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Effect of biopesticides on the lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* (Guenée) (Insecta: Lepidoptera: Pyralidae)

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Abstract

The effects of bacterial toxins (*Bacillus thuringiensis*) and botanical insecticides (*Azadirachta indica* and *Vitex negundo*) on lactate dehydrogenase (LDH) activity in *Cnaphalocrocis medinalis* (Guenée) (the rice leaffolder) were evaluated. Bacterial toxins and botanical insecticides affected the LDH activity individually and in combination. When they were combined, the effect was more severe at low concentration. There was a decrease in enzyme activity over controls at all concentrations tested. The combined effect of the three biopesticides resulted in a considerable decrease in enzyme activity, indicating strong enzyme inhibition. Clear dose–response relationships were established with respect to enzyme activity.

Keywords: Biopesticides; Botanical insecticide; Neem; *Vitex*; *Btk*; Bioassay; Toxicity; Enzyme; LDH; Metabolism

1. Introduction

Insecticide resistance has limited the effectiveness of many chemical insecticides. Consequently, an intensive effort has been made to find alternative methods of control (Senthil Nathan et al., 2004). Botanical insecticides and microbial pesticides are highly effective, safe, and ecologically acceptable. Plants produce a diversity of biologically active substances that affect the growth and development of other organisms and can provide protection against herbivory. These plant products discourage or prevent attack from nonadapted organisms and play an important role in the ecology and physiology of phytophagous insects (Sukumar, 1993). *Bacillus thuringiensis* (*Bt*) Berliner is a naturally occurring Gram-positive, spore-forming soil bacterium, and preparations containing *Bt* are widely used as microbial pesticides in agriculture and forestry (Gill et al., 1992).

Chemical preparations from the leaves and seeds of the Indian neem tree, *Azadirachta indica* A. Juss. (Meliaceae), have been shown to have deleterious effects on insects (Schmutterer, 1990). Neem seed kernel extracts (NSKEs) have suppressed insect feeding, growth, and reproduction (Ascher et al., 1984) and have been used in many integrated pest management (IPM) programs (Schmutterer, 1990). *Vitex negundo* L. (Verbenaceae) is an important aromatic and medicinal plant with pesticidal properties (Kirthikar and Basu, 1981).

Lactate dehydrogenase (LDH) (EC 1.1.1.28) is an important glycolytic enzyme that is present in virtually all tissues (Kaplan and Pesce, 1996). It is involved in

Abbreviations: RLF, rice leaffolder; LDH, lactate dehydrogenase; *Btk*, *Bacillus thuringiensis* Berliner sub sp. *kurstaki*; NSKE, neem seed kernel extract; VNLE, *Vitex negundo* leaf extract; SE, standard error

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carbohydrate metabolism and has been used as an indicative criterion of exposure to chemical stress (Diamantino et al., 2001; Wu and Lam, 1997). LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue, and organ damage. However, the potential of this enzyme as an indicative criterion in invertebrate toxicity tests has been scarcely explored (Ribeiro et al., 1999). To examine the combined effects of botanical insecticides and bacterial toxins on the insect midgut, we conducted a study of the activity of LDH in a susceptible insect pest, the rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae).

2. Materials and methods

2.1. Laboratory mass culture of Cnaphalocrocis medinalis

C. medinalis larvae were collected from the paddy fields in and around Coimbatore district, Tamil Nadu, India, 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 $27 \pm 2\,^{\circ}C$ in a 14:10 light: dark photoperiod and 85% relative humidity. Rice plants were grown in earthenware pots, 18 cm tall with a 20-cm-diam. top. Each pot held 15 plants and gave 62 tillers. Pots were placed in about 10 cm of water in a metal tray (Senthil Nathan, 2000; Senthil Nathan et al., 2004). The culture was initiated with partly grown larvae from the field. Thereafter, newly hatched larvae were placed on ca. 60-day-old plants of the rice variety "TN1".

Twelve recently emerged adult females and 13 males were placed in an oviposition cage containing one potted plant. Moths were fed a 10% sucrose solution fortified with a few drops of vitamin mixture (Multidec drops, Ashok Pharmaceutical, Chennai-24, India) to enhance oviposition. After 2 days, leaves containing eggs were clipped and placed on moist filter paper in Petri dishes. These eggs were used to establish the colony of *C. medinalis*.

2.2. Preparation of Bacillus thuringiensis subsp. kurstaki (Btk)

Delfin WG, which contains *Btk*, serotype 3a, 3b, 85% and dispersing agents 15%, potency: min 53,000 SU/mg (Sandoz (India) Ltd., 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.

2.3. Preparation of neem seed kernel and Vitex negundo leaf extracts

Neem seed kernel and *V. negundo* were collected from forests in the Marudamalai Hills, Bharathiar University, Coimbatore, Tamil Nadu, India. Samples of 50 g each of seed kernels of *A. indica* (from three trees) and *V. negundo* (from five plants) leaves were washed and oven-dried to constant weight at 55 °C. The dried seeds and leaves were ground separately into powders by a mixer grinder. The powders were then each mixed with 100 mL of water in a Soxhlet apparatus to prepare a stock solution of each plant extract (100 mg/mL). From the stock solution, the required concentrations, 0.1, 0.25, 0.5, 1, and 2%, were prepared using water. The combined extracts were prepared by mixing equal volumes of NSKE and *V. negundo* leaf extract (VNLE) (1:1, v/v).

2.4. Bioassay and treatments

Bioassays were performed with second to fifth instars of *C. medinalis* using concentrations of 0.25%, 0.5%, 1%, and 2% of NSKE and VNLE and 0.5, 1, and 2 μg/mL of *Btk*. Control leaves were treated with distilled water. A minimum of 10 larvae/concentration were used for all the experiments, and the experiments were replicated five times (total $n = 50$). The effective concentration (EC_{50}) was calculated using the Probit analysis (Finney, 1971) using the above experiments.

Fresh rice leaves (*Oryza sativa* L) were sprayed with different concentrations of *Btk*, NSKE, and VNLE and allowed to air-dry. The formulations were applied to leaves with a regulator-controlled spray applicator for all the experiments. Control leaves were treated with distilled water alone. Second to fifth instar larvae were starved for 4 h and then fed leaves treated with different concentrations of *Btk*, NSKE, or VNLE. After being exposed to the treatment for 24 h, each larva was transferred to an untreated diet and then enzyme assays were carried out for respective instars. A minimum of 10 larvae/concentration were used in each experiment and all experiments were replicated five times (total $n = 50$).

2.5. Preparation of enzyme extract

Second to fifth instars of treated *C. medinalis* were used to quantify the enzyme activities. Enzyme extracts were prepared by the method of Applebaum (1964) and Applebaum et al. (1961). Individuals were anesthetized with 5×5 mm 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 (foregut, midgut, and hindgut) and weighed and each region was homogenized for 3 min at 4 °C in ice-cold citrate-phosphate buffer (pH 6.8) using a tissue grinder. Homogenized gut sections were suspended in ice-cold buffer and diluted to 1 mL. The homogenate was centrifuged at 500 rpm for 15 min and the supernatant was used as the enzyme source.

2.6. Estimation of lactate dehydrogenase (LDH) (EC 1.1.1.27)

To standardize volumes, 0.2 mL of NAD+ solution was added to the 'test' and 0.2 mL of water was added to the control test tubes, each containing 1 mL of the buffered substrate; 0.01 mL of the sample was also added to the 'test'. Test tube samples were incubated for exactly 15 min at 37 °C and then arrested by adding 1 mL of color reagent (2,4-dinitrophenylhydrazine reagent) to each tube and the incubation was continued for an additional 15 min. After the contents were cooled to room temperature, 10 mL of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline to maximize development of hydrazine. Exactly 60 s after the addition of alkali to each tube, the intensity of color was measured at 440 nm. Replicated blanks with standards were run through the same procedure. Inclusion of the calculated amount of reduced coenzyme in the standard makes allowance for the chromogenicity of $NADH_2$ formed in the test. The enzyme activity is expressed as multi-International Units (mIU) per mg protein per minute (King, 1965).

A mIU is defined as the amount of enzyme that is required to catalyze the conversion of 1 μL of lactate to pyruvate or pyruvate to lactate per minute per mL of the sample under the prescribed assay conditions.

2.7. Statistical analysis

The effective concentration was calculated using Probit analysis (Finney, 1971). Data from enzyme activity were subjected to analysis of variance (ANOVA of arcsine square root transformed percentages). Differences between the treatments were evaluated by Tukey's multiple range test ($P \leqslant 0.05$) (Snedecor and Cochran, 1989; SAS Institute, 2001).

3. Results

Our results show that botanical insecticides and bacterial toxin affected the LDH activity of *C. medinalis* at several doses. Treatment with botanical insecticides and bacterial toxin significantly decreased the activity of the gut enzymes after either individual and combined treatments. The maximal suppression of gut enzyme activity was obtained by combination of botanical insecticides and bacterial toxin at 1 μg/mL *Btk*, 0.1% NSKE, and 0.1% VNLE in all larval instars. An EC_{50} value of *Btk*, NSKE, and VNLE against rice leaffolder is shown in Fig. 1. NSKE was most potent in all experiments, with lowest EC_{50} (0.4%, 0.8%, 1.2%, and 1.4%, second to fifth instars, respectively).

The LDH activity in the second instar (control insect) was 10.51 mIU/mg/protein/min. It was reduced to 5.41 mIU/mg/protein/min (50%) by NSKE treatment (0.5%), and it was further reduced to 0.92 mIU/mg/protein/min (72%) by a combination of botanical insecticides and bacterial toxin at 1 μg/mL *Btk,* 0.1% NSKE, and 0.1% VNLE. Similarly, there were significant reductions in the activities of LDH in third instar (maximum of 67%), fourth instar (maximum of 59%), and fifth instar (maximum of 54%) in the combined treatment of botanical insecticides and bacterial toxin (Table 1, Figs. 2–5). LDH activity was considerably decreased when the insects were fed on leaves treated with both *Btk* and botanical insecticides, compared to control treatment. LDH activity significantly decreased with increasing concentration of *Btk*, NSKE, and VNLE. There were statistically significant differences ($P \leqslant 0.05$) in LDH activities between individual and combined treatments (Table 1).

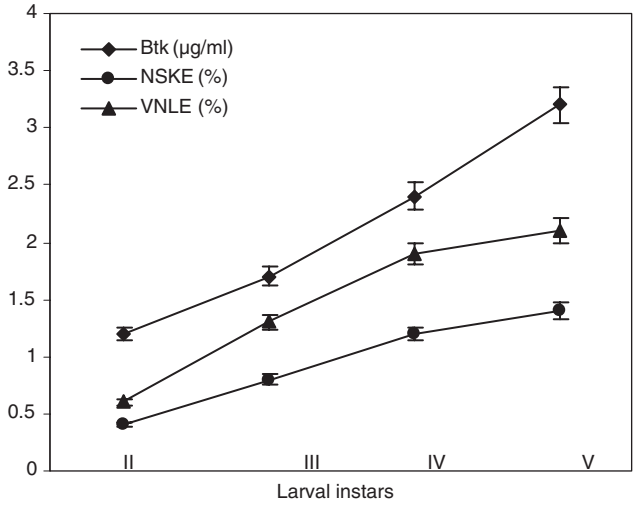

Fig. 1. Effective concentrations (EC_{50}) of *Btk*, NSKE, and VNLE against first to fifth instar larvae of *C .medinalis*. Values are means (±SE) of five replicates.

4. Discussion

Changes in metabolism and physiology and decreases in the gut enzyme activity of neem-treated individuals may be expected to affect enzyme titers and activities (see review by Schmutterer (1990) and Mordue and Blackwell (1993)). The doses of botanical insecticides and bacterial toxin affected the LDH activity of the larvae. Our data support this hypothesis (Table 1 and Figs. 2–5), because 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 enzyme–substrate complex and inhibition of peristaltic movement of the gut might have inhibited the enzyme activity in the treated insects (Chapman, 1985; Senthil Nathan et al., 2005c–e). We demonstrate

Table 1
Lactate dehydrogenase activity (mIU/mg/protein/min) of *C. medinalis* after the treatment with *Btk,* NSKE, and VNLE

Treatments	Larval instar			
	II[a]	III[a]	IV[a]	V[a]
Control	10.51±1.23[a]	16.26±1.64[a]	26.32±1.85 [a]	30.00±3.21[a]
Btk (μg/mL)				
1.5	8.22±1.04[a]	14.77±1.41[a]	23.19±1.88[a]	27.63±2.03[a]
3.0	6.12±0.86[b]	10.64±1.18[c]	19.41±1.63[a]	23.89±2.15[b]
NSKE (%)				
0.25	7.21±0.54[ab]	12.79±1.34[b]	21.95±1.72[b]	24.18±2.13[b]
0.50	5.41±0.43[b]	8.16±0.96[c]	17.54±1.46[bc]	21.34±2.06[b]
VNLE (%)				
0.25	7.32±0.93[ab]	13.21±1.37[ab]	22.61±1.95[ab]	24.46±2.74[b]
0.50	5.93±0.63[b]	9.51±1.35[c]	18.03±1.54[b]	21.64±1.95[b]
Btk(μg/mL)+NSKE (%)				
1.0+0.10	5.90±0.65[b]	12.31±1.18[b]	18.51±1.71[b]	21.68±1.87[b]
2.0+0.25	3.46±0.50[c]	7.95±0.89[c]	15.64±1.25[bc]	17.28±1.36[c]
Btk(μg/mL)+VNLE (%)				
1.0+0.10	6.21±0.90[b]	13.01±1.26[ab]	18.91±1.65[b]	22.48±1.95[b]
2.0+0.25	4.32±0.54[bc]	8.05±0.85[c]	15.81±1.16[bc]	18.57±1.36[c]
NSKE (%)+VNLE (%)				
0.10+0.10	5.24±0.43[b]	11.56±1.14[b]	17.50±1.56[bc]	19.73±1.85[c]
0.25+0.25	2.91±0.41[c]	7.58±0.86[d]	14.31±0.96[c]	17.48±1.06[c]
Btk (μg/mL)+NSKE (%)+VNLE (%)				
1.0+0.10+0.10	0.92±0.21[d]	5.24±0.53[e]	10.56±0.95[d]	13.51±1.65[d]

[a]Means within columns followed by the same letter are not significantly different (Tukey's test, $P \leqslant 0.05$) (±SE—standard error).

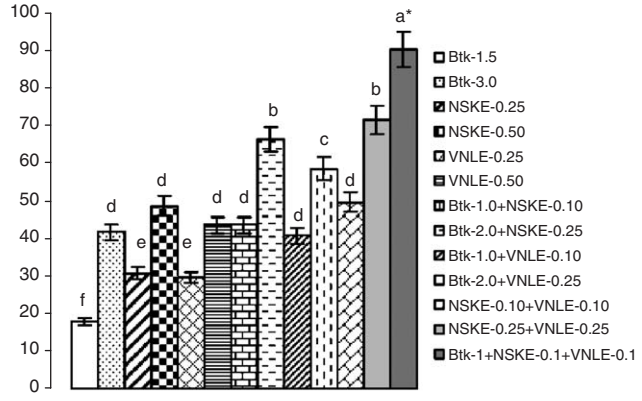

Fig. 2. Percentage reduction of LDH activity in second instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. Means (±SE) followed by the same letters within bars indicate no significant difference ($P \leqslant 0.05$) in a Tukey test. Treatments: *Btk* in μg/mL, NSKE and VNLE in % (±SE—standard error).

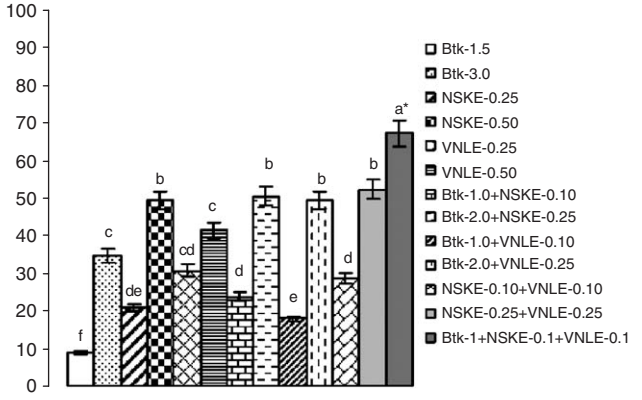

Fig. 3. Percentage reduction of LDH activity in third instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. Means (±SE) followed by the same letters within bars indicate no significant difference ($P \leqslant 0.05$) in a Tukey test. Treatments: *Btk* in μg/mL, NSKE and VNLE in % (±SE—standard error).

significant differences ($P \leqslant 0.05$) in activity among individual and combined treatments. Our data show that combined treatments in larval diet highly influence LDH activity in larvae. This effect is more pronounced among second instar larvae than for the rest of the instars. Our data demonstrate that suppression of the LDH activity is among the signs of toxicity that are observed following exposure to these bioinsecticides.

It is well documented that neem derivatives and *Vitex* are enzyme and feeding inhibitors (Senthil Nathan et al., 2004, 2005a–c). Neem derivatives, in which azadirachtin is the main active ingredient, have been demonstrated to be effective crop insecticides against several insect species (Mordue and Blackwell, 1993; Schmutterer, 1990; Schmutterer and Ascher, 1987; Senthil Nathan et al., 2005a–c; Shafeek et al., 2004).

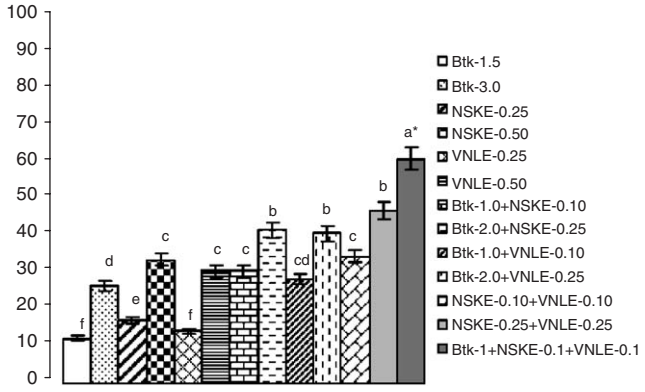

Fig. 4. Percentage reduction of LDH activity in fourth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. Means (±SE) followed by the same letters within bars indicate no significant difference ($P \leqslant 0.05$) in a Tukey test. Treatments: *Btk* in μg/mL, NSKE and VNLE in % (±SE—standard error).

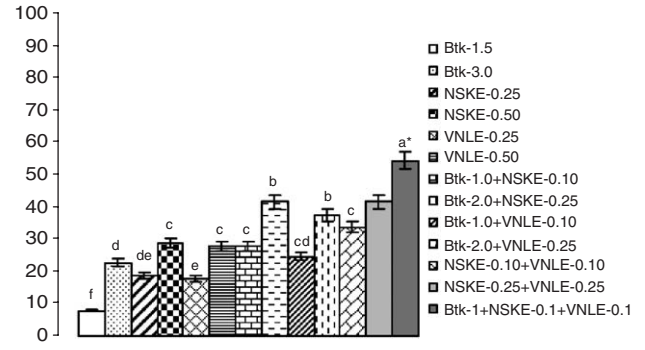

Fig. 5. Percentage reduction of LDH activity in fifth instar larvae of *C. medinalis* after treatment with *Btk,* NSKE, and VNLE. Means (±SE) followed by the same letters within bars indicate no significant difference ($P \leqslant 0.05$) in a Tukey test. Treatments: *Btk* in μg/mL, NSKE and VNLE in % (±SE—standard error).

LDH is involved in the production of energy, being particularly important when a considerable amount of additional energy is rapidly required. A negative correlation between LDH activity and ambient oxygen levels has been reported for some aquatic organisms, suggesting a possible biochemical adjustment in response to the lowered oxygen levels (Augenfeld, 1966; Bidlack and Lockshin, 1976; Diamantino et al., 2001). This probably occurs also in situations of chemical stress. Therefore, this enzyme may be a sensitive criterion for pesticide exposure (Diamantino et al., 2001; Wu and Lam, 1997). After *Btk*, NSKE, and VNLE treatments, a decrease in LDH activity denotes reduced metabolism in the insect and may be due to the toxic effects of neem derivatives on membrane permeability, especially on the gut epithelium (Senthil Nathan et al., 2004, 2005a; Smirle et al., 1996).

Our data also suggest that both botanical insecticides and bacterial toxin had a stronger effect on enzyme activities in early instars. Botanical insecticides such as neem may interfere with the production of certain types of proteins. This activity is apparently strongest during pupation; pupae were strongly affected after larval exposure (Senthil Nathan et al., 2004; Senthil Nathan et al., 2005c–e). Results of the present study may be of some importance for the continued development and use of biopesticide combinations in the future.

In conclusion, neem and *Vitex* have significant effects on LDH activity of RLF and appear to act synergistically with *Bt* toxin causing a greater reduction of enzyme activity.

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