

ORIGINAL ARTICLE

Comparative bioefficacy of *Bacillus thuringiensis* var. *kurstaki* and neem on American white moth, *Hyphantria cunea* Drury

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Vol. 66, No. 2: 244–254, 2026

DOI: 10.24425/jppr.2026.158072

Received: December 28, 2024

Accepted: March 17, 2025

Online publication: April 28, 2026

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Responsible Editor:

Beata Borowiak-Sobkowiak

Abstract

The American white moth, *Hyphantria cunea* Drury, is a polyphagous insect pest that feeds on a wide range of fruit and forest trees. In the present study, the potential of the most commonly used biological pesticide *Bacillus thuringiensis* var. *kurstaki* (Btk) and the botanical-derived insecticide neem Achook® and their combination on the mortality and physiological disruptions of *H. cunea* was investigated. The LC₃₀ (1.200 and 13.350 ppm for Bt and neem, respectively), LC₅₀ (3.103 and 31.753 ppm for Bt and neem, respectively), and their combinations were considered in all biochemical assays. The combination of biopesticides showed a synergistic phenomenon in all treatments at different concentrations. To explore the underlying mechanisms, we assessed the main biochemical compounds, including the activity of digestive and detoxifying enzymes of the moth larvae. Significant reductions in the activities of protease, amylase, lipase, α-glucosidase, and β-glucosidase were realized compared to the control ($p < 0.05$). The activities of detoxifying enzymes, specifically α- and β-esterases, glutathione S-transferase, and phenol oxidase, exhibited significant increases in the treated groups. Conversely, the activity of acetylcholine esterase was found to be decreased across all treatment conditions. The treatments administered resulted in a statistically significant reduction in pupal weight ($p < 0.05$). The lowest average pupal weight was recorded for the combination of *Bacillus thuringiensis* (Bt) and neem at their concentration (LC₅₀) of 98.98 mg. This research demonstrated that using Bt and neem had combined synergistic pesticidal effects that can be proposed for integrated pest management of *H. cunea*.

Keywords: biopesticides, compatibility, digestive enzymes, detoxification enzymes, pupal weight

Introduction

The fall webworm, or American white moth, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), with its origin in North America has gradually spread to central Europe and Asia (Su *et al.* 2008). As one of the key and polyphagous insect pests, *H. cunea* can cause severe damage to forests, urban trees, and crops (Kim and Kil 2012). In Iran, *H. cunea* was first recorded in August 2002 near Lasht-e-Nesha, Guilan province and it is a major pest in Northern provinces like Guilan,

Mazandaran, and Ardebil (Rezaei *et al.* 2004). The populations of this pest have become widely established. They feed voraciously on a wide range of forest and fruit trees, shrubs, and herbaceous plants. More than 600 plant species have now been identified as hosts (Rezaei *et al.* 2006). The caterpillars exhibit gregarious behavior, often feeding together in large groups, which allows them to skeletonize leaves within their nests before expanding the webs, sometimes

covering entire branches (Trajković and Žikić 2023). This leads to reduced yields, aesthetic value, and plant health, with repeated damage potentially killing individual plants. The impact of the pest on fruit and berry crops can last for multiple years. The availability of food sources influences the pest's development and population dynamics, affecting its preference for certain plant species (Yanar *et al.* 2021).

Although chemical pesticides play an important role in pest control, they contaminate the environment, have adverse effects on humans and non-target organisms, and cause the emergence of pesticide-resistant populations (Kaur *et al.* 2024). As a result, there is a growing interest in shifting towards more efficient and eco-friendly alternatives, such as biopesticides, which can significantly reduce the reliance on synthetic chemicals and mitigate these negative impacts (Mawcha *et al.* 2025).

Recent trends in pest control have led to the development of many biopesticides from microorganisms (bacteria, fungi, viruses, etc.), plant and animal-derived products (pheromones, hormones, insect-specific toxins, etc.), and are being encouraged worldwide for insect pest management (Sabbour and E-Abd-El-Aziz 2010; Ayilara *et al.* 2023). The appropriate use of eco-friendly microbial pesticides can play an important role in a sustainable and scalable pest management program (Mawcha *et al.* 2025). In integrated pest management (IPM), biological control with entomopathogens is a potentially important means of reducing pest populations (Geedi and Reddy 2023). *Bacillus thuringiensis* is one of the Gram-positive spore-forming bacteria belonging to the *Bacillus cereus* group of bacilli. It produces proteinaceous crystals during sporulation which is a feature which distinguishes it from other members. Some of these crystals are toxic to the detriment of insects, nematodes, and even human cancer cells (Palma *et al.* 2014). Regarding insects, the crystals are solubilized in the midgut of insect larvae and subsequently activated by proteases. The activated toxins bind to midgut epithelial cells, forming pores and causing osmotic cell lysis, leading to insect death (Guerrero 2023). The insecticidal potential of Bt against destructive Lepidopteran pests has been documented in recent studies. For example, the susceptibility of the cutworm (*Agrotis exclamationis* L.), the velvet bean caterpillar (*Anticarsia gemmatilis* (Hübner)), the armyworm (*Spodoptera cosmioides* (Walker)), soybean looper (*Chrysodeixis includens* (Walker)), the sugarcane borer (*Diatraea saccharalis* (Fabricius)), the diamondback moth (*Plutella xylostella* (L.)), and the nettle caterpillar (*Euprosterna elaeasa* Dyar) larvae to Bt has been demonstrated (Quintero *et al.* 2020; Pinheiro and Valicente 2021; Navya *et al.* 2022; Baranek *et al.* 2023).

Several bioactive insecticidal compounds have been extracted from different parts of the neem tree (*Azadirachta indica* A. Juss.) (Sapindales: Meliaceae), of which azadirachtin is the dominant active ingredient. Multiple insecticidal effects, from repellency to molting disruption, growth reduction, development and oviposition inhibition, and high mortality of neem-based agents against several insect pests have been reported in previous studies (Valizadeh *et al.* 2013; Allahvaisi *et al.* 2021; Luo *et al.* 2023). Neem-based agents can also be used in combination with other pesticides or entomopathogens (Amizadeh *et al.* 2019; Hiremath *et al.* 2020; Bharti *et al.* 2023).

In addition to their direct lethal effects on pests, biopesticides can influence insect physiology in various ways, including altering the activity of digestive and detoxifying enzymes (Oftadeh *et al.* 2020). These agents often negatively impact insect digestive enzyme activity through direct enzyme inhibition, damage to gut epithelial cells, changes in neurotransmitter activity, and disruption of gut microbiota (Chaudhari *et al.* 2021). Detoxification enzymes, including cytochrome P₄₅₀ enzymes, carboxylesterases, and glutathione S-transferases, play a critical role in insect adaptation and detoxification processes (Zibae and Bandani 2010). These enzymes help insects convert lipophilic external toxins into hydrophilic compounds, effectively neutralizing the toxic effects of pesticides (Yao *et al.* 2025). These effects highlight the potential of these compounds as effective natural pest control agents.

Ensuring the compatibility of products used against target pests is a critical aspect of developing an effective pest management strategy. This involves avoiding highly toxic substances or applying them during periods when their impact on natural control agents is minimized. Studies have demonstrated that combining compatible products with entomopathogenic agents can enhance pest control efficacy, reduce the quantity of insecticides needed, lower costs, and decrease the risk of pest resistance (Sabbahi *et al.* 2022). This approach aligns with the principles of integrated pest management (IPM), emphasizing sustainable and environmentally friendly methods to effectively control pest populations.

Neem and Bt are therefore promising biopesticides for the development of integrated pest management tools. Since both Bt and neem target the digestive system of insects, their potential synergistic effects warrant investigation. Therefore, this study aimed to evaluate the effect of commercial Bt and neem products, alone and in combination, on *H. cunea*, in which, along with lethality, their effects on digestive and detoxifying enzymes were assessed. The current research was conducted to minimize or avoid the use of

chemical pesticides for the sustainable management of *H. cunea*.

Materials and Methods

Insect rearing

Eggs of *H. cunea* were collected from the forests of Rasht (37°19'N, 49°37'E.), Guilan province, Iran. The hatched larvae were reared on mulberry leaves (Kenmochi variety) under laboratory conditions: 25 ± 1°C, 75% relative humidity (RH), and 16 : 8 (light : darkness). Third-instar larvae were selected for the experiments due to their lower hair cover than older instars and high potential for damage. Body length and head capsule width were used to identify larval instars based on Dyar's law. The body length and head capsule width for third-instar larvae of this pest have been estimated to be 6–10 mm and 0.6–0.743 mm, respectively, with a Dyar's ratio of 1.631 (Mohammadi *et al.* 2010).

Insecticides

In this study, the commercial neem formulation Achook® (containing 0.03% azadirachtin), produced by Bahar Agrochem & Feeds Pvt. Ltd., Maharashtra, and marketed by Godrej Agrovat Ltd., Gujarat, India, was used. A stock suspension of *Bacillus thuringiensis* var. *kurstaki* (10⁹ spore · ml⁻¹) was provided by Giah Company (Tehran, Iran), and a serial concentration was prepared using distilled water.

Bioassays

Insect bioassay was performed by the leaf disc method on the newly emerged third-instar larvae of *H. cunea* (Ebadollahi *et al.* 2013). Initial tests were conducted to select the appropriate range of concentrations. Then, five concentrations were selected based on logarithmic intervals: 3.33, 7.033, 14.67, 13.33, and 66.60 mg · l⁻¹ for neem, and 0.156, 0.512, 1.25, 2.5, and 5 mg · l⁻¹ for *Bt*. Each leaf (8 cm in diameter) was separately immersed in a tested concentration for 10 s, and placed in Petri dishes (150 ml) for 30 min to be dried under laboratory conditions. Control leaves were treated with distilled water. For each treatment, 10 larvae per Petri dish were used in three replicates, and mortality was documented after 24 h. The lethal concentrations (LC₃₀, LC₅₀ and LC₉₀: lethal concentrations to kill 30, 50 and 90% of tested insects, respectively) were determined using Probit analysis (Finney 1971).

Determination of the synergistic effects of Bt and neem

Based on the aforementioned method, the larvae were treated separately with the mixtures of Bt + neem: LC₃₀ + LC₃₀; LC₃₀ + LC₅₀; LC₅₀ + LC₃₀, respectively). After 24 h, the larval mortality was recorded and calculated based on the following formulas:

$$Em = Oa + Ob (1 - Oa),$$

where: *Em* – the expected mortality, *Oa* and *Ob* – the obtained mortalities of tested agents at the given concentration.

The insecticidal properties of combinations were identified as either antagonistic or synergistic by analysis using comparisons of χ^2 :

$$\chi^2 = (Om - Em)^2 / Em,$$

where: *Om* – the obtained mortality due to the combinations, *Em* – the expected mortality, and $\chi^2 - 3.84$ with $\alpha = 0.05$ and *df* = 1. A value of $\chi^2 > 3.84$ and higher-than-expected mortality was considered to reveal synergistic effects, while $\chi^2 < 3.84$ demonstrated additive effects. If the observed mortality is lower than the expected mortality, it indicates an antagonistic effect between the compounds (Trisnyono and Whalon 1999).

Biochemical assessments

Third-instar larvae of *H. cunea*, after treatment and in the control insects, were collected after 48 h from the onset of the experiments and then frozen at -20°C. The whole larval body was centrifuged (Thermo Fisher Scientific, Asheville, NC, USA) for 10 min at 28 600 g. The supernatant was transferred to new tubes and stored at -20°C until used. Each biochemical analysis, including digestive enzymes and detoxification enzymes, was repeated three times.

α -Amylase activity

The α -amylase activity was measured based on the method of Bernfeld (1955), using 1% soluble starch as substrate. The reaction was performed by sodium phosphate buffer (Sigma, St. Louis, MO, USA) at 35°C with 10 ml of the enzyme, 40 ml substrate, and 40 ml sodium phosphate buffer (pH 9) for 30 min. To stop the reaction, 100 ml dinitrosalicylic acid (DNS, Sigma, St. Louis, MO, USA) was added and heated in boiling water for 10 min. The absorbance was read at 540 nm using Elisa Reader (Awareness, USA). One unit of α -amylase activity was defined as the amount of enzyme required to produce 1 mg maltose as a standard curve in 30 min at 35°C.

α -Glucosidase and β -glucosidase activity

For solubilization of membrane hydrolyses (α - and β -glucosidases) in Triton X-100, membrane preparations were exposed to Triton X-100 for 20 h at 40°C, at a ratio of 10 mg of Triton X-100/mg of protein, before being centrifuged at 15,000 rpm for 30 min. No sediment was visible after centrifuging this supernatant at 22 000 g for 60 min. The activity of the enzymes remains unchanged, at -20°C, for periods of at least a month (Ferreira and Terra 1983). Incubating 50 μ l of enzyme solution with 75 μ l of p-nitrophenyl- α -D-glucopyranoside (pNaG, Sigma-Aldrich, St. Louis, MO, USA) (5 mM), p-nitrophenyl- β -D-glucopyranoside (pN β G, Sigma-Aldrich, St. Louis, MO, USA) (5 mM), and 125 μ l of 100 mM universal buffer (pH 5.0) at 37°C for 10 min was done. The reaction was stopped by adding 2 ml of sodium carbonate (1 M) and was read at 450 nm (Ferreira and Terra 1983).

Lipase activity

The determination of lipases was conducted using the method outlined by Tsujita *et al.* (1989). In this procedure, a 10 μ l homogenate solution was combined with 18 μ l of p-nitrophenyl butyrate (Sigma-Aldrich, St. Louis, MO, USA) (50 mM) and 172 μ l of buffer (1M, pH 7). The mixture was incubated at 37°C, and the absorbance was measured at 405 nm.

Protease activity

The general protease activity of midguts was determined using azocasein as a substrate (Elpidina *et al.* 2001). The reaction mixture was 80 μ l of 2% azocasein solution (Sigma-Aldrich, St. Louis, MO, USA) in 40 mM universal buffer of specified pH and 30 μ l enzyme. The reaction mixture was incubated at 37°C for 60 min. Proteolysis was stopped by the addition of 300 μ l of 10% trichloroacetic acid (TCA, Sigma-Aldrich, St. Louis, MO, USA). Appropriate blanks in which TCA was added first to the substrate were prepared for each assay. Precipitation was achieved by cooling at 4°C for 120 min, and the reaction mixture was centrifuged at 16,000 rpm for 10 min. An equal volume of 1 N NaOH (Sigma-Aldrich, St. Louis, MO, USA) was added to the supernatant, and the absorbance was recorded at 440 nm.

Detoxification enzymes

To measure the activity of glutathione S-transferases according to Habing *et al.* (1974), CDNB (1-chloro-2,4-dinitrobenzene) (Sigma-Aldrich, St. Louis, MO, USA) (20 mM) was used as the substrate to measure the enzyme activity. The absorbance was recorded at 340 nm. Esterase (EST) activity was measured according to the method described by Han *et al.* (1998). For the assay, 15 μ l of α -NA (10 mM) or β -NA (10 mM) was added

separately to 25 μ l of fast blue RR Salt (1 mM) and 15 μ l of the enzyme sample.

Phenol oxidase activity assay

Ten μ l of hemolymph was diluted with 90 μ l of cold-sterile phosphate buffered solution and vortexed for 10 min. It was then frozen at -20°C for 48 h, after which the samples were used for phenol oxidase (PO) activity assay. The method of Cotter *et al.* (2004) was used for the assay. Centrifugation was done (5000 g at 4°C for 5 min). The supernatant (50 μ l) was collected and mixed with 150 μ l of L-3,4-dihydroxyphenylalanine (10 mM) (LDOPA, Sigma-Aldrich, St. Louis, MO, USA). The activity of phenol oxidase was recorded on a linear basis of phasic reactions at 490 nm. The method of Parkinson and Weaver (1999) was incorporated to determine the specific activity of PO.

Effect on pupal weight

Two-day-old third-instar larvae of *H. cunea* were fed treated leaves for 24 h. Then, until the end of the larval period (on average 10–16 days), fresh mulberry leaves were provided to the larvae every day. Each treatment had four replications (10 larvae). The weight of the formed pupae was measured with a digital scale and compared with the control group (Wang *et al.* 2009).

Statistical analysis

The probit analysis was done using the POLO-PC software (2002). In this case, significant differences between the concentrations were recorded when 95% confidence intervals (CI) did not overlap. Other data were considered for one-way analysis of variance (ANOVA) followed by Tukey's test to find significant differences between means using the SAS software (SAS Institute, Cary, Cary, NC, USA, 1997). The synergistic effect between the neem and Bt was evaluated by using χ^2 .

Results

Single and combined effect of Bt and neem on *Hyphantria cunea* mortality

Significant mortality was observed in the mortality of *H. cunea* larvae treated by Bt ($F = 14.98$; $df = 3$; $p < 0.05$) and neem ($F = 17.76$; $df = 3$; $p < 0.05$). Results of the probit analysis of the lethality of Bt and neem on *H. cunea* larvae are given in Table 1, in which LC_{30} , LC_{50} and LC_{90} values were 1.20, 3.10, and 31.18 ppm for Bt and 13.33, 31.75 and 102.28 ppm for neem (Table 1). Based on low LC_{50} values and non-overlapping

Table 1. Lethal concentrations, confidence limits (95%) and regression slope lines of the effects of Bt and neem against *Hyphantria cunea* larvae after 24 h

Treatments	Slope $\pm$ SE	χ^2 (df = 3)	Lethal concentrations [ppm]		
			LC ₅₀	LC ₃₀	LC ₉₀
Bt	1.280 $\pm$ 253	0.515	3.103 (2.270–6.160)	1.20 (0.740–1.831)	31.18 (12.418–228.696)
Neem	1.394 $\pm$ 5.009	3.424	31.753 (16.628–216.369)	13.350 (4.943–29.284)	102.277 (45.049–1034.604)

confidence limits, *H. cunea* larvae were more susceptible to Bt than neem.

Bioassays using binary mixtures of Bt and neem demonstrated that their combination resulted in synergistic phenomena on the toxicity against *H. cunea* larvae in all the used ratios (Table 2).

Digestive enzyme inhibitory effects

Results showed that Bt and neem affected the digestive enzymatic profiles of *H. cunea* larvae in the tested

concentrations. When larvae fed on leaves treated with Bt and neem, the activity of all digestive enzymes, including protease, α -amylase, lipase, α -glucosidase, and β -glucosidase, was decreased related to the studied concentrations (Table 3). By increasing the time from 24 h to 72 h, the activity level of enzymes gradually decreased in all treatments. The anti-nutritional effect of leaves treated with neem and Bt caused a decrease in the activity of digestive enzymes in the days after feeding. The activity of these enzymes was the lowest in the combination treatments of Bt and neem (Table 3).

Table 2. Results of binary mixture of neem and Bt against the larvae of *Hyphantria cunea*

Binary mixtures	Ratios	Larval mortality [%]				χ^2	Effect
		pure compound		binary mixtures			
		O _a	O _b	E _m	O _m		
Bt + Neem	LC ₃₀ + LC ₃₀	24	18	37.68	60	13.22	synergy
Bt + Neem	LC ₃₀ + LC ₅₀	24	34	49.84	76	13.73	synergy
Bt + Neem	LC ₅₀ + LC ₃₀	40	18	50.8	88	27.19	synergy

O_a – observed mortality of the first compound; O_b – observed mortality of the second compound; E_m – expected mortality; O_m – observed mortality

Table 3. Effect of Bt and neem on the activity of digestive enzymes of *Hyphantria cunea* larvae

Treatments	Protease [U · mg ⁻¹]	α -Amylase [U · mg ⁻¹]	Lipase [U · mg ⁻¹]	α -Glucosidase [U · mg ⁻¹]	β -Glucosidase [mg · dl ⁻¹]
Control	3.608 $\pm$ 0.0045 a	2.7195 $\pm$ 0.0045 a	3.805 $\pm$ 0.0045 a	2.0841 $\pm$ 0.005 a	2.8900 $\pm$ 0.0191 a
Neem LC ₃₀ 24 h	2.2541 $\pm$ 0.0065 b	2.22 $\pm$ 0.00212 b	2.969 $\pm$ 0.0015 b	2.0048 $\pm$ 0.0191 a	2.0707 $\pm$ 0.0191 b
Neem LC ₅₀ 24 h	2.0232 $\pm$ 0.005 c	2.150 $\pm$ 0.0021 b	2.3537 $\pm$ 0.0276 b	2.220 $\pm$ 0.0151 a	2.0075 $\pm$ 0.0525 b
Control	3.4386 $\pm$ 0.0045 a	2.5557 $\pm$ 0.0007 a	4.0018 $\pm$ 0.0341 a	1.9879 $\pm$ 0.0154a	2.7707 $\pm$ 0.0100 a
Neem LC ₃₀ 48 h	1.4098 $\pm$ 0.0045 b	2.1162 $\pm$ 0.0102 b	1.9744 $\pm$ 0.0012 ab	1.2895 $\pm$ 0.0141 b	1.4707 $\pm$ 0.0145 b
Neem LC ₅₀ 48 h	1.3819 $\pm$ 0.0082 b	2.0965 $\pm$ 0.0003 b	1.7924 $\pm$ 0.0005 b	1.2943 $\pm$ 0.0137 b	1.1703 $\pm$ 0.0261 c
Control	3.889 $\pm$ 0.0085 a	2.0785 $\pm$ 0.0003 a	3.6046 $\pm$ 0.0007 a	1.9303 $\pm$ 0.0033 a	3.0734 $\pm$ 0.0091 a
Neem LC ₃₀ 72 h	0.9882 $\pm$ 0.0002 b	1.273 $\pm$ 0.0056 b	1.115 $\pm$ 0.0018 b	1.4580 $\pm$ 0.0195 b	1.5707 $\pm$ 0.0198 b
Neem LC ₅₀ 72 h	0.748 $\pm$ 0.0285 c	1.260 $\pm$ 0.0038 b	1.0101 $\pm$ 0.0002 b	1.2451 $\pm$ 0.0143c	1.2707 $\pm$ 0.0089 c
Control	3.231 $\pm$ 0.012 a	2.765 $\pm$ 0.0054 a	3.912 $\pm$ 0.0032 a	2.1287 $\pm$ 0.0003 a	2.7632 $\pm$ 0.0054 a
Bt LC ₃₀ 24 h	1.876 $\pm$ 0.0087 b	1.54 $\pm$ 0.0012 b	2.876 $\pm$ 0.0034 b	1.9854 $\pm$ 0.0098 b	2.00 $\pm$ 0.0075 b
Bt LC ₅₀ 24 h	1.1265 $\pm$ 0.008c	1.065 $\pm$ 0.0004 c	1.5437 $\pm$ 0.0864 c	1.054 $\pm$ 0.0043 c	1.4325 $\pm$ 0.0235 c
Control	3.1298 $\pm$ 0.0098a	2.4321 $\pm$ 0.0023 a	4.000 $\pm$ 0.0432 a	2.021 $\pm$ 0.0087 a	2.6532 $\pm$ 0.01876 a
Bt LC ₃₀ 48 h	1.2002 $\pm$ 0.0076 b	2.0132 $\pm$ 0.0076 b	2.9864 $\pm$ 0.0008 b	1.1432 $\pm$ 0.0008 b	1.287 $\pm$ 0.0153 b
Bt LC ₅₀ 48h	0.8197 $\pm$ 0.0043 c	1.9565 $\pm$ 0.0043 b	1.4321 $\pm$ 0.0054 c	0.998 $\pm$ 0.0076 c	1.0721 $\pm$ 0.0054 b

Table 3. Effect of Bt and neem on the activity of digestive enzymes of *Hyphantria cunea* larvae – continued

Treatments	Protease [U · mg ⁻¹]	α-Amylase [U · mg ⁻¹]	Lipase [U · mg ⁻¹]	α-Glucosidase [U · mg ⁻¹]	β-Glucosidase [mg · dl ⁻¹]
Control	3.354 ± 0.0032 a	2.2136 ± 0.0032 a	3.654 ± 0.0023 a	2.213 ± 0.0214 a	3.123 ± 0.0032 a
Bt LC ₃₀ 72 h	0.7652 ± 0.0076 b	1.013 ± 0.0032 b	1.081 ± 0.0009 b	1.087 ± 0.0032 b	1.1208 ± 0.0021 b
Bt LC ₅₀ 72 h	0.423 ± 0.0011 c	0.7632 ± 0.0003 c	0.712 ± 0.0054 c	0.6432 ± 0.0065 c	0.5432 ± 0.0005 c
Control (Neem + Bt 72 h)	3.81037 ± 0.002 a	1.865 ± 0.0545 a	3.5577 ± 0.0026 a	2.0064 ± 0.0151 a	3.127 ± 0.0049 a
Neem (LC ₃₀) + Bt (LC ₃₀)	0.610 ± 0.0065 b	1.189 ± 0.0038 b	0.9134 ± 0.0006 b	1.4290 ± 0.0022 b	2.6707 ± 0.0097 b
Neem (LC ₃₀) + Bt (LC ₅₀)	0.5893 ± 0.0076 bc	0.9762 ± 0.0038 c	0.7115 ± 0.0005 bc	1.0612 ± 0.0051 bc	2.2706 ± 0.0159 bc
Neem (LC ₅₀) + Bt (LC ₃₀)	0.548 ± 0.0088 c	0.948 ± 0.0088 c	0.5063 ± 0.0053 c	0.935 ± 0.0176 c	1.6697 ± 0.0093 c

Means ± SE followed by the same letters indicate no significant difference for a given column in each treatment according to the Tukey test ($p < 0.05$)

Detoxification enzyme inhibitory effects

It was observed that treatment of the larvae by neem and Bt, alone and in combinations, caused significant changes in the activity of detoxifying esterase enzymes compared to the control group ($F = 21.43$, $df = 3$, $p < 0.0001$). The LC₃₀ of neem caused a significant increase in the esterase activity regarding the β-naphthylacetate substrate. Also, an increase in the activity of general esterases (both α- and β-naphthylacetate substrates) was observed in the combination of LC₃₀ values of two insecticides. Mixing LC₅₀ and LC₃₀ of neem and Bt, respectively, caused a significant increase in esterase activity based on the β-naphthylacetate substrate (Fig. 1).

Glutathione S-transferase activity was also increased in all treatments compared to the control ($F = 27.11$, $df = 3$, $p < 0.0001$) (Fig. 2). The highest activity of this enzyme was observed in the LC₅₀ of Bt in the test with DCNB. In contrast, the highest activity of

this enzyme with CDNB as substrate was observed in the combination of two insecticides at different concentrations.

Regarding acetylcholine esterase activity, unlike the other detoxifying enzymes, the enzyme activity was decreased in the larvae treated by all treatments ($F = 34.22$, $df = 3$, $p < 0.0001$). The greatest inhibition of the activity of acetylcholine esterase was detected by the combination of LC₃₀ values of neem and Bt (Fig. 3).

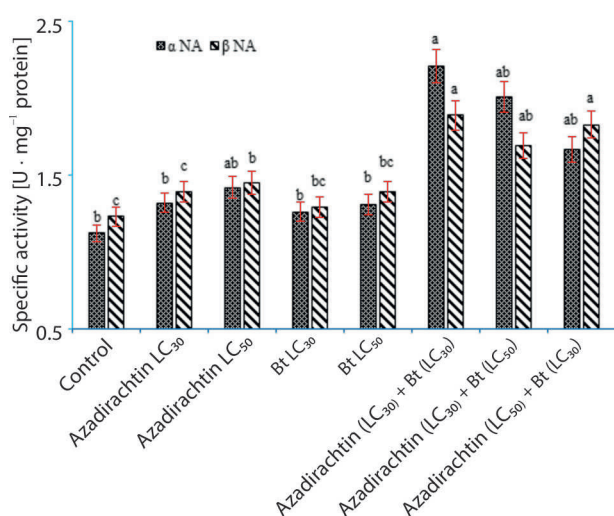


Fig. 1. Activity of general esterases in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). α NA: α-naphthylacetate; β NA: β-naphthylacetate

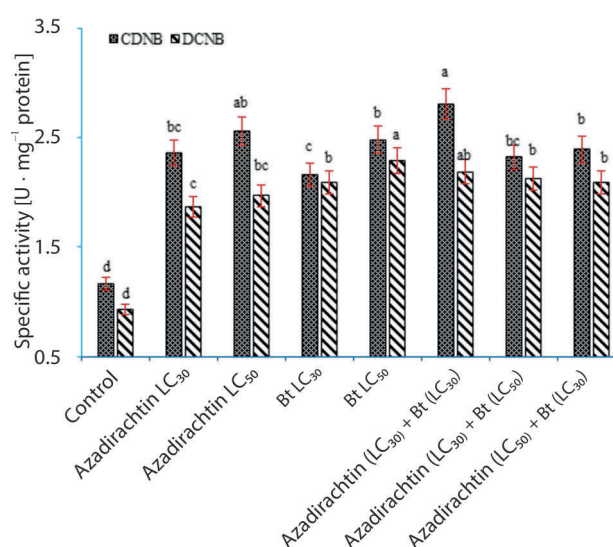


Fig. 2. Activity of glutathione S-transferases in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). CDNB: 1-chloro-2,4-dinitrobenzene; DCNB: 1,2-dichloro-4-nitrobenzene

Effect on phenol oxidase activity

According to Figure 4, the activity of phenol oxidase was increased in all treatments compared to the control group ($F = 21.11$, $df = 3$, $p < 0.0001$), in which the highest activity was detected in the larvae treated with neem and Bt LC₃₀ – LC₅₀ combinations.

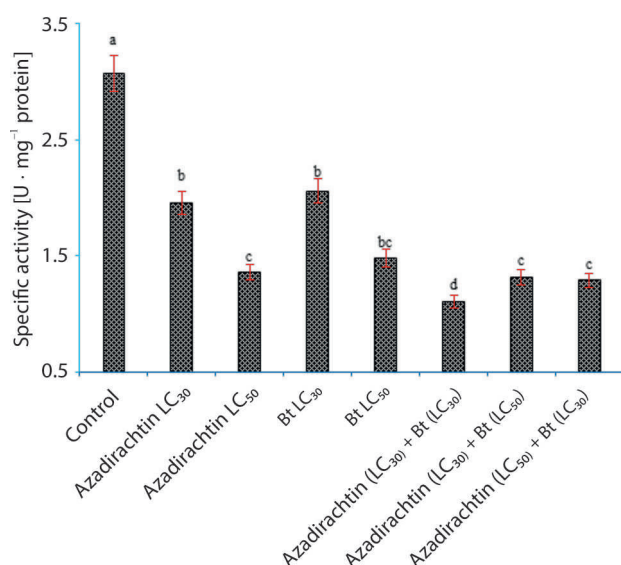


Fig. 3. Activity of acetylcholine esterase in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$)

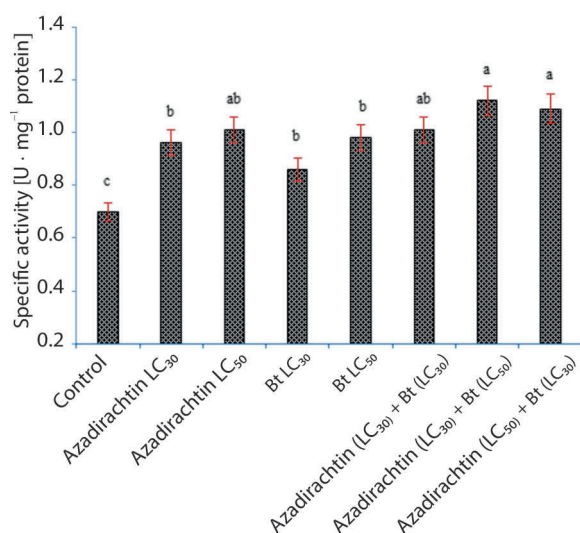


Fig. 4. Activity of phenol oxidase in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$)

Effect on pupal weight

According to Table 4, the weight of *H. cunea* pupae emerged from larvae affected by all separate and combined treatments of neem and Bt was significantly decreased compared to the control group ($F = 68.67$, $df = 3$, $p < 0.0001$). The neem LC₅₀ treatment and the combination of neem LC₅₀ + Bt LC₃₀ resulted in the most significant reduction in pupal weight compared to all other treatments. Indeed, neem, present in all treatments, had a high inhibitory effect on the feeding of larvae, and therefore, incomplete pupae with low weights emerged from weak larvae.

Table 4. Pupal weight (mg $\pm$ SE) emerged from *Hyphantria cunea* larvae affected by neem and Bt (mean of both sexes)

Treatments	Pupa weight [mg]
Control	207.08 $\pm$ 0.87 a
Neem (LC ₃₀)	127.23 $\pm$ 3.37 bc
Neem (LC ₅₀)	110.31 $\pm$ 5.09 cd
Bt (LC ₃₀)	172.65 $\pm$ 3.12 b
Bt (LC ₅₀)	130.72 $\pm$ 3.33 bc
Neem (LC ₃₀) + Bt (LC ₃₀)	130.54 $\pm$ 11.07 bc
Neem (LC ₃₀) + Bt (LC ₅₀)	116.08 $\pm$ 5.32 c
Neem (LC ₅₀) + Bt (LC ₃₀)	98.98 $\pm$ 0.07 d

Means $\pm$ SE followed by the same letters indicate no significant difference for a given column in each treatment according to the Tukey test ($p < 0.05$)

Discussion

Biorational pesticides are environmentally friendly as they possess biodegradable and low-risk features compared to classic synthetic pesticides (Horowitz *et al.* 2009). Furthermore, due to minimal residual effects, predators, parasitoids, and pollinators are the least affected, and accordingly, they are compatible with IPM programs (Murugesan *et al.* 2023). Biopesticides, with their multiple modes of action and diverse insecticidal targets, provide significant advantages in managing pest resistance, driving growing interest among researchers (Birch *et al.* 2011). In the present study, the high mortality rate, the inhibition of digestive enzymes and acetylcholine esterase activity, and the reduction in emerged pupae weight were revealed on *H. cunea* larvae treated with Bt and neem, either individually or in combination.

According to the present findings, the combination of different concentrations (LC₃₀ and LC₅₀ values) of Bt and neem resulted in a synergistic phenomenon in the mortality of *H. cunea* larvae. Similarly, Singh *et al.* (2007) presented a positive interaction between Bt (Delfin® WG™ (Certis USA, LLC) and neem (500 ppm azadirachtin (AI)) on the larval mortality of the cotton bollworm *Helicoverpa armigera* Hübner. Although they concluded that neem facilitated the action of Bt, the combined action was not synergistic. Abedi *et al.* (2014) showed that a combination of neem and Bt can be more effective on *H. armigera* than using them alone to control this pest. In another study, positive interactions of Bt and neem on larvae of the Indian meal moth *Plodia interpunctella* Hübner were reported (Nouri-Ganbalani *et al.* 2016). The results of these studies emphasize the present findings about enhancing the insecticidal potential of Bt and neem by their combination. However, differences in lethal

concentrations and synergistic or additive phenomena can be justified by different studied insect pests.

The activity of enzymes reflects the absorption, digestion, and positive transport of nutrients through the midgut. The Bt has been reported to cause damage to the epithelial cells of the midgut through crystalline parasporal bodies, which release the active toxin after digestion by serine proteases under alkaline conditions in the intestinal juice; the damage to the midgut causes a decrease in enzyme activities (Mathavan *et al.* 1989; Miao 2002). Diet treatment with botanical insecticides can also affect several enzyme activities in larvae of *H. cunea* by interfering with the production of certain types of proteins (Senthil-Nathan *et al.* 2006). Therefore, it may be expected that such damage to the midgut would cause a serious reduction in the activity of digestive enzymes.

The study revealed that Bt and neem treatments significantly reduced protease activity, suggesting disruptions in both digestive enzyme production and midgut epithelial integrity. Proteases play a critical role in insect digestive processes and metabolic functions. The observed decline in protease activity likely impairs nutrient absorption and disrupts cellular homeostasis, highlighting the profound physiological impact of these treatments on target insects (Klowden 2007). Lipases, vital for growth, reproduction, and pathogen defense, are reduced post-treatment with Bt and neem, indicating a blockage in the conversion of phytosterols to lipase building blocks (Klowden 2007). There is a reduction in the activity level of α - and β -glucosidases. This may be due to a drop in consumption rates and leveling off or a decline in food conversion efficiencies (Zibae *et al.* 2009). The observed reduction in α -amylase in this study may result from damage to the midgut epithelium, which is responsible for enzyme production (Senthil-Nathan *et al.* 2006). Bt and neem have been shown to significantly reduce the activity of digestive enzymes in various insect species, thereby impairing their ability to process nutrients effectively (Senthil-Nathan *et al.* 2009). Studies have demonstrated that these botanical products can decrease the activity of proteases, amylases, and lipases in other insects such as southern armyworm *Spodoptera eridania* Stoll and the gypsy moth *Lymantria dispar* (L.) (Shannag *et al.* 2015; Zou *et al.* 2019). This reduction in enzyme activity not only impairs nutrient absorption but also leads to antifeedant effects, reduced food consumption, and slower larval growth (Mordue(Luntz) and Nisbet 2000). These effects, observed in previous studies on applying botanical compounds, highlight the disruption of digestive enzyme synthesis in response to substrate presence in the midgut (Oftadeh *et al.* 2020, 2021).

Increases in glutathione S-transferase (GST) activity and decreases in the AChE activity as important

detoxifying enzymes by the combination of neem and Bt were observed in the present study. Potentially, this indicates that while GST increased to detoxify the biopesticides, an inhibitory effect was observed with AChE. Similar results were observed in the application of the essential oil of Ajwain (*Carum copticum*, L.) on *Chilo suppressalis* Walker (Basij *et al.* 2023). War *et al.* (2014) observed that a combined treatment of neem oil formulation and endosulfan reduced esterase activity but increased glutathione-S-transferase activity in *H. armigera* larvae. The application of neem and Bt has been shown to increase the activity of some detoxification enzymes in insects as part of their defense mechanisms. For instance, Bt treatment in two commercial formulations in *Spodoptera litura* (F.) has been found to elevate phenol oxidase activity after 48 h, which plays a crucial role in melanization and pathogen defense (Kamel *et al.* 2010). Similarly, exposure to neem in the palmetto weevil *Rhynchophorus cruentatus* F. led to increased levels of detoxification enzymes such as glutathione S-transferase, and superoxide dismutase, aiding in the breakdown of toxic compounds (Gabr *et al.* 2022). In this study, despite these increases, both neem and Bt reduced the activity of acetylcholinesterase, an enzyme critical for nerve function, thereby impairing the insect's ability to develop resistance, particularly when these compounds were used in combination.

Bt and neem induced significant insecticidal effects on third-instar larvae of *H. cunea*. Higher mortality was observed when the larvae fed on leaves treated with Bt and neem combinations than larvae that fed only on a diet containing one of these insecticides. Furthermore, Bt and neem significantly reduced the activity of digestive enzymes and pupae weight along with an increase in the activity of acetylcholine esterase. Exposure to a combination of Bt and neem led to a greater effect of these parameters. It can be suggested that the use of neem as a synergist to a Bt with multiple modes of action may result in a more effective management strategy against *H. cunea*. However, to apply the research results, future research should focus on field-level studies and investigate the combination of weather and temperature on these natural products to find the suitable time for using or protecting these compounds. Such studies could provide new insights into the use of botanical agents as additives to Bt, which may play a more prominent role in pest control programs in the future.

Conclusions

Hyphantria cunea is a relevant insect pest causing significant damages to fruit, ornamental plants, forest

trees and other crops. The extensive use of synthetic insecticides to control this pest raises environmental and health concerns, highlighting the need for alternative methods. Investigating bio-pesticides is critical to support IPM programs, as they offer a safer and more sustainable approach to pest control. This study found that the combination of neem and Bt was effective in suppressing *H. cunea* due to their synergistic effect. The potential benefits of this synergistic effect on IPM strategies are substantial: the combination of neem and Bt has been shown to be effective against *H. cunea* larvae, particularly in reducing digestive enzyme activity and pupal weight. Treated larvae also showed a significant decrease in detoxifying enzyme activity. Conducting such analyses will provide valuable insights to optimize pest control while prioritizing safety and efficacy.

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