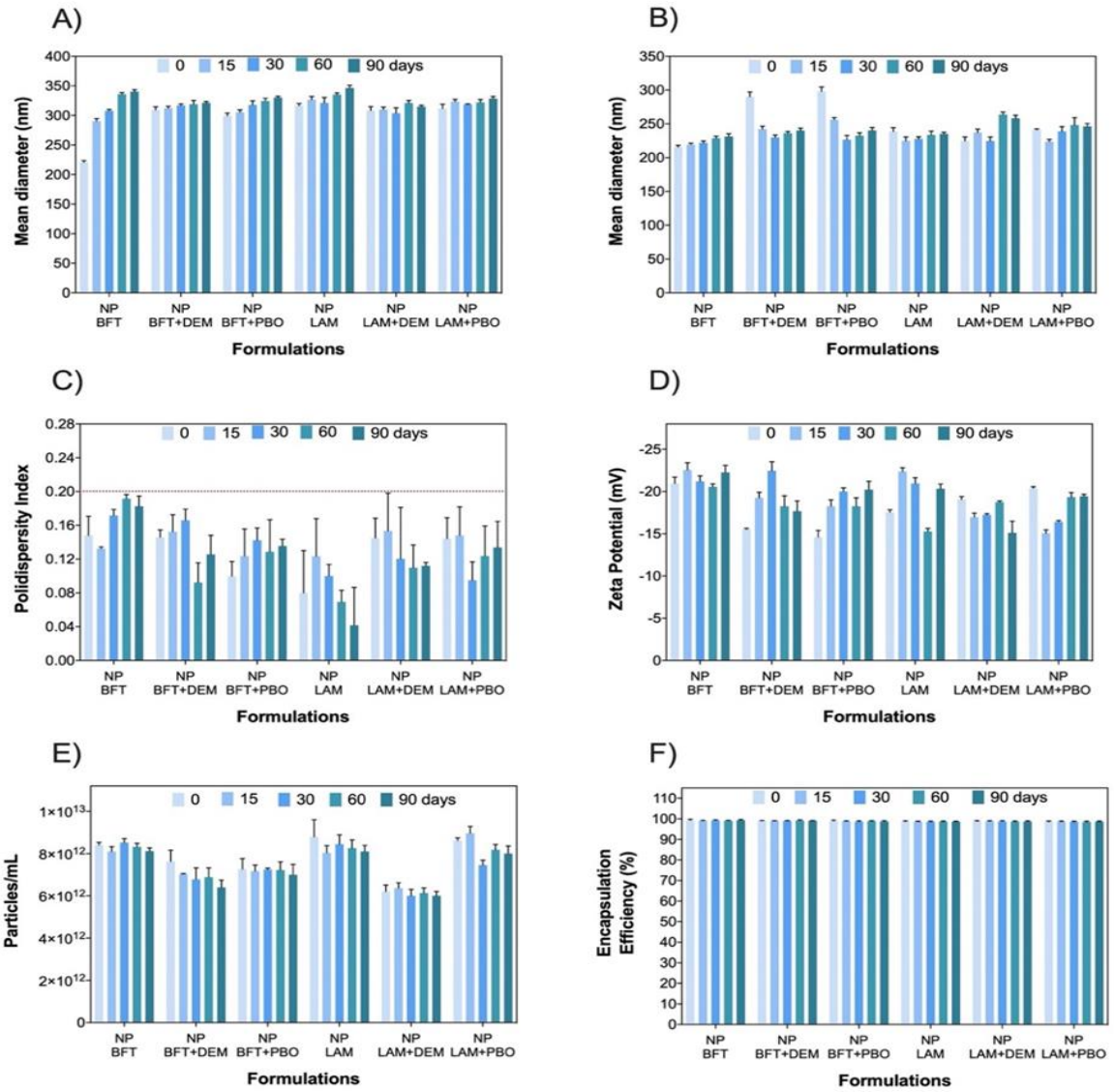

Figure 2. Physical-chemical parameters and stability evaluation of NLC containing bifenthrin (BFT) and λ-cyhalothrin (LAM). The stability evaluation was performed for 90 d. (A) mean diameter (nm) by DLS, (B) mean diamter (nm) by NTA, (C) polydispersity index, (D) zeta potential (mV), (E) nanoparticles concentration (particles/mL) and (F) Encapsulation efficiency (%). All analyses were performed in triplicate, at 25ºC.

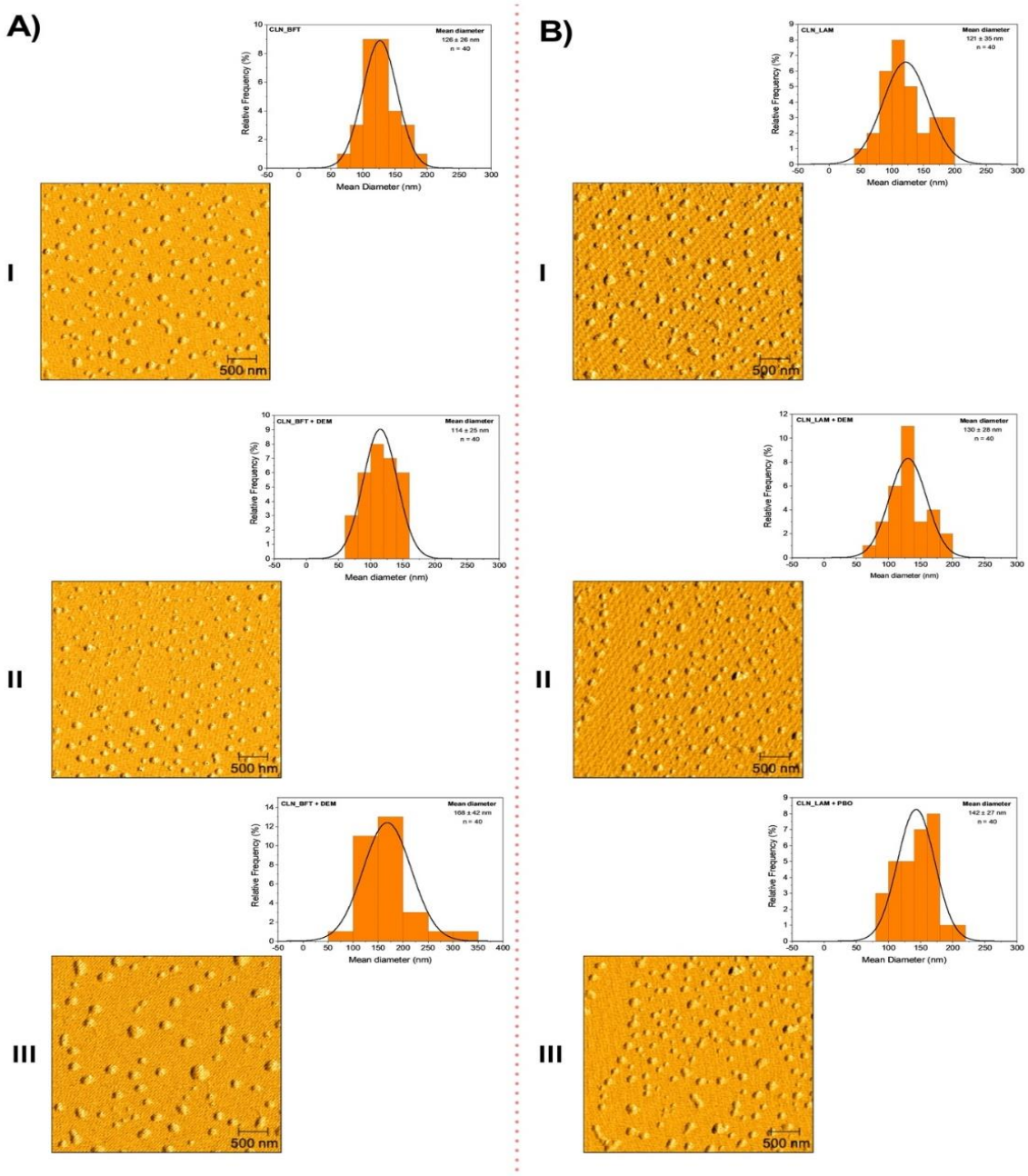

Figure 3. Micrographs obtained through AFM analysis for NLC formulations containing bifenthrin-BFT (A) and λ-cyhalothrin-LAM (B), being A–I) NLC containing BFT in the absence of PBO and DEM, A–II) NLC containing BFT and DEM, A–III) NLC containing BFT and PBO, BI) NLC containing LAM in the absence of PBO and DEM, B–II) NLC containing LAM and DEM, B–III) NLC containing LAM and PBO. In the *inset* of each graph

are found the size distribution graphs obtained through the micrographs (*n* = 40 nanoparticles).

No studies that had encapsulated these actives in nanostructured lipid carriers were found in the literature, however these results corroborate with other nanoformulations. Liu et al. (2008) encapsulated the BFT compound in polyvinylpyrrolidone (PVP) based nanoparticles through the nanoprecipitation technique. The authors obtained particles with sizes from 200 to 350 nm, which showed changes in mean diameter values over 30 days of storage. The authors also obtained high EE values (>90%). Moradi et al. (2019) prepared nanoliposomes containing a mixture of the actives imidacloprid and LAM, obtaining formulations with a size of 57 nm, and a coating with chitosan provided greater stability with zeta potential values above 30 mV.

3.2 Toxicity of nanoencapsulated BFT and LAM with synergists in E. heros

The estimated LC_{50} values of nanoencapsulated BFT with PBO or DEM against *E. heros* did not differ from those values of nanoencapsulated BFT only, indicating a synergistic ratio ranging from 1.17- to 1.55-fold (Table 2). However, the LC_{90} values were significantly lower for *E. heros* treated with nanoencapsulated BFT containing PBO or DEM, revealing a synergistic ratio up to 3.50-fold, in relation to insects treated only with BFT without synergists. In contrast, the LCs values of nanoencapsulated LAM with and without PBO or DEM did not differ (based in the overlap of 95% CIs) in lethality to *E. heros* compared with this active ingredient without encapsulation, indicating a maximum synergistic ratio of 1.45-fold (Table 2).

Table 2. Lethal doses of encapsulated BFT and LAM in the presence and absence of synergists PBO and DEM against *E. heros* in topical bioassays.

Treatment	*n*	Fit of probit lines			LC_{50}[b,c]	LC_{90}[b,c]	SR_{50}[d]	SR_{90}[d]
		Slope ± SE	χ^2 (*df* [a])	*P*				
BFT	175	1.69 ± 0.31	1.22 (4)	0.87	0.25 (0.15–0.36) a	1.47 (0.93–3.36) a	–	–
BFT + PBO	175	1.85 ± 0.32	0.93 (4)	0.91	0.22 (0.13–0.30) a	1.09 (0.73–2.16) a	1.17	1.35
BFT + DEM	150	3.10 ± 0.59	0.71 (3)	0.86	0.16 (0.11–0.21) a	0.42 (0.32–0.68) b	1.55	3.50
LAM	200	1.65 ± 0.28	3.00 (5)	0.69	0.32 (0.18–0.47) a	1.92 (1.22–4.04) a	–	–
LAM + PBO	175	1.58 ± 0.30	0.96 (4)	0.92	0.30 (0.14–0.48) a	1.97 (1.22–4.44) a	1.05	0.97
LAM + DEM	175	1.94 ± 0.42	0.18 (3)	0.98	0.28 (0.13–0.43) a	1.31 (0.86–2.84) a	1.12	1.45

[a]Degrees of freedom.

[b]LC_{50}: dose required to kill 50% of stink bugs in the observation period of 72 h. Similarly, LC_{90} is the dose of insecticide required to kill 90% of stink bugs tested.

[c]LC_{50} and LC_{90} values designated by different letters within a column are significantly different from each other through non-overlap of 95% CIs.

[d]Synergist ratio (SR_{50} and SR_{90}) = LC_{50} and LC_{90} of insects treated with nanoencapsulated BFT and LAM/LC_{50} and LC_{90} of insects treated with BFT and LAM nanoencapsulated with PBO and DEM.

There was a significant increase in mortality of *E. heros* exposed to a single dose of nanoencapsulated BFT with PBO and DEM (68% and 72%, respectively) compared to the same dose of the commercial product containing BFT only ($F = 12.53$; $df = 5, 45$; $P < 0.0001$) (Fig. 4A). A significant increase mortality (>60%) of *E. heros* was also observed when insects were exposed to nanoencapsulated LAM with PBO and DEM ($F = 11.05$; $df = 5, 38$; $P < 0.0001$), whereas no differences in mortality occurred for insects treated with nanoencapsulated LAM + PBO in relation to the commercial product LAM used against stink bugs previously treated with PBO (Fig. 4B).

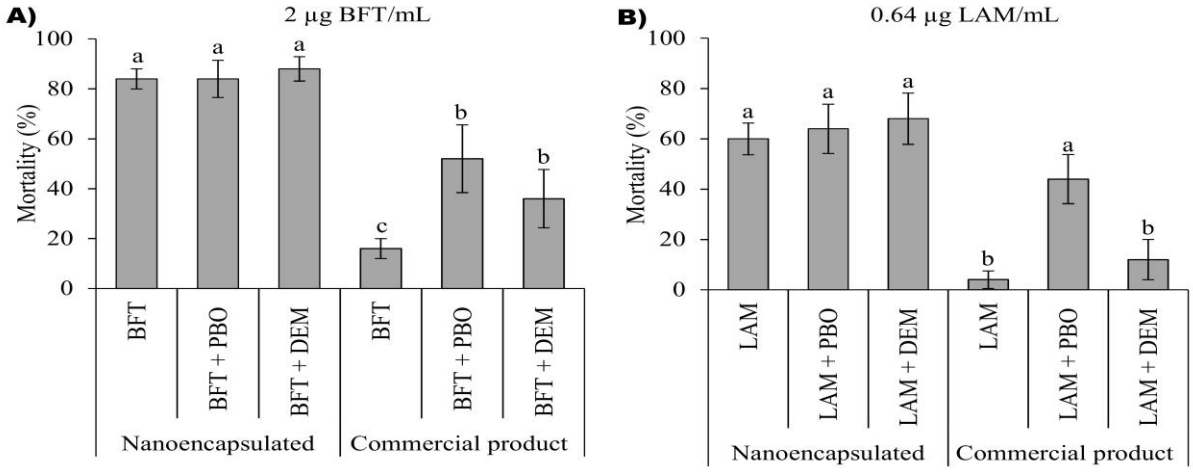

Figure 4. Mortality of *E. heros* exposed to nanoencapsulated BFT (A) or LAM (B) with PBO and DEM and commercial products containing BFT and LAM with t synergists. Bars (± SE) with the same letters do not differ significantly.

The susceptibility of *E. heros* to BFT and LAM increased when these insecticides were nanoencapsulated with PBO and DEM in relation to commercially available products containing BTF and LAM. This high susceptibility can be explained by the reduced metabolic activity of P-450 cytochrome, glutathione S-transferases, and esterases enzymes, which are the main responsible for the metabolic detoxification of these insecticides in *E. heros*. These results corroborate previous studies that demonstrated that the low susceptibility of *E. heros* to methamidophos, endosulfan, and acephate was associated with high expression of α-esterase, β-esterase, and glutathione S-transferases enzymes (Sosa-Gómez et al., 2009, 2019). Similarly, studies with *Cimex* (Hemiptera: Cimicidae) also indicated that the resistance mechanism to λ-cyhalothrin and deltamethrin has been associated with the overexpression of cytochrome P-450 (How and Lee, 2011; Lilly et al., 2016). In summary, our findings indicate that nanoencapsulated formulations of BFT and LAM with PBO and DEM have the potential of improving control and restoring the susceptibility of *E. heros* to pyrethroid-based insecticides.

3.3 Toxicity of nanoencapsulated BFT and LAM with synergists in C. includens

The estimated LC_{50} values of nanoencapsulated BFT and LAM with PBO or DEM against *C. includens* did not differ from nanoencapsulated BFT without synergists, indicating a synergistic ratio <1.77-fold (Table 3). In contrast, the LC_{90} values of nanoencapsulated BFT containing PBO were significantly higher than BFT only, revealing a synergistic ratio up to 2.16-fold. Similarly, the LC_{90} values were significantly lower when *C. includens* were exposed to nanoencapsulated LAM containing PBO in relation to larvae exposed only to LAM; synergistic ratio up to 1.70-fold (Table 3).

Table 3. Lethal doses of encapsulated BFT and LAM in the presence and absence of synergists PBO and DEM against *C. includens* in ingestion bioassays.

Treatment	*n*	Fit of probit lines			LC_{50}[b,c]	LC_{90}[b,c]	SR_{50}[d]	SR_{90}[d]
		Slope ± SE	χ^2 (*df* [a])	*P*				
BFT	240	2.21 ± 0.44	0.95 (3)	0.63	1.76 (0.73–2.71) a	6.14 (4.44–8.59) a	–	–
BFT + PBO	240	2.63 ± 0.41	3.70 (3)	0.15	0.99 (0.64–1.33) a	2.84 (2.15–4.06) b	1.77	2.16
BFT + DEM	240	2.12 ± 0.34	3.20 (3)	0.20	1.64 (0.83–2.46) a	6.10 (4.44–8.61) a	1.07	1.00
LAM	240	2.77 ± 0.52	0.95 (3)	0.61	0.87 (0.56–1.15) a	2.51 (1.87–4.09) a	–	–
LAM + PBO	240	4.46± 0.92	0.42 (3)	0.81	0.80 (0.56–0.96) a	1.48 (1.27–1.85) b	1.08	1.70
LAM + DEM	240	4.36 ± 0.96	0.85 (3)	0.65	0.84 (0.58–1.01) a	1.59 (1.36–2.06) ab	1.03	1.57

[a]Degrees of freedom.

[b]LC_{50}: dose required to kill 50% of larvae in the observation period of 48 h. Similarly, LC_{90} is the dose of insecticide required to kill 90% of larvae tested.

[c]LC_{50} and LC_{90} values designated by different letters within a column are significantly different from each other through non-overlap of 95% CIs.

[d]Synergist ratio (SR_{50} and SR_{90}) = LC_{50} and LC_{90} of insects treated with nanoencapsulated BFT and LAM/LC_{50} and LC_{90} of insects treated with BFT and LAM nanoencapsulated with PBO and DEM

A significant increase in mortality of *C. includens* occurred when larvae were exposed to a single dose of nanoencapsulated BFT with PBO (70% mortality) compared to the same dose of the commercial product containing BFT (37% mortality) or commercial BTF + synergists (<50% mortality) (F = 15.40 df = 5, 30; P < 0.0001) (Fig. 5A). A high mortality of *C. includens* was also observed when larvae were exposed to nanoencapsulated LAM with PBO and DEM (54% and 52% mortality, respectively) in comparison to commercial product with LAM (37% mortality) (F = 11.71; df = 5, 22; P < 0.0001) (Fig. 5B). However, the mortality of *C. includens* exposed to nanoencapsulated BFT with DEM and LAM with PBO was similar of those larvae exposed to commercial BFT and LAM pre-exposed to DEM and PBO, respectively (Fig. 5A, B).

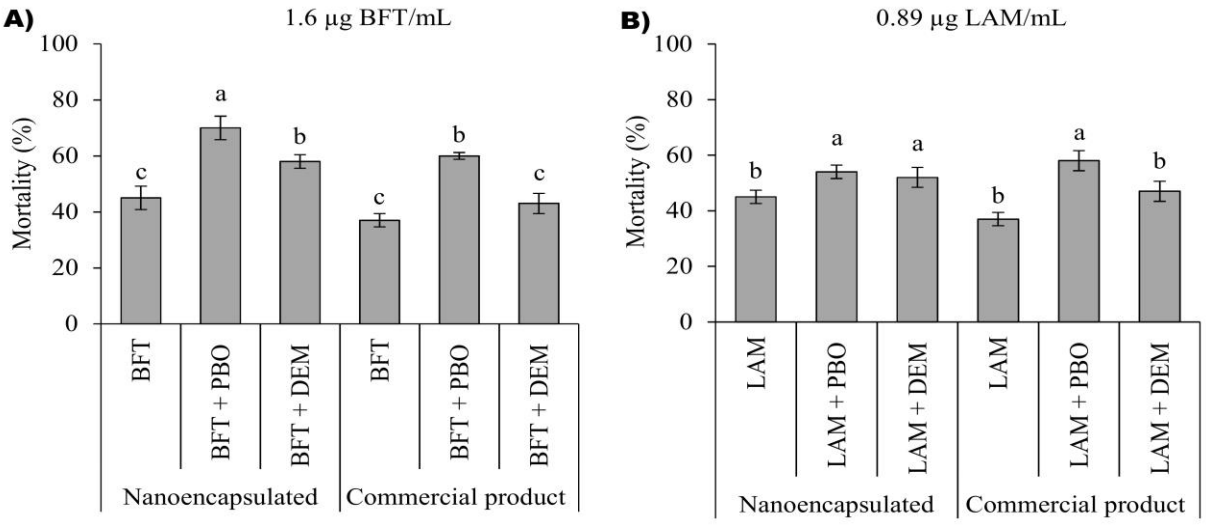

Figure 5. Mortality of *C. includens* exposed to nanoencapsulated BFT (A) or LAM (B) with PBO and DEM and commercial products containing BFT and LAM with synergists in ingestion bioassays. Bars (± SE) with the same letters do not differ significantly.

The increased susceptibility of *C. includens* to nanoencapsulated BFT and LAM with PBO and DEM can be explained by the effect of this synergist reducing the activity of metabolic

detoxification enzymes. In a previous study, the detoxification of LAM by *C. includens* was also linked with the overexpression of enzymes from P-450 cytochrome (Perini et al., 2021). Similar detoxification mechanism was also reported in *S. litura* and *S. exigua* in which the synergist PBO reduced the resistance to deltamethrin (Ishtiaq et al., 2012; Cheema et al., 2020) and in *H. armigera* increasing its susceptibility to deltamethrin and fenvalerate (Durigan et al., 2017). The synergist DEM also enhanced the susceptibly to pyrethroids in lepidopteran pests as in *S. exigua* and *S. litura* to cypermethrin and deltamethrin, respectively, revealing that the high expression of glutathione S-transferases enzymes are involved with the resistance to these pyrethroids (Kaur et al., 2015; Hu et al., 2019).Our results suggested that nanoencapsulated formulations of BFT and LAM with synergists has the potential to improve control and reverse the resistance of *C. includens* to pyrethroids.

4. Conclusion

The present study provides the first evidence that insecticides BFT and LAM can be nanoformulated with sinergistic compounds for the resistance management of pest species. The insecticides BFT and LAM nanoencapsulated with PBO and DEM present great physical-chemical characteristics and are stable. Furthermore, the nanoformulations of BFT and LAM with PBO and DEM increased the susceptibility of *E. heros* and *C. includens* to these pyrethroids, suggesting that these nanoformulations reduce the activity of the detoxification enzymes improving its control. From an insect management perspective, our findings provide new insights for designing more effective insecticide formulations for controlling insect pests in several crops.

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4 DISCUSSÃO

O manejo de *E. heros* e *C. includens* na cultura da soja tem sido comumente realizado com o uso de inseticidas, dentre eles os moduladores dos canais de sódio (piretroides) (PANIZZI, 2013; BUENO et al., 2020). Contudo, estudos prévios reportaram uma baixa suscetibilidade de populações de ambas as espécies aos piretroides λ-cialotrina e bifentrina (TUELHER et al., 2018; STACKE et al., 2019; SOMAVILLA et al., 2020; STACKE et al., 2020; PERINI et al., 2021). Os resultados deste estudo indicam que a baixa suscetibilidade a λ-cialotrina e bifentrina em *E. heros* e *C. includens* está associada a enzimas metabólicas que desintoxicam os inseticidas, pois quando do uso de sinergistas, que reduzem a atividade enzimática, houve aumento na suscetibilidade aos piretroides testados. No primeiro estudo, as populações de campo de *E. heros* e *C. includes* apresentaram maior suscetibilidade a λ-cialotrina e bifentrina na presença de PBO e DEM, indicando que as enzimas P-450 monooxigenases, glutationa S-transferases e esterases estão diretamente envolvidas no processo de metabolização e desintoxicação dos piretroides avaliados. No segundo estudo, desenvolveu-se nanoformulações de bifentrina e λ-cialotrina com PBO e DEM e contatou-se que as nanoformulações aumentaram a letalidade de bifentrina e λ-cialotrina para *E. heros* e *C. includens*.

O aumento na suscetibilidade de *E. heros* de *C. includens* na presença de DEM e PBO, respectivamente, indica que, ao se reduzir a atividade das enzimas glutationa S-transferases, esterases e P-450, uma maior quantidade de ingrediente ativo chega ao sítio de ação (canais de sódio), resultando em maior letalidade. O uso de sinergistas para o manejo da resistência de insetos se enquadra na clássica estratégia denominada “manejo por saturação”. Essa estratégia tem o intuito de reduzir as vantagens adaptativas dos resistentes mediante o uso de sinergistas que bloqueiam ou inibem a desintoxicação metabólica de inseticidas (METCALF, 1967; GONZALEZ-MORALES; ROMERO, 2019; NAUEN et al., 2021).

O uso de nanoformulações bifentrina e λ-cialotrina com PBO e DEM aumentou a letalidade dos inseticidas para populações de campo de *E. heros* e *C. includens*. Do ponto de vista prático, as nanoformulações de piretroides com sinergistas tem o potencial de prolongar a vida útil das moléculas, especialmente para o manejo de sugadores na cultura da soja, na qual há poucos modos de ação registrados (piretroides, organofosforados, neonicotinoides e fenilpirazois) (AGROFIT, 2021). Essa limitação de ingredientes ativos expõe os insetos mais frequentemente ao mesmo modo de ação, favorecendo a evolução da resistência. Entretanto,

além do controle químico ainda se faz necessário a adoção de outras práticas de manejo, tais como (i) usar inseticidas somente quando o nível de controle for atingido, (ii) utilizar ingredientes ativos com baixa taxa de resistência, (iii) reduzir o uso de piretroides, (iv) rotacionar inseticidas com modo de ação distintos e (v) usar outras táticas de controle.

Em resumo, os resultados do presente estudo indicam que mecanismos metabólicos estão envolvidos com a resistência de *E. heros* e *C. includens* a piretroides e que é possível desenvolver formulações de bifentrina e lambda-cialotrina nanoencapsuladas com os sinergistas PBO e DEM para controle e manejo da resistência dessas espécies-praga.

5 CONCLUSÕES

Os sinergistas PBO e DEM aumentam a letalidade de λ-cialotrina e bifentrina para *E. heros*, enquanto PBO aumenta a letalidade de ambos os inseticidas para *C. includens.*

Nanoformulações de bifentrina com DEM e bifentrina e λ-cialotrina com PBO e DEM reduziram a resistência de *E. heros* e *C. includens* a ambos os piretroides.

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