Effect of Botanical Insecticides and Bacterial Toxins on the Gut Enzyme of the Rice Leaffolder *Cnaphalocrocis medinalis*

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The effect of botanical insecticides and bacterial toxins on gut enzyme activity of larvae of the rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (Insecta: Lepidoptera: Pyralidae) was investigated. Gut enzyme activities were affected by botanical insecticides and bacterial toxin individually and in combination. When fed a diet of rice leaves treated with botanical insecticides and bacterial toxins, in bioassays the activities of gut tissue enzymes - acid phosphatases (ACP), alkaline phosphatases (ALP) and adenosine triphosphatases (ATPase) – of rice leaffolder larvae were affected. When combined, the effect was more severe at a low concentration. Larvae that were chronically exposed to botanical insecticides and bacterial toxins showed a reduction in weight (59–89%) and exhibited a significant reduction in ACP, ALP and ATPase activities. The combination of *Bacillus thuringiensis kurstaki* and botanical insecticides caused a decrease of twofold in enzyme activity even at reduced concentration. A synergistic effect was found when botanical insecticides and bacterial toxins were combined at low doses. These effects were most pronounced in early instars. Clear dose–response relationships were established with respect to enzyme activity. In conclusion: (i) biopesticides are relatively safe and biodegradable; (ii) a synergistic effect of botanical insecticides and bacterial toxins was found; (iii) less expensive, readily available and naturally occurring biopesticides could be an alternative for organic and inorganic pesticides in controlling RLF.
KEY WORDS: Biopesticide; *Bacillus thuringiensis*; *Bt*; neem; *Vitex negundo*; *Cnaphalocrocis medinalis;* enzyme; ACP; ALP; ATPase.

INTRODUCTION

The rice leaffolder (RLF) *Cnaphalocrocis medinalis* (Guenée) (Insecta: Lepidoptera: Pyralidae) is a major insect pest of rice (*Oryza sativa* L). It appears to have become increasingly important with the spread of high-yield rice varieties and the accompanying changes in cultural practices. Outbreaks of serious RLF infestations have been reported in many Asian countries including India, Korea, Japan, China, Malaysia, Sri Lanka and Vietnam (10,15). Bautista *et al.* (4) have shown that losses due to RLF are positively related to the percentage of damaged leaves. In their studies, 17.5% damaged leaves resulted in 16.5% yield loss, and a 21.3% yield loss corresponded to 26.6% damaged leaves. Rice farmers control this pest primarily through the use of broad-spectrum chemical insecticides such as methyl parathion, monocrotophos and endosulfan. Misuse of chemical insecticides

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increased the RLF population because the sprayed insecticides reduce populations of natural enemies of RLF and its biological control in the field (10). Expanded rice areas with new irrigation systems, multiple rice cropping and application of high levels of nitrogenous fertilizers have further compounded the leaffolder problem. The larvae feed by scraping the green mesophyll tissues of rice leaves, thus producing linear pale white stripe damage. The general vigor and photosynthetic ability of an infested rice plant is greatly reduced. The damaged leaves also serve as entry points for fungal and bacterial infections. In recent years the use of synthetic organic insecticides in crop pest control programs around the world has resulted in damage to the environment, pest resurgence, pest resistance to insecticides and lethal effects on non-target organisms.

Management of this insect pest using synthetic chemicals has failed because of insecticide resistance, pest resurgence and environmental pollution. Consequently an intensive effort has been made to find alternative methods of control. Botanical insecticides and microbial pesticides are highly effective, safe and ecologically acceptable. The common trend in the past two decades towards reducing reliance on synthetic insecticides for control of insect pests in agriculture, forestry and human health has renewed world-wide interest in *Bacillus thuringiensis* (*Bt*) Berliner as an environmentally desirable alternative. It has been tested successfully against more than 137 insect species including Lepidoptera, Hymenoptera, Diptera and Coleoptera. *Bt* can effectively replace chemical insecticides and has become established in control programs against defoliator larval moths in Europe and North America (6). Commercially available *Bt* products contain endotoxins, a class of *Bt* proteins that are seldom toxic to mammals. In recent years the use of synthetic organic insecticides in crop pest control programs around the world has resulted in disturbance of the environment, pest resurgence, pest resistance to insecticides, lethal effects on non-target organisms, and environmental pollution. In recent years an increasing number of reports on the development of resistance to *Bt* in agriculture have been published; the lethal dose of *Bt* is also instar-dependent and the susceptibility of mature larvae is very low (25,34). The combination of *Bt* with baculovius has been tested (24), but a combination with other biopesticides is very necessary.

The Indian neem tree, *Azadirachta indica* (A. Juss.), has been found to be a promising source of natural pesticides and several constituents of its leaves and seed show marked insect control potential. Neem seed kernel extract suppresses the feeding, growth and reproduction of insects. Due to their relative selectivity, neem products can be recommended for many Integrated Pest Management (IPM) programs (29). Butterworth and Morgan (8) first isolated tertranotriterpenoid azadirachtin from *A. indica* seed, which primarily showed antifeedance and subsequently also regulatory effects on larval development and metamorphosis. *Bt* products break down very rapidly when exposed to sunlight. *Vitex negundo* L. (Verbenaceae) showed a pesticidal activity against insects and is used predominantly for its pest control properties. It is apparent that botanical insecticides affect insect physiology in many different ways and that other modes of action are still to be discovered. Alkaline phosphatase (ALP, E.C.3.1.3.1) and acid phosphatase (ACP, E.C.3.1.3.2) are hydrolytic enzymes, which hydrolyze phosphomonoesters under acid or alkaline conditions, respectively. These enzymes are located in the midgut, Malpighian tube, muscles and nerve fibers of lepidopteran insects (17). The midgut has higher ALP and ACP activity than the other tissues (11,21). The ALP and ACP activities were found to be low during the larval molting stage and to increase gradually after molting (20). The

highest activity appeared before the full appetite gluttonous stage of the 5th instar and the lowest activity was found at the mature larval stage (20). The objectives of this study were to examine the ALP, ACP and adenosine triphosphatase (ATPase) activities in the midgut of RLF and to determine the effects of bacterial toxin and botanical insecticides on enzyme activity. In this paper are described experiments that examine potential effects of botanical insecticides and bacterial toxin on activities of gut enzymes in the rice leaffolder.

MATERIALS AND METHODS

Laboratory mass culture of *Cnaphalocrocis medinalis* *Cnaphalocrocis medinalis* larvae were collected from the paddy fields in and around Coimbatore district, Tamil Nadu, and the Paddy Breeding Station (PBS), Tamil Nadu Agricultural University, Coimbatore, India. Larvae were reared in a greenhouse on potted rice plants covered with mesh sleeves at 27±20°C, 10:14LD, and 85% r.h. Rice plants were grown in earthenware pots, 18 cm tall, 20 cm diam at the top. Each pot held 15 plants and gave 62 tillers. The pots were placed in ∼10 cm of water in a metal tray in the greenhouse. The culture was initiated with partly grown larvae from the field. Thereafter, newly hatched larvae were placed on ∼60-day-old plants of the rice variety TN1.

After pupation, adults emerged on plants in the sleeves. To maintain the culture, 12 female and 13 male moths were placed in an oviposition cage containing one potted plant. The moths were fed with 10% sucrose solution fortified with a few drops of vitamin A + D mixture (Multidec®drops, Ashok Pharmaceuticals, Chennai, India) to enhance oviposition. After 2 days the potted plants were removed from the oviposition cage. The leaf portions containing the eggs were clipped and placed on moist filter paper in a petri dish. These eggs were used to maintain the culture.

Preparation of *Bacillus thuringiensis* subsp. *kurstaki* Delfin WG, which contains *Bacillus thuringiensis kurstaki (Btk)*, serotype 3a, 3b 85%, and dispersing agents 15%, with a minimum potency of 53,000 SU/mg (Sandoz (India) Limited, Mumbai, India), was used. The required quantity of *Btk* crystal was thoroughly mixed with distilled water to prepare various concentrations, ranging from 1 to 15 μg ml^{-1}.

Preparation of neem seed kernel and *Vitex negundo* leaf extracts Neem seed kernel and *Vitex negundo* were collected from forests in the Marudamalai hills, near Bharathiar University, Coimbatore. Fifty grams of seed kernels of *A. indica* and *V. negundo* were washed and oven-dried to constant weight at 55°C. The dried seeds and leaves were ground into powder. The powder was then mixed with 100 ml of water in a Soxhlet apparatus to prepare a stock solution, from which several concentrations ranging from 0.05% to 3.0% were made up using water. The combined extracts were prepared by mixing equal volumes of neem seed kernel extract (NSKE) and *V. negundo* leaf extract (VNLE) (1:1,v/v).

Treatments Fresh rice leaves were coated with different concentrations of *Btk*, NSKE and VNLE and air-dried. Control leaves were treated with distilled water alone. The various larval instars were starved for 4 h and then individually fed with different concentrations of *Btk*, NSKE and VNLE. The uneaten leaves were removed every 24 h, and replaced with fresh treated leaves. A minimum of 30 larvae were used per concentration for all the experiments, which were replicated five times.

Preparation of enzyme extract Two-day-old 3rd, 4th and 5th instars of treated *C. medinalis* were used to quantify the enzyme activities. The method used to prepare the

enzyme extract was that of Applebaum (1) and Applebaum *et al.* (2). Individuals were anaesthetized with cotton pads soaked in ether and the entire digestive tract was dissected out in ice cold insect Ringer's solution. The Malpighian tubules, adhering tissues and gut contents were removed. The gut was split into regions, weighed (accuracy in mg) and homogenized for 3 min at 4°C in ice-cold citrate-phosphate buffer (pH 6.8) using a tissue grinder. Homogenized gut was suspended in ice-cold buffer and made up to 1 ml. The homogenate was centrifuged at 500 rpm for 15 min and the supernatant was used as the enzyme source.

Estimation of ACP and ALP The enzyme assays were carried out as described by Bessey *et al.* (5). The buffered substrate was incubated with tissue extract for 30 min. Alkali was added to stop the reaction and to adjust the pH for the determination of the concentration of the product formed. The spectral absorbance of *p*-nitrophenolate was maximal at 310 nm. The molar absorbance of *p*-nitrophenolate at 400 nm is about double that of *P*-nitrophenyl phosphate at 310 nm. Upon converting the *p*-nitrophenolate into *p*-nitrophenol by acidification, the absorption maximum is shifted to ∼320 nm with no detectable absorption at 400 nm.

Estimation of ATPase The specific activity of sodium- and potassium-dependent ATPase in the gut was assayed according to the method described by Shiosaka *et al.* (31). ATP catalyzes the conversion of ATP to ADP. During this conversion, one molecule of phosphorus is liberated.

$$Adenosine\ triphosphate \xrightarrow{ATPase} Adenosine\ diphosphate\ +\ P$$

The inorganic phosphorus liberated was assayed according to the method of Fiske and Subbarow (12), by which the protein is precipitated with trichloroacetic acid. The protein-free filtrate is treated with acid molybdate solution and the phosphoric acid formed is reduced by the addition of 1-amino–2-napthol-4-sulphonic acid (ANSA) reagent to produce a blue color; the color intensity is proportional to the amount of phosphorus present.

Statistical analysis Data from all experiments were subjected to analysis of variance (ANOVA) and means were separated using Tukey's test (28).

RESULTS

The efficacy and mechanism of action of two botanical insecticides and a bacterial toxin were evaluated. The study showed that these materials affected the physiology of *C. medinalis* at several doses. The insect is able to survive at low doses (individual or combination treatments), which produce external symptoms such as sluggish movement, change in color, etc. Sublethal effects on larvae may greatly hinder the survival and fitness of leaffolder adults. Tables 1–3 demonstrate the efficacy and insecticidal activity of botanical insecticides and bacterial toxin against gut enzyme activity of rice leaffolder. Their activity and potency was dose- and pesticide-combination-dependent. Generally, combining several insecticides was more effective than individual treatments, even at low doses. The decrease in enzyme production by the gut was dose-dependent. However, the effect on ACP, ALP and ATPase activities by botanical insecticides and bacterial toxin was concentration-dependent (Tables 1–3).

Differences in ACP, ALP and ATPase activities in the gut between the control and treated 3rd instar larvae are shown in Table 1. The treatments significantly decreased

TABLE 1. Enzyme activities (in μmol/mg h^{-1}) of 3rd instar larvae of *Cnaphalocrocis medinalis* after treatment with *Btk,* NSKE and VNLE[z]

Treatments	Acid phosphatase (ACP)	Alkaline phosphatase (ALP)	Adenosine triphosphatase (ATPase)
Control	[y]7.62 ±0.41a	11.21±0.81a	61.46±5.29a
Btk (μg ml^{-1})			
1.0	6.54±0.34b	9.95±0.76b	56.16±4.12ab
2.0	4.98±0.21d	8.24±0.65c	50.23±4.23b
3.0	3.51±0.14fg	6.89±0.43d	46.31±3.84c
NSKE (%)			
0.10	6.29±0.37b	9.43±0.75b	53.42±4.71b
0.25	4.18±0.21d	8.06±0.65c	45.21±3.78cd
0.50	3.09±0.15f	6.27±0.43d	38.64±3.00e
VNLE (%)			
0.10	6.37±0.42b	9.71±0.72b	55.19±4.12b
0.25	4.51±0.26d	8.15±0.65c	48.25±3.95c
0.50	3.24±0.15f	6.65±0.39d	41.22±3.11e
Btk (μg ml^{-1}) + NSKE (%)			
1.0+0.10	5.79±0.31c	8.31±0.62c	42.22±3.26d
2.0+0.25	2.98±0.11g	5.56±0.35e	28.61±1.56g
Btk (μg ml^{-1}) + VNLE (%)			
1.0+0.10	6.09±0.37bc	8.71±0.61c	44.23±2.95d
2.0+0.25	4.05±0.23e	5.93±0.35e	31.62±1.96f
NSKE (%) + VNLE (%)			
0.10+0.10	5.14±0.26c	8.05±0.63c	40.75±2.98de
0.25+0.25	2.45±0.14gh	5.40±0.34e	26.62±2.24gh
Btk (μg ml^{-1}) + NSKE (%) +VNLE (%)			
1.0+0.10+0.10	1.63±0.00i	3.49±0.14f	17.84±1.56i

[z] *Btk* = *Bacillus thuringiensis kurstaki;* NSKE = neem seed kernel extract; VNLE = *Vitex negundo* leaf extract.
[y] Within columns, means followed by a common letter do not differ significantly (Tukey's test, $P<0.05$).

the activity of the gut enzymes after individual and combined treatments. The maximal suppression of activity was obtained by the combination of 1 μg ml^{-1} *Btk* 0.1% NSKE and 0.1% VNLE in all larval instars. The ACP activity in the 3rd instar (control insect) was 7.62 μmol/mg h^{-1}. ACP activity was reduced to 3.51μmol/mg h^{-1} (53.9%) by *Btk* treatment (3 μg ml^{-1}) and to 1.63 μmol/mg h^{-1} (78.6%) by combining the three materials (*Btk* 1 μg ml^{-1}, VNLE 0.10% and NSKE 0.010%). Similarly, there was a significant reduction in the activities of ALP (max. of 68.8%) and ATPase (max. of 70.9%) in combined treatment. Gut tissue enzyme activity was considerably decreased when the insects were fed on leaves treated with both *Btk* and botanical insecticides, compared to control treatment. Gut tissue enzyme activities decreased significantly with increasing concentrations of *Btk*, NSKE and VNLE.

The adverse effect of *Btk*, NSKE and VNLE on the activity of the gut enzymes of *C. medinalis* was evident during the feeding. Furthermore, the growth rate of *C. medinalis* was reduced by *Btk*, and by a combined treatment of *Btk*, NSKE and VNLE. ALP activity of 4th instar larva was markedly reduced, to 7.91 μmol/mg h^{-1} (46.4%) in combined treatment of *Btk* (2 μg ml^{-1}) and VNLE (0.25%), and the combination of *Btk*, NSKE and VNLE at 1 μg ml^{-1} + 0.10% + 0.10%, respectively, drastically decreased the enzyme activity to 4.78 μmol/mg h^{-1} (67.6%). A significant reduction in activity of ATPase (64.3%) was observed in the combined treatment of *Btk*, NSKE and VNLE at, respectively, 1.0 μg ml^{-1}

TABLE 2. Enzyme activities (in μmol/mg h^{-1}) of 4th instar larvae of *Cnaphalocrocis medinalis* after treatment with *Btk,* NSKE and VNLE[z]

Treatments	Acid phosphatase (ACP)	Alkaline phosphatase (ALP)	Adenosine triphosphatase (ATPase)
Control	[y]9.91±0.78a	14.76±1.26a	76.39±6.95a
Btk (μg ml^{-1})			
1.0	8.75±0.67b	13.84±1.30ab	70.65±6.21b
2.0	7.63±0.55c	12.51±1.25b	68.77±5.96b
3.0	6.21±0.49d	11.68±0.98bc	65.51±5.79bc
NSKE (%)			
0.10	7.53±0.56c	12.57±1.12b	67.37±5.62b
0.25	5.93±0.37d	10.90±0.89c	61.72±5.12c
0.50	4.91±0.24ef	9.42±0.84d	55.42±4.23d
VNLE (%)			
0.10	7.89±0.52c	12.79±1.00b	68.54±5.86b
0.25	6.01±0.48d	11.01±0.86c	64.21±5.46c
0.50	5.23±0.37de	9.68±0.76d	56.28±4.75d
Btk (μg ml^{-1}) + NSKE (%)			
1.0+0.10	7.10±0.62c	11.21±0.95c	58.82±4.25cd
2.0+0.25	4.98±0.35ef	7.56±0.59e	41.47±3.76ef
Btk (μg ml^{-1}) + VNLE (%)			
1.0+0.10	7.21±0.61c	11.43±0.97c	61.52±5.64c
2.0+0.25	5.12±0.38e	7.91±0.59e	45.37±3.79e
NSKE (%) + VNLE (%)			
0.10+0.10	6.93±0.56cd	11.01±0.94c	54.63±4.26d
0.25+0.25	4.89±0.34ef	7.21±0.56e	38.42±3.54f
Btk (μg ml^{-1}) + NSKE (%) + VNLE (%)			
1.0+0.10+0.10	2.88±0.12g	4.78±0.29f	30.24±2.56g

[z] *Btk* = *Bacillus thuringiensis kurstaki;* NSKE = neem seed kernel extract; VNLE = *Vitex negundo* leaf extract.
[y] Within columns, means followed by a common letter do not differ significantly (Tukey's test, $P<0.05$).

+ 0.10% + 0.10% (Table 2). There were statistically significant differences ($P<0.05$) in enzyme activities between individual and combined treatments (Tables 1–3). Our data demonstrate that, among the signs of toxicity that were observed following exposure to these bioinsecticides, is suppression of the gut enzyme activity.

The activities of ACP, ALP and ATPase were significantly decreased in the midgut of 4th and 5th instar larvae of *C. medinalis* after treatment with *Btk*, NSKE and VNLE (Tables 2 and 3). The highest activity of the gut enzymes was found in controls of 5th instar larvae: 12.36 μmol/mg h^{-1} (ACP), 18.64 μmol/mg h^{-1} (ALP) and 88.12 μmol/mg h^{-1} (ATPase). Insects fed with 1 μg ml^{-1} *Btk*, 0.1% NSKE and 0.1% VNLE showed maximum reduction in ACP (70.9% in 4th instar and 67.6% in 5th instar), ALP (67.6% in 4th instar and 67% in 5th instar) and ATPase (64.3% in 4th instar and 58.8% in 5th instar) when compared with controls (Tables 3 and 4). The combined treatment of *Btk*, NSKE and VNLE affected the enzyme activity to a greater extent, and in a dose-dependent manner. The last instar RLF larvae were able better to withstand the botanical insecticides and bacterial toxin-treated leaves. Botanical insecticides and biocides do not affect the last larval instar more severely than the initial instars.

Larval weight was gradually decreased in 1st to 5th instar larvae due to treatment with *Btk*, NSKE and VNLE, individually and in combination. Larval body weight was 32.2 mg in 5th instar (larvae control). At 0.5% VNLE, it dropped to 21.4 mg (33.5%) and was

TABLE 3. Enzyme activities (in μmol/mg h^{-1}) of 5th instar larvae of *Cnaphalocrocis medinalis* after treatment with *Btk,* NSKE and VNLE[z]

Treatments	Acid phosphatase (ACP)	Alkaline phosphatase (ALP)	Adenosine triphosphatase (ATPase)
Control	[y]12.36±1.21a	18.64±1.74a	88.12±8.45a
Btk (μg ml^{-1})			
1.0	11.96 ±9.75ab	17.43±1.63a	86.73±7.56a
2.0	10.78±9.12b	16.81±1.42ab	81.59±7.95ab
3.0	9.64±8.45c	15.92±1.54a	76.24±7.15b
NSKE (%)			
0.10	10.41±9.58b	16.24±1.59b	81.53±6.58ab
0.25	9.15±7.86cd	15.18±1.52b	74.21±7.41b
0.50	8.09±7.16d	14.08±1.32c	68.35±6.23c
VNLE (%)			
0.10	10.56±9.25b	16.49±1.49b	82.94±7.52a
0.25	9.23±8.94c	15.21±1.42b	76.23±7.12b
0.50	8.18±7.12d	14.11±1.37c	70.19±6.52c
Btk (μg ml^{-1}) + NSKE (%)			
1.0+0.10	9.31±8.68c	14.92±1.32bc	68.21±6.21c
2.0+0.25	6.38±5.24e	9.68±9.12e	51.43±4.89e
Btk (μg ml^{-1}) + VNLE (%)			
1.0+0.10	9.43±8.45c	15.21±1.42b	69.44±6.56c
2.0+0.25	6.45±2.23e	10.93±9.21d	53.65±5.12e
NSKE (%) + VNLE (%)			
0.10+0.10	9.21±8.54c	14.21±1.26c	61.57±5.12d
0.25+0.25	6.10±5.24e	9.15±8.42e	44.32±3.78f
Btk (μg ml^{-1}) + NSKE (%) + VNLE (%)			
1.0+0.10+0.10	4.0±2.76f	6.14±4.78f	36.24±2.91g

[z] *Btk* = *Bacillus thuringiensis kurstaki;* NSKE = neem seed kernel extract; VNLE = *Vitex negundo* leaf extract.
[y] Within columns, means followed by a common letter do not differ significantly (Tukey's test, $P<0.05$).

further reduced to 12.9 mg (59.9%) in combined treatment of *Btk*, NSKE and VNLE at 1.0 μg ml^{-1}, 0.10% and 0.10%, respectively (Table 4). There was a gradual decrease in the pupal weight of insects fed on *Btk*, NSKE and VNLE separately, but after a combined treatment the effect was more pronounced. For example, the average pupal weight in the control was 22.7 mg, but after treating with *Btk*, NSKE and VNLE at 1.0 μg ml^{-1}, 0.10% and 0.10%, it was reduced to 4.3 mg (81%) (Table 4).

DISCUSSION

As a consequence of concern about the persistence of synthetic pesticides in the environment and their potential toxicity to humans, beneficial insects and domestic animals, research has focused on more natural products for pest control. Naturally occurring biopesticides seem a logical choice for further investigation. They determine the acceptability of plants to herbivores – vertebrates and invertebrates. They also prevent fungal infection, and deter parasitic nematodes. Many of these metabolites can be classified as allelochemicals, plant compounds involved in biological interspecies interactions (14).

Leaffolder larvae that were fed *Btk* become flaccid and gradually turn blackish in color. Soon after death, the integument ruptures, allowing the liquefied internal *Btk* crystal content to be released. Such symptoms were observed as early as 3 days after larvae were placed on a diet containing high concentrations of *Bt*, and mortality occurred on

TABLE 4. Larval and pupal weights (in mg) of *Cnaphalocrocis medinalis* after treatment with *Btk*, NSKE and VNLE[z]

Treatments	Larval weight (10 larvae)					Pupal weight (10 pupae)
	Larval instars					
	I	II	III	IV	V	
Control	[y]10.2±1.04a	13.4±1.12a	16.8±1.23a	23.4±1.68a	32.2±2.05a	22.7±1.45a
Btk (μg ml^{-1})						
1.0	8.5±0.72b	11.8±0.95b	15.0±1.18c	21.9±1.46ab	30.1±1.98ab	19.3±1.27b
2.0	7.6±0.56c	10.5±0.91c	13.7±1.11c	19.3±1.19b	27.5±1.90b	18.2±1.07bc
3.0	5.4±0.32d	8.4±0.64d	11.5±0.81b	17.2±1.18c	24.1±1.74bc	11.3±0.86b
NSKE (%)						
0.10	8.1±0.65b	11.3±0.75e	14.5±1.16b	20.0±1.51b	28.2±1.95b	18.3±1.35b
0.25	7.2±0.52c	10.1±0.71c	13.2±0.12c	18.5±1.29b	23.1±1.95c	15.2±0.93d
0.50	5.0±0.38d	7.6±0.50c	11.2±0.85d	15.3±1.19d	19.2±1.54de	8.6±0.56g
VNLE (%)						
0.10	8.3±0.61b	11.5±0.90d	14.1±1.18b	21.3±1.63b	29.5±2.09ab	19.1±1.32b
0.25	7.4±0.54c	10.3±0.75c	12.9±0.91cd	19.1±1.11b	25.4±1.92b	16.4±0.86cd
0.50	5.2±0.38d	8.2±0.60d	10.7±0.78d	16.0±1.21cd	21.4±1.55d	9.3±0.62f
Btk (μg ml^{-1}) + NSKE (%)						
1.0+0.10	7.5±0.51c	10.8±0.78bc	13.5±0.14c	19.0±1.33b	23.2±1.78c	16.1±0.91cd
2.0+0.25	4.1±0.29e	7.0±0.49e	8.7±0.57e	13.5±1.13e	17.6±1.31ef	7.9±0.42gh
Btk (μg ml^{-1}) + VNLE (%)						
1.0+0.10	7.6±0.53de	11.0±0.83b	14.1±1.21b	19.6±1.39b	24.5±2.05c	17.2±1.25c
2.0+0.25	4.5±0.28e	7.3±0.50e	9.5±0.66f	14.1±1.15e	18.4±1.55e	8.2±0.59g
NSKE (%) + VNLE (%)						
0.10+0.10	7.2±0.48g	10.5±0.76c	13.1±1.15c	18.3±1.05b	21.8±1.53cd	15.2±1.08d
0.25+0.25	3.8±0.21f	6.7±0.45ef	8.1±0.51e	12.1±1.06e	16.2±1.18f	7.0±0.45h
Btk (μg ml^{-1}) + NSK (%) + VNLE (%)						
1.0+0.10+0.10	1.8±0.1g	4.5±0.29g	6.1±0.29g	9.0±0.42g	12.9±0.74g	4.3±0.25i

[z]*Btk* = *Bacillus thuringiensis kurstaki;* NSKE = neem seed kernel extract; VNLE = *Vitex negundo* leaf extract.
[y]Within columns, means followed by a common letter do not differ significantly (Tukey's test, $P<0.05$).

or before the 5th day post-treatment, depending on the age and stage of the larvae when they were exposed to the toxin (21,24,25). The general antinutritive effects of botanical insecticides can cause disruption of growth regulation, decreased growth, and low feeding efficiency. The present findings that increasing amounts of NSKE and VNLE consumed reduce conversion efficiency and larval body weight, suggest that, in addition to antifeedant properties, NSKE and VNLE contain digestive components inhibiting enzyme activities and reducing conversion rate. Several studies have shown that feeding is necessary for the stimulation of enzyme activities (7,32). Exposure of *C. medinalis* larvae to sublethal doses of *Btk* in the laboratory prolonged larval development and reduced pupal weight (26). In the field, delayed phenology of surviving larvae and reduced pupal weight are common occurrences after treatment with *Btk* (22). The direct and indirect contribution of such effects to treatment efficacy through reduced larval feeding and fitness need to be better understood in order to improve the use of *Btk* for management of *C. medinalis*. Higher enzyme activity in the midgut of control insects is most probably due to consumption as well as utilization of large quantities of food. Imbalance in the enzyme–substrate complex and inhibition of peristaltic movement of the gut (16) might have inhibited the enzyme

activity in the treated insects. Chapman (9) reported that enzyme production is clearly related to the feeding behavior (amount of food that passes through the alimentary canal). The activity of these enzymes is related to the physiological situation of RLF and reflects the absorption, digestion and positive transport of nutrients in the midgut. *Btk* 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 the alkaline conditions in the intestinal juice; the damage to the midgut caused a decrease in enzyme activities (11,19,21).

Changes in ALP and ACP activities after treatment with botanical insecticides and bacterial toxin indicate that changing the physiological balance of the midgut might affect these enzymes. ALP is found mainly in the intestinal epithelium of animals and its primary function is to provide phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes (27). ALP is involved in the transphosphorylation reaction. In the present study, the decrease in the activity of these enzymes after *Btk*, NSKE and VNLE were fed to *C. medinalis* suggests that these materials affect gut physiological events (*i.e.,* ion transport) that might influence these enzymes.

Decreased level of ACP at higher concentration of *Btk*, NSKE and VNLE suggests a reduced phosphorus liberation for energy metabolism, decreased rate of metabolism, as well as decreased rate of transport of metabolites, and may be due to the direct effect of *Btk*, NSKE and VNLE on enzyme regulation. In the present study, the biochemical parameters and enzymatic profiles were markedly affected following NSKE and VNLE treatment. The significant decrease in the rate of maturation caused by NSKE and VNLE treatment appears to be due to poor nutrition in the treated insects and also related to altered feeding physiology.

ATPases are essential for transport of glucose, amino acids, etc. Any impairment in their activity will affect the physiology of the gut. ATPases are membrane-bound enzymes. The role of membrane lipids and their micro-environmental changes at the physical and chemical levels may be responsible for the differential response observed at the level of ATPase activity after *Btk*, NSKE and VNLE feeding treatment. Membrane ATPase, especially in the intestinal epithelium, assists transport and reabsorption of metabolites and nutrients and also secondary transport of ions and non-electrolytes (13,18). Babu *et al.* (3) showed that the ATPase activity in the gut of *Helicoverpa armigera* was significantly decreased, due to toxic effects of azadirachtin. In the present study, the ATPase activity in the gut was also significantly reduced by *Btk*, NSKE and VNLE treatments. ATPase inhibition may affect active ion transport, leading to alteration in electrolyte regulation. After *Btk*, NSKE and VNLE treatments, a decrease in enzymatic activity denotes reduced metabolism in the insect and may be due to the toxic effects of *Btk*, NSKE and VNLE on membrane permeability, especially on the gut epithelium (23).

It is evident that exposure to botanical insecticides in the larval diet has significant effects on the activity of several enzymes found in the late instar larvae and adult *C. medinalis*. Botanical insecticides such as neem may interfere with the production of certain types of proteins. This activity is apparently strongest during pupation; pupae were very susceptible after larvae were exposed to the botanical insecticides. Previous reports by several groups found that treatment with neem and botanical insecticides induced similar signs of toxicity (30,33). Active principles present in neem and *Vitex* (azadirachtin, vitexin, etc.) are responsible for such effects.

In conclusion, neem and *Vitex* had significant effects on larvae and pupae of *C. medinalis* and acted synergistically with *Bt* toxin, causing a reduction in weight and in enzyme activity. The adult physiology is thus impaired after larvae are exposed to botanical insecticides and bacterial toxin These botanical insecticides and *Btk* may therefore serve as effective alternatives to conventional synthetic insecticides in the control of agricultural pests.

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